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NATIONAL INSTITUTE OF PUBLIC HEALTH AND THE ENVIRONMENT

RIVM report 289202 028

Comparison of methods for enumeration of total coliforms and *Escherichia coli* in water samples in the Netherlands

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March 2001

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This investigation has been performed by order and for the account of the General Directorate for Environmental Protection, directorate of Drinking Water, Water and Agriculture, within the framework of project 289202, Watermicrobiology (MGB project MGB143).

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Abstract

The new European Drinking Water Directive (December 1998) defines a reference method for estimating the concentration of coliforms and *Escherichia coli* in drinking water. Laboratories may use other methods, but should demonstrate that results obtained are at least as reliable as those produced with the reference method. Three Dutch laboratories participated in a European trial, in which a protocol for comparing enumeration methods for total coliform bacteria and *Escherichia coli* in water samples was tested. Besides the membrane filtration method on Lactose TTC agar with Tergitol 7 (LTTC) described in ISO 9308-1, and the Colilert® method, the Dutch laboratories included membrane filtration methods on Laurylsulphate Agar (LSA), Chromocult® Coliform Agar (CCA) and the *E. coli* Direct Plating method. On LTTC37 significantly more total coliforms were enumerated than on LSA37; however, the LTTC method was found suitable for analysing very clean (drinking) water samples only, due to its lack of selectivity. The DP method (or Rapid Test in ISO 9308-1) is the best method for enumeration of *E. coli,* while the Colilert® method produces 12,5 % false negative *E. coli* results. Recommendations for making a choice for a method for detection and enumeration of total coliforms and/or *E. coli* in water samples with different contamination levels are given.

Samenvatting

De nieuwe Europese Drinkwater Richtlijn (Anonymous, 1998) schrijft een methode voor voor de bepaling van het aantal bacteriën van de coligroep en *Escherichia coli* in drinkwater. Laboratoria mogen andere methoden gebruiken, maar moeten aantonen dat de resultaten die met deze methoden verkregen worden ten minste zo betrouwbaar zijn als die verkregen worden met de referentiemethode.
Er is een protocol voor het vergelijken van referentie en alternatieve methoden ontwikkeld (Fricker, Thames Water Utilities, Engeland; Appendix 1) en een aantal Europese laboratoria heeft dit protocol getest. De referentie methode voor het bepalen van bacteriën van de coligroep en *E. coli* in water was hier Lactose TTC agar met Tergitol 7 (LTTC), die beschreven staat in ISO 9308-1 (Anonymous, 2000). Colilert® (IDEXX Laboratories) werd gebruikt als alternatieve methode.
De drie Nederlandse laboratoria die deelnamen aan het onderzoek (RIVM-MGB, Kiwa, PWN) gebruikten naast bovengenoemde methoden ook drie methoden die nu in Nederland in gebruik zijn. Er werden monsters met een verwacht besmettingsniveau van 1-50 bacteriën van de coligroep per 100 ml geselecteerd. Een totaal aantal van 179 monsters ((verdund) oppervlaktewater, drinkwater (halfproducten)) werd geanalyseerd met behulp van de Direct Plating methode (als snelle test beschreven in ISO 9308-1), Chromocult® Coliform Agar (CCA, Merck), Colilert® en LTTC om *E. coli* te bepalen. Laurylsulfaat agar (LSA37, Anonymous, 1982)), CCA, Colilert® en LTTC werden gebruikt om bacteriën van de coligroep te bepalen. Van membraanfilters met groei op LTTC en LSA37 werden karakteristieke kolonies bevestigd en uit positieve Colilert® wells werden reincultures geïsoleerd en bevestigd. Dit rapport beschrijft de resultaten van de Nederlandse deelnemers. Uit paarsgewijze vergelijking van de tellingen met behulp van de "sign test" en de "signed (Wilcoxon) rank test", bleek dat significant ($P<0.05$) meer bacteriën van de coligroep werden geteld op LTTC37 dan op LSA37. Alle drie de laboratoria rapporteerden echter achtergrond groei die 50-100 % van het oppervlak van membraanfilters, geïncubeerd op LTTC bij 37 °C (LTTC37), bedekte. Dit maakte het tellen van de karakteristieke gele kolonies tussen grote aantallen oranje tot bruine kolonies erg lastig. Incubatie van LTTC bij 44 °C (LTTC44) verbeterde dit gebrek aan selectiviteit enigzins. Karakteristieke kolonies die oxidase positief waren en door één laboratorium bevestigd werden als *Aeromonas*, werden regelmatig aangetroffen op LTTC37. De deelnemende laboratoria gebruiken de LSA37 methode routinematig en vonden deze eenvoudig uit te voeren en af te lezen. Er werd wel melding gemaakt van de aanwezigheid van enige storende achtergrondgroei (kleine rose kolonies) bij de analyse van oppervlaktewater monsters. Gemiddeld werd 80 tot 85 % van de isolaten verkregen van resp. LTTC37 en LSA37 bevestigd als bacteriën van de coligroep.
Colilert®, de enige MPN methode in dit onderzoek, was erg gebruikersvriendelijk: zowel het analyseren van de monsters als het aflezen van de resultaten was eenvoudig. De drie deelnemers telden significant ($P<0.05$) hogere aantallen bacteriën van de coligroep met deze methode dan met de andere methoden. Verschillen in tellingen verkregen met de membraanfiltratie methoden op lactosehoudende media en Colilert®, kunnen verklaard

worden uit het gebruik van verschillende substraten, wat resulteert in de detectie van verschillende groepen coliformen: op lactosehoudende media vormen bacteriën die zowel het *lacY* als het *lacZ* gen bezitten (coderend voor lactose fermentatie) karakteristieke gele kolonies; bacteriën die alleen het *lacZ* gen hebben kunnen lactose niet gebruiken en vormen geen gele kolonies, maar geven wel een positief resultaat in Colilert®.
Met Colilert® werden significant ($P<0.05$) lagere aantallen *E. coli* gevonden dan met LTTC37, LTTC44 en de Direct Plating methode, bovendien werden ook 12,5 % vals negatieve resultaten voor *E. coli* waargenomen. Dit bevestigt resultaten uit eerdere onderzoeken. Hieruit bleek dat een deel van de *E. coli* populatie niet in staat is het Colilert® substraat (een ander substraat dan in de overige methoden) te gebruiken. Het bevestigingspercentage voor *E. coli* was zeer hoog: 96 % van de isolaten uit wells die karakteristieke reacties voor *E. coli* vertoonden werd als *E. coli* bevestigd.
Concluderend: m.b.v. LTTC37 werden significant meer bacteriën van de coligroep gedetecteerd dan m.b.v. LSA37. Het bevestigingspercentage voor bacteriën van de coligroep was voor beide methoden vergelijkbaar, maar door gebrek aan selectiviteit is LTTC alleen geschikt voor het onderzoeken van zeer schoon (drink)water. Voor bepaling van *E. coli* is de Direct Plating methode het meest geschikt; deze methode is eenvoudig uit te voeren en detecteerde meer *E. coli* dan LTTC37, LTTC44 en Colilert®. Colilert® is gebruikersvriendelijk, maar vanwege het hoge percentage vals negatieven minder geschikt voor bepaling van *E. coli*.
Er worden aanbevelingen gegeven voor het maken van een keuze voor een methode voor bepaling van bacteriën van de coligroep en/of *E. coli* in monsters water met verschillend besmettingsniveau.

Summary

The new European Drinking Water Directive (Anonymous, 1998) defines a reference method for the estimation of the concentration of total coliforms and *Escherichia coli* in drinking water. Laboratories may use other methods, but should demonstrate that the results obtained are at least as reliable as those produced by the reference method.
A protocol for comparison of reference and alternative methods was developed (Fricker, Thames Water Utilities, UK; Appendix 1) and a number of European laboratories tested this protocol. The reference method for determination of total coliforms and *E. coli* in water was Lactose TTC agar with Tergitol 7 (LTTC), which is described in ISO 9308-1 (Anonymous, 2000); Colilert® (IDEXX Laboratories) was used as an alternative method.
The three Dutch laboratories (RIVM-MGB, Kiwa, PWN) that joined in the trial also included three methods that are currently used in the Netherlands. Samples with an expected contamination level of 1-50 coliforms per 100 ml were selected. A total number of 179 samples ((diluted) surface water, drinking water (half products)) were analysed by using the Direct Plating method (described as Rapid Test in ISO 9308-1), Chromocult® Coliform Agar (CCA, Merck), Colilert® and LTTC to enumerate *E. coli*. Laurylsulphate agar (LSA37, Anonymous, 1982), CCA, Colilert® and LTTC were used to enumerate total coliforms. From positive membrane filters on LTTC and LSA37, characteristic colonies were confirmed and from positive Colilert® wells pure cultures were obtained and confirmed. This report describes the results obtained by the Dutch participants.
Pairwise comparison of the count results by using the sign test and the signed rank (Wilcoxon) rank test, showed that LTTC37 enumerated significantly more ($P<0.05$) total coliforms than LSA37. However, all three laboratories reported background growth as high as 50-100 % coverage of membrane filters that were incubated on LTTC at 37 °C (LTTC37). This made counting of the characteristic yellow colonies amongst a crowd of orange to brown colonies very difficult. Incubation at 44 °C (LTTC44) slightly improved this lack of selectivity. Characteristic yellow colonies being oxidase positive (and confirmed as *Aeromonas* by one laboratory) were regularly observed on LTTC37. The participating laboratories use the LSA37 method routinely and found it easy to perform, although some disturbing background growth (small pink colonies) was reported when analysing surface water samples. An average of 80 to 85 % of the isolates obtained from resp. LTTC37 and LSA37 was confirmed as total coliforms.
Colilert®, the only MPN method included in the trial, was very convenient: analysing samples and reading results was easy. The three participants enumerated significantly ($P<0.05$) higher numbers of total coliforms with this method than with the other methods. Differences in counts obtained with membrane filtration methods on lactose containing media and Colilert® can be explained from the different substrates that are used resulting in the detection of different groups of coliform bacteria: on lactose containing media bacteria having both the *lacY* and the *lacZ* coding for lactose fermentation form charcteristic yellow colonies; bacteria having only the *lacZ* gene cannot use lactose and do not form yellow colonies, but do give a positive result in Colilert®.

Colilert® enumerated significantly ($P<0.05$) less *E. coli* than LTTC37, LTTC44 and the Direct Plating method; 12.5 % false negative *E. coli* results were also observed. This confirms results from previous research, which showed that a part of the *E. coli* population was not able to use the Colilert® substrate for *E. coli* detection, which differs from substrates in the other methods. From 96 % of the Colilert® wells that showed a positive reaction for *E. coli, E. coli* could be isolated.

In conclusion: LTTC37 detected significantly more total coliforms than LSA37, confirmation rates were comparable, but due to lack of selectivity LTTC is suitable for monitoring of very clean (drinking) water samples only.

For enumeration of *E. coli*, the Direct Plating method is the most suitable method: the method is easy to perform and detected more *E. coli* than LTTC37, LTTC44 and Colilert®. Colilert® is userfriendly, but due to a high percentage false negative results considered less suitable for enumeration of *E. coli*.

Recommendations for making a choice for a method for detection and enumeration of total coliforms and/or *E. coli* in water samples with different contamination levels are given.

Abbreviations

BBLB	Brilliant Green Bile Lactose Broth
CCA	Chromocult® Coliform Agar
DP	Direct Plating
DW	Drinking Water
DWI	Drinking Water Inspectorate
EN	Europese Norm
ISO	International Organisation for Standardisation
LSA	Laurylsulphate Agar
LTTC	Lactose TTC agar with Tergitol 7
MPN	Most Probable Number
MUG	4-methyllumbelliferyl-β-D-glucuronide
ONPG	ortho-nitrofenyl-β-α-galactopyranoside
PS	Peptone Saline
PWN	Water Supply Company North Holland
RIVM-MGB	National Institute of Public Health and the Environment - Microbiological Laboratory for Health Protection
TB	Tryptophane Broth
TBA	Tryptone Bile Agar
TSA	Tryptone Soy Agar
totcol	Total Coliforms

Contents

1. Introduction

The new European Drinking Water Directive (Anonymous, 1998) defines reference methods for the enumeration of microbiological parameters in drinking water. Member states may use alternative methods, in case they have demonstrated that these methods produce results that are at least as reliable as those produced by the reference methods. Most of the reference methods chosen are ISO (International Oragnisation for Standardisation) and EN (Europese Norm) methods, some of them recently revised.

For many member states the methods defined as reference methods in the new Drinking Water Directive, are different from the methods currently used in their laboratories. Laboratories that wish to continue the use of their 'own' methods will be regularly confronted with the problem of having to prove these methods being as good as the reference method. This resulted in the need for a protocol that describes how such a test should be performed to give a statistically reliable outcome. Within ISO, a working group (ISO TC147/SC4/WG12 "Analytical Quality control") is in charge of producing an ISO document for comparing microbiological methods titled 'Equivalence of microbiological quantitative cultural methods'. For the purpose of the comparison of methods for total coliforms and *Escherichia coli* in this study, a protocol was developed on basis of a Drinking Water Inspectorate (DWI, UK) document ("Comparison of Methods for Drinking Water Bacteriology – Cultural Techniques') by C. Fricker (Thames Water Utilities, UK) in cooperation with the participants in this study. The applied protocol is inserted in Appendix 1 of this report.

According to this protocol, methods are compared based on the recovery of the target organisms and the number of false positive results. An alternative method will be rejected if it shows significantly lower average counts than the reference method. It will be accepted if it is better or if it is "no different", and the 95 % confidence interval for the average difference lies entirely above the value which would indicate that the alternative method was finding " 10 % fewer organisms than the reference method. Comparison of the fraction of false positive results can be obtained by estimating the true positive rate of the methods. In order to determine this true positive rate, colonies should be confirmed. The true positive rate is the fraction of the tested typical colonies confirmed positive.

Participating countries in the test trial of the "Draft protocol for comparison of the EU reference method for coliforms and *E. coli* with alternative culture methods" (Appendix 1) were to examine 150 samples with an expected total coliform count of 10-50 per 100 ml and 50 samples with an expected total coliform count of 1-10 per 100 ml. For each country, this workload could be divided over a number of participating laboratories. Methods to be used were the membrane filtration method described in ISO 9308-1 (Anonymous, 2000), in which membrane filters were incubated on Lactose TTC agar with Tergitol 7 (LTTC) and the commercially available Colilert®18/QuantiTray (Colilert®) system which has a Most Probable Number (MPN) format. To suppress expected background growth due to lack of selectivity of the LTTC medium, for each sample a membrane filter was incubated on LTTC

at (44 ± 0.5) °C, in addition to the usual incubation at (36 ± 2) °C. Participating laboratories were to confirm at least 100 colonies from LTTC and 200 positive wells from Colilert®.

Three Dutch laboratories participated in the European trial: National Institute of Public Health and the Environment, Microbiological Laboratory for Health Protection (RIVM-MGB), Kiwa Research and Consultancy (Kiwa) and Water Supply Company North Holland (PWN). Besides the methods prescribed in the protocol, they also analysed their samples with three methods that are currently used in the Netherlands for the enumeration of total coliforms and *E. coli*, which differ from the methods included in the European trial. They analysed a total of 179 samples using LTTC, Colilert®, the *E.coli* Direct Plating method, Laurylsulphate Agar (LSA) and Chromocult® Coliform Agar (CCA). This report describes the results obtained by the Dutch laboratories. The methods used were compared on basis of their count results and confirmation rates, but practical aspects such as readability of results and user friendliness were also considered.

The final report of the European Trial, in which 20 laboratories participated and used the methods prescribed in the protocol only, is inserted in Appendix 8 of this report.

2. Materials and methods

2.1 Samples

Samples were taken according to NEN 6559 (Anonymous, 1992), cooled and transported to the laboratories, where they were stored at 2-8 °C , if necessary, and analysed within 24 hours. Samples were taken at different sites, a description of each sample site and the sample type is summarized in Appendix 2. A total of 179 samples was analysed (RIVM-MGB 80, Kiwa 38, PWN 61). Surface water samples were diluted in peptone saline (PS: 0.1 % peptone in 0.09 % saline) to obtain an expected count of 1-50 total coliforms per 100 ml. Diluted samples with this target were considered natural samples and 100 ml of these samples was analysed per method. Counts were not corrected for the dilution.

2.2 Methods per laboratory

RIVM-MGB, Kiwa and PWN enumerated total coliforms by using Colilert®, Lactose TTC with Tergitol 7 (LTTC) and Laurylsulphate Agar (LSA). Kiwa used Colilert® and LTTC (both at (36 ± 2) and at (44 ± 0.5) °C) to enumerate *E. coli*, RIVM-MGB added the *E. coli* Direct Plating (DP) method to these methods, whereas PWN added Chromocult® Coliform Agar (CCA) to enumerate both total coliforms and *E. coli*. The methods were performed according to the brief descriptions in the next paragraph.

2.3 Method description

2.3.1 Colilert®

100 ml water sample is mixed with a fixed amount of Colilert® reagent, the mixture is poured into a Quanti-Tray, which has a 51-well MPN format. The tray is sealed and incubated at (37 ± 1) °C for 18-22 h. Total coliform positive wells display a yellow color, whereas *E. coli* positive wells are yellow and fluoresce under UV light (365 nm). By means of a MPN table (provided with the system) counts of the number of positive wells can be transferred to a Most Probable Number (MPN) of the target organisms.
The Colilert® method is described in detail in the user instruction that is inserted in Appendix 3.

2.3.2 Lactose TTC with Tergitol 7 (LTTC)

The sample is filtered through a 0.45 µm pore size membrane filter, which is incubated on LTTC for (21 ± 3) h at (36 ± 2) °C. Typical lactose positive colonies are yellow. A representative number of typical colonies (at least 10) is subcultured onto Tryptone Soy Agar (TSA), which is incubated at (36 ± 2) °C for (21 ± 3) h. Material from the TSA plate is used for oxidase tests and is also inoculated into Tryptophane Broth (TB), which is incubated for (21 ± 3) h at (44 ± 0.5) °C and examined for the production of indole by adding 0.2-0.3 ml

Kovacs' reagent. Lactose positive colonies being oxidase negative are total coliforms; lactose positive colonies being oxidase negative and indole positive are *E. coli*.
This method and the composition of the used media are described in detail in ISO 9308-1 (Anonymous, 2000).

2.3.3 Laurylsulphate Agar (LSA)

A water sample is filtered through a 0.45 µm pore size membrane filter, which is incubated on LSA for (5 ± 1) h at (25 ± 1) °C, followed by (14 ± 2) h at (37 ± 1) °C. At least 5 typical yellow colonies are selected for confirmation in Brillant Green Bile Lactose Broth (BBLB), which is incubated at (37 ± 1) °C. The tubes are examined for gas production after (22 ± 2) h and (44 ± 4) h. Yellow colonies which produce gas in BBLB are total coliforms.
This method and the composition of the used media are described in detail in NEN 6571 (Anonymous, 1982).

2.3.4 Chromocult® Coliform Agar (CCA)

Filtration of a sample through a 0.45 µm pore size membrane filter is followed by incubation of the membrane filter on CCA. PWN incubated the agar plates for (5 ± 1) h at (25 ± 1) °C and (14 ± 2) h at (37 ± 1)°C, which is different form the incubation prescribed by the manufacturer. Total coliforms produce salmon to red colonies, whereas *E. coli* forms dark-blue to violet colonies. For confirmation of presumptive *E. coli* the dark-blue colonies are overlayed with a drop of Kovacs' reagent. A positive reaction (cherry-red coloring of the colony) confirms the presence of *E. coli*.
A detailed describtion of this method is inserted in Appendix 4.

2.3.5 *E. coli* Direct Plating Method (DP)

After filtration of a sample through a 0.45 µm poresize membrane filter, the membrane filter is incubated on TSA for 4 –5 h at (36 ± 2) °C, transferred to Tryptone Bile Agar (TBA) and incubated for 19-20 h at (44 ± 0.5) °C. Colonies are stained with James reagent (bioMerieux); cherry-red colonies are *E. coli*.
The *E. coli* DP method is described as Rapid Test in ISO 9308-1 (Anonymous, 2000).

2.4 Quality control

The participating Dutch laboratories used positive and negative controls and blanc samples as prescribed by their own individual quality control systems. They all used *E. coli* WR1 and *Enterobacter cloacae* WR3 as reference strains. Quality control data are not shown since there were no deviations.

2.5 Additional confirmation

To assess the true positive rate, which is the fraction of the tested typical colonies confirmed positive, for each method (whenever applicable) additional confirmation tests were performed. LSA37 and CCA were confirmed as described in paragraph 2.3.3 and 2.3.4; *E.coli* DP counts were not further confirmed, since the method already includes testing for indole production. From total coliform positive (yellow) and *E. coli* positive (yellow and fluorescence) Colilert® wells material was subcultured on MacConkey agar no. 3 (Oxoid) to obtain pure cultures which were confirmed according to the flow chart in Figure 1. Characteristic colonies from LTTC incubated at (36 ± 2) °C (LTTC37) and at (44 ± 0.5) °C (LTTC44) were confirmed following the flow chart in Figure 2.

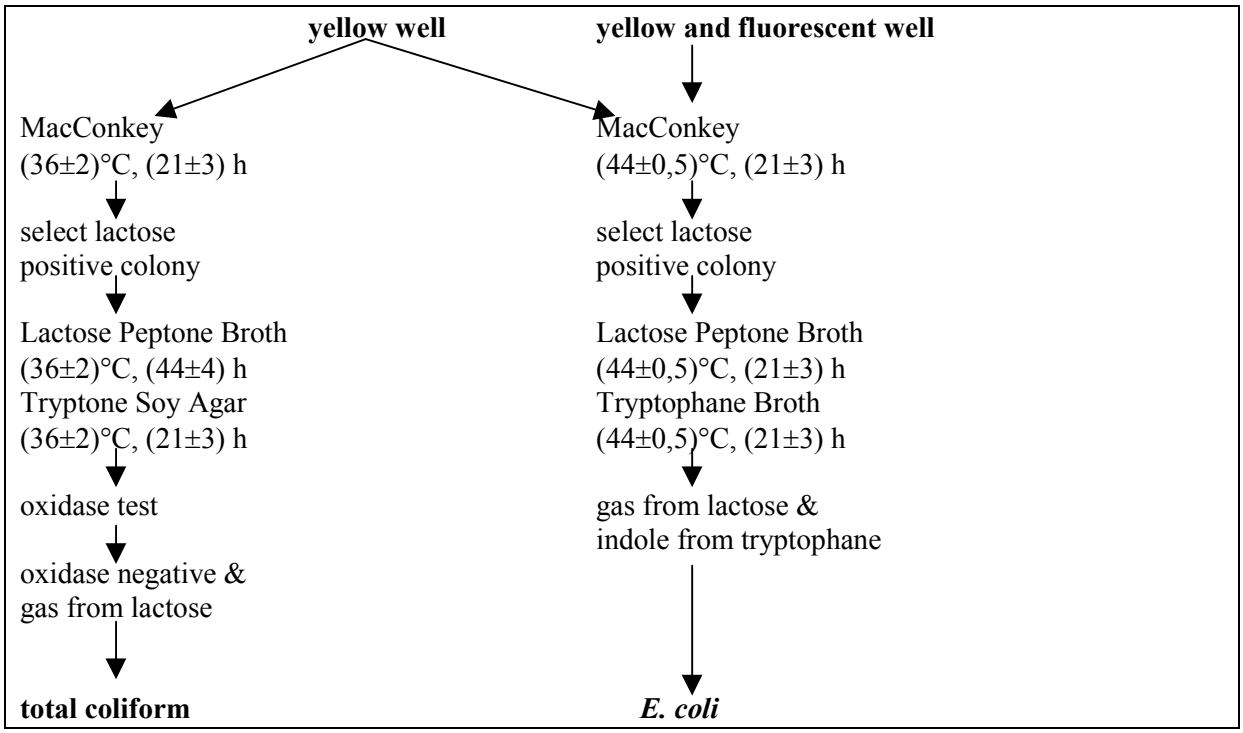


Figure 1 Confirmation procedure of total coliform positive (yellow) and E. coli positive (yellow and fluorescence) Colilert® wells.

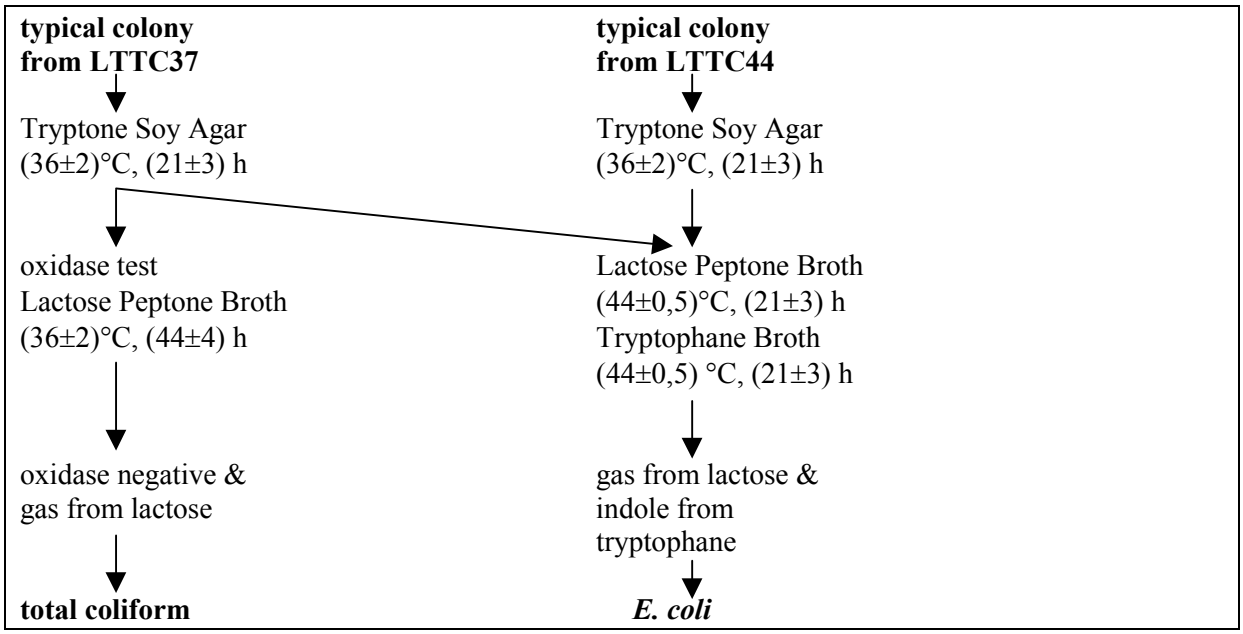


Figure 2 Confirmation procedure of typical colonies from Lactose TTC agar with Tergitol 7 incubated at 37 °C (LTTC37) or at 44 °C (LTTC44).

2.6 Statistical evaluation

To compare the counts obtained by using different methods, counts were compared pairwise in SAS 6.12. Two statistical tests were used: the sign test and the signed rank (Wilcoxon) test. These tests test whether the pairwise difference between two values differs significantly from zero.

As some counts were censored (*i.e.* only a minimum value for the counts was known due to *e.g.* overgrowth of the plates), exact differences could not always be calculated and thus direct application of these tests was not possible. Therefore the following steps were taken to calculate a "difference-score" delta between two counts:

1. If one of the counts was missing, delta was recorded as missing.
2. When both counts were known exactly, delta was recorded as the arithmetic difference.
3. If, of two values, the larger one was censored, delta was recorded as the difference between the value of the smaller count and the minimum of the larger one. Thus delta would be the minimum of what the true difference could be. The sign of delta (positive or negative), however, thereby corresponds with that of the true difference.
4. If, of two values, only the minimum of the smaller one was known, delta was recorded as 0. This was done, to reflect the uncertainty on which of the values was the larger one.

3. Results

3.1 Count results

Confirmed (whenever applicable) count results are summarized in Appendix 5 (Kiwa), 6 (PWN) and 7 (RIVM-MGB). These results were used to compare a set of methods that enumerated total coliforms and a set of methods that enumerated *E.coli*. Comparisons were made for each laboratory individually. The count results of individual samples were summed per laboratory and an average count per 100 ml was calculated for each method. The results of these calculations are displayed in Table 1.
All laboratories had highest average total coliform counts with Colilert®, whereas LTTC37 and LSA37 gave similar average counts. Both Kiwa and PWN found highest average *E. coli* counts on LTTC37. However, at RIVM-MGB average *E. coli* counts on LTTC37, LTTC44 and with Colilert® were similar, but the *E.coli* DP method gave a higher average count.
The number of samples that was found positive for total coliforms or *E. coli* was calculated; the membrane filtration methods done by all laboratories (LSA37 and LTTC37 for total coliforms and LTTC37 and LTTC44 for *E. coli*) were compared with Colilert® (Table 2).

Table 1 Average total coliform counts and E. coli counts in a set of samples, calculated for each laboratory and per method used (nd = not done);LSA37, LTTC37 and LTTC44 counts are confirmed counts, all others are not.

total coliforms

laboratory	average count per 100 ml with			
	LSA37	LTTC37	Colilert®	CCA
Kiwa	32.5	36.6	50.1	nd
PWN	6.1	7.4	30.3	9.1
RIVM-MGB	18.9	14.9	72.2	nd

E. coli

laboratory	average count per 100 ml with				
	LTTC37	LTTC44	Colilert®	CCA	DP
Kiwa	20.7	18.2	14.5	nd	nd
PWN	5.3	3.7	4.1	2.8	nd
RIVM-MGB	13.7	13.8	14.0	nd	21.4

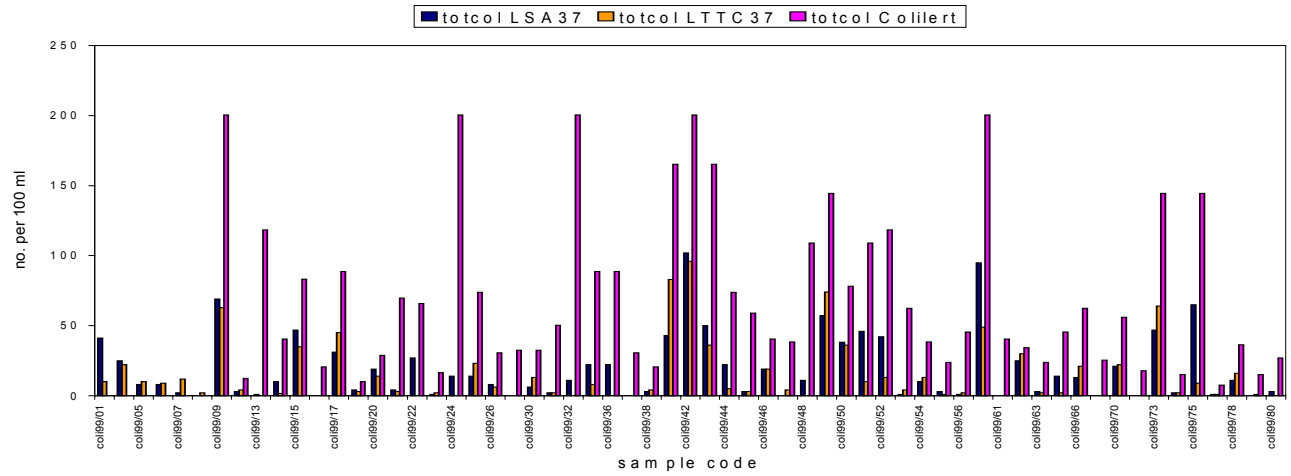
Table 2 The number of samples positive or negative for total coliforms or E. coli with membrane filtration methods (mf; LSA37 and LTTC37 for total coliforms, LTTC37 and LTTC44 for E. coli) or Colilert®.

	total coliforms		***E. coli***	
	mf positive	mf negative	mf positive	mf negative
Colilert® positive	144	21	129	2
Colilert® negative	1	5	13	27

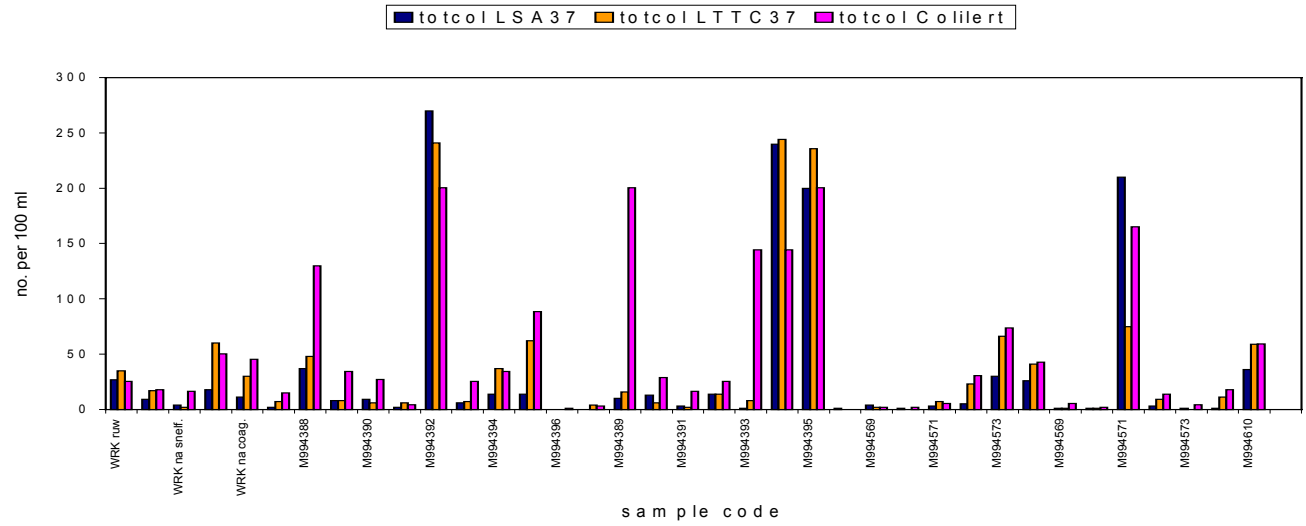
3.2 Comparing count results per laboratory

For each laboratory the count results inserted in Appendices 5-7 were used to compare the methods. In these data sets all results with a smaller than (<) sign were replaced by zero's and all samples that had infinite counts (indicated by >) with one or more methods were discarded. Results are displayed in simple bar charts with the sample code on the X-axis and the number of target organisms on the Y-axis to visualize trends in the performance of the methods. Bar charts of total coliform counts are displayed in Figure 3; they show that the membrane filtration methods (LTTC37, LSA37, CCA) give similar results and that the MPN method Colilert® gives higher counts, especially at RIVM-MGB; both at PWN and at Kiwa incidental peaks are observed. *E. coli* bar charts (Figure 4) show that there are no distinct differences between LTTC37, LTTC44, CCA and Colilert®, but that most DP data points are above the data points obtained with the other methods.
The results are also displayed in scatter charts with log transformed LTTC37 counts on the X-axis as reference method, log transformed counts obtained with the other methods on the Y-axis and a line of equality drawn in the chart. Figure 5 shows that LSA37 and LTTC37 perform almost similar, although LSA37 shows a tendency towards somewhat lower counts. For all three laboratories Colilert® counts are above the line of equality, indicating that they are higher than LTTC37 counts. Counts on CCA obtained by PWN vary and are sometimes higher, sometimes lower than LTTC37 counts. PWN appears to have highest *E. coli* counts with LTTC37, since most data points obtained with other methods appear below the line of equality (Figure 6C). Kiwa obtained comparable results with the methods they used (Figure 6B) and for RIVM-MGB again DP counts were higher (Figure 6A).

A.



B.



C.

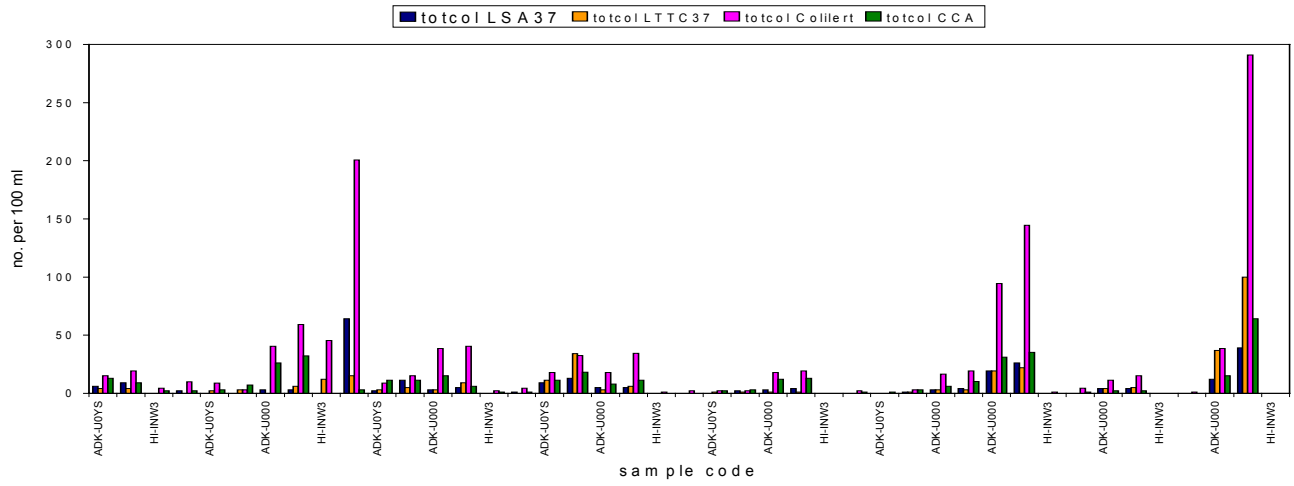


Figure 3 The number of total coliforms (totcol) per 100 ml in water samples analysed with different methods by different laboratories (A=RIVM-MGB, B=Kiwa, C=PWN); LSA37 and LTTC37 counts are confirmed counts, Colilert® and CCA counts are not.

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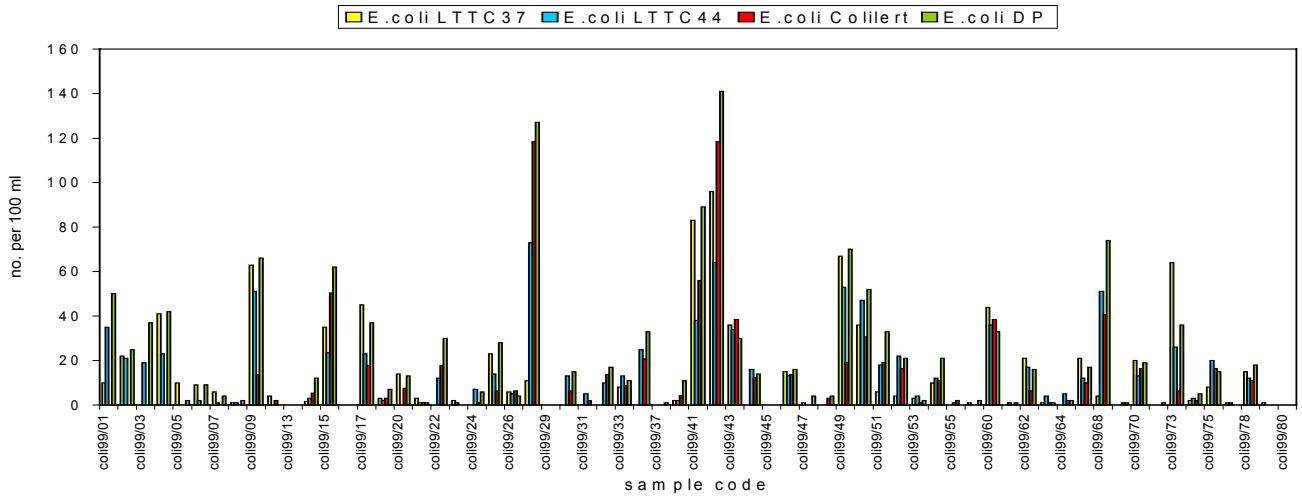

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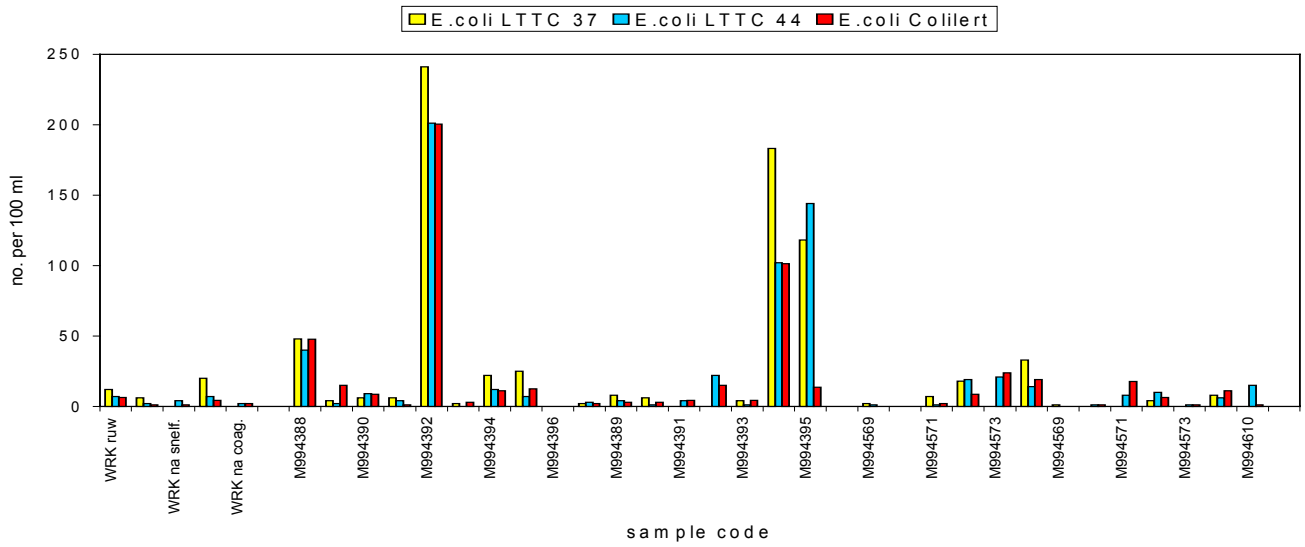

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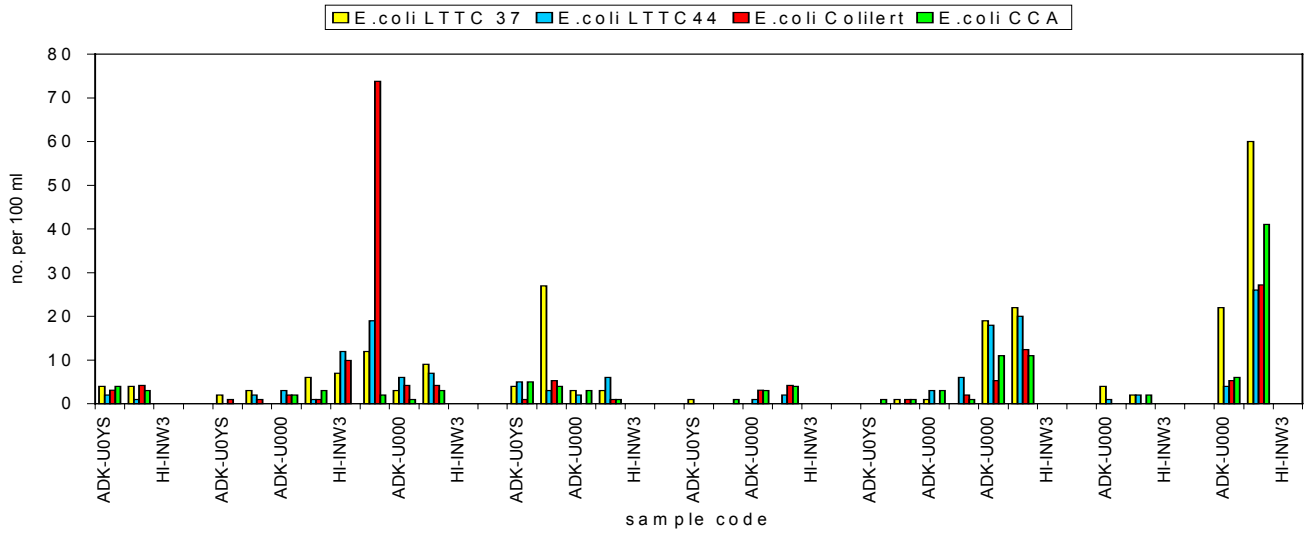

Figure 4 The number of E. coli per 100 ml in water samples analysed with different methods by different laboratories (A=RIVM-MGB, B=Kiwa, C=PWN); LTTC37,LTTC44 and CCA counts are confirmed counts, Colilert® counts are not.

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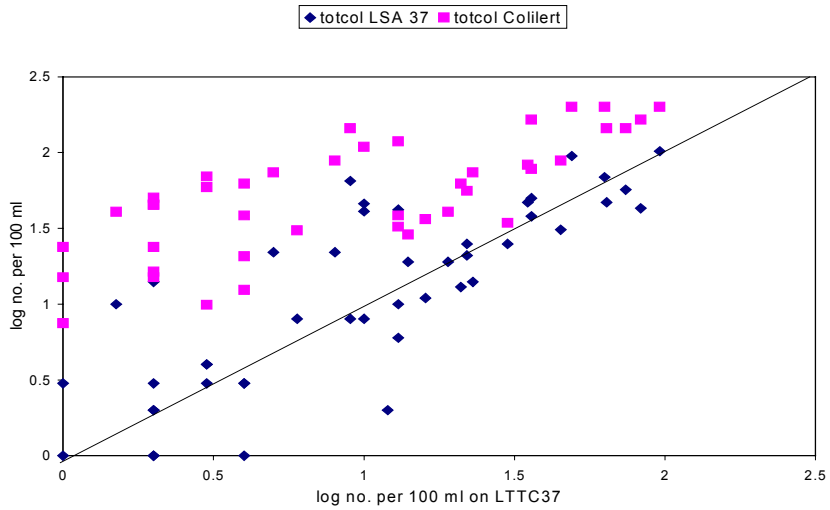

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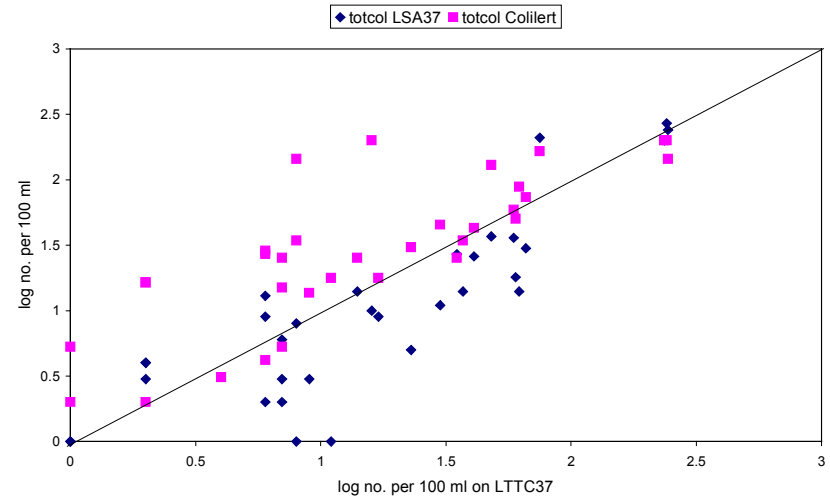

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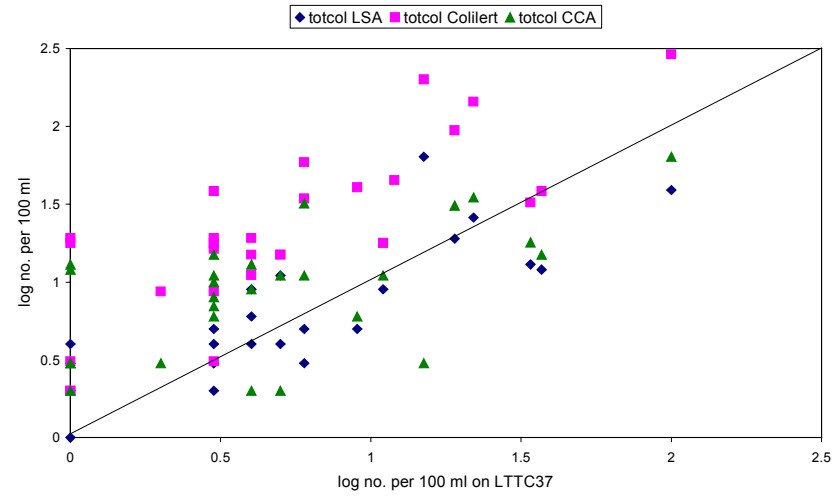
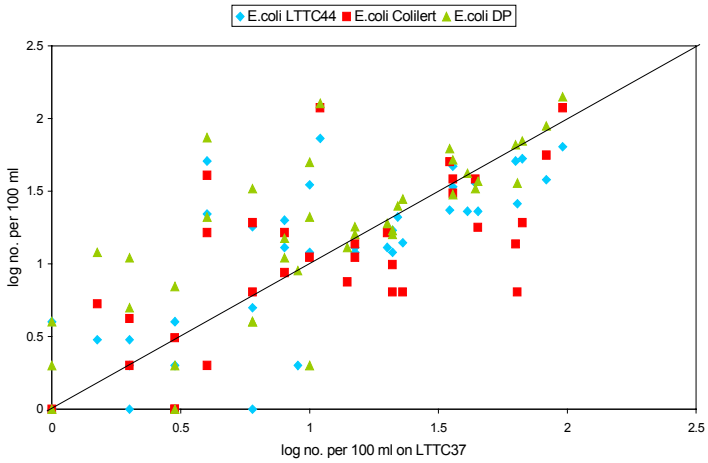


Figure 5 *The number of total coliforms (totcol) in water samples enumerated by different laboratories with alternative methods (LSA37, Colilert®, CCA) compared to the reference method (LTTC37) (A=RIVM-MGB, B=Kiwa, C=PWN); LSA37 and LTTC37counts are confirmed counts, Colilert® and CCA counts are not.*

A



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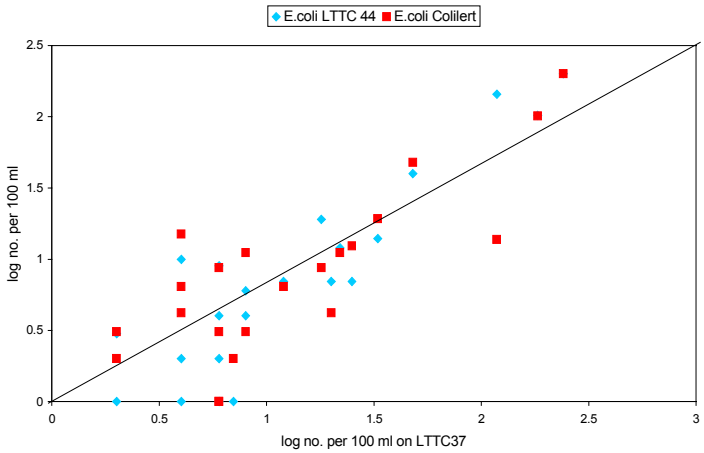

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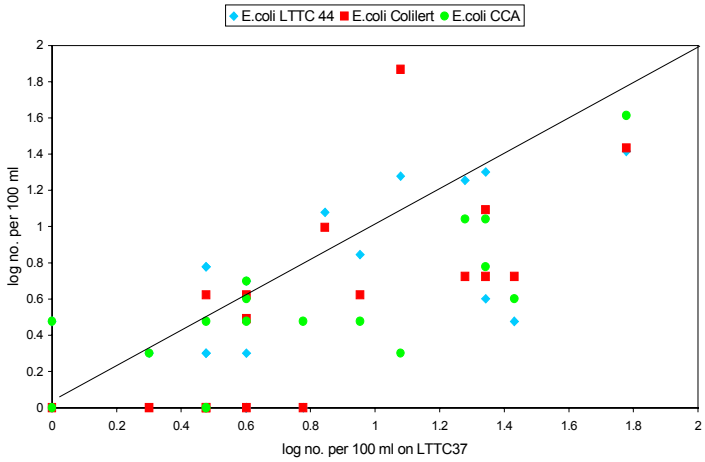


Figure 6 The number of E. coli in water samples enumerated by different laboratories with alternative methods (LTTC44, Colilert®, DP, CCA) compared to the reference method (LTTC37) (A=RIVM-MGB, B=Kiwa, C=PWN); LTTC3 , LTTC44 and CCA counts are confirmed counts, Colilert® and DP counts are not.

3.3 Confirmation

The results of the confirmation tests that were performed on isolates obtained with the different methods by the participating laboratories are summarized in Table 3. The average confirmation percentage of characteristic yellow colonies on LSA37 is about 85 %. A comparable percentage of characteristic colonies from LTTC37 (average 80 %) could be confirmed as total coliforms, whereas, as expected since they are a part of the coliform population, a lower percentage (average 55 %) could be confirmed as *E. coli*. The fraction of the characteristic colonies that was confirmed as *E. coli* increased to an average of 79 % when LTTC was incubated at 44 °C (LTTC44). Confirmation percentages of yellow Colilert® wells (average 55 %) are below the total coliform confirmation percentages obtained with the other methods and from some yellow but non-fluorescent wells (average 12.5 %) *E.coli* was isolated, indicating the occurrence of false negative *E. coli* results. In 96 % of the Colilert® wells that showed yellow coloration and fluorescence, *E. coli* was found. The average total coliform and *E. coli* counts that were generated per laboratory and per method (Table 1), were corrected for the results of the additional confirmation and multiplied with the confirmation rates per laboratory and per method (Table 3). Results are shown in Table 4.

Table 3 Results of confirmation tests: the number and percentage of isolates that was confirmed as total coliforms (totcol) and E. coli calculated per method and per laboratory

laboratory	**method**	**total no. isolates tested**	**no. confirmed totcol**	**(%)**	**no. confirmed *E. coli***	**(%)**
RIVM-MGB	LSA37	522	449	(86.0)	nd	(nd)
Kiwa	LSA37	123	96	(78.0)	nd	(nd)
PWN	LSA37	190	172	(90.5)	nd	(nd)
RIVM-MGB	LTTC37	265	218	(82.3)	178	(67.2)
Kiwa	LTTC37	116	105	(90.5)	55	(47.4)
PWN	LTTC37	134	90	(67.2)	68	(50.7)
RIVM-MGB	LTTC44	313	nd	(nd)	269	(85.9)
Kiwa	LTTC44	115	nd	(nd)	86	(74.8)
PWN	LTTC44	129	nd	(nd)	100	(77.5)
RIVM-MGB	Colilert®-y	70	30	(42.9)	9	(12.9)
Kiwa	Colilert®-y	35	24	(68.6)	6	(17.1)
PWN	Colilert®-y	93	51	(54.8)	7	(7.5)
RIVM-MGB	Colilert®-yf	58	nd	(nd)	56	(96.6)
Kiwa	Colilert®-yf	31	nd	(nd)	29	(93.5)
PWN	Colilert®-yf	50	nd	(nd)	49	(98.0)

nd = not done; Colilert®-y = yellow Colilert® well; Colilert®-yf = yellow and fluorescing Colilert® well

Table 4 Average counts, corrected for additional confirmation of total coliforms and E. coli in a set of samples, calculated for each laboratory and per method used.

total coliforms

laboratory	average confirmed count per 100 ml with		
	LSA37	LTTC37	Colilert®
Kiwa	32.5	36.6	34.4
PWN	6.1	7.4	16.6
RIVM-MGB	18.9	14.9	31.0

E. coli

laboratory	average confirmed count per 100 ml with		
	LTTC37	LTTC44	Colilert®
Kiwa	20.7	18.2	13.6
PWN	5.3	3.7	4.0
RIVM-MGB	13.7	13.8	13.5

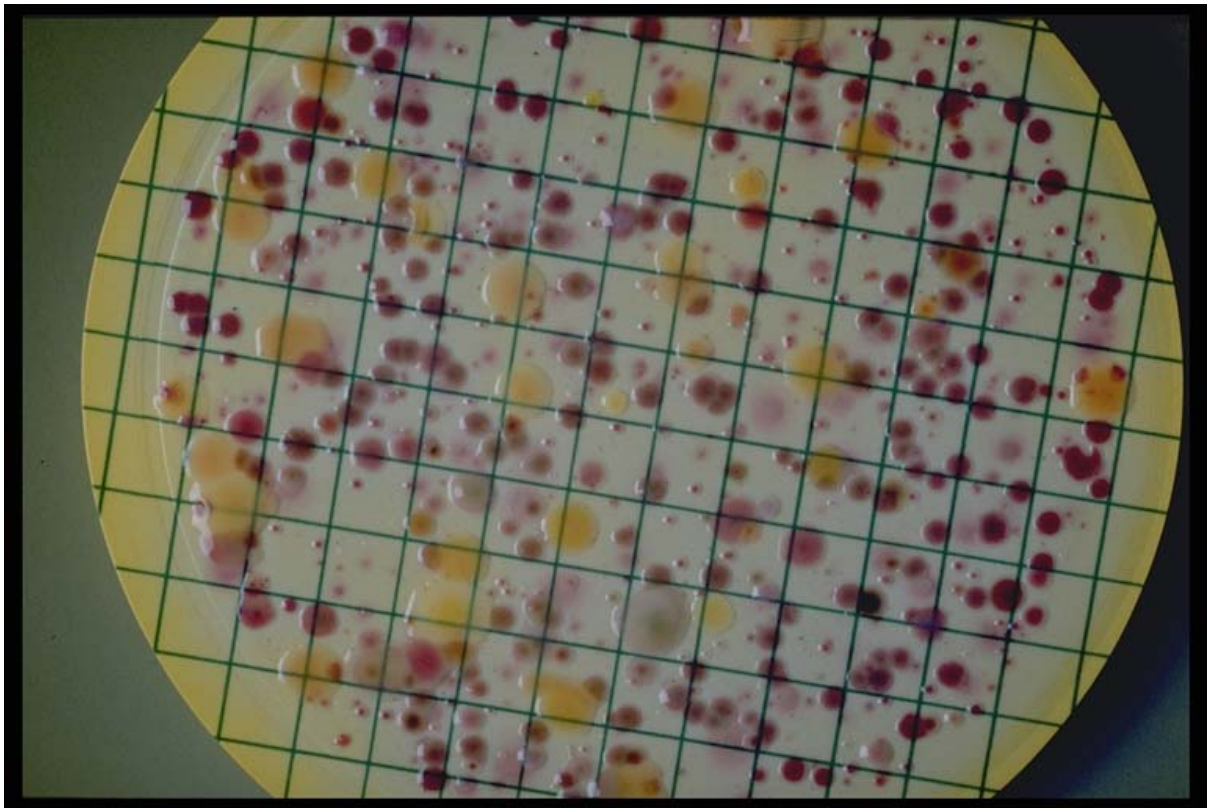
3.4 Practical aspects of methods

The methods that were used in this trial differed in their practical application. The participating laboratories use the LSA37 method routinely and found it easy to perform. Some disturbing background growth of small pink colonies was however reported, especially when surface water samples with higher contamination levels were analysed. The *E. coli* DP method and the CCA method were easy to perform and gave direct and clear results.
All participants reported heavy background growth on membrane filters that were incubated on LTTC at 37 °C. Background growth was reported as the percentage of the membrane filter surface that was covered with background flora. Four categories were defined: less than 10 %, 10-50 %, 50-100 % and 100 % coverage. The results are summarized in Table 5.
In 85 % (132/155) of the samples tested, 50-100 % of the membrane filter surface was covered with orange to brown colonies, amongst which the counting of the characteristic yellow colonies was extremely difficult. Incubation of membrane filters on LTCC at (44 ± 0.5) °C slightly improved this lack of selectivity, but in 52 % (80/155) of the samples the membrane filter surface was still covered with disturbing background flora. The photographs in Figure 7 illustrate the above. Characteristic colonies being oxidase positive were regularly observed on LTTC incubated at (36 ± 2) °C. Kiwa confirmed these oxidase positive colonies as *Aeromonas*. Colilert®, as the only MPN method included in the test, was found very convenient: analysing of samples and reading of results was easy.

Table 5 Background growth on membrane filters incubated on LTTC, incubated at (36 ± 2) °C (37 °) and at (44 ± 0.5) °C (44 °), indicated as the percentage coverage of the membrane filter surface

laboratory	no. tested	no. of samples with % coverage of membrane filter surface							
		<10		10-50		50-100		100	
		37 °	44 °	37 °	44 °	37 °	44 °	37 °	44 °
RIVM-MGB	72	0	16	18	24	39	26	15	6
Kiwa	35	2	23	3	12	24	0	6	0
PWN	48	0	0	0	0	2	32	46	16

A



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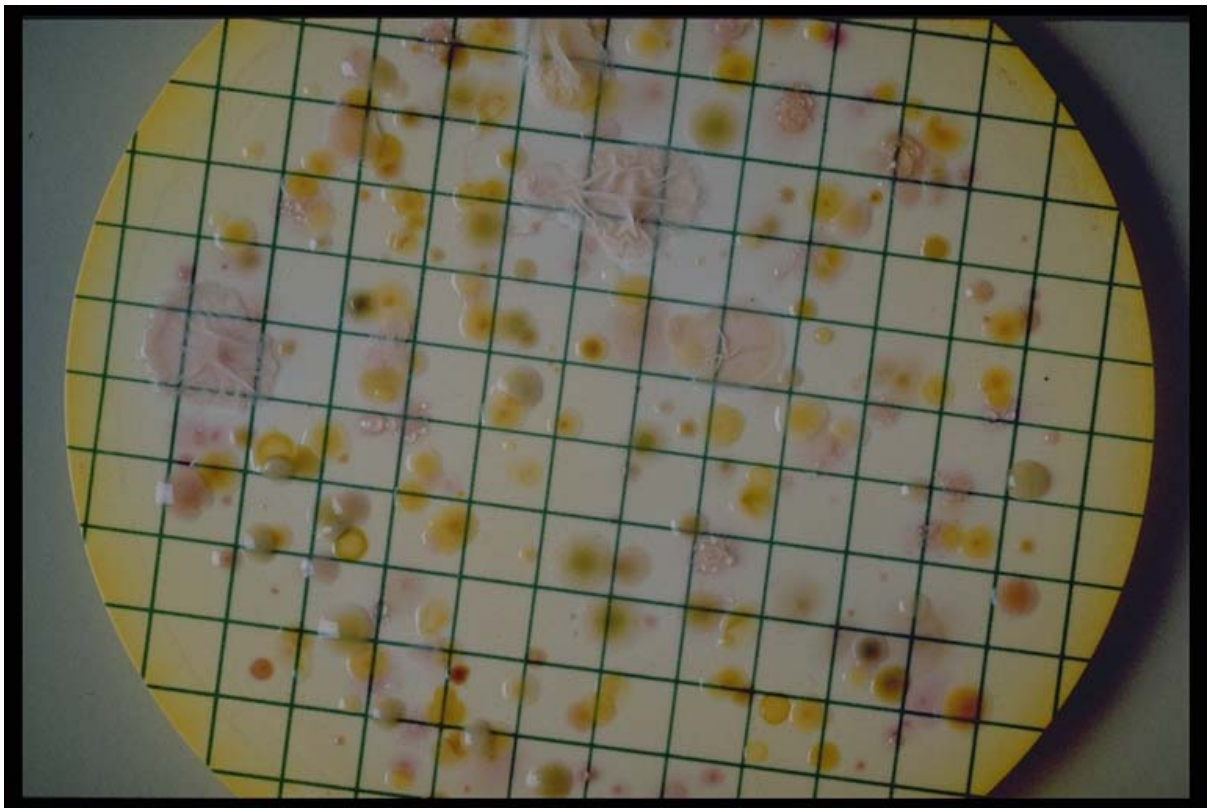

Figure 7 Membrane filter incubated on LTTC at (36 ± 2) °C (A) and membrane filter of the same sample incubated on LTTC at (44 ± 0.5) °C (B); yellow colonies are characteristic.

4. Discussion

All participating laboratories enumerated much higher numbers of total coliforms with Colilert® than with LSA37 and LTTC37; differences were significant ($P<0.05$). Differences in counts obtained with these membrane filtration methods and Colilert® can be explained from the different substrates that are used, resulting in the detection of two different groups of coliform bacteria. Coliforms growing on lactose-containing agar media, need two enzymes to ferment lactose: β-galactoside-permease, for active transport of lactose into the bacterial cells and β-galactosidase, to use lactose, resulting in the production of acid and gas. The genes that code for these enzymes are *lacY* and *lacZ,* respectively. Coliforms that lack *lacY* do not form characteristic yellow colonies on lactose-containing agar media, unless lactose is present in extremely high concentrations. When *lacZ* is however present, the Colilert® substrate ortho-nitrofenyl-β-α-galactopyranoside (ONPG) can be used. Coliforms that have both genes can use lactose and ONPG.

From our results it is clear that a larger population of bacteria contains only the *lacZ* gene resulting in higher counts with Colilert®. Fricker et al. (1997) also reported the occurrence of non-lactosefermenting coliforms: approximately 10 % of the coliforms they isolated from potable source water did not ferment lactose due to lack of β-galactosidepermease. The Chromocult® method is based on the same detection principle as Colilert®, but CCA counts were however significantly ($P<0.05$) lower than Colilert® counts. This might be due to a different resuscitation capacity of liquid media (Colilert®) and solid agar media (CCA). CCA total coliform counts were higher ($P<0.05$) than LSA37 counts, but were equal to LTTC37 counts ($P>0.05$).

Total coliform counts on LSA37 were significantly ($P<0.05$) lower than total coliform counts on LTTC37. Confirmation percentages of these membrane filtration methods were comparable: LSA37 85 % and LTTC37 80 %. The presence of disturbing background growth is experienced as a serious drawback of the LTTC37 method. Presence of some disturbing background growth (small pink colonies) on LSA37 was reported, particularly when highly contaminated surface water samples were analysed, but this was not considered a major problem and no attempt was made to quantify the proportion of background growth. The abundance of organisms other than the target organisms on LTTC37 reduces readability of the membrane filters and makes it almost impossible to use the criterium that is stated in ISO 9308-1: ”… count as lactose-positive bacteria all typical colonies irrespective of size which show a yellow color development in the medium under the membrane.”. With so many other organisms present, the medium under the membrane filters is almost completely yellow. In this way counts are less accurate and can only be properly made when only a few target organisms are present and the amount of background growth is equally reduced. We noticed that heavy background growth appeared in surface water with higher total coliform contamination levels (most RIVM-MGB samples, some PWN samples), but that it could also be abundant in samples with rather low total coliform contamination levels (most Kiwa samples, some PWN samples). Incubation of LTTC at 44 °C slightly reduced the amount of background growth, but a different type of background bacteria, the so called ‘spreaders’

(usually members of the genus *Bacillus*) appeared. There were low numbers of these bacteria present, but they spread along the membrane filters and hindered the counting of the target organisms. Incubation of membrane filters on LTTC at 44 °C also suppresses the growth of some of the members of the total coliform group and results in the detection of fecal coliforms in stead of total coliforms. Incubation at 44 °C did not alter the *E. coli* count results in the analysed samples.
Confirmation of isolates from yellow (total coliform positive) Colilert® wells showed that only 55 % (range 43 – 69 %) of the isolates were total coliforms, when total coliforms were defined as lactosefermenting organisms. The confirmation procedure that was followed, included direct streaking of material from yellow Colilert® wells on MacConkey agar in which lactose is present. In quite a few cases this direct streaking on MacConkey agar resulted in no or very poor growth due to the lack of the *lacY* gene in a part of the isolates from Colilert®, resulting in a low confirmation rate. As Colilert® and the tested membrane filtration methods enumerated different groups of coliform bacteria, counts obtained with these methods can only be fairly compared when results are confirmed and the criteria used to define the group of bacteria called 'total coliforms'are identical. In an attempt to get an indication, the average total coliform and *E. coli* counts were corrected for the obtained confirmation rates. These calculations suggest that a larger number of lactose fermenting total coliforms is detected with Colilert® than with LSA37 and LTTC37.
LTTC37, LTTC44 and DP enumerated significantly ($P<0.05$) more *E. coli* than Colilert® and CCA. Colilert® and CCA performed similar ($P>0.05$). A high percentage false negative *E. coli* results was found with Colilert®: from 12,5 % (range 7,5-17,1 %) of wells that were positive for total coliforms but negative for *E. coli*, *i.e.* showing yellow coloration but no fluorescence, *E. coli* was isolated. The lower counts with Colilert® and CCA, which include 4-methylumbelliferyl-β-D-glucuronide (MUG) as a substrate for the enzyme β-glucuronidase for *E. coli* detection, and the high percentage false negative results with Colilert® in this study show that testing water samples for β-glucuronidase activity will underestimate the number of *E. coli* present in a sample. Present results confirm results from previous research (Schets and Havelaar, 1991) in which 14 % (range 4-26 %) of isolated *E. coli* strains was found β-glucuronidase negative at 44 °C. A fraction of these isolates was β-glucuronidase positive at 37 °C. An indole test will give a more reliable count of the number *E. coli* in samples, since only 2-4 % of *E. coli* strains are indole negative (Schets and Havelaar, 1991; Schets et al., 1993). The *E. coli* DP method is based on testing for indole production and includes a resuscitation step on a non-selective medium (Havelaar and During, 1988). In this study the method was used by RIVM-MGB only and gave higher counts than all other methods used by RIVM-MGB, differences were significant ($P<0.05$) for LTTC44 and Colilert®, but not for LTTC37.

5. Conclusions

- Membrane filtration methods based on transport and use of lactose used in this study (LTTC37 and LSA37) have similar true positive rates for total coliforms, but total coliform counts on LSA37 are significantly lower than on LTTC37.
- Due to lack of selectivity, LTTC37 is however suitable for monitoring of very clean (drinking) water samples only.
- Colilert® and CCA enumerate a different group of coliform bacteria than the other methods used; counts can only be fairly compared when results are confirmed and the criteria used to define the group of bacteria called 'total coliforms' are identical.
- For enumeration of ONPG positive total coliforms Colilert® might be a suitable alternative for membrane filtration methods.
- For enumeration of *E. coli*, the DP method (or Rapid Test in ISO/FDIS 9308-1) appeared to be most suitable, although the method was only used by one laboratory in this study;

6. Recommendations

For enumeration of total coliforms, drinking water companies can use the EU reference method on LTTC agar, but due to its lack of selectivity this method can only be used for monitoring of very clean drinking water samples. This forces the water companies to have a second method for enumeration of total coliforms operable, for analysis of *e.g.* surface water samples, drinking water half products or repair samples. The LSA method, extensively used in the Netherlands, is more selective than the LTTC method and can therefore be used for analysis of water samples with various contamination levels, ranging from surface water to drinking water. Some disturbing background growth may however occur when certain surface water samples are analysed. The elevated selectivity of LSA results in lower total coliform counts.

Colilert® can be used as alternative for the above mentioned membrane filtration methods: it is a convenient method, giving final results within 18 h and can be used for analysis of all water types. It should be borne in mind that this method enumerates a broader group of coliform bacteria than the membrane filtration methods, resulting in higher counts.

When a drinking water sample is found positive for total coliforms with either LTTC, LSA or Colilert®, subculturing for *E. coli* should be performed; for Colilert® this should be done regardless of the presence of fluorescence in wells. Colilert® is considered less suitable for direct detection of *E. coli* because of the occurrence of a high percentage false negative results due to the inability of a fraction of the *E. coli* population to use the substrate incorporated in Colilert® for *E. coli* detection. Resulting form the present study, the DP method is the most suitable method for detection and enumeration of *E. coli*: it is easy to perform and detected more *E. coli* than the other methods.

Acknowledgements

The authors thank Nico Nagelkerke (RIVM-IMA) for statistical analysis of data.

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Appendices

Appendix 1 A draft protocol for comparison of the EU reference method for coliforms and *E.coli* with alternative culture methods

Introduction

The European Drinking Water Directive (1998) defines a reference method for the estimation of the concentration of coliforms and *E.coli* in drinking water. Whilst it is not mandatory that member states use this method, there is an obligation to demonstrate that any alternative method which is to be used has a similar performance. The exact nature of the similarity is not defined, nor is the methodology to be used for comparison of methods. It is generally accepted however, that alternative methods should have a performance which is equivalent or better. In this context, "equivalent or better" must be defined. A method is normally considered to be "equivalent" if the recovery of target organisms is not significantly lower than the reference method. However, "significantly lower" also requires definition and for the purposes of comparisons such as this, a definition of overall not significantly lower than 10% of the reference method is suitable.

In addition to testing the recovery of organisms using the two test methods, the number of false positive results should be determined. This can done by estimating the true positive rate of the methods. Since the EU reference method does not require that colonies be "confirmed" all colonies which fulfil the criteria described in the method should be counted. In order to determine the true positive rate, all colonies should be confirmed using an appropriate methodology. However, this is not feasible for a study of this size and therefore a compromise must be used. In this case, it is reasonable for each laboratory to "confirm" the identity of one hundred colonies selected at random from a range of water types. Any positive results which do not confirm by traditional methodologies should be sent to single reference laboratory (in this case Thames Water) for accurate identification. In addition to the 100 colonies identified from the EU reference method, 100 positive wells from Colilert should be examined. This can be done using the protocol given below.

Tests to be performed by individual laboratories

Each laboratory or group of laboratories, will perform a minimum of 200 tests in total. These will be split into two phases. In phase one, a minimum of five and a maximum of ten sites will be selected which will give 10-50 colonies of coliforms per 100 ml. A total of 150 samples from this type of source should be examined, with the samples being spread evenly over the different sites. The type of water sample should be chosen according to the type of sample normally examined by individual laboratories. For example, laboratories whose normal samples are from surface water treatment plants which receive disinfection, then sewage effluent disinfected with chlorine should be used to generate the samples, according to the protocol given below. Laboratories who examine samples of water which receives no disinfection should use contaminated groundwater or good quality surface water for this phase of the study.

Results should be reported on the forms given in this document. The site, type of sample, date of analysis, and results should be presented as shown in the example.

For the second phase of the study, samples should be selected to give results in the range 1-10 coliforms per 100 ml. All results should be reported, even where the result is zero. A minimum of 30 samples where at least one organism is recovered by one or other method or both should be reported.
In reporting results, no attempt should be made to correct the results in view of the findings of confirmation tests, unless the method being compared specifically states that results should be confirmed. In this trial of Colilert and the EU reference method, neither method requires confirmation. When the results are analysed, account will be taken of the true results obtained by examination of the 100 organisms identified as described above.

Samples
Samples should be taken into bottles of no less than 500 ml. Aliquots (100 ml) should be used for each of the three tests, i.e. Colilert, membrane filtration for coliforms and membrane filtration *E.coli*. Each aliquot **must** be taken from the same bottle. On no account must subsamples be taken from different bottles. Samples must be adequately mixed by repeated inversion of the sample bottle.

Disinfection of sewage effluent
Disinfected sewage effluent has been used in several studies to good effect to determine the efficiency of different methods for recovering coliforms and *E.coli*. The protocol uses "good quality" sewage effluents i.e. with suspended solids of less than 30 mg per litre.
The method for producing samples from sewage effluent is as follows:

Day 1

1. Fill a 10 litre container with tap water warm to 37^{o}C for two hours and then place at 4^{o}C overnight.

Day 2

2. Select a good quality sewage effluent (one litre) and allow to stand for 2 hours to allow large particulates to sediment.
3. Carefully pour off 500 ml of effluent into a clean container.
4. Add the 500 ml of sewage effluent to the tap water, mix by shaking and place on a magnetic stirrer.
5. Prepare a solution of chlorine using chlorine generating tablets (Instachlor, Palintest) containing in total 10-15 mg of chlorine.
6. Add the chlorine solution to the diluted sewage effluent.
7. Allow to mix for 5 minutes.
8. Take one litre samples of the chlorinated effluent into sterile bottles containing 5 ml of 18% sodium thiosulphate solution at one minute intervals (i.e. 5-14 minutes contact time) and mix thoroughly.
9. Examine 100 ml of the chlorinated sewage effluent from each time point using Colilert 18 and the Colilert 2000 QuantiTrays.
10. Store all chlorinated samples at 4^{o}C overnight.

Day 3

11. Read the QuantiTrays and record the results

12. Select samples which give 15-70 coliforms per 100 ml. These can be used directly to compare the methods and duplicate samples can be analysed.
13. Select samples which yield 150-700 coliforms per 100 ml.
14. These can be diluted 1:10 in de-chlorinated water to yield one litre samples. Tap water can be de-chlorinated by adding 5 ml of 18% sodium thiosulphate to 900 ml of water. Five replicate one litre samples can be prepared from each sample which yielded 150-700 coliforms. These samples can each be used to compare the methods and samples from each bottle can be analysed in duplicate.
15. Analyse samples using EU reference method to be incubated for recovery of total and faecal coliforms (i.e. incubate one membrane at 37^{o}C and 44^{o}C) and the Colilert 18 method.
16. Incubate samples at appropriate temperatures for the relevant time.
17. Examine samples after incubation and record the count from each sample on the Results Sheet.
18. Organisms can be selected at this stage for determination of the "True Positive Rate" for each method according to the protocol given below.

Protocol for determining the "True Positive Rate"

To determine the "True Positive Rate" 100 organisms from each test will be identified. For methods which produce colonies these must be selected at random from a variety of sites. **Selecting colonies at random is essential and no attempt to should be made to select colonies on the basis of whether they are "typical" or "atypical".** For Colilert, select one hundred wells which are yellow with no fluorescence and one hundred which are yellow and fluorescent.

1. Colonies from membranes

i. Select a well isolated colony and streak out onto nutrient agar, incubate overnight at 37^{o}C
ii. After incubation, inoculate a single colony into lactose peptone water and incubate at 37^{o}C for 48 hours and record the result. For membranes intended to detect faecal coliforms, inoculate a single colony into lactose peptone water and tryptone water and incubate at 44^{o}C for 24 hours. Test the tryptone water for indole production using Kovacs reagent and record the result. Record the result from the lactose peptone water.
iii. Test the growth from the nutrient agar plate for cytochrome oxidase production and record the result

2. For Colilert samples

i. Select wells showing yellow colour or yellow colour plus fluorescence as appropriate.
ii. Puncture the back of the well with a sterile hypodermic needle or sterile pipette tip and withdraw a small volume (approximately 10 µl) of liquid.
iii. Inoculate the liquid on to a MacConkey agar plate and streak out for single colonies. Incubate plates from fluorescing wells at 44^{o}C and those from wells which are yellow only at 37^{o}C overnight.

iv. Select a well isolated lactose fermenting colony. Isolates from fluorescing wells should be inoculated into lactose peptone water and tryptone water for incubation at 44°C as described above. Isolates from yellow only wells should be inoculated into lactose peptone water and incubated at 37°C for 48 hours and onto a nutrient agar plate for examination for cytochrome oxidase.
v. Results should be recorded on the appropriate sheet.

Quality Assurance
It is essential that all laboratories taking part in this study must have appropriate quality control systems in place. Some specific quality control points are listed below in relation to the use of the EU reference medium. It is also beneficial if laboratories can provide results from an external quality assurance scheme.

Incubators
All incubators to be used in this study must be monitored daily to ensure compliance with the target temperature. The temperature required is ±1 degree C of the target temperature or as specified within the specific method (i.e. the Colilert package insert and the EU reference method).

Media
Culture media should be made and stored in accordance with the manufacturers instructions. With regard to the EU reference media, all media should be used within seven working days of preparation.
A quality control procedure, based on that used by Institut Pasteur, Lille is provided. This must be used with every batch of media produced.

Protocol for quality control of media

I / Field of application
This protocol describes the quality controls to be performed on each production batch of media used in microbiology laboratories.

The sensitivity, pH, selectivity and/or performance are studied (performance of a positive strain comparing to a reference medium and non selective medium, selection characteristics of a positive strain for confirmation media).

II / Definitions
2.1/ Batch : a batch is a group of containers, tubes or flasks, containing medium, coming from the same production lot, in other words a « series » in the case of media prepared with an automatic preparator.
2.2/ Sterility : absence of any culture at the temperature and timing normally used for the medium studied.

III/ Control

3.1/Presumptive media

3.1.1/ general principle

Take :

- 1% of the production (at different stages of production, with a minimum of 3) to control the pH,
- 1% of the production (at different stages of production, with a minimum of 3) to control the sterility,
- 1% of the production (at different stages of production, with a minimum of 3) to control the performance of a positive control
- 2 samples for the negative control (at the beginning and at the end of the production), so in total 3% of the production and 2 samples.

To control the pH, a pH-meter is used. The mean of measurements must be calculated. The pH should fall within the limits set by the manufacturer. If this is not the case, then the media must be rejected.

The negative control (a strain of *Aeromonas*) must not grow on both plates or must not be/look like the target strains identified on the media.

To test the performance of the medium, a reference strain should be examined on the "reference medium" and on nutrient agar. The recovery on the reference medium should be between 55 and 120% of that found on the nutrient agar. For this purpose, lenticules will be supplied. These should be rehydrated in 500 ml of sterile distilled water. Four membrane filters are then used to concentrate 100 ml of the material and two incubated on nutrient agar (one at 37^{o}C and the other at 44^{o}C) and two on the reference medium (one at 37^{o}C and the other at 44^{o}C). Colonies are then counted after incubation and the results recorded.

If results do not demonstrate that the medie are equivalent in performance then a second set of controls should be run. If these do not give acceptable results then the batch of medium should be discarded.

3.2/ Confirmation media

3.2.1/ general principle

Take :

- 1% of the production (at different stages of production, with a minimum of 3) to control the pH,
- 1% of the production (at different stages of production, with a minimum of 3) to control the sterility,

- 1% of the production (at different stages of production, with a minimum of 3) to control the performance of a positive control
  so in total 3% of the production

This applies to tryptone water and lactose peptone water.

To control the pH, a pH-meter is used. The results must fall within the range specified by the media manufacturer.

The sterility is controlled with plates or tubes, incubated at the specified temperature and the timing normally used for the medium. No growth in all samples is the only acceptable result.

Each medium is incubated with a strain of *E.coli* and a strain of *Aeromonas spp.* To ensure correct performance. The *E.coli* strain should ferment lactose at both 37 and 44°C and produce indole from tryptophane at 44°C. The strain of *Aeromonas* should give negative results in all tests.

Results are reported in the file of results. If results do not meet fixed criteria of quality, a new control is done. If the second control gives the same results, the batch is discarded a new batch is made.

3.3/ Nutrient agar

At each production, we take 1% of the production (at different stages with a minimum of 3 flasks) to control the sterility, the pH and the performance.

For each flask, prepare 2 boxes (1 for the control of the sterility and 1 for the pH). Keep the rest at 50°C to control the performance.

To control the sterility, put the plates at 20°C. No growth after an incubation of 72 hours.
To control the pH, a pH-meter is used. The mean must be within the limits specified by the manufacturer.

If results do not follow the fixed criteria of quality, a new control is done. If the second control gives the same results, the batch is refused and a new production is made.

Quality Control samples
On each day that analyses are being performed, a lenticule should be added to 500 ml of sterile deionised water. After appropriate mixing, samples should be analysed by membrane filtration and incubated on tergitol TTC medium at 37 and 44°C and on nutrient agar at 37 and 44°C. Counts should be recorded and sent in with the other analytical results.

Appendix 2 Short description of sample sites

sample code	sample site	sample type and dilution
coli99/01, /09, /17, /25, /33, /41, /49, /57, /65, /73	De Oude Pol – Nunspeet	surface water, 10 x in PS
coli99/02, /10, /18, /26, /34, /42, /50, /58, /66, /74	Strand Horst – Harderwijk	surface water, 10 x in PS
coli99/03, /11, /19, /27, /35, /43, /51, /59, /67, /75	Salmsteke – Lopik	surface water, 10 x in PS
coli99/04, /12, /20, /28, /36, /44, /52, /60, /68, /76	River Lek – Vianen	surface water, 10 x in PS
coli99/05, /13, /21, /29, /37, /45, /53, /61, /69, /77	Maarseveenseplassen – Molenpolder	surface water, 10 x in PS
coli99/06, /14, /22, /30, /38, /46, /54, /62, /70, /78	De Strook – Tienhoven	surface water, 10 x in PS
coli99/07, /15, /23, /31, /39, /47, /55, /63, /71, /79	De Wijde Blik – Kortenhoef	surface water, 10 x in PS
coli99/08, /16, /24, /32, /40, /48, /56, /64, /72, /80	Vinkeveenseplassen – Vinkeveen	surface water, 10 x in PS
WRK ruw	WRK Nieuwegein	surface water
WRK coag, M994391, M994572	WRK Nieuwegein	drinking water proces, after coagulation
WRK snelf	WRK Nieuwegein	drinking water proces, after rapid filtration
M994388, M994390, M994392, M994571, M994573, M994574	not specified	surface water, after reservoir storage
M994389, M994393, M994369, M994570,	not specified	drinking water proces, after filtration
M994394, M994395	not specified	artificial contaminated drinking water
M994610, M994611		pilotplant after filtration
AUOY1	Lake IJsselmeer	surface water, 10 x in DW
AUOY2	Lake IJsselmeer	surface water, 10 x in DW
AUOY3	Lake IJsselmeer	surface water
AUOY4	Lake IJsselmeer	surface water, after 1 week storage at 4°C
AUOO1	Lake IJsselmeer	raw water, 10 x in DW
AUOO2	Lake IJsselmeer	surface water, 10 x in DW
AUOO3	Lake IJsselmeer	raw water
AUOO4	Lake IJsselmeer	raw water, after 1 week storage at 4°C
HI-INW1	WRK Andijk	surface water
HI-INW2	WRK Andijk	surface water, after 1 week storage at 4°C

PS = peptone saline, DW = drinking water

Appendix 3 Description of Colilert®-18

Colilert®-18 Test Kit
Introduction and Product Use
Colilert-18 is used for the simultaneous detection and confirmation of total coliforms and *E. coli* in fresh and marine waters. It is based on IDEXX's patented Defined Substrate Technology's (DST=). When total coliforms metabolize Colilert-18's nutrient-indicator, ONPG, the sample turns yellow. When *E. coli* metabolize Colilert-18's nutrient-indicator, MUG, the sample fluoresces. Colilert-18 can simultaneously detect these bacteria at 1 cfu/100 ml within 18 hours even with as many as 2 million heterotrophic bacteria per 100 ml present.
Materials
Catalog # WP020-18 and WP200-18 contain 20 and 200 Snap Packs respectively, each containing sufficient Colilert-18 reagent for a 100 ml water sample.
Storage
Store at 4-25°C away from light.
Presence/Absence (P/A) Procedure
1. Add contents of one pack to a 100 ml sample in a sterile, transparent, non-fluorescing vessel
2. Cap vessel and shake
3. If sample is not already at 33-38°C, then place vessel in a 35°C waterbath for 20 minutes or, alternatively, a 44.5°C waterbath for a minimum of 7 and a maximum of 10 minutes.
4. Incubate at 35±0.5°C for the remainder of 18 hours.
5. Read results according to Result Interpretation table below.

Quanti-Tray" Enumeration
Procedure
1. Add contents of one pack to a 100 ml, room temperature water sample in a sterile vessel, Vessel does not need to be transparent or non-fluorescing.
2. Cap vessel and shake until dissolved
3. Pour sample/reagent mixture into a Quanti-Tray or Quanti-Tray/2000 and seal in an IDEXX Quanti-Tray Sealer.
4. Place the sealed Tray in a 35±0.5°C incubator for 18 hours (this 18 hours includes warming time).
5. Read results according to the Result Interpretation table below. Count the number of positive wells and refer to the MPN table provided with the Trays to obtain a Most Probable Number.

Result Interpretation

Appearance	**Result**
Colorless or slight tinge	negative for total coliforms and *E. coli*
Yellow equal to or greater than the comparator*	positive for total colitorms
Yellow and fluorescence equal to or greater than the comparator	positive for *E. coli*

- Look for fluorescence with a 6 watt, 365 nm, UV light within 5 inches of the sample. Face light away from your eyes and towards the sample.
- Samples are negative if at any time after 18 hours there is no yellow and/or fluorescence.
- Yellow or yellow/fluorescence observed before 18 hours is a valid positive. However, after 22 hours from inoculation, heterotrophs may overwhelm Colilert-18's inhibition system. Therefore, yellow or yellow/fluorescence first observed after 22 hours from inoculation is not a valid positive.

Procedural Notes
- **A slight tinge maybe observed when Colilert-18 is added to the sample.**
- Colilert-18 can be run in any multiple tube format. Standard Methods for the **Examination of Water and** Wastewater **MPN tables** should be used to find Most Probable Numbers (MPN's). .
- Some water samples containing humic material may have an innate color. If a water sample has some background color, compare inoculated Colilert-18 sample to a control blank of the same water sample.
- Do not dilute sample in buffered water. Colilert-18 is already buffered.
- Colilert-18 is a primary water test. Colilert-18 performance characteristics do not apply to samples altered by any pre-enrichment or concentration.
- Marine water samples must be diluted at least ten-fold with sterile fresh water (e.g. 10 ml sample added to 90 ml sterile fresh water).
- In samples with excessive chlorine, a blue flash may be seen when adding Colilert-18. If this is seen, consider sample invalid and discontinue testing
- Aseptic technique should be always be followed when using Colilert-18. Dispose of in accordance with Good Laboratory Practices.

Quality Control Procedures
The following quality control procedure is recommended for each lot of Colilert-18, or more often as regulations require.
1. Inoculate 3 sterile vessels filled with 100 ml sterile water with the following:
   A one with Quanti-Cult°"" *E. coli* or a sterile loop of ATCC"` 25922 or 11775 (*E. coli*)
   B. one with Quanti-Cult *Klebsiella pneumoniae* or a sterile loop of ATCC 31488 (totalcoliform)
   C. one with Quanti-Cult *Pseudomonas aeruginosa* or a sterile loop of ATCC 10145 or 27853 (non-coliform)
2. Follow the P/A Procedure or Quanti-Tray Enumeration Procedure above.
3. Results should match the Result Interpretation table above

Appendix 4 Description of the Chromocult® Coliform method

Chromocult® Coliform Agar

Selective agar for the simultaneous detection of total coliforms and E. coli in water and food samples.

Mode of Action
In the first instance, the interaction of selected peptones, pyruvate, sorbitol and a phosphate buffer guarantees rapid colony growth, even for the subletally injured coliforms. The growth of Gram-positive bacteria as well as some Gram-negative bacteria is largely inhibited by the content of Tergitol® 7 which has no negative effect on the growth of the coliform bacteria.
For the second stage, Merck has developed a new combination of two chromogenic substrates which allow for the simultaneous detection of total coliforms and E. coli.

Coliform identification
The characteristic enzyme for coliforms, [3-D-galactosidase cleaves the Salmon-GAL substrate and causes a salmon to red colour of the coliform colonies.

E. coli identification
The substrate X-glucuronide is used for the identification of (3-D-glucuronidase which is characteristic for E. coli.

E. coli cleaves broth Salmon-GAL and X-glucuronide, so that positive colonies take on a dark-blue to violet colour. These are easily distinguished from the other coliform colonies which have a salmon to red colour.

As part of an additional confirmation of E. coli, the inclusion of tryptophane improves the indole reaction, thereby increasing detection reliability when it is used in combination with the Salmon-GAL and X-glucuronide reaction.

Typical Composition (g/litre)
Peptones 3.0; sodium chloride 5.0; sodium di-hydrogen phosphate 2.2; di-sodium hydrogen phosphate 2.7; sodium pyruvate 1 .0; tryptophane 1 .0; agar-agar 10.0; Sorbitol 1 .0; Tergitol~ 7 0.15; chromogenic mixture 0.4.

Preparation
Suspend 26.5 g in 1 litre of demin. water by heating in a boiling water bath or in a flowing steam. Stir the concents to assist dissolution (approx. 35 min). Some turbidity may occur, but this does not effect the performance!
• **Do not autoclave! Do not overheat!** pH: 6.8 ± 0.2 at 25 °C.

Note: if high accompanying flora is expected, especially Pseudomonas spp. and Aeromonas spp., let the medium cool to 45-50 °C and add Cefsoludin solution (5 mg Cefsulodin in 1 ml of demin. water in case of surface and pourplate method and 10 mg Cefsulodin in 2 ml of demin. water in case of membrane-filter-technique) to 1 litre of the medium. Homogenize by gently shaking and pour into plates.

The plates are turbid and yellowish. Store in a refrigerator and protect from light. To prevent plates from becoming dry seal in plastic-pouches or bags. Shelf-life under these conditions: 6 months.

Experimental Procedure
Inoculate the medium by the pour-plate method or by spreading the sample material on the surface of the plates. In addition the membrane filter technique can also be used. Incubation: 24 hours at 35-37 °C.

Evaluation
E. coli: dark-blue to violet colonies (Salmon-GAL and X-Glucuronicle reaction).
Total coliforms: salmon to red colonies (Salmon-GAL reaction) and dark-blue to violet colonies (E. coli).

Other Gram-negatives: colourless colonies, except for some organisms which possess P-D-glucuronidase activity. Theses colonies appear light-blue to turquoise In order to confirm E. coli, overlay the dark-blue to violet colonies with a drop of KOVACS' indole reagent. If the reagent turns to a cherry-red colouring after some seconds, a positive reaction confirms the presence of E. coli.

Cat. **No. 1.10426.0100/0500**
(100 g, 500 g)

Appendix 5 The number of total coliforms (totcol) and *E. coli* per 100 ml in samples analysed by Kiwa with different methods

sample date	sample code	totcol LSA37	totcol LTTC37	totcol Colilert	*E.coli* LTTC37	*E.coli* LTTC44	*E.coli* Colilert
3-11-99	WRK ruw	27	35	25,4	12	7	6,4
3-11-99	WRK na coag.	9	17	17,8	6	2	1,0
3-11-99	WRK na snelf.	4	2	16,4	<2	4	1,0
9-11-99	WRK ruw	18	60	50,4	20	7	4,2
9-11-99	WRK na coag.	11	30	45,3	<6	2	2,0
9-11-99	WRK na snelf.	2	7	15,0	<4	0	0,0
16-11-99	M994388	37	48	129,8	48	40	47,8
16-11-99	M994389	8	8	34,4	4	2	15,0
16-11-99	M994390	9	6	27,1	6	9	8,7
16-11-99	M994391	2	6	4,2	6	4	1,0
16-11-99	M994392	270	241	>200,5	241	201	>200,5
16-11-99	M994393	6	7	25,4	2	<1	3,1
16-11-99	M994394	14	37	34,4	22	12	11,1
16-11-99	M994395	14	62	88,5	25	7	12,4
16-11-99	M994396	0	0	1,0	0	0	0,0
18-11-99	M994388	0	4	3,1	2	3	2,0
18-11-99	M994389	10	16	>200,5	8	4	3,1
18-11-99	M994390	13	6	28,8	6	1	3,1
18-11-99	M994391	3	2	16,4	<1	4	4,2
18-11-99	M994392	14	14	25,4	<7	22	15,0
18-11-99	M994393	1	8	144,5	4	1	4,2
18-11-99	M994394	240	244	144,5	183	102	101,3
18-11-99	M994395	200	236	>200,5	118	144	13,7
18-11-99	M994396	1	0	0,0	0	<2	0,0
23-11-99	M994569	4	2	2,0	2	1	0,0
23-11-99	M994570	1	<1	2,0	<1	0	0,0
23-11-99	M994571	3	7	5,3	7	1	2,0
23-11-99	M994572	5	23	30,6	18	19	8,7
23-11-99	M994573	30	66	73,8	<13	21	23,8
23-11-99	M994574	26	41	42,9	33	14	19,2
25-11-99	M994569	1	1	5,3	1	0	0,0
25-11-99	M994570	1	1	2,0	<1	1	1,0
25-11-99	M994571	210	75	165,2	<19	8	17,8
25-11-99	M994572	3	9	13,7	4	10	6,4
25-11-99	M994573	1	0	4,2	0	1	1,0
25-11-99	M994574	1	11	17,8	8	6	11,1
25-11-99	M994610	36	59	59,1	<12	15	1,0
25-11-99	M994611	0	0	0,0	0	0	0,0

Note: LSA37, LTTC37 and LTTC44 counts are confirmed counts, Colilert counts are not

Appendix 6 The number of total coliforms (totcol) and *E. coli* per 100 ml in samples analysed by PWN with different methods

sample date	sample code	totcol LSA37	totcol LTTC37	totcol Colilert	totcol CCA	*E.coli* LTTC37	*E.coli* LTTC44	*E.coli* Colilert	*E.coli* CCA
12-10-99	ADK-U0YS	6	4	15	13	4	2	3,1	4
12-10-99	ADK-U0YS	9	4	19,2	9	4	1	4,2	3
12-10-99	ADK-U0YS	44	>	144,5	57	>	>	34,4	29
12-10-99	ADK-U0YS	59	>	165,2	54	>	>	34,4	38
12-10-99	ADK-U000	68	>	200,5	>100	>	38	36,4	87
12-10-99	ADK-U000	73	>	>200,5	>100	>	42	30,6	41
12-10-99	HI-INW3	0	0	4,2	2	0	0	0,0	0
12-10-99	HI-INW3	2	0	9,9	2	0	0	0,0	0
15-10-99	ADK-U0YS	0	2	8,7	3	2	0	1,0	0
15-10-99	ADK-U0YS	0	3	3,1	7	3	2	1,0	0
15-10-99	ADK-U0YS	7	>	56,0	39	>	>	2,0	1
15-10-99	ADK-U0YS	6	>	62,4	44	>	>	3,1	4
15-10-99	ADK-U000	3	0	40,6	26	0	3	2,0	2
15-10-99	ADK-U000	3	6	59,1	32	6	1	1,0	3
15-10-99	ADK-U000	21	>	>200,5	180	>	19	17,8	18
15-10-99	ADK-U000	26	>	69,7	190	>	19	11,1	23
15-10-99	HI-INW3	0	12	45,3	0	7	12	9,9	0
15-10-99	HI-INW3	64	15	200,5	3	12	19	73,8	2
19-10-99	ADK-U0YS	2	3	8,7	11	3	>	2,0	0
19-10-99	ADK-U0YS	11	5	15	11	5	>	3,1	0
19-10-99	ADK-U000	3	3	38,4	15	3	6	4,2	1
19-10-99	ADK-U000	5	9	40,6	6	9	7	4,2	3
19-10-99	HI-INW3	0	0	2	1	0	0	0,0	0
19-10-99	HI-INW3	1	0	4,2	1	0	0	0,0	0
21-10-99	ADK-U0YS	9	11	17,8	11	4	5	1,0	5
21-10-99	ADK-U0YS	13	34	32,4	18	27	3	5,3	4
21-10-99	ADK-U000	5	3	17,8	8	3	2	0,0	3
21-10-99	ADK-U000	5	6	34,4	11	3	6	1,0	1
21-10-99	HI-INW3	0	0	1,0	0	0	0	0,0	0
21-10-99	HI-INW3	0	0	2,0	0	0	0	0,0	0
28-10-99	ADK-U0YS	0	1	2,0	2	1	0	0,0	0
28-10-99	ADK-U0YS	2	1	2,0	3	0	0	0,0	1
28-10-99	ADK-U000	3	1	17,8	12	0	1	3,1	3
28-10-99	ADK-U000	4	1	19,2	13	0	2	4,2	4
28-10-99	HI-INW3	0	0	0,0	0	0	0	0,0	0
28-10-99	HI-INW3	0	0	2,0	1	0	0	0,0	0
2-11-99	ADK-U0YS	0	0	0,0	1	0	0	0,0	1
2-11-99	ADK-U0YS	1	1	3,1	3	1	0	1,0	1
2-11-99	ADK-U0YS	3	>	5,3	5	>	>	1,0	3
2-11-99	ADK-U0YS	3	>	6,4	6	>	>	1,0	4
2-11-99	ADK-U000	3	3	16,4	6	1	3	0,0	3
2-11-99	ADK-U000	4	3	19,2	10	0	6	2,0	1
2-11-99	ADK-U000	19	19	94,5	31	19	18	5,3	11
2-11-99	ADK-U000	26	22	144,5	35	22	20	12,4	11
2-11-99	HI-INW3	0	0	1,0	0	0	0	0,0	0
2-11-99	HI-INW3	0	0	4,2	1	0	0	0,0	0
4-11-99	ADK-U0YS	1	>	3,1	1	>	>	1,0	1
4-11-99	ADK-U0YS	0	>	6,4	1	>	>	2,0	1
4-11-99	ADK-U0YS	5	>	19,2	2	>	>	5,3	3
4-11-99	ADK-U0YS	7	>	15	5	>	>	1,0	4

continued

Appendix 6 (continued)

sample date	sample code	totcol LSA37	totcol LTTC37	totcol Colilert	totcol CCA	*E.coli* LTTC37	*E.coli* LTTC44	*E.coli* Colilert	*E.coli* CCA
4-11-99	ADK-U000	4	4	11,1	2	4	1	0,0	0
4-11-99	ADK-U000	4	5	15,0	2	2	2	0,0	2
4-11-99	ADK-U000	22	>	131	78	>	.>	3,1	3
4-11-99	ADK-U000	24	>	125	84	>	>	12,4	9
4-11-99	HI-INW3	0	0	0,0	0	0	0	0,0	0
4-11-99	HI-INW3	0	0	1,0	0	0	0	0,0	0
9-11-99	ADK-U0YS	190	>	>200,5	90	>	>	65,9	75
9-11-99	ADK-U0YS	>	>	2400	>200	>	68	517	>200
9-11-99	ADK-U000	12	37	38,4	15	22	4	5,3	6
9-11-99	ADK-U000	39	100	291	64	60	26	27	41
9-11-99	HI-INW3	0	0	0,0	0	0	0	0,0	0

Note: LSA37, LTTC37, LTTC44 and CCA E. coli counts are confirmed counts, Colilert and CCA totcol counts are not ; > = too numerous to count

Appendix 7 The number of total coliforms (totcol) and *E. coli* per 100 ml in samples analysed by RIVM-MGB with different methods

sample date	sample code	totcol LSA37	totcol LTTC37	totcol Colilert	*E.coli* LTTC37	*E.coli* LTTC44	*E.coli* Colilert	*E.coli* DP
11-10-99	coli99/01	41	10	*	10	35	*	50
11-10-99	coli99/02	25	22	*	22	21	*	25
11-10-99	coli99/03	>	43	*	0	19	*	37
11-10-99	coli99/04	>	41	*	41	23	*	42
11-10-99	coli99/05	8	10	*	10	0	*	2
11-10-99	coli99/06	8	9	*	9	2	*	9
11-10-99	coli99/07	2	12	*	6	1	*	4
11-10-99	coli99/08	0	2	*	1	1	*	2
13-10-99	coli99/09	69	63	>200.5	63	51	13.7	66
13-10-99	coli99/10	3	4	12.4	4	<1	2.0	0
13-10-99	coli99/11	>	>	>200.5	>	75	144.5	92
13-10-99	coli99/12	69	>	200.5	>	13	20.7	35
13-10-99	coli99/13	1	<1	118.4	<1	0	0.0	0
13-10-99	coli99/14	10	1.5	40.6	1.5	3	5.3	12
13-10-99	coli99/15	47	35	83.1	35	23.5	50.4	62
13-10-99	coli99/16	0	0	20.7	0	0	0.0	0
18-10-99	coli99/17	31	45	88.5	45	23	17.8	37
18-10-99	coli99/18	4	3	9.9	3	2	3.1	7
18-10-99	coli99/19	>	>	200.5	>	26	34.4	26
18-10-99	coli99/20	19	14	28.8	14	<13	7.5	13
18-10-99	coli99/21	4	3	69.7	3	1	1.0	1
18-10-99	coli99/22	27	<10	65.7	<10	12	17.8	30
18-10-99	coli99/23	1	2	16.4	2	1	0.0	0
18-10-99	coli99/24	14	<5	200.5	<5	7	1.0	6
25-10-99	coli99/25	14	23	73.8	23	14	6.4	28
25-10-99	coli99/26	8	6	30.6	6	5	6.4	4
25-10-99	coli99/27	>	>	>200.5	>	69	165.2	>
25-10-99	coli99/28	>	11	>200.5	11	73	118.4	127
25-10-99	coli99/29	0	<1	32.4	<1	<1	0.0	0
25-10-99	coli99/30	6	13	32.4	<13	13	6.4	15
25-10-99	coli99/31	2	2	50.4	<2	5	2.0	0
25-10-99	coli99/32	11	0	>200.5	0	10	13.7	17
27-10-99	coli99/33	22	8	88.5	8	13	8.7	11
27-10-99	coli99/34	>	>	>200.5	>	>	>200.5	>
27-10-99	coli99/35	>	>	>200.5	>	25	101.3	121
27-10-99	coli99/36	22	<1	88.5	<1	25	20.7	33
27-10-99	coli99/37	0	0	30.6	0	0	0.0	1
27-10-99	coli99/38	3	4	20.7	2	2	4.2	11
27-10-99	coli99/39	5	>	>200.5	>	>	2.0	3
27-10-99	coli99/40	>	>	>200.5	>	>	8.7	3
1-11-99	coli99/41	43	83	165.2	83	38	56.0	89
1-11-99	coli99/42	102	96	>200.5	96	64	118.4	141
1-11-99	coli99/43	50	36	165.2	36	34	38.4	30
1-11-99	coli99/44	22	5	73.8	<1	16	12.4	14
1-11-99	coli99/45	3	3	59.1	<1	<1	0.0	0
1-11-99	coli99/46	19	19	40.6	15	13	13.7	16
1-11-99	coli99/47	<1	4	38.4	1	0	0.0	4
1-11-99	coli99/48	11	0	109.1	0	<1	3.1	4
3-11-99	coli99/49	57	74	144.5	67	53	19.2	70

continued

Appendix 7 (continued)

sample date	sample code	totcol LSA37	totcol LTTC37	totcol Colilert	*E.coli* LTTC37	*E.coli* LTTC44	*E.coli* Colilert	*E.coli* DP
3-11-99	coli99/50	38	36	78.2	36	47	30.6	52
3-11-99	coli99/51	46	10	109.1	6	18	19.2	33
3-11-99	coli99/52	42	13	118.4	4	22	16.4	21
3-11-99	coli99/53	1	4	62.4	3	4	1.0	2
3-11-99	coli99/54	10	13	38.4	10	12	11.1	21
3-11-99	coli99/55	3	1	23.8	<1	1	2.0	0
3-11-99	coli99/56	1	2	45.3	1	0	0.0	2
8-11-99	coli99/57	>	>	>200.5	>	62	94.5	110
8-11-99	coli99/58	>	>	>200.5	>	>	>200.5	>
8-11-99	coli99/59	>	>	>200.5	>	51	78.2	76
8-11-99	coli99/60	95	49	200.5	44	36	38.4	33
8-11-99	coli99/61	0	<1	40.6	<1	1	0.0	1
8-11-99	coli99/62	25	30	34.4	21	17	6.4	16
8-11-99	coli99/63	3	2	23.8	1	4	1.0	1
8-11-99	coli99/64	14	2	45.3	<1	5	2.0	2
10-11-99	coli99/65	81	>	>200.5	>	>	40.6	154
10-11-99	coli99/66	13	21	62.4	21	12	9.9	17
10-11-99	coli99/67	58	>	>200.5	>	not done	59.1	49
10-11-99	coli99/68	71	7	>200.5	4	51	40.6	74
10-11-99	coli99/69	0	<1	25.4	<1	0	1.0	1
10-11-99	coli99/70	21	22	56.0	20	13	16.4	19
10-11-99	coli99/71	0	0	17.8	0	0	0.0	1
10-11-99	coli99/72	83	>	>200.5	>	22	20.7	25
15-11-99	coli99/73	47	64	144.5	64	26	6.4	36
15-11-99	coli99/74	2	2	15.0	2	3	2.0	5
15-11-99	coli99/75	65	9	144.5	8	20	16.4	15
15-11-99	coli99/76	41	>	165.2	>	22	32.4	22
15-11-99	coli99/77	1	1	7.5	1	1	0.0	0
15-11-99	coli99/78	11	16	36.4	15	12	11.1	18
15-11-99	coli99/79	0	1	15.0	1	0	0.0	0
15-11-99	coli99/80	3	0	27.1	0	0	0.0	0

*Note: LSA37, LTTC37 and LTTC44 counts are confirmed counts, Colilert and DP counts are not; * = analysis failed, > = too numerous to count*

Appendix 8 European method comparison trial: final report
Colin R. Fricker, Seppo I. Niemela & John L. Lee, September 2000

Summary

A large trial has been performed with twenty participating laboratories from thirteen European countries. The aim of the trial was to test whether a protocol for comparing two microbiological methods was suitable for its purpose. The ISO reference procedure for detection of coliforms and *E. coli* in water was compared with the Colilert 18/QuantiTray method. Analysis of the data was carried out independently and not by any of the participating laboratories.

Examination of the data showed that the use of "confirmation coefficients" or "true positive rates" to compare the two methods showed a degree of uncertainty. The data produced by multiplying presumptive counts with laboratory-specific confirmation factors is not Normal enough (based on Gaussian statistics) to permit a parametric test. Non-parametric tests do not address comparison of the true means. Therefore, the statistically inefficient compromise of testing the sums of 20 laboratories must be accepted.

Data collected in one laboratory where all colonies from a large number of samples were confirmed showed that the use of confirmation coefficients led to an underestimate of the performance of Colilert compared to the ISO method. In that particular laboratory, whilst confirming all isolates showed that Colilert recovered significantly more *E. coli* than the reference method, use of a confirmation coefficient, would have led to the conclusion that the reference method recovered significantly more *E. coli* than Colilert. Comparing the differences between individual samples can be misleading. However, it was clear from the data that the confirmation rate for Colilert was significantly higher than that for the reference method in most laboratories and in most cases Colilert results could be used without confirmation. The confirmation rate for Colilert was generally under estimated because fermentation of lactose was used in the definition of a coliform. It is clear that many coliforms are unable to ferment lactose within 48 hours but are able to cleave ONPG through the use of β-D-galactosidase. If the definition of a coliform had been possession of β-D-galactosidase then the confirmation rates for Colilert would have been higher.

Comparisons have been made of the relative difference of the sums of organisms detected in each laboratory and the mean relative difference. These comparisons demonstrate that Colilert recovered significantly more coliforms than the ISO method and that there was no apparent difference between Colilert and the ISO method for recovery of *E. coli*. In order to determine if the use of confirmation coefficients is acceptable and to provide more useful data for *E. coli* further work was performed in five laboratories. This work involved confirmation of all colonies obtained from a small number of samples and subsequent statistical analysis of the results demonstrated that Colilert was significantly better than the ISO reference method in some laboratories and was as good in the remaining laboratories. It is concluded that Colilert is a suitable alternative procedure for the detection of *E. coli* and coliforms in water.

A protocol has been developed which has been shown to be suitable for comparing two microbiological methods. The basis of the protocol is that a relatively small number of laboratories (five is suggested) from different geographical locations and with different water types should be used. Samples should not be "drinking water" but should be drinking water contaminated with sewage effluent which is then chlorinated or drinking water seeded with river water, depending on whether the laboratory normally analyses chlorinated or

unchlorinated drinking water. The need for very large numbers of samples can be avoided in this way. Whilst large numbers of samples are not required, it is important that a large amount of confirmation data is obtained and for this reason, samples with relatively low numbers of target organisms are preferable such that all isolates can be confirmed.

Introduction

The microbiological quality of drinking water is safeguarded through a program of frequent analyses of water samples leaving the treatment works, in the distribution system and at customers' taps. These frequent analyses for the indicator organisms, coliforms, *Escherichia coli*, faecal streptococci and *Clostridium perfringens* are prescribed in the European Union Drinking Water Directive (EUDWD) and will be incorporated into the national law of member states. The EUDWD specifies a method to be used for the detection of coliforms and *E.coli* that involves membrane filtration and incubation on a tergitol-TTC agar medium. Whilst it is not mandatory that member states use this method, there is an obligation to demonstrate that any alternative method which is to be used has a similar performance.

The exact nature of the similarity is not defined, nor is the methodology to be used for comparison of methods. In Article 7, Part 5b, the European Directive specifically states, "Methods other than those specified in Annex III, Part 1, may be used, providing it can be demonstrated that the results obtained are at least as reliable as those produced by the methods specified. Member States which have recourse to alternative methods shall provide the Commission with all relevant information concerning such methods and their equivalence."

It is generally accepted that alternative methods should have a performance, which is equivalent or better. In this context "equivalent or better" must be defined. A method is normally considered to be "equivalent, if the (confirmed) recovery of target organisms is not significantly lower than the reference method. However, "significantly lower" also requires definition. "Equivalence" may also incorporate factors other than sensitivity. When comparing two methods it is often the case that the practicality of the tests may also be considered. For example a test which takes one day to complete and has a similar sensitivity to another test which takes three days to complete may be considered "better" or at least more appropriate.

In order to try and develop a protocol for comparison of quantitative microbiological cultural methods, a large team of laboratories from thirteen European countries was established (Appendix 1). A detailed protocol for performing the comparison between the European reference method and Colilert 18/QuantiTray (Colilert) was developed and agreed to by all participants (Appendix 2). Quality control procedures were also established. Colilert was chosen, as the method for comparison as it is easy to use, is quick to perform, requires very little training and allows a wide range of laboratory personnel to perform microbiological tests.

The Trial

The Initial Trial

Initial tests were performed over a period of several months during the latter part of 1999. Individual laboratories used the types of water which they would normally expect to examine during routine operation. Thus those laboratories which routinely examine unchlorinated waters used these for comparison whilst those which typically examine chlorinated waters used, for the most part, disinfected sewage effluent, using a procedure developed at Thames Water and subsequently modified by the Public Health Laboratory Service, UK. A training session was held before the commencement of the comparison where the personnel who would actually perform the tests were trained in how to perform both tests and, where necessary, how to prepare disinfected sewage effluents. In addition, trained personnel made visits to individual laboratories for further training and advice where necessary.

Each laboratory performed a small number of confirmations as described in Appendix 2 and also sent strains to a central laboratory for independent confirmation. One laboratory performed confirmations on all samples where the result for one of the two tests was ten organisms or less per 100 ml of water. All these confirmations of isolates from the various laboratories were performed in an effort to determine the "true positive rate" for each test in each laboratory.

The Second Stage of the Trial

A second stage of the trial, where samples were analyzed for *E.coli* only was performed during June and July of 2000. This part of the trial was performed in five laboratories and all isolates (either colonies on membranes or positive wells from Colilert) were confirmed. All samples were analyzed by the same personnel who visited each laboratory to perform the work.

Results were collated at one laboratory and subsequently independently analyzed by Professor Seppo Niemela.

Results

The Initial Trial

Table 1 shows the number of useable data points from each laboratory. Samples where both methods gave a negative result (0/0) were removed, as were any samples where results were reported as "greater than" or "too numerous to count".

Table 1 The number of samples available for method comparisons

Laboratory	Number of samples for Total Coliforms	Number of samples for *Escherichia coli*
1	218	170
2	633	625
3	110	64
4	30	31
5	44	50
6	60	63
7	76	35
8	38	31
9	61	63
10	29	44
11	24	12
12	45	38
13	153	150
14	138	133
15	38	29
16	52	59
17	200	173
18	132	125
19	727	790
20	50	50
Total:	2921	2672

The amount of information for the method comparison is directly proportional to the number of colonies observed.

Table 2 Numbers of presumptive colonies (and corresponding Colilert counts) observed

Laboratory	Presumptive Colilert TC	Presumptive Tergitol TC	Presumptive Colilert EC	Presumptive Tergitol EC
1	8378	6982	230	475
2	23220	13637	3728	6291
3	1310	1084	108	511
4	1147	378	316	331
5	1753	1524	588	889
6	1383	1471	449	940
7	2323	1333	141	483
8	1525	1047	512	477
9	1715	2624	655	1018
10	183	245	715	186
11	443	259	24	44
12	1447	611	182	265
13	5068	5137	542	762
14	2940	1376	373	622
15	1011	209	266	270
16	3337	571	1518	1349
17	4328	4235	602	1256
18	2509	1609	468	1257
19	13838	13592	2531	4888
20	909	974	211	786
Total:	78770	58898	14160	23100

The amount of information contributed by each laboratory differs considerably.

The amount of information available for the comparison of the total coliform methods is almost four times that available for comparing the *E.coli* methods. The difference becomes even greater after the confirmation coefficients have been applied (see Tables 6 and 8).

For the determination of the confirmation coefficients, each laboratory isolated a number of cultures for confirmation. The reliability of the coefficient is related to the number of cultures tested.

Table 3 *The numbers of cultures tested in each laboratory to determine an average confirmation coefficient for each of the four methods. The coefficients themselves are presented in Table 4*

Laboratory	Colilert TC	Tergitol TC	Colilert EC	Tergitol EC
1	148	109	97	125
2	356	310	323	308
3	99	109	57	60
4	52	22	22	25
5	50	50	50	50
6	28	30	28	28
7	51	46	29	41
8	39	133	41	129
9	20	18	18	19
10	38	32	12	24
11	30	28	7	14
12	34	34	24	31
13	113	107	114	118
14	101	96	68	82
15	89	52	48	48
16	70	55	58	59
17	100	55	100	42
18	26	27	25	27
19	1894	2265	1766	3806
20	25	25	25	25
Total:	3363	3603	2912	5061

The numbers of cultures isolated in each laboratory were very different and small numbers lead to imprecise coefficients, thus increasing the random differences between laboratories.

The “confirmation coefficients” (the proportion of isolates which were detected by each method and subsequently shown by confirmation tests to be target organisms) for each laboratory have been calculated and are shown in Table 4.

Table 4 Table of confirmation coefficients

Laboratory	Colilert TC	Tergitol TC	Colilert EC	Tergitol EC
1	0.53	0.21	0.56	0.18
2	0.77	0.48	0.86	0.59
3	0.91	0.83	0.93	0.78
4	0.90	0.82	0.91	0.64
5	0.90	0.86	0.92	0.70
6	1.00	1.00	0.96	1.00
7	0.75	0.74	0.90	0.46
8	0.95	0.90	0.88	0.76
9	0.75	0.67	0.83	0.63
10	1.00	0.66	0.92	0.46
11	0.90	0.93	1.00	0.50
12	0.62	0.91	0.92	0.94
13	0.88	0.65	0.97	0.85
14	0.83	0.77	0.75	0.30
15	0.57	0.60	1.00	0.56
16	0.60	0.82	0.97	0.83
17	0.91	0.67	0.97	0.26
18	0.92	0.96	0.88	0.41
19	0.97	0.74	0.99	0.42
20	1.00	0.92	1.00	0.44

It is clear that there are considerable differences between the confirmation coefficients obtained in different laboratories and between methods. The ranges, means, standard deviations and standard error of the means of the confirmation coefficients are shown in

Table 5 Range, mean, standard deviations and standard error of the means of all participating laboratories

Method	Range of confirmation coeff	Mean confirmation coeff	Standard deviation	Standard error of the mean
Colilert total coliforms	0.53-1.0	0.83	0.15	0.034
Tergitol total coliforms	0.21-1.0	0.76	0.19	0.043
Colilert E.coli	0.56-1.0	0.91	0.10	0.022
Tergitol E.coli	0.18-1.0	0.59	0.23	0.051

The means have been calculated simply by summing the confirmation coefficient for each laboratory and dividing by 20 (the number of laboratories which submitted confirmation data). In most cases, Colilert had higher confirmation coefficients for both coliforms and *E.coli*.

Although it was noticeable that in some laboratories the confirmation coefficient for coliforms was slightly lower with Colilert than for the reference method. This phenomenon was investigated in one laboratory and it was shown to be due to the presence of coliforms, which were unable to ferment lactose, but were ONPG positive. These organisms are coliforms but the procedure used in the trial would not confirm them as confirmation relied on fermentation of lactose. These "environmental" coliforms form a substantial part of the natural flora in some waters and have been shown to be common in other countries (Fricker et al., 1997). Therefore the confirmation coefficient for Colilert was probably underestimated in many laboratories.

Table 6 shows the comparison of Colilert with the reference method for total coliforms based on confirmed counts and their relative differences.

Table 6 Comparison of Colilert and Tergitol total coliform methods using confirmed counts and their relative differences

Laboratory	Confirmed Colilert TC sum	Confirmed Tergitol TC sum	Relative difference of sums %
1	4444	1466	100.7
2	17880	6545	92.8
3	1192	899	28.0
4	1032	310	107.6
5	1577	1310	18.5
6	1383	1471	-6.2
7	1742	986	55.4
8	1448	942	42.4
9	1286	1758	-31.0
10	182	161	12.3
11	398	240	49.4
12	897	556	47.0
13	4459	3339	28.7
14	2440	1059	78.9
15	576	125	128.5
16	2002	615	105.9
17	3938	2837	32.5
18	2308	1544	39.6
19	13475	10072	28.9
20	908	896	1.4
Grand Total:	63570	37140	
Mean	3178	1857	48.06

Where values are expressed as minus (-) this means that the tergitol TTC medium recovered more coliforms than Colilert.

The unweighted mean per cent difference of the 20 laboratory sums was 48.06% and happens to be close to the true relative difference based on the grand totals or means of the confirmed counts:

Relative difference = 200(63570-37140)/(63570+37140)= 52.49% (calculated from grand total) or
Relative difference = 200(3178.5-1857.0)/(3178.5+1857.0)= 52.49% (calculated from mean)

The range of variation between the laboratories is so great (from a few negative per cent to more than 100 per cent positive) that a much greater difference might have occurred by chance. The main reasons for the large differences are the uncertainty of the confirmation coefficients, the effect of crowding of colonies in some samples and the random variation due to the statistics of particle distribution.
The difference of the order of 50% looks real and significant. There is no legitimate statistical test for the comparison of the difference between the grand totals (or means, for that matter) but a one sample t test can be applied to the values on the last column, provided that the data are Normal (see Table 7). “Normal” when written with a “N” refers to the Gaussian Normal distribution.

If the mean relative difference should be proven statistically significant (i.e. significantly different from zero) the result would mean that Colilert gives significantly more total coliforms than Tergitol.
The results of performing the one sample t test on the 20 values of the last column of Table 6 are shown in Table 7.

Table 7 *Results of t tests for relative difference of sums for coliform detection in twenty laboratories*

Parameter	Relative Difference of Sums %
Mean	48.06
S.E. of the Mean	9.48
t value	5.07
Probability (with 19 d.f.)	0.0001
95% C.I. low	28.2
95% C.I. high	67.9
Wilk-Shapiro (Normality)	0.968 (good)

The observed relative differences allowed the t test because the data appeared sufficiently Normal. Apart from the t value and its probability, the most important ‘parameter’ is the lower limit of the 95% Confidence Interval (95% C.I.). The Confidence Interval does not include the value -10%, which is likely to be taken as the decision limit in the ISO document, describing how to compare microbiological methods. The 10% value is important because it is likely that the ISO document will state that a method can be considered to be equivalent to the reference method if it gives results which are no worse than 10% lower. This means that the data are adequate to demonstrate that the trial method is equal or better than the reference method.

Table 8 shows the comparison of Colilert with the reference method for *E.coli* based on confirmed counts and their relative differences based on laboratory sums.

Table 8 Comparison of Colilert and Tergitol E. coli methods using confirmed counts and their relative differences

Laboratory	Confirmed Colilert sum	Confirmed Tergitol sum	Relative difference of sums %
1	129	85	40.6
2	3206	3711	-14.6
3	100	398	-119.3
4	287	211	30.3
5	541	622	-14.0
6	431	940	-74.2
7	126	222	-54.6
8	450	362	21.7
9	544	641	-16.4
10	657	85	154.0
11	24	22	8.7
12	167	249	-39.2
13	525	647	-20.9
14	279	192	36.7
15	266	151	55.0
16	1472	1119	27.2
17	583	326	56.5
18	411	522	-23.7
19	2509	2010	22.09
20	210	345	-48.7
Grand Total:	12930	12870	
Mean	646.3	643.5	1.36

The true relative difference based on the grand totals:

The relative difference can be calculated using the general formula 200 (A-B/A+B).

Thus the Relative difference = 200(12930-12870)/(12930+12870)= 0.47%

The true difference cannot be statistically tested because the grand totals or the means do not represent genuine samples from the Poisson distribution. The unweighted mean relative difference based on the sums is close to the true value. This number can be statistically tested, provided that the data do not deviate from Normal.

Results of performing the one sample t test on the 20 values of the last column of Table 8 are shown in Table 9.

Table 9 Results from one sample t tests from Table 8

Parameter	Relative Difference of Sums %
Mean	1.36
S.E. of the Mean	12.97
t value	0.10
Probability	0.9176
95% C.I. low	-25.8
95% C.I. high	28.5
Wilk-Shapiro (Normality)	0.941 (sufficient)

The null hypothesis of no difference between the methods has not been disproved. This means that the methods should be considered equivalent unless further data show otherwise. The data are Normal enough for the test to be acceptable.

Due to the very great variation between laboratories the mean is very uncertain and its standard error remains high. The Confidence Interval contains the value -10%. That means more or better quality data are required to definitively determine that the -10% limit is not included in the 95% C.I.

The ideal situation of basing a t test on all the 2671 degrees of freedom (see Table 1) from paired samples is not realized.

After completion of the second stage of the study in which all isolates were confirmed it became apparent that an alternative statistical analysis would be preferable. Data were therefore re-examined using the same statistical approach as was used for the second stage, for both coliforms and *E.coli*. The data have been 'edited' by omitting cases (samples) as follows:

1. All cases (samples) with either Colilert or Tregitol counts zero
2. Colilert counts higher than 201 (This is a safety measure because such high counts are impossible with Colilert 18 unless dilutions are involved. There is some doubt whether this was taken into account with the accompanying Tergitol results. This affects only 20 total coliform and no faecal coliform cases.)
3. Cases with Tergitol counts higher than 200.

The evaluations are based on decision rules adapted from the ISO document. The words 'better' and 'worse' contain a value judgement wider than mere quantitative comparison and are not used. The following evaluation includes only the quantitative comparison of mean differences between confirmed counts. The evaluation includes four alternatives:

1. 'higher' is used when the mean relative difference is positive (Colilert counts higher) and the difference is statistically significant. Statistical significance is judged by observing that the lower 95% C.I. is higher than zero.
2. 'lower' is used when the mean relative difference is negative (Colilert counts lower) and the upper 95% C.I. is less than zero (negative).
3. 'no difference' is used when the confidence interval includes zero but the lower 95% C.I. is higher than –10%.

4. ‘more data’ are requested when the 95% C.I. includes both 0% and –10% (lower 95% C.I. less than –10%).

The following text describes the reasons for taking the particular statistical approach that was taken and the approach itself. Statistical evaluation of the relative method performance applying the decision rules detailed above was based on the average relative difference and its 95% Confidence Interval.

The **weighted mean relative difference** (relative difference of the means) is believed to be the best measure of the relative performance of two methods. It is defined as the arithmetic difference between the mean counts of the two methods divided by their mean. It can be calculated in two equivalent ways, with the one based on the sums being less affected by rounding errors.

Weighted mean relative difference $(\overline{w})$ calculated from the means of counts with methods A and B

$$\overline{w} = \frac{\overline{a} - \overline{b}}{(\overline{a} + \overline{b})/2} = \frac{2(\overline{a} - \overline{b})}{\overline{a} + \overline{b}}$$

The same calculated from the sums of n observations

$$\overline{w} = \frac{2(\Sigma a_i - \Sigma b_i)}{(\Sigma a_i + \Sigma b_i)}$$

The estimate, being based on a single figure (sum or mean) per method, does not have an empirical standard deviation. Its standard deviation can only be estimated by so called Type B estimation (ISO 1995). Lacking a Type B estimate at the time of this report the empirical standard deviation of the **unweighted relative mean** ($\overline{u}$) was used as an approximation. The unweighted mean is the arithmetic mean of *n* individual relative mean values

$$\overline{u} = \frac{1}{n}\sum_{i=1}^{n}\frac{2(a_i - b_i)}{(a_i + b_i)} = \frac{2}{n}\sum_{i=1}^{n}\frac{d_i}{a_i + b_i}$$

a_i= confirmed count of method A in the i:th sample
b_i= confirmed count of method B in the i:th sample
$d_i = a_i - b_i$

The standard deviation of the weighted mean ($\overline{w}$) was assumed to be sufficiently closely approximated by the sample standard deviation of the unweighted mean ($\overline{u}$). Therefore,

$$s_{\overline{w}} \approx s_{\overline{u}} = \frac{s(u)}{\sqrt{n}}$$

The 95% confidence interval (C.I.) was calculated from

$$95\%C.I. = \pm t_{(n-1)}\, s_{\overline{u}}$$

Finding the necessary values of the *t* distribution for sometimes very large numbers of degrees of freedom (*n*-1) may be difficult. Standard statistical software was used for calculating the 95% C.I. of the unweighted mean. As the unweighted and weighted means are likely to differ the 95% C.I. could not be directly used. The problem was overcome by calculating the half width (upper 95% C.I. - lower 95% C.I.)/2 of the confidence interval and centering it around the weighted mean.

Table 10 *E.coli results from the initial trial*

Laboratory	**N**	**Weighted mean RD%**	**Half width of 95% C.I.**	**Lower 95% C.I.**	**Upper 95% C.I.**	**Evaluation**
1	85	56.3	14.3	42.0	70.6	higher
2	482	-14.3	5.8	-20.1	-8.4	lower
3	28	-88.4	23.4	-111.8	-65.0	lower
4	13	19.2	49.2	-30.0	68.3	more samples
5	50	-14.0	17.7	-31.7	3.7	more samples
6	56	-69.9	11.5	-81.4	-58.4	lower
7	20	-30.6	29.8	-60.5	-0.8	lower
8	29	24.0	29.5	-5.5	53,5	no diff.
9.	50	-9.4	18.9	-28.4	9.5	more samples
10	25	146.2	30.2	116.0	176.5	higher
11	8	20.7	45.1	-24.4	65.8	more samples
12	28	-37.9	24.9	-68.8	-13.1	lower
13	131	-18.9	12.0	-31.0	-6.9	lower
14	85	58.7	15.9	42.8	74.7	higher
15	23	57.1	31.2	25.9	88.3	higher
16	53	27.7	18.4	9.2	46.1	higher
17	134	63.1	11.9	51.2	75.1	higher
18	113	-17.4	12.7	-30.1	-4.7	lower
19	51	55.8	8.9	47.0	64.7	higher
20	46	-41.1	18.4	-59.5	-22.7	lower
Total	**1510**	**2.2**	**3.9**	**-1.7**	**6.1**	**no diff.**

Table 11 Total coliform results from the initial trial. Tergitol counts up to 200 considered

Laboratory	**N**	**Weighted mean RD%**	**Half width of 95% C.I.**	**Lower 95% C.I.**	**Upper 95% C.I.**	**Evaluation**
1	216	100.9	7.9	93.0	108.8	higher
2	590	90.0	6.9	83.0	96.9	higher
3	84	21.9	11.9	10.0	33.9	higher
4	16	57.2	53.7	3.5	110.9	higher
5	44	18.5	19.9	-1.4	38.4	no difference
6	60	-6.2	14.9	-21.0	8.7	more samples
7	66	54.5	25.4	29.1	79.8	higher
8	37	41.9	19.3	22.7	81.2	higher
9	59	-30.5	11.2	-41.7	-19.3	lower
10	15	17.4	48.3	-30.6	65.9	more samples
11	22	35.5	28.5	7.0	63.9	higher
12	45	23.5	21.4	2.1	44.9	higher
13	153	28.7	9.2	19.6	37.9	higher
14	133	79.8	10.2	69.7	90.0	higher
15	27	124.1	22.0	102.1	146.0	higher
16	47	101.3	18.7	82.6	119.9	higher
17	199	32.4	9.2	23.2	41.5	higher
18	126	40.7	9.9	30.9	50.6	higher
19	472	32.3	4.2	28.1	36.6	higher
20	50	1.4	18.1	-16.7	19.5	more samples
Total	**2461**	**52.0**	**2.8**	**49.2**	**54.8**	**higher**

Stage two

In stage two of the trial, five laboratories analyzed samples for *E.coli* only and all isolates were confirmed. The results of those analyses are shown in Table 12.

Table 12 Results of E.coli studies during stage two

Laboratory	TTC presumptive	TTC confirmed	Colilert presumptive	Colilert confirmed	Confirmation rate Colilert	Confirmation rate TTC
2	384	327	475	472	99%	85%
5	805	596	593	580	98%	74%
12	521	448	515	502	97%	86%
14	516	450	512	496	97%	87%
17	221	187	518	451	87%	85%
Total	2447	2008	2613	2501	96%	82%

Whilst the confirmation rate for Colilert in laboratory 17 was found to be only 87% this was due to an unusually high proportion of *E.coli* (as confirmed by other phentoypic tests) which were unable to grow at 44^{o}C. Thus whilst Colilert accurately identified these organisms as being *E.coli*, the confirmation procedures used by the reference method would have incorrectly classified them as non-*E.coli*.

The results are presented in the form of a table based on the confirmed counts (Table 13). RD% is the weighted average relative difference. The average RD can be obtained by calculating the value from the means (or sums) of confirmed counts in a series of samples. This can be called the weighted mean.

The half width of the 95% C.I. is based on the unweighted RD% values. The half width of the 95% confidence interval, when subtracted from the average RD% gives the lower 95% C.I. The 95% C.I. is based on the standard deviation and the number of samples. Thus the solution is that the 95% C.I. of the mean is based on the unweighted counts but the estimate is centered around the weighted mean RD. Thus it is necessary to calculate the half width of the 95% C.I..

An estimate of the standard deviation (SD) of the weighted mean cannot be calculated whereas the standard deviation of the unweighted mean is obtainable by standard statistical procedures from the series of individual RD values.

An evaluation of the significance of the difference is based on the principles developed by ISO.

Table 13 *Relative differences (RD%) and 95% confidence intervals (C.I.) for E.coli. Complete data including zero counts. Analyses made individually for each data set and collectively with (Total) and without (Subtotal) laboratory 19 results which were generated during the initial trial*

Data Set	**N**	**RD%**	**Half Width of 95% C.I.**	**Lower 95% C.I.**	**Evaluation**
2	75	36.1	22.2	13.9	Colilert higher
5	49	-2.7	10.9	-13.6	More samples
12	60	11.4	17.0	-5.6	No different
14	30	9.7	16.6	-6.9	No different
17	45	82.8	20.5	62.3	Colilert higher
Subtotal	**259**	**21.8**	**9.5**	**12.3**	**Colilert higher**
19	545	10.1	9.5	0.6	Colilert higher
Total	**804**	**16.8**	**7.2**	**9.6**	**Colilert higher**

How did confirmation coefficients (or true positive rates) vary between laboratories?

Confirmation coefficients were generally higher for Colilert than for the reference method for both coliforms and *E.coli* and in many cases this difference was statistically significant ($p<0.05$). This means that presumptive data generated using Colilert was more accurate than that generated using the reference method. In fact in many cases the presumptive Colilert

data could in effect be used as confirmed data. Some laboratories reported low confirmation coefficients for coliforms using Colilert but this appeared to be largely due to the presence of non-lactose fermenting coliforms.

This is an important difference as it means that data generated within 24 hours of receipt of a sample can be used to make public health or operational decisions. For *E.coli*, the confirmation coefficient was higher for Colilert than for the reference method in all but two laboratories during the initial trial. In the second phase of the study both the confirmed and presumptive Colilert results were equivalent or higher than the confirmed results with the reference method demonstrating that use of "presumptive" Colilert results is acceptable when analyzing drinking water. One laboratory had a relatively low confirmation rate for *E.coli*, found to be due to the presence of an unusually high proportion of non-thermotolerant *E.coli.*

Are there problems with using confirmation coefficients to determine the confirmed count?

Confirmation coefficients (or true positive rates) based on random isolates were used in an attempt to determine the confirmed count of coliforms and *E.coli* because of the huge amount of work necessary to confirm all isolates. One major problem with this approach, which was not foreseen, was that any sample that contained presumptive organisms gave a confirmed count. For example if a sample in a laboratory contained 1 cfu/100 ml and the confirmation coefficient was 0.6 then the "confirmed" count would be calculated as 0.6 cfu/100 ml. This clearly cannot be correct, as the true value must be either 0 or 1. Thus the method which is least selective would generally give higher values.

The situation is further complicated by the presence of thermotolerant coliforms. In a sample that contained 10 cfu/100 ml of thermotolerant coliforms but no *E.coli*, 10 colonies would be counted by the reference method as a presumptive count and none as *E. coli* by Colilert. Application of confirmation coefficients to these data (using for example the mean values calculated for all participants in the trial of Colilert 0.88, reference method 0.55) would generate "confirmed results" of Colilert 0 and the reference method 5.5 cfu/100 ml. Thus although the Colilert result would be correct, the reference method would apparently be significantly better. In essence then, whilst false negative results are taken into account, false positive results are much more difficult to detect using this approach.

The mathematical problems, such as loss of Normality and unrealistic means, caused by the use of global confirmation coefficients can only be mitigated by omitting all samples where zero results appear.

How good are the confirmation coefficients?

Analysis of the data used to generate the confirmation coefficients showed that there was a large degree of uncertainty around the data and examination of the data from laboratory 19 where all isolates were confirmed showed that confirmation coefficients for the reference method varied from zero to one. Therefore despite the large amount of data collected, the degree of uncertainty surrounding the confirmation coefficient makes the "confirmed" count data for *E.coli* very difficult to interpret. There are many possible reasons for the uncertainty and for the large variation between laboratories. These include differences in experience between laboratories and between individual staff within a laboratory, differences in sample type and the organisms within samples, subjectivity when reading the tergitol membranes and in selecting colonies from MacConkey plates produced by subculturing Colilert. These differences are impossible to quantify but are likely to have contributed to the large degree of

uncertainty. Such differences can only be eliminated by confirming all colonies and by using the same personnel to perform the work in each laboratory.

How do the data generated using confirmation coefficients compare with those generated using actual confirmation?

In laboratory 19, 676 data sets were collected for *E.coli*, where all colonies were confirmed. Of these samples, 239 had presumptive *E.coli* on the reference medium that subsequently did not confirm. Thus by confirming all colonies 239 samples were shown to give false positive presumptive counts. Had the procedure of using a confirmation coefficient been used then all of these samples would then have had a positive value. The effect of this would have been dramatic. In laboratory 19, Colilert was found to be significantly better than the reference method for both coliforms and *E.coli*, if the confirmation coefficient concept was used, then the reference method would have appeared to have been significantly better than Colilert for *E.coli* which would have been incorrect.

Data from Stage Two

Due to the difficulty in interpreting the data from the *E.coli* part of the initial trial, the differences seen in the data generated in laboratory 19 using "true positive rates", and confirmation of all isolates, stage two consisted of five laboratories performing a comparison for *E.coli* only and confirming all isolates. This data showed that in two laboratories Colilert was significantly better than the ISO reference method, in two laboratories there was no significant difference and in one laboratory there was insufficient data to draw conclusions. However, in all laboratories, Colilert had a significantly higher confirmation rate and overall, when all data were combined Colilert recovered significantly more *E.coli* than did the reference method.

A major problem encountered when attempting to compare two methods is the definitions used for coliforms and *E.coli.* In this study, the definition of coliforms for the purposes of the ISO reference method is that the organisms should be able to ferment lactose. Colilert does not contain lactose but detects coliforms on the basis of their ability to cleave ONPG using the enzyme β-D-galactosidase. Similarly Colilert detects *E.coli* on the basis of possession of the enzyme β-D-glucuronidase whereas the reference methods relies on the ability of *E.coli* to grow at 44^{o}C and ferment lactose and produce indole from tryptophan. Thus the two methods will undoubtedly be different.

Many organisms will be detected by both methods but only one or the other of the methods will detect a subset. For example glucuronidase negative *E.coli* will not be detected by Colilert but may be detected by the reference method. However, lactose negative *E.coli* will not be detected by the reference method but may be detected by Colilert. In this study, the criteria used for confirmation were those incorporated into the reference method which will result in a proportion of coliforms and *E.coli* that was detected by Colilert being recorded as "false positives" despite the fact that they were indeed the target organisms.

It is clear from the two separate stages of work that the key to successful methods comparison is not necessarily the absolute number of total samples tested, but the confirmation of all isolates. Use of this approach enables an accurate assessment of the sensitivity and specificity of both methods.

Conclusions

From this study several important conclusions can be drawn:

1. When comparing two methods, in order to generate statistically significant data, the key is not necessarily to test a huge number of samples, but to confirm as many isolates as possible. Confirmation of all presumptive positive organisms is the preferred option.
2. There is no need to utilize large numbers of laboratories for inter-laboratory studies. In the second stage of the trial statistically significant data was generated using five laboratories.
3. Single laboratory validations however are not recommended, as there are often significant differences between the results generated in different laboratories. This may be due to difference in sample types or some other differences within the laboratory.
4. It is suggested that future comparative studies should utilize five laboratories in different geographical locations. The study design should incorporate a variety of water types (e.g. chlorinated and unchlorinated) and that all isolates should be confirmed. This recommendation will be incorporated into the ISO document describing the basis for comparison of methods.
5. Colilert gave significantly higher recoveries of both coliforms and *E.coli* than did the ISO reference method even when the ISO reference procedure definitions were used for confirmation.
6. Confirmation rates for Colilert, when all isolates were confirmed were significantly higher than those for the reference method and in many cases approached 100%.
7. The confirmation rate is dependent on the criteria used for defining the target organism. During this study, Colilert isolated many coliforms which did not "confirm" because the criteria used included fermentation of lactose. Many coliforms are unable to ferment lactose in 48 hours. Similarly, some strains of *E.coli* were isolated by Colilert, which did not subsequently confirm because they were unable to grow at 44 $^{\circ}$C, did not ferment lactose or failed to produce indole from tryptophan. Despite this the confirmation rate for Colilert was still higher.
8. Based on the results of this study, Colilert can be recommended as being at least as good as the ISO reference method for the detection of *E.coli* and coliforms and can be used without the need for confirmations.

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Appendix 9 Mailing list

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