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EU Drinking Water Directive reference methods for enumeration of total coliforms and *Escherichia coli* compared with alternative methods

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Aims: The reference methods for enumeration of total coliforms and *Escherichia coli* as stated in the European Drinking Water Directive were compared with alternative methods.

Methods and Results: Laboratories used the reference method on Lactose TTC agar (LTTC), the Colilert®/18 system, Laurysulphate Agar (LSA), Chromocult® Coliform Agar and the *E. coli* Direct Plating (DP) method. They enumerated more total coliforms on LTTC than on LSA.

Conclusions: LTTC is suitable for analysis of very clean water samples only, due to heavy background growth. Colilert®/18 is a good alternative but it enumerates a broader group of total coliforms, resulting in higher counts. The DP method appeared to be the best choice for enumeration of *E. coli* because Colilert®/18 produces lower counts and false-negative results.

Significance and Impact of the Study: This study shows the limitations of the EU reference method on LTTC due to lack of selectivity and suggests alternative methods for the enumeration of total coliforms and *E. coli*.

INTRODUCTION

The new European Drinking Water Directive (EU DWD) (Anon. 1998) defines reference methods for the enumeration of microbiological parameters in drinking water. Member states may use alternative methods in cases where they have demonstrated that these methods produce results that are at least as reliable as those produced by the reference methods. Within the ISO, a working group (ISO TC147/SC4/WG12 'Analytical Quality Control') is in charge of producing a standard for comparing microbiological methods. On the basis of a UK Drinking Water Inspectorate document (Anon. 2000a), a protocol for comparison of enumeration methods for total coliforms and *Escherichia coli* was developed. This protocol was tested in a European trial, in which methods were compared based on the recovery of the target organisms and the number of false-positive results. Participating countries were to examine 150 samples with an expected total coliform count of 10–50 100 ml^{-1} and 50 samples with an expected total coliform count of 1–10 100 ml^{-1}. Methods to be used were the membrane filtration method on Lactose TTC agar with Tergitol 7 (LTTC), as described in ISO 9308-1 (Anon. 2000b) and stated as a reference method in the EU DWD, and the Colilert®/18 QuantiTray system as an alternative method. The three participating Dutch laboratories (RIVM-MGB, Kiwa and PWN) also analysed their samples with three methods currently used in the Netherlands. Here, the results of the Dutch laboratories are presented. The methods used were compared on the basis of their count results and confirmation rates, but practical aspects, such as readability of results and user friendliness, were also considered. The results of the European trial, including the evaluation of the value of the tested protocol for comparing microbiological enumeration methods, are described elsewhere (Schets *et al.* 2001, Fricker *et al.* in preparation).

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MATERIALS AND METHODS

Samples

Samples were taken at different sites, according to NEN 6559 (Anon. 1992), and included surface water, drinking water (half products) and drinking water artificially contaminated with surface water. Samples were cooled on melting ice and analysed within 24 h. A total of 179 samples was analysed (RIVM-MGB 80, Kiwa 38 and PWN 61). Surface water samples were diluted in 0·1% peptone saline to obtain an expected count of 1–50 total coliforms 100 ml^{-1}.

Colilert®18/QuantiTray system

The Colilert®/18 system (IDEXX Laboratories, Inc., Westbrook, ME, USA) was used according to the manufacturer's instructions. Sample and substrate were mixed and incubated in a 51 well QuantiTray for 18–22 h at 37 ± 1°C. Total coliform-positive wells displayed a yellow colour, whereas *E. coli*-positive wells were yellow and fluoresced under u.v. light (365 nm). By means of a table provided with the system, counts of the number of positive wells were transferred to a Most Probable Number (MPN) of the target organisms.

Lactose TTC with Tergitol 7 (LTTC) method

This method was performed according to ISO 9308-1 (Anon. 2000b). Membrane filtration of the samples was followed by incubation of the membrane filters on LTTC for 21 ± 3 h at 36 ± 2°C (LTTC37). Ten typical, yellow, lactose-positive colonies were confirmed for oxidase activity and production of indole from tryptophane broth at 44 ± 0·5°C. Oxidase-negative colonies were considered total coliforms, those being oxidase-negative and indole-positive, *E. coli*. To suppress expected background growth due to lack of selectivity of LTTC, LTTC was also incubated at 44 ± 0·5°C (LTTC44).

Laurylsulphate Agar (LSA) method

Samples were filtered and membrane filters were incubated on LSA for 5 ± 1 h at 25 ± 1°C, followed by 14 ± 2 h at 37 ± 1°C (Anon. 1982). Ten typical yellow colonies were confirmed in Brilliant Green Bile Lactose Broth (BBLB) at 37 ± 1°C. Yellow colonies that produced gas in BBLB after 22 ± 2 h or 44 ± 4 h were considered total coliforms.

Chromocult® Coliform Agar (CCA) method

After filtration of the sample, membrane filters were incubated on CCA (Merck) for 5 ± 1 h at 25 ± 1°C followed by 14 ± 2 h at 37 ± 1°C. Total coliforms produced salmon to red colonies, whereas presumptive *E. coli* formed dark blue to violet colonies. Presumptive *E. coli* were confirmed by overlaying the colonies with a drop of Kovacs' reagent (indole test), resulting in the development of a cherry red colour when *E. coli* was present.

E. coli Direct Plating (DP) method

The *E. coli* DP method is described as a Rapid Test in ISO 9308-1 (Anon. 2000b). After filtration of the sample, membrane filters were incubated on Tryptone Soy Agar for 4–5 h at 36 ± 2°C, followed by incubation on Tryptone Bile Agar for 19–20 h at 44 0·5°C. Colonies were stained with James® reagent (BioMerieux); cherry red colonies were considered *E. coli*.

Quality control

The Dutch laboratories used positive and negative controls and blank samples as prescribed by their own quality control systems. They all used *E. coli* WR1 (NCTC 13167) and *Enterobacter cloacae* WR3 (NCTC 13168) as reference strains. Quality control data did not show deviations.

Additional confirmation

LSA and CCA counts were confirmed as described, whereas DP counts were not confirmed further since the method already includes testing for indole production. LTTC37 and LTTC44 counts were confirmed as described for LTTC37 counts. Characteristic colonies from LTTC37 and LTTC44 were additionally tested for production of gas from lactose broth at 37 ± 1°C and 44 ± 0·5°C, respectively. Material from yellow and yellow with fluorescence Colilert®/18 wells was sub-cultured on MacConkey agar no. 3 (Oxoid), which was incubated for 21 ± 3 h at 36 ± 2°C and 44 ± 0·5°C, respectively. Pure cultures were confirmed as described for colonies from LTTC37 and LTTC44, respectively.

Statistical analysis

Count data were compared in pairs, using the sign test and the signed rank (Wilcoxon) test in SAS 6.12 (SAS Institute Inc., Cary, NC, USA), to test whether the pairwise difference between two values differed significantly (95% confidence level) from zero.

RESULTS

Counts

Count results from individual samples were summed per laboratory and an average count per 100 ml was calculated

for each method. Samples that gave over 80 colonies on membrane filters were excluded from the calculations; zero counts were included. All three laboratories reported higher average total coliform counts with Colilert®/18 than with any of the other methods (Table 1). Figure 1 shows that most of the Colilert®/18 data points are above the line of equality and most of the data points obtained with LSA and CCA. Differences in average *E. coli* counts were not very distinct, except for the results obtained by RIVM-MGB, who found a higher average count with the DP method (Table 1). Figure 2 illustrates these findings.

Confirmation

Confirmation tests were performed on 835 isolates from LSA, 515 from LTTC37 and 557 from LTTC44. Total coliform confirmation rates for LSA and LTTC37 are in the same order of magnitude, 86% and 80%, respectively. Elevation of the incubation temperature from 37 to 44°C resulted in an increase in the confirmation of characteristic colonies from LTTC as *E. coli* from 58 to 82%.

Only 53% of the 198 isolates from yellow Colilert®/18 wells that were tested could be confirmed as total coliforms. From some yellow but non-fluorescent wells (11%), *E. coli* was isolated, indicating the occurrence of false-negative *E. coli* results. Ninety-six percent of the 139 isolates from yellow and fluorescent Colilert®/18 wells tested were confirmed as *E. coli*.

Practical aspects

The Dutch laboratories use the LSA method routinely and find it easy to perform. Some disturbing background growth of small pink colonies was, however, reported, especially when highly contaminated surface water samples were analysed, but this was not considered a major problem. The *E. coli* DP method and the CCA method were easy to perform and gave direct and clear results; the Colilert®/18 system was found to be very convenient. All participants reported heavy background growth on membrane filters incubated on LTTC37. Background growth was reported as the percentage of the membrane filter surface that was covered with background flora. 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 LTTC at 44 ± 0·5°C slightly improved this lack of selectivity, but in 52% (80/155) of the samples, 50–100% of the membrane filter surface remained covered with disturbing background flora. Incubation at 44°C did not alter the *E. coli* count results in the analysed samples.

Statistical analysis

Colilert®/18 enumerated significantly more total coliforms than LSA, LTTC37 and CCA. Total coliform counts on LTTC37 were significantly higher than on LSA and equalled those on CCA. LTTC37, LTTC44 and DP enumerated significantly more *E. coli* than Colilert®/18 and CCA; the latter two performed similarly. *Escherichia coli* DP counts were significantly higher than LTTC44 counts, whereas differences with LTTC37 were not significant.

DISCUSSION

The presence of disturbing background growth is considered a serious drawback of the LTTC method. Heavy background growth appeared when analysing surface water

Table 1 Average total coliform and *Escherichia coli* counts in a set of samples, calculated for each laboratory and per method used. LSA counts are confirmed and LTTC37 and LTTC44 counts are additionally confirmed; all other counts are unconfirmed

		Average count per 100 ml with				
Laboratory	No. of samples	LSA	LTTC37	Colilert®	CCA	
Total coliforms						
Kiwa	38	32·5	36·6	50·1	nd*	
PWN	43	6·1	7·4	30·3	9·1	
RIVM-MGB	58	18·9	14·9	72·2	nd	
		Average count per 100 ml with				
Laboratory	No. of samples	LTTC37	LTTC44	Colilert®	CCA	DP
Escherichia coli						
Kiwa	38	20·7	18·2	14·5	nd	nd
PWN	41	5·3	3·7	4·1	2·8	nd
RIVM-MGB	58	13·7	13·8	14·0	nd	21·4

*Not done.

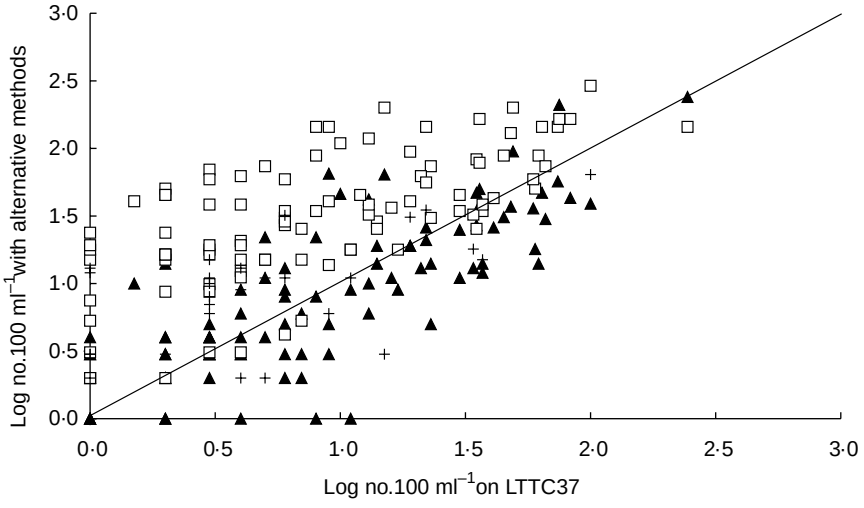

Fig. 1 Total coliform counts on LTTC37, obtained by RIVM-MGB, Kiwa and PWN, compared with counts on LSA (▲), CCA (+) and with Colilert®/18 (□)

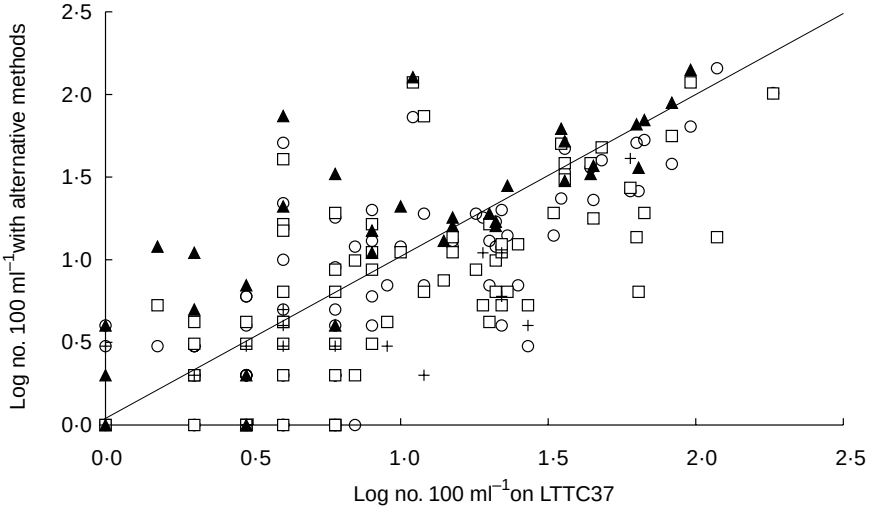

Fig. 2 *Escherichia coli* counts on LTTC37, obtained by RIVM-MGB, Kiwa and PWN, compared with counts on LTTC44 (○), CCA (+) and with Colilert®/18 (□) and DP (▲)

with high total coliform levels, but also when samples with rather low total coliform contamination levels were analysed (e.g. drinking water half products). Incubation of LTTC at 44°C slightly suppressed background growth, but it resulted in the detection of faecal coliforms due to suppression of some of the total coliforms. For enumeration of total coliforms, drinking water companies can use the LTTC method as stated in the EU DWD, but due to its lack of selectivity, this method can only be used for the monitoring of very clean drinking water samples. This forces the water companies to operate a second method for the analysis of, for example, 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. In this study, isolates from LSA and LTTC were tested for gas production from lactose. BBLB, used for isolates from LSA, is more selective than lactose broth, which was used for isolates from LTTC. The elevated selectivity of LSA combined with a more selective confirmation step has resulted in lower total coliform counts.

Colilert®/18 can be used as an 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 noted that this method enumerates a broader group of coliform bacteria than the membrane filtration methods. Coliform bacteria having both the *lacY* (for β-galactosidepermease) and *lacZ* (for β-galactosidase) genes form characteristic colonies on lactose-containing agar media such as LSA and LTTC. Coliforms that lack *lacY* but do have *lacZ* do not form characteristic colonies on those media, unless lactose is present in extremely high concentrations, but can use the Colilert®/18 substrate *ortho*-nitrophenyl-β-α- galactopyranoside (ONPG). Our results indicate that a larger

population of bacteria contains only the *lacZ* gene, resulting in higher total coliform counts with Colilert®/18. As a result of the confirmation procedure used, testing for both genes, the Colilert®/18 total coliform confirmation rate was low. Fricker *et al*. (1997) also reported the occurrence of non-lactose-fermenting coliforms; approximately 10% of the coliforms isolated from potable source water did not ferment lactose due to lack of β-galactosidepermease. The lower counts with CCA, which is based on the same detection principle as Colilert®/18, may be due to differences in the composition of the media. Colilert®/18 is considered less suitable for direct detection of *E. coli* because of the lower *E. coli* counts and the high percentage (11%) of false-negative results found in this study. These findings indicate the need for sub-culturing for *E. coli*, regardless of the presence of fluorescence in wells, and show that testing water samples for β-glucuronidase activity for *E. coli* detection will underestimate the number of *E. coli* in a sample. The present results confirm those from previous research (Schets and Havelaar 1991) in which 14% of isolated *E. coli* strains were found to be β-glucuronidase-negative at 44°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). Since the DP method detected more *E. coli* than the other methods in this study and is based on testing for indole production, it appears to be the most suitable method for detection and enumeration of *E. coli*. The higher *E. coli* counts with the DP method are not likely to be due to false-positive reactions caused by indole-positive *Plesiomonas shigelloides*, although this bacterium may cause false-positive results. In a moderate climate, it is only present at elevated water temperatures during the summer months and can be sufficiently suppressed by the addition of the vibriostatic agent O/129 to the selective isolation medium (Schets *et al*. 1998). In this trial, samples were analysed in October–November, when water temperatures are too low for *P. shigelloides* growth.

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