Literature Cover Sheet

IDEXX Library #: 6H

Topic: Colilert and Quanti-Tray Compared to the French Standard Methods

Title: "Comparison of Field and Standardized Techniques For The Enumeration of Total Coliforms and Eschericia Coli in Water"

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Highlights:

- Four methods were compared:
  1. Colilert and Quanti-Tray
  2. The French total coliform method (NF T90-414) – membrane filtration (MF) followed by 44 hours at 37C on lactose agar with triphenyl tetrazolium chloride (TTC) and Tergitol. Typical colonies are subcultured and verified as being oxidase negative.
  3. The French *E. coli* method (NF T90-414) – membrane filtration (MF) followed by 44 hours of incubation at 44C and then tested for typtophanase activity (indole positive).
  4. The French Microplate Method (MP) (AFNOR XP T90-433) 96-well microplate incubated with MUG at 44C for 36 hours
- Colilert & Quanti-Tray were found to be equivalent to MF
- MF was shown to be better than the MP method for *E. coli.*
- "The combined Colilert/Quanti-Tray system was easy to apply and allowed the quantification of *E. coli* and total coliforms within 24 hours."
- Colilert's false positive and false negative rates were found to be low: 2.4 and 3.85% respectively.

COMPARISON OF FIELD AND STANDARDIZED TECHNIQUES FOR THE

ENUMERATION OF TOTAL COLIFORMS AND ESCHERICHIA COLI IN WATER

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ABSTRACT

The French standard method (MF) for the detection of total and fecal coliforms involves membrane filtration of water through two separate membranes which are incubated on TTC and tergitol lactose agar at 37°C for total coliforms and 44°C for fecal coliforms, respectively; a test for tryptophanase (IND) can be performed to identify *E. coli* among the fecal coliforms. For surface waters a miniaturized (Most Probable Number) method (MP) also exists, based on inoculation in a liquid medium containing the MUG substrate (for revelation of glucuronidase) and incubating at 44°C. Similar methods are being standardized at the European and International levels. Quanti-Tray (IDEXX) is a device to quantify total coliforms and *E.coli* in water samples using Colilert, by dividing a 100ml sample into 51 individual wells and using the MPN technique for enumeration of total coliforms and *E.coli*.
Samples have been run in parallel with the three methods in order to compare the results.

For the enumeration of *E.coli*, the following conclusions were derived :
- no statistically significant difference between the results of the QT and MF methods
- lower results when using the MP than the MF method

In addition, the QT method was found reliable with a false-positive rate of 2.4% and a false negative rate of 3.85%.

For total coliforms the results obtained with Quanti-tray were significantly higher than the membrane filtration results. The difference between the results of the two methods could be ascribed to two causes:
(i) the growth in the QT wells of β-galactosidase positive but lactose negative coliforms, which do not form typical colonies on the lactose agar used in the MF method.
(ii) the sporadic but rare growth of oxidase positive bacteria in the QT wells (false positive results)

In this study, the QT method was easy to apply and gave satisfactory results for the enumeration of total coliforms and *E.coli* in water.

INTRODUCTION

The microbiological control of drinking water relies on the enumeration of total and fecal coliforms in most countries. For surface (recreational) waters, the parameters used to follow water quality are *Escherichia coli* and *Enterococci.*
The applicability of the thermotolerant group is being questionned since it is known that some of these bacteria do not have a fecal origin [1]. Among the thermotolerant coliform group, *E.coli* is considered to be a more specific indicator of fecal contamination [2]

The French Membrane Filtration standard method for the enumeration of coliforms relies on (1) incubation of the membrane on lactose agar containing Triphenyl tetrazolium chloride (TTC) and Tergitol at 37°C for total and 44°C for thermotolerant coliforms and, (2) confirmation of the oxidase negative character of total coliforms. For fecal coliforms, a proposed (but not compulsory) test is the search for tryptophanase activity in order to identify *E.coli* [3]. A similar method is being considered for European and international standardization.

For surface (recreational) waters, a microplate test based on incubation at 44°C and selection of β-glucuronidase positive strains has been standardized at the French level and is in the process of standardization at the international level. This method has been validated in an collaborative study by Hernandez *et al.*[4].

Beside these standard methods, the Colilert® field technique that rely on Defined Substrate Technology based on the detection of β-galactosidase and β-glucuronidase by chromogenic substances have been introduced by Edberg *et al.* [5]. This method can be used as a Presence/Absence (P/A) version, or its results can be quantified by a multiple tube Most Probable Number (MPN) version. Recently, a device called Quanti-Tray™ separating the sample into 51 wells has been as well introduced [6]. The P/A and MPN methods have been compared with US standard methods and methods in use in other countries in a number of studie [7-13].

The objective of the present study is to assess the reliability of the Colilert/Quanti-Tray method for the enumeration of total coliforms and *E. coli* and to compare the results obtained with those of the membrane filtration and the microplate methods.

MATERIALS AND METHODS

Samples

230 samples of distributed waters were used in this study. In order to find positive samples, source waters, surface waters from Paris and different other regions of France, as well as partially treated water samples (settled, sand or granulated active carbon filtered waters) were analysed.

French (AFNOR) membrane filtration method [3] (NF T90-414) (MF) and enumeration of E.coli

200 mL of water (100 mL per membrane) are filtered and the membranes are incubated for 44 hours on lactose agar with triphenyl tetrazolium chloride (TTC) and Tergitol. The incubation is carried out at 37°C for total coliforms and 44°C for thermotolerant coliforms. Typical lactose positive colonies growing at 37°C are subcultured and tested for the oxidase activity. Colonies giving a negative oxidase reaction are enumerated as total coliforms.

Typical colonies grown at 44°C were tested for tryptophanase activity (IND) by growing them in a tryptophan broth incubated overnight at 44°C. A positive reaction with James reagent proved the presence of IND activity. Unless otherwise stated, the enumeration of *E.coli* with the AFNOR method was carried out with this method. When no colony was present at 44°C, colonies at 37°C were tested for IND activity.

In order to compare the tryptophanase and β-glucuronidase reactions, for some of the samples, colonies were inoculated into sterile water and tested with the Colilert method for the MUG reaction (see below).

AFNOR microplate method [3](MP)(XP T90-433)

The sample (or its dilutions) is distributed into a 96-well microplate and incubated at 44°C for 36 hours. The results are calculated using the Most Probable Number method. The enumeration of *E.coli* is based on 4-methyl-umbelliferyl-β-D-glucuronide (MUG) hydrolysis, which shows the presence of β-glucuronidase activity.

Quanti-Tray/Colilert (QT) method

The Colilert method is based on the hydrolysis of ortho-nitrophenyl-β-D galactoside which is cleaved by the β-galactosidase present in coliforms, giving a yellow coloration, and the hydrolysis by *E.coli* of the 4-methyl-umbelliferyl-β-D-glucuronide (MUG). After hydrolysis the MUG substrate releases a blue fluorescence under UV radiation at 360 nm [5].

Quanti-Tray/colilert is a device designed to quantify total coliforms and *E.coli* in water samples using Colilert [6]. The Colilert reagent is added to the sample, and Quanti-Tray automatically divides a 100 mL sample into 51 individual wells. After incubating 24 hours at 37°C, the number of wells giving the yellow or fluorescent reaction is counted and the results evaluated with the MPN method. The quantification range is from 1 to 200.

Identification of bacteria from Quanti-Tray positive wells

50µl of broth were sampled and streaked on lactose agar with TTC and tergitol. Typical colonies (or other when typical colonies were not present) were cultured on nutrient agar and tested for the oxidase reaction. The positive Colilert reaction was verified by inoculating the bacteria into a Colilert flask filled with distilled water and incubating this flask for 24 hours at 37°C. Identification of bacteria was performed using the API 32 identification system (Bio-Mérieux, France).

RESULTS

230 distributed water samples were analysed in parallel with the AFNOR membrane filtration (MF) and the Colilert/Quanti-Tray (QT) methods. All the results were negative with both techniques.
In order to carry out a quantitative comparison of both techniques, the present study was conducted with the use of samples from river water (diluted or not), source waters, and partially treated waters within production facilities.

Comparison of methods for the enumeration of *E.coli*

1-Tryptophanase (IND) versus β-glucuronidase(MUG) tests

Two of the methods tested for *E. coli* enumeration rely on the MUG test (the microplate and the Quanti-Tray tests). In order to find out if a bias is expected in the results because of the different tests used, a preliminary experiment was carried out to compare the occurrence of the IND and MUG activities in fecal coliform strains.

487 colonies grown at 44°C on lactose agar with TTC and tergitol were tested for tryptophanase (IND) and β-glucuronidase (MUG) activities. 442 (90.6%) were found to give the same result. Among the colonies giving divergent results, 7% were IND positive and MUG negative, and 2.4% IND negative and MUG positive (Table 1). Colonies showing only one positive activity (IND or MUG) were identified as *E.coli* in 78% of the cases ; the other genera giving either IND or MUG positive activity are also listed in Table 2.

These results show a good concordance between the IND and MUG tests ; thus , the comparison of the three methods (MF, QT and MP) is not expected to be affected by the fact that different tests are used for *E.coli* enumeration.

TABLE 1 : Relationship between tryptophonase (IND) and β-Glucuronidase (MUG) activities in thermotolerant coliforms.

	IND + MUG +	IND - MUG -	IND + MUG -	IND - MUG +	TOTAL
Nb colonies	286	156	33	12	487
%	58.6 %	32 %	7 %	2.4 %	100 %
% convergence or divergence	90.6 % convergent		9.4 % divergent		

The strains tested were isolated from samples of river water or partially treated water from the Paris suburbs. The 487 strains were isolated at 44°C on lactose agar with TTC and Tergitol.

TABLE 2 : Identification of colonies with divergent IND and MUG activities.

Genera/species	IND + MUG -	IND - MUG +	Total
E.coli	25	10	35 (78%)
Enterobacter	3	1	
Klebsiella	2		
Citrobacter	2		10 (22%)
Rahnella		1	
Aeromonas	1		
Total	33	12	45 (100%)

a) Concordance/discordance analysis

Quanti-Tray (QT) versus Membrane Filtration (MF) method

62 samples were analysed in paralled with the QT method and the MF method completed with the IND and the MUG tests. Results were considered to be convergent if the Most Probable Number obtained by QT lied within the 95% confidence interval of the MF outcome (assuming Poisson distribution). The following conclusions can be derived from the results shown in Table 3:

(i) using the IND or the MUG test does not generate significant differences in the results;

(ii) 13% (IND) or 15% (MUG) of the results were found to be divergent with the QT method ; and

(iii) all of the divergent results were higher with the QT than the MF method.

Consequently, the QT method is likely to be more sensitive than the MF method for the detection of *E. coli* in a number of samples.

Microplate (MP) versus Membrane Filtration (MF) method

The same study was performed for comparing the MP with the MF method: 59 results from samples analyzed with the MP and MF methods were compared.

Out of 15 divergent results (25%), 9 results (15%) were higher with the MP method, and 6 (10%) with the MF method, regardless of the test (IND or MUG) used to complete the MF method (Table 3). This outcome confirms the above observation that no important bias is introduced by comparing methods based on different enzymatic activities. Moreover, the rate of results disagreeing with the MF outcome is significantly higher with the MP than with the QT method.

TABLE 3 : Convergence analysis Quanti-Tray (QT) and microplate (MP) versus Membrane Filtration (MF) methods for *E. coli* enumeration.

	Nb Convergent (%)	Nb Divergent QT > MF (%)	Nb divergent MF > QT (%)	Total
QT versus MF + IND	54 (87 %)	8 (13 %)	0	62
QT versus MF + MUG	53 (85 %)	9 (15 %)	0	62
MP versus MF + IND	44 (75 %)	9 (15 %)	6 (10 %)	59
MP versus MF + MUG	44 (75 %)	9 (15 %)	6 (10 %)	59

Sample of river waters or partially treated waters : 53 from the Paris suburbs, 9 from other regions of France

b) Statistical analysis

Comparison tests

The general statistics for the observations are given in Table 4.

TABLE 4 : Statistics for Quanti-Tray (QT) versus Membrane Filtration (MF) and Microplates (MP) versus Membrane Filtration (MF) comparison for *E. coli*.

Number of observation	Variable	Means of *E.coli* enumerations	Std Deviation	Median	Mini	Maxi
58	**MF**	35.07	63.83	3.0	0	270
	QT	34.16	56.69	4.75	0	200.5
65	**MF**	68.17	126.48	4.00	0	620
	MP	44.24	82.24	5.23	0	380

River water samples or partially treated water samples:
QT versus MF :46 samples from the Paris suburbs and 12 from other regions of France.
MP versus MF: 53 samples from the Paris suburbs and 12 from other regions of France.

The data were analysed using the paired t-test. The basic assumption for this test is that the difference between the outcomes from the two techniques is normally distributed. If the distribution of the difference is not significantly skewed and the number of observations is large, then the paired t-test retains its relative efficiency.

The statistics on Table 5 indicate that there is no significant difference between the MF and QT methods, whereas the difference between MP and MF methods is significant at the 1% level. Furthermore, the negative value of mean (table 5, line 2) indicates that the membrane filtration method yielded higher results.

TABLE 5 : Paired t-test analysis of results from Quanti-Tray (QT), Microplates (MP) and Membrane Filtration (MF) for the enumeration of *E. coli.*

	Number of observations	MEAN	STD	t	T prob. > | t |
QT versus MF	58	- 0.875	4.01	- 0.218	0.828
MP versus MF	65	- 23.93	8.52	- 2.89	0.006 **

** *significant at the 1 % level.*

The normality assumption of the difference variable has been tested with the Kolmogorov goodness of fit test. From this test statistic it was found that the distribution of the difference variable was not normal. To confirm the above results a nonparametric test such as the Wilcoxon signed rank test has been applied. A comparison of the relative efficiency of the paired t-test to that of the Wilcoxon signed ranks test under various population shapes indicates that the nonparametric test is a more efficient test statistic than its nonparametric counterpart in non normal situations and that the power of the Wilcoxon test in non normal situations increases with increasement in sample size [14]. The statistics in Table 6 confirm the above conclusions, i.e. : (i) the difference between the MF and QT results is not statistically significant (ii) significantly higher results are likely to be obtained with the MF than with the MP method.

TABLE 6 : Wilcoxon signed-ranks test statistics for *E. coli* enumaration by-MF, QT and MP methods.

	Number of observations	SIGN RANK	T Prob > | s |
QT versus MF	58	2.5	0.9717
MP versus MF	65	-404	0.0001 **

** *Significant at the 1 % level.*

Correlation analysis

The experimental techniques (QT versus MF and MP versus MF) are highly correlated since the Spearman correlation coefficients are significant at the 1% level (Table 7).

TABLE 7 : Spearman correlation coefficient (SCC) for Quanti-Tray (QT) versus Membrane Filtration (MF) and microplate (MP) versus Membrane Filtration (MF) methods for *E. coli* enumeration.

	Number of observations	SCC	Prob = 0
QT versus MF	58	0.9154	0.00001 **
MP versus MF	65	0.9192	0.0001 **

** *Significant at 1 % level.*

c) False positive/false negative results

52 MUG positive Quanti-Tray wells were analysed for the presence of *E.coli*. In 50 of thoses wells, the presence of *E.coli* was demonstrated ; one *Citrobacter freundii* and one *Rahnella aquatilis* were found in the two remaining MUG positive wells. Consequently, the false-positive occurrence of the MUG reaction was 2.4%.

False negative responses of the QT method were also tested by analysing 335 ONPG positive but MUG negative wells for the presence of *E.coli*. *E.coli* were found in 8 of these wells, giving a false negative rate of 3.85% .

Comparison of techniques for the enumeration of total coliforms

1.Comparison of results

29 out of 79 samples (36.7%) gave divergent results when analyzed with the QT and MF methods in parallel, 23 of them being higher with the QT method (Table 8)

TABLE 8 : Convergence analysis between Quanti-Tray (QT) and Membrane Filtration (MF) methods for total coliform enumeration.

Nb convergent	Nb divergent QT > MF	Nb divergent MF > QT	Total
50 (63.3 %)	23 (29.1 %)	6 (7.6 %)	79 (100 %)

Surface waters, source waters and partially treated waters
59 samples from the Paris suburbs, 20 samples from other region of France

The paired t-test analysis showed a difference which was significant at the 1% level, with the QT method yielding higher results. This conclusion was confirmed from the Wilcoxon signed-ranks test (Table 9). In addition, the results were highly correlated (Spearman correlation coefficient 0.656, p=0.00001).

TABLE 9 : Paired t-test analysis and Wilcoxon signed rank statisticfor the results from Quanti-Tray (QT) and Membrane Filtration (MF) methods for total coliforms.

	N#	Mean of total coliform enumerations	STD	T	T Prob > | T |	Wilcoxon signed rank statistic	Test Statistic T Prob > | S |
QT versus MF	76	+ 8.95	3.17	2.819	0.0061 **	420	0.0063 **

number of samples
Surface waters, source waters and partially treated waters
57 samples from the Paris suburbs, 19 samples from other region of France

2. Analysis of causes for divergence

In order to find out the cause for divergent results, all the positive Quanti-tray wells from 16 samples giving divergent results were analysed for the presence of coliforms. Two main causes for divergence were identified to be the presence of :
- oxidase positive bacteria (*Plesiomonas* or *Aeromonas*) giving a positive ONPG response (false positive) ; or
- lactose negative but ONPG positive bacteria, probably lacking the permease.

It should be mentioned that lactose negative bacteria do not give the typical colour on the MF method. This is not however considered to be a false positive response for coliform enumeration, since coliform bacteria are defined by the presence of β-galactosidase [15]. The results were recalculated after withdrawing the positive responses attributed to the two above causes. The MF and QT results (QT1) related to these 16 samples were shown to be statistically different. After recalculation as described above (QT2) , the QT2 and MF results were not different at the 1% or 5% levels (Table 10).

TABLE 10 : Total coliforms: Statistics for comparing Membrane Filtration (MF) with Quanti-Tray results (QT1) and Quanti-Tray results recalculated after withdrawing the number of positive wells due to oxidase positive or lactose negative bacteria (QT2)

	N#	Mean of coliform enume-rations	STD	T	T Prob > | t |	Signed rank statistic	T Prob > | s |
QT1 vs MF	16	42.39	10.11	4.19	0.0008 **	68	0.00076 **
QT2 vs MF	16	6.58	4.37	1.50	0.153	27.5	0.057

*** significant at the 1% level*

 # number of samples

Surface waters, source waters and partially treated waters

6 samples from the Paris suburbs, 10 samples from other region of France

Based on these observations, it can be concluded that the two above mentionned causes (i.e. the growth of oxidase positive and of lactose negative bacteria in the QT wells) are likely to be the main causes for generating discrepancies between the results of the MF and QT methods.

DISCUSSION AND CONCLUSIONS

The combined Colilert/Quanti-Tray system was easy to apply and allowed the quantification of *E.coli* and total coliforms in water within 24 hours.

For *E.coli*, the results with Colilert/Quanti-Tray were shown not to be significantly different from those obtained with the French membrane filtration standard method. Results of Colilert similar to the British standard method [12,13] have been found by other authors. The rate of false positive and false negative responses in this study was low : 2.4 and 3.85%, respectively. The validity of β-glucuronidase for detecting *E.coli* has been confirmed in many studies [16,17]. Hernandez *et al.* [4] found a rate of MUG negative *E.coli* strains below 1%, and Cowburn *et al.*[13] below 2%.

With the samples tested in this study, the microplate results were lower than the membrane filtration method. However, due to the high number of interfering bacteria in surface and recreational waters, the microplate method is easier to perform out and allows enumeration of a wide range of contamination levels with a small confidence interval. Thus, it remains the method of choice for this type of water.

For total coliforms, the combined Colilert/Quanti-Tray system was more sensitive than the Membrane Filtration method, mainly because of the enumeration of β-galactosidase positive but lactose negative coliforms which do not appear as typical colonies on the medium used in the membrane filtration method. The occurrence of such strains and their detection had been mentionned by Fricker [18]. Edberg *et al.*[7] also found a trend of Colilert to give a higher response than Membrane Filtration methods. However, in their study, the difference was not statistically significant.

The present study demonstrated the occurrence of false positive responses (growth of oxidase positive bacteria). The possible growth of oxidase positive bacteria: *Flavobacterium*, *Aeromonas* and *Pseudomonas* in the Colilert broth was mentionned by Edberg *et al.* [7]. However, according to these authors, growth was reported to occur only if the concentration of these bacteria was very high (more than 20 000 per mL. An enumeration of the *Aeromonas* and *Plesiomonas* in the samples where these bacteria gave ONPG positive responses was not performed. In addition, it should be mentionned that the occurrence of false positive responses with the Colilert method was rare ; only in two samples of surface waters were the results strongly biased by this cause.

The results analysed originate from samples of surface , source, and partially treated waters, because only negative outcomes were found from the analysis of 230 distributed water samples. In order to use the Colilert/Quanti-Tray system for the analysis of distributed water, more observations are needed from disinfected water samples giving a positive response. Artificially contaminated waters could help to verify the detection of stressed bacteria by this technique.

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