Use of the ISO 9308-1 procedure for the detection of *E. coli* in water utilizing two incubation temperatures and two confirmation procedures and comparison with defined substrate technology

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ABSTRACT

Disinfected and non-disinfected samples have been used to determine the accuracy of the ISO procedure (ISO 9308-1) for detection of *E. coli* in drinking water. Samples were analysed using the ISO procedure at both 36 and 44°C and using the defined substrate technology method Colilert-18®/Quanti-Tray® (Colilert-18). Utilizing the confirmation procedure described in ISO 9308-1, large numbers of false positive *E. coli* results were obtained using the ISO primary isolation procedure at 36°C. However, when glucuronidase production was used as the confirmation procedure, the 'confirmed' count of *E. coli* was lowest with ISO 9308-1 performed at 36°C. When TTC medium was incubated at 36°C confirmation using production of indole at 44°C resulted in 29% more '*E. coli*' being recovered than when confirmation was performed using production of glucuronidase. When 44°C was used as the primary isolation temperature the difference between the number of 'confirmed' *E. coli* identified using the two confirmation procedures was less than 1% and was not significant. Identification of isolates which 'confirmed' when using production of indole at 44°C as the test method but °which failed to produce β-D-glucuronidase, showed that the majority of these isolates were *Klebsiella oxytoca.*

Key words | Colilert-18, drinking water, false positive *E. coli*, ISO procedure, tergitol TTC

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INTRODUCTION

Consumption of contaminated drinking water continues to cause significant human disease in both developed and developing countries. Consequently, regulatory authorities around the world frequently review drinking water regulations, and the methodologies used to comply with these regulations. Within the European Union, the Drinking Water Directive (98/83/EC) specifies the parameters which must be tested for, the frequency of sampling and recommends the laboratory methods to be used. Frequently the specified methods are ISO standards and are listed as reference methods in the Directive. Individual member states may use procedures other than the reference methods providing they have data which demonstrates that the alternative procedure produces results that are 'at least as reliable' as the reference method (European Union 1998).

This concept is not well defined but the ISO procedure for comparing quantitative microbiological methods (ISO 17994) considers that methods which do not differ by more than 5% from the reference method may be considered equivalent. A difference of 5% has been suggested as the smallest significant value, although this level of concordance has not been arrived at by any scientific methodology. It is explained within ISO 17994 thus: 'Fixing a value for the maximum acceptable deviation (D) implies indirectly that the smallest average difference (L) to be considered significant is one half that value'. It is not clear from the European

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legislation if a method which has a sensitivity which results in more than a 5% increase in detection of the target organism should be considered 'at least as reliable'. Clearly, if the aim of the legislation is to standardize the methodology used across all member states and to enable meaningful comparisons to be made, then methods with similar sensitivity and specificity are required. However, if the principal reason for specifying a methodology is to ensure that a minimum level of sensitivity is achieved, then the use of methods which are more sensitive should be encouraged.

The methodology specified in the European Directive for the detection of *Escherichia coli* is the method described by ISO (ISO 9308-1) and is a membrane filtration procedure using a lactose-based medium (TTC medium, Chapman 1951). This medium is relatively non-selective and is only recommended for use with high quality waters. In a previous study which compared the sensitivity of the ISO 9308-1 procedure with defined substrate technology (Niemela *et al.* 2003), many laboratories reported that TTC medium failed to suppress the growth of non-target organisms resulting in overgrowth and an inability to accurately detect and enumerate coliforms and *E. coli*. The ISO procedure describes the use of the medium at 36°C for the recovery of both coliforms and *E. coli* while suggesting that a temperature of 44°C may be beneficial for the enumeration of *E. coli* when high levels of background flora are present. In practice, many laboratories have found that the use of the higher temperature is essential for the enumeration of *E. coli* and it was for this reason that in the study described by Niemela *et al.* (2003) membranes intended for the recovery of *E. coli* were incubated at 44°C. While the use of the higher temperature enabled membranes to be read more easily, it has been suggested that incubation at 44°C may significantly reduce the number of *E. coli* detected. Indeed, a recent study in Sweden and Finland concluded that the incubation of membranes at 44°C may result in an underestimation of *E. coli* of up to 30% (Slapokas, personal communication). The conclusions drawn from that study prompted the investigation described here which sought to compare the efficiency of recovery of *E. coli* from water using both 36°C and 44°C as incubation temperatures and to compare these with the recovery of *E. coli* using the defined substrate technology method, Colilert-18.

In the previous study described by Niemela *et al.* (2003) the importance of confirmation of the identity of all 'presumptive' target organisms was demonstrated. The ISO procedure for *E. coli* states that only a cytochrome oxidase test and a test for the production of indole from tryptophan at 44°C are required for confirmation of the presence of *E. coli*. All organisms which apparently ferment lactose on the primary isolation medium, give a negative cytochrome oxidase test and produce indole from tryptophan at 44°C are considered to be *E. coli*. Our experience is that there are other organisms which could fulfil these criteria (notably *Klebsiella oxytoca*) and for this reason we included an additional confirmation test, the production of the enzyme β-D-glucuronidase. While this test is not completely specific for *E. coli* (Rice *et al.* 1991; Fricker & Fricker 1996*a*) other studies (Fricker, unpublished) have suggested that this test is a more useful confirmatory test than production of indole from tryptophan at 44°C. Furthermore, these studies have shown that in excess of 99.5% of strains of *E. coli* recovered from water have the ability to produce β-D-glucuronidase and that up to 3% of strains fail to produce indole from tryptophan at 44°C.

The Colilert-18 method uses production of the enzyme β-D-glucuronidase together with production of β-D-galactosidase as being indicative of the presence of *E. coli*. Numerous studies have demonstrated that confirmation of positive results is not required (Cowburn *et al.* 1994; Fricker *et al.* 1997; Niemela *et al.* 2003). However, in this study we chose to confirm the identity of all presumptive positive results using tests for cytochrome oxidase, production of β-D-glucuronidase and production of indole from tryptophan at 44°C.

ISO publishes reference procedures against which other methods can be compared to determine their suitability for use for a particular purpose. It is important that these methods perform well and in the case of ISO 9308-1 that the sensitivity and specificity of the method is high. Methods which lack specificity or sensitivity are not suitable for use as reference procedures.

This study was designed to test the performance of the TTC medium at the two temperatures described in ISO 9308-1, 36 and 44°C and to compare them with the performance of the defined substrate technology method, Colilert-18. In addition, the use of two confirmation

procedures was compared: production of indole from tryptophan at 44°C and production of β-D-glucuronidase. Thus six actual methodologies were compared during this study.

MATERIALS AND METHODS

Samples

The samples used during this study were derived from municipal sewage effluent and were both disinfected and non-disinfected. Non-disinfected samples (76) were diluted in tap water dechlorinated by the addition of sodium thiosulphate. Samples of effluent were allowed to settle for at least one hour before being carefully decanted. Serial tenfold dilutions were made and these were examined using Colilert-18®/Quanti-Tray® (Colilert-18, IDEXX, Maine) and the effluent samples stored at 2–8°C. After incubation, dilutions which gave *E. coli* counts in the range 10–80 were used to produce samples for the comparison of the three methods.

For disinfected samples (49), sewage effluents were diluted approximately 1:10 to produce 10 litres of diluted sample and chlorine solution containing 10–15 mg free chlorine added. Samples were mixed well by shaking and placed on a magnetic stirrer. Aliquots (500 ml) of the diluted sewage/chlorine mixture were removed at one minute intervals from 5–15 minutes and the remaining chlorine neutralized with sodium thiosulphate. The disinfected samples were examined using Colilert-18®/Quanti-Tray® and stored at 2–8°C. After incubation, dilutions which gave *E. coli* counts in the range 10–80 were used to produce samples for the comparison of the three methods.

Media comparison

Dilutions yielding 10–80 MPN 100 ml^{-1} *E. coli* using the Colilert-18 system were prepared from the refrigerated samples and used to compare the performance of the ISO 9308-1 method at 36 and 44°C and the Colilert-18 method. For the ISO methods, two volumes (100 ml) were filtered through 0.45 μm membranes and incubated at 36 and 44°C for 20–24 hours on tergitol TTC agar (Oxoid, Basingstoke, UK). For Colilert-18, samples were prepared and incubated at 37°C for 18–22 hours as described in the manufacturer's instructions. The membrane filters were examined for colonies showing lactose fermentation and the number of lactose fermenting colonies recorded as the 'presumptive' count. Quanti-Trays® were examined under ultraviolet light (366 nm) and wells which were yellow and showed blue fluorescence were recorded as presumptive positive *E. coli*.

Confirmation of presumptive results

'Presumptive' *E. coli* colonies from the membranes and a loopful of Colilert-18 from fluorescing wells were subcultured to TBX (Oxoid, Basingstoke, UK) plates which were incubated at 36°C for 18–24 hours. The plates were then examined for blue-green colonies (indicating production of β-D-glucuronidase) which were subcultured to nutrient agar plates and used to inoculate tubes of tryptone water. Where no blue-green colonies were present, the most predominant colonial type was subcultured to nutrient agar and inoculated into tryptone water. The β-D-glucuronidase activity was recorded. Nutrient agar plates were incubated at 36°C and the resulting growth used to perform tests for production of cytochrome oxidase. Tubes of tryptone water were incubated at 44°C for 18–24 hours and tested for indole production with Kovacs reagent. Isolates which are cytochrome oxidase negative and produce indole from tryptone water incubated at 44°C are considered by ISO 9308–1 to be *E. coli*.

Biochemical identification of selected isolates

In order to determine the accuracy of the confirmation procedures, a number of isolates were selected for biochemical identification using the Vitek 2 instrument (Biomerieux, Basingstoke, UK). Isolates were selected according to the original primary isolation medium and the phenotypic characteristics displayed in the confirmation tests. Isolates were sorted according to phenotype and then selected at random from each phenotypic group.

Statistical approach

Preliminary statistical analysis of the data was conducted using the Wilk-Shapiro test for Normality and ANOVA for

determining any difference in the performance of the different analytical methods with chlorinated and non-chlorinated samples. The whole data set was analysed according to ISO 17994. Data from samples which yielded a confirmed result of zero or a presumptive result greater than 50 cfu 100 ml^{-1} with any of the methods was excluded from this study. It was considered that samples yielding a presumptive result of greater than 50 cfu 100 ml^{-1} by membrane filtration were unreadable. Conformity of the data with the Poisson distribution was assessed by examining the standard deviations of the root differences.

The relative difference ($x = 100[\ln(a) - \ln(b)]$) for each sample for every pair of methods and the relative performance of the methods was computed according to ISO 17994. A value of 10% difference between methods was used to determine whether statistically significant differences occurred between methods.

The effects of the two different confirmation procedures (production of indole from tryptophan at 44°C and production of β-D-glucuronidase) were examined using the sign test.

RESULTS

The normality of the relative differences data appeared satisfactory by Rankit plots. All Wilk-Shapiro coefficients were above 0.96 and no substantial deviations from the Normal distribution were observable in histograms of the relative differences. The value of the standard deviation of the root difference of two counts ($z = \sqrt{a} - \sqrt{b}$), which is expected to be about 0.7 in perfectly mixed suspensions, was found to vary in the range 0.51 − 0.70.

A one-way analysis of variance by sample type (disinfected vs. non-disinfected) showed that there was no need to analyse the two types separately (F-values 0.02–0.67 and p values of 0.41–0.88) and consequently all subsequent analyses utilized all 125 samples for which data sets were available.

Examination of the data in Table 1 suggests that the comparison of 125 samples in this study showed that TTC incubated at 36°C recovered significantly more *E. coli* than either of the other two methods. Similarly Colilert-18 recovered significantly more *E. coli* than TTC incubated at 44°C when the standard ISO 9308-1 confirmation procedure was followed. When assessed using the procedures outlined in ISO 17994, every mean value of the relative differences was significantly different from zero.

However, when using an alternative confirmation procedure (production of β-D-glucuronidase), the situation was quite different (Table 2). Colilert-18 appeared to recover significantly more confirmed *E. coli* than either other method, and TTC incubated at 44°C recovered significantly more than TTC incubated at 36°C.

Comparison of the results presented in Tables 1 and 2 clearly shows the importance of the method of confirmation, in fact a change in the confirmation procedure completely changes the outcome of the methods comparison. Further statistical analysis of the data is presented in Table 3. Here the data are presented to show which confirmation procedure gave the highest 'confirmed' counts with each method. For Colilert-18 and TTC incubated at 44°C, confirmation with production of β-D-glucuronidase as the critical test gave the highest number of confirmed isolates. For TTC incubated at 36°C production of indole

Table 1 | Mean relative differences between methods (percentage) based on 125 samples; confirmation by production of indole from tryptophan at 44°C

	Colilert-18 vs. TTC 36°C	Colilert-18 vs. TTC 44°C	TTC 44°C vs. TTC 36°C
N	125	125	125
Mean	−16.6	+7.7	−24.3
Standard deviation	43.07	41.44	55.36
Standard error of the mean	3.85	3.71	4.95
U (expanded uncertainty)	7.70	7.42	9.90
Evaluation	Different	Different	Different
Best sensitivity	TTC 36°C	Colilert-18	TTC 44°C

Table 2 | Mean relative differences between methods (percentage) based on 125 samples; confirmation by production of β-glucuronidase

	Colilert-18 vs. TTC 36°C	Colilert-18 vs. TTC 44°C	TTC 44°C vs. TTC 36°C
N	125	125	125
Mean	+25.3	+10.8	+14.5
Standard deviation	48.30	41.66	56.76
Standard error of the mean	4.32	3.73	5.08
U	8.64	7.46	10.16
Evaluation	Different	Different	Different
Best sensitivity	Colilert-18	Colilert-18	TTC 44°C

Table 3 | Descriptive statistics of the presumptive and confirmed counts; sums of counts of all 125 samples

	Total number of isolates			Number of differences			
Method	Presumptive	IND	B-GLU	IND count lower	IND count higher	No difference	Probability
Colilert-18	1,236	1,194	1,225	23	6	96	0.0012
TTC 36°C	3,487	1,405	1,048	0	106	19	0.0000
TTC 44°C	1,408	1,162	1,153	8	16	101	0.0758

IND, confirmed using production of indole at 44°C; B-GLU, confirmed using production of β-D-glucuronidase.

from tryptophan at 44°C gave the highest 'confirmed' count. Clearly the combination of the primary isolation method and the confirmation procedure was causing the difference in performance.

Table 3 shows that, for Colilert-18, 23 of 125 samples gave a higher confirmed count when β-D-glucuronidase was used as the confirmation procedure while 6 of 125 were higher when production of indole from tryptophan at 44°C was the confirmation method. For TTC incubated at 44°C, 16 samples gave higher confirmed counts when production of indole at 44°C was used as the confirmation criterion and 8 gave higher counts when production of β-D-glucuronidase was the confirmation method. However, for TTC incubated at 36°C, 106 of 125 samples produced higher results when production of indole at 44°C was used as the confirmation procedure and none was higher when production of β-D-glucuronidase was the confirmation method. The statistical comparison of the two confirmation procedures shows that, when using Colilert-18, confirmation using production of β-D-glucuronidase results in significantly more samples having a higher confirmed count of *E. coli* than when using production of indole from tryptophan at 44°C. The converse is true for TTC incubated at 36°C, while with TTC incubated at 44°C there was no significant difference in the number of samples giving higher counts whichever confirmation method was used.

According to the sign test it is clear that confirmation by indole at 44°C gave fewer positive results with Colilert-18 than confirmation by glucuronidase. The difference of 2.6% was not large but was significant. With TTC incubated at 36°C the converse was true, significantly more (29%) apparent *E. coli* were recovered by confirmation with indole at 44°C. When the primary isolation medium was incubated at 44°C the difference was small (less than 1%) and not statistically significant.

The confirmation rates differed significantly between methods. The confirmation rates obtained are shown in Table 4. It is clear from the data presented in Table 4 that the 'presumptive' results obtained with TTC incubated at 36°C cannot be used without confirmation while those

Table 4 | Confirmation rates of presumptive results

	Confirmation procedure	
Basic method	IND +	GLU +
Colilert-18	0.966	0.991
TC 36°C	0.403	0.301
TTC 44°C	0.825	0.819

obtained with Colilert-18 do not require confirmation. Incubation of TTC at 44°C gave presumptive results which approximated to the confirmed results although confirmation would be required. The similarity between the confirmed and presumptive counts may be reduced when sewage is not the primary source of contaminating organisms and environmental 'thermotolerant' coliforms are more numerous than *E. coli*.

Differences between confirmed mean counts

The paired comparisons performed according to ISO 17994 test the relative performance of two methods in isolation, without regard to the recovery of the other methods at the same time. It is of considerable interest to compare the recoveries of all methods. Table 5 shows the mean confirmed count obtained using the six procedures studied.

The highest 'confirmed' count was seen with TTC incubated at 36°C confirmed by production of indole at 44°C and the lowest with TTC incubated at 36°C and confirmed by production of β-D-glucuronidase. Clearly the confirmation procedure had a marked effect on the results.

Isolates were examined to determine the accuracy of the two confirmation procedures, production of indole from tryptophan at 44°C and production of the enzyme β-D-glucuronidase. In addition, additional isolates recovered from Colilert-18 were identified to determine the specificity of the method and the need to confirm isolates when the method is in routine use. Table 6 shows the identification obtained using the Vitek 2 instrument of isolates recovered using different primary isolation methods and displaying different phenotypic characteristics in the confirmation tests.

The isolates selected for identification were mostly chosen from those with the phenotypes IND + GLUC + and IND + GLUC-. Those with the phenotype IND- GLUC + were also identified although these were not encountered frequently and mainly from Colilert-18. Isolates with the phenotype IND + GLUC + were all identified as *E. coli*. However, those with the phenotype IND + GLUC- were primarily identified as *Klebsiella oxytoca* (94%). More importantly, of the 59 isolates which were isolated on TTC at 36°C and were indole positive, only 33 (55.9%) were identified biochemically as *E. coli*, meaning that 44.1% of indole positive isolates from TTC incubated at 36°C would be incorrectly identified as *E. coli* when using the confirmation procedures described in ISO 9308-1.When the medium was incubated at 44°C very few organisms with the phenotype IND + GLUC- were encountered, indicating that the organisms responsible for these 'false positive' *E. coli* results did not grow at 44°C on the medium. It is clear from Table 6 that the majority of organisms with the phenotype IND + GLUC- were *Klebsiella oxytoca*.

Table 7 shows the accuracy of Colilert-18 with respect to correctly identifying coliforms and *E. coli* compared with the identification given by Vitek 2. It can be seen from Table 7 that Colilert-18 performed well with regard to correctly identifying the organisms present. Only one isolate of those identified from wells indicative of the presence of a coliform organism was not identified as a coliform (0.5%). Three organisms identified as *E. coli* did not produce fluorescence in Colilert-18 giving an apparent false negative rate of 1.1% for *E. coli*. Of the total number of organisms which produced fluorescence in Colilert-18, 9 of 268 (3.4%) were not identified as *E. coli*. In fact all of these organisms were identified as *Citrobacter* spp. Further tests (based on molecular techniques) would be required to determine whether the identification of *Citrobacter* was correct.

Table 5 | Mean confirmed counts by six methods compared in this study

Method	Mean confirmed count
Colilert-18 IND 44	9.55
Colilert-18 GLUC	9.80
TTC 36 IND 44	11.24
TTC 36 GLUC	8.38
TTC 44 IND 44	9.30
TTC 44 GLUC	9.22

DISCUSSION

The detection of *E. coli* and, to a lesser extent, total coliforms in drinking water has significant consequences for water providers, health care professionals and regulators. Consequently, the choice of methodology for detecting these organisms is of paramount importance and the use of non-specific procedures, which yield a high rate of false positive reactions, can lead to complacency and inappropriate responses to data supplied by the micro-

Table 6 | Results of biochemical identification of selected isolates

Method	Confirmation results	*E. coli*	*K. oxytoca*	Other coliform	Non-coliform	Total
TTC 36	IND + GLUC +	32	0	0	0	32
TTC 36	IND + GLUC −	1	25	1	0	27
TTC 36	IND - GLUC +	2	0	2	1	5
TTC 36	IND - GLUC −	0	4	25	2	31
TTC 44	IND + GLUC +	46	0	0	0	46
TTC 44	IND + GLUC −	1	4	0	0	5
TTC 44	IND − GLUC +	2	0	1	1	4
TTC 44	IND − GLUC −	0	1	0	1	2
Colilert-18	IND + GLUC +	249	0	0	0	249
Colilert-18	IND + GLUC −	2	38	1	0	41
Colilert-18	IND − GLUC +	11	0	9	0	20
Colilert-18	IND − GLUC −	0	49	109	1	159
Total		346	121	148	6	621

biology laboratory. A plethora of methods have been described in the literature and it is impossible for individual laboratories to investigate their suitability for a specific purpose. It is a role of the International Standards Organization to develop standards for the detection of individual analytes. The procedure for the detection of *E. coli* in drinking water is specified in ISO 9308-1 and describes the use of TTC agar incubated at 36°C followed by testing for the production of cytochrome oxidase and indole production from tryptophan at 44°C. The European Drinking Water Directive specifies that this method should be utilized for the examination of drinking water for regulatory purposes unless member states supply specific data to demonstrate that an alternative method produces 'comparable' results.

TTC agar has been used as a medium for the detection and differentiation of coliforms and *E. coli* from water for many years (Chapman 1951) after the initial observation that tergitol inhibited Gram-positive bacteria (Chapman 1947). It is widely used in France and in other parts of southern Europe as the primary isolation medium for coliforms and *E. coli* for regulatory purposes and was adopted as the ISO reference procedure for detection of *E. coli* in ISO standard 9308-1. However, in previous studies, concern has been raised over the sensitivity and particularly the specificity of the medium (Niemela *et al.* 2003). In that study, several participating laboratories reported that growth of non-target organisms impeded the ability to detect *E. coli* when the medium was incubated at 36°C, while incubation at 44°C reduced background growth sufficiently to facilitate recovery of *E. coli*. Similar findings were seen during the study reported here and many samples were excluded due to high background growth. Nonetheless, the use of TTC medium incubated at 36°C yielded the lowest rate of recovery of confirmed *E. coli* when the production of β-D-glucuronidase was used as the confirmation procedure.

Clearly the observation that fewer *E. coli* were isolated at 36°C than at 44°C is an artefact caused by the growth of non-target organisms. Strains of *E. coli* which are recovered at 44°C will also grow at 36°C. The only reasonable explanation for the lower counts observed when TTC was incubated at 36°C and confirmed by production of

Table 7 | Identification of isolates from Colilert-18

Appearance in well	Colilert-18 ID	*E. coli*	Non-*E. coli* coliform	Non-coliform
Yellow no fluorescence	Coliform	3	197	1
Yellow fluorescence	*E. coli*	259	9	0

β-D-glucuronidase is the growth of non-target organisms. This may be due to inhibition of growth of *E. coli* by other organisms or a change in the appearance of *E. coli* colonies which renders them unrecognizable as *E. coli* on the primary isolation medium.

The difference in confirmation rate depending on the method used to identify the organism was of particular interest. Confirmation using production of β-D-glucuronidase as the definitive test gave slightly higher confirmation rates with Colilert-18 and TTC incubated at 44°C, while the converse was true with TTC incubated at 36°C where use of indole production as the definitive confirmatory test increased the confirmation rate by 29% over that obtained when using production of β-D-glucuronidase. It is clear by examination of Table 6 that the cause of these false positive *E. coli* results was the presence of *K. oxytoca* which has the ability to produce indole from tryptophan at 44°C. Of the isolates identified using Vitek, 26 of 59 (44.1%) indole positive organisms were non-*E. coli*. Ideally, all organisms recovered during this study would have been identified biochemically, but this was not possible for reasons of practicality and cost. Nonetheless, it is very clear that the use of TTC agar incubated at 36°C followed by confirmation using production of indole from tryptophan at 44°C results in a significant proportion of false positive *E. coli* results. The magnitude of the false positive confirmations will depend on the source of the water being examined and the frequency of occurrence of *K. oxytoca*. In this study the water samples used were derived from sewage where the ratio of *E. coli* to *K. oxytoca* is likely to be high. In other situations where *K. oxytoca* may be more predominant than *E. coli*, the extent of false positive reactions may be higher.

The realization that *K. oxytoca* can be responsible for false positive *E. coli* results is not new. Niemi and colleagues (Niemi *et al.* 2001) demonstrated that when indole production from tryptophan at 44°C was used exclusively for the confirmation of *E. coli*, significant numbers of false positive reactions occurred. They concluded that an additional or alternative method for confirmation was required. Conversely, the production of β-D-glucuronidase has been used effectively for the confirmation of *E. coli.* Venkateswaran and colleagues (Venkateswaran *et al.* 1996) showed that of 200 *E. coli* isolates only two (1%) were β-D-glucuronidase negative and that only 2 of 90 (2.2%) non-*E. coli* isolates produced the enzyme. The results presented here concur with those of Venkateswaran. Other workers have described production of β-D-glucuronidase by other non-*E. coli* organisms (Gauthier *et al.* 1991; Fricker & Fricker 1994; Alonso *et al.* 1996; Manafi 2000) but the use of this test as a confirmatory test for *E. coli* has become well accepted. Schindler (1991), in a large study, concluded that there was no benefit in using production of indole as a confirmatory test when the organism had been shown to produce β-D-glucuronidase. Similarly, Cakir *et al.* (2002) showed that 2,493 of 2,520 of β-D- glucuronidase positive organisms were indole positive. Some of the initial biochemical identifications performed in this study used a methodology which did not include production of β-D-glucuronidase in the battery of tests used. These tests often misidentified indole positive organisms as *E. coli* whereas more extensive biochemical testing, together with molecular methods (Fricker & Fricker 1996*b*; Prescott & Fricker 1999) showed that the commercial systems that included production of β-D-glucuronidase as one of the tests employed, correctly discriminated between *E. coli* and other coliforms.

While it is clear from the data presented here that organisms isolated on TTC agar, particularly when it has been incubated at 36°C, require appropriate confirmation, the use of indole production at 44°C is not an adequately specific test. Conversely, the specificity of the Colilert-18 medium has been demonstrated for both total coliforms and *E. coli* and further confirmation is not required. The current ISO procedure for the detection of *E. coli* describes a method where tergitol TTC agar is incubated at 36°C. A note in the procedure allows for an additional membrane to be incubated at 44°C for detection of *E. coli* in heavily contaminated samples. It is clear that incubation at 36°C results in an unacceptably high number of false positive isolations when the production of indole at 44°C is used as the confirmatory procedure. Therefore it is important when using the method to look for *E. coli* in drinking water that a membrane incubated at 44°C is included. Such a change significantly reduces the numbers of false positive results obtained. Alternatively, confirmation of the results using a test for production of β-D-glucuronidase can be used to minimize false positive results. A change to the ISO protocol should be adopted in order to minimize the number of erroneous results encountered by laboratories examining water for the presence of *E. coli*.

CONCLUSIONS

Incubation of TTC at 36°C followed by confirmation of 'presumptive' colonies using production of indole from tryptophan at 44°C, results in significant numbers of false positive identifications of *E. coli*.

Use of production of β-D-glucuronidase as a marker for *E. coli* gives more accurate results than use of tests for indole production at 44°C.

The ISO procedure for detection of *E. coli* in water should be modified to either require incubation at 44°C for primary isolation or to use production of β-D-glucuronidase as the confirmation procedure for 'presumptive' colonies.

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