IDEXX
Literature Cover Sheet

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Topic: Colilert, Colilert-18 and Enterolert vs Swedish Standard Methods in Drinking and Bathing Waters

Title: "Comparison of Membrane Filtration and Multiple-Tube Fermentation by the Colilert and Enterolert Methods for Detection of Waterborne Coliform Bacteria, Escherichia coli, and Enterococci Used in Drinking and Bathing Water Quality Monitoring in Southern Sweden"

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Highlights:

- On the drinking water coliform/*E. coli* study:
  - 261 well, drinking, waste and surface water samples were tested with Colilert, Colilert-18 and either MF (mEndo-LES at 35+/-1C, 24+/-4h followed by the MTF steps) or MTF (LB at 35+/-1C for 48+/-4h then BGLB at 35+/-1C for 48+/- 4h and LTLSB at 44+/-0.5C for 24+/-3h then Kovac's)
  - The methods had correlation coefficients of 0.77 for coliforms and 0.84 for *E. coli*.
- On the Bathing Water study:
  - 33 freshwater samples and 45 salt water samples were tested by Colilert, Colilert-18, Enterolert, MTF (see above) and MF (mEnterococcus agar at 44C for 48+/-4h)
  - The methods had correlation coefficients of 0.954 for *E. coli* and 0.68 for enterococci.

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Comparison of Membrane Filtration and Multiple-Tube Fermentation by the Colilert and Enterolert Methods for Detection of Waterborne Coliform Bacteria, *Escherichia coli*, and Enterococci Used in Drinking and Bathing Water Quality Monitoring in Southern Sweden

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A total of 338 water samples, 261 drinking water samples and 77 bathing water samples, obtained for routine testing were analyzed in duplicate by Swedish standard methods using multiple-tube fermentation or membrane filtration and by the Colilert and/or Enterolert methods. Water samples came from a wide variety of sources in southern Sweden (Skåne). The Colilert method was found to be more sensitive than Swedish standard methods for detecting coliform bacteria and of equal sensitivity for detecting *Escherichia coli* when all drinking water samples were grouped together. Based on these results, Swedac, the Swedish laboratory accreditation body, approved for the first time in Sweden use of the Colilert method at this laboratory for the analysis of all water sources not falling under public water regulations (A-krav). The coliform detection study of bathing water yielded anomalous results due to confirmation difficulties. *E. coli* detection in bathing water was similar by both the Colilert and Swedish standard methods as was fecal streptococcus and enterococcus detection by both the Enterolert and Swedish standard methods.

Water can be considered the foodstuff consumed in the greatest quantity around the world. Therefore, it comes as no surprise that the health risks associated with consumption of contaminated water are of great interest. Methods were being developed already in the early 1900s to assess water quality with regard to public health (7) by enumerating coliforms and *Escherichia coli* cells in water as indicators of water purity.

Typically, these tests for coliforms and *E. coli* come in two formats, a most-probable-number (MPN) multiple-tube fermentation based on lactose fermentation with production of acid and gas within 48 h and a membrane filtration method also based on lactose fermentation. If the water sample yields presumptively positive results, confirmation taking an extra 24 to 48 h of incubation time is required. *E. coli* is detected with these same methods, but often by using elevated temperature, different medium formulations, and a test for indole production in the multiple-tube fermentation method.

Coliforms and *E. coli* possess the enzyme β-D-galactosidase, giving them the ability to degrade *ortho*-nitrophenyl-β-D-galactopyranoside (ONPG), producing yellow-colored product *o*-nitrophenol. *E. coli* also has the ability to cleave methylumbelliferyl-β-glucuronide (MUG), resulting in the formation of the fluorescent product 4-methylumbelliferone (12). These characteristics were first developed for identification purposes (5, 16). They have recently been exploited by new, rapid methods for environmental testing. One such rapid method, Colilert, developed by IDEXX simultaneously detects coliforms and *E. coli* in water, within 24 h for Colilert and within 18 h for Colilert-18, with sensitivities and specificities equivalent to or better than those of the standard multiple-tube lactose fermentation method or membrane filtration method (1, 6, 8, 11). However, the Colilert and Colilert-18 methods have not been performed in parallel with and compared to Swedish standard methods.

Drinking water testing regulations in Sweden require that the membrane filtration and the multiple-tube fermentation methods be used for communal drinking water. There is concern that the Colilert method may not yield equivalent results because the methodologies are based on two different mechanisms. Furthermore, bathing water regulations prescribe membrane filtration for fecal streptococci and enterococci and the multiple-tube fermentation method for coliform bacteria and *E. coli*. IDEXX has also developed a defined substrate technology for rapid detection of enterococci in water. The method is based on the β-glucosidase activity of enterococci to produce methylumbelliferylone from 4-methylumbelliferyl-β-D-glucoside when incubated at 41°C for 24 h. Detection is also based on fluorescence at 366 nm. Likewise, there is concern that the Colilert and Enterolert methods may not yield results equivalent to those of the traditional methods when used on bathing water samples. To date there has been a study of enterococcus detection in river water performed in England (10) indicating good correlation and no significant difference in detection between the Enterolert method and traditional membrane filtration methods. This study was designed to address these concerns and to compare the performance of the Colilert and Enterolert methods with that of the Swedish standard methods for the enumeration of coliforms, *E. coli* cells, and fecal streptococci and enterococci in water.

The drinking water study consisted of a total of 261 water samples obtained for routine testing. The samples were analyzed in duplicate by both Swedish standard methods and the Colilert method. A total of 247 valid analytical results were obtained for the analysis of coliform data and 257 valid results were obtained for *E. coli* analysis. Samples were eliminated from analysis if they exceeded detection levels, making a comparison impossible. Water samples came from a wide variety of sources including raw and treated drinking waters, private well

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vaters, waste waters, and surface waters. All samples were obained from communities in southern Sweden (Skåne).

Raw water and drinking water for communities were analyzed by membrane filtration and incubation of the filter on mEndo-LES agar at 35°C for 24 ± 4 h (13). Colonies exhibiting typical characteristics for suspect coliforms were confirmed by being streaked on yeast peptone agar (YPA) for purification. YPA plates were incubated at 35°C for 18 h. Colonies were tested for an oxidase reaction. Oxidase-negative isolates were inoculated into lactose broth (LB) and lactose tryptose lauryl sulfate broth (LTLSB). LB was incubated at 35 ± 1°C for 48 ± 4 h and LTLSB was incubated at 44 ± 0.5°C for 24 ± 3 h.

Private well waters were analyzed by a five-tube, three-dilution MPN method employing LB (14). Waste and surface waters were analyzed by a five-tube, five-dilution MPN method employing LB. Ten microliters from tubes of LB exhibiting acid and gas production was loop inoculated to LTLSB and to brilliant green LB (BG). BG was incubated at 35 ± 1°C for 48 ± 4 h, and LTLSB was incubated at 44 ± 0.5°C for 24 ± 3 h followed by the addition of Kovac's reagent to LTLSB gas-positive tubes to determine the indole reaction.

The Colilert method was performed according to manufacturer's instructions. First, 100-ml sample volumes were added to IDEXX's dehydrated media in the sterile jars supplied. Samples were then shaken by hand two or three times over 5 min to dissolve the media. The contents of the jars were poured into sterile Quanti-Trays (IDEXX), trays with wells for enumeration of bacteria, and heat sealed. Quanti-Trays were incubated according to the manufacturer's instructions at 35°C for 24 h for Colilert and for 18 h for Colilert-18. After incubation, the yellow wells were counted and by using an MPN table the number of coliforms was calculated. Then the fluorescing wells (366 nm) were counted, and the number of *E. coli* cells was calculated.

Confirmation of results by Swedish standard filtration and multiple-tube fermentation methods is described above.

Although not part of the routine Colilert water testing protocol, testing of all water samples yielding positive results by the Colilert method was performed. A total of 10 to 100% of the individual presumptively coliform positive wells were confirmed, and 100% of the wells presumptively positive for *E. coli* were confirmed. Colilert results were confirmed by removing 0.5 ml of the well contents with a sterile syringe and inoculating LB and LTLSB with 0.25 ml each. In accordance with Swedish standard methods, gas and acid production in LB was the confirmation criterion for coliforms and gas and indole production in LTLSB was the criterion for *E. coli*. If the confirmation results did not agree with the Colilert results, the broths were streaked to mEndo-LES agar and isolated colonies were subcultured on yeast peptone. Purified colonies were identified with API 20E strips.

A total of 78 water samples consisting of 33 freshwater and 45 saltwater samples obtained for routine testing were analyzed in duplicate by Swedish standard methods and Colilert and Enterolert methods for the bathing water study. A total of 77 valid analytical results, 33 for freshwater and 44 for saltwater, were obtained for the analysis of coliform, *E. coli*, and enterococcus data. Samples were eliminated from analysis if they exceeded detection levels making a comparison impossible.

Bathing waters were analyzed for coliform bacteria and *E. coli* by using a five-tube, five-dilution MPN method employing LB (14). Ten microliters from tubes of LB exhibiting acid and gas production was loop inoculated to LTLSB and to BG. BG was incubated at 35 ± 1°C for 48 ± 4 h, and LTLSB was incubated at 44 ± 0.5°C for 24 ± 3 h, followed by the addition of Kovac's reagent to gas-positive tubes containing LTLSB to determine the indole reaction.

Bathing waters analyzed for the presence of fecal streptococci were tested by membrane filtration and incubation of the filter on mEnterococcus agar at 44°C for 48 ± 4 h. Although the Swedish method (15) states that confirmation is usually not necessary, colonies exhibiting typical characteristics for suspect fecal streptococci were checked by a combination of Swedish Standards Institution (SIS) and Nordisk Metodikkommitté för Livsmedel (NMKL) methods including streaking on yeast peptone agar (YPA) for purification. YPA plates were incubated at 37 ± 0.5°C for 18 h. Colonies were tested for growth in 6.5% salt brain heart infusion broth and pH 9.6 brain heart infusion broth at 37°C; colonies were also tested for a catalase reaction and Gram staining.

Colilert and Enterolert methods were performed according to manufacturer's instructions. First, 90-ml volumes of sterile deionized water were added to IDEXX's dehydrated media in the sterile jars supplied. Samples were shaken by hand two or three times over 5 min to dissolve the media. Then 10-ml water sample volumes were added to the solutions and the solutions were shaken. The contents of the jars were poured into sterile Quanti-Tray 2000 trays and heat sealed. Quanti-Trays for coliform bacteria and *E. coli* were incubated according to manufacturer's instructions at 35 ± 0.5°C for 24 h for the Colilert method and for 18 h for the Colilert-18 method. After incubation the yellow wells were counted and an MPN table was used to calculate the number of coliforms. Then the fluorescing wells (366 nm) were counted and the number of *E. coli* cells was calculated. Quanti-Tray 2000 trays for enterococci were incubated according to the manufacturer's instructions at 41 ± 0.5°C for 24 h. The fluorescing wells (366 nm) were counted, and the number of enterococci was calculated from the MPN table supplied.

The confirmation of results by Swedish standard filtration, multiple-tube fermentation, and Colilert methods was as described above. Enterolert-positive samples were extracted from the heat-sealed wells with a sterile syringe and confirmed by testing for growth in 6.5% salt brain heart infusion broth and pH 9.6 brain heart infusion broth at 37°C; samples were also tested for a catalase reaction and Gram staining.

Sample results were defined as equivalent if the Colilert result obtained lay within the confidence interval around the Swedish multiple-tube fermentation method result or if the confirmation step from mEndo-LES agar or mEnterococcus agar yielded the same number of confirmed isolates ± 0.25 log units. If a sample exceeded the sensitivities of both methods, the sample was discarded from the analysis. Statistics used for analysis included general descriptive statistics and the Spearman rank correlation coefficient.

The following definitions were used for this study. A coliform as defined by the Colilert method was an organism capable of cleaving ONPG to produce the yellow-colored product *o*-nitrophenol in the Colilert Defined Substrate Technology (DST) medium within 24 (for Colilert) or 18 h (for Colilert-18). An *E. coli* cell was defined by the Colilert method as an organism able to split MUG resulting in the formation of the fluorescent product 4-methylumbelliferone in the Colilert DST medium within 24 h (for Colilert) or 18 h (for Colilert-18) at 35 ± 1°C.

Coliforms as defined by the Swedish membrane filtration reference method were organisms which exhibited a yellow-green metallic sheen on mEndo-LES agar incubated at 35°C for 24 ± 4 h and which then were confirmed as oxidase-negative organisms producing acid and gas in LB incubated at 35 ± 1°C for 48 ± 4 h. *E. coli* cells were defined as organisms

TABLE 1. Detection of coliform bacteria in drinking water by SIS and Colilert methods

Standard method used for comparison and water type	Total no. of samples	No. of samples with:			
		Equivalent, positive results	Higher recovery by the Colilert method	Higher recovery by the standard method	Equivalent, negative results
All	247	83	51	9	104
Multiple-tube fermentation					
Storm water runoff	1	0	1	0	0
Groundwater	7	2	3	0	2
Waste leachate	2	1	0	1	0
Raw, pretreated	13	9	1	1	2
Private well	130	61	38	5	26
Surface	4	4	0	0	0
Membrane filtration					
Raw, pretreated	42	5	5	1	31
Under production	4	0	0	0	4
Treated	44	1	3	1	39

which exhibited the same characteristics as coliforms on mEndo-LES agar and which were confirmed as oxidase-negative organisms generating gas in LTLSB at 44 ± 0.5°C after 24 ± 3 h and producing a positive indole reaction.

Coliforms as defined by the Swedish MPN reference method were those organisms which exhibited acid and gas production in LB when incubated at 35 ± 1°C for 48 ± 4 h and which then produced gas in BG when incubated at 35 ± 1°C for 48 ± 4 h. *E. coli* cells were defined by the Swedish MPN reference method as those organisms generating gas in LTLSB at 44 ± 0.5°C after 24 ± 3 h and producing a positive indole reaction.

Thus, samples containing β-D-galactosidase-negative coliforms or MUG-negative *E. coli* were negative by Colilert. Samples containing nonaerogenic or non-lactose-fermenting coliforms and nonaerogenic or indole-negative *E. coli* were judged negative by standard methods.

The results indicated that the Colilert method was more sensitive than Swedish standard methods for detecting coliforms (Table 1) and of equal sensitivity for detecting *E. coli* (Table 2) when all drinking water samples were grouped together, but not always by individual water types because of the small sampling size.

Statistical analysis indicated that the results could be correlated with the Spearman rank correlation coefficient. Correlation coefficients of 0.77 and 0.84 were obtained for coliforms and *E. coli*, respectively. The paired *t* test indicated that the Colilert method was as sensitive in detecting *E. coli* as the Swedish standard method but that Colilert was slightly more sensitive in detecting coliform bacteria than the Swedish standard method ($P = 0.10$). This slightly better sensitivity in detecting coliforms mirrors performance characteristics noted in previous studies in the United States (6) and the United Kingdom (1, 11). Correlation coefficients were also similar to those observed in the aforementioned studies.

Thus, based on this equivalent performance of methods for testing drinking water, Swedac, the Swedish laboratory accreditation body, granted this laboratory site in Helsingborg approval for use of this method on all private drinking waters and waters not under public water regulations (A-krav) for the first time ever in Sweden.

Bathing water samples came from approximately 25 beaches in southern Sweden (Skåne) from both freshwater lakes and along the west coast of Sweden from approximately Helsingborg to 10 km north of Båstad.

Comparison of coliform detection between the methods was difficult due to problems confirming Colilert-positive samples. Samples of 0.25 ml were inoculated into 5 ml of LB and incubated for 48 h at 35°C as described in the drinking water

TABLE 2. Detection of *E. coli* in drinking water by SIS and Colilert methods

Standard method used for comparison and water type	Total no. of samples	No. of samples with:			
		Equivalent, positive results	Higher recovery by the Colilert method	Higher recovery by the standard method	Equivalent, negative results
All	257	28	2	1	226
Multiple-tube fermentation					
Storm water runoff	1	1	0	0	0
Groundwater	7	2	0	0	5
Waste leachate	3	2	0	0	1
Raw, pretreated	13	1	0	0	12
Private well	137	14	2	1	120
Surface	6	6	0	0	0
Membrane filtration					
Raw, pretreated	42	2	0	0	40
Under production	4	0	0	0	4
Treated	44	0	0	0	44

TABLE 3. Detection of *E. coli* in bathing water by SIS and Colilert methods

Water type	Total no. of samples	No. of samples with:			
		Equivalent, positive results	Higher recovery by the Colilert method	Higher recovery by the standard method	Equivalent, negative results[a]
All	77	60	4	5	8
Saltwater	44	36	4	3	1
Freshwater	33	24	0	2	7

[a] Both methods yielded results that were under minimum detection levels (<2 MPN/ml for the standard method, <10 MPN/ml for the Colilert method).

study. The tubes were always acid positive but often gas negative. Streaking on various coliform-selective media yielded growth, and growth on yeast peptone yielded gram-negative, oxidase-negative strains, which could sometimes be confirmed with API 20E strips as belonging to the family *Enterobacteriaceae* but frequently could not be identified (data not shown). After this had occurred with circa 25 samples, confirmation of coliform-positive results was abandoned as confirmation according to Swedish methods requires gas and acid production within 48 h at 35°C from oxidase-negative isolates. Previous studies (9, 11) used acid production from LB at 37°C plus oxidase-negative results to confirm coliforms. These different confirmation routines and definitions of what is a confirmed result can be one reason for the anomalous coliform bacterium confirmation results obtained in this study. Coliform bacteria can also maintain enzymatic activity even though they are nonculturable (3). Another potential cause for the difficulty in isolating and identifying coliforms is interference from algal β-D-galactosidase and β-D-glucuronidase (4) or from marine vibrios (2).

The Colilert method was of equal sensitivity to Swedish standard methods for detecting *E. coli* in bathing water samples (Table 3). All *E. coli*-positive Colilert results could be confirmed.

The Enterolert method was of higher sensitivity than Swedish standard methods for detecting fecal streptococci and enterococci in bathing water samples (Table 4).

Statistical analysis indicated that the bathing water results could be correlated with the Spearman rank correlation coefficient. Correlation coefficients of 0.954 and 0.68 were obtained for *E. coli* and enterococci, respectively. The paired *t* test indicated that the Colilert and the Swedish standard methods were equally sensitive in detecting *E. coli*, although slightly more enterococci were detected with Enterolert than with the Swedish method. On two occasions typical enterococcus-type colonies were isolated by the Swedish membrane filtration method, but these turned out to be false-positive reactions.

TABLE 4. Detection of fecal streptococci in bathing water by SIS and Enterolert methods

Water type	Total no. of samples	No. of samples with:			
		Equivalent, positive results	Higher recovery by the Enterolert method	Higher recovery by the standard method	Equivalent, negative results[a]
All	77	40	14	3	20
Saltwater	44	22	9	0	13
Freshwater	33	18	5	3	7

[a] Both methods yielded results that were under minimum detection levels (<1 CFU/ml for the standard method, <10 MPN/ml for the Enterolert method).

These results are similar to those of previous studies in that no significant differences in the recovery of *E. coli* and enterococci were noted (9, 10). This study yielded a higher correlation coefficient for *E. coli* than that previously reported (9) but a lower correlation coefficient for enterococci (10). This is likely a result of the smaller number of bathing water samples in this study and the different detection levels for the methods (<2 for the MPN method and <10 for the Colilert and Enterolert methods). An analysis of variance ($P \leq 0.05$) indicated no differences between saltwater and freshwater other than a higher level of *E. coli* and enterococci in the freshwater bathing samples than in saltwater samples.

The Colilert method offers several advantages compared to Swedish standard methods for drinking water analysis. Of primary importance is the public health benefit of shortened analysis and response time should coliforms or *E. coli* be present in the water. It is in the interest of both private persons owning wells and water utilities to have shorter time delays before a confirmed result is obtained. The elimination of the confirmation steps of traditional methods saves approximately 48 h and eliminates the need to either act on presumptive, nondifferentiated results or delay action in situations where remedial action is required. From the laboratory viewpoint the test is easy to use and saves time by eliminating confirmations. In theory this would create time for extra testing or more frequent analyses. Community public health officials in Sweden would benefit from more-rapid turnaround times.

Similar advantages of shortened analysis and response times compared to Swedish standard methods could result from using Colilert and Enterolert on bathing water samples. There was more difficulty in confirming coliform bacteria results than was encountered in other studies, but this may be due to different definitions of a confirmed coliform result and a greater variety of microorganisms in these samples than in drinking water.

Enterolert also possessed one significant practical advantage when used for water samples with high particulate content. It was often difficult or impossible to filter 100 ml through the membrane filter by the traditional method (15) due to membrane filter clogging by particulate matter. The particulate matter did not interfere with reading results for the Enterolert and Colilert methods.

In conclusion, the data presented in this study confirm recent studies in the United States and the United Kingdom. Performance of the Colilert method was statistically at least as good as, if not superior to, the reference Swedish multiple-tube fermentation and membrane filtration methods for determining numbers of coliforms and *E. coli* cells in drinking water. These results suggest that Colilert could be a viable alternative method for statutory water quality testing for coliforms and *E. coli* in drinking water and other types of freshwater in Sweden. Furthermore, performance of the Colilert and Enterolert methods was statistically at least as good as, if not superior to, the reference Swedish multiple-tube fermentation and membrane filtration methods for determining numbers of *E. coli* cells and enterococci in bathing water, although there were inconsistencies in confirming coliform results with these samples. Based on these findings it is recommended that a collaborative study be performed to assess performance of both Colilert and Enterolert on all water types.

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