

# Literature Cover Sheet

**IDEXX Library #:** 6G

**Topic:** Colilert-18 vs M-Endo and m-FC in Norwegian Source Waters

**Title:** “Coliform bacteria and *Eschericia coli* in Norwegian drinking water sources – Comparison of methods based on the fermentation of lactose and methods based on the activity of specific enzymes”

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**Date:** November 8, 2000

**Source:** AWWA Water Quality Technology Conference, Salt Lake City, Utah, USA

## **Highlights:**

- Source water samples were taken from 6 rivers feeding into drinking water reservoirs
- Colilert®-18 and Quanti-Tray® were compared to LES Endo agar and m-FC
- Only 60% of LES Endo agar presumptive positives could be confirmed with gas production in lactose peptone water
- only 73% of m-FC *E. coli* were confirmed with gas production in Lactose Peptone Water and indole production in tryptone water.
- 100% of Colilert-18 *E. coli* were found to be gas positive and 96% were indole positive.

**Coliform bacteria and *Escherichia coli* in Norwegian drinking water  
sources -  
Comparison of methods based on the fermentation of lactose and methods  
based on the activity of specific enzymes**

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**Introduction**

Total coliform bacteria (TC), faecal coliform bacteria (FC) and *Escherichia coli* are the most common bacterial indicators of sanitary significance in water microbiology<sup>1</sup>. Coliform organisms comprise a heterogeneous group of lactose positive bacteria belonging to the family *Enterobacteriaceae*<sup>2</sup>. The main coliform genera are *Citrobacter*, *Enterobacter*, *Escherichia* and *Klebsiella*. Members of the coliform group may be either of environmental or faecal origin<sup>3</sup>. *E. coli* is a normal faecal bacterium of humans and warm-blooded animals and indicates the potential presence of bacterial pathogens. However, the limited survival of *E. coli* in water does not make this bacterium a sufficient indicator of protozoan cysts or important pathogenic virus<sup>1</sup>.

In Norway, surface waters are the main source of drinking water. Approximately 1800 water works supply a population of 4 million people. The current National standards for TC and FC analyses are quite similar to the membrane filtration and multiple tube fermentation methods given by ISO<sup>4,5</sup>. Both methods use fermentation of lactose to indicate the presence of target bacteria.

Methods based upon the fermentation of lactose have a long tradition worldwide, and exist as international and national standards<sup>4-6</sup>. More recently, methods based on the activity of  $\beta$ -D-galactosidase (GAD) and  $\beta$ -D-glucuronidase (GUD) has been developed for TC and *E. coli*, respectively<sup>6,7</sup>. To visualise activity of these enzymes chromogenic and fluorogenic substrates are used<sup>8</sup>.

Lactose and enzyme methods use different key reactions when defining TC, FC and *E. coli*<sup>4-6</sup>. One or few specific tests comprise a small fragment of the morphological and biochemical characteristics used in systematic microbiology. When only a few reactions to identify groups of bacteria is used, the possibility of false positives and negatives must be considered. In drinking water microbiology false test results may be of great importance, because a correct diagnosis is essential for proper management.

In Norway preliminary studies with enzyme-based methods have been carried out during the last three years. The method examined is based on defined substrate technology (Colilert-18/Quantitray™). As an introduction to a quantitative comparative study, the confirmation of TC, FC and *E. coli* by lactose and indole tests was compared with the activity of the specific enzymes GAD and GUD.

## Materials and methods

### *Samples.*

In this study untreated surface water was used. Water samples from 6 rivers draining into drinking water reservoirs were examined. Samples were collected in sterile 1000 ml glass bottles, and kept refrigerated until analysis.

### *Microbiological methods*

Lactose methods. TC, FC (Thermotolerant Coliform Bacteria) were isolated by membrane filtration as outlined in ISO 9308-1<sup>4</sup>. The isolation media used for TC and FC were LES Endo agar (Difco) and mFC (Difco), respectively.

Enzyme method. Colilert-18/QuantaTray™ (IDEXX) was used for the examination of CB and EC. The manufacturer's description of the method was followed and the incubation was carried out at 37±1°C for 18 hours.

### *Confirmation*

Coliform bacteria. All dark red colonies on LES Endo agar were regarded as possible coliform colonies. According to the common definition of presumptive coliform colonies on LES Endo agar<sup>4</sup>, this study includes both typical (dark red with golden sheen) and atypical (dark red without golden sheen) colonies. Dark red colonies were split into two groups, typical and atypical. Each of these groups was subdivided as large (1-2mm) or small (<1mm) colonies. The colony diameter was related to the 3x3mm grid on the membrane filters used. Membrane filters containing up to 30 target colonies were chosen in the confirmation study. The proportion between the number of large and small golden sheen colonies was shown to vary in different water sources. In two different samples the percent small colonies were 38% and 78%. A total of 237 colonies belonging to the four morphologic groups were picked randomly, subcultured on Nutrient agar and examined for the production cytochrome oxidase. Two hundred and nineteen oxidase negative colonies were examined for gas production from lactose in lactose peptone water (LPW) at 37°C for 24-48 hours. In addition, 106 oxidase negative colonies, classified in the same morphologic groups as defined above, were examined for β-D-galactosidase activity, (ONPG-test). Sixteen strains of anaerogenic bacteria from LES Endo agar were identified using MICRO-ID® (Remel, Santa Fe Drive, Lenexa, USA).

Colilert. The intensity of the yellow colour that developed after incubation showed considerable variation. The majority of positive wells in this study were classified as light yellow, and the reference colour comparator (IDEXX) was used to differentiate between positive and negative wells. A total number of 334 yellow wells with different colour intensity were examined. From each well 0,1 ml was transferred to LPW and examined for production of acid and gas from lactose.

Faecal coliforms. Blue or blue-green colonies on mFC agar were divided in two morphologic groups, large (2-3mm) and small (~1mm). A total of 162 colonies were picked randomly and examined for the production of gas from lactose in LPW, and indole production at 44°C for 24 hours. Presumptive *E. coli* was defined as FC that produced gas in LPW and indole from tryptophane within 24 h within at 44°C<sup>4</sup>. In addition, 171 colonies were tested for β-D-glucuronidase activity, (MUG-test).

*E. coli.* Eighty-four yellow and fluorescent Colilert-18 wells were examined for acid and gas production from lactose in LPW and the production of indole from tryptophane in tryptone water at 44°C.

## Results

The confirmation of morphologically different colonies from LES Endo agar with lactose fermentation and activity of  $\beta$ -D-galactosidase are summarised in Table 1 and 3.

TABLE 1. *Coliform bacteria. Confirmation of oxidase negative colonies from LES Endo agar with gas production from lactose in lactose peptone water.*

Colony morphology	Number	Confirmed	% Confirmed
Dark red - golden sheen			
- 1-3mm	99	93	94
- < 1mm	43	8	19
- Total	142	101	71
Dark red - no sheen			
- 1-3mm	44	29	66
- < 1mm	33	2	6
- Total	77	31	40
Total	219	132	60



Using gas production in LPW as criterion for verification, the results showed marked differences related to colony morphology (Table 1). Seventy one percent of the examined dark red colonies with a golden sheen were confirmed. For large and small colonies the percent was 94 and 19, respectively. Among the 40% confirmed dark red colonies without a golden sheen, 66% of the large colonies, but only 6% of the small colonies were confirmed. Sixteen anaerogenic strains isolated from colonies with different morphology on LES Endo agar were identified by MICRO-id (Table 2).

TABLE 2. *Identification of oxidase negative, anaerogenic bacteria from LES Endo agar.*

Colony morphology	Diagnosis	Number
Dark red - golden sheen	<i>Citrobacter diversus</i>	4
	<i>Enterobacter agglomerans</i>	2
	<i>Enterobacter cloacae</i>	1
	<i>Hafnia alvei</i>	1
	<i>Klebsiella ozaenae</i>	2
Dark red - no sheen	<i>Citrobacter freundii</i>	2
	<i>Enterobacter agglomerans</i>	1
	<i>Klebsiella oxytoca</i>	1
	<i>Serratia rubroidea</i>	2

The MICRO-ID results showed that 13 of the strains were classified as *Citrobacter*, *Enterobacter* or *Klebsiella*, the common coliform genera. Two strains were identified as *Serratia rubroidea*, and one strain as *Hafnia alvei*.

When  $\beta$ -D-galactosidase was used as criterion for confirmation of morphologically different colonies on LES Endo agar, 96% of the examined colonies showed a positive ONPG test (Table 3).

TABLE 3. *Coliform bacteria. Confirmation of oxidase negative colonies from LES Endo agar with  $\beta$ -D-galactosidase activity, ONPG test.*

Colony morphology	Number	Confirmed	% Confirmed
Dark red - golden sheen			
- 1-3mm	52	52	100
- <1mm	23	23	100
- Total	75	75	100
Dark red - no sheen			
- 1-3mm	21	21	100
- <1mm	10	6	60
- Total	31	27	87
Total	106	102	96

All dark red colonies with a golden sheen were ONPG positive, and no difference between large and small colonies was observed. The results from the ONPG test on dark red colonies without a golden sheen showed that 87% of the examined colonies were ONPG positive. For this group of colonies 100% of the large and 60% of the small colonies were confirmed, respectively.

The ability of  $\beta$ -D-galactosidase positive bacteria (coliform bacteria) from Colilert-18 to produce acid and gas in LPW was examined, and 67% of the 334 examined yellow wells were acid and gas positive. In this study, the fermenting properties of GAD-positive bacteria were not related to the intensity of the yellow colour produced in the positive wells.

*E. coli* was confirmed from blue or blue-green colonies on mFC agar with lactose fermentation combined with indole production, and  $\beta$ -D-glucuronidase activity (Table 4 and 5). Gas production in LPW was observed in 81% of the large colonies, and 88% were indole positive (Table 4).

TABLE 4. *Escherichia coli*. Confirmation of blue or blue green colonies from mFC agar with gas production in Lactose Peptone Water and indole production in tryptone water at 44 °C.

Morphology	Number	Confirmed	% Confirmed
Large	147	114	78
Small	15	4	27
Total	162	118	73

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When both lactose fermentation and indole production were used as criteria for confirmation, 78% of the examined large colonies were verified as *E. coli*. Sixteen, (11%) indole positive colonies were anaerogenic. From blue or blue green colonies on mFC classified as small, 87% produced acid and gas in LPW, and 27% were indole positive. Four small indole positive colonies were all gas positive in LPW.

When  $\beta$ -D-glucuronidase activity was used to confirm the presence of *E. coli*, 89% of large blue or blue-green colonies, and 36% small colonies were verified (Table 5).

TABLE 5. *Escherichia coli*. Confirmation of blue or blue-green colonies on mFC agar with  $\beta$ -D-glucuronidase activity, MUG-test.

Morphology	Number	Confirmed	% Confirmed
Large	171	152	89
Small	11	4	36
Total	182	156	86

From 84 yellow and fluorescent wells in Colilert-18, the ability of MUG-positive bacteria (*E. coli*) to produce gas in LPW and indole in tryptone water was examined. All wells examined were gas positive and 96% were indole positive.

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### Discussion

In this study the percent confirmed presumptive coliform colonies from LES Endo agar was higher using  $\beta$ -D-galactosidase compared with gas production from lactose. The number of anaerogenic bacteria from yellow Colilert-18 wells was in agreement with the results from LES Endo agar. The identification of gas negative coliform bacteria (Table 3) indicates that anaerogenic strains of coliform bacteria are common in Norwegian surface waters, and that the use of one single criterion to confirm target bacteria may result in false negative interpretations.

On LES Endo agar presumptive coliform colonies are defined as dark red with a golden sheen<sup>4</sup>. The ability of coliform bacteria to ferment lactose was related to colony morphology on this medium. In this study, 71% of the golden sheen colonies were confirmed as coliform bacteria on basis of lactose fermentation. The number of anaerogenic small colonies was high, 81%, but only 6% of the large colonies were gas negative. Non-confirmed bacteria produced a weak acid reaction in LPW, but the most distinct observation was the negative gas reaction. The ability of coliform bacteria to produce the typical golden sheen does not seem to be constant, and the appearance of golden sheen from small colonies can be difficult to observe. When dark red colonies without a golden sheen on LES Endo agar were examined for gas production in LPW, 40 % of these non-target colonies fulfilled the criterion for coliform bacteria. This observation indicates a considerable number of false negative colonies when the definition in ISO 9308-1 is used<sup>4</sup>.

Geldreich<sup>9</sup> studied the production of gas from lactose at 44.5°C with coliform bacteria from different sources. From human faeces 2.5% anaerogenic strains were observed. From soil and stormwater samples there was 20.4 and 38.6% anaerogenic strains, respectively. According to Bergey's Manual<sup>3</sup> the ability of different coliform bacteria to ferment lactose vary from 26 to 100%. The results in the present study indicate a high degree of environmental coliform bacteria in the samples examined.

When golden sheen colonies from LES Endo agar were examined for  $\beta$ -D-galactosidase activity, this study showed that 100% of the strains were ONPG-positive. No difference was observed between large and small colonies. The  $\beta$ -D-galactosidase test reflects the potential ability for coliform bacteria to ferment lactose<sup>2</sup>. Despite the presence of the enzyme, many environmental strains of coliform bacteria are unable to carry out complete fermentation of lactose<sup>9</sup>. Colilert-18 detects ONPG-positive coliform bacteria with different abilities to ferment lactose. When 334 yellow wells were examined for gas production in LPW, 33% of the wells contained anaerogenic bacteria. This result agrees quite well with the frequency of colonies from LES Endo agar that produced gas in LPW. Gas production from lactose as criterion for confirmation of presumptive coliform colonies on LES Endo agar<sup>4</sup> eliminates anaerogenic strains, and consequently affects the verified coliform count from surface waters. Edberg<sup>10</sup> concluded that Colilert was favourable for source water when enumeration is required.

Interestingly, the two morphologically different groups of target colonies on LES Endo agar showed marked differences in the ability to produce gas from lactose. One question that can be raised is to what extent large colonies are composed by faecal strains and small colonies of environmental strains. The results from Geldreich<sup>9</sup> point to differences in the frequency of anaerogenic coliform bacteria from various sources. The present results indicate that the observed ratio between large (mainly faecal) and small (mainly environmental) colonies may suggest an environmental or faecal origin of the coliform population.

Confirmation of *E. coli* from mFC agar includes both gas production from lactose in LPW and indole production from tryptophane at 44°C<sup>4</sup>. In this study 73% of the target colonies were confirmed. From large colonies 88% were indole positive. Of the 130 indole positive strains, 11% were anaerogenic. According to Bergey's Manual<sup>3</sup> the percent lactose-negative *E. coli* is 0-10%. Field studies report approximately 10% anaerogenic strains of *E. coli*<sup>10,11</sup>. Using  $\beta$ -D-glucuronidase to confirm *E. coli* from mFC, 86% showed a positive MUG-test. When yellow and fluorescent wells on Colilert-18 were examined for indole production from tryptophane, 4% indole negative *E. coli* were found. *E. coli* identification made on Colilert-18 include both anaerogenic and indole negative strains, and the diagnosis is made after 18 hours of incubation. A rapid diagnosis of faecal indicator bacteria is important demand in drinking water microbiology.

In a proposed revised ISO 9308-1<sup>12</sup> gas production from lactose is removed as a confirmatory criterion for both coliform bacteria and *E. coli*. In this standard coliform bacteria are defined as oxidase negative, lactose positive bacteria on Lactose TTC agar with Tergitol 7. *E. coli* is defined as indole-positive coliform bacteria. The results from the present investigation support the removal of gas production from lactose as a confirmatory step.

This study has focused on the identification of coliform bacteria and *E. coli*, and the presence of false positive reactions. There is need for further examinations, primarily on the rate of false negative reactions. A quantitative comparative study between the membrane filtration methods and Colilert-18 has been carried out recently (unpublished data). Preliminary results indicate that the recovery of target bacteria is comparable in the two methods. There was a higher recovery of coliform bacteria with Colilert-18, and equal amounts of *E. coli* were recovered. These results are consistent with a large comparative study carried out in Great Britain<sup>13</sup> and a study carried out in Sweden<sup>14</sup>.

Coliform bacteria and *E. coli* are regularly present in Norwegian surface waters used as drinking water. The results in the present study show that Colilert-18 is a specific method to identify coliform bacteria and *E. coli*. However, information from colony morphology on solid media is lost. Using methods based on the GAD and GUD principle, the interpretation of morphologic different colonies on LES Endo agar, anaerogenic strains of coliform bacteria and *E. coli* and indole negative *E. coli* are avoided.

The results from the present study agree with several comparative studies on recovery of coliform bacteria and *E. coli* from various types of waters, showing that Colilert-18 is both specific and sensitive. The Colilert-18/Quantitray™ method seems to have advantages compared with traditional membrane filtration methods, and should be considered when alternative methods are evaluated.

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