Gas Chromatographic Presumptive Test for Coliform Bacteria in Water

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A gas chromatographic procedure which shows promise as a presumptive test for coliform bacteria in water is described. Total coliform bacteria concentrations were determined from the incubation times at 37 C required for ethanol to be produced. Fecal coliform densities were determined in a similar manner at 44.5 C. The culture medium was filter sterilized M-9 salts supplemented with 1% lactose, 0.1% Casamino Acids, and 0.1% yeast extract. Best results were obtained when the initial total coliform concentrations were 5 per ml or higher and when fecal coliform concentrations were 50 per ml or higher. Minimum detection times at these concentrations were 9 and 12 h, respectively.

Detection and enumeration of coliform bacteria of fecal and nonfecal origin are among the most commonly used microbiological indicators of quality in food and water. Whereas there are numerous analytical methods for detecting coliform bacteria quantitatively, the primarily used techniques require up to 2 days before results are available. Such delays impose obvious health hazzards, and, in the case of food, economic burdens since microbiological results often determine safety for human consumption or storage time. There is, then, need for techniques which make possible rapid assessment of the microbiological quality of water and foods.

In an earlier report from this laboratory the detection of Escherichia coli by gas chromatography was described (3). The method was based on the Eijkman concept and detection was accomplished by analyzing for the presence of metabolically produced ethanol in cultures incubated at 44.5 C. According to the Eijkman concept coliform bacteria can be differentiated on the basis of growth at elevated temperatures. Coliform bacteria of fecal origin grow and ferment lactose at 44 to 46 C and are usually + + + + or + + + + + + IMViC types, whereas coliform bacteria from other sources are rarely able to grow in the 44 to 46 C temperature range (4). In this report we describe the application of the Eijkman principle to a gas chromatographic method for the presumptive detection and estimation of coliform bacteria. By incubating cultures inoculated with water samples at 37 or 44.5 C total coliform and fecal coliform concentrations can be estimated.

MATERIALS AND METHODS

The pure cultures used in this study were a laboratory strain of Escherichia coli B and coliform bacteria isolated from the effluent of the local sewage treatment plant. In experiments involving water samples, the samples were inoculated directly into culture medium, and the indigenous coliforms were allowed to grow out.

The growth medium was M-9 salt mixture (6) supplemented with 1.0% lactose, 0.1% Casamino Acids, and 0.1% yeast extract at pH 7.2. The medium was made double strength and sterilized by membrane filtration. For experiments 1 ml of medium was inoculated with an equal volume of water sample, and the cultures were incubated in water baths at 37 or 44.5 C. Best results were obtained when the cultures were stirred with small Teflon-coated magnets and an immersible magnetic stirrer.

Coliform concentrations in water samples were determined by the multiple-tube most-probable number method (MPN) in lauryl sulfate tryptose broth (LST) or by the membrane filter procedure (MFC) with M-endobroth both as described in Standard Methods (1). The presence of coliforms was verified by plating positive cultures on EMB agar and by IMViC tests.

Gas chromatographic analyses of ethanol in the cultures were done with a Varian model 1200 gas chromatograph equipped with a hydrogen flame ionization detector. The column was 5 feet by 1/8 inch outer diameter (ca. 152 by 0.32 cm) aluminum tubing packed with 80/100 mesh porapak Q8 (Waters Associates) with nitrogen at 30 cm\(^2\) per min as the carrier gas. Other operating parameters were a column temperature of 170 C, injector temperature of 190 C, and the detector at 200 C. The electrometer was operated at 1 × 10\(^{-19}\) A full scale on a 1-mV 10-inch (ca. 25 cm) recorder. The instrument was standardized daily by injecting 2 µl of a 0.01% aqueous ethanol solution.
Detector response was linear between 0 and 0.01% ethanol (Fig. 1). Analysis of cultures was accomplished by injecting 2 μl of culture directly onto the column. The retention time for ethanol was 45 s. Between each analysis the column was purged with 5 μl of water.

RESULTS

An important consideration in high-sensitivity gas chromatography is a sample free of interfering compounds contributed by the culture medium. In preliminary experiments it was found that autoclaving the culture medium resulted in thermal degradation of lactose and yeast extract which resulted in numerous volatile compounds which chromatographed with metabolically produced ethanol. Filter sterilization minimized interfering compounds contributed by the medium although as shown in the 0- to 420-min panel in Fig. 2 some background peaks were present. The compounds associated with these peaks were found to be associated with lactose; fortunately, the peaks did not interfere with ethanol analysis (Fig. 2). As indicated, one of the chromatographable medium compounds was identified as acetic acid.

Also shown in Fig. 2 are the chromatographic changes in the culture medium caused by coliform metabolic activity. It can be seen that concomitant with ethanol formation the acetic acid peak also increased in size. However, acetic acid formation was not used as an indicator of coliform bacteria since it was difficult to reliably detect small increases in peak size. Occasionally a water sample contained microorganisms which grew in the test cultures but did not produce ethanol or other detectable chromatographic changes in the medium. Subculture showed these organisms were non-lactose

Fig. 1. Detector response to different ethanol concentrations. Chromatographic operating conditions are given in the text.

Fig. 2. Chromatographic changes in 1 ml of culture medium inoculated in 1 ml of a water sample initially containing approximately 80 coliform bacteria per ml. Incubation temperature was 37°C. The water sample contained fecal and nonfecal coliforms.
fermentors. Apparently utilization of other medium constituents did not produce metabolic end products which were detectable under the analytical conditions used in this study. Similarly, when lactose was omitted from the culture medium no chromatographic changes in the medium as a result of microbial activity were detected in 24-h cultures incubated at 37 or 44.5 C.

Whereas our initial research indicated that laboratory cultures of E. coli produced ethanol from lactose fermentation, it was of interest to determine if this was also true for coliform bacteria isolated from contaminated water. Water samples taken over a 10-day period were inoculated directly into duplicate LST broth tubes, and the tubes were incubated for 24 h at 37 and 44.5 C. Coliform bacteria were isolated from positive tubes on EMB agar, and the isolates were IMViC typed. The coliform isolates were inoculated into the M-9 lactose medium described above and incubated at 37 and 44.5 C. Results summarized in Table 1 show that at 37 C all the organisms grew and produced ethanol. At 44.5 C all the +++ group and 16 out of 18 of the ++-- group grew and produced ethanol. Only 8 out of 68 coliform isolates with --++ characteristics grew and produced ethanol at 44.5 C. The data in Table 1 show that relatively few --++ coliform bacteria grew at 44.5 C in the M-9 lactose medium. However, when temperature-tolerant organisms with nonfecal IMViC classifications are present in water samples a positive ethanol test results. Consequently, in gas chromatographic experiments ethanol-positive 44.5 C samples were streaked on EMB agar and colonies with the typical sheen tested on citrate agar or IMViC typed. Only when a positive culture was shown to contain citrate-negative coliforms or coliforms with +++-- or ++-- characteristics was the test considered positive. In practice we did not find any 44.5 C positive samples which did not contain fecal coliforms, although mixed flora composed of both fecal and nonfecal types were not uncommon.

The kinetics of formation of ethanol were the same for pure cultures of E. coli and for cultures inoculated with water samples (Fig. 3). However, as would be expected the time period between inoculation of the cultures and the formation of detectable amounts of ethanol was dependent on the initial coliform concentration of the inoculum. Under the analytical conditions used in the study the minimum detectable ethanol concentration in cultures was 1 to 2 ng. Theoretically the minimum detectable ethanol concentration was 0.1 ng; however, we were not able to attain this level of sensitivity due to baseline instability.

To determine the relationship between the initial coliform count and the incubation time required for ethanol formation, 1-ml aliquots of diluted water samples were inoculated into equal volumes of 2× culture media and incubated at 37 or 44.5 C. Total coliform concentrations in water samples were determined by MPN tests in LST broth at 37 C after 48 h and fecal coliform concentrations were determined by the MFC method at 44.5 C after 24 h. Typical 37 C results (Table 2) show that coliform analysis by ethanol formation was comparable to results from MPN tests. Samples for gas chromatographic analysis for ethanol were taken at 30-min intervals, and the tests were terminated when ethanol was detected or after 12 h. The detection times for ethanol were plotted as functions of the corresponding results of the MPN or MFC tests. Results of these experi-

| IMViC type | Isolation temp (C) | No. tested | 37 C | 44.5 C |
|------------|-------------------|------------|------|--------|
| +++--      | 37                | 42         | 42   | 42     |
| +++--      | 44.5              | 32         | 32   | 32     |
| +---       | 7                 | 7          | 7    | 5      |
| +---       | 44.5              | 11         | 11   | 11     |
| ++--       | 37                | 54         | 54   | 2      |
| --++       | 44.5              | 14         | 14   | 6      |

**Fig. 3.** Comparison of ethanol formation at 37 C in 1 ml of culture medium inoculated with 1 ml of a water sample (A) and 1 ml of washed E. coli B cells (B). The water sample initially contained approximately 100 coliforms. The E. coli B suspension contained approximately 100 organisms per ml.
Table 2. Coliform analysis of a water sample* by the MPN test and ethanol formation at 37 C

| Method    | Coliform-positive tubes |
|-----------|-------------------------|
|           | 10<sup>6</sup> | 10<sup>5</sup> | 10<sup>4</sup> |
| MPN<sup>b</sup> | 3/3       | 2/3        | 0/3          |
| Ethanol<sup>c</sup> | 3/3       | 2/3        | 0/3          |

* Water sample MPN = 9.3 per ml.
<sup>b</sup> Tubes were inoculated with 1 ml of each dilution.
<sup>c</sup> Ethanol was determined by gas chromatography. Cultures contained 1 ml of water sample and 1 ml of 2 × M-9 lactose medium.

ments (Fig. 4) show that initial coliform counts were exponential functions of the incubation times required for ethanol formation. Coliform detection required less time at 37 than at 44.5 C for reasons which are discussed below. With respect to sensitivity, at 37 C it was possible to consistently detect differences in the incubation time required for ethanol formation when the initial number of organisms differed by a factor of two or more at initial counts of 10 per ml or higher. When the initial count was less than 10 coliforms per ml of sample, variability tended to reduce reliability. Detection of small numbers of fecal coliforms at 44.5 C was more variable than total coliform detection at 37 C. In part this was due to longer lag times at 44.5 C. In addition we found that when the initial fecal coliform count was less than 10 per ml the cultures frequently failed to grow at all. Apparently, fecal coliforms in water samples have difficulty in adapting to 44.5 C; however, we found that once adapted to this temperature the organisms grew at the same rates as at 37 C.

It is worth noting that in the experiments summarized in Fig. 4 the presence of coliform bacteria was confirmed in 79 out of 80 water samples positive for ethanol. As might be expected, coliforms in water samples cultured at 37 C were usually mixed IMViC types, whereas at 44.5 C fecal IMViC types predominated. At 37 C the tests could be terminated after 12 h of incubation since cultures negative for ethanol after 12 h were negative for coliform bacteria at 24 and 48 h. At 44.5 C the cutoff time for ethanol formation was 14 h.

**DISCUSSION**

The gas chromatographic procedure described here could serve as a useful presumptive test in cases where a specific coliform limit is established. For example if the limit was 10 coliforms or less per ml then a test culture containing 1 ml of the sample should not contain detectable ethanol after 9 h of incubation at 37 C or 11 h at 44.5 C. Cultures which were positive for ethanol could be confirmed by conventional methods. If greater sensitivity or shorter detection times are required samples can be concentrated by centrifugation or membrane filtration. We have obtained the same detection times with concentrated samples as with unconcentrated samples both at the same initial coliform density. Shorter detection times would also be possible by operating the gas chromatograph at maximum analytical sensitivity. If this had been possible coliform detection times would have been shortened by 1 h or more. With respect to rapidity the gas chromatographic method detection times are comparable with those reported for radiorespirometry (2, 5) and calorimetry (5).

At present, incubation at 44.5 C is not completely selective for fecal coliform bacteria since mixed coliform populations were occasionally isolated from water samples cultured at 44.5 C.
in M-9 lactose medium. This was also seen when samples were cultured in LST broth for MPN determinations at 44.5°C. It is not known if the nonfecal coliform organisms were fermenting lactose and producing ethanol at 44.5°C or growing synergistically with fecal types.

An advantage of the method described here is that coliform analyses can be done without sample preparation. Direct analysis of culture medium is possible since the column packing has ideal characteristics for separating polar compounds such as water and alcohols. Columns are relatively unaffected by accumulation of nonvolatile culture medium constituents or bacterial cell components since peak shapes and retention times were the same after 2,800 analyses on the same column. We are aware of the possibilities of automating the gas chromatographic procedure and this problem is currently under investigation. We are also investigating application of the method to determine coliform contents and total bacterial counts in foods. The results of these studies will be reported later.

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