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Rapid Enumeration of Fecal Coliforms in Water by a Colorimetric β-Galactosidase Assay

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The colorimetric β-galactosidase assay is based upon the enzymatic hydrolysis of the substrate *o*-nitrophenyl-β-D-galactoside (ONPG) by fecal coliforms. This technique provides an estimate of the fecal coliform concentration within 8 to 20 h. A 100-ml portion of test sample was passed through a 0.45-μm membrane filter. This filter was then incubated at 37°C for 1 h in EC medium followed by the addition of filter-sterilized ONPG. The incubation was continued at 44.5°C until a half-maximum absorbance (at 420 nm) was reached. The time between the start of incubation and the half-maximum absorbance was proportional to the concentration of fecal coliforms present. *Escherichia coli* (K-12) was used to measure the kinetics of substrate hydrolysis and the response time of different cell concentrations. High cell densities produced an immediate response, whereas 1 cell/ml will produce a response in less than 20 h. In field studies in which samples were taken from a range of grossly polluted streams to relatively clean lake water, a linear correlation between ONPG hydrolysis times and fecal coliform most-probable-number values was established. A total of 302 isolates randomly selected from positive ONPG-EC media, which were derived from 11 different habitats, were identified as *E. coli* (96.69%), *Enterobacter cloacae* (2.32%), *Klebsiella pneumoniae* (0.66%), and *Citrobacter freundii* (0.33%).

There is often an urgent need to evaluate quickly the bacterial quality of water. Natural disasters, malfunctions in potable water distribution and sanitary waste transmission systems, or contamination of recreation waters from urban and agricultural wastes in runoff illustrate some instances where the 22 or 72 h required to complete the fecal coliform analysis is too long to analyze the problem and select the most reasonable solution. The appearance of sewage sludge at Long Island beaches in June 1976 just hours before the summer season began is an example of such a situation.

The fecal coliform index has proven to be one of the most important indicators of health hazard due to fecal pollution in water (6). This report presents a technique in which the fecal coliform concentration can be estimated within 8 to 20 h. The possibility exists that the assay time may be reduced by an increase in sample volume or decrease in incubation temperature. The assay makes use of the chromogenic substance *o*-nitrophenyl-β-D-galactoside (ONPG) in conjunction with the 44.5°C temperature requirement of the fecal coliform test.

The basis for this assay is a modification of Eijkman's observation that coliform bacteria of fecal origin could produce gas in a glucose medium at 46°C, whereas coliform bacteria of nonfecal origin are rarely able to do so (5). The Eijkman test is positive when gas generated by the formic hydrogenlyase system (10) is detected, whereas in this assay the enzymatic hydrolysis of ONPG is employed. Theoretically, the time required to achieve detectable ONPG hydrolysis in the inoculated medium is proportional to the quantity of fecal coliforms in the inoculum.

MATERIALS AND METHODS

Escherichia coli strain ATCC 14948 (K-12) was the principal laboratory culture used in the initial phase of this study, although strains ATCC 9637 and 9723 were also used. Fecal coliforms isolated from various water sources were identified as *E. coli* by growth and gas production in EC broth in 24 h at 44.5°C, characteristic colonies on EMB agar, and IMViC type ++−−. Fecal coliform isolates that were not IMViC ++−− or which formed uncharacteristic colonies on EMB agar were identified by the use of the Analytical Profiles Index (API 20E, Analytab Products, Inc., Plainview, N.Y.).

EC medium (Difco, 24.67 g/liter) with 0.022 M ONPG (Sigma) was used throughout the study. EC medium was chosen because: the medium contains bile salts, which inhibit most spore-forming or gram-positive bacteria capable of fermenting lactose; it is a well buffered, complex medium that meets the require-

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ments of β-galactosidase synthesis; the medium has little color interference with the ONPG hydrolysis product; and *E. coli* grows very rapidly in this medium. The ONPG was dissolved in distilled water, filter sterilized, and added to the medium shortly before inoculation to minimize nonenzymatic hydrolysis of the galactoside. All other media were Difco products. The hypothesis was tested using a more dilute EC medium than is normally used in fecal coliform measurement (1) to reduce optical interference and possibly the time required for preincubation.

Portions of 1 ml each of various dilutions of a washed, 12-h culture of *E. coli* K-12 were added to 9 ml of the ONPG-EC medium in standard screw-cap culture tubes. The initial cell density was determined by a spread-plate procedure on plate count agar after serial dilution in phosphate-buffered water blanks (1). All inoculated tubes were incubated in a circulating water bath with temperature (44.5 ± 0.1°C) calibrated against a National Bureau of Standards certified thermometer. The rate of color production from the ONPG substrate was determined by periodically removing culture tubes. The enzymatic reaction was stopped by the addition of 3 ml of 1 M Na_2CO_3, and the absorbance was read on a Bausch and Lomb Spectronic 20 at a wavelength of 420 nm. The 100% transmission value for the spectrophotometer was set with uninoculated ONPG-EC medium, which was incubated at 44.5°C for a period equal to that of the inoculated media to account for spontaneous ONPG hydrolysis. The Na_2CO_3 developed the full color of the liberated *o*-nitrophenol and eliminated any turbidity caused by the bacterial cells in the medium. The experiment was repeated with different concentrations of bacteria to evaluate how time of color production was related to inoculum size, which varied from 10^2 to 10^6 cells.

To evaluate the ONPG method in natural aquatic systems, grab samples were tested from water sources ranging from a grossly polluted, low-flow stream to lake and river water of very high bacteriological quality. Three to five replicates of 100-ml replicates of each sample were filtered through 0.45-μm Gelman GN-6 filters. The filters were aseptically folded and inserted into culture tubes containing EC medium. The samples were then preincubated at 37°C for 1 h before beginning the 44.5°C incubation. Filter-sterilized ONPG was added at the end of the preincubation period. The incubation at 44.5°C was terminated when the absorbance was judged to be between 0.1 and 1.0. The experiment was complete when: a portion of the medium was streaked on plate count agar for subsequent culture identification; the reaction was stopped; the length of incubation was recorded; and the absorbance was measured as described above.

Each water sample that was examined by the ONPG method was also tested for fecal coliform density by traditional most-probable-number (MPN) procedures (1). A variety of isolates from the confirmed fecal coliform MPN tubes were tested in the ONPG-EC medium.

RESULTS

The rate of ONPG hydrolysis after different concentrations of *E. coli* K-12 were added to the ONPG-EC medium and incubated at 44.5°C is shown in Fig. 1. A sigmoid curve was shown in all cases except in the inoculum containing 10^6 cells/ml. In these cases the inoculum was sufficient to permit immediate observable enzymatic hydrolysis, which yielded a sigmoid curve after a slowly ascending front edge. Detectable levels of the *o*-nitrophenyl indicator were produced by the 10^5, 10^4, 10^3, and 10^2 cells/ml inocula at approximately 4.25, 6, 7.75, and 9.25 h, respectively. The time from first detectable color appearance to complete color development was approximately 1 h. The curves shown in Fig. 1 (excluding 6.0×10^6 and 3.4×10^6 cells/ml) were used to generate a standard curve using an SAS General Linear Models nonlinear regression procedure (3). Since the R-square value of the regression analysis was 0.893, it was concluded that the rate of ONPG hydrolysis in the ONPG-EC medium was consistent once a minimum number of cells was present.

The equation that describes the shape of the standard curve is:

$$\text{absorbance} = \frac{1.3312}{1 + e^{(2.24397 - 0.03216 \text{ time [h]})}}$$

Membrane filters were very useful in concentrating fecal coliforms from grab samples from the different aquatic habitats tested, but the usual cautions must be applied to prevent damage to the cells (9). The relative recovery effectiveness of different types of membrane filters was not studied. Furthermore, the fecal coliforms collected from the different habitats may be in different states of physiological vigor, which are quite different from that of the fresh, mid-log *E. coli* K-12 cells used to generate the data in Fig. 1. Therefore, a preincubation period at 37°C (before ONPG addition) was used to minimize the physiological shock of the medium and high temperature. A preincubation period of 1 h was more desirable than one of 2, 3, or 4 h because there was more statistical variation between repetitions in the longer incubation pe-

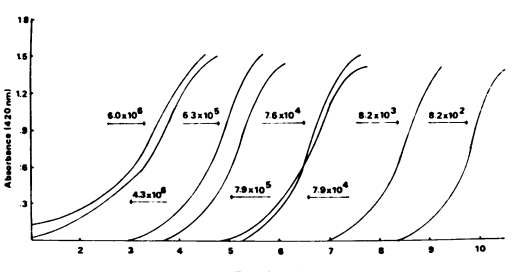

FIG. 1. *Rates of ONPG hydrolysis by varying sized inocula of E. coli K-12 in ONPG-EC medium at 44.5°C.*

riods; there was no evidence that a significantly greater quantity of cells was recovered with the longer preincubation period. Without preincubation, samples of very low fecal coliform levels (1 to 50/100 ml) sometimes yielded negative results after 24 to 48 h of incubation. Samples of 100 ml each from these relatively clean habitats always yielded ONPG activity after a long lag when the 1-h preincubation step was used.

Preliminary studies showed that ONPG hydrolysis of mixed cultures of fecal coliforms from raw sewage and the habitats used in this study proceeded at rates similar to those of *E. coli* K-12. Therefore, to estimate the fecal coliform content of a given water sample, the color development of the inoculated ONPG-EC medium was arrested (preferably near the midpoint), the length of incubation was recorded, and the absorbance was measured. This absorbance was located on the standard curve described above, and the difference between it and the half-maximum absorbance (0.7) was noted. This difference can be translated on the X axis to a time correction so that all values are compared to the time required to reach half-maximum absorbance after preincubation. This correction permits the investigator to check samples at set intervals, such as every hour, and still obtain consistent results.

To test the hypothesis that the time required to reach half-maximum absorbance of ONPG hydrolysis in the ONPG-EC medium is dependent on the number of fecal coliforms in a natural sample, a variety of samples were examined and the results were compared to traditional MPN fecal coliform values obtained with the same samples. These data are shown in Fig. 2. Each point represents the average of three to five replicates. Table 1 briefly describes the habitats from which the samples were collected and presents the numerical data from which Fig. 2 was constructed. The linear equation:

$$\text{time (h) of half-maximum ONPG absorbance} = 17.468 - 2.341 \log_{10} \text{fecal coliform MPN}$$

was determined by an SAS General Linear Models linear regression procedure (3). An R-square value of 0.602 was computed, and the 95% confidence limits of the regression line are shown. Data points n and s were deleted from the regression analysis for reasons to be discussed. Those data points, which depart from the linear function, reflect the strengths and weaknesses of the two methods used and will be discussed in that light.

The usefulness of the ONPG method is based on the premise that the test is relatively specific yet does not exclude bacteria that fit the accepted fecal coliform definition. To test this hypothesis, bacteria that grew in the ONPG-EC medium at 44.5°C were isolated by streak plate isolation on plate count agar and identified. These data (Table 2) indicate that the test is relatively specific for *E. coli*, although some members of the genera *Enterobacter, Citrobacter,* and *Klebsiella* were isolated. To determine if this test was underestimating the fecal coliform concentration, bacteria isolated on plate count agar from the fecal coliform MPN procedure were examined for their ability to grow and hydrolyze ONPG in the ONPG-EC medium at 44.5°C. Of the 656 isolates that produced gas in EC broth at 44.5°C, 638 grew in the ONPG-EC medium and hydrolyzed ONPG at a rate comparable to that of *E. coli* K-12. A total of 18 isolates were "atypical" in that only a slight color production was noted and very little growth was observed. Of these 18 atypical isolates, 11 were identified as *K. pneumoniae*, 2 as *E. coli*, and the remaining 5 were not identified. Some strains of *E. coli* such as ATCC 9723 and 9637 responded to the ONPG-EC medium in the same manner as the *K. pneumoniae* isolates.

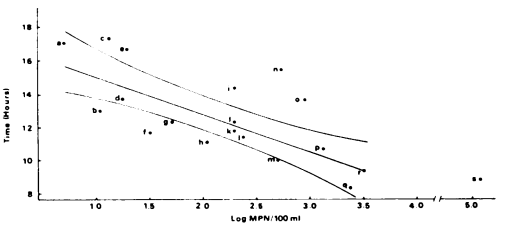

FIG. 2. *Regression analysis of fecal coliform MPN and time required for half-maximum absorbance after preincubation. The 19 samples were taken from 12 different habitats. Each point is an average of three to five repetitions. R-square = 0.602. Each lower case letter refers to a sample obtained from habitats described in footnote b, Table 1.*

DISCUSSION

The hypothesis that a short-time fecal coliform test could be developed based on the rate of hydrolysis of ONPG was established with the laboratory data derived from cultures of *E. coli* K-12 and field data from water samples taken from various habitats. The test was selective for the detection of *E. coli*, and an estimate of the fecal coliforms population could be obtained in 8 to 20 h at densities frequently encountered in natural samples. Very consistent results were obtained with replicate runs of *E. coli* K-12, indicating that the experimental procedure was effective for detecting very high and low numbers of bacteria in log growth phase.

In the regression analysis of field samples (Fig.

2), the values that fall outside the 95% confidence limits of the regression line reflect some of the differences in physiological conditions and genetic diversity within the group of bacteria that qualify as fecal coliforms. Some of the habitats (c, e) had higher fecal coliform MPN values than would be predicted by the ONPG hydrolysis time. These habitats share the common characteristic of being distant from the most likely sources of fecal contamination. Furthermore, the water at these locations was well aerated and low in temperature (less than 5°C) and organic nutrients. Several other habitats (i, o), which have been designated as critical areas in the Potomac River Basin because of high total and fecal coliform levels (15), also had higher MPN values than predicted by the ONPG hydrolysis time. Both of these habitats were located a short distance below sewage treatment plants discharging chlorinated effluent. It is possible that the fecal coliforms from these sites were damaged by chlorination or some toxic agent.

TABLE 1. *Description of sites examined in field study*

	Location[a]	Habitat[b]	Fecal coliforms/100 ml	Half-maximum ONPG activity	
				Time (h)	Standard deviation
a	North River	1	5	16.93	2.360
b	Claytor Lake	2	11	13.15	1.084
c	Back Creek	4	13	17.28	3.060
d	Lewis Creek	3	17	13.52	0.295
e	Shenandoah River	4	20	16.67	3.396
f	Factory	5	33	11.67	0.059
g	Claytor Lake	2	49	12.17	0.234
h	Hawksbill Creek	6	110	11.02	0.308
i	South River	1	200	14.20	0.488
j	Duck Pond	7	200	12.07	0.313
k	Duck Pond	7	200	11.92	0.133
l	Claytor Lake	2	240	11.48	0.359
m	Duck Pond	7	500	10.13	0.223
n	Front Royal	8	700	15.33	0.559
o	North Fork of Shenandoah River	1	918	13.57	0.687
p	North Fork of Shenandoah River	1	1,300	10.40	0.209
q	Factory	5	2,300	8.28	0.119
r	Hawksbill Creek	6	3,300	9.23	0.243
s	Front Royal	8	130,000	9.13	0.533

[a] Locations a, c, d, e, f, h, i, n, o, p, q, r, and s are in the Shenandoah River Basin of Virginia. Locations b, g, j, k, l, and m are in or near Blacksburg, Va.

[b] Habitat codes: (1) Well aerated, downstream of chlorinated sewage treatment plant effluent; (2) water supply and hydroelectric reservoir; (3) moderate flow stream, receives agricultural runoff and urban sewage discharge; (4) fast flowing, well aerated stream, far from any known point source; (5) textile plant effluent, warm temperature, high nutrient; (6) moderate flow stream, low dissolved oxygen, high levels of tannery wastes; (7) small, highly eutrophic pond, receives extensive urban and rural runoff; (8) low flow, foul-smelling stream receiving cannery wastes.

TABLE 2. *Indentification of randomly picked isolates from ONPG-EC medium by the IMViC procedure and Analytical Profiles Index (API 20E)*

Species	No. isolated	% of total
E. coli	292	96.69
E. cloacae	7	2.32
K. pneumoniae	2	0.66
C. freundii	1	0.33

Two samples (f, q) were obtained from habitats that had a water temperature of approximately 30°C and a high organic and inorganic nutrient status. The fecal coliforms in these samples were apparently near their optimum physiological state.

The data points n and s were deleted from the regression analysis because of the unusual microbial population at this site. These samples were taken from a foul-smelling, anoxic stream receiving cannery wastes. *K. pneumoniae* was found to dominate the fecal coliform MPN culture tubes. No *K. pneumoniae* strains were isolated from the ONPG-EC tubes from the same site. In these tubes *E. coli* was the only organism isolated. *K. pneumoniae* has been found in high numbers in forest-related samples and fresh produce (4). Strains of *K. pneumoniae*, which were of human or paper mill effluent origin were not tested to determine if they were ONPG positive at 44.5°C. A more extensive testing program with field samples and pure cultures of known history must be examined before the specificity of this method can be evaluated.

The possibility exists that the relationship between ONPG hydrolysis time and the real number of fecal coliforms present (approximated by the MPN) is nonlinear, but a far greater number of samples covering a broader range of fecal coliform values than was possible in this investigation are needed to prove or disprove this. However, analysis of the available data indicates that the relationship is linear (F test fails to reject linearity at $\alpha = 0.50$). A polynomial regression using a χ^2 term failed to significantly improve the R-square term.

Geldreich et al. (8) have shown that 96.4% of the human fecal coliform strains they examined were detected by EC broth when incubated at

44.5°C. Our data (Table 2) are in agreement with Geldreich's findings and suggest that the ONPG test is specific for *E. coli* yet does not exclude a significant portion of the fecal coliform population. The non-*E. coli* isolates are not likely to impair the usefulness of the test since their growth in the ONPG-EC medium was slow and their ONPG hydrolysis was limited.

The autocytotoxic effects of some β-D-galactosides have been described (11; F. Whitehouse and H. Proctor, Bacteriol. Proc., p. 148, 1969). We observed the autocytotoxic phenomenon with *E. coli* ATCC strains 9723 and 9637 as well as with the *K. pneumoniae* isolates. The effect was not noted with *E. coli* K-12, and very few field isolates exhibited autocytotoxicity. Most of the *E. coli* isolates demonstrated growth patterns similar to that shown by *E. coli* K-12. *E. coli* ATCC strains 9723 and 9637 did produce luxuriant growth and ONPG hydrolysis at temperatures of up to 43°C, but not at 44.5°C. Van Donsel et al. (D. J. Van Donsel, R. M. Twedt, and E. E. Geldreich, Bacteriol. Proc., p. 25, 1969) have shown the optimum temperature for growth of fecal coliforms to be between 40 and 44°C. Our preliminary experiments with *E. coli* K-12 were conducted at 43.5°C, and we observed significantly faster rates of ONPG hydrolysis at that temperature. This study was done at 44.5°C because we wished to retain maximum specificity, but a lower temperature is clearly more desirable if specificity is not reduced. It may be possible to gain a 10 to 15% decrease in the incubation time by lowering the temperature 1 to 2°C.

In addition to modifying the temperature of incubation, this method may be improved by modifying the composition of the medium or making the medium less selective during the preincubation phase. The preincubation procedure used in this study appeared to be adequate for the recovery of fecal coliforms from most water samples. However, the data obtained from samples below sewage treatment plants, where cells had experienced possible chlorine damage, indicated that growth and ONPG hydrolysis were delayed because some cells were physiologically debilitated. The procedure proposed by Lin (13) to improve membrane filter recovery of fecal coliforms from chlorinated sewage effluents could be adapted to the ONPG method. Simply withholding bile salts from the EC medium until after the preincubation may allow greater recovery of damaged cells. It should also be possible to achieve more rapid results by increasing the quantity of water filtered per test. In this study, 100-ml amounts were filtered with little or no difficulty, but some samples were too turbid to permit a larger sample size. A high-volume sampling apparatus such as that described by Levin et al. (12) should give more rapid results with no decrease in sensitivity.

Reasoner and Geldreich (16) have stated that the cost per test for rapid bacteriological assays of water may necessarily be higher than those for conventional methods. This would certainly be the case for radiometric methods using labeled substrates (2), the glutamate decarboxylase method (17), or the gas chromatographic presumptive test for coliforms (14). The ONPG method is not only highly specific but, since a relatively small amount of media is required and the cost of ONPG is negligible, its cost may be equal to or lower than that of conventional methods.

The ONPG method described shows promise as a rapid, highly specific test for fecal contamination in water. It is especially promising for use when a specific fecal coliform limit has been established. For example, if the limit is to be 200 fecal coliforms or less per 100 ml, no detectable enzymatic hydrolysis of the ONPG should be observed in less than 11 h (using the protocol of this investigation). The test is a departure from most recent rapid methods in that simplicity, low cost, and specificity have been retained while providing an assessment of the bacteriological quality of water in a shorter time period than is possible with conventional methods.

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