RAPID COLORIMETRIC TECHNIQUE FOR THE ENUMERATION OF FECAL COLIFORMS IN WATER

by

LELAND STANFORD WARREN

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APPROVED:

R. E. Benoit, Chairman

N. R. Krieg | C. W. Randall

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INTRODUCTION

There is often an urgent need to evaluate the bacterial quality of water quickly. 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 to 72 hours 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 (FC) index has proven to be one of the most important indicators of health hazard due to fecal pollution in water (7). In this report we present a technique in which the FC concentration can be estimated within 8 to 20 hours. 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, ortho-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 non-fecal origin are rarely able to do so (5). The Eijkman test is positive when gas generated by the formic

hydrogenylase 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 principle laboratory culture used in the initial phase of this study, although strains ATCC 9637 and 9723 were also used. FC isolated from various water sources were identified as *E. coli* by growth and gas production in EC broth in 24 hours at 44.5 C, characteristic colonies on EMB agar, and IMViC type ++--. FC isolates which were not IMViC ++-- or formed uncharacteristic colonies on EMB agar were identified by the use of the Analytical Profiles Index (API 20E, Analytab Products, Inc.).

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 sporeforming or Gram positive bacteria capable of fermenting lactose; it is a well buffered, complex medium which meets the requirements 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 in order to minimize the non-enzymatic hydrolysis of the galactoside.

All other media: lactose broth, plate count agar, tryptophane broth, MR-VP broth, eosine methylene blue agar, and Simmons citrate agar; were purchased from Difco.

One ml portions of various dilutions of a washed, 12 hour 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 (0.000312 M) buffered water blanks. The inoculated medium was incubated in a Forma Scientific Model 2095 water bath and circulator set at 44.5 C. The water temperature fluctuated less than 0.1 C. The bath temperature was set with a thermometer calibrated at the National Bureau of Standards. 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 molar Na_2CO_3 and the absorbance read on a Bausch and Lomb Spectronic 20 at a wavelength of 420 nm. The 100 percent transmission value for the spectrophotometer was set with uninoculated ONPG/EC medium which was incubated at 44.5 C for a period equal to the inoculated media in order 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 in order to evaluate how time of color production was related to inoculum size, which varied from 10^2 to 10^6 cells.

In order to evaluate the ONPG method in natural aquatic systems, grab samples were tested from water sources which

varied from a grossly polluted, low flow stream to lake and river water of very high bacteriological quality. Three to five replicates of 100 ml quantities of each sample was filtered through 0.45 μm Gelman GN-6 filters using an all glass filtration system. The filters were aseptically folded and inserted into culture tubes containing EC medium. The samples were then preincubated at 37 C for a prescribed time 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 recorded; and the absorbance measured as described above.

Each water sample that was examined by the ONPG method was also tested for FC density using the traditional MPN procedures (1). A variety of isolates from the confirmed FC MPN tubes were tested in the ONPG/EC medium.

RESULTS

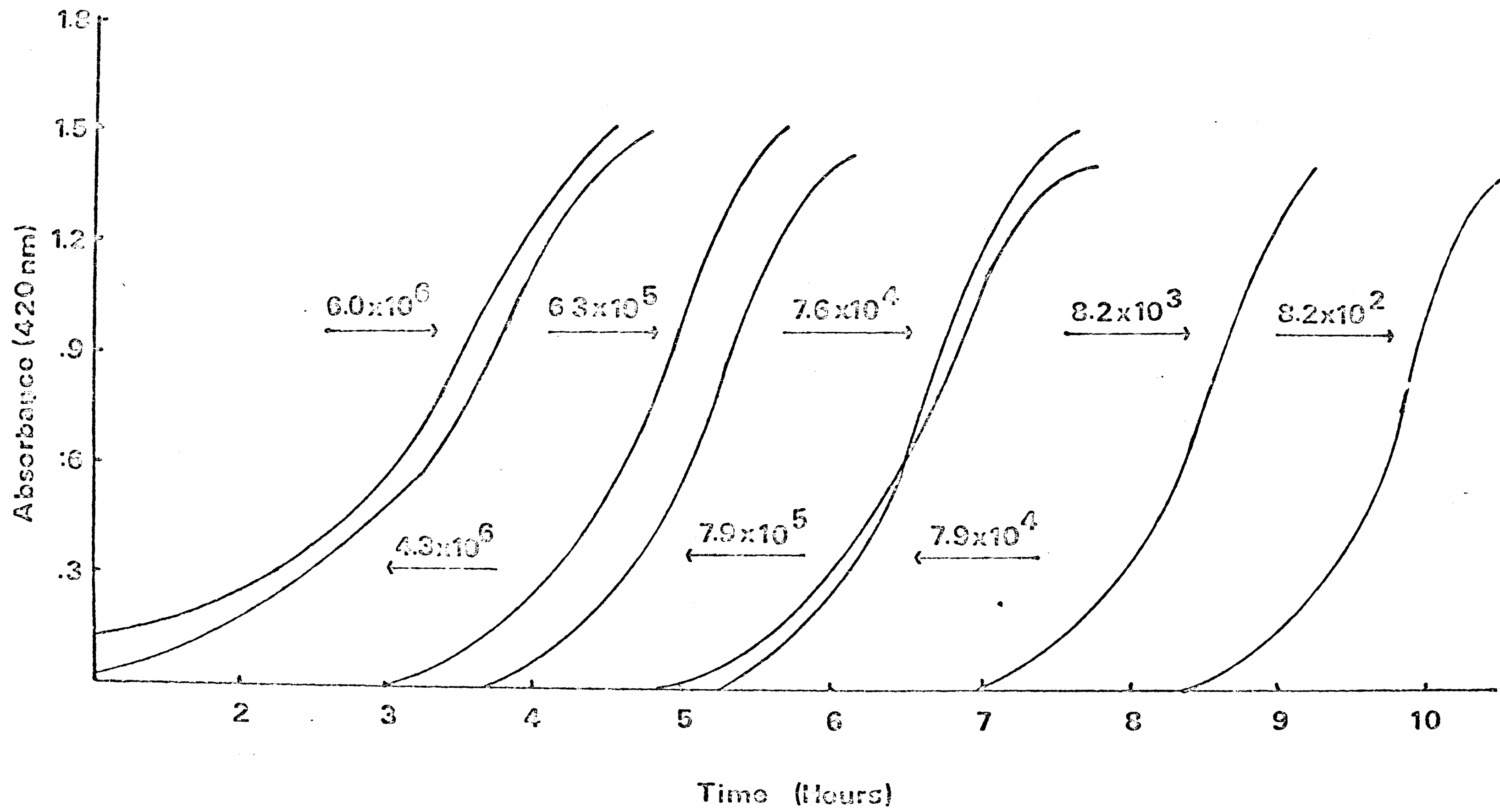
The rate of ONPG hydrolysis after different concentrations of E. coli K-12 were added to the ONPG/EC medium and incubated at 44.5 C is shown in Fig. 1. A sigmoid curve was shown in all cases except the 10^6 cells/ml inoculum. In these cases the inoculum was sufficient to permit immediate observable enzymatic hydrolysis which yielded a sigmoid curve after a slowly ascending front edge. Detectable levels of the ONP indicator were produced by the 10^5, 10^4, 10^3, and 10^2 cell/ml inocula at approximately 4.25, 6, 7.75 and 9.25 hours respectively. The time from first detectable color appearance to complete color development was approximately 1 hr. The curves shown in Fig. 1 (excluding 6.0 and 3.4 x 10^6 cells/ml) were used to generate a standard curve using a SAS General Linear Models non-linear 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 were present.

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

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

Membrane filters were very useful in concentrating FC from grab samples from the different aquatic habitats tested,

Figure 1. Rates of ONPG hydrolysis by different size inocula of *E. coli* K-12 in ONPG/EC medium at 44.5 C.



1.8
1.5
1.2
.9
.6
.3
Absorbance (420 nm)
6.0x10^6
6.3x10^5
7.6x10^4
8.2x10^3
8.2x10^2
4.3x10^6
7.9x10^5
7.9x10^4
2
3
4
5
6
7
8
9
10
Time (Hours)

but the usual cautions must be applied to prevent damage to the cells (8). The relative effectiveness of different types of membrane filters was not attempted in this study. Furthermore, the FC collected from the different habitats may be in different states of physiological vigor which are quite different than 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 hr was more desirable than 2, 3, or 4 hr preincubation because there was more statistical variation between repetitions in the longer incubation periods; and there was no evidence that a significantly greater quantity of cells were recovered with the longer preincubation period. Without preincubation, samples of very low FC levels (1 - 50/100 ml) sometimes yielded negative results after 24 to 48 hr of incubation. One hundred ml samples from these relatively clean habitats always yielded ONPG activity after a long lag when the one hr preincubation step was used.

Preliminary studies showed that ONPG hydrolysis of mixed cultures of FC 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 FC content of a given water sample the color development of the inoculated ONPG/EC medium was arrested (preferably near the mid-point), the length of

incubation recorded and the absorbance measured. This absorbance was located on the standard curve described above and the difference between it and the 1/2 maximum absorbance (0.7) 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 1/2 maximum absorbance. This correction permits the experimentor to check samples at set intervals such as every hour and still obtain consistent results.

In order to test the hypothesis that the time required to reach 1/2 maximum absorbance of ONPG hydrolysis in the ONPG/EC medium is dependent on the number of FC in a natural sample, a variety of samples were examined and the results compared to traditional MPN FC values obtained with the same samples. These data are shown in Fig. 2. Each point represents the average of 3 to 5 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:

$$\text{time (hr) of 1/2 maximum ONPG absorbance} = 17.468 - 2.341 \log_{10} \text{FC MPN}$$

was determined by a 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

Figure 2. Regression analysis of fecal coliform MPN and time required for 1/2 maximum absorbance. The 19 samples were taken from 12 different habitats. Each point is an average of 3 to 5 repetitions. R-square = 0.602. Each small case letter refers to a sample obtained from habitats described in Table 1.

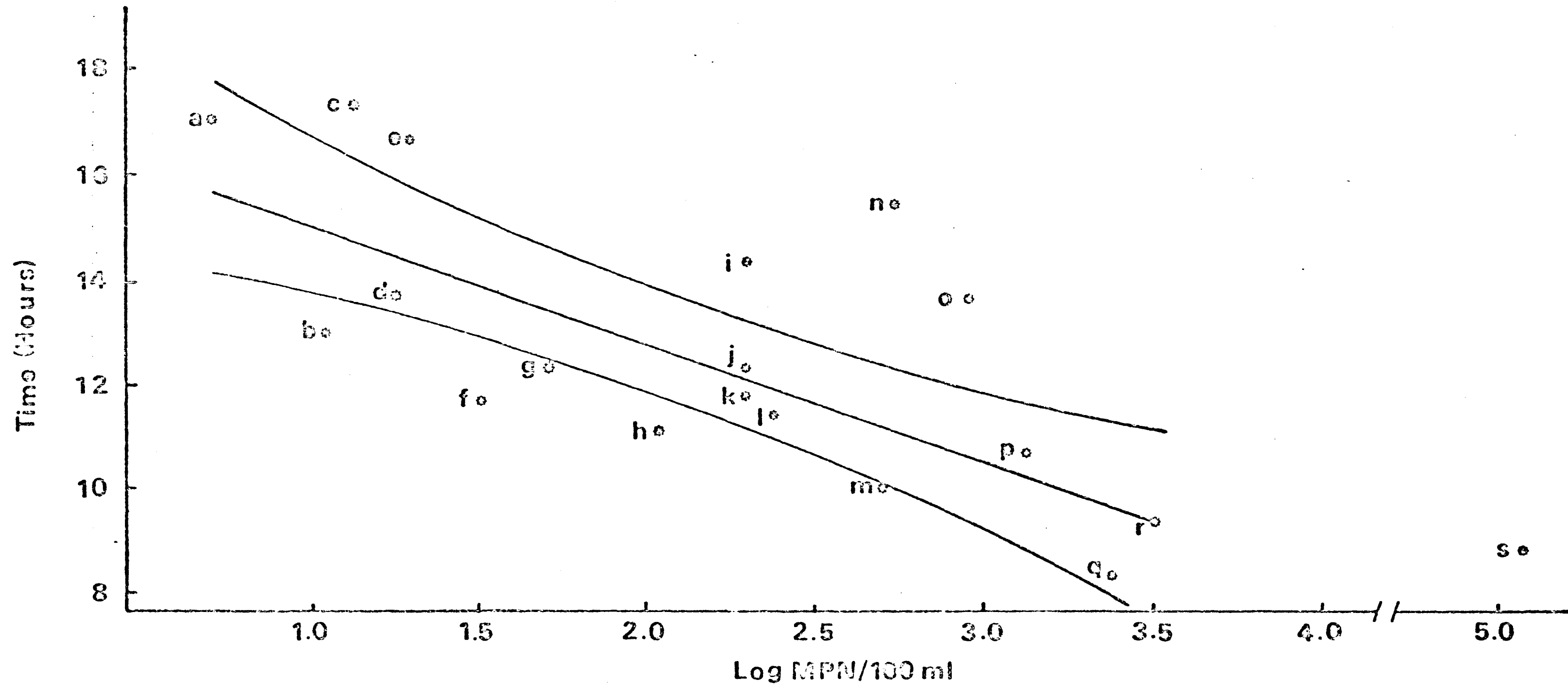

Time (Hours)
18
16
14
12
10
8
1.0
1.5
2.0
2.5
3.0
3.5
4.0
5.0
Log MPN/100 ml
a
b
c
d
e
f
g
h
i
j
k
l
m
n
o
p
q
r
s

Table 1

	Location**	Habitat*	Fecal Coliforms/ 100 ml	Corrected Time	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 the Shenandoah River	1	918	13.57	0.687
p	North Fork of the Shenandoah River	1	1300	10.40	0.209
q	Factory	5	2300	8.28	0.119
r	Hawksbill Creek	6	3300	9.23	0.243

Table 1 (Continued)

	Location**	Habitat*	Fecal Coliforms/ 100 ml	Corrected Time	Standard Deviation
s	Front Royal	8	130,000	9.13	0.533

*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.

**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, Virginia.

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 which fit the accepted fecal coliform definition. In order to test this hypothesis bacteria which 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. In order to determine if this test was underestimating the FC concentration, bacteria isolated on plate count agar from the FC 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 which produced gas in EC broth at 44.5 C, 638 grew in the ONPG/EC medium and hydrolyzed ONPG at a rate comparable to *E. coli* K-12. Eighteen isolates were "atypical" isolates in that only a slight color production was noted and very little growth observed. Of these 18 "atypical" isolates, 11 were identified as *Klebsiella 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.

Table 3. Identification of randomly picked isolates from ONPG/EC medium using IMViC procedure and Analytical Profiles Index (API 20E).

Species	Number Isolated	Percent of Total
E. coli	292	96.69
E. cloacae	7	2.32
K. pneumoniae	2	0.66
C. freundii	1	0.33

DISCUSSION

The hypothesis that a short time FC 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 FC population could be obtained in 8 to 20 hours at densities frequently encountered in natural samples. Very consistent results were obtained with replicate runs of *E. coli* K-12, indicating 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 MPN valued than would be predicted by the ONPG hydrolysis time. These habitats share the common characteristic of being miles away from most likely sources of fecal contamination. Furthermore, the water at these locations was well aerated, low temperature (less than 5 C) and low in 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 FC from these sites were damaged by chlorination or some toxic agent.

Two samples (f, q) were obtained from habitats which had a water temperature of approximately 30 C and a high organic and inorganic nutrient status. The FC present 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. *Klebsiella pneumoniae* was found to dominate the FC MPN culture tubes. No *K. pneumoniae* 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 from forest related samples and from fresh produce (4). Although the organism fits the definition of a FC, it may be capable of misrepresenting the extent of recent fecal pollution because it has the ability to thrive in an aquatic, soil, or plant environment. Therefore, by not detecting *K. pneumoniae*, the ONPG/EC method may be more useful in evaluating water quality than the current FC MPN technique for some types of effluents.

The possibility exists that the relationship between ONPG hydrolysis time and the real number of FC present

(approximated by the MPN) is non-linear, but a far greater number of samples covering a broader range of FC 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 an x^2 term failed to improve the R-square term significantly.

Geldreich et al (6) have shown that 96.4 percent 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 FC 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 limited.

The autocytotoxic effects of some β-D-galactosides have been described (11, 19). 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 up to 43 C but not at 44.5 C. Van Donsel et al (18) has shown the optimum

temperature for growth of fecal coliforms to be between 40 and 44 C. Our preliminary experiments 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 percent 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 FC 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 was delayed because some cells were physiologically debilitated. The procedure proposed by Lin (13) to improve membrane filter recovery of FC 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 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 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 FC limit has been established. For example, if the limit is to be 200 FC or less per 100 ml, no detectable enzymatic hydrolysis of the ONPG should be observed in less than 11 hours (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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RAPID COLORIMETRIC TECHNIQUE FOR THE ENUMERATION OF FECAL COLIFORMS IN WATER

by

Leland Stanford Warren

(ABSTRACT)

A new colorimetric method for the detection of fecal coliforms (FC) in water has been developed. The method is based upon the enzymatic hydrolysis of the substrate o-nitrophenyl-β-D-galactoside (ONPG) by FC. This technique provides an estimate of the FC concentration within 8 to 20 hr. One hundred ml of test sample is passed through a 0.45 μm membrane filter. This filter is then incubated at 37 C for one hr in EC medium followed by the addition of filter sterilized ONPG. The incubation is continued at 44.5 C until 1/2 maximum absorbance (420 nm) is reached. The time between the start of incubation and the 1/2 maximum absorbance is proportional to the concentration of FC 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 hr. In field studies, using samples which ranged from grossly polluted streams to relatively clean lake water, a linear relation between ONPG hydrolysis times and FC MPN values was established.