A liquid, colorimetric presence-absence coliphage detection method

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Summary

A liquid, colorimetric presence-absence coliphage detection method based on the induction of β-galactosidase by Escherichia coli is described. The release of β-galactosidase in the medium due to lytic cell infections by coliphages permits the hydrolysis of a yellow chromogenic substrate that develops into a distinct red coliphage positive sample, while a coliphage negative sample remains yellow. This method has proven to be rapid, simpler to perform than an agar medium assay, easy to read and interpret, inexpensive, and highly sensitive.

β-Galactosidase; Coliphage; Chlorophenol red β-D-galactopyranoside; Escherichia coli; Isopropyl-β-D-thiogalactoside

Currently, only one method has been proposed by the American Public Health Association (APHA, 1992) for the enumeration of coliphages from ground and surface waters. This method is the single-agar-layer plaque technique which is analogous to colony counting in bacteria, and allows for the enumeration of coliphages. The APHA (1992) specifies the use of Escherichia coli strain C (American Type Culture Collection, (ATCC) 13706) as the host and the addition of 2,3,5-triphenyl tetrazolium chloride that is added to the agar to aid in counting plaques. The three main limitations associated with the use of this agar-based method includes poor plaque visibility (Ijzerman and Hagedorn, 1992), low coliphage detection sensitivity (Kott, 1966), and the potential of false positive results due to the appearance of plaque-like cytotoxic areas in the agar caused by nonviral toxic material (Sobsey, 1982).

The liquid, colorimetric presence-absence coliphage detection method was...
developed to overcome the limitations imposed by the APHA (1992) method. The first of its kind to be developed in the field of public health virology, the liquid method provides an alternative to traditional plaque counting techniques. We propose that the liquid method be used alone or in combination with an agar-based coliphage detection method, such as either that proposed by the APHA method or the improved agar-based method developed by Ijzerman and Hagedorn (1992). The liquid method can provide a quick and easy way to evaluate large numbers of water samples and provide a presence-absence decision for those samples that may need to be further evaluated with an agar-based method in order to estimate coliphage density. Under laboratory conditions, the liquid, colorimetric presence-absence coliphage detection method has proven to be rapid, simpler to perform than an agar medium assay, easy to read and interpret, inexpensive, and highly sensitive.

The liquid, colorimetric coliphage detection assay is based on the phage-induced lysis and release of induced β-galactosidase from *E. coli* strain C. β-Galactosidase, encoded by the *lacZ* gene of the lactose operon, is an essential enzyme in the metabolism of lactose and can be induced by a number of chemically synthesized analogs that mimic lactose, the most powerful inducer being isopropyl-β-D-thiogalactoside (IPTG). In the absence of an inducer, β-galactosidase levels have been detected to be fewer than ten molecules per cell, while in the presence of IPTG, β-galactosidase levels are increased 1000-fold (Beckwith, 1987). Due to a lytic infection by coliphages present in the water sample, β-galactosidase is released from the cell. The released β-galactosidase hydrolyzes β-galactosides coupled to chromogen molecules that results in the release of the chromogen and the formation of a unique color. The chromogenic substrate, chlorophenol red β-D-galactopyranoside (CPRG), was used in the liquid assay because CPRG is a water-soluble substrate for the detection of β-galactosidase; CPRG is 10-times more sensitive than 2-nitrophenyl-β-D-galactopyranoside (ONPG) in the kinetic assay of β-galactosidase; and a distinct chlorophenol red color appears when hydrolyzed by the enzyme (Eustice et al., 1991).

The liquid, colorimetric coliphage detection method was developed with *E. coli* strain C (ATCC-13706), an F− (recipient) that lacks restriction and modification systems and is sensitive to a broad spectrum of sewage phages (Havelaar and Hogeboom, 1983). The common sewage coliphage (ATCC-13706-B2) was used to determine the sensitivity of the method.

*E. coli* strain C was grown (24 h at 37°C) and stored no longer than 30 days at 4°C on Luria-Bertani (LB) agar (Sambrook et al., 1989). A single colony was aseptically transferred into 10 ml LB broth supplemented with 5 mmol CaCl₂·2H₂O (Fisher Scientific, NJ) after autoclaving, and 1.25 mmol MgSO₄·7H₂O (Sigma Chemical Co., St. Louis, MO) prior to autoclaving. The culture was incubated on a rotary shaker (Lab-line Orbit Environ-Shaker) at 37°C and 200 revolutions per minute (rpm) for 12 h.

One hundred and twenty-five μl of the 12 h *E. coli* strain C culture
(O.D. ≥ 1.80 at a wavelength of 520 nm; Milton Roy Spectronic 20D) was aseptically transferred into 25 ml (20–25°C) LB broth supplemented with 5 mmol CaCl₂·2H₂O after autoclaving and 1.25 mmol MgSO₄·7H₂O prior to autoclaving. The culture was incubated at 37°C and 200 rpm for 1 h. After 1 h, the culture was aseptically inoculated with 25 μl of IPTG (Boehringer Mannheim Biochemicals, Indianapolis, IN; 0.1 M stock) and incubated at 37°C and 200 rpm for an additional 30 min. At the end of 30 min, a concentrated water sample (SM buffer (Sambrook et al., 1989) seeded with coliphage) no greater than 1.25 ml was added to the culture. Sample concentration was first performed using the CH2 Ultrafiltration System equipped with a 100 000 molecular weight (MW) cut-off spiral-wound membrane cartridge (S1Y100; Amicon, Inc., MA), and second using the Centriprep-100 concentrator (Amicon, Inc., MA) in order to further concentrate the sample to 1.25 ml. The culture was swirled and maintained at room temperature without shaking for 10–15 min and then returned to the 37°C shaker for an additional 105 min at 200 rpm.

The culture was added to a sterile 50 ml centrifuge tube (Perfector Scientific, Atascadero, CA) and centrifuged (Beckman Model TJ-6) at 6°C, 5000 rpm for 15 min. The supernatant was vacuum-filtered through a 0.2 μm low protein-binding filter (Spor-200, Gelman Sciences, Ann Arbor, MI) into a sterile (20 × 150 mm) test tube. To 9 ml of Z buffer (Miller, 1977), 1 ml of filtrate was added plus 100 μl of CPRG (Boehringer Mannheim Biochemicals, Indianapolis, IN; 5 mmol stock) and incubated at 37°C for 30 min.

A positive coliphage test can be observed by the immediate development of red, in an initially yellow solution, that intensifies over a 15-min period and further develops into a uniform purple-red color by 30 min. A negative coliphage test is visually observed by a uniform yellow throughout the test tube at the end of the 30 min incubation period. Sometimes due to carry-over of small amounts of enzyme in the filtration apparatus, a slight red band of color will appear at the meniscus of the liquid in an otherwise yellow sample. Such a sample should still be regarded as a negative result, since only a uniform red throughout the tube is positive.

The liquid, calorimetric presence-absence coliphage detection method can be completed and the results obtained in 4.5 h. The liquid method is simpler to perform than an agar medium assay primarily due to the absence of agar and the difficulties associated with rapid hardening. The results obtained by the liquid method are easy to read and interpret with a uniform red indicating a positive coliphage test, and a uniform yellow indicating a negative coliphage test. The final results in this method are obtained without the need of a spectrophotometer. The estimated cost to perform the liquid method based on the price of the two main ingredients, IPTG and CPRG, is $0.07 (U.S.) per 1.25 ml concentrated water sample.

Theoretically, the liquid, calorimetric presence-absence coliphage detection method can detect as few as 2 PFU/μl (Table 1), which is an improvement over the APHA agar-based method that can only detect as few as 5 PFU/100 ml. At
TABLE I
The detection sensitivity of the liquid, colorimetric presence-absence coliphage detection method using *E. coli* strain C as host

<table>
<thead>
<tr>
<th>Number (PFU)</th>
<th>Positive Samples (30 min)</th>
<th>Total No. of Samples</th>
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<tbody>
<tr>
<td>60</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>30</td>
<td>23</td>
<td>23</td>
</tr>
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<td>22</td>
<td>16</td>
<td>16</td>
</tr>
<tr>
<td>11</td>
<td>15</td>
<td>15</td>
</tr>
<tr>
<td>6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>15</td>
<td>15</td>
</tr>
<tr>
<td>4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>12 (15)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>15</td>
</tr>
<tr>
<td>2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>8 (11)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>15</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>39</td>
</tr>
</tbody>
</table>

<sup>a</sup>2 ml of filtrate was added to 8 ml of Z buffer, while 1 ml of filtrate plus 9 ml of Z buffer was used for titers > 10 PFU, and negative controls (0 PFU) were done both ways.

<sup>b</sup>An additional three samples turned positive 1.5 h after the end of the designated 30 min incubation period.

<sup>c</sup>An additional three samples turned positive 1.5 h after the end of the designated 30 min incubation period.

no time throughout the entire study did the liquid method produce a false positive result and the incidence of false negative results only became apparent at extremely low coliphage titers (Table 1). In order to reduce the rate of false negative results under conditions of low coliphage titers (0–10 PFU), it is advised that 2 ml of filtrate be added to 8 ml of Z buffer and the incubation time extended for a period not to exceed a total of 2 h. It is also important to note that, due to the high sensitivity of the assay, all materials used in this method, including hands, must be thoroughly cleansed of any residual coliphage particles that could possibly lead to a false positive test. In all experiments, positive and negative controls should be used to ensure proper functioning of the liquid colorimetric detection method. Although this method was developed using *E. coli* strain C as the host, this procedure is perfectly suitable for use with other coliphage host strains including donor *E. coli* strains used for the detection of F<sup>+</sup>-specific bacteriophage strains.

The liquid, colorimetric presence-absence coliphage detection method can be proportioned to accommodate a wide range of water sample volumes by keeping all chemicals in ratio to the 1.25 ml concentrated water sample volume described here. For example, if the final volume of water sample after concentration is approximately 5 ml but no greater, inoculate 100 ml LB broth with 500 µl of the 12 h *E. coli* strain C culture, and add 100 µl IPTG. Keep the CPRG and Z buffer volumes and all incubation times the same as described. If water concentration is not feasible, unconcentrated water samples can be analyzed by proportioning the assay to fit a larger sample volume, or by using multiple replication of a smaller volume, thereby ensuring proper representation of the entire volume of sample collected.

The liquid coliphage detection method is not intended to replace an agar
medium coliphage detection assay. Its purpose is to test for the presence or absence of coliphages in surface and ground water samples much in the same way as the Autoanalysis Colilert Test (Environetics, Inc., CT; Edberg et al., 1988) is used for detecting the presence or absence of coliform bacteria in water samples. A plaque assay using an agar medium is still required to quantify coliphage numbers.

Our liquid coliphage detection method would provide the means for public and private water quality laboratories to more thoroughly and accurately test for the presence of enteric viruses, through the use of a coliphage indicator, than is currently available. The increased detection sensitivity afforded by the liquid assay would reduce the potential for human infection by enteric viruses in contaminated water, thereby increasing the overall safety of potable water supplies.

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References