DEVELOPMENT OF A RAPID COLIPHAGE DETECTION ASSAY

by

James Emmett Stanek

Thesis submitted to the Faculty of the

Virginia Polytechnic Institute and State University

in partial fulfillment of the requirements for the degree of

Master of Science

in

Microbiology

APPROVED:

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Joseph O. Falkinham III, Chairman

_____ _____

Stephen M. Boyle George H. Lacy

January 24, 1997

Blacksburg, Virginia

keywords: coliphage, virus, water, method

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by
James Emmett Stanek
Joseph O Falkinham III, Chairman
Microbiology and Genetics Section, Biology Department

(Abstract)

A rapid coliphage detection assay (RCDA), based on the phage-induced release of β-galactosidase from cells of ***Escherichia coli*** (Ijzerman, M., J.O. Falkinham III and C. Hagedorn. (1993) [A liquid, colorimetric presence-absence coliphage detection method. J. Virol. Meth. **45:**229-234] was modified to reduce the number of steps required to perform the assay, remove the need for specialized media and buffers, reduce the volumes required, and simplify growth and reaction conditions. Tolerances of the assay were defined at each step of the assay. The number of steps has been reduced from 12 to 7. The β-galactosidase reaction buffer was eliminated. Culture volumes were reduced from 25 ml to 5 ml and reaction volumes were reduced from 10 ml to 0.5 ml. Optimal growth conditions were 37^{o}C with orbital shaking at 200 rpm, a one hour subculture time and an incubation of subculture with water sample for two hours. Color development occurred at 37^{o}C in 30 minutes. The changes and modifications of the assay increased the ease of its performance without sacrificing the ability of the assay to detect as few as two phage particles per sample. By understanding the tolerances of the assay, technical support representatives of companies producing kits modeled after the assay will be prepared to answer questions from customers concerning possible kit failures or user error.

ACKNOWLEDGMENTS

I have been a member of the Virginia Tech community since 1987, when I arrived as a freshman. Over the years, it has been my pleasure to meet many fine people and, I would like to take this opportunity to thank them for the impact they have had on shaping my life.

First, I would like to thank Joe Falkinham for allowing me the honor of working in his lab and, to double the thanks for his patience with me as I wrote this thesis. Sometimes, the demands Joe made upon me were difficult and did not seem to make sense at the time, but now that I am working in the "real-world" his hard won lessons have prepared me well. Thank you.

I would also like to thank Drs. George Lacy and Stephen Boyle for serving on my committee. I appreciated their ideas, encouragements and, their willingness to allow me to move in different directions.

There are several faculty members who were selflessly giving to me while I conducted my work. To Drs. Muriel Lederman, Brenda Shirley, Bill Claus, Noel Krieg and Bruce Parker, thank you for sharing your ideas, help, and sympathy. I could not have done it alone.

Laura Via described graduate school as a circus in her dissertation. With the group of friends I made while working in 4014 Derring Hall I would have to agree. Thanks to all the graduate students in the microbiology section for their friendship and help, especially; John Mayo, Twilla Eaton, Donna Jensen, Laura Via, Marcus Jucker, Ross Zirkle, Cody Cain, and Lori Brookman.

The three people who helped me to complete the work in this thesis are Dr. Jane Duncan, Scott Burian, and Whitney Emsch. These three people helped me get my start in this project and with the collection of data. I would also like to thank Dominion BioSciences for funding this work.

I would like to thank my parents, Larry and Donna Stanek, for their love and support and, the occasional kick in the pants to keep me on track. I hope I have made you proud. I love you both.

Finally, I owe a tremendous debt to my wife Shannon. She always encouraged my successes and nursed me through my failures. Simple words cannot express my gratitude to you, "I love you" will have to do.

I would like to dedicate this work to the memory of Dr. John L. Johnson. Dr. Johnson best represents science to me. He was a gentleman and a dedicated scientist who shared his passion with students freely. He should serve as a role model for us all.

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Literature Review

The American Public Health Service first adopted fecal and total coliforms, the coliform bacterial group, as the bio-indicator of fecally-contaminated water in 1914 (Gerba, 1987). The coliform bacterial group remains the standard indicator for fecal contamination even though it includes bacteria of other than those of fecal origin, [e.g. from plants and soils (Berg, 1978)]. Although the coliform bacterial group and ***Escherichia coli*** specifically serve as adequate indicators of bacterial contamination in water, they are not adequate as indicators of enteric viruses (Havelaar, 1993; Kott ***et al.***, 1974, Melnick ***et al.***, 1978, Stetler, 1984).

Over 120 different types of enteric viruses that cause disease in humans are excreted in human feces and urine (Melnick, 1984). The enteric viruses isolated from human feces include: adenoviruses, caliciviruses, cytomegaloviruses, enteroviruses, Norwalk viruses, reoviruses, and rotaviruses (Havelaar, 1993; Rao and Melnick, 1986). These viruses cause a variety of illnesses including: congenital heart anomalies, conjunctivitis, diarrhea, encephalitis, fever, gastroenteritis, hepatitis, meningitis, myocarditis, nephritis, paralysis, pericarditis, respiratory illnesses, and skin rashes (Havelaar, 1993; Rao and Melnick, 1986). Epidemiologically, the most important enteric viruses involved in human illnesses are the hepatitis A virus, rotaviruses, fecal adenoviruses, and caliciviruses (Havelaar, 1993). Viruses are important etiological agents due to the low number of infectious particles required to cause disease. For example, as few as two plaque forming units (PFU) of the virus can cause the illness (Ward and Akin, 1984).

Viruses are more resistant to chlorination and other means of water disinfection than ***E. coli*** (Berg ***et al,***, 1978). The simple structure of virus particles, a nucleic acid genome surrounded by a protein coat, accounts for their greater resistance to methods of water disinfection (Melnick, 1984). Gerba and Rose (1990) isolated viruses from finished drinking waters supplies that were free of indicator bacteria. These findings have prompted development of testing methods for enteric viruses in treated waters. Only a few methods are available (Havelaar, 1993). Traditional methods for detecting enteric viruses in water rely on tissue culture with the Buffalo Green Monkey cell culture line and take several days to yield results (Havelaar, 1993; Kott ***et al.***, 1974). Primary tissue culture methods require a high degree of technical ability, and are too expensive to be used practically in many areas of the world (Kott ***et al.***, 1974; Havelaar, 1993).

The latest methods to be used in detecting human enteric viruses in water are based on the polymerase chain reaction (PCR) (Schwab ***et al.***, 1993), nucleic acid hybridization (Margolin ***et al.***, 1989) and immunological methods (Kfir and Genthe, 1993). The use of nucleic acid probes and PCR to detect viruses in water has several limitations (Alvarez, ***et al.***, 1993). The use of these

methods requires high levels of technical skill, knowledge and expensive reagents which are prohibitive in most laboratories (Alvarez ***et al.***, 1993).

Virus detection methods share a common step of concentrating a large water sample (up to 2000 liters) to as little as a few milliliters. One of these methods involves binding viruses to membrane filters and eluting viruses from the membrane with a beef extract solution (Stetler, 1984). Concentration is required because virus concentrations are low and samples must be of a manageable size. Unfortunately, beef extract contains high levels of protein and salts that interfere with molecular biological methods of detecting coliphage by stabilizing non-specific DNA binding or by destruction of immunoglobulins by proteases. The acceptance by diagnostic laboratories of beef extract as the virus eluant, makes the use of nucleic acid hybridization and PCR techniques even more difficult and time consuming to perform by requiring low salt, and protease-free samples (Schwab ***et al.***, 1993). Due to the high degree of specificity inherent in molecular techniques, a battery of probes or PCR primers would have to be used to detect a variety of enteric viruses in waters because of the lack of agreement on an indicator enteric virus (Alvarez ***et al.***, 1993).

The use of immunological techniques such as enzyme linked immuno-sorbant assay (ELISA), radio-immuno assay (RIA) and immuno-fluorescent (IF) microscopy also present considerable problems (Kfir and Genthe, 1993). The ELISA method utilizes color-formation rather than radioisotopes used in RIA for detecting viruses. Both the ELISA and the RIA methods are insensitive. They can only detect between 10^5 and 10^6 PFU/ ml as a minimum (Kfir and Genthe, 1993). Further, both methods are expensive to perform and frequently yield false positive results are obtained due to non-specific binding of antibody (Kfir and Genthe, 1993). Immuno-fluorescent techniques are as sensitive as other histochemical methods for detecting bacterial pathogens, although they have a limited range of viral detection (Kfir and Genthe, 1993). A disadvantage of both the immunological and DNA-based technologies is their inability to distinguish between active and inactivated virus particles (Kfir and Genthe, 1993).

It has been proposed that coliphages, bacteriophages that infect ***E. coli***, could serve as indicators for the presence of enteric viruses in a variety of waters (Havelaar, 1994; Palmateer ***et al.***, 1991; Stetler, 1984; Sikova and Cervenka, 1981; Kott ***et al.***, 1974). Coliphages may be better suited as indicators of enteric viruses than indicator bacteria because their structure is more similar to animal viruses than indicator bacteria and, they persist in the environment longer than do indicator bacteria (Grabow ***et al.***, 1984). The acellular nature of coliphages (coliphages consist of a nucleic acid genome surrounded by a protective protein coat) affords them resistance to environmental and water treatment methods equal to resistance levels in enteric viruses (Stetler, 1984).

In order for coliphage to serve as an adequate indicator for enteric viruses, coliphages must be: (1) recovered from the same sources as enteric viruses, (2) recovered in numbers greater than or equal to numbers of enteric viruses, (3) share the same susceptibility to harsh environmental conditions and water treatment processes as enteric viruses and (4) detected more rapidly and more inexpensively than enteric viruses (Kott ***et al.***, 1984).

Stetler (1984) showed that coliphage were always recovered along with enteric viruses throughout a water purification facility. ***E. coli*** hosts for both somatic and donor-specific coliphages were used. Somatic coliphage host ***E. coli*** strain C recovered 2.9 times more coliphage than the donor-phage-specific host ***E. coli*** strain A-19 (Stetler, 1984). Moreover, coliphage enumerated with ***E. coli*** strain C were typically 13,870 times greater than enteric virus numbers (Stetler, 1984) Coliphage numbers diminished as water passed through stages of the purification facility just as the enteric viruses did. Coliphage exhibited the same seasonal variation in numbers as did the enteric viruses (Stetler, 1984). Havelaar ***et al.*** (1986) reported that somatic coliphages detected by ***E. coli*** strain C were recovered in <70% of their samples of animal and human feces while donor specific phages weren't detected using ***E. coli*** strain HfrH. Donor specific coliphage occurred mainly in young domestic farm animals and are isolated in human feces only to a limited extent (Palmateer ***et al***, 1991). In an Egyptian study of potable waters using ***E. coli*** strain C, 78 of 147 (53%) samples yielded coliphages from drinking water samples that were free of fecal and total coliforms (El-Abagy ***et al.***, 1988) Coliphage were recovered in 65 of 147 (44%) of samples that were negative for total coliforms, and fecal coliforms. Four of 147 (3%) of the samples were positive for both coliphage and coliforms (El-Abagy ***et al.***, 1988).

Coliphage have also been proposed as indicator organisms of fecal contamination in recreational and shell-fish harvesting waters (Palmateer ***et al.***, 1991; Vaughn and Metcalf, 1975). In 600 natural water samples numbers of, fecal coliform, total coliform and, coliphage exhibited the same linear response (Wentzel ***et al.***, 1982). Coliphage were detected in 5 of 5 (100%) Canadian beaches tested for the presence of coliphage, fecal coliforms and ***E. coli*** (Palmateer ***et al.***, 1991). Coliphage numbers in black mussels in shellfish-harvesting waters paralleled those of the fecal bacteria in the waters surrounding them (Lucena ***et al.***, 1994). Additionally, Vaughn and Metcalf (1975) showed an equal linear response of coliphage and coxsackievirus in estuarine shell-fish raising waters during a year. Coliphage were always recovered even after coxsackievirus was no longer detectable (Vaughn and Metcalf, 1975).

The method first proposed for the detection of coliphage was described in the 17th edition of ***Standard Methods for the Examination of Water and Wastewater*** (APHA, 1989). It is a single-agar-layer infectivity method in which plaques are visualized by the addition of 2,3,5-triphenyl tetrazolium chloride

(TPTZ). Coliphage are adsorbed to the ***E. coli*** strain C host cell; following injection of their nucleic acid into the host cell, where it replicates and directs the synthesis of phage components and phage are released in a lytic burst. The lysis of host cells by coliphage results in a cleared zone, a plaque, on a lawn of ***E. coli*** on the surface of a solid medium. In the APHA (1989) method, a suspension of ***E. coli*** C (ATCC 13706) able to detect only somatic coliphage, is added to molten modified TSA. Then a water sample is added to the bacterial-agar suspension, mixed, and poured into a sterile Petri dish. Plaques are more easily visualized by the reduction of TPTZ to a pinkish color on the lawn of ***E. coli*** strain C. Plaques containing the contents of lysed cells are darker than the ***E. coli*** strain C lawn around them. ***E. coli*** strain C is used because it lacks a restriction modification system and is susceptible to a wide range of sewage coliphages (Havelaar and Hogeboom, 1983). The lack of a restriction-modification system prevents the ***E. coli*** strain C from destroying injected DNA (Havelaar and Hogeboom, 1983).

Although this method is simple to perform, the interpretation of the data obtained is not straight forward. The formation of plaque like zones due to non-viral cytotoxic compounds in the water sample can yield false-positive results (Ijzerman and Hagedorn, 1992). Further, plaques are not easily visualized even with TPTZ A modified agar agar-based plaque assay was developed based on the release of β-galactosidase from coliphage-infected cells (Ijzerman and Hagedorn, 1992). In that method, Ijzerman and Hagedorn (1992) used the overproduction of β-galactosidase and its coliphage-induced release to cleave 5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside (X-gal), a chromogenic β-galactosidase substrate, in the agar medium. When X-gal is cleaved by β-galactosidase, the dark blue indoyl product accumulates in the medium inside and surrounding the plaque (Ijzerman and Hagedorn, 1992). Though, twice as many coliphage were detected using the Ijzerman and Hagedorn (1992) method than in the APHA (1989) method in 5 parallel experiments, the assay still required a 24 hour incubation.

A liquid based, presumptive coliphage detection assay, also based on the phage-induced release of induced β-galactosidase, has also been developed (Ijzerman ***et al***, 1993) The liquid colorimetric presence-absence (LCPA) assay was developed to avoid the problem of false positive plaque like areas and to shorten the time required for detection (Ijzerman ***et al.***, 1993).

IPTG was used as the inducer for over-expression of β-galactosidase in the ***E. coli*** C (Miller, 1978). IPTG is a lactose analogue and it is not a substrate of β-galactosidase, unlike lactose. β-Galactosidase acts on lactose to produce allo-lactose, an isomer of lactose, that binds to the repressor of ***lac*** operon expression and modifies its structure to prevent binding to the ***lac*** operator region, thus allowing the transcription of the ***lac*** operon. Free lactose is cleaved to galactose and glucose by β-galactosidase. IPTG binds to the ***lac*** operon repressor as avidly as allo-lactose and induced β-galactosidase levels

are 10-fold higher than those found in lactose-induced cells at a 100 fold lower molar concentration (Barkley and Bourgeois, 1970, Miller, 1978).

Because β-galactosidase is a large oligomeric protein it cannot diffuse through the cell envelope of ***E. coli***. Therefore, β-galactosidase in the medium can only come from lysed or damaged cells (Zabin and Fowler, 1970). In the LCPA method, cultures were filtered and 1 ml samples of the filtrate were added to 9 ml of Z buffer to assay β-galactosidase activity (Miller, 1972). Chlorophenol red -β-D galactopyranoside (CPRG) was added as a chromogenic substrate for β-galactosidase. Filtrates were incubated at 37^{o}C. CPRG is yellow and when cleaved by β-galactosidase releases the purple colored chlorophenol red. Thus, any reaction that turns purple contains β-galactosidase while any reaction that remains yellow does not. Because β-galactosidase release requires the coliphage induced lysis, β-galactosidase activity is a reflection of coliphage in a sample. When using a concentrated water sample, the LCPA method can detect as few as 2 plaque forming units/ liter, compared to the APHA's (1989) sensitivity to 5 PFU/ 100 ml (Ijzerman ***et al***, 1993). In field trials of the LCPA and APHA assays, of 90 samples of tap, spring, creek and wastewater samples the LCPA produced only 3.3% (3 of 90) false negative results compared to the APHA (1989) method. The false negative results only occured in samples with a very low coliphage titer (Ijzerman ***et al***, 1994). The LCPA method was determined to be as sensitive as the APHA (1989) method and the LCPA yielded results in 4.5 hours compared to the 24 hours required to perform the APHA method.(Ijzerman ***et al***, 1994).

The LCPA method offers several advantages over the APHA (1989) method. First, it is simpler to perform due to its lack of melted agar that can harden early resulting in a clear area easily mistaken for a plaque and make interpretation of data more difficult. Second, it is more sensitive and detects fewer coliphage. And, finally, the LCPA method does not have the false-positive results associated with the APHA method.

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Chapter II

IMPROVEMENTS OF AN EXISTING COLIPHAGE DETECTION ASSAY

Introduction

The first proposed method for the detection of coliphage was introduced in the 17th edition of ***Standard Methods for the Examination of Water and Wastewater*** (APHA, 1989). It is a single-agar-layer infectivity method in which plaques are visualized with the addition of 2,3,5-triphenyl tetrazolium chloride (TPTZ). In the APHA (1989) method, a suspension of ***E. coli*** strain C (ATCC 13706) is added to molten (55^{o} C) modified T-soy agar. Then a water sample is added to the agar, mixed, and poured into a sterile Petri dish. Clear areas, plaques, with a pinkish tinge, form on the lawn of ***E. coli*** where coliphage have infected, replicated, and lysed cells releasing progeny phage.

The problems with the APHA (1989) method include low sensitivity, difficulty in visualizing plaques, and formation of plaque-like zones forming due to non-viral cytotoxic compounds in the water sample (Ijzerman and Hagedorn, 1992). To avoid these problems a liquid colorimetric presence-absence coliphage detection (LCPA) method was developed (Ijzerman ***et al.***, 1994). The LCPA method was based on the phage-induced release of β-galactosidase from isopropyl-β-D-galactopyranoside (IPTG)-induced ***Escherichia. coli*** strain C (ATCC 13706) cells.

The use of IPTG as the inducer for β-galactosidase in the ***E. coli*** C hosts is due to the virtually irreversible nature of its binding to the ***lac I*** repressor molecule (Miller, 1978). IPTG is a lactose analogue and it is not a substrate of β-galactosidase, unlike lactose. β-galactosidase acts on lactose to produce allo-lactose, an isomer of lactose, which binds to the lac repressor effecting a conformational change preventing its binding to the operator region of the ***lac*** operon and allowing the transcription of the ***lac*** operon. Free lactose is cleaved to galactose and glucose by β-galactosidase. Because IPTG is not a substrate of β-galactosidase it cannot be removed from the repressor protein. This prevents repressor binding to the operator DNA and allows for production of β-galactosidase in ***E. coli*** induced with IPTG (Barkley and Bourgeois, 1970). IPTG binds to the repressor with comparable affinity as allo-lactose and increases β-galactosidase levels in the cell 10-fold higher than found in lactose-induced cells (Miller, 1978) (Barkley and Bourgeois, 1970).

Because β-galactosidase is a large oligomeric protein (~116 KDa) molecule and it cannot diffuse out of ***E. coli,*** extracellular β-galactosidase in the medium can only come from lysed or damaged cells (Zabin and Fowler, 1970). Cultures were filtered and 1 ml samples of the filtrate added to 9 ml of Z buffer to detect β-galactosidase activity (Miller, 1972); chlorophenol red -β-D

galactopyranoside (CPRG) was added as a chromogenic substrate for β-galactosidase. Filtrates were allowed to incubate at 37^{o}C. CPRG is yellow and when cleaved by β-galactosidase releases the purple colored chlorophenol red. Thus, any reaction that turns purple indicates coliphage while any reaction that remains yellow indicates a lack of coliphage.

A liquid colorimetric, presence-absence coliphage detection method was modified for ease of use and lower cost. The objective of this study was to remove all unnecessary steps and minimize the amount of time required to perform the assay. These two goals would have the net effect of lowering the cost of performing the assay by removing unnecessary reagents and by reducing the amount of hands on time required to perform the assay.

Materials and Methods

Bacteria and phage. ***E. coli*** strain C (ATCC 13706) used in the LCPA method was chosen as the host organism for the RCDA due to its susceptibility to a wide range of sewage coliphages because it lacks restriction-modification system (Havelaar and Hogeboom, 1983).

The common sewage coliphage (ATCC 13706 β2) was the phage used.

Media preparation. Luria Broth (LB) and Luria Agar (LA) were prepared and sterilized as described in Sambrook ***et al.*** (1989). A modified LB (mLB) was prepared by adding 0.15 g magnesium sulfate ($MgSO_4$)/ liter prior to autoclaving. Sterilized media were stored at 25^{o}C.

A 0.25 M calcium chloride ($CaCl_2$) solution was prepared by dissolving 0.277 g $CaCl_2$ in 10 ml distilled water, sterilized by filtration through a 0.2 µm filter and stored at 4^{o}C.

A 10 mM solution of isopropyl β-D thiogalactoside (IPTG) (Gibco BRL, Gaithersburg, MD) was prepared by dissolving 23 mg in 10 ml distilled water, sterilized by filtration through a 0.2 µm filter and stored at 4^o C.

Z buffer for the β-galactosidase assay was made following the protocol of Miller (1972) and stored at 4^o C..

A 5 mM solution of chlorophenol red β-D galactopyranoside (CPRG) (Boehringer-Mannheim Biochemicals, Indianapolis, IN) was made by dissolving 3 mg in 10 ml distilled water, sterilized by filtration through a 0.2 µm filter and, stored at 4^o C.

Growth of bacteria. ***E. coli*** C was grown at 37^{o}C on LB agar medium (Sambrook ***et al.***, 1989) from lyophilized stocks purchased from the American Type Culture Collection (Rockville, MD). A single isolated colony was used to inoculate 5 ml LB broth (Sambrook ***et al.***, 1989) in a 13 x 150 mm screw-capped tube and grown overnight at 37^{o}C at 200 rpm. This overnight culture was used to inoculate 100 ml of LB broth in a 250 ml Erlenmeyer and grown overnight at 37^{o}C to late log phase.

Storage of bacteria. Glycerol (Fisher Scientific, NJ) was added to LB to a final concentration of 20% (v/v) and 1 ml aliquots of ***E. coli*** strain C cell suspension were flash frozen in liquid nitrogen and stored at -70^{o}C. Fresh stock plates of ***E. coli*** strain C were made from a thawed frozen sample streaked onto LB agar and incubated at 37^o C for isolated colonies.

Coliphage assay.

(1.) Each assay was started by inoculating a single isolated colony (1-2 mm diameter) into a 13 x 150 mm screw-capped tube containing 5 ml mLB broth.
(2.) The culture was incubated overnight for 17 hours at 37^{o}C at 200 rpm.
(3.) 0.05 ml of the overnight was subcultured into 5 ml of mLB in a 13 x 150 mm screw-capped tube for each sample as well as for the controls.
(4.) To each subculture 0.1 ml of a solution containing 0.25 M $CaCl_2$ (Fisher Scientific, NJ) and 10 mM IPTG (Gibco, BRL, Gaithersburg, MD) was added.

(5.) The resulting cultures were incubated at 37^{o}C at 200 rpm.
(6.) After 1 hour incubation between 0.1 and 10 ml of a suspension of phage was added. A volume of sterile water equal to the volume of the phage-containing sample was added for a negative control. Suspensions were mixed thoroughly by inversion and incubated at 37^{o}C with shaking (200 rpm).
(7.) After 2 hours, a 0.5 ml sample of each culture was placed in the filtration unit of a 0.2 µm Spin-X tube (Corning Costar, Cambridge, MA). The separate collection unit of the Spin-X tube contained 0.1 ml of the 5 mM CPRG.
(8.) The Spin-X tube was centrifuged at 16,000 x g in an Eppendorf microcentrifuge (Brinkman Instruments, Westbury, NY) for 2 minutes at room temperature to separate whole cells from the medium.
(9.) The Spin-X tubes containing the filtrate and CPRG were incubated at 37^{o}C for 30 minutes and then observed by eye for a color change. A tube with a deep red to purple color was scored a positive and any tube that was yellow or slightly orange in color was scored as negative for the presence of coliphage (Fig. 1).
(10). To insure that coliphage were present in Spin-X tubes that were purple and not present in tubes that were yellow, a 0.01 ml sample from each tube was spotted onto a lawn of ***E. coli*** C on LB agar and incubated overnight at 37^{o} C to see if a plaque would form in areas spotted with positive and negative reacting filtrate. The protocol for the assay is presented on Table 2-1.

Rationale for Changes

The modifications of the LCPA method have increased the ease of the assays performance without sacrificing the sensitivity of the assay (Table 2-2). An initial goal was to reduce the culture size from 25 ml in the LCPA method to 5 ml. The benefit of reducing culture volumes allows the assay to be performed in 16 x 150 mm screw-capped tubes rather than 125 ml Erlenmeyer shake flasks and thus more samples to be analyzed in a limited space.

A second goal of this project was to reduce the number of steps and manipulations required to perform the assay. In attaining this goal the possibility of contamination would be lowered by reducing the number of steps and also by removing the need for micropipetors that become phage contaminated due to aerosolization of phage suspensions. Also, a 0.1 ml sample allows for the use of a 1 ml glass pipet and not a micropipetor that could contaminate each tube with phage.

Results and Discussion

Culture volume. The culture volume was scaled down from 25 ml to 5 ml and the assay remained able to detect as few as two coliphage particles; this allows for a more manageable transportation of cultures to and from the incubator in screw-capped tubes rather than in 125 ml Erlenmeyer flasks (Table 2-2).

Media additives. The LCPA method required the addition of $CaCl_2$ followed by a one hour incubation and then the addition of IPTG and a 30 minute incubation to induce the β-galactosidase. In the RCDA method, the $CaCl_2$ and IPTG were combined into one solution and the concentration of the stock was adjusted so addition of 0.1 ml would yield 5 mM $CaCl_2$ and 1 mM IPTG. This modification removed one pipeting step from the LCPA method and allowed for the use of a single 1 ml glass pipet. In combining the media additives into one solution the chance for introducing contaminating coliphage is decreased. By modifying the concentration of the additives a single 1 ml pipet is used. A goal of the assay was to use only one size of pipet thus reducing the possibility of pipeting an incorrect volume from another size pipet.

Subculture time. The initial incubation of the subculture was reduced from a total of 1.5 hours to 1 hour to maintain the cells in early log phase rather than in late log phase growth. We found that the longer incubation time increased the number of false positive results (Table 2-3) when the subculture incubation period exceeds 1.5 hours. A 1 hour subculture gave sufficient numbers of cells to give a positive reaction with 5×10^3 coliphage particles. The culture would continue to grow at low coliphage titers to generate more host cells as more coliphage particles are released upon lysis of each infected

host. The culture provided reproducible data for both high and low titer samples with the initial incubation time reduced to 1 hour (Table 2-2). The 1 hour subculture time was used to fine tune the assay in the smaller culture volume to allow enough cells to be present to yield positive results in highly contaminated (high coliphage titer) samples, while allowing enough cells to be produced to yield a positive result in less contaminated (low coliphage titer) samples.

Sample size and pre-incubation. The coliphage sample was modified to a volume between 0.1 ml and 5 ml in the RCDA and gave accurate and reproducible data (Table 2-4). Increasing the sample volume to a volume equal to the culture did not yield false positive results in phage-free tests or false negative results in phage containing tests. Further, the ratio of sample volume to culture volume did not lower the sensitivity of the assay. As long as two coliphage particles were present in a water sample of between 0.1 and 5 ml, a positive result was consistently obtained. In the 10 ml sample volume, false negative results were seen in the phage containing samples. In none of the tests performed (N= 64) were any false positive results observed in phage free samples. Also, the water samples were returned to the shaker without the 15 minute-room temperature incubation as used in the LCPA. Presumably, in the LCPA method that was used to allow coliphage to adsorb to the host cells. It was not required for the RCDA. By removing this step another source for human error was removed from the assay. The only sample volume stipulated in the LCPA method was 1.25 ml.

Sample incubation. The incubation of water sample with the culture was maintained for a total of 2 hours in both methods. This length of time was found to be essential in maintaining the assay's sensitivity level of two coliphage particles per water sample. The incubation period of 2 hours used in the LCPA was maintained to allow for several lytic cycles to occur to increase the amount of released β-galactosidase in the medium to insure positive results in low titer samples. Longer periods of incubation were avoided to prevent false positive results from occurring due to non-coliphage-induced cell lysis.

Centrifugation. Centrifugation of the culture prior to filtration as was performed in the LCPA method was found unnecessary in the RCDA method (Table 1-5). In the LCPA method, the centrifugation was required to remove cells and cellular debris from the media prior to filtration of 25 ml of culture for use in the β-galactosidase assay. In the RCDA, pre-centrifugation was not required and no false negative results were seen in phage containing samples (0 of 41) and no false positive results were seen in phage-free samples (0 of 41). A 0.5 ml sample of culture material was filtered in a microcentrifuge filter unit (0.2 µm pore diameter) in the RCDA, rather than 25 ml of supernatant filtered as required in the LCPA. Both of these modifications reduce the amount of time needed to perform the assay because a 15 minute

centrifugation is eliminated from the LCPA method. Further, as many as 18 filtrations may be done all at once in a microcentrifuge, rather than individually by vacuum filtration. This also reduced the amount of glassware that required sterilization. The necessity for centrifugation prior to filtration was not required in the RCDA because the smaller volume of filtrate used in the assay did not clog up the membrane in the filter unit of the Spin-X column.

β**-galactosidase buffer.** The requirement for a β-galactosidase reaction buffer (Z-buffer, Miller, 1972) was found to be unnecessary and removed from the assay. The medium alone had a sufficient buffer capacity and allowed for β-galactosidase activity. Combination of 0.5 ml of filtrate and 0.1 ml of a 5 mM CPRG solution consistently yielded a purple color in reactions containing coliphage (37 of 37). A yellow color was seen in samples containing no coliphage (37 of 37). In 37 tests performed without Z-buffer none of the experiments yielded a false negative result in phage-containing samples. There were no false positive results in 37 phage-free tests performed in parallel. The 30 minute incubation of culture filtrate with CPRG was maintained to allow for color development. By removing the necessity for a β-galactosidase reaction buffer, the cost of the assay was lowered and removes the necessity for another tube for the color development reaction.

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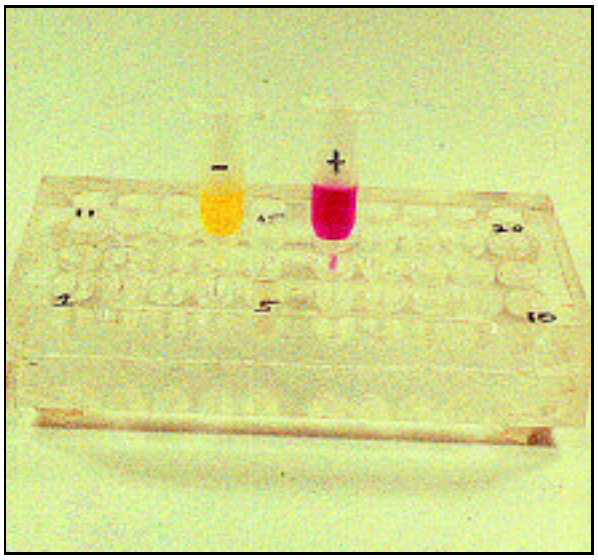

Fig. 1. A photograph of a coliphage negative test (left) and a coliphage positive test (right) using the rapid coliphage detection assay.

Table 2-1. Protocol for performing the rapid coliphage detection method.

Step	
1.	Streak a working plate of ***E. coli*** C from a stock plate and incubate overnight at 37°C.
2.	Inoculate 5 ml mLB with a single isolated colony of ***E. coli*** C no larger than 2 mm in diameter and incubate overnight at 37°C with rotation (200 rpm)
3.	To each 5 ml mLB used in the assay add 0.1 ml of the $CaCl_2$ + IPTG solution.
4.	Inoculate each 5 ml mLB + $CaCl_2$ + IPTG with 0.05 ml of the overnight culture of ***E. coli*** C.
5.	Incubate cultures for 1 hour at 37°C with rotation at 200 rpm.
6.	Add 0.2 ml control phage suspension to the positive control tube.
7.	Add from 0.1 to 5 ml water sample to each sample tube.
8.	Add a volume of sterile water equal to the sample volume to the negative control tube.
9.	Incubate all tubes for 2 hours at 37°C with rotation at 200 rpm.
10.	Place 0.1 ml CPRG solution in the bottom of each Spin-X tube and return the filter unit to the tube.
11.	Apply 0.5 ml of each culture to the filter unit of each Spin-X tube.
12.	Centrifuge the tubes for 2 minutes at 16,000 x g.
13.	Discard the filter units and incubate the tubes for 30 minutes at 37°C to allow for color development.

Table 2-2. Detection sensitivity of the rapid coliphage detection assay using ***E. coli*** C as the host contained in a 5 ml mLB broth contained in a 13 x 150 mm screw-capped tube.

Number coliphage (PFU)	Positive Samples	Total Samples
5000 -100	39	39
87	5	5
12	4	4
8	2	5
6	6	6
5	5 (14)[a]	15
4	4	5
3	4	4
2	4	9
0	0	92

[a] 14 of 15 samples were positive within 45 minutes

Table 2-3. Effects of different incubation periods of the ***E. coli*** strain C culture prior to the addition of sample phage on the rapid coliphage detection assay.

Time (hours)	Number tested	Number positive	Percent positive
0.5	3	0	0
0.75	3	3	100
1.0	37	37	100
1.5	3	3	100
2.0	3	3	100[a]
2.5	3	3	100[a]

[a] false positive resulted in phage-free controls

Table 2-4. Effects of different coliphage sample volumes on the rapid coliphage detection assay.

ml of sample	Number tested	Number positive	Percent positive
0.1	6	6	100
0.2	37	37	100
1.0	6	6	100
5.0	8	8	100
10	8	7	85[a]

[a] false negative results in phage-containing control

Table 2-5. Effects of different pre-centrifugation periods of culture + sample suspensions prior to the addition of CPRG on the rapid coliphage detection assay.

Time centrifuged (min)	Number tested	Number positive	Percent positive
0	37	37	100
10	2	2	100
15	2	2	100
30	2	2	100

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Chapter III

DEFINING THE TOLERANCES OF A RAPID COLIPHAGE DETECTION ASSAY

Introduction

A liquid colorimetric coliphage detection assay (LCPA) (Ijzerman ***et al.***, 1994) was modified with the objective of simplifying its performance and reducing unnecessary steps and reagents. Further study of each parameter in the Rapid Coliphage Detection Assay (RCDA) was performed to define the acceptable ranges at which the assay will still yield accurate and reproducible results and be as sensitive as the original test.

The objective of this study was to vary each parameter in the assay(Table 3-1) to determine its effects on the results of the assay. The results of this study define the acceptable parameters at which the RCDA may be performed, and accurate and reproducible results obtained without sacrificing sensitivity.

Materials and Methods

Bacteria and phage. ***E. coli*** strain C (ATCC 13706), used in the LCPA method, remained the host organism for the RCDA because of its susceptibility to a wide range of sewage coliphages as it lacks a restriction-modification system (Havelaar and Hogeboom, 1983).

The common sewage coliphage (ATCC 13706 β2) was used as the phage.

Media preparation. Luria Broth (LB) and Luria Agar (LA) were prepared and sterilized as described in Sambrook ***et al.*** (1989). A modified LB (mLB) was prepared by adding 0.15 g magnesium sulfate ($MgSO_4$)/ liter prior to autoclaving. Media were stored at 25^{o}C.

A 0.25 M calcium chloride ($CaCl_2$) solution was prepared by dissolving 0.277 g $CaCl_2$ in 10 ml distilled water, sterilized by filtration through a 0.2 mm filter and, stored at 4^{o}C.

A 10 mM solution of isopropyl β-D thiogalactoside (IPTG) (Gibco BRL, Gaithersburg, MD) was prepared by dissolving 23 mg in 10 ml distilled water, sterilized by filtration through a 0.2 µm filter and, stored at 4^o C.

Z buffer for the β-galactosidase assay was made following the protocol of Miller (1972) and stored at 4^o C..

A 5 mM solution of chlorophenol red β-D galactopyranoside (CPRG) (Boehringer-Mannheim Biochemicals, Indianapolis, IN) was made by dissolving 3 mg in 10 ml distilled water, sterilized by filtration through a 0.2 µm filter and, stored at 4^o C.

Growth of bacteria. ***E. coli*** strain C was grown on LB agar medium (Sambrook ***et al.***, 1989) from lyophilized stocks purchased from the American Type Culture Collection (Rockville, MD). A single isolated colony was used to inoculate 5 ml LB broth (Sambrook ***et al.***, 1989) and grown overnight at 37^{o}C with rotation at 200 rpm. This overnight was used to inoculate 100 ml of LB broth and grown overnight to late log phase.

Storage of bacteria. Glycerol (Fisher Scientific, NJ) was added to LB to a final concentration of 20% (v/v) and 1 ml aliquots of ***E. coli*** strain C cell suspension were flash frozen in liquid nitrogen and stored at -70^{o}C. Fresh stock plates of ***E. coli*** C were made from a thawed frozen sample streaked onto LB agar for isolated colonies.

Coliphage assay.

(1.) Each assay was started from inoculating a 1-2 mm diameter single isolated colony into a 13 x 150 mm screw-capped tube containing 5 ml mLB broth.

(2.) The culture was incubated overnight for 17 hours at 37^{o}C with shaking (200 rpm).

(3.) 0.05 ml of the overnight was subcultured into 5 ml of mLB for each sample and for the controls.

(4.) To each subculture 0.1 ml of a solution containing 0.25 M $CaCl_2$ (Fisher Scientific, NJ) and 10 mM IPTG (Gibco, BRL, Gaithersburg, MD) was added.
(5.) The resulting cultures were incubated at 37^{o}C with shaking (200 rpm).
(6.) After 1 hour incubation between 0.1 and 10 ml of a suspension of phage was added. A volume of sterile water equal to the volume of the phage-containing sample was added for a negative control. Suspensions were mixed thoroughly by inversion and incubated at 37^{o}C with shaking (200 rpm).
(7.) After 2 hours, a 0.5 ml sample of each culture was placed in the filtration unit of a 0.2 µm Spin-X tube (Corning Costar, Cambridge, MA). The separate collection unit of the Spin-X tube contained 0.1 ml of the 5 mM CPRG.
(8.) The Spin-X tube was centrifuged at 16,000 x g in an Eppendorf microcentrifuge (Brinkman Instruments, Westbury, NY) for 2 minutes at room temperature to separate whole cells from the medium.
(9.) The Spin-X tubes containing the filtrate and CPRG were incubated at 37^{o}C for 30 minutes and then read for a color change. A tube with a deep red to purple color was scored a positive and any tube that was yellow or slightly orange in color was scored as negative for the presence of coliphage. The protocol for the assay is presented on Table 3-1.

Experimental Variables Investigated. The parameters tested, the ranges tested, and the standard values for each parameter are listed on Table 3-2. In each experiment performed, the single step being varied was compared with an assay performed under the standard conditions listed in Table 3-2. Each experiment was performed using the same ***E. coli*** overnight culture to inoculate each subculture and, only a single step was varied in each experiment. A false positive result is scored when the phage-free control exhibit a purple (positive) color. A false negative result is scored when the phage-containing control exhibits a yellow (negative) color.

Stability of IPTG. A 10 mM IPTG solution was divided and samples stored at 4^{o} C, 25^{o} C, and 37^{o} C to determine the effects of these temperatures on its stability. Weekly samples were taken and used in the RCDA following standard assay protocol. Fresh IPTG was used as the control.

Stability of CPRG. A 5 mM CPRG solution was divided and samples stored at 4^{o} C, 25^{o} C, and 37^{o} C to determine the effects of these temperatures on its stability. Weekly samples were taken, absorbances measured, and used in the RCDA following standard assay protocol. Fresh CPRG was used as the control.

Stability of *E. coli* strain C Lyophiles. A 5 ml ***E. coli*** strain C was lyophilized in 0.005 ml samples following the ATCC method (Simone and Brown, 1991) using a suspension of cells in either 20% (wt/vol) skim milk solution (Carnation,)or by diluting a culture 1:1 with fresh LB. Lyophiles were stored at 4^{o} C. Lyophiles were rehydrated weekly with LB diluted and enumerated on LB agar to determine survival and a resulting colony from each storage type was used in the RCDA.

Stability of *E. coli* strain C suspensions. Stocks of ***E. coli*** strain C were prepared from a overnight broth by inoculating LB agar plates, LB agar slants, by washing 5 ml broth in 0.1 M $MgSO_4$ and suspending the washed cells in 0.5 ml 0.1 M $MgSO_4$. Each was tested for viability by streaking an LB agar plate weekly and using a single isolated colony from each resulting plate for use in the RCDA. All stocks were stored at 4^oC.

Results

Culture Agitation. The speed of rotation used to agitate the cultures during each incubation step was examined. The standard rate of rotation was 200 rpm. Assays were performed in a 37^{o}C incubator with or without rotation (orbital 200 rpm). The shaken culture was used as the standard control. The data from those experiments is presented in Table 3-3. Orbital shaking was not required for the assay to perform acceptably. The only difference was that the time required for a positive reaction to appear in tests performed without rotation was retarded by 5-10 minutes. However, a positive reaction was seen within 30 minutes.

Temperature. The effects of temperature were examined to determine if high temperature or low temperature affected the appearance of results compared to the control. The standard temperature used in the assay and as the comparison control in each experiment was 37^{o}C. The data are presented in Table 3-4. The assays performed at 35^{o} and 40^{o} C were the same as the control performed at 37^{o}C. Temperatures below 35^{o}C gave false negative results in phage-containing experiments and temperatures above 40^{o}C gave false positive results in phage-free controls experiments.

Age of Overnight Cultures. The age of the overnight culture was tested to determine if the age of the culture used to inoculate the subcultures would effect the assay results. The data are presented on Table 3-5. Overnight cultures from between 8 and 41 hours were compared to the standard 17 hr overnight control.

Volume of $CaCl_2$. The volume of $CaCl_2$ was examined to see if the molarity of $CaCl_2$, which is required for coliphage adsorption (Adams, 1959), would effect the assay's performance (Table 3-6). The standard volume of 0.1 ml of 25 mM $CaCl_2$ solution was used in the comparison control assay. Levels of $CaCl_2$ below 0.03 ml (1.5 mM $CaCl_2$) gave false positive results. Volumes between 0.04 and 0.06 ml (2 mM and 3 mM) of $CaCl_2$ yielded false negative result. Volumes of more than 0.07 ml (3.5 mM) of $CaCl_2$ yielded results identical to the standard volume.

Concentration of Inducer. The effects of different volumes of IPTG on the assay results were examined (Table 3-7). The standard volume of 0.1 ml of a 10 mM IPTG was used as the comparison control (0.2 mM IPTG final concentration). Both 0.2 or 1.0 ml (0.4 mM and 2 mM) yielded results identical to the results with the standard volume. The color development reaction was slowed by 5-10 minutes when 0.01, 0.2 and 1.0 ml were used. However, these volumes still gave a positive result within 30 minutes.

Volume of Overnight. The volume of overnight was varied to see if a higher or lower inoculum would adversely affect the assay. The standard volume of 0.05 ml of overnight was used to inoculate the comparison controls.

The data are presented in Table 3-8. Inocula of 0.01, 0.02, or 0.1 ml yielded results identical to the standard volume.

Subculture Incubation Period. The period of time the subculture was incubated with $CaCl_2$ and IPTG was examined to determine its effect on the assay. The standard incubation time of 1 hr was used in the comparison control. The data are presented in Table 3-9. If the culture was incubated less than 0.75 hr, there was no color change when coliphage were present. If the cells were incubated for over 1.5 hr, false positive results were seen in the absence of coliphage.

Sample Volume. The volume of water sample was varied to measure its effect on phage-containing (positive) or phage-free (negative) assays. The data are presented in Table 3-10. The standard volume of 0.2 ml was used as the comparison control. As long as there were at least 10 plaque forming units present in sample volume of up to 10 ml, the assay yielded results identical to the standard control (***i.e.,*** 0.2 ml) within 30 minutes. When 10 ml of water sample was used in the assay color development was slowed by 5-15 minutes but was still positive within 30 minutes in phage-containing experiments.

Length of Incubation of Induced Cells and Phage. The length of incubation of the mixture of induced cells and phage on the assay was measured. The standard incubation time of 2 hr was used in the comparison control. The data are presented in Table 3-11. Incubation periods less than 2 hr were frequently negative in phage-containing experiments. Incubation periods of 2 and 2.5 hr had normal color development. When the incubation was increased beyond 2.5 hr, color developed in phage-free experiments (false positive).

Volume of Filtrate. The amount of culture and water sample that was required to be filtered into the CPRG solution was varied to determine its effects on the results. The standard volume of 0.5 ml was used in the comparison control. The data are presented on Table 3-12. As little as 0.05 ml of filtrate gave a deep purple color comparable to the standard control within 30 minutes with approximately 100 coliphage per sample. Volumes greater than 0.5 ml were not examined because they would exceed the capacity of the filtration unit of the Spin-X tube.

Volume of CPRG. The amount of CPRG was varied to determine its effect on the assay results. The standard volume of 0.1 ml of the 5 mM CPRG solution was used in the comparison control. The data are presented on Table 3-13. As little as 0.01 ml yielded a color change in 30 minutes, although the color was not as intense as the standard control due lower concentration of CPRG.

Alternative Inducer. The effect of using lactose as an inducer for the assay was performed. The standard inducer IPTG was used as the comparison control. In the four experiments tested using 1 mM lactose (final concentration) only one yielded results identical to the standard IPTG control.

Alternative Substrates. The effect of using 5 mM 5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside (Bluo-Gal, Boehringer-Mannheim Biochemicals, Indianapolis, IN) as a substrate for β-galactosidase on the assay was measured. The standard control of 0.1 ml 5 mM CPRG was used as the standard comparison. In the three experiments performed the Bluo-gal yielded a color change from clear to blue following the color changes in the standard CPRG assay.

Stability of IPTG. Two assays were performed using a sample of IPTG from each storage condition weekly for 124 days. All assays performed with IPTG stored at 4^{o}C and at room temperature yielded results identical to the fresh IPTG control results. Sample of IPTG stored at 37^{o}C yielded false negative results in half (1 out of 2) assays performed weekly after 8 days.

Stability of CPRG. Two assays were performed using a sample of CPRG from each storage condition weekly for 126 days. Results from both assays performed weekly on CPRG stored at 4^{o} C and room temperature were identical to the fresh CPRG controls results. CPRG stored at 37^{o}C yielded false negative results in half of each (1 of 2) weekly assays after 15 days.

Stability of *E. coli* strain C Lyophiles. The initial concentration of ***E. coli*** in the 20% skim milk suspension prior to lyophilization was 2.4×10^{9} colony forming units/ ml (cfu/ ml). The initial concentration of cells suspended in LB prior to lyophilization was 2.0×10^{9} cfu/ ml. Immediately following lyophilization of 20% skim milk and LB suspensions, the concentrations were 8.3×10^{8} cfu/ ml and 4.4×10^{6} cfu/ ml, yielding survival percentages of 35 and 0.22, respectively. After 56 days the survival of 20% skim milk stored lyophiles remained approximately 10%. After 56 days the survival of LB stored lyophiles remained approximately 1%.

Stability of *E. coli* strain C stocks. All media provided adequate storage for up to 57 days except the LB broth. The LB broth failed to yield any colonies when streaked onto LB agar plates after 21 days. The assay performed normally with each storage type (LB agar plate, LB agar slant, LB broth, and $MgSO_4$ suspension) whenever colonies resulted from inoculated LB agar plates from and were used in the RCDA.

Discussion

The acceptable ranges at which the assay may be performed were studied by assessing the effects of varying each step of the assay.

Agitation. Although agitation was not required for the assay to perform normally, agitation is beneficial in that the ***E. coli*** host cells grow at a more rapid rate because of better aeration. The higher growth rate will provide greater numbers of host cells for infection by coliphage in a shorter amount of time. Therefore, it is recommended that all culture steps be performed with agitation (200 rpm).

Temperature. The effects of temperature on the assay were very pronounced. At temperatures below 35^{o}C the assay frequently gave false negative results in phage-containing experiments. This is possibly due to the host cell producing an extracellular slime that prevents phage adsorption. At temperatures above 40^{o}C, the assay always gave false positive (color change) in phage-free experiments. The higher temperatures appear to make the cells leaky and induced β-galactosidase is released into the medium from cells that have not been lysed by coliphage.

Overnight Culture Age. As little as 8 hr were required to yield sufficient growth in the overnight to provide an adequate inoculum for the assay. In addition, an overnight up to 41 hr old still functioned well in the assay.

Volume of $CaCl_2$. The volume of $CaCl_2$ added to the assay was critical. If volumes less than 0.03 ml of the 25 mM $CaCl_2$ solution were added to the assay, a color change was consistently observed in phage-free experiments possibly due to cells leaking β-galactosidase due to a lack of calcium. Volumes between 0.04-0.06 ml (2 mM and 3 mM) of $CaCl_2$ consistently yielded no color change in phage-containing experiments due to a lack of calcium ion which is required for some coliphage for adsorption to the host cell (Adams, 1959). Only when volumes greater than 0.07 ml (3.5 mM) of the $CaCl_2$ solution were added to the assay were the results identical to standard results. Adding 0.5 ml (25 mM) of $CaCl_2$ gave identical results to the standard control.

Volume of Inducer. The volume of IPTG was not as critical to the assay as was the $CaCl_2$. Volumes between 0.01 and 1.0 ml (0.02 mM and 2.0 mM) of IPTG yielded results identical to the standard. By having the $CaCl_2$ and IPTG solutions combined, the volume required for the assay to perform optimally is dependent upon the $CaCl_2$ concentration (see Volume of $CaCl_2$ above). As long as 0.1 ml of the combined solution is added the assay will work optimally.

Volume of Overnight Culture. Volumes between 0.02 and 0.1 ml of ***E. coli*** overnight culture yielded results identical to the standard results. When 0.01 ml of overnight was used as an inoculum, the assay gave a false negative result in phage-containing experiments. Possibly this was due to too few host cells being lysed and not enough β-galactosidase released to be detected.

Length of Subculture Incubation. Incubation times between 0.75 and 1.5 hr gave results identical to the standard results. When the subculture was incubated for 0.5 hr, a false negative result was seen in the phage-containing experiments due to too few host cells being present. If the subculture was incubated for longer than 1.5 hr, by the end of the assay enough cells had lysed or leaked their β-galactosidase to yield false positive results in the phage-free experiments.

Volume of Sample. Volumes of water sample between 0.1-5.0 ml yielded results identical to the standard results. When a 10 ml sample contained as many plaque forming units as the standard control, color development was usually slower by 5-15 minutes and occasionally yielded in a false negative result within 30 minutes.

Length of Incubation of Induced Cells and Phage. The length of incubation of induced cells with the water sample is critical. Incubation times less than 2 hr yielded false negative results in phage-containing experiments. Possibly too few cells had been lysed and not enough β-galactosidase had been released to yield a color change. When the incubation lasted longer that 2.5 hr too many cells had lysed or leaked their β-galactosidase even in the phage-free experiments to yield a false positive result.

Volume of Filtrate. Filtrate volumes between 0.05 and 0.5 ml gave color changes identical to the standard results. If the assay is performed as described in the Materials and Methods section, results will be accurately and reproducibly observed.

Volume of CPRG. Volumes of 5 mM CPRG between 0.01-0.1 ml yielded identical results to the standard results. When as little as 0.01 ml of CPRG is added to the filtrate a color change is observed in the phage-containing experiments. However, the color was not as intense as in the standard results due to a lower concentration of chlorophenol red being released upon cleavage by β-galactosidase.

Alternative Inducer. The use of lactose as inducer is not favorable to the assay. IPTG binds the ***lac*** repressor with equal affinity as lactose (Miller, 1978) and results in 10 times more β-galactosidase induction compared to lactose (Miller, 1978). The use of lactose as inducer in the assay yielded false negative results in phage-containing tests due to a lowered level of β-galactosidase production in ***E. coli***. Alternatively, it could be metabolized and not be available as an inducer.

Alternative Substrate. Although Bluo-Gal yielded results identical to the CPRG standard, Bluo-Gal is not recommended for use in the assay because it must be dissolved in dimethyl formamide while CPRG is soluble in water. Using Bluo-Gal would only increase the expense of performing the assay by requiring an additional reagent.

Stability of IPTG. Temperatures above room temperature are not recommended for the storage of IPTG. At temperatures above room

temperature IPTG may possibly hydrolyze and lose its ability to induce the production of β-galactosidase in ***E. coli*** C. Storage of IPTG at 4^{o} C is recommended to maintain exact control over the storage conditions as room temperatures may vary and adversely affect the IPTG.

Stability of CPRG. Use of CPRG stored for more than 15 days at 37^{o}C yielded false negative results in half of the samples tested. The samples that failed to yield acceptable results (***i.e.***, false negative in phage-containing samples) were stored in a cardboard box at 37^{o}C, while the samples of CPRG stored at 37^{o}C that gave acceptable results (***i.e.,*** identical to the fresh CPRG control) were stored in a screw-capped polypropylene bottle. A possible explanation for the false negative results of samples stored in the cardboard box could be in the extra oxidation from the constant exposure to fresh air and accelerated desiccation of the CPRG samples contained in the box. Oxidation of the CPRG could possibly prevent β-galactosidase from being capable of cleaving it.

Stability of *E. coli* strain C lyophiles. Although, the 20% skim milk-stored lyophiles had a higher survival percentage than the LB-stored lyophiles both methods yielded viable cultures for up to 70 days that could be used in the RCDA.

Stability of *E. coli* strain C stocks. ***E. coli*** C was successfully recovered for at least 57 days from LB agar plates or slants stored at 4^{o} C , and from cells washed in 0.1 M $MgSO_4$ and stored at 4^{o} C. The use of LB broth to store the stock culture is not recommended because no viable cells were recovered after 21 days.

The goal of defining the tolerances of the RCDA was to provide a commercial producer of the RCDA with a list of acceptable ranges that could be used by a technical support staff (Table 14). If a user performed the test differently than in the standard method, they could call a technical support representative and describe how the assay had been performed incorrectly. If the incorrect procedure fell within the acceptable parameters of the assay, the incorrectly performed test could be accepted as giving acceptable results. This would prevent the user from repeating the assay and would save both time and money. If the user performed the test outside of the acceptable parameters the technical support representative would recommend the assay be repeated.

Table 3-1. Protocol for performing the rapid coliphage detection method.

Step	
1.	Streak a working plate of ***E. coli*** C from a stock plate and incubate overnight at 37°C.
2.	Inoculate 5 ml mLB in a 13 x 150 mm screw-capped tube with a single isolated colony of ***E. coli*** C no larger than 2 mm in diameter and incubate overnight at 37°C at 200 rpm
3.	To each 5 ml mLB (13 x 150 mm screw-capped tube) used in the assay add 0.1 ml of the $CaCl_2$ + IPTG solution.
4.	Inoculate each 5 ml mLB + $CaCl_2$ + IPTG with 0.05 ml of the overnight culture of ***E. coli*** C.
5.	Incubate cultures for 1 hour at 37°C at 200 rpm.
6.	Add 0.2 ml control phage suspension to the positive control tube.
7.	Add from 0.1 to 5 ml water sample to each sample tube.
8.	Add a volume of sterile water equal to the sample volume to the negative control tube.
9.	Incubate all tubes for 2 hours at 37°C with rotation (200 rpm).
10.	Place 0.1 ml CPRG solution in the bottom of each Spin-X tube and return the filter unit to the tube.
11.	Apply 0.5 ml of each culture to the filter unit of each Spin-X tube.
12.	Centrifuge the tubes for 2 minutes at 16,000 x g.
13.	Discard the filter units and incubate the tubes for 30 minutes at 37°C to allow for color development.

Table 3-2. Ranges of parameters tested in the rapid coliphage detection method

Variable	Range tested	Standard value
Rotation	0 - 200 rpm	200 rpm
Temperature	30 -44^{o} C	37^{o}C
Age of ***E. coli*** C culture	8 - 41 hours	17 hours
Volume $CaCl_2$ + IPTG	0.01- 0.5 ml	0.1 ml
Volume ***E. coli*** C inoculum ml	0.01 - 0.1	0.05
Subculture time	0.5 - 2.5 hours	1 hour
Water Sample	0.1 - 10 ml	0.2 ml
Length of sample incubation	0.5 3 hours	2 hours
Volume of culture filtered	0.1 - 0.5 ml	0.5 ml
Volume CPRG	0.01 - 0.1 ml	0.1 ml
Length of color development	30 - 45 minutes	30 minutes

Table 3-3. Effects of culture rotation (rpm) on the rapid coliphage detection assay.

Speed of rotation (rpm)	Number tested	Number positive	Percent positive
200	37	37	100
0	5	5	100

Table 3-4. Effects of temperature of incubation on the rapid coliphage detection assay.

Temperature (^{o}C)	Number tested	Number positive	Percent positive
30	6	1	17[a]
32	3	0	0[a]
35	3	3	100
37	37	37	100
40	3	3	100
42	3	0	0[b]
44	3	0	0[b]

[a] false negative results seen in phage-containing samples
[b] false positive results seen in phage-free samples

Table 3-5. Effects of age of ***E. coli*** culture on the rapid coliphage detection assay.

Overnight age (hours)	Number tested	Number positive	Percent positive
8	3	3	100
17	37	37	100
41	6	6	100

Table 3-6. Effects of $CaCl_2$ concentration on the rapid coliphage detection assay.

25 mM $CaCl_2$ Percent volume (ml)	Concentration (mM)	Number tested	Number positive	Percent positive
0.02	1.0	8	3	37.5 [b]
0.03	1.5	9	3	33 [b]
0.04	2.0	9	5	56 [a]
0.05	2.5	8	4	50 [a]
0.06	3.0	3	1	33 [a]
0.07	3.5	5	4	80 [a]
0.08	4.0	3	2	66 [a]
0.1	5.0	37	37	100
0.5	25.0	3	3	100

[a] false negative results seen in phage-containing samples
[b] false positive results seen in phage-free samples

Table 3-7. Effects of IPTG concentration on the rapid coliphage detection assay.

10 mM IPTG volume (ml)	Concentration (mM)	Number tested	Number positive	Percent positive
0.01	0.02	4	3	75 [a]
0.1	0.2	37	37	100
0.2	0.4	5	5	100
1.0	2.0	4	4	100

[a] false negative results seen in phage-containing samples

Table 3-8. Effects of inoculum volume of ***E. coli*** C on the rapid coliphage detection assay.

Volume overnight (ml)	Number tested	Number positive	Percent positive
0.01	4	3	75 [a]
0.02	4	4	100
0.05	37	37	100
0.1	2	2	100

[a] false negative results seen in phage-containing samples

Table 3-9. Effects of different subculture incubation periods on the rapid coliphage detection assay.

Subculture time (hours)	Number tested	Number positive	Percent positive
0.5	3	0	0 [a]
0.75	3	3	100
1.0	37	37	100
1.5	3	3	100
2.0	3	3	100 [b]
2.5	3	3	100 [b]

[a] false negative results seen in phage-containing samples
[b] false positive results seen in phage-free samples

TABLE 3-10. Effects of sample volume on the rapid coliphage detection assay.

Sample volume (ml)	Number tested	Number positive	Percent positive
0.1	6	6	100
0.2	37	37	100
1.0	6	6	100
5.0	8	8	100
10	8	7	85 [a]

[a] false negative results seen in phage-containing samples

Table 3-11. Effects of different lengths of phage + culture incubation periods on the rapid coliphage detection assay.

Incubation (hours)	Number tested	Number positive	Percent positive
0.5	5	0	0 [a]
1.0	5	4	80 [a]
1.5	4	1	25 [a]
2.0	37	37	100
2.5	5	5	100
3.0	4	4	75 [b]

[a] false negative results seen in phage-containing samples
[b] false positive results seen in phage-free samples

Table 3-12. Effects of different volumes of filtrate added to CPRG on the color development.

Filtrate volume (ml)	Number tested	Number positive	Percent positive
0.05	3	3	100
0.1	4	4	100
0.2	4	4	100
0.5	37	37	100

Table 3-13. Effects of different CPRG concentrations on the rapid coliphage detection assay.

CPRG volume (ml)	Concentration (mM)	Number tested	Number positive	Percent positive
0.01	0.08	3	3	100
0.1	0.83	37	37	100

Table 3-14. Summary of acceptable ranges for the rapid coliphage detection assay.

Parameter	Acceptable Range	Standard Value
Rotation	0 - 200 rpm	200 rpm
Temperature	35 - 40^{o} C	37^{o} C
Age of ***E. coli*** C culture	8 - 41 hours	17 hours
Volume $CaCl_2$ + IPTG	0.1 - 1 ml	0.1 ml
Volume ***E. coli*** C inoculum	0.02 - 0.1	0.05 ml
Subculture time	0.75 - 1.5 hours	1 hour
Water Sample	0.1 - 5 ml	0.2 ml
Length of sample incubation	2 - 2.5 hours	2 hours
Volume of culture filtered	0.05 - 0.5 ml	0.5 ml
Volume CPRG	0.01 - 0.1 ml	0.1 ml
Length of color development	30 - 45 minutes	30 minutes

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Curricula Vitae

James Emmett Stanek

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549 Tannery Lane #35 · Elkton, VA 22827 · (540) 298 1740

Education:

Masters of Science, Microbiology and Genetics, January 1997. Virginia Tech.
Thesis: Development of a Rapid Coliphage Detection Assay
Bachelor of Science, Biology, May 1992. Virginia Tech

Coursework:

Topics in Microbial Genetics
Microbial Physiology
Microbial Physiology Lab
Molecular Biology for the Life Science
Prokaryotic Gene Regulation
Virology
Nucleic Acids with Lab
Aquatic Microbiology with Lab

Teaching Experience:

Graduate Teaching Assistant, General Microbiology Lab, Biology Dept. Virginia Tech 1993-94.
Instructed four sections (~30 students per section) of the introductory microbiology lab course. Responsibilities included; assigning and grading projects, and determining final course grades.

Grants Received:

"Use of Mini-Transposons to Create a ***Mycobacterium avium - Mycobacterium smegmatis*** Shuttle Vector" Sigma Xi 1992. $600 with an additional $500 from the Virginia Tech Biology Dept.

Papers Presented:

"Evaluation of an epifluorescence based method for enumeration of microorganisms in pharmaceutical grade water." Merck and Co., Inc. 50th Meeting of the Paraenternal Drug Association, Philadelphia, November 1996.

Posters Presented:

"Development of a Rapid Coliphage Detection Assay" J. Stanek, M. Ijzerman, C. Hagedorn, and J. Falkinham. Virginia Polytechnic Inst. and State Univ. and US Environmental Protection Agency. American Society of Microbiologist General Meeting, Washington DC, May 1995

Work Experience:

Temporary Microbiologist, Merck & Co., Inc., 1995-Present.
Performed limit of detection assays on various microorganisms using the Chemunex Chem***Scan*** instrument to detect viable organisms on a membrane filter. Correlated data from the Chem***Scan*** with standard plate count method used in water quality testing to generate data for instrument evaluation.

Organized records and maintained a photographic log of results. Designed adaptation of the Chem***Scan*** protocol to detect desiccated bacterial spores. Collected water samples and, evaluated data for comparison of the Chem***Scan*** system to standard plate counts. Wrote SOPs for the Chem***Scan*** system.

Undergraduate Supervisor, Biology Dept., Virginia Tech, 1994-1995.

Supervised and directed two undergraduates who assisted in collecting data for the Rapid Coliphage Detection Method project.

Graduate Research Asst., Biology Dept., Virginia Tech, 1992-1995. Initiated and performed experiments using molecular biological techniques including: restriction analysis, gel electrophoresis, DNA isolation, Southern blotting, and electroporation. Initiated and performed microbiological experiments including: pure culture isolation, water sampling, spectrophotometry, phage isolation, and protocol optimization.

Business Manager, Student Publications Photo Staff, Virginia Tech, 1991-1993. Wrote and managed ~ $25,000 and ~ $20,000 annual budgets and assisted in training of photographers. Managed accounts for the Student Media Board of Virginia Tech.

Editor, Student Publications Photo Staff, Virginia Tech, 1989-1991. Trained ~30 photographers in photographic protocols and techniques and issued photographic assignments for the semi-weekly university newspaper, ***The Collegiate Times*** (circulation 14,000). Evaluated photographs for publication and assisted in newspaper production

Co-op Student, Virginia Power, Chesapeake, VA & Mt. Storm, WV 1988-1989.
Performed daily spectrophotometric assays of powerplant water; entered data into the company mainframe; monitored ash ponds, metal ponds and a small waste-water treatment plant; and performed standard coal analysis.