Comparison of PCR and Plaque Assay for Detection and Enumeration of Coliphage in Polluted Marine Waters

JOAN B. ROSE,* XINTING ZHOU, DALE W. GRIFFIN, AND JOHN H. PAUL

Department of Marine Science, University of South Florida, Tampa, Florida 33624

Received 7 August 1996/Accepted 30 July 1997

A total of 68 marine samples from various sites impacted by sewage and storm waters were analyzed by both the plaque assay and a reverse transcriptase (RT) PCR technique for F⁺-specific coliphage. The coliphage levels detected by the plaque assay averaged 1.90×10^4 PFU/100.0 ml. Using a most probable number (MPN) PCR approach, the levels averaged 2.40×10^6 MPN-PCR units/100.0 ml. Two samples were positive by RT-PCR but negative by plaque assay, and 12 samples were positive by plaque assay but negative by RT-PCR (levels lower than 11.00 PFU/100.0 ml). The host system used for the plaque assay may detect somatic coliphage in addition to the F⁺-specific coliphage. When it is used as an indicator of pollution, contamination may be missed with more restrictive systems. The difference in results may be due to the sensitivity, specificity, or inhibition of RT-PCR in marine samples. This study provides information on quantifying PCR results by an MPN method and insights into interpretation of PCR data for detection of viruses in marine environments.

We had previously demonstrated as part of a multidisciplinary program to study contamination in Mamala Bay, Hawaii, that coliphage and phage infective for *Vibrio parahaemolyticus* could be found at sites impacted by sewage and freshwater inputs, indicating fecal contamination and eutrophication, respectively (16). We report here the comparison of a reverse transcriptase (RT) PCR protocol with a standard plaque assay to detect coliphage as an indicator of fecal pollution.

Coliphage have been used as indicators of fecal pollution, and F-specific RNA coliphage have been found to be consistently associated with *Escherichia coli* and are abundantly present in wastewaters of domestic origin. F-specific RNA coliphage have also been proposed as human enteric virus indicators because of similar behavior and survival in water treatment processes and in the aquatic environment (5, 7–9, 15, 17, 18). While the plaque assay is used to detect coliphage, there has been no consensus on which *E. coli* host to use, with different hosts giving varying results. In addition, not all coliphage are capable of initiating a plaque; therefore, this assay may underestimate contamination.

RT-PCR has been used for the detection and/or differentiation of enteric viruses in freshwater, marine waters, and shellfish (1–3, 11–14). Comparison of data generated by more routine cultivation techniques and RT-PCR for environmental monitoring has not been widely reported. This study presents a comparison of RT-PCR and a plaque assay for detection and enumeration of coliphage in polluted marine waters.

MATERIALS AND METHODS

Sampling sites ranged from onshore to offshore in several bay sites and watersheds in Mamala Bay, Hawaii, including Diamond Head (as an offshore control site), Manoa Stream, Sand Island sewage effluent, Pearl Harbor, and sites possibly affected by Manoa Stream and/or Sand Island sewage effluent (16).

Manoa Stream originates from a remote protected mountain and passes through the city of Honolulu, the largest urban area in the Hawaiian Islands. It finally discharges into Mamala Bay after traveling nine miles from its sources. Sampling site MS1 was on the stream near the University of Hawaii campus, just upstream of where Manoa Stream discharges into Mamala Bay. Ala Wai Canal merges with Manoa Stream close to the site where the two then discharge into

Mamala Bay. Sites AW1 and AW2 were the onshore and offshore sites of the Ala Wai Canal, respectively. Sites W1 and W2 were at Waikiki Beach and Waikiki Beach offshore sites, respectively, and site AM1 was at Ali Manoa Beach. Each of the five marine water sites was impacted by freshwater inputs.

Four sites, D0, S1, D2, and D3, from nearshore to offshore, were chosen around the Sand Island outfall. Site D2 was at the sewage outfall site. In order to evaluate the contamination of the outfall at different levels of water, water from three depths was collected at all four sites. Sites SB1 and HH1 were also chosen for evaluation of sewage contamination at the west side of the sewage outfall.

Pearl Harbor receives one hospital sewage discharge. Sites C1, C2, C3, EW1, EW2, and HU1 were located from near shore to offshore in Pearl Harbor.

Several samples were collected at a remote site off of Diamond Head (offshore E4), which does not have freshwater inputs and was considered a control site. Three depths of water were sampled to evaluate the water quality at this site.

Twenty liters of water was concentrated to between 45.0 and 65.0 ml (Benchmark Gx Flow Filtration System; Membrex Inc., Garfield, N.J.) (15). The concentrate was analyzed by plaque assay using a soft agar overlay technique and *E. coli* HS(pFamp)R as a host (courteously offered by Roger Fujioka, University of Hawaii) (4). Serial dilutions were made and PFU per 100.0 ml of the unconcentrated water were calculated.

For PCR analysis, samples were passed through a Sephadex G-100 (Pharmacia, Uppsala, Sweden) and Chelex-100 (Bio-Rad, Hercules, Calif.) combined column to decrease inhibition associated with environmental samples (1). The concentrate (300.0 μ l) was loaded onto the column (placed inside a 15.0-ml centrifuge tube) and centrifuged at $1,800 \times g$ for 3.5 min (Centra-8 centrifuge, with a swinging bucket rotor; International Equipment Co., Needham, Mass.). The filtrate was collected, 10-fold dilutions were made in triplicate, and 30.0 μ l of each of the undiluted and diluted samples was transferred into 0.60-ml polypropylene PCR tubes (Perkin-Elmer Cetus, Norwalk, Conn.). Master mixes were formulated as follows: (i) for nucleic acid release, 1.0 mM deoxynucleoside triphosphates and 5.0 mM MgCl₂; (ii) for reverse transcription, 0.50 μ g of random hexamer, 8.0 μ g of RNase inhibitor, and 8.0 μ g of avian myeloblastosis virus RT; and (iii) for amplification, 300.0 nM (each) primer and 2.50 U of *Taq* polymerase. The primer sets were designed to amplify the replicase gene which is conservative for the entire F-specific RNA coliphage group. [Sequences were courtesy of Kelly Reynolds, University of Arizona. The upstream primer started at base 2175: 5'-CAA GTT GCA (G) GGA TGC AGC GCC-3'; the downstream primer started at base 2375: 3'-GCC CGA CGG ACA TTC CTC GG0-5'.] The PCR profiles were run (model 480; Perkin-Elmer Cetus) with 1 cycle of 100.0°C for 5 min and 4.0°C for at least 5 min (profile 1) and 1 cycle of 24.0°C for 10 min, 42.0°C for 50 min, 99.0°C for 5 min, and 4.0°C for 5 min (profile 2), followed by 1 cycle of 95.0°C for 2 min, 50.0°C for 30 s, and 72.0°C for 45 s and then 30 cycles of 95.0°C for 1 min, 50.0°C for 45 s, and 72.0°C for 45 s (profile 3). The results were visualized with 1.50% agarose gels (stained with ethidium bromide) and UV light.

Triplicate analyses were run for each sample by using the RT-PCR protocol for each dilution, as previously described. Negative and positive signals observed on agarose gels were recorded, and quantitative results were calculated by a most probable number (MPN) program (3-dilution, 3-tube approach; Microsoft Basic, version 2.02, 1982).

* Corresponding author. Phone: (813) 553-3928. Fax: (813) 893-9189. E-mail: jrose@seas.marine.usf.edu.

TABLE 1. Differing results in coliphage enumeration by plaque assay and MPN-PCR^a

Sampling site	Plaque assay result (PFU/100.0 ml)	MPN-PCR result (MPN-PCR units/100.0 ml)
D1S	1.90	—
D3S	0.55	—
AW1	11.12	—
C3	0.08	—
D2S	2.47	—
D2M	7.77	—
D3S	—	203.60
D3B	0.92	—
D0S	0.46	—
C1	0.63	—
D3B	0.54	—
E4S	0.09	—
HB1	—	238.40
D2S	1.06	—

^a —, negative result.

RESULTS AND DISCUSSION

Once optimized, the level of sensitivity of the RT-PCR protocol for RNA coliphage detection was 0.10 PFU of laboratory-prepared MS2 per tube. The protocol was also applied to analysis of seeded water samples with other groups of F-specific coliphage. The results showed that the primers can amplify all four groups of F-specific coliphage but did not amplify the somatic DNA phages (i.e., T2) or the bacterium *E. coli* [strains C-3000 and HS(pFamp)R and natural isolates]. In five of six trials, improvement in sensitivity was demonstrated in seeded filtered concentrates with use of the Sephadex G-100–Chelex-100 combination.

A total of 68 samples were analyzed by both plaque assay and RT-PCR. Results were cataloged via PFU and MPN-PCR units. With the exception of the sewage and canal samples that were diluted from direct grab samples, the volumes used for the assay came from the Membrex concentrate and all values were calculated per equivalent volume of 100.0 ml of water. The levels detected by plaque assay ranged from 0.09 to 2.60×10^5 PFU/100.0 ml (average of 1.90×10^4 and median of 3.93 PFU/100.0 ml), while the MPN-PCR levels ranged from 50.0 to 8.0×10^7 MPN-PCR units/100.0 ml (average of 2.40×10^6 and median of 2.28×10^5 MPN-PCR units/100.0 ml). Volumes of 4.0 ml were assayed by plaque assay, and the limits of detection corresponded to between 1,000.0 and 1,428.0 ml. The RT-PCR technique was limited to 30.0 μ l, which corresponded to a detection limit of 10.0 to 14.0 ml.

Coliphage were detected by both methods in 33.80% of the samples (23 of 68), and coliphage were not detected by either method in 45.60% of the samples (31 of 68). Two samples were positive by RT-PCR but negative by plaque assay. This may reflect the ability of PCR techniques to pick up noncultivable virus particles. The 12 samples which were positive by plaque assay but negative by RT-PCR had levels lower than 11.0 PFU/100.0 ml and appear to be indicative of the sensitivity of the method (Table 1). Complete agreement was observed when the two methods were used for analysis of freshwater samples (sewage and Manoa Stream); however, these samples also had the highest numbers of coliphage. The negative samples by RT-PCR occurred only in marine waters. The marine water samples were concentrates, which may have increased the inhibition potential for RT-PCR. We demonstrated clearly during seeded studies, as others have, that RT-PCR can be inhibited and false-negative results can be obtained. There-

FIG. 1. Association between PFU and MPN-PCR units for naturally occurring coliphage in marine waters.

fore, the difference in results may be due to the sensitivity, specificity, or inhibition of RT-PCR in marine samples. In addition, the host system used for the plaque assay may have detected somatic coliphage in addition to the F⁺-specific coliphage. Thus when this system is used as an indicator of pollution, contamination may be missed with more restrictive systems.

Figure 1 shows PFU plotted against MPN-PCR unit levels; after log₁₀ transformation and analysis the correlation coefficient was 0.75. Though statistically significant, the inability of RT-PCR to detect low levels of coliphage suggests that this technique may underestimate the contamination of shellfish and marine waters when used for noncultivable human viruses such as Norwalk, human calicivirus, and small round-structured viruses (11–14). Perhaps false negatives can be overcome by analyzing twice as many samples. The levels estimated with the MPN-PCR method were on average 100- to 1000-fold higher than the plaque assay levels. Similar ratios have been reported in other studies on virus particle-to-PFU ratios with electron microscopy (6).

Evaluations of fecal contamination in marine waters by the plaque assay and the RT-PCR method are comparable and were associated with freshwater inputs from Manoa Stream and Ala Wai Canal and discharge from wastewater treatment facilities (Sand Island and Pearl Harbor) (Table 2). Concentrations of coliphage in Sand Island wastewater prior to discharge through an outfall were consistent in all four quarterly collections, averaging 10^5 PFU to 10^7 MPN-PCR units. After discharge, the levels were diluted 10,000- to 100,000-fold, with very low levels seen on the surface and higher concentrations found at the greater sampling depths. Although coliphage levels were not as high in Manoa Stream (2.51×10^2 PFU/100.0 ml and 1.24×10^5 MPN-PCR units/100.0 ml), the impact on other waters was apparent with less dilution. Although no coliphage were detected in the samples collected from the beach sites, only four samples were analyzed. It is possible that low-level contamination is present, originating from either the outfall, Manoa Stream, or the recreational bathers themselves, and would be detected with a more intensive sampling program. Contamination in Pearl Harbor associated with other wastewater discharges was evident; however, this was the only site where apparently noncultivable coliphage were detected in much larger concentrations in two of the collections.

Compared to the standard plaque assay for coliphage detec-

TABLE 2. Concentrations of coliphage indicative of pollution in Mamala Bay

Sampling site (designation; no. of samples)	Avg results, October 1993–November 1994	
	PFU/100.0 ml ^a	MPN-PCR units/ 100.0 ml ^b
Manoa Stream (MS; 4)	251.30 (75.0)	1.24×10^5 (75.0)
Sites possibly affected by Manoa Stream (AW1 and AW2; 7)	201.60 (43.0)	3,537.0 (29.0)
Beach sites (W1 and W2; 4)	<0.10 (0.0)	<10.0 (0.0)
Sand Island sewage effluent (S1; 4)	1.75×10^5 (100.0)	2.2×10^7 (100.0)
Sites possibly affected by Sand Island (D0, ^c D1, D2, and D3; 24)	48.0 (75.0)	392.0 (46.0)
Pearl Harbor (C1, C2, and C3; 9)	2.40 (44.0)	1.75×10^4 (22.0)
Diamond Head control site (E4; 10)	0.09 (10.0)	<10.0 (0.0)

^a Plaque assay. Values in parentheses are percentages of positive samples.

^b MPN-PCR method. Values in parentheses are percentages of positive samples.

^c Closest to shore.

tion, the RT-PCR procedure has the following advantages. (i) The RT-PCR method can be used to detect F-specific coliphage within 6 h, batch processing is possible, samples can be stored in a freezer for extended periods of time prior to analysis without affecting sensitivity, and the assay is not dependent on the growth and maintenance of a bacterial host. (ii) RT-PCR can detect fewer than 1.0 PFU and in some situations can detect high levels of contamination. There are apparently large numbers of noncultivable coliphage, as determined by the plaque assay, and this phenomenon is not clearly understood. (iii) RT-PCR primers can be designed to detect only F-specific coliphage or, even more specifically, some subgroups which may be useful in distinguishing human from animal fecal contamination (10). The problems related to detection of coliphage by RT-PCR include (i) existence of RT-PCR inhibitors which can interfere with the reaction, (ii) the decreased limit of sensitivity for detection of low levels of coliphage or other viruses, and (iii) lack of a method to quantify PCR analysis. The MPN method used in this project is one possible approach for quantifying PCR results.

ACKNOWLEDGMENTS

This work was supported by a grant from the Mamala Bay Study Commission.

We thank Roger Fujioka and Kimberly Roll for assistance in laboratory and field logistics.

REFERENCES

1. Abbazadegan, M., M. S. Huber, C. P. Gerba, and I. L. Pepper. 1993. Detection of enteroviruses in groundwater with the polymerase chain reaction. *Appl. Environ. Microbiol.* **59**:1318–1324.
2. Atmar, R. L., T. G. Metcalf, F. H. Neill, and M. K. Estes. 1993. Detection of enteric viruses in oysters by using the polymerase chain reaction. *Appl. Environ. Microbiol.* **59**:631–635.
3. Atmar, R. L., F. H. Neill, C. M. Woodley, R. Manger, G. S. Fout, W. Burkhardt, L. Leja, E. R. McGovern, F. Le Guyader, T. G. Metcalf, and M. K. Estes. 1996. Collaborative evaluation of a method for the detection of Norwalk virus in shellfish tissues by PCR. *Appl. Environ. Microbiol.* **62**:254–258.
4. Debartolomeis, J., and V. J. Cabelli. 1991. Evaluation of an *Escherichia coli* host strain for enumeration of F male-specific bacteriophages. *Appl. Environ. Microbiol.* **57**:1301–1305.
5. Dhillon, T. S., E. K. S. Dhillon, M. C. Chau, W. K. Li, and A. H. S. Tsang. 1976. Studies on bacteriophage distribution: virulent and temperate bacteriophage content of mammalian feces. *Appl. Environ. Microbiol.* **32**:68–74.
6. Dulbecco, R., and H. S. Ginsberg (ed.). 1980. *Virology*. Harper & Row, Publishers, Inc., Philadelphia, Pa.
7. Havelaar, A. H., K. Furuse, and W. M. Hogeboom. 1986. Bacteriophages and indicator bacteria in human and animal faeces. *J. Appl. Bacteriol.* **60**:255–262.
8. Havelaar, A. H., M. van Olphen, and Y. C. Drost. 1993. F-specific RNA bacteriophages are adequate model organisms for enteric viruses in fresh water. *Appl. Environ. Microbiol.* **59**:2956–2962.
9. Havelaar, A. H., and W. M. Pot-Hogeboom. 1988. F-specific coliphage as model viruses in water hygiene: ecological aspects. *Water Sci. Technol.* **20**:399–407.
10. Hsu, F. C., S. C. Shieh, J. Van Duin, M. J. Beekwilder, and M. D. Sobsey. 1995. Genotyping male-specific RNA coliphages by hybridization with oligonucleotide probes. *Appl. Environ. Microbiol.* **61**:3960–3966.
11. Jaykus, L.-A., R. De Leon, and M. D. Sobsey. 1996. A virion concentration method for detection of human enteric viruses in oysters by PCR and oligoprobe hybridization. *Appl. Environ. Microbiol.* **62**:2074–2080.
12. Kopecka, H., S. Dubrou, J. Prevot, J. Marechal, and J. M. Lopez-Pila. 1993. Detection of naturally occurring enteroviruses in waters by reverse transcription, polymerase chain reaction, and hybridization. *Appl. Environ. Microbiol.* **59**:1213–1219.
13. Lees, D. N., K. Henshilwood, and W. J. Dore. 1994. Development of a method for detection of enteroviruses in shellfish by PCR with poliovirus as a model. *Appl. Environ. Microbiol.* **60**:2999–3005.
14. Le Guyader, F., F. H. Neill, M. K. Estes, S. S. Monroe, T. Ando, and R. L. Atmar. 1996. Detection and analysis of a small round-structured virus strain in oysters implicated in an outbreak of acute gastroenteritis. *Appl. Environ. Microbiol.* **62**:4268–4272.
15. Osawa, S., K. Furuse, and I. Watanabe. 1981. Distribution of ribonucleic acid coliphages in animals. *Appl. Environ. Microbiol.* **41**:164–168.
16. Paul, J. H., J. B. Rose, S. C. Jiang, P. London, X. Zhou, and C. Kellogg. 1997. Coliphage and indigenous phage in Mamala Bay, Oahu, Hawaii. *Appl. Environ. Microbiol.* **63**:133–138.
17. Reynolds, K. A. 1992. Evaluation of methods for the recovery and quantitation of bacteriophage from marine waters and sediment. M.S. thesis. University of South Florida, Tampa, Fla.
18. Stetler, R. E. 1984. Coliphages as indicators of enteroviruses. *Appl. Environ. Microbiol.* **49**:259–264.