APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Sept. 1986, p. 430–433
0099-2240/86/090430-04$02.00/0
Copyright © 1986, American Society for Microbiology
Vol. 52, No. 3

Practical Direct Plaque Assay for Coliphages in 100-ml Samples of Drinking Water

W. O. K. GRABOW* AND P. COUBROUGH

National Institute for Water Research, Council for Scientific and Industrial Research, Pretoria 0001, South Africa

Received 4 December 1985/Accepted 2 June 1986

A practical single-agar-layer plaque assay for the direct detection of coliphages in 100-ml samples of water was designed and evaluated. With this assay a 100-ml sample of water, an agar medium containing divalent cations, and the host *Escherichia coli* C (ATCC 13706) were mixed in a single container, and the mixture was plated on 10 14-cm-diameter petri dishes. It was more sensitive, reliable, and accurate than various other methods and proved rapid, simple, and economic.

Coliphages are valuable indicators of the presence of human enteric viruses in water because, in general, they outnumber viruses; they are at least as resistant to conditions in natural water environments and water treatment processes; and they are detectable by simple, inexpensive, and rapid techniques (4, 6, 9, 11, 16, 24). Coliphages are particularly useful for assessment of the safety of treated drinking water supplies because their absence has proven to be a reliable indication of acceptable virological and general hygienic quality (4, 6, 8, 13, 21, 23, 25). Based on the incidence and behavior of coliphages and enteric viruses in raw water sources, various treatment and disinfection processes, and final supplies, we have proposed a coliphage limit of 0/100 ml for drinking water, including supplies directly reclaimed from wastewater (4–6).

Coliphages are generally detected by the double-agar-layer method of Adams (1) with 1-ml test samples. Havelaar and Hogeboom (9) obtained higher counts for 1-ml samples by means of a single-agar-layer method. In another single-agar-layer method, four 5-ml fractions of a 20-ml sample were tested, and the result was used to calculate the count of coliphages per 100 ml at a theoretical minimum detection limit of 5 PFU/100 ml (2, 12). A variation of this method, using a double-agar-layer detection technique, has been described previously (23). Kott (15) designed a most probable number assay in which 65-ml samples are tested and the result is used to calculate the count of coliphages per 100 ml at a theoretical minimum detection limit of 2 PFU/100 ml. A number of techniques have been described for the recovery of phages from large volumes of water, usually detecting the phages by means of the double-agar-layer method with different host strains, growth media, selection procedures, and incubation conditions, depending on the objectives of the test (3, 7, 10, 14, 17–19, 22). The wide variety of methods used for the enumeration of coliphages frequently yields contradictory results which lead to confusion and underestimation of the value of coliphage tests because the methods differ in sensitivity, selectivity, and reproducibility (4, 7, 11, 14, 19, 23).

In this report we describe a practical single-agar-layer plaque assay for directly testing 100-ml samples of water at a theoretical minimum detection limit of 1 PFU/100 ml. Variations of the growth medium were evaluated, and the efficiency of detection of naturally occurring coliphages in a variety of fresh- and seawater samples was compared with that of selected other methods.

* Corresponding author.

MATERIALS AND METHODS

Host cultures. *Escherichia coli* C (ATCC 13706) (WG4) and its nalidixic acid-resistant (WG5) and histidine-arginine-auxotrophic (C603) (6) mutants were used. Overnight cultures (about 18 h) were grown at 35 to 37°C in nutrient broth (Difco Laboratories, Detroit, Mich.) without shaking, except for strain C603, which was incubated with shaking (6).

Media and reagents. Phage agar concentrate (PAC) was a modification of a medium described by Havelaar and Hogeboom (9) and prepared as follows. A total of 14 g of meat extract (Lab Lemco powder; Oxoid Ltd., Basingstoke, England), 4 g of yeast extract powder (Oxoid), 4 g of sodium chloride, 12 g of peptone (Difco), 1 g of sodium carbonate, 1 g of magnesium chloride, and 12 g of agar were mixed in 1,000 ml of distilled water. The mixture was heated to dissolve the agar; 100-ml quantities were mixed in suitable bottles, e.g., 250-ml screw-cap medical flats; and the solution was autoclaved (121°C, 15 min) and stored (pH 7.2). Calcium chloride solution (Ca) was prepared by autoclaving a solution of 13 g of $CaCl_2 \cdot 2H_2O$ in 100 ml of distilled water. Nalidixic acid solution (NA) was prepared by dissolving 0.7 g of nalidixic acid in 20 ml of sterile distilled water followed by membrane filtration (0.45-μm-pore-size membranes).

Phage assay. The single-agar-layer plaque assay was carried out as follows. A bottle of PAC was steamed to liquefy the agar and was adjusted to 48°C in a water bath. The 100-ml water sample to be tested was poured into a convenient sterile glass container, e.g., a 200-ml screw-cap medical flat, and 1 ml of Ca solution was added. If interference by microbial growth in water sample is expected, 1 ml of NA should be added. The water sample was adjusted to 48°C in a water bath, and 5 ml of overnight host culture was added, e.g., *E. coli* WG4 in the absence and *E. coli* WG5 in the presence of NA solution. The mixture of water sample and host culture was kept at 48°C for 3 min, and then the mixture was gently added to the bottle of PAC and gently mixed to avoid the formation of bubbles by slowly inverting once. The mixture then was poured swiftly but gently in equal volumes into 10 14-cm-diameter disposable petri dishes (without a

bottom agar layer) and allowed to solidify with the lids partly open. The inverted plates were incubated overnight at 35 to 37°C and plaques were counted (all plaques were generally visible after 8 h).

Evaluation of different phage assays. Phage counts obtained by means of the following six assays were compared in tests on homologous portions of selected water samples containing naturally occurring coliphages: NS, the new single-agar-layer assay using the plating medium described above; HS, the NS assay using the medium of Havelaar and Hogeboom (9); GS, the NS assay using the medium of Grabow et al. (6); SS, the standard methods single-agar-layer assay (2); MS, the SS method using the modified nutrient agar of the American Society for Testing and Materials (9); MD, a modified double-agar-layer assay (6). Phage counts per 100 ml were obtained as follows: NS, HS, and GS methods, plaques were counted on 10 plates; MS and SS methods, plaques on four plates were counted and multiplied by 5; MD method, plaques on 10 plates were counted and multiplied by 10. The enhancement of plaque visibility by 2,3,5-triphenyl tetrazolium chloride (tetrazolium) was investigated by applying it as described previously (2).

Samples. The collection, transportation, and processing of samples, as well as details on river water (Apies and Vaal Rivers) and tertiary treated wastewater (sand-filtered effluent in Pretoria and maturation pond effluent in Windhoek), have been described previously (6). Seawater samples were collected near wastewater outfalls at Port Elizabeth and East London. A variety of drinking water supplies (6, 7) has been analyzed.

Statistical analyses. The statistical assessment of differences in counts obtained by means of various assays was done as described by Siegel (20). Because counts were not normally distributed, the nonparametric Friedman two-way analysis of variance by ranks was used to determine the significance of differences at the 95% confidence limit (P = 0.05).

RESULTS

Typical results of tests in which counts obtained by various assays on homologous samples were compared are summarized in Tables 1 to 4. In each case results of only those comparative tests in which at least one of the assays yielded a positive result were recorded. Similar results were obtained in comparative tests on samples of water from other sources, including fresh- and seawater. The comparative tests represented in Tables 1 to 4 were selected to illustrate the following important features of the various assays. The NS method generally yielded the highest average and median counts, and more often it yielded positive results and higher counts than those obtained by the MS, SS, and MD methods (Tables 1, 2, and 4). However, differences in counts were significant only in one case (Table 1), in which NS counts were significantly higher than GS, MS, and MD counts, while the latter three counts did not differ significantly. Differences between NS and HS counts were marginal (Table 2).

The related group of NS, HS, and GS methods generally yielded higher average and median counts, and more often they yielded positive results and higher counts than those obtained by the related MS and SS methods (Tables 1 to 4). The MD method consistently had the lowest number of positive results, generally the lowest median count, occasionally the largest number of highest counts and average counts, and without exception the widest range of counts and the highest coefficient of variation (Tables 1 to 3). The extent to which MD counts were higher than the others tended to increase with increasing numbers of coliphages in the water tested.

Differences in counts obtained with the three *E. coli* C host strains were marginal when WG5 was used in the absence of NA (Table 4). In the presence of NA, however, WG5 counts were significantly lower than those of strain 603 (Table 5).

Counts were higher on thin than on thick layers of plating medium. Differences between counts on NS plating medium in 10 14-cm- and 10 9-cm-diameter petri dishes (Table 6) were significant. Counts on 8, 10, and 15 14-cm-diameter petri dishes did not differ significantly, but these counts were

TABLE 1. Counts of coliphages per 100 ml in samples of Vaal River water obtained by four different assays with *E. coli* WG5 as host

Parameter	Coliphage count/100 ml by:			
	NS	GS	MS	MD
No. of tests	31	31	31	31
No. of positive tests[a]	30	30	27	25
No. of highest count[b]	20	4	1	6
Avg count	129	96	65	81
Median count	102	67	40	60
Range of counts	0–348	0–289	0–245	0–370
Coefficient of variation (%)	77	83	99	120

[a] No. of positive tests, the number of tests in which a method yielded plaques.
[b] No. of highest count, the number of tests in which a method yielded higher counts than the other methods.

TABLE 2. Counts of coliphages per 100 ml in sand-filtered wastewater effluent obtained by four different assays with *E. coli* C603 as host

Parameter	Coliphage count/100 ml by:			
	NS	HS	SS	MD
No. of tests	19	19	19	19
No. of positive tests[a]	19	19	15	12
No. of highest count[b]	3	4	4	7
Avg count	34	31	31	57
Median count	11	9	10	10
Range of counts	1–161	1–128	0–185	0–370
Coefficient of variation (%)	142	130	159	180

[a] See footnote *a*, Table 1.
[b] See footnote *b*, Table 1.

TABLE 3. Counts of coliphages per 100 ml in samples of Apies River water obtained by three different assays with *E. coli* C603 as host

Parameter	Coliphage count/100 ml by:		
	GS	MS	MD
No. of tests	26	26	26
No. of positive tests[a]	24	21	19
No. of highest count[b]	11	7	8
Avg count	151	127	164
Median count	55	45	30
Range of counts	0–1,000	0–590	0–2,130
Coefficient of variation (%)	158	138	248

[a] See footnote *a*, Table 1.
[b] See footnote *b*, Table 1.

TABLE 4. Counts of coliphages per 100 ml in sand-filtered wastewater effluent obtained by two different assays with three strains of *E. coli* C as hosts

Parameter	Coliphage count/100 ml with the indicated strains by the following assay:					
	NS			SS		
	603	WG4	WG5	603	WG4	WG5
No. of tests	6	6	6	6	6	6
No. of positive tests[a]	6	6	6	6	6	5
No. of highest count[b]	3	0	0	1	1	1
Avg count	124	144	150	108	141	123
Median count	64	83	66	40	38	75
Range of counts	16–348	12–428	15–489	5–285	10–495	0–305
Coefficient of variation (%)	98	104	114	110	126	103

[a] See footnote *a*, Table 1.
[b] See footnote *b*, Table 1.

significantly higher than those on 4 14-cm-diameter petri dishes (Table 7).

In a comparison of NS assays using various volumes of host culture, plaque counts were not significantly affected, but the clearest plaques were obtained with an inoculum of 5 ml. There was no detectable difference in counts or plaque morphology in NS assays using hosts in the form of stored frozen cultures (2, 9, 12) or overnight broth cultures. Plaque visibility in NS assays was not appreciably enhanced by tetrazolium.

Results of tests on drinking water supplies were not recorded because they were generally negative for all tests and were not suitable for purposes of comparison. However, the NS assay proved convenient, simple, and quick to carry out in the routine assessment of drinking water quality.

DISCUSSION

The NS assay proved superior to the other methods for the detection of coliphages in 100-ml samples of water because it was more sensitive and accurate, generally yielded the highest average and median counts, and was simple and inexpensive. The sensitivity was probably due to the fact that all 100 ml of a sample was directly tested and not smaller volumes directly tested followed by theoretical calculation of the count per 100 ml as in some other assays. Adequate quantities of selected cations in the plating medium to enhance the adsorption of phages to the host probably also contributed to efficiency (9). The assay was quick and simple because the handling of tubes in a water bath was eliminated and pipetting was reduced to a minimum.

The finding that the ingredients in the plating medium can be used in concentrations lower than those described by Havelaar and Hogeboom (9) implies a savings in the cost of the assay. The application of commonly used chemicals and commercial dehydrated media, eliminating compounds such as strontium nitrate (2, 12), was also advantageous. Furthermore, cost and labor was reduced by the elimination of tetrazolium for the enhancement of plaque visibility (2, 12).

Assays other than those included in this study, which use growth media not supplemented with divalent cations to facilitate phage adsorption, were not considered because they can be expected to be less sensitive (9).

Assays which included chloroform treatment for the inactivation of interfering organisms were not considered because chloroform inactivates many coliphages (9). In the case of drinking water supplies, for which the NS assay is primarily designed, interfering growth is generally not a problem.

Assays based on the recovery of coliphages from large volumes of water followed by enumeration of the phages in the concentrate by means of conventional methods were not considered because the recovery procedures have limited and variable efficiencies (3, 17–19, 22), which implies that these methods can be expected to be less sensitive than a direct testing procedure.

Hosts other than *E. coli* C were not considered because this strain generally yields the highest count of coliphages in water (2, 6, 9, 12, 19). The observation that the auxotroph *E. coli* C603 yields counts similar to *E. coli* WG4 (the original strain ATCC 13706) (9) implies that counts obtained in earlier studies with strain C603 (6, 7, 19) are comparable to those in which strain WG4 was used.

TABLE 5. Counts of coliphages per 100 ml in samples of Vaal River water obtained by the GS method with *E. coli* WG5 and C603 as hosts

Parameter	Coliphage count/100 ml with:	
	WG5	603
No. of tests	26	26
No. of positive tests[a]	26	26
No. of highest count[b]	7	18
Avg count	95	111
Median count	69	89
Range of counts	1–289	1–318
Coefficient of variation (%)	55.54	55.56

[a] See footnote *a*, Table 1.
[b] See footnote *b*, Table 1.

TABLE 6. Counts of coliphages per 100 ml in Vaal River water obtained by the NS method with *E. coli* C603 as host[a]

Parameter	Coliphage counts/100 ml on dishes with the following diam:	
	14 cm	9 cm
No. of tests	4	4
No. of highest count[b]	4	0
Avg count	240	158
Median count	155	122
Range of counts	139–513	71–317
Coefficient of variation (%)	66	60

[a] Agar medium was plated in 10 14-cm-diameter or 10 9-cm-diameter petri dishes.
[b] See footnote *b*, Table 1.

TABLE 7. Counts of coliphages per 100 ml in sand-filtered wastewater effluent obtained by the NS method with *E. coli* WG4 as host and plating the medium in various numbers of petri dishes

Parameter	Coliphage counts/100 ml in:			
	4 plates	8 plates	10 plates	15 plates
No. of tests	15	15	15	15
No. of highest count[a]	0	1	7	7
Avg count	77	115	135	131
Median count	73	106	119	123
Range of counts	4–184	19–230	22–298	18–246
Coefficient of variation (%)	61	41	48	46

[a] See footnote *b*, Table 1.

ACKNOWLEDGMENTS

Thanks are due to A. H. Havelaar for personal advice and *E. coli* cultures and to D. C. Grobler for statistical analyses.

LITERATURE CITED

1. **Adams, M. H.** 1959. Bacteriophages. Interscience Publishers, New York.
2. **American Public Health Association.** 1985. Standard methods for the examination of water and wastewater, 16th ed. American Public Health Association, Inc., Washington, D.C.
3. **Farber, F. E., S. E. Gradwohl, P. B. Sanford, M. J. Tobin, K. J. Lee, and C. P. Gerba.** 1983. Bacteriophage concentration from water by filter chromatography. J. Virol. Methods **7:** 297–304.
4. **Grabow, W. O. K., J. S. Burger, and E. M. Nupen.** 1980. Evaluation of acid-fast bacteria, *Candida albicans*, enteric viruses and conventional indicators for monitoring wastewater reclamation systems. Prog. Water Technol. **12:**803–817.
5. **Grabow, W. O. K., P. Coubrough, C. Hilner, and B. W. Bateman.** 1984. Inactivation of hepatitis A virus, other enteric viruses and indicator organisms in water by chlorination. Water Sci. Technol. **17:**657–664.
6. **Grabow, W. O. K., P. Coubrough, E. M. Nupen, and B. W. Bateman.** 1984. Evaluation of coliphages as indicators of the virological quality of sewage-polluted water. Water S.A. **10:** 7–14.
7. **Grabow, W. O. K., E. M. Nupen, and B. W. Bateman.** 1984. South African research on enteric viruses in drinking water. Monogr. Virol. **15:**146–155.
8. **Guy, M. D., J. D. McIver, and M. J. Lewis.** 1977. The removal of virus by a pilot treatment plant. Water Res. **11:**421–428.
9. **Havelaar, A. H., and W. M. Hogeboom.** 1983. Factors affecting the enumeration of coliphages in sewage and sewage-polluted waters. Antonie van Leeuwenhoek J. Microbiol. **49:**387–397.
10. **Havelaar, A. H., W. M. Hogeboom, and R. Pot.** 1984. F specific RNA bacteriophages in sewage: methodology and occurrence. Water Sci. Technol. **17:**645–655.
11. **International Association on Water Pollution Research and Control Study Group on Water Virology.** 1983. The health significance of viruses in water. Water Res. **17:**121–132.
12. **Isbister, J. D., J. A. Simmons, W. M. Scott, and J. F. Kitchens.** 1983. A simplified method for coliphage detection in natural waters. Acta Microbiol. Pol. **32:**197–206.
13. **Kenard, R. P., and R. S. Valentine.** 1974. Rapid determination of the presence of enteric bacteria in water. Appl. Microbiol. **27:**484–487.
14. **Kennedy, J. E., Jr., G. Bitton, and J. L. Oblinger.** 1985. Comparison of selective media for assay of coliphages in sewage effluent and lake water. Appl. Environ. Microbiol. **49:**33–36.
15. **Kott, Y.** 1966. Estimation of low numbers of *Escherichia coli* bacteriophage by use of the most probable number method. Appl. Microbiol. **14:**141–144.
16. **Kott, Y., N. Roze, S. Sperber, and N. Betzer.** 1974. Bacteriophages as viral pollution indicators. Water Res. **8:**165–171.
17. **Purdy, R. N., B. N. Dancer, M. J. Day, and D. J. Stickler.** 1985. A note on a membrane filtration method for the concentration and enumeration of bacteriophages from water. J. Appl. Bacteriol. **58:**231–233.
18. **Schulze, E., and J. Lenk.** 1983. Concentration of coliphages from drinking water by $Mg(OH)_2$ flocculation. Naturwissenschaften **70:**612.
19. **Seeley, N. D., and S. B. Primrose.** 1982. The isolation of bacteriophages from the environment. J. Appl. Bacteriol. **53:**1–17.
20. **Siegel, S.** 1956. Nonparametric statistics for the behavioral sciences. McGraw Hill, New York.
21. **Simkova, A., and J. Cervenka.** 1981. Coliphages as ecological indicators of enteroviruses in various water systems. Bull. W.H.O. **59:**611–618.
22. **Singh, S. N., and C. P. Gerba.** 1983. Concentration of coliphage from water and sewage with charge-modified filter aid. Appl. Environ. Microbiol. **45:**232–237.
23. **Wentsel, R. S., P. E. O'Neill, and J. F. Kitchens.** 1982. Evaluation of coliphage detection as a rapid indicator of water quality. Appl. Environ. Microbiol. **43:**430–434.
24. **Yates, M. V., C. P. Gerba, and L. M. Kelley.** 1985. Virus persistence in groundwater. Appl. Environ. Microbiol. **49:** 778–781.
25. **Zaiss, U.** 1982. Coliphagen und coliforme Bakterien in Fliessgewässern unterschiedlicher Güte. J. Wasser Abwasser Forsch. **15:**171–177.