APPLIED AND ENVIRONMENTAL MICROBIOLOGY, May 2004, p. 2801–2805 Vol. 70, No. 5
0099-2240/04/$08.00+0 DOI: 10.1128/AEM.70.5.2801–2805.2004

Double-Layer Plaque Assay for Quantification of Enteroviruses

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Received 13 November 2003/Accepted 14 January 2004

We describe here a double-layer plaque assay for the quantification of enteroviruses, combining a monolayer plaque assay and a suspended-cell plaque assay. The double-layer assay provides significantly greater counts than other methods of virus quantification of both suspensions of pure culture viruses and naturally occurring viruses. The counts obtained by this method are approximately one order of magnitude greater than those obtained with the more commonly used method, the monolayer plaque assay. We conclude that the methods available for quantifying viruses rank in efficiency as follows: double-layer plaque assay ≥ suspended-cell plaque assay > counting cytopathogenic virus adsorbed to cellulose nitrate membrane filters ≥ most probable number of cytopathogenic units > monolayer plaque assay. Moreover, the double-layer plaque assay allows the use of two different cell lines in the two layers. Using the human colonic carcinoma cell line CaCo2 facilitates the recovery of a greater number and diversity of naturally occurring enteroviruses in water than the monolayer agar method. In addition, the pretreatment of cells with 5-iodo-2′-deoxyuridine (IDU) prior to the quantification of enteroviruses by the double-layer plaque assay provides significantly higher recoveries than the use of IDU does with the other methods of quantification.

One of the most commonly used methods for the quantification of infectious viruses in suspensions is the infection of susceptible cell cultures with dilutions of the viral suspensions, followed by an analysis of viral replication. Two types of infecting assays should be distinguished: quantitative and quantal.

Quantitative assays are based on the use of solid or semisolid culture media, utilizing the principle that each viable virus multiplies to produce a localized area of cells destroyed or killed by viral action. These discrete clones are known as plaques; each plaque is considered to be derived from an infectious virus particle, or a clump of viruses, in the inoculum. The plaque assay was introduced in animal virology by Dulbecco (6), and a few years later Hsiung and Melnick (9) established the principles of the monolayer plaque assay for enteroviruses as used today. This method is currently the most commonly used technique. However, other methods of plaque assay have been described, including an assay on suspended cells (4) and one on viruses adsorbed to and cells growing on membrane filters (the VIRADEN method, for virus adsorption enumeration) (14). Both methods have been described as providing higher counts than the standard monolayer plaque assay.

The second type of infectivity assay is not quantitative, but quantal, i.e., it does not quantify the number of infectious virus particles in the inoculum but, rather, their presence in a given amount of a given sample. These assays, based on liquid culture media, rely on the development of cytopathogenic changes induced in monolayers of susceptible host cells and detectable by microscopic observation. The presence of infecting viruses can be demonstrated in some instances by the adsorption of red blood cells with fluorescent (or otherwise labeled) antibody or by molecular probes. The most frequently used quantal assays are the 50% tissue culture infectious dose and the most probable number of cytopathogenic units (MP NCPU) (3). Quantal methods have been reported to provide higher counts than the monolayer plaque assay (12, 15).

Maximizing the number and types of infectious viruses detected is crucial in some areas of study, as, for example, in the detection of enteric viruses in water and food samples. In addition to improving the detection methods themselves, the sensitivity of cell detection can be increased by treating the viruses or cells with chemicals or by coculturing different cell lines. Thus, the addition of 5-iodo-2′-deoxyuridine (IDU) to growing cells prior to infection has been described as increasing the sensitivity of different cell lines to enteroviruses (1). Mixed cultures have also been used, with various degrees of success, to detect enteroviruses from different water sources (2, 18) and adenoviruses (2), although the numbers of viruses recovered was generally higher than that obtained by other methods.

Here we report a new virus infectivity assay in cell cultures that combines the monolayer plaque assay and the plaque assay on suspended cells. We define it as a double-layer plaque assay, since it combines the cell monolayer on the bottom surface of the cell culture plates and a thin (about 1- to 2-mm) semisolid layer containing suspended cells. The method applied to enteroviruses has been compared with existing methods to quantify suspensions of pure cultures of enteroviruses and enteroviruses naturally occurring in environmental water samples.

The effect of IDU and the potential use of different cells in the two layers have also been explored in the newly described method. Finally, the viruses detected in natural samples when either one or two cell lines were used were compared by restriction fragment length polymorphism (RFLP) of genomic fragments amplified by reverse transcription (RT)-PCR.

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MATERIALS AND METHODS

Cells and viruses. Viruses used were the attenuated poliovirus type 1 strain Lsc-2ab, coxsackievirus B5, and echovirus 6, the latter two of which were isolated from sewage in our laboratory and identified by RFLP and sequencing.

The cell lines used were a Buffalo green monkey (BGM) kidney continuous cell line (European Collection of Animal Cell Cultures accession no. 90092601), human colonic carcinoma cell line CaCo2 (ATCC HTB37), and human hepatoma cell line PLC/PRF/5 (ATCC CRL 8024).

Culture media. BGM cells were grown in Eagle's minimum essential medium (MEM) (ICN Biomedicals, Inc., Aurora, Ohio) containing 5% fetal bovine serum (FBS), 2 mM L-glutamine, 26.8 mM $NaHCO_3$, 100 U of penicillin ml^{-1} and 100 μg of streptomycin ml^{-1}. Instead of 5% FBS, 10% FBS and nonessential amino acids were added to the medium used to grow the CaCo2 and PLC/PRF/5 cells.

The overlay medium used for the standard monolayer plaque assay was MEM supplemented with 1% FBS, 2 mM L-glutamine, 26.8 mM $NaHCO_3$, 100 U of penicillin ml^{-1}, and 100 μg of streptomycin ml^{-1}. The overlay medium was prepared in a double concentration and mixed with an equal volume of 1.5% purified agar (Oxoid, Hampshire, United Kingdom).

The overlay medium used for the suspended cell assay was MEM supplemented with 3% FBS, 2 mM L-glutamine, 26.8 mM $NaHCO_3$, 100 U of penicillin ml^{-1} and 100 μg of streptomycin ml^{-1}, 0.01% magnesium chloride, 50 U of nystatin ml^{-1}, 20 μg of gentamicin ml^{-1}, and 0.05% neutral red.

The overlay medium used for the VIRADEN method was MEM supplemented with 3% FBS, 2 mM L-glutamine, 26.8 mM $NaHCO_3$, 100 U of penicillin ml^{-1} and 100 μg of streptomycin ml^{-1}, 0.01% magnesium chloride, 50 U of nystatin ml^{-1}, 50 μg of gentamicin ml^{-1}, and 20 μg of ceftazidime ml^{-1}, as described previously (14).

The overlay medium used for the double-layer plaque assay was the same as for the VIRADEN method.

In the previous three methods, the overlay medium was prepared in a double concentration and mixed with an equal volume of 2% purified agar.

The liquid overlay medium used for the MPNCPU method was MEM supplemented with 1% FBS.

Suspensions of viruses. (i) Pure-culture viruses. Suspensions containing 10^7 to 10^8 PFU of pure-culture viruses ml^{-1} grown on BGM cells were obtained by freezing and thawing the infected cell culture, followed by filtration through 0.22-μm-pore-size, low-protein binding polyether sulfone (PES) membrane filters (Millipore Corp., Bedford, Mass.) to remove viral aggregates (20). The suspensions were then diluted in PBS to obtain 30 ± 10 PFU per ml (enumerated by the standard monolayer plaque assay) and finally distributed in 2-ml aliquots. Control charts of these suspensions were made prior to and during the quantification experiments as described by van Dommelen (19).

(ii) Naturally occurring viruses. Raw sewage samples collected in a wastewater treatment plant and filtered through 0.22-μm-pore-size PES membrane filters (SLGP R25 LS; Millipore Corp.) were used as suspensions of naturally occurring viruses in sewage. Suspensions of naturally occurring viruses from river water were obtained after concentration by electropositive cartridge (17), elution with 0.25 M glycine buffer (pH 10.5), and reconcentration by organic flocculation (10), followed by suspension in 50 ml of PBS and decontamination by filtration through 0.22-μm-pore-size PES membrane filters.

Virus quantification. (i) Monolayer plaque assay. Virus suspensions were quantified by the plaque assay method in confluent monolayers of BGM cells grown in cell culture plates as described elsewhere (16).

(ii) VIRADEN. Virus suspensions were quantified by the VIRADEN method in BGM cells as previously described (13). Briefly, the virus suspension was amended by the addition of $MgCl_2 \cdot 6H_2O$ to a final concentration of 0.05 M $MgCl_2$. The suspension was then filtered through a 47-mm-diameter, 3-μm-pore-size cellulose nitrate membrane filter at a flow rate not exceeding 200 $ml \cdot min^{-1}$. When the entire sample had been filtered (1 to 500 ml, depending on the kind of sample), the membrane filter was washed with 100 ml of sterile 0.05 M $MgCl_2$. Finally, the viruses adsorbed on the membrane were counted in a BGM cell monolayer as follows. The growth medium in a 60-mm-diameter cell culture plate with a confluent monolayer was discarded, and 100 μl of a suspension of BGM cells in MEM supplemented with antibiotics containing $(1.75 \pm 0.25) \times 10^7$ cells $\cdot$ ml^{-1} was placed in the center of the plate. The membrane with the adsorbed viruses was then carefully placed upside down on top of the cell suspension and the cell monolayer; finally, 5 ml of overlay medium was poured slowly onto the center of the membrane filter and spread over the whole plate. As with the standard plaque assay, the agar was then allowed to set, and the plates were incubated under the same conditions upside down. The agar and the membrane were then removed simultaneously, and the monolayer was stained with 0.1% crystal violet.

When a second (different) cell line was used in the membrane filter, the method was as follows. After the virus suspension was filtered, 100 μl of the second (different) cell line (CaCo2 or PLC/PRF/5) with the same cell concentration was placed in the center of the plate on the BGM confluent monolayer, and the membrane filter was placed on top. The overlay medium was poured slowly, spread all over the plate, and allowed to set.

(iii) Suspended-cell plaque assay. Virus suspensions were quantified by a suspended-cell plaque assay using the BGM cell line according to the method described by Morris and Waite (12).

(iv) MPNCPU. Viral quantifications were performed according to the MPN method by using multiwell microplates as described by Gantzer et al. (6).

(v) Double-layer plaque assay. Virus suspensions were quantified by the double-layer method as follows. Up to 2 ml of virus suspension was placed in sterile tubes, and 260 μl of cell suspension containing $(1.75 \pm 0.25) \times 10^7$ cells $\cdot$ ml^{-1} was added to each tube. Two milliliters of 2× MEM containing extra antibiotics (final concentrations: gentamicin, 50 $\mu g \cdot ml^{-1}$; nystatin, 50 $\mu g \cdot ml^{-1}$; and ceftazidime, 20 $\mu g \cdot ml^{-1}$) was then added to each tube. Finally, 2 ml of 2% agar melted and kept at 55°C was added, and, after mixing, the contents of the tube were immediately poured onto a preprepared confluent monolayer on a 90-mm-diameter petri dish. Prior to the pouring of the mixture described above, the cell growth medium was discarded. The plates were then incubated right side up at 37°C in the presence of 5% CO_2 at a relative humidity of more than 80% for 36 to 96 h. The agar was subsequently removed, and the monolayer was stained with 0.1% crystal violet as it was for the monolayer assay. As in the case of the VIRADEN method, a second cell line can be used in the upper layer.

RFLP. Plaques selected by statistical randomization were subcultured on BGM cells, and when 100% cytopathogenic effect was evident, the isolates were subjected to freezing and thawing once. For RFLP analysis, the method described by Kuan (11) was used. Briefly, RNA was extracted by using a QIAamp viral RNA mini kit (QIAGEN GmbH, Hilden, Germany) and amplified by RT-PCR using the primers EP1 and EP4 described by Gow et al. (7). The amplified DNA was then digested with BglI, StyI, and XmnI as indicated by Kuan (11). Finally, the restriction patterns were compared to the patterns obtained from the GenBank database (National Center for Biotechnology Information) sequences.

Effect of IDU. The pretreatment of cells with IDU (Sigma, St. Louis, Mo.) was performed as indicated by Benton and Ward (1). Briefly, plates were seeded with BGM cells, and after 24 h the growth medium was supplemented with IDU in Hanks' balanced salts (Sigma) at a final concentration of 50 $\mu g \cdot ml^{-1}$. The plates were then incubated for 48 to 72 h at 37°C in the presence of 5% CO_2 at a relative humidity of more than 80% to allow the formation of monolayers. When the monolayers were confluent, the medium was removed, and the cells were washed twice with PBS. The plaque assays were then performed as described above.

Statistical analysis. Data comparisons were performed by using an analysis of variance (ANOVA) and Student's *t* test and the SPSS software package (SPSS Inc., Chicago, Ill.). Simpson's diversity index (9) was used to compare the diversity of the enteroviruses detected in sewage by the different cell lines.

RESULTS

Virus numbers counted by the different methods. (i) Suspensions of pure cultures of viruses. The results reported in Table 1 show the comparative recoveries of three different viruses when they were quantified by the different methods. Both the double-layer plaque assay and the VIRADEN method provided significantly higher counts than the monolayer plaque assay in enumerating each of the three virus types ($P < 0.05$ by Student's *t* test). In addition, the double-layer plaque assay gave significantly higher counts than VIRADEN for all the viruses tested ($P < 0.05$ by Student's *t* test). We encountered difficulties in visualizing the plaques in the suspended-cell tests, making it possible that we underestimated the number of plaques by this method. Nevertheless, the numbers of polioviruses obtained were significantly higher than those provided by the monolayer plaque assay ($P < 0.05$ by Student's *t* test).

TABLE 1. Comparison of numbers of viruses obtained by different assay methods

Assay methods compared (method 1 vs method 2)	No. of viruses detected by method 1/no. of viruses detected by method 2 (±SD)[a]				
	Pure-culture viruses			Naturally occurring viruses in:	
	Poliovirus 1	Coxsackie B5	Echovirus 6	Sewage	River water
VIRADEN vs monolayer	3.2 (1.5)	5.5 (1.6)	6.6 (2.3)	2.5 (1.9)	ND
Double layer vs monolayer	12.0 (6.3)	8.8 (2.5)	15.2 (0.7)	7.7 (4.0)	>7.9[b]
Double layer vs VIRADEN	3.6 (1.4)	1.4 (0.1)	2.3 (0.7)	4.5 (2.8)	ND
Suspended cell vs monolayer	2.1 (0.6)	ND		ND	ND
VIRADEN vs MPN	ND	ND		0.5 (0.4)	ND
Double layer vs MPN	ND	ND		1.3 (0.6)	ND

[a] Values are average ratios. All results for pure-culture viruses are the average of a minimum of 6 independent assays made in duplicate; averages for naturally occurring viruses are based on 9 assays for sewage and 19 assays for river water. ND, not done.
[b] For the double-layer plaque assay, 84% of samples were positive versus 20% positive for the monolayer plaque assay.

(ii) Naturally occurring viruses. Counts obtained from filtered sewage are also shown in Table 1. Again, both VIRADEN and the double-layer plaque assay gave significantly higher counts than the monolayer plaque assay ($P < 0.01$ by Student's t test), with increases that averaged 7.7, almost one order of magnitude. The double-layer plaque assay method also generated numbers of viruses significantly higher than the counts obtained by the VIRADEN method ($P < 0.01$ by Student's t test). Thus, the performance of these methods with viruses occurring naturally in sewage was similar to that with pure-culture viruses. In this case viruses were also quantified by the MPN quantal method. Numbers obtained were similar to those obtained by the VIRADEN method ($P > 0.05$ by Student's t test) and significantly lower ($P < 0.05$ by Student's t test) than those obtained by the double-layer plaque assay.

Naturally occurring viruses in a virus concentrate of river water were quantified by monolayer and double-layer plaque assays. Again, the double-layer plaque assay provided significantly higher counts than the monolayer plaque assay, with increases similar to those observed for viruses in sewage ($P < 0.01$ by Student's t test). Similarly, the percentage of samples in which viruses were detected by the double-layer plaque assay was much higher, 84 versus 20%, than the percentage provided by the monolayer plaque assay.

Naturally occurring viruses counted in the two cell layers. All of the experiments were performed with BGM cells in the lower layer, since this cell line forms better monolayers than the others. Using CaCo2 cells in the upper layer provided better recoveries in terms of both the percentage of positive samples and the counts (Table 2). Indeed, using CaCo2 cells in the upper layer provided significantly higher counts (7.4 and 4.8 times higher, respectively) than counts with either BGM or PLC/PRF/5 cells ($P < 0.01$ by ANOVA). The use of BGM or PLC/PRF/5 cells in the upper layer, however, did not significantly alter the recovery of naturally occurring viruses ($P > 0.05$ by ANOVA).

Naturally occurring viruses detected by using different cells in the two cell layers. Fifty-six viral isolates from sewage, isolated in each one of the cell lines, were typed by RFLP. Two restriction patterns were detected, accounting for 13 of the 168 virus isolates typed, which did not fit any of the enteroviruses for which restriction patterns or RNA sequences are available. The other isolates could be successfully assigned. These results are summarized in Table 3. CaCo2 cells allowed the detection of 10 different viruses, PLC/PRF/5 cells allowed detection of 8, and BGM cells allowed detection of 6. All cells detected echovirus 6 as the most abundant. The Simpson diversity index was 0.56 for BGM cells, 0.72 for PLC/PRF/5 cells, and 0.82 for CaCo2 cells.

Effect of IDU. The results shown in Table 4 indicate that IDU had a significant effect, regardless of the quantification method or cell culture used. In all cases, counts obtained after pregrowing the cells in the presence of IDU were significantly

TABLE 2. Comparison of results with different cell types in the layers of the double-layer plaque assay of naturally occurring viruses

Sets of cell layer combinations compared (set 1 vs set 2)	No. of samples	% Positive (set 1 vs set 2)	Avg ratio of set 1/set 2 (SD)
CaCo2 over BGM vs BGM over BGM	43	89 vs 75	7.4 (5.1)
PLC/PRF/5 over BGM vs BGM over BGM	38	64 vs 75	1.8 (1.5)
CaCo2 over BGM vs PLC/PRF/5 over BGM	39	89 vs 64	4.8 (3.6)

TABLE 3. Percentages of enteroviruses isolated from sewage with different cell lines in the top layer

Enterovirus(es) isolated	No. (%) of viruses isolated (no. of viruses detected)		
	BGM (6)	CaCo2 (10)	PLC/PRF/5 (8)
Pattern RFLP 1	19.6	4	
Pattern RFLP 9		2	
Poliovirus 1	14.3	8	7.1
Poliovirus 2-echovirus 7		6	7.1
Poliovirus 3	1.8		1.8
Coxsackievirus B1			3.6
Coxsackievirus B2–B3, echovirus 1, echovirus 5	1.8	8.9	
Coxsackievirus B4-echovirus 4		2	
Coxsackievirus B5 (Faulkner)			5.4
Coxsackievirus B6	1.8		
Echovirus 6	60.7	36	37.5
Echovirus 11 (Metcalf)		26	21.4
Echovirus 12		2	
Echovirus 30		4	16.1

TABLE 4. Effect on quantification of enteroviruses of addition of IDU to growing cells

Assay method	No. of viruses detected in cells grown with IDU/no. of viruses detected in cells grown without IDU (SD)[a]			
	Poliovirus 1	Coxsackievirus B5	Echovirus 6	Naturally occurring viruses in sewage
Monolayer plaque assay	1.54 (0.3)	ND	ND	ND
VIRADEN	1.6 (0.5)	3.1 (1.4)	ND	4.2 (3.1)
Double-layer plaque assay	1.6 (0.3)	ND	3.1 (1.4)	2.4 (1.1)

[a] Values are average ratios. ND, not done.

higher than those obtained without IDU pretreatment ($P < 0.01$ by Student's t test).

DISCUSSION

In the experiments performed here, the double-layer method provided significantly higher counts than any of the other methods tested. Results obtained regarding the performance of the previously described methods agree with those reported previously elsewhere (12, 14), with the exception of the results of the suspended-cell assay. As described above, we encountered problems in visualizing plaques in the suspended-cell assay. However, a comparison of our results with comparisons described elsewhere of the suspended-cell assay with the standard monolayer plaque assay (12, 14) or the suspended-cell assay with the VIRADEN method (14) indicates that the double-layer plaque assay should provide plaques counts at least as high as the suspended-cell method does. Thus, we conclude that the different methods available for quantifying enteroviruses rank in efficiency (as measured by higher counts) as follows: double-layer plaque assay $\geq$ suspended-cell plaque assay $>$ VIRADEN $\geq$ MPNCPU $>$ monolayer plaque assay.

In comparison to other assay methods, the double-layer plaque assay seems to respond similarly to strategies applied to increase sensitivity, such as the pretreatment of cells with IDU (1) or the use of mixed cell cultures (2, 18).

Using two different cell lines is appropriate for both the double-layer plaque assay and the VIRADEN assay. The counts in natural samples increased significantly when CaCo2 cells were used in the upper layer in the double-layer plaque assay or in the membrane in the VIRADEN assay. However, numbers were not significantly higher when PLC/PRF/5 cells were used in the upper layer in the double-layer plaque assay or in the membrane in the VIRADEN assay. The use of CaCo2 cells in the upper layer or in the membrane increased both the counts of viruses and the diversity of viruses counted, as indicated by the characterization of the isolates by RFLP. Several explanations could account for this observation, including the ability of CaCo2 cells to detect viruses not detected by BGM cells and of CaCo2 cells to recover some partially damaged viruses which are not recovered by the BGM cells. However, the percentages of the different viruses isolated by using CaCo2 over BGM cells or by using BGM over BGM cells do not fully support the suggestion that the ability of CaCo2 cells to detect viruses not detected by BGM cells accounts for the increase in numbers. Further studies will be necessary to clarify this issue. However, from a practical point of view, the use of CaCo2 cells in the upper layer is recommended since it clearly increases the recovery of viruses from water samples.

In summary, we have shown that the double-layer assay method provides higher counts of viruses, is very easy and time efficient as there is no adsorption period, and allows a combination of cells and the isolation of viruses by plaque purification. Although this method requires more cells than the monolayer plaque assay (but fewer than the suspended-cell plaque assay method), the higher recoveries might allow testing of smaller volumes of samples and, thus, compensate for the need of extra cells.

The results reported here indicate that care should be taken when comparing the numbers of viruses in natural samples obtained by previously used methods of quantification. In addition, observations of very low infectious doses of viruses or of infectious viruses detected by PCR or RT-PCR should be interpreted cautiously since they will largely depend on the quantification method used. We have demonstrated in this study that there may be differences up to one $\log_{10}$ unit, depending on the method used for the quantification of infectious viruses.

ACKNOWLEDGMENTS

This study was supported by grant 2001 SGR 00099 from the Generalitat de Catalunya and CeRBA (Centre de Referència en Biotecnologia de la Generalitat de Catalunya) and by CICYT grant REN2002-04035-C03-03/HID. L. Mocé-Llivina is the recipient of a fellowship from the Generalitat de Catalunya.

We thank Susana Calle for excellent technical assistance and the Group of Enteric Viruses of the University of Barcelona for providing CaCo2 cells.

REFERENCES

1. **Benton, W. H., and R. L. Ward.** 1982. Induction of cytopathogenicity in mammalian cell lines challenged with culturable enteric viruses and its enhancement by 5-iododeoxyuridine. Appl. Environ. Microbiol. **43:**861–868.
2. **Benton, W. H., and C. J. Hurst.** 1986. Evaluation of mixed cell types and 5′-iodo-2′-deoxyuridine treatment upon plaque assay titers of human enteric viruses. Appl. Environ. Microbiol. **51:**1036–1040.
3. **Clesceri, L. S., A. E. Greenberg, and A. D. Eaton (ed.).** 1998. Standard methods for the examination of water and wastewater, 20th ed. American Public Health Association, Washington, D.C.
4. **Cooper, P. D.** 1961. An improved agar cell-suspension plaque assay for poliovirus: some factors affecting efficiency of plaquing. Virology **134:**153–157.
5. **Dulbecco, R.** 1952. Production of plaques in monolayer tissue cultures by single particles of an animal virus. Proc. Natl. Acad. Sci. USA **38:**747–752.
6. **Gantzer, C., Y. Levi, and L. Schwartzbrod.** 1996. Effect of heat on the survival of infectious coxsakievirus B3 and its genome in water. Zentbl. Hyg. Umweltmed. **199:**76–83.
7. **Gow, J. W., W. M. H. Behan, G. B. Clements, C. Woodall, M. Riding, and P. O. Behan.** 1991. Enterovirus RNA sequences detected by polymerase chain reaction in muscle of patients with postviral fatigue syndrome. Br. Med. J. **302:**692–696.
8. **Hsiung, G. D., and J. L. Melnick.** 1958. Adsoprtion, multiplication, and cytopathogenicity of enteroviruses (poliomyelitis, Coxsackie, and ECHO groups) in susceptible and resistant monkey kidney cells. J. Immunol. **80:**45–52.
9. **Hunter, P. R., and M. A. Gaston.** 1988. Numeric index of the discriminatory ability of typing systems: an application of Simpson's index of diversity. J. Clin. Microbiol. **26:**2465–2466.
10. **Kattzenelson, E., B. Fattal, and T. Hostovesky.** 1976. Organic flocculation: an efficient second step concentration method for the detection of viruses in tap water. Appl. Environ. Microbiol. **30:**638–639.
11. **Kuan, M. M.** 1997. Detection and rapid differentiation of human enteroviruses following genomic amplification. J. Clin. Microbiol. **35:**2598–2601.
12. **Morris, R., and W. M. Waite.** 1980. Evaluation of procedures for recovery of viruses from water. II. Detection systems. Water Res. **14:**795–798.
13. **Papageorgiou, G. T., L. Mocé-Llivina, C. G. Christodoulou, F. Lucena, D.**

Akkelidou, E. Ioannou, and J. Jofre. 2000. A simple methodological approach for counting and identifying culturable viruses adsorbed to cellulose nitrate membrane filters. Appl. Environ. Microbiol. **66:**194–198.
14. **Papageorgiou, G. T.** 2000. Description of a simple method for the enumeration and identification of viruses adsorbed to cellulose nitrate membranes. Ph.D. thesis. University of Barcelona, Barcelona, Spain.
15. **Schmidt N. J., H. Hoh, J. L. Riggs, and E. H. Lenette.** 1978. Comparative sensitivity of various cell culture systems for isolation of viruses from wastewater and fecal samples. Appl. Environ. Microbiol. **36:**480–486.
16. **Smith, E. M., and C. P. Gerba.** 1982. Laboratory methods for the growth and detection of animal viruses, p. 15–47. *In* C. P. Gerba and S. M. Goyal (ed.), Methods in environmental virology. Marcel Dekker, Inc., New York, N.Y.
17. **Sobsey, M. D., and J. S. Glass.** 1980. Poliovirus concentration from tap water with electropositive adsorbent filters. Appl. Environ. Microbiol. **40:** 201–210.
18. **Tougianidou, D., M. Jacob, K. Herbold, T. Kahn, B. Flehmig, and K. Botzenhart.** 1989. Assessment of various cell lines (including mixed cell cultures) for the detection of various enteric viruses in different water sources. Water Sci. Technol. **21:**311–314.
19. **van Dommelen, J. A.** 1995. Statistics for the use of microbiological (certified) reference materials. Report 281 008008. National Public Health and Environmental Protection, Bilthoven, The Netherlands.
20. **Wallis, C., and J. L. Melnick.** 1967. Viral aggregation as the cause of the nonneutralizable persistent fraction. J. Virol. **1:**478–488.