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Optimization of the BGM Cell Line Culture and Viral Assay Procedures for Monitoring Viruses in the Environment

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An in-depth study of the continuous cell line designated BGM is described herein, and recommendations are made for standardizing cell culture and viral assay procedures. Based on data gathered from a survey of 58 laboratories using this cell line, a research plan was developed that included the study of growth media, sera, $NaHCO_3$ levels, culture bottles, cell concentration, overlay media, agar, virus infection conditions, and cell-dissociating agents. Additionally, a comparative virus isolation study with BGM cells and nine other cell types was conducted with 37 sewage samples collected from nine different geographic areas. The results of the study indicated that the BGM cell line is superior for virus isolation when compared with the other cell types and that certain media and additives tend to increase BGM cell sensitivity to a specific group of viruses. A standardized procedure for cultivation of BGM cells is described which provides a more effective enterovirus assay system.

In 1962 Almen L. Barron cloned a continuous line from African green monkey kidney cells which he designated BGM (Buffalo green monkey) (1). Subsequent studies of the cell line in this laboratory revealed that it was far more sensitive to enteroviruses isolated from environmental samples than were primary rhesus or African green monkey kidney cells (8). As a result of this 1974 publication, the BGM cell line was requested by numerous laboratories throughout the world. Although many of these laboratories confirmed the cell line's sensitivity, a number of reports indicated that all laboratories had not met with the same success. It was suspected that these inconsistencies resulted from different cell culture practices.

Consequently, 98 laboratories in 16 countries were queried about their procedures in cultivating the BGM cell line (10). Responses were received from 58 laboratories indicating that there were sufficient differences in cell culture practices to assure marked disparities in virus recoveries. The comparative testing program described in this report was undertaken to maximize the BGM cell line sensitivity to enteric viruses in monitoring environmental samples.

MATERIALS AND METHODS

Viruses and viral assays. Strains of poliovirus 1 (Mahoney LP), echovirus 7 (Wallace), echovirus 11 (Gregory), echovirus 12 (Travis), echovirus 14 (Tow), echovirus 27 (SEC), coxsackievirus B2 (Taylor), coxsackievirus B5 (Faulkner), coxsackievirus A9 (CME456), and coxsackievirus A16 (natural sewage isolate) were assayed by the plaque method of Dahling et al. (8); reovirus 1 (Lang) was assayed by the method of Wallis et al. (36); and simian rotavirus SA-11 was monitored by the procedure of Smith et al. (33, 34). In assaying for the rotavirus, each 100 ml of overlay medium was supplemented with 0.2 ml of a 1% DEAE–dextran solution and 2.0 ml of a stock pancreatin solution, prepared as previously described (8). Fetal calf serum, $MgCl_2$ and milk were deleted from the overlay but replaced with sterile distilled water.

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Cell cultures. During the final phase of this study, the virus sensitivities of 10 different cell cultures, including BGM cells, were compared by using 37 sewage samples collected from various parts of the United States. Table 1 lists the different cells tested and media used to culture each.

All cells tested were continuous lines with the exception of the primary African green and rhesus cells, which were purchased from Flow Laboratories (Rockville, Md.). The BGM cells routinely used in our laboratory were originally obtained in 1971 from A. L. Barron. MDBK, HeLa, HEp-2, Vero, and L-132 cell lines were purchased from the American Type Culture Collection and passaged initially on their suggested media to establish growth. Once established, all were successfully transferred to the growth medium used for the BGM cells. RD cells were obtained from W. Benton (3), Health Effects Research Laboratory, U.S. Environmental Protection Agency, Cincinnati, Ohio, and maintained on the RPMI 1640 medium because of better growth than on BGM growth medium. MA104 cells were purchased from Microbiological Associates and carried on the medium listed in Table 1.

Media. The majority of the effort expended in this study was devoted to the evaluation of different growth media. A total of 48 different media (15, 25, 27), 42 individual media and 6 combination media (see Tables 2 and 3), were tested for their ability to support growth of BGM cells, which in turn were used to assay a series of 12 viruses and one sewage sample. All media were purchased from either GIBCO Laboratories or Flow Laboratories and prepared in accordance with their specifications.

Of the media tested, 33 were prepared from powder and 15 were liquid. Stock cultures of BGM cells were planted in each medium, supplemented with 10% fetal calf serum and $NaHCO_3$ as required by each individual medium. Four days later each medium was replaced with fresh medium containing 5% fetal calf serum. The only exception was the serumless medium, which did not support cell growth without serum; however, the addition of 5% fetal calf serum was sufficient for cell growth, whereas 2% was suitable for maintenance.

TABLE 1. Summary of cell cultures tested

Cell culture designation	Type of cells	Passage levels used in study	Growth medium	References
BGM	African green monkey kidney	106 through 210	Equal parts of MEM (Eagle) with Hanks salts, L-glutamine, and NEAA and Leibovitz L-15 with L-glutamine; supplemented with 0.22% $NaHCO_3$ and 10% fetal calf serum	1, 8, 9
MDBK	Bovine kidney	94 through 106	Same medium	19
HeLa	Human cervix epitheloid carcinoma	93-105 through 93-113	Same medium	20
HEp-2	Human larynx epidermoid	363 through 375	Same medium	24
Vero	African green monkey kidney	125 through 137	Same medium	11
L-132	Human embryonic lung	Unknown	Same medium	(E. V. Davis, and V. S. Bolin, Fed. Proc. **19**:386, 1960)
RD	Human embryonal rhabdomyosarcoma	37 through 49	Medium RPMI 1640 supplemented with 0.22% $NaHCO_3$ and 10% fetal calf serum	2, 3, 30
MA104	Embryonic rhesus monkey kidney[a]	47 through 60	MEM (Eagle) with Hanks salts, L-glutamine, and NEAA; supplemented with 10% fetal calf serum, 5% tryptose phosphate broth, 0.5% of a 50% solution of glucose and 7.5% $NaHCO_3$	33
Primary	African green monkey kidney	NA[b]	Hanks salts with 0.5% lactalbumin hydrolysate supplemented with 0.22% $NaHCO_3$ and 5% calf serum	4
Primary	Rhesus monkey kidney	NA	Same medium	4

[a] Recently determined to be African green monkey kidney cell line by Microbiological Associates.
[b] NA, Not applicable.

Cells were planted at a concentration of 1×10^7 cells for 0.95-liter (32-oz) bottles, 9×10^7 cells for roller bottles (690 cm^2), and 5×10^6 cells for 0.18 liter (6-oz) bottles. All stock cultures were passed weekly for a period of 10 weeks to ensure full acclimation to each medium. Cultures for virus testing were prepared at passages 6, 8, and 10.

Sera. Fetal calf, calf, newborn calf, and horse sera were tested in conjunction with cell sensitivity to viral infection and cell growth. They were tested in various forms, i.e., untreated, heat inactivated, dialyzed, gamma globulin free, and irradiated (see Table 4). A serum substitute from a nonfat dry milk filtrate, prepared as described elsewhere (13), and a replacement serum, Zeta Sera produced by AMF Cuno, were also tested. The newborn calf serum was unavailable in dialyzed form, and the calf and horse sera were unavailable in irradiated form.

A culture of BGM cells was prepared and planted in samples of minimum essential medium (MEM)–L-15 medium; each sample contained one of the sera to be tested (with the exception of the milk filtrate). All sera and serum substitutes were added at a 10% concentration. Stock cultures were grown in 0.95-liter (32-oz) glass bottles planted at a density of 1×10^7 cells and passed weekly for 12 weeks to determine whether any sera failed to support adequate cell growth. Stock cultures could not be carried on the milk supplement beyond passage four; therefore test cultures for milk were prepared at the same time as the other test cultures. Cells cultured in irradiated newborn and fetal calf serum failed to replicate beyond passage levels 8 and 9, respectively, whereas those grown on GG-free newborn calf serum failed to replicate beyond passage 5. Test cultures were prepared at passages 3, 5, 9, and 12 for all sera with the following exceptions: the fetal calf irradiated, newborn calf irradiated, and GG-free test cells were prepared only at passages 3 and 5. All test cultures were challenged by the 11 test viruses and one sewage sample. The SA-11 rotavirus was not tested because it was unavailable at the time.

Sodium bicarbonate and cell concentration levels. Survey results (10) indicated that a wide range of bicarbonate levels and cell concentration levels were utilized by the laboratories. For this current study the lowest and highest levels were selected and filled in between with those other levels most often used. Bicarbonate levels tested are listed in Table 5, and Table 6 lists the range of cell concentrations tested in the 0.18-liter culture bottles.

Overlay media and agars. Survey results (10) indicated that 11 different media and 12 different agars were being used for overlay purposes (see Tables 7 and 8). With the overlay method for routine plaque assays (8), cultures of BGM cells infected with the various test viruses and sewage samples were first tested with the 11 different overlay media without the addition of HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) buffer and subsequently against the same overlays with 0.02 M HEPES added. In all of these trials the agar base remained constant. Once the overlay medium of choice was determined, it was used to test the effect of the various agar types on virus recovery. The overlay combination found most effective was used in the final comparative testing phase of the study for all plaque assays.

Culture vessels and microcarriers. For the cultivation of cells, four types of glass and two types of plastic roller bottles, four types of plastic and one type of glass flat bottles, and four different microcarriers were investigated (see Table 9). The plastic type culture vessels tested were as follows: Costar 150-cm^2 flask (no. 3150) and 25-cm^2 triangular flask (no. 3050); Falcon 175-cm^2 flask (no. 3028) and 25-cm^2 flask (no. 3013); Nunc 175-cm^2 flask (no. 156502) and 25-cm^2 flask (no. 163371); Corning 150-cm^2 canted-neck flask (no. 25120-150), 25-cm^2 canted-neck flask (no. 25100-25) and

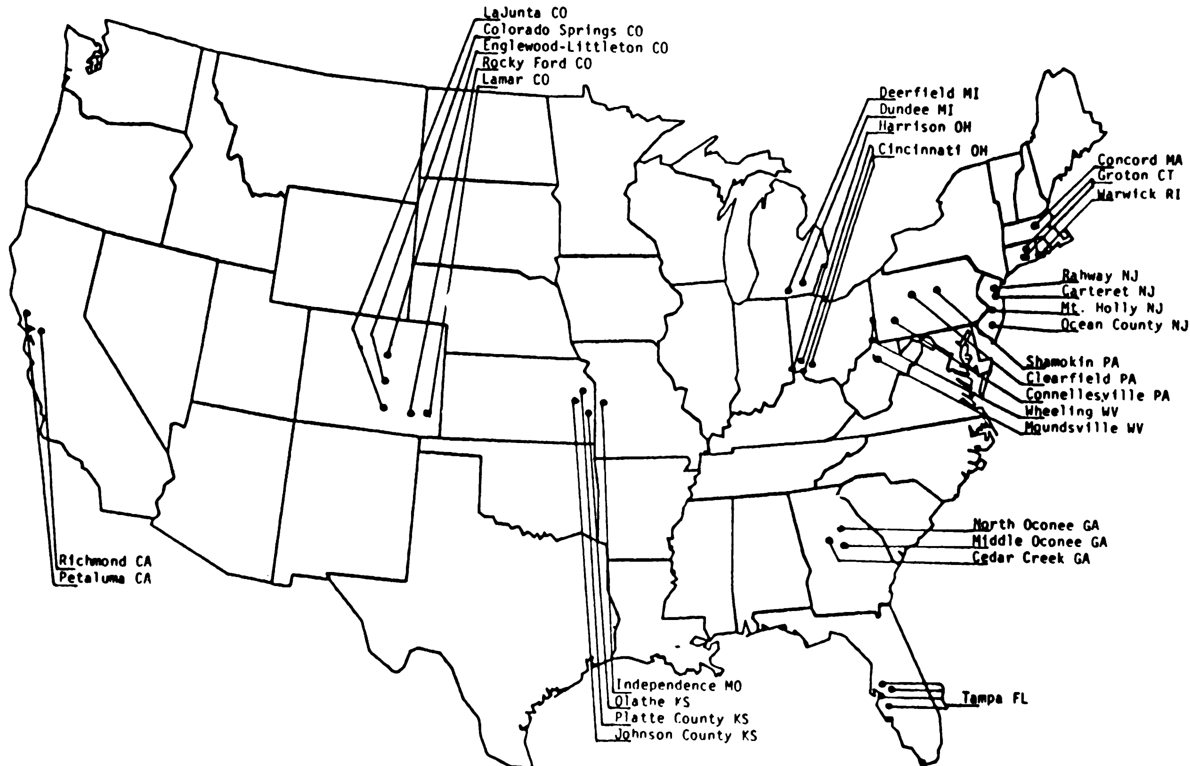


FIG. 1. Location of sample sites.

850-cm^2 roller bottle (no. 25140-850); and Falcon 850-cm^2 roller bottle (no. 3027). Glass bottles tested included the Brockway 0.95-liter (32-oz). Sani-glass bottles with rubber-lined screw caps (no. 1076-09A) and 0.18-liter (6-oz.) flint glass bottles with rubber-lined screw caps (no. 1925-11A) and the Bellco disposable 690-cm^2 roller bottles (no. 7730-38260) and reusable borosilicate 1,585-cm^2 roller bottle (no. 7730-38585).

BGM cells were planted in MEM–L-15 medium at the densities described above, and test cultures were prepared in comparable glass or plastic flasks after 3, 5, 9, and 12 weeks of passage and challenged with the 12 viruses and one sewage sample.

An additional study was conducted in which stock cells grown in disposable glass roller bottles were planted into plastic 25-cm^2 flasks, whereas comparable stock cells were grown in Corning plastic roller bottles and planted into 0.18-liter glass bottles.

A study was also conducted with the 1,585-cm^2 Bellco reusable bottles. These bottles were first brush washed with soap, rinsed, cleaned with chromic acid, and washed on a glassware washer followed by an additional distilled water rinse. Bottles were dry heat sterilized at 250°C for 2 h and then treated with either Vitrogen 100 (Flow Laboratories), as directed by the manufacturer, or with magnesium acetate (23). A control set of these bottles was not treated after cleaning and sterilization.

In a limited study four different biocarriers, Biosilon from Nunc, Cytodex 1 from Pharmacia, Bio-Carriers from Bio-Rad Laboratories, and Superbeads from Flow Laboratories, were tested. Only the Superbeads were ready for use. The others were prepared in accordance with manufacturer's instructions. Experiments were carried out in 250-ml Wheaton Spinner flasks by using MEM–L-15 growth medium. The bead concentration used was as specified by manufacturer (14, 16–18). Test cultures were prepared as above in 0.18-liter glass bottles.

Trypsin. Six different trypsin solutions (see Table 10) were tested for their ability to disperse cell monolayers and for any possible effect on cell sensitivity to infection. Five commercial trypsin preparations tested included the following: VMF trypsin and lyophilized trypsin (Millipore Corp.) prepared following the manufacturer's directions, with the exception that the final dilution for both was changed from 1:100 to 1:50; Enzar trypsin (Reheis) prepared and used as suggested by the manufacturer; trypsin 1× solution and 10× EDTA trypsin (K.C. Biological) with the 10× preparation used at a final dilution of 1:5 instead of the 1:10 recommended by the manufacturer. The sixth trypsin preparation (trypsin-EDTA) was formulated in this laboratory (0.8% NaCl, 0.02% KCl, 0.02% KH_2PO_4, 0.115% $Na_2HPO_4 \cdot 7H_2O$, 0.5% glucose, 0.125% EDTA sodium salt, and 0.3% trypsin; Difco; 1:250).

Six separate cultures of BGM cells were cultivated on MEM–L-15 medium for 12 weeks. Every week, each of the six cultures of cells was removed with the same trypsin solution. Virus test cultures were prepared at weeks 3, 5, 9, and 12, for challenge by the 12 test viruses and one sewage sample as stated above.

Test procedure. Those factors with potential effects on cell growth and subsequent virus sensitivity were tested by growing BGM cells in MEM–L-15 medium containing 10% fetal calf serum for 12 weekly passages under test conditions. These cultures were then plaque assayed against 10 enteroviruses, reovirus 1, rotavirus SA-11, and a combined sewage sample. Test cultures were inoculated with 0.5 ml of

TABLE 2. Media and combination media tested for the propagation of BGM cells

Medium	Catalog no.[a]	Type of medium		Avg cell increase[b]	Type of culture bottle capable of sustaining adequate stock cell growth	
		Powder	Liquid		Glass roller bottle	Glass 32-oz. flask
Swim's S-77	430-2000	X		4.7		X
Serumless (Neuman and Tytell)	320-1630		X	4.6		X
Waymouth 705/1	320-1925		X	4.6	X	X
Medium 199 Hanks base 50%, MEM Hanks base (NEAA) 50%	400-1200, 410-1600	X		4.4		X
BGJ_b (modified)	320-2591		X	3.6	X	X
BGJ_b (original)	320-2581		X	3.4	X	X
RPMI 1640	320-1875		X	3.3	X	X
McCoy 5A (modified)	320-6600		X	3.1	X	X
RPMI 1634	320-1865		X	3.1		X
McCoy 5A (modified), Hanks base	320-6601		X	3.0		X
MEM Hanks base (NEAA) 50%, Leibovitz L-15 50%	430-1300, 410-1600	X		2.9	X	X
Leibovitz L-15 50%, BME Hanks base 50%	430-1300, 420-1200	X		2.9	X	X
Nutrient mixture F-12 (HAM) 50%, Dulbecco modified Eagle 50%	430-1700, 430-2100	X		2.8	X	X
MEM Earle base	410-1100	X		2.8	X	X
Earles (ELH)	460-1100	X		2.8		X
BME (Diploid)	420-1300	X		2.7	X	X
Dulbecco modified Eagle	430-1600	X		2.7	X	X
MEM Hanks base	410-1200	X		2.6	X	X
McCoy 5a (modified)	430-1500	X		2.5	X	X
RPMI 1640	430-1800	X		2.5	X	X
Waymouth MB 752/1	430-1400	X		2.5	X	X
Dulbecco modified Eagle	430-2100	X		2.3	X	X
Waymouth MB 752/1	330-1445		X	2.3	X	X
BME (Diploid) 50%, Dulbecco modified Eagle 50%	420-1300, 430-1600	X		2.2	X	X
Medium 199 Earle base	400-1100	X		2.2	X	X
Glasgow MEM with tryptose phosphate broth	410-2200	X		2.2	X	X
Hanks (HLH)	460-1200	X		2.2		X
MEM Hanks base (NEAA) 75%, Leibovitz L-15 25%	410-1600, 430-1300	X		2.1	X	X
Nutrient mixture F-12 (HAM)	430-1700	X		2.0	X	X
Leibovitz L-15	430-1300	X		1.9	X	X
Glasgow MEM	410-2100	X		1.9	X	X
Swim 67-G	320-2480		X	1.9		X
MEM Earle base Auto-Pow	11-100	X		1.8	X	X
MEM Earle base (NEAA)	410-1500	X		1.8	X	X
BME Earle base autoclavable	420-1400	X		1.7	X	X
BME Hanks base	420-1200	X		1.7	X	X
MEM Earle base autoclavable	410-1700	X		1.6	X	X
RPMI 1630	320-1855		X	1.6		X
BME Earle base Auto-Pow	11-000[a]	X		1.6	X	X
MEM Hanks base (NEAA)	410-1600	X		1.5	X	X
Medium 199 Hanks base	400-1200	X		1.5		X
Williams D	320-2541		X	1.5		X
Williams E	320-2551		X	1.5		X
CMRL 1066	330-1545		X	1.5	X	X
Nutrient mixture F-10 (HAM)	430-1200	X		1.4	X	X
BME Earle base	420-1100	X		1.4	X	X
Trowell T8	320-1490		X	1.4		X
NCTC-135	440-1100	X		1.1	X	X

[a] Products of GIBCO Laboratories (Grand Island, N.Y.), except for 11-100, which is a product of Flow Laboratories (Rockville, Md.).
[b] Represents the average multiplicity of cell increase over initial cell inoculum in stock cultures measured over 10 weeks of passage in each medium.

known virus and 1.0 ml of sewage sample in four bottle replicates. When test bottles other than 0.18-liter bottles were used, the inoculum was adjusted according to surface area so that the virus/cell ratio equaled that in glass 0.18-liter bottles.

Sewage samples for comparative cell line testing. Thirty-seven sewage samples were collected from 14 different states (Fig. 1). Four liters of raw sewage were obtained from each collection point and processed for viruses by the addition of $MgCl_2$ to a concentration of 0.05 M and pH adjustment to 3.5 before filtration through 0.45-μm filters as previously described (8). Initial viral assays were performed on each sample in BGM cultures to determine whether dilution was necessary due to either toxicity or the number of viruses present. Of the 37 samples, it was necessary to dilute 8 to reduce plaque numbers to make plaque picking possible; 1 was diluted due to toxicity. The processed viral concentrate from each sample was stored at −70°C until needed. For each cell line tested against the 37 sewage samples, plaques were picked based on the following criteria: of counts over

TABLE 3. Virus titrations on BGM cells grown in various media

Medium	Ranking	Virus titer (PFU/ml × 10^7)																									
		Poliovirus 1		Echovirus										Coxsackievirus								Reovirus 1		Rotavirus SA-11		Sewage	
				7		11		12		14		27		CB2		CB5		CA9		CA16							
		PFU	CV[a]	PFU	CV	PFU	CV	PFU	CV	PFU	CV	PFU	CV	PFU	CV	PFU	CV	PFU	CV	PFU	CV	PFU	CV	PFU	CV	PFU/ml	CV
MEM Hanks base (NEAA) 50%, Leibovitz L-15 50%	4.5	37	50	139	63	255	8.3	435	34	8.9	66	60	118	176	92	520	2.7	52	54	505	49	1.8	74	3.8	140	63	42
Medium 199 Hanks base 50%, MEM Hanks base (NEAA) 50%	5.0	40	102	132	94	392	132	410	66	7	81	25	96	110	91	560	78	50	68	345	84	0.8	82	17	125	53	56
BME Hanks base 50%, Leibovitz L-15 50%	5.9	46	49	124	53	75	86	458	44	8	20	46	3.1	140	40	375	21	45	43	440	26	2.0	25	4.1	139	58	9.8
—[b]																											
MEM Hanks base 75%, Leibovitz L-15 25%	8.5	25	101	103	79	210	61	425	68	5.7	49	27	96	91	92	260	16	34	100	253	99	1.0	76	9.7	132	32	88
McCoy 5a (modified)	9.1	21	59	90	31	225	67	345	6.2	4.6	37	20	74	67	70	600	28	27	58	190	60	2.6	87	4.7	141	47	30
Serumless (Neuman and Tytell)	10.3	17	58	27	2.7	9	1	270	5	1.8	28	36	18	160	44	340	4	8.6	24	530	1.3	23.0	9.4	15	34	114	27
BME (diploid) 50%, Dulbecco modified Eagle 50%	10.8	21	98	78	34	310	14	285	12	3.7	14	8	35	136	47	155	50	29	92	157	94	4.0	72	14	92	32	65
RPMI 1640	12.6	4.5	17	11	6.7	72	76	94	24	10	77	23	22	156	112	145	63	21	100	140	61	0.8	20	12	84	60	27
Nutrient mixture F-12 (HAM) 50%, Dulbecco modified Eagle 50%	12.8	18	31	53	6.7	0	0	198	52	5.4	24	33	98	136	104	330	21	38	52	410	52	0.2	124	1.4	106	46	65
BGJ_b (modified)	14.0	9.2	28	21	134	140	102	160	5	4.1	103	2.7	16	14	102	150	34	42	127	122	134	1.1	59	7.8	13	50	27
McCoy 5A (modified)	15.3	4.4	3	11	13	46	77	67	8.4	7.1	50	13	5.7	148	111	130	22	21	97	123	43	0.8	31	11	81	61	26
Waymouth 705/1	15.5	11	22	21	81	10	128	150	15	7.6	129	5.8	92	60	1	106	100	7	3.1	180	47	1.0	93	13	17	53	18
BME Earle base autoclavable	15.5	7.2	116	112	136	78	76	466	124	3.8	28	14	18	30	55	235	45	5.2	130	67	54	1.0	93	1.4	82	39	40
Medium 199 Earle base	15.6	3	5	9.6	21	67	91	86	23	9.4	69	22	16	77	116	96	51	20	112	96	35	0.8	31	6.2	72	50	16
MEM Earle base Auto-Pow	16.1	6.4	123	112	138	260	114	65	60	6.3	24	9.7	127	43	63	220	19	5.1	135	91	46	1.1	58	1.1	62	53	43
Nutrient mixture F-12 (HAM)	16.2	3.1	26	23	6.2	82	66	100	13	4.9	84	22	63	57	101	96	3	32	131	80	12	0.7	46	15	86	33	33
Nutrient mixture F-10 (HAM)	16.8	3.7	70	37	68	66	44	175	45	4.9	106	17	72	21	97	124	32	5.1	22	101	55	0.4	131	16	102	37	33
MEM Earle base (NEAA)	17.2	4.9	32	20	16	53	105	505	25	4	5.2	11	88	14	124	95	11	36	121	90	43	0.5	97	7.7	69	43	94
RPMI 1640	17.9	5.3	96	23	47	42	120	39	135	4	101	9.2	0.8	20	103	102	68	23	101	93	103	0.4	35	18	100	23	28
MEM Earle base	18.2	4.9	75	18	16	40	50	97	5	6.6	7	8.5	21	18	79	92	74	41	126	104	137	0.4	127	15	141	41	26
MEM Earle base autoclavable	18.8	8.3	116	72	133	51	72	76	65	4.2	36	8.7	1.6	45	74	95	33	5.2	121	115	6.2	1.0	84	1.2	91	64	31
RPMI 1630	19.7	8.4	3	16	50	30	26	260	36	0.4	35	2.8	33	29	12	106	33	63	5.7	125	5.7	1.3	17	10	13	38	22
Dulbecco modified Eagle	19.8	3.7	54	15	28	32	43	73	3.9	3.8	77	14	55	23	68	82	17	34	54	107	17	0.6	29	8.5	42	45	19
Waymouth MB 752/1	19.8	2.2	84	4.5	41	67	91	48	55	4.2	8.5	12	24	20	51	184	90	2.6	117	170	8.3	0.7	64	17	62	50	16
BME Earle base Auto-Pow	19.8	6	120	92	137	79	73	57	26	3.7	38	6.7	5.3	30	47	180	24	5.4	120	71	24	1.0	76	1.7	81	29	72

Medium																											
BME (diploid)	19.8	3.9	9	6.6	30	52	82	73	0	7.1	27	22	69	64	122	93	27	21	102	62	2.2	0.8	27	4.8	61	40	30
Dulbecco modified Eagle	20.4	5.2	8	9.2	7.7	32	97	32	25	3.3	24	15	61	67	93	86	74	21	83	93	57	1.0	8.2	9.8	17	42	13
BGJ$_b$ (original)	21.2	6.6	14	22	62	60	67	127	37	1.7	95	10	13	13	51	135	47	4.2	19	62	121	0.8	133	6.1	39	27	77
Leibovitz L-15	21.2	2.6	71	4	7.1	42	13	50	4.3	3	75	11	61	23	97	69	76	3.5	13	125	28	0.4	39	14	42	63	9
MEM Hanks base (NEAA)	22.2	3.8	32	18	16	53	105	94	25	1.4	5.2	8.7	88	59	124	93	11	23	121	77	43	0.4	97	1.6	70	33	94
Glasgow MEM with tryptose phosphate broth	22.6	2.6	2.8	5.5	7.7	0	0	68	7.3	4.6	18	9.1	2.4	78	112	50	57	7	64	185	4	0.3	25	0	0	80	4.5
Waymouth MB 752/1	22.8	2.2	58	6.7	61	44	124	59	17	8	126	11	75	17	88	77	61	3.2	15	80	90	0.7	98	12	51	37	46
NCTC-135	23.5	3.5	69	17	17	62	56	112	35	2.3	43	7.8	41	7.7	42	89	34	21	124	43	23	0.5	44	7.3	61	26	38
Glasgow MEM	24.0	1.8	63	5	51	54	44	43	15	4.1	93	12	68	18	75	73	33	3.1	90	53	107	0.4	72	23	42	50	7.1
MEM Hanks base	24.2	5.7	76	19	45	45	25	119	62	2.1	107	7.7	11	14	64	86	41	5.1	15	86	15	0.1	126	L[c]		33	26
Swim S-77	24.6	2.5	17	8.1	29	51	58	86	5.8	6.5	27	9.6	21	8.1	51	81	14	3.7	19	32	16	0.9	25	8.1	38	26	16
BME Earle base	25.1	2.7	13	6.3	17	59	26	59	14	3.2	92	8.7	38	9.7	14	61	33	32	93	87	19	0.4	71	6.4	46	46	26
Medium 199 Hanks base	25.2	2.7	16	12	6.2	23	9.4	99	1.4	3.4	15	9.2	7.7	28	7.7	81	6.2	4.1	5.2	64	4.4	0.8	16	ND[d]		39	11
Hanks (HLH)	25.8	2.0	33	3.7	33	68	110	56	50	2.2	83	4.3	33	10	50	120	12	3.3	77	69	52	0.4	4.9	16	61	24	100
Swim 67/G	26.3	2.2	39	13	25	30	75	149	87	0.7	3	6.5	2	14	88	36	78	11	112	19	77	0.4	61	4.5	97	16	18
Williams D	28.5	3.9	25	13	17	20	55	190	4	0.3	20	4.1	5	4.5	11	20	11	3.8	11	66	1.8	0.5	1	0.4	43	23	18
Trowell T8	28.5	2.3	22	8.4	7	30	2	91	7	0.7	68	5	64	16	104	43	81	21	45	50	85	0.4	112	3.5	19	22	13
Williams E	29.2	2.8	35	9.5	1	20	3	290	67	0.3	2	4.5	11	2.9	72	33	28	1.9	4	58	15	0.5	14	2.4	18	20	11
CMRL 1066	30.5	1.5	28	5	32	20	57	53	26	0.6	33	3.8	11	32	121	56	98	7	81	31	16	0.6	2.4	L		22	82
McCoy 5A (modified) Hanks base	31.7	1.6	53	3	65	2.8	35	37	33	0.3	2.4	1.9	54	24	132	16	44	10	114	10	13	0.5	23	6.3	28	3	141
Earle (ELH)	31.8	1.1	35	2.9	37	31	2.3	36	50	0.6	80	3.4	117	14	128	39	79	1.4	64	49	116	0.4	125	6.1	82	27	110
BME Hanks base	32.8	1.2	12	3.5	48	26	71	37	76	1.4	14	3.4	116	8.7	119	19	37	1	52	53	119	0.2	20	5.4	49	20	57
RPMI 1634	34.5	1.6	50	2.3	66	5	97	43	5	0.04	5	1	14	0.4	26	9	16	1.9	42	2.6	31	1.2	12	3.7	61	3	28

[a] CV, Percent relative standard deviation.
[b] Media above this point were significantly better statistically than those below this point.
[c] L, Lysed due to nature of overlay.
[d] ND, No data.

TABLE 4. Effect of various sera on BGM cell sensitivity to virus infection

Type of serum	Ranking	Virus titer (PFU/ml, 10^7)											
		Poliovirus		Echovirus									
		1		7		11		12		14		27	
		PFU	CV[a]	PFU	CV	PFU	CV	PFU	CV	PFU	CV	PFU	CV
Newborn calf, GG-free	3.1	52	26	130	24	360	32	500	25	7.4	36	36	58
Fetal calf, dialyzed	3.3	53	25	60	92	200	78	500	23	4.1	36	28	97
Newborn calf, irradiated	5.3	36	19	61	48	220	102	370	23	5.8	56	18	63
Fetal calf	5.4	24	43	51	103	180	66	260	54	5.0	147	27	70
Fetal calf, heat inactivated	5.7	30	35	51	68	69	34	240	62	8.1	136	22	82
Fetal calf, GG free	6.0	33	19	46	90	230	54	270	32	2.8	117	24	82
Fetal calf, irradiated	6.3	32	47	64	46	77	26	190	43	3.3	72	15	82
Calf, GG free	6.5	37	30	79	13	290	38	360	38	84.0	38	2.1	81
—[c]													
Newborn calf, heat inactivated	7.8	25	41	68	14	190	39	300	41	5.8	47	17	91
Newborn calf	7.9	27	50	8	16	96	63	380	96	5.0	56	18	100
Horse	10.2	22	21	49	65	74	81	160	38	1.2	113	4.2	34
Calf	10.3	17	37	22	68	170	47	89	27	4.9	36	24	60
Zeta Sera	10.3	21	13	65	23	220	6	155	41	2.4	39	22	81
Calf, heat inactivated	10.4	13	65	27	35	100	108	120	58	4.8	36	10	64
Horse, heat inactivated	11.4	20	58	48	28	39	26	170	66	1.0	70	2.7	72
Calf, dialyzed	11.6	17	56	28	37	23	46	93	51	4.1	45	7.4	54
Milk filtrate	12.4	8.9	111	47	14	91	105	115	18	2.7	72	11	18
Horse, dialyzed	13.5	12	94	40	66	85	124	170	46	0.4	93	3.2	33
Horse, GG free	15.4	9.8	23	20	60	20	60	63	24	0.2	78	3.1	39

[a] CV, percent relative standard deviation.
[b] Number represents the average cell increase as measured against initial inoculum.
[c] Sera above this point were significantly better statistically than those below this point.
[d] ND, no data; stock cells could not be carried on media with milk filtrate substituted for serum.

150, at least 30% of the plaques were picked; of counts under 150, all plaques were picked if possible. Virus isolates were identified from antiserum pools by a microtiter system (5).

Statistical evaluation. Comparative Tables 3 through 11 and Table 13 have a column showing the numerical ranking of each medium, additive, or material being tested. The tables are arranged by a ranking procedure based on virus titer. Rankings were determined by ranking each vertical column as follows: the highest titer received a number 1, the lowest titer received the highest number, usually the number of parameters being tested, such as 48 in the media being tested. In cases in which identical titers occurred, these numbers both received the same ranking number. Once each vertical column had been ranked, the ranking numbers for each parameter were totaled across the table and divided by either 12 or 13 depending on whether SA-11 was assayed. In a ranking system where 1.0 would indicate the highest ranking attainable, the medium, additive, or material with a numerical rank value closest to 1.0 would be listed first and considered to have given the best results. All tables were prepared by using means (compiled from four replicate samples) from at least three replicate tests, unless otherwise noted, along with their relative standard deviations. In addition, Tables 3 and 4, 7 to 11, and 14 and 15 were compared within themselves by the paired *t* test at the 95% confidence limit, whereas the data in Tables 5 and 6 were compared by a repeated-measures analysis of variance and the Duncan multiple-range test (35). These in turn were used to prepare Fig. 2 through 5. The data in Table 12 were compared by a repeated-measures analysis of variance and linear regression analysis, for which Fig. 6 was prepared. These analyses followed standard checks for normality and homogeneity of variance.

RESULTS

Growth media. Table 2 lists the average cell increase obtained with the 48 growth media tested. Comparing cell counts at the time of planting to those when cells were trypsinized yielded a cell multiplicity that ranged from a low of 1.1 for medium NCTC-135 to a high of 4.7 for medium Swim S-77. Thirteen of the media failed to support sufficient cell growth in roller bottles and were only used for stationary culture. These can be identified in Table 2 as they have no mark in the roller-bottle column.

Table 3 lists the results of virus titrations on BGM cells grown on the 48 media listed in Table 2. The top-ranked medium, composed of equal parts of MEM and L-15, ranked first in titer for three of the viruses, second for two, and third for three. The second ranked medium had only one first ranking, three seconds and two thirds. When compared with the paired *t* test, no significant difference could be found between the top three media; however, the difference between the first medium and all those ranked lower was significant. Based on the ranking and the average virus titers obtained, the MEM–L-15 combination medium is recommended for regular use with the BGM cells. It is interesting to note that 6 of the first 10 media were combination media, and that Swim S-77 medium, which produced the best average cell growth, ranked only 36th by virus titer. Also note that Glasgow MEM with tryptose phosphate broth medium and combined F-12–Dulbecco modified Eagle medium produced no echovirus 11 plaques 7 days under overlay, and that separate cultures grown on MEM Hanks base medium and CMRL-1066 medium lysed under rotavirus overlay, whereas those cultures grown on Glasgow MEM with tryptose phosphate broth medium never produced rotavirus plaques.

TABLE 4—*Continued*

Virus titer (PFU/ml, 10^7)												
Coxsackievirus								Reovirus		Sewage		Avg cell increase[b]
82		B5		A9		A16		1				
PFU	CV	PFU	CV	PFU	CV	PFU	CV	PFU	CV	PFU/ml	CV	
65	7.1	280	83	58	40	260	31	1.5	80	137	18	4.4
300	138	500	30	63	21	260	61	2.1	100	115	16	4.1
54	51	590	56	30	16	280	23	0.9	74	110	27	3.6
370	123	430	25	36	29	200	88	4.5	47	100	36	4.5
300	145	370	39	37	26	260	54	3.7	106	109	24	4.4
240	144	300	34	43	30	170	37	2.8	116	99	14	4.1
87	16	430	24	35	19	210	97	17.0	132	111	30	4.0
58	39	200	62	35	26	160	62	2.4	95	108	44	3.3
51	51	300	57	26	31	170	45	0.9	25	91	19	3.8
67	43	240	101	42	29	270	44	0.7	70	84	33	4.0
150	128	98	81	31	11	140	56	0.3	90	45	22	3.2
63	35	270	30	19	35	190	54	0.6	104	88	23	3.5
57	12	68	110	39	29	137	27	0.5	31	32	29	1.6
61	27	330	25	14	34	260	24	0.5	70	94	34	3.4
120	133	210	154	27	31	150	91	0.8	141	54	72	3.2
65	41	260	32	17	68	180	35	0.5	100	97	20	3.4
25	14	200	71	22	0	137	75	0.5	69	52	21	ND[d]
27	55	72	39	20	31	82	103	0.2	72	33	27	3.0
26	133	7.9	93	22	27	23	36	0.01	141	17	101	1.5

Sera and serum substitutes. Table 4 lists the 17 sera and two serum substitutes tested. As previously mentioned, during 12 weeks of cultivation, cells grown on irradiated newborn calf and irradiated fetal calf sera as well as on GG-free newborn calf serum failed to replicate continuously throughout the test period. Therefore, these sera would be considered unsuitable for routine passage of the BGM cell line. Based on comparison by the paired *t* test, no difference could be shown between the first eight sera; however, the GG-free and irradiated newborn calf sera failed to support continuous cell growth.

Dialyzed fetal calf serum was considered the serum of choice, based on average mean titers and its ranking, either first or second in 7 of 12 virus titrations. The cost of the dialyzed fetal calf serum would undoubtedly be prohibitive for most laboratories, in which case we would consider the gamma globulin-free calf serum to be the next best alternative. It is only slightly higher in cost than heat-inactivated newborn calf serum or newborn calf serum, which fall just below the top eight recommended sera. The Zeta Sera serum substitute and the milk filtrate preparation fell well below the top-rated group; the milk filtrate also failed to sustain stock cell growth beyond four passages.

Effect of $NaHCO_3$ concentration. Table 5 lists the effect of $NaHCO_3$ concentrations on virus sensitivity of BGM cells infected when cultures were 3 to 7 days old. Titers of known viruses generally decreased with cell age for poliovirus and echovirus, and the decline became statistically significant at 6 days (Fig. 2). With natural isolates from sewage, no trend in virus counts was observed with increasing cell culture age beyond 4 days. Therefore, the use of 3- to 4-day-old cell cultures is recommended. Analysis was done by a repeated-measures analysis of variance statistical program and the Duncan multiple-range test.

These programs also showed that recovery of known viruses from cultures grown in media containing $NaHCO_3$ at 667 mg/liter was not significantly different than at the other concentrations tested (Fig. 3), but ranked higher in four of five cell ages. Based on this information, plus the number one ranking on 4 of 5 days with sewage samples, the 667-mg/liter concentration is recommended as well as use of the cultures before day 6. The data on the 200-mg/liter concentration of $NaHCO_3$ were not tested statistically because the cells turned acid quickly and lysed by day 6 due to the low buffering capacity at this concentration.

Cell concentration. The cell densities tested ranged from 1.9×10^6 to 13.1×10^6 cells per 0.18-liter glass bottle, and (Table 6) densities of 4.3×10^6 and above generally yielded the highest virus recoveries in isolates from sewage (Fig. 4). As with the $NaHCO_3$ data, the analysis of variance statistical program and the Duncan multiple-range test were applied to this data. The poliovirus and echovirus isolation recovery showed no trends with cell densities.

The effect of cell culture age on virus recovery was also studied. No significant pattern emerged with age and cell concentration (Fig. 5) as did with the $NaHCO_3$ data.

Overlay media and agar. Table 7 lists the rankings of the 11 overlay media tested with and without HEPES buffer against 11 known viruses and one mixed virus sample. When compared by the paired *t* test, no significant differences could be revealed between overlays. However, with the addition of 0.02 M HEPES buffer, of a total of 121 media-sample combinations with the known viruses, 70 showed increased titers, 2 remained unchanged, and 49 had lower titers. With the sewage samples, 9 of 11 showed increased virus titers. When viral recoveries from sewage samples alone were compared by the paired *t* test, there were significantly higher recoveries for those with HEPES added to the overlay as opposed to those without it. The one MEM Earle salt base medium (autoclavable medium by GIBCO) showed the greatest number of increased titers overall, 10 of 12, whereas the MEM Hanks salts showed the least change with only 2 of 12 higher when HEPES was present in the overlay. Ranking the overlay media with HEPES buffer added, medium 199 with Earle salts ranked first. Comparing the individual titers, it ranked either first or second in 7 of the 12 test agents; when

TABLE 5. Effect of concentration of $NaHCO_3$ on virus titers in BGM cells

$NaHCO_3$ concn[a] (mg/liter)	Cell culture age (days)	Poliovirus 1			Echovirus 7			Sewage		
		PFU/ml (10^7)	CV[b]	Numerical rank[c]	PFU/ml (10^7)	CV	Numerical rank	PFU/ml	CV	Numerical rank
200	3	18.0	40	5	31	18	7	30	32	6
	4	13.0	67	7	31	72	6	29	58	6
	5	8.8	103	7	18	53	7	27	39	7
	6	L[d]			L			L		
	7	L			L			L		
667	3	17.0	38	6	69	5	5	45	26	3
	4	27.0	24	1	77	16	3	61	18	1
	5	18.0	52	1	71	24	3	62	30	1
	6	9.8	32	4	66	35	3	62	9	1
	7	7.8	26	5	56	26	1	65	5	1
800	3	18.0	58	5	72	4	4	40	38	4
	4	20.0	61	2	86	13	1	41	64	3
	5	13.0	70	5	74	25	2	42	77	3
	6	9.9	36	3	68	31	2	48	34	3
	7	7.9	22	4	56	30	1	47	55	3
1,200	3	23.0	48	3	82	11	1	52	48	1
	4	18.0	47	4	81	5	2	46	55	2
	5	12.0	69	6	75	33	1	52	66	2
	6	7.0	40	5	62	19	4	52	19	2
	7	7.0	17	6	51	41	2	49	81	2
1,500	3	28.0	16	1	32	49	6	46	12	2
	4	19.0	32	3	74	39	4	39	50	4
	5	14.0	42	4	64	31	4	29	37	6
	6	13.0	60	2	50	62	5	36	65	5
	7	11.0	58	1	27	38	5	39	36	5
1,800	3	26.0	25	2	78	51	2	32	41	5
	4	17.0	20	5	81	53	2	38	49	5
	5	15.0	56	3	62	35	6	32	27	5
	6	14.0	44	1	44	71	6	33	43	6
	7	8.9	21	2	43	68	3	37	64	6
3,000	3	19.0	71	4	75	46	3	32	38	5
	4	14.0	17	6	41	34	5	41	29	3
	5	13.0	41	2	63	31	5	35	39	4
	6	9.9	28	3	43	96	1	38	30	4
	7	8.3	44	3	28	41	4	42	10	4

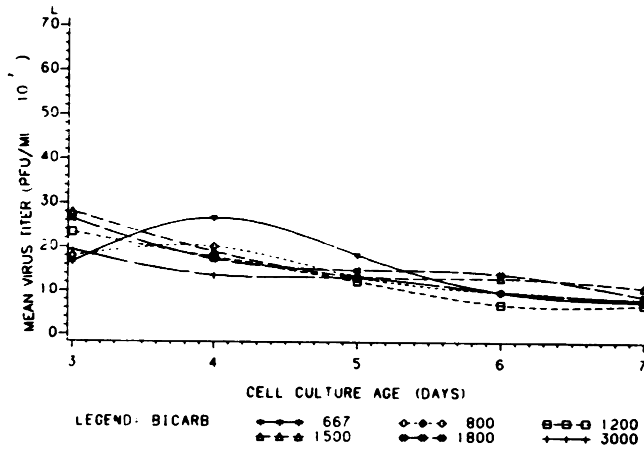
[a] Level of $NaHCO_3$ in growth medium when test cell cultures were planted.
[b] CV, percent relative standard deviation.
[c] Numerical ranking is the rank of any one sample on a given day versus all others in that group on the same day.
[d] L, Lysis due to acidity caused by low buffering capacity of $NaHCO_3$ concentration.

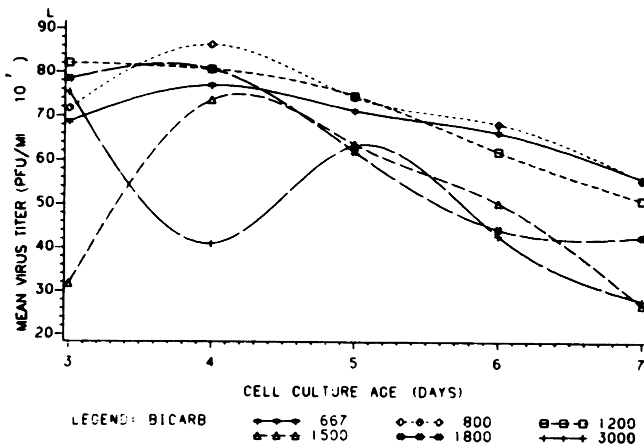
compared with the BME Earle salts overlay (ranked first without HEPES) it also yielded higher titers in 9 of the 12 agents tested.

Table 8 lists the 12 agars tested, in descending order of sensitivity to virus. Statistically, the paired t test showed significant differences only between the first-ranked agar and the last two, indicating that any of the first 10 agars will give similar overall results; however, the Difco Bacto-Agar, Oxoid purified, Oxoid no. 1, and GIBCO agarose all gave lower virus recoveries from sewage samples. Therefore, based on the data in Tables 7 and 8, we recommend the use of medium 199 with Earle salts, the addition of 0.02 M HEPES buffer to the overlay medium, and any one of the following: GIBCO purified or bacteriological, Oxoid technical no. 3, Difco flake, purified, or technical bacteriological, or Inolex bacteriological agars for the overlay medium components.

Culture vessels and microcarriers. Table 9 lists the results of tests conducted to determine on which type of bottle BGM cells should be grown to obtain maximum sensitivity when these cells are subsequently planted and assayed on comparable test flasks. Based on the paired t test, no difference could be shown between the first eight culture vessels listed in Table 9; however, those ranked below the eighth vessel (Brockway 0.95-liter glass bottles) showed a significant difference. Most of the vessels used for stationary culture growth were in this lower category, with the exception of the Falcon 175-cm^2 flask and the Brockway glass 0.95-liter bottle. Average cell multiplicity was 4.7 for the eight types of roller bottles tested and 7.4 for the five stationary flasks tested.

Trypsin. In an effort to determine whether various types of trypsin preparations affected subsequent virus titrations in BGM cells, five commercial preparations and one of our own





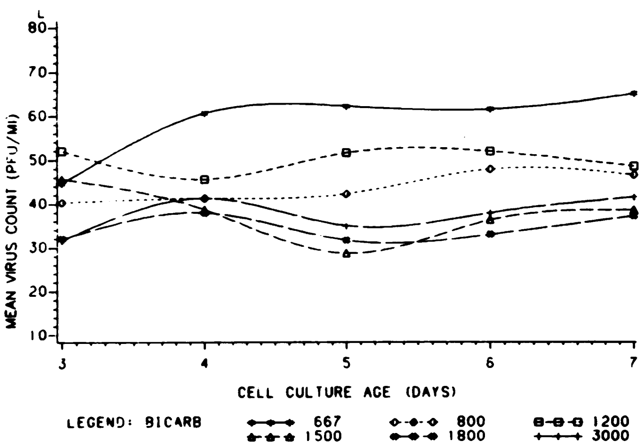
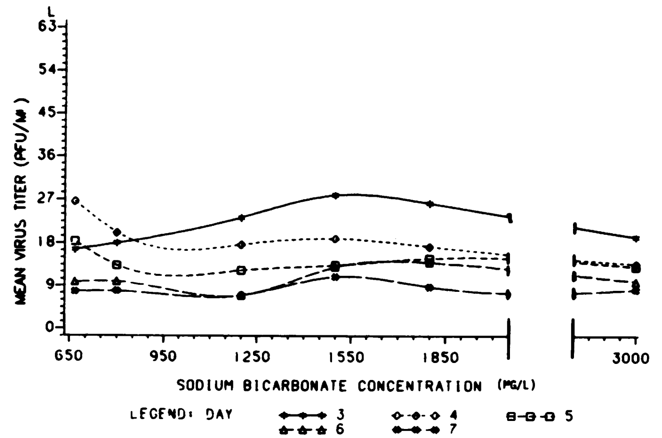
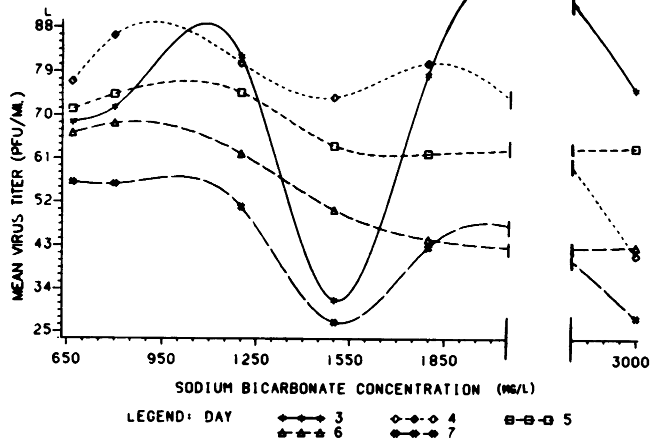


FIG. 2. Effect of cell culture age on virus recovery.

formulation (Table 10) were tested. The trypsins are listed in descending order of rank, and average cell increases are given for the 12-week study. Based on the paired t test, no significant difference between any of the preparations tested was observed; however, the Enzar T preparation yielded the highest titer in 5 of the 13 virus titrations.





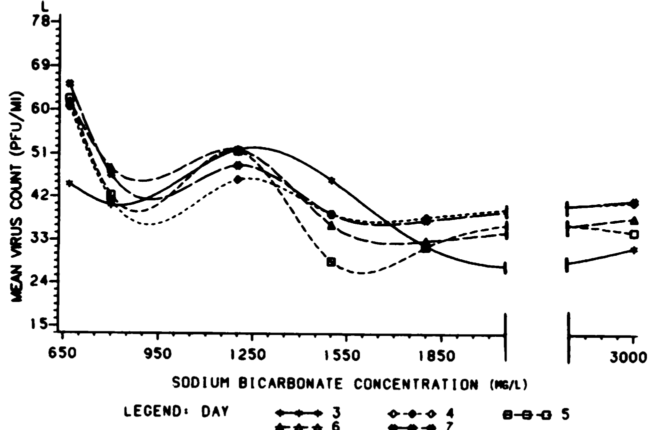


FIG. 3. Effect of sodium bicarbonate concentration on virus recovery.

Cell culture washing before virus inoculation. The results of washing cell cultures just before inoculation of viruses are given in Table 11. Cells washed with Earle basic salt solution yielded enterovirus titers from 10 to 66% higher than those not washed. When compared by the paired t test the differ-

TABLE 6. Effect of BGM cell density on virus titer and recovery from sewage samples

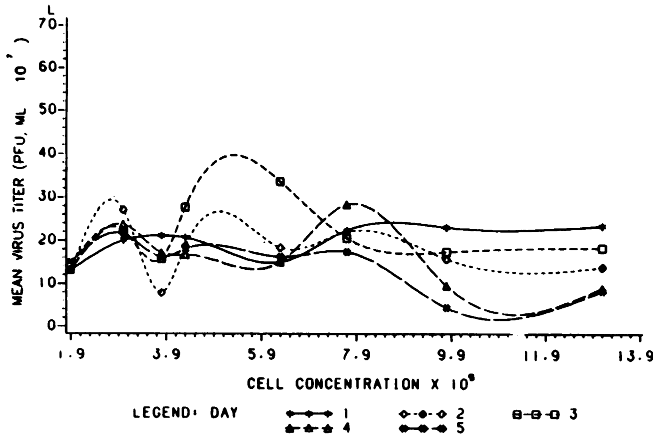
Cell concn[a] (10^6)	Cell culture age (days)	Poliovirus 1			Echovirus 7			Sewage		
		PFU/ml (10^7)	CV[b]	Numerical rank[c]	PFU/ml (10^7)	CV	Numerical rank	PFU/ml	CV	Numerical rank
1.9	3	13.0	62	6	67	57.0	2	25	26.0	7
	4	13.0	65	7	54	36.0	7	30	13.0	7
	5	14.0	69	8	57	37.0	6	32	19.0	8
	6	13.0	62	5	50	10.0	5	21	13.0	8
	7	15.0	65	5	63	65.0	1	16	30.0	8
3.0	3	20.0	43	4	56	15.0	4	32	32.0	6
	4	27.0	57	1	69	10.0	3	32	36.0	6
	5	22.0	64	3	61	11.0	3	43	2.7	7
	6	24.0	61	2	53	16.0	2	41	6.5	7
	7	22.0	57	1	51	26.0	6	32	8.3	7
3.8	3	21.0	56	3	67	38.0	2	34	51.0	5
	4	7.9	76	6	82	9.0	1	50	62.0	5
	5	16.0	54	7	59	14.0	5	38	30.0	6
	6	17.0	46	3	47	11.0	6	38	38.0	6
	7	16.0	48	4	55	12.0	2	34	40.0	6
4.3	3	21.0	27	3	81	16.0	1	46	44.0	4
	4	19.0	24	3	76	10.0	2	54	41.0	4
	5	28.0	13	2	63	2.0	1	47	31.0	5
	6	17.0	57	3	47	42.0	6	47	33.0	5
	7	18.0	39	2	44	22.0	7	31	37.0	5
6.3	3	15.0	52	5	64	52.0	3	64	9.0	1
	4	18.0	40	4	62	14.0	4	72	27.0	1
	5	34.0	47	1	62	15.0	2	73	5.6	1
	6	15.0	86	4	52	35.0	3	68	17.0	2
	7	16.0	61	4	49	10.0	5	57	24.0	3
7.7	3	22.0	45	2	64	6.3	3	56	11.0	3
	4	22.0	45	2	52	37.0	8	62	2.0	3
	5	21.0	55	4	57	15.0	6	63	2.0	3
	6	28.0	22	1	55	20.0	1	55	5.0	4
	7	17.0	47	3	43	4.7	8	52	24.0	4
9.8	3	23.0	26	1	43	74.0	5	46	44.0	3
	4	16.0	35	5	59	13.0	6	61	32.0	2
	5	17.0	61	6	55	6.0	7	47	54.0	4
	6	9.5	3	6	51	19.0	4	55	55.0	3
	7	4.4	70	7	50	14.0	4	49	49.0	2
13.1	3	23.0	30	1	81	22.0	1	63	8.0	2
	4	14.0	106	6	61	24.0	5	70	37.0	2
	5	18.0	65	5	60	19.0	4	69	30.0	2
	6	9.0	12	7	46	10.0	7	71	27.0	1
	7	8.0	95	6	53	16.0	3	76	21.0	1

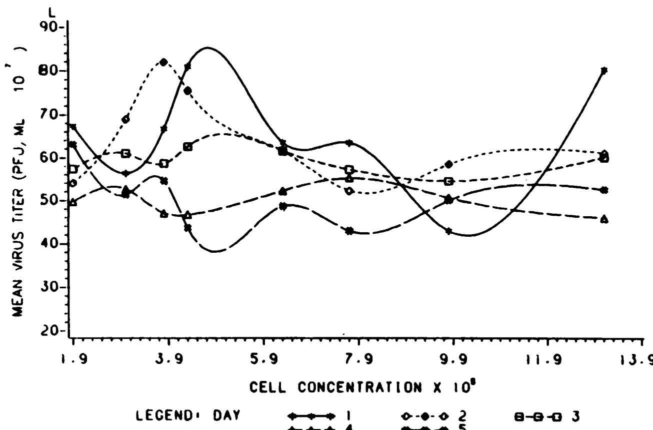
[a] Concentration of cells planted into 6-oz. glass bottles with 45-cm^2 surface area.
[b] CV, Percent relative standard deviation.
[c] Numerical rank is the rank of any one sample on a given day versus all others in that group on the same day.

ences were statistically significant. With reovirus and rotavirus titrations, no virus recovery occurred unless the cells were first washed. Washes are essential for these two viruses inasmuch as they serve to remove serum and other protein materials that normally inactivate pancreatin, the additive necessary for plaque development of these viruses. Washing cells with growth medium (MEM–L-15) without serum did not enhance virus titers above those obtained when cultures were washed with Earle basic salt solution.

Virus infection time and temperature. Table 12 and Fig. 6 show the effects on virus recovery of varied time and temperature conditions during the infection process before agar overlay. The highest virus counts were obtained in those samples incubated on the cell cultures for 120 min at 25°C, indicating that it was the better temperature. At 25°C, the lowest counts occurred at 10 min of exposure. Regression analysis with a lack-of-fit test indicated that the data were suitably modeled by a linear relationship and that a statistically significant trend of increased viral counts existed with time but not with temperature. These analyses showed that virus-to-cell exposure times less than 80 min gave significantly lower counts ($P = 0.95$) than exposures of 80 to 120 min. Holding cultures for up to 120 min resulted in continued increases in counts, but the increases were not statistically significant.

Cell line comparison. The results of virus sensitivity testing





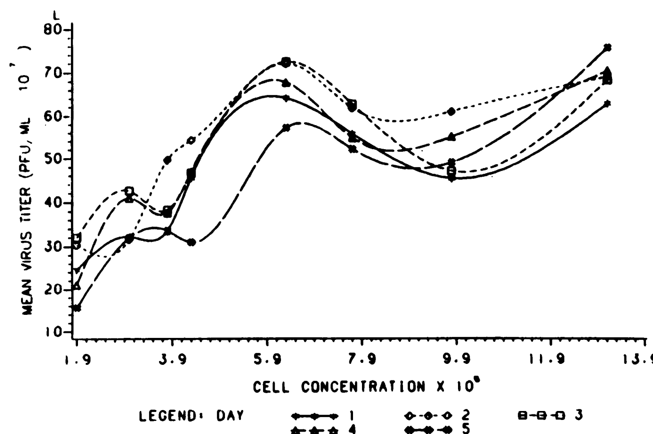
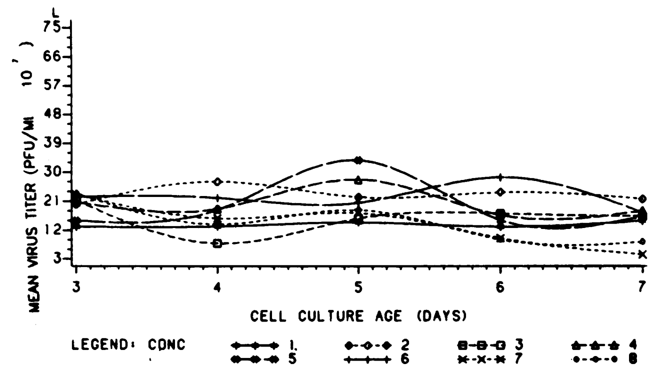
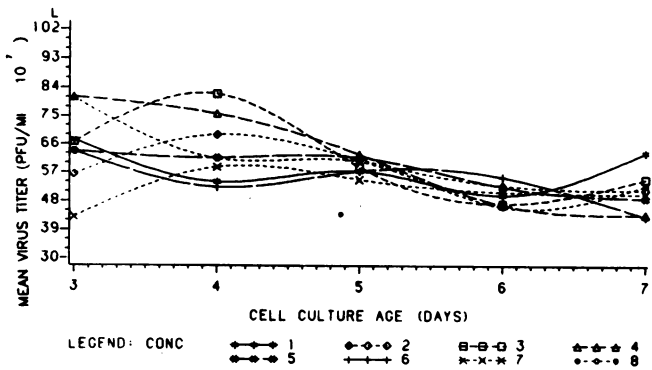


FIG. 4. Effect of initial cell concentration on virus recovery.





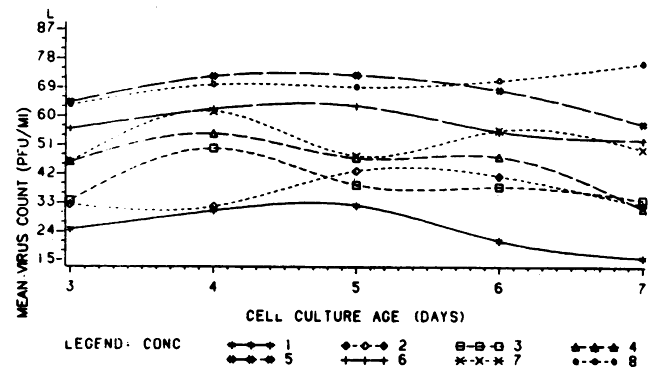


FIG. 5. Effect of cell culture age on virus recovery.

of eight continuous cell lines (Table 1) against 12 known viruses and one sewage sample are given in Table 13. Under the condition used in this assay the overall rank of the BGM cells was twice as high as the next highest cell line. BGM titers were higher in all categories except for reovirus and rotavirus, in which the BGM cells ranked second. Statistical evaluation by the paired t test showed a significant difference between BGM and all other cell lines tested. We recognize that one or more of the other cell lines could be as sensitive as BGM if their culture conditions and assays were optimized.

The sensitivity of these eight continuous cell lines, along with two primary cell cultures, to human enteric viruses contained in sewage samples is listed in Table 14. The BGM cell line far exceeded the other tested cell lines in overall

TABLE 7. Effect of various overlay media on virus titers in BGM cell cultures

Overlay medium[a]	HEPES buffer[b]	Virus titers (PFU/ml, 10^7)[c]									
		Poliovirus 1		Echoviruses							
				7		11		12		14	
		PFU	CV[d]	PFU	CV	PFU	CV	PFU	CV	PFU	CV
BME Earle salts	w/o	19	50	83	16	375	25	270	5	6.4	73
	w	33	42	70	6.5	98	20	290	11	7.8	62
MEM Hanks salts	w/o	17	63	62	13	505	57	225	50	5.1	69
	w	13	49	61	42	470	56	260	61	5.2	51
BME Hanks salts	w/o	24	3	85	0.8	230	61	290	20	2.7	13
	w	30	4.5	73	3.4	11	43	250	18	7.9	12
Medium 199 Earle salts	w/o	16	32	90	11	400	0	225	3	6	67
	w	26	19	74	8	340	22	210	4	9.9	10
Medium 199 Hanks salts	w/o	20	50	80	6	460	9.2	335	19	2.9	44
	w	29	48	75	12	290	15	210	21	9.9	31
BME[e] Earle salts	w/o	14	20	72	21	102	68	225	2.8	3.2	13
	w	25	45	58	22	75	24	260	9.5	6	68
MEM[f] Earle salts	w/o	11	21	60	11	125	28	135	5	6.1	127
	w	13	26	62	16	280	31	200	44	4.2	26
MEM Earle salts with NEAA	w/o	12	24	62	20	190	37	180	39	5.2	33
	w	9.4	31	63	32	350	20	270	42	3.3	28
MEM[e] Earle salts	w/o	18	39	89	6	213	92	255	8	3.1	2
	w	22	41	73	12	130	96	310	6.5	7	4
MEM Earle salts	w/o	13	51	82	2.6	155	132	183	37	1.9	60
	w	9.9	26	68	5	250	92	260	85	3.9	46
L-15 Medium (Leibovitz)	w/o	8.5	24	62	14	137	118	215	56	2.1	123
	w	7.5	21	39	20	23	29	160	48	4.9	98

[a] All base media were ordered without phenol red.
[b] w/o, media without HEPES; w, media with HEPES.
[c] Based on three replicate samples.
[d] CV, percent relative standard deviation.
[e] Autoclavable medium by GIBCO.
[f] Autoclavable medium by Flow Laboratories (Auto-Pow).
[g] L, Lysed cell sheet due to medium applied.

virus sensitivity, which was twice that of the second-ranked cell line and over 400 times that of the last-ranked cell line. Also, the BGM cell line had higher plaque counts in 26 of the 37 samples tested and showed no effects of toxicity due to inoculum as did other cell lines with some of the samples. (Also, the paired *t* test showed the BGM cell line to be significantly better than all others.)

Identification of confirmed plaques is given in Table 15. Isolates from the MDBK line were not included, but were subsequently tested by the immunofluorescence procedure (29); all were identified as reovirus types. Excluding the reoviruses, a total of 30 different viruses were isolated from the 37 sewage samples.

The initial inoculation of sewage sample number 2 onto BGM cells yielded only coxsackievirus B3, whereas viruses isolated from the L-132 cells were 88% echovirus types 5, 11, and 17, and 12% coxsackievirus B3. However, when a like sample of sewage inoculum from sample number 2 was pretreated with coxsackievirus B3 antiserum and then inoculated onto BGM cells, 39% of the isolates were found to be other coxsackievirus B types, whereas the remaining 61% were echovirus types 4, 5, 7, 11, 13, 15, 17, 24, and 25.

DISCUSSION

The development of an improved procedure for growth of BGM cells and the subsequent viral assay will do much to increase the precision of data acquired with this cell line. The need for standardization in culturing techniques was pointed out in the survey (10) of known users of the BGM cell line and by participants in a recent round-robin study (21). Based on information from the survey, nine factors affecting cell growth and virus assays were studied by comparing BGM cells with nine other cell lines. This study was conducted with the monolayer plaque system; consequently the use of some of these conditions may not be optimum for BGM cells in a suspended cell culture system. Thirty-one percent (18 of 58) of the laboratories responding to the survey used the combination medium MEM–L-15, which ranked first in this study. The MEM medium, used by 34% (20 of 58) of the responding laboratories, only ranked 35th in this study, whereas 57% (33 of 58) of those responding used media for cell growth which ranked eighth or lower.

Ninety-one percent (52 of 58) of those responding to the survey used fetal calf serum. Current studies showed that

TABLE 7—*Continued*

Virus titers (PFU/ml, 10^7)[c]														
Coxsackieviruses										Reovirus 1		Sewage		Medium ranking
27		B2		B5		CA9		CA16						
PFU	CV	PFU	CV	PFU	CV	PFU	CV	PFU	CV	PFU	CV	PFU/ml	CV	
22	84	85	110	260	65	24	21	118	59	0.6	75	50	48	3.0
45	69	73	82	490	48	35	43	310	41	1.8	28	91	39	3.3
21	86	48	109	410	62	24	69	175	110	1.6	5	57	46	3.0
8.9	64	12	92	160	58	13	43	63	49	0.3	15	38	21	7.2
11	35	29	12	340	4	22	23	85	8	1.0	0	43	2	4.6
36	28	76	14	510	6.5	24	21	280	10	1.9	26	99	3.5	3.9
17	76	29	0	230	80	28	8	76	11	0.1	24	31	7	4.8
62	53	930	46	510	46	370	12	340	14	1.1	18	102	62	2.5
12	46	33	28	275	13	23	0	63	6	0.1	26	37	38	5.1
43	39	790	14	480	14	23	0	370	4.5	0.2	24	86	62	3.8
12	12	19	19	365	41	26	11	119	38	1.4	16	38	3.7	5.4
47	82	72	92	630	67	24	19	320	43	2.4	72	103	36	4.1
19	57	32	88	365	48	19	70	173	93	1.3	28	47	54	5.5
11	41	13	7	110	29	11	28	51	82	0.3	41	39	26	7.5
19	69	39	90	365	41	18	55	31	68	1.7	21	49	60	5.5
9.3	52	13	82	75	52	13	49	68	59	0.3	32	56	41	7.1
12	6	17	33	200	21	19	7	67	34	1.3	0	42	12	5.8
48	5.5	74	22	620	38	23	15	290	29	1.5	2.5	90	15	3.7
5.8	125	15	70	255	8.3	23	37	32	109	1.4	5.2	37	15	7.1
9	22	13	85	200	14	19	22	41	38	1.4	5.2	40	29	7.0
12	127	20	90	91	92	14	71	78	76	L[g]		13	119	8.1
14	111	43	22	270	84	21	46	92	41	0.3	5.5	46	38	7.3

GG-free newborn calf serum ranked first of the 19 sera and serum substitutes tested; however, it was incapable of sustaining prolonged cell passage. Therefore, the second-ranked dialyzed fetal calf serum is recommended for the preparation of BGM stock cultures. Test cultures prepared for monitoring of viruses from environmental samples should be prepared with dialyzed fetal calf or GG-free newborn calf serum since these cultures will not be further passed. The same serum should also be used in the overlay medium when required. For test cultures prepared for everyday laboratory work with seeded samples used in methods development work or nonmonitoring sampling, GG-free calf serum would be a suitable substitute and would be less costly.

The survey also indicated that the $NaHCO_3$ concentration varied more than any other factor among laboratories. In all, 15 different concentrations were reported, ranging from 200 to 3,000 mg/liter. Seven bicarbonate concentrations were selected for testing; the 667-mg/liter concentration ranked first overall and first on 4 of 5 days with virus isolates from sewage samples (Fig. 2 and 3; Table 5).

The effect of cell culture age and, to a lesser extent, cell concentration was somewhat more noticeable. Peak sensitivities were achieved at a cell concentation of 1.4×10^5 cells per cm^2 of surface area and with cell cultures 4 to 5 days old. Three-day-old cultures showed good sensitivity to polioviruses and echoviruses but not to sewage samples. This may be related to the presence of other substances such as heavy metals and humic acids that inhibit initial virus growth or reduce cell sensitivity in the younger cell cultures.

The age at which cell cultures were used for virus assay could not be ascertained from the survey (10). However, if assays were conducted with cultures 3 to 5 days old, in which there was provided a 667-mg/liter concentration of $NaHCO_3$ and a planting of 1.4×10^5 cells per cm^2 of surface area, improved virus isolations and uniform results among laboratories would be obtained.

The addition of HEPES buffer did not significantly increase viral titers between individual overlay media; however, significantly higher counts ($P = 0.95$) were observed with sewage samples containing HEPES buffer as opposed to those without it. The general increase in virus titers with the use of HEPES buffer is no doubt caused by the better control of oxygen tension and subsequently pH in the media, which others have shown to be critical (7, 12, 28). Results of this study would recommend its use.

The agar for use in overlay medium as recommended by this study would be any of the following: Difco flake, purified, or technical bacteriological; GIBCO bacteriological or purified; Oxoid technical no. 3; or Inolex bacteriological. All of these gave good virus counts with the sewage samples.

Because no basic differences were observed between culture vessels used to grow the BGM cell cultures, the choice would depend on which type of vessel was found

TABLE 8. Effects of agar type (in overlay medium) on recovery of viruses in BGM cell cultures

Agar tested	Ranking	Virus titer (PFU/ml, 10^7)																									
		Poliovirus 1		Echovirus										Coxsackievirus								Reovirus 1		Rotavirus SA-11		Sewage	
				7		11		12		14		27		B2		B5		CA9		CA16							
		PFU	CV[a]	PFU	CV	PFU	CV	PFU	CV	PFU	CV	PFU	CV	PFU	CV	PFU	CV	PFU	CV	PFU	CV	PFU	CV	PFU	CV	PFU/ml	CV
GIBCO bacteriological	3.8	21	45	81	40	180	65	310	50	4.7	93	24	124	85	68	500	8	25	33	260	55	1.6	51	0.1	56	51	35
Oxoid technical no. 3	3.9	26	55	70	9.4	250	89	330	8	6.2	20	22	60	56	58	400	32	280	68	250	71	1.2	62	0.08	72	51	37
Difco flake	4.5	25	50	63	42	280	61	400	8	7.6	49	27	50	69	63	370	66	26	156	260	80	1.2	20	0.02	47	74	56
GIBCO purified	4.5	21	54	76	13	120	48	280	34	5.5	32	25	50	76	34	440	37	33	25	290	37	1.0	50	0.3	37	56	33
Inolex bacteriological	4.6	23	56	62	38	530	23	290	19	4.5	14	12	93	340	159	440	38	150	146	190	88	1.1	21	6.3	62	52	67
Difco Bacto-Agar	4.6	23	23	95	33	330	26	440	21	4.1	80	14	103	54	20	520	10	28	15	210	52	70.0	172	3.2	169	44	9.1
Difco technical bacteriological	4.7	24	58	74	33	290	74	240	80	6.7	37	18	58	56	84	350	69	250	56	270	72	1.1	27	0.03	90	66	62
Difco purified	5.5	23	38	75	39	110	97	260	33	6.1	24	18	26	210	119	370	20	120	136	270	19	0.4	29	0.02	61	74	37
Oxoid purified	6.0	12	64	96	6.6	210	39	310	9.7	2.6	49	11	25	58	34	370	45	330	36	310	51	0.7	58	0.08	25	34	34
Oxoid no. 1 bacteriological	6.5	19	61	74	24	360	53	260	19	3.3	18	9.4	38	60	58	380	11	30	18	240	70	0.8	52	0.09	90	39	4
Difco K	7.4	24	74	64	44	290	6.9	310	21	4.4	47	9.2	24	30	58	290	67	24	14	130	51	0.9	37	0.05	22	49	24
GIBCO agarose	8.4	12	24	76	20	120	92	220	50	1.1	101	3	94	40	18	450	45	15	53	52	44	0.5	86	0.1	97	34	13

[a] CV, Percent relative standard deviation.

easiest to handle and work with. Roller bottles are easy to use and generally require fewer bottles and less medium; however, the basic equipment is expensive. In addition, cultures in roller bottles must be subcultured weekly, whereas those grown in stationary culture may be carried longer between passages if necessary. The only other consideration would be cost; however, this changes often so cost comparison is not practical, although at this time the cost of glass bottles is about half that of comparable plastic bottles. The data in Table 9 do indicate that growing stock cells in glass or plastic with subsequent subculture of test cells into the opposite type of container (glass to plastic, plastic to glass) results in lower virus recoveries, although the loss is not statistically significant.

Of the microcarriers tested, all ranked low and not only had lower virus titers, but also had lower weekly cell increases. They were difficult to work with compared with roller bottles or stationary flasks, and the cells could not be removed easily and entirely from them.

The survey also indicated that most investigators used trypsin or a mixture of trypsin-EDTA to free cells from culture vessels (10). In this study, no significant difference between the six different trypsin preparations tested was observed. The contact time between cells and trypsin may affect sensitivity, but this was not investigated.

Before inoculation of cell cultures with virus, washing the cells with Earle basic salt solution without serum is recommended. This serves several purposes. It removes cellular debris, conditions cells to a more stable physiological state, and removes serum and other protein materials which interfere with plaque production.

Once cell cultures have been inoculated with virus they must be incubated before overlay to allow infection to take place. Study data showed that virus recovery increased with exposure time throughout the period examined (120 min). Forty-three percent (25 of 58) of the survey respondents (10) allowed 60 min for infection, whereas 21% (12 of 58) used 45 min or less. Sixty-four percent (37 of 58) of the respondents could significantly increase their virus recovery (17% for poliovirus, 6% for echovirus, 15% for coxsackievirus) by allowing the infection process to take place for at least 80 min. Increases in counts obtained with longer exposure periods were slight and were not statistically significant.

The final phase of this study involved comparing the sensitivity of the optimized BGM method to that of various cell lines (whose cultivation was previously reported [Table 1] but may not be totally optimized) to known viruses and naturally occurring viruses in 37 sewage samples. Data show that the BGM cell line was superior by a 2:1 margin over the next best cell line. Of the 37 sewage samples tested, 65% showed a predominance of coxsackievirus type B with the BGM line. This is not in accord with isolation patterns described elsewhere (26, 32) and is thought by some to be a characteristic of the BGM cell line (22, 31). However, it may to a large degree be more related to the type of infection dominant in the community at the time the samples were collected. As reported by the Centers for Disease Control (6), type B coxsackieviruses have been endemic in the United States over the past 10 years, which could account for the large numbers of these viruses that are consistently isolated. Additionally, reoviruses were detected in one-third of the samples, which is similar to that reported elsewhere (32). All reovirus isolations were made on the MDBK cell line, which, as previously reported (29), was best for isolation of this virus. This shows the potential of this cell line as

TABLE 9. Effect of growing BGM cells in various culture vessels and microcarriers on sensitivity to virus infection

Type of culture bottle or microcarrier	Ranking	Virus titer (PFU/ml, 10^7)																										Avg cell increase[a]
		Poliovirus 1		Echovirus										Coxsackievirus								Reovirus 1		Rotavirus SA-11		Sewage		
				7		11		12		14		27		CB2		CB5		CA9		CA16								
		PFU	CV[b]	PFU	CV	PFU	CV	PFU	CV	PFU	CV	PFU	CV	PFU	CV	PFU	CV	PFU	CV	PFU	CV	PFU	CV	PFU	CV	PFU/ml	CV	
Glass disposable roller bottle	3.2	47	31	119	45	200	75	330	35	7.8	97	48	92	143	11	440	43	56	17	530	26	3.7	56	5.6	87	70	20	3.5
Corning plastic roller bottle	3.8	42	31	111	23	142	86	377	36	8.3	89	58	91	133	19	513	44	43	18	523	14	2.9	60	5.8	95	83	32	2.9
Bellco reusable glass roller bottle[c]	3.9	38	31	109	29	128	76	457	22	7.5	86	41	56	120	36	560	48	49	21	453	36	4.2	8.6	10	18	58	11	7.2
Bellco reusable glass roller bottle[d]	4.5	35	45	98	32	191	79	463	2.5	7.5	30	35	56	109	26	550	23	44	24	413	23	4.8	15	10	12	51	25	5.6
Corning plastic roller bottle[e]	5.8	35	23	84	10	105	66	280	16	7.4	13	66	46	135	40	360	40	40	42	457	26	2.9	62	13	113	60	28	2.9
Falcon 175-cm^2 culture flask	6.3	25	66	74	44	280	101	260	71	8.5	84	57	86	107	51	370	73	27	69	450	65	3.5	69	5.4	111	55	44	7.7
Falcon plastic roller bottle	6.6	28	47	92	27	126	36	317	75	10	85	40	83	119	35	360	56	41	18	413	43	2.8	40	5.6	106	55	21	4.0
Brockway 32-oz. glass bottle	7.6	25	46	80	21	229	65	403	18	4.8	82	45	127	78	12	430	25	28	14	327	17	3.4	66	6.1	106	43	4.8	6.6
—[f]																												
Disposable glass roller bottle[g]	7.6	21	48	87	27	114	95	210	11	9.8	23	43	60	85	27	330	34	34	43	303	50	3.6	62	9	28	58	14	3.5
Nunclon 175-cm^2 culture flask	8.2	20	83	65	40	246	100	230	75	5.5	120	41	118	106	34	303	81	23	57	403	30	2.9	45	8.3	97	64	37	7.5
Bellco reusable glass roller bottle[h]	8.3	22	41	71	34	137	74	303	19	6.2	32	28	58	92	37	473	36	35	8.3	347	93	4.2	26	5	91	47	26	7.7
Costar 150-cm^2 culture flask	8.5	15	35	72	69	143	16	208	88	6.6	89	34	97	75	65	473	56	22	71	457	87	3.4	63	4.6	86	56	47	7.7
Corning 150-cm^2 culture flask	9.6	21	75	63	64	250	56	190	65	5.8	109	24	125	97	48	333	92	21	46	303	68	3.3	70	5.4	84	50	31	7.3
Flow microcarriers[i]	11.5	12		48		140		210		6.6		35		53		270		11		150		2.1		1.2		45		1.6
Nunclon microcarriers[i]	13.2	11		56		120		110		3.7		8.3		21		67		21		72		0.3		1.3		30		1.8
Bio-Rad Bio-Carriers[i]	14.5	2.6		41		79		93		2.1		4.6		3.7		29		7.1		36		0.005		0.3		32		1.4
Cytodex microcarriers[i]	15.3	1.6		40		110		76		1.2		2.6		3.1		13		6.8		28		0.06		0.2		31		1.3

[a] Number represents the average cell increase as measured against initial inoculum.
[b] CV, Percent relative standard deviation.
[c] Bottles treated with Vitrogen after sterilization.
[d] Bottles not treated after sterilization.
[e] Stock cultures grown on plastic roller bottles; test cultures grown on glass bottles.
[f] Bottles or flasks listed above this point were significantly better statistically than those below this point.
[g] Stock cultures grown on disposable glass roller bottles; test cultures grown on plastic flasks.
[h] Bottles treated with $(CH_3CO_2)_2Mg \cdot 4H_2O$ after sterilization.
[i] Titers based on only one titration; all others based on average of three titrations.

TABLE 10. Effect of trypsin type on virus titers in BGM cell culture

Type of trypsin	Avg cell increase[a]	Ranking	Virus titer (PFU/ml, 10^7)																									
			Poliovirus 1		Echovirus										Coxsackievirus								Reovirus 1		Rotavirus SA-11		Sewage	
					7		11		12		14		27		CB2		CB5		CA9		CA16							
			PFU	CV[b]	PFU	CV	PFU	CV	PFU	CV	PFU	CV	PFU	CV	PFU	CV	PFU	CV	PFU	CV	PFU	CV	PFU	CV	PFU	CV	PFU/ml	CV
Enzar T[c]	2.0	2.1	18	34	69	16	600	18	380	13	8.6	38	23	63	86	12	210	20	20	66	180	22	1.4	40	8.5	8.3	58	23
EDTA-trypsin[d]	2.4	2.6	19	67	57	37	560	3.8	275	2.6	10	20	37	38	84	27	290	34	150	94	200	31	2.1	102	7.3	25	49	31
Trypsin 1×[e]	2.0	3.5	11	36	41	41	500	21	280	0	3.8	76	23	27	71	12	375	17	14	85	230	42	0.5	45	7.7	17	57	7.4
EDTA-Trypsin 10×[e]	2.0	3.9	17	47	57	37	390	1.8	330	19	3.9	15	7.4	25	47	12	320	13	15	55	220	28	0.5	83	3.6	12	53	13
Lyophilized[f]	2.1	4.0	18	27	65	21	230	112	350	12	6.3	5.6	240	59	74	31	410	38	20	10	240	36	1.2	80	3.2	62	60	24
VMF[f]	2.5	4.8	7.5	65	39	51	410	28	190	85	4.8	61	8.6	43	18	114	370	29	11	32	130	26	1	141	1.4	140	44	0

[a] Number represents the average cell increase as measured against initial inoculum.
[b] CV, Percent relative standard deviation.
[c] Product of Armour Pharmaceutical Co.
[d] Prepared in U.S. Environmental Protection Agency laboratory.
[e] Product of K.C. Biological Co.
[f] Product of Millipore Corp.

TABLE 11. Effect of medium change (before inoculation) on BGM sensitivity to virus infection

Treatment	Ranking	Virus titer (PFU/ml, 10^7)																									
		Poliovirus 1		Echovirus										Coxsackievirus								Reovirus 1		Rotavirus SA-11		Sewage	
				7		11		12		14		27		CB2		CB5		CA9		CA16							
		PFU	CV[a]	PFU	CV	PFU	CV	PFU	CV	PFU	CV	PFU	CV	PFU	CV	PFU	CV	PFU	CV	PFU	CV	PFU	CV	PFU	CV	PFU/ml	CV
Test bottles changed with EBSS[b]	1.3	19	45	80	26	308	32	348	31	9.8	42	41	83	83	55	400	35	23	54	318	62	1.1	41	2.4	193	68	22
Test bottles changed with MEM–L-15 medium	1.8	17	6	57	14	335	43	233	40	10	55	40	67	69	61	365	40	24	54	337	65	1.0	18	0.8	48	61	37
Test bottles not changed	2.8	16	40	27	41	168	20	131	41	6.2	38	24	14	72	37	303	19	19	57	283	56	0	0	0	0	61	32

[a] CV, Percent relative standard deviation.
[b] EBSS, Earle balanced salt solution.

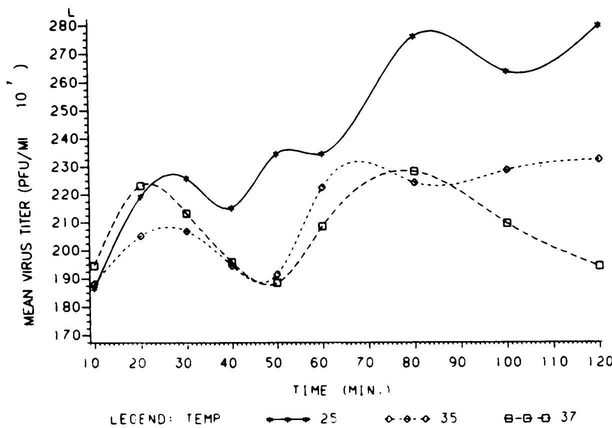
TABLE 12. Effects of contact time and incubation temperature during virus infection of BGM cells (before overlay) on virus recovery[a]

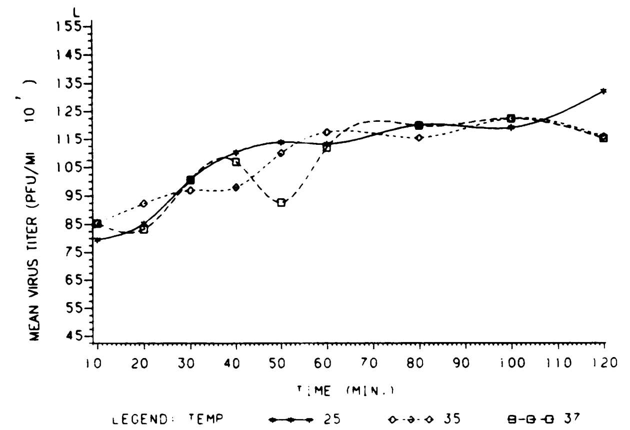
Virus	Temp (°C)	Virus recovery (PFU/ml) after cell virus infection after incubation for:																	
		10 min		20 min		30 min		40 min		50 min		60 min		80 min		100 min		120 min	
		PFU	CV[b]	PFU	CV	PFU	CV	PFU	CV	PFU	CV	PFU	CV	PFU	CV	PFU	CV	PFU	CV
Poliovirus 1	37	195	12.0	223	10.0	213	5.5	196	20.0	189	26.0	209	33.0	**228**[c]	23.0	210	22.0	194	33.0
	35	188	8.2	205	15.0	207	16.0	195	20.0	192	24.0	223	19.0	224	11.0	229	20.0	**232**	16.0
	25	187	15.0	219	24.0	226	22.0	215	26.0	235	25.0	235	28.0	276	14.0	264	14.0	**280**	24.0
Echovirus 7	37	85	6.5	83	2.8	101	17.0	107	28.0	93	13.0	112	14.0	120	15.0	**123**	10.0	115	2.2
	35	86	21.0	92	24.0	97	26.0	98	22.0	110	12.0	118	21.0	116	19.0	**123**	15.0	116	20.0
	25	80	13.0	85	12.0	101	27.0	110	27.0	114	22.0	113	6.6	120	20.0	119	27.0	**132**	28.0
Coxsackievirus A9	37	227	13.0	232	12.0	234	9.0	240	18.0	215	29.0	235	40.0	**250**	29.0	244	46.0	233	54.0
	35	240	21.0	231	14.0	238	17.0	229	16.0	235	23.0	227	29.0	228	21.0	244	30.0	**259**	30.0
	25	207	15.0	235	17.0	232	22.0	241	13.0	243	7.0	231	23.0	265	26.0	282	27.0	**290**	29.0

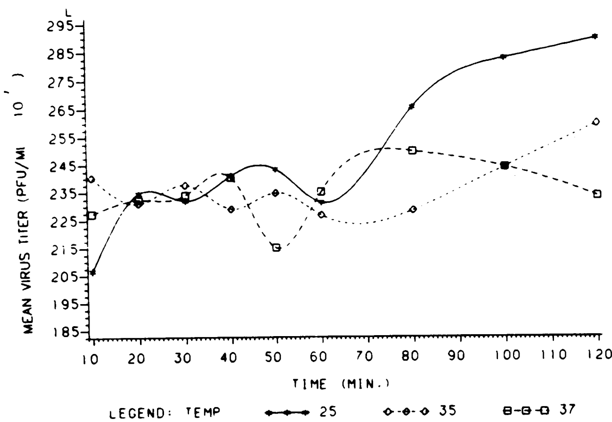
[a] Data represent the averages of three replicate samples for each time and temperature.
[b] CV, Percent relative standard deviation.
[c] Numbers in boldface type represent highest titer in series.

an added environmental monitoring tool, since it does not plaque the human enteroviruses.

Subsequent studies comparing virus isolations on BGM cells to those on RD cells showed that BGM cells isolated polioviruses in 15 samples to 12 for RD cells; BGM cells isolated echoviruses in 18 samples to 3 for RD cells; and BGM cells isolated type A coxsackieviruses in 2 samples to 1 for RD cells. This shows that BGM cells isolate other virus







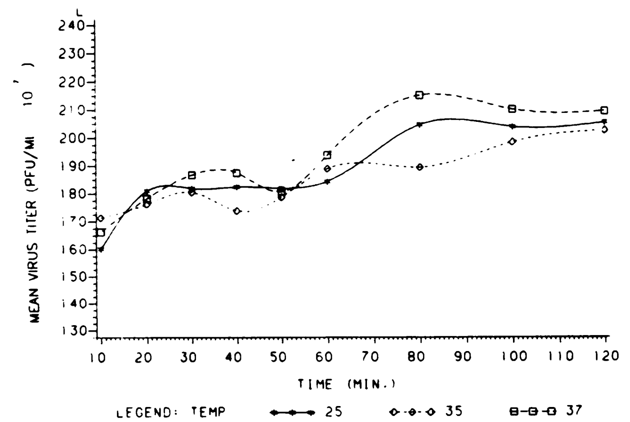


FIG. 6. Effect of virus-to-cell contact time (before overlay) during the infection period on mean virus titer for three temperatures (25, 35, and 37°C).

TABLE 13. Comparative sensitivity of eight cell lines to various viruses

Cell line	Ranking	Virus titer (PFU/ml, 10^7)[a]												
		Poliovirus 1	Echovirus					Coxsackievirus				Reovirus 1	Rotavirus SA-11	Sewage PFU/ml
			7	11	12	14	27	CB2	CB5	CA9	CA16			
BGM	1.2	38	130	250	450	8.9	46	99	470	77	370	5.6	12	68
MA104	2.4	24	43	64	150	0.1	2.4	49	140	29	29	4.3	20	31
RD	3.0	32	23	1	29	0.03	1.8	67	200	42	240	2.2	1.5	18
HEp-2	5.0	4.1	<0.1	<1	<1	<0.01	<0.1	1.4	13	6.6	44	2.7	0.4	0
Vero	5.4	4.7	33	0	98	0	0	3.4	130	2.7	7.2	1.9	0.3	2
L132	5.5	14	<0.001	<0.01	<0.01	<0.0001	<0.01	1	6	18	<0.01	110	2.4	5
HeLa	6.5	3.7	<0.001	<0.0001	<0.0001	0	<0.01	<0.0001	<0.001	0.4	3	2	0	0
MDBK	6.9	0	0	0	0	0	0	0	0	0	0	2.8	1.5	0

[a] Based on only one assay of four replicates, conducted on all cell lines on the same day.

TABLE 14. Comparative sensitivity of cell lines to infection by indigenous viruses in 37 wastewater samples

Sample no.	No. of virus plaques confirmed on each cell line										Location of sample site
	BGM	MDBK	RD	MA104	L-132	Primary African green monkey	Primary rhesus monkey	HEp-2	Vero	HeLa	
1	11	113	8	4	6	7	0	1	0	0	Groton (Town Plant), Conn.
2	52	73	20	3	87	9	7	25	8	0	Groton (City Plant), Conn.
3	9	2	3	5	2	2	0	0	0	0	Warwick, R.I.
4	35	30	27	0	0	2	1	0	0	0	Concord, Mass.
5	25	0	12	17	0	1	1	0	0	0	North Oconee, Ga.
6	9	L[a]	5	L	0	L	0	0	0	0	Cedar Creek, Ga.
7	64	0	33	15	0	3	2	1	2	0	Middle Oconee, Ga.
8	82	2	27	13	3	3	5	2	1	0	Independence, Mo.
9	28	0	8	7	0	4	0	1	0	0	Johnson County, Kans.
10	67	0	19	19	3	10	6	5	2	0	Olathe, Kans.
11	23	L	0	L	L	L	0	L	0	0	Platte County, Kans.
12	5	0	0	0	0	0	0	0	0	0	Tampa, Fla.
13	0	0	2	0	0	0	0	0	0	0	Tampa, Fla.
14	1	0	0	0	0	0	2	0	0	0	Tampa, Fla.
15	3	0	0	0	1	0	0	0	0	0	Tampa, Fla.
16	27	0	18	7	0	4	4	0	2	0	Lamar, Colo.
17	18	13	1	2	0	7	2	1	1	1	Rocky Ford, Colo.
18	37	7	15	26	0	12	4	0	0	0	Englewood-Littleton, Colo.
19	49	0	20	18	14	3	5	1	0	0	Colorado Springs, Colo.
20	7	0	4	1	0	1	1	0	0	0	La Junta, Colo.
21	0	0	0	0	0	0	0	0	0	0	Clearfield, Pa.
22	31	0	7	1	27	0	1	3	0	1	Shannokin, Pa.
23	34	0	13	0	0	0	0	3	0	0	Connellesville, Pa.
24	17	0	7	0	2	0	0	0	0	0	Wheeling, W.Va.
25	12	0	6	18	2	9	7	1	5	0	Moundsville, W.Va.
26	59	0	19	8	13	7	5	1	3	0	Richmond, Calif.
27	37	0	12	7	5	11	5	2	0	0	Petaluma, Calif.
28	14	146	7	6	0	2	2	L	0	0	Dundee, Mich.
29	15	16	7	0	0	3	7	L	1	0	Deerfield, Mich.
30	6	0	3	0	0	0	0	1	0	0	Carteret, N.J.
31	19	5	4	6	7	1	3	1	3	0	Mt. Holly, N.J.
32	3	2	0	3	0	0	0	3	0	0	Rathway Valley, N.J.
33	0	0	0	0	0	0	0	0	0	0	Ocean County, N.J.
34	2	4	2	3	0	1	1	1	0	0	Cincinnati, Ohio
35	3	0	0	0	0	1	0	1	0	0	Cincinnati, Ohio
36	21	0	2	2	0	2	4	0	1	0	Cincinnati, Ohio
37	22	0	11	10	0	11	2	3	2	0	Harrison, Ohio
Total plaques	847	413	322	301	172	116	77	57	31	2	
No. picked	279	24	117	106	65	106	74	42	25	2	
No. identified	274	24	109	95	37	86	55	38	19	2	
No. unidentifiable	3	0	2	7	5	6	3	1	0	0	
No. negative picks	2	0	6	4	23	14	16	3	6	0	
% Positive picks	99	100	95	96	65	87	78	93	76	100	

[a] Lysed cell sheet due to sample.

TABLE 15. Identification of virus isolates

Sewage sample	Number and types[a] of viruses isolated on cell lines																	
	BGM		RD		MA104		L-132		Primary African green monkey		Primary rhesus monkey		HEp-2		Vero		HeLa	
	Type	No.	Type	No.	Type	No.	Type	No.	Type	No.	Type	No.	Type	No.	Type	No.	Type	No.
1	CB1	1	P2	1	CB3	1	E17	2	CB1	1			CB3	1				
	CB2	3	P3	2					CB3	3								
	CB3	2							CB5	2								
2a	CB3	13	CB3	5			CB3	1	CB2	1	CB3	1	CB3	4				
							E5	1	CB3	3	CB4	1	CB4	6				
							E11	1			E17	1	CB5	1				
							E17	5										
2b[b]	CB1	1																
	CB2	10																
	CB4	7																
	CB5	1																
	E4	1																
	E5	7																
	E7	1																
	E11	11																
	E13	1																
	E15	2																
	E17	4																
	E24	2																
	E25	1																
3	P2	1	P3	1			E17	1	CB1	1	CB2	1						
	CB1	4							CB3	1								
	CB2	1																
4	CB1	8	CB1	2	CB3	1			CB1	1	CB2	1						
	CB2	1	CB3	3					CB5	1								
			E15	1														
5	CB2	8	CB2	6	CB2	3			CB2	1	CB2	1						
	CB3	2	CB3	2	CB3	6												
	E4	1																
	E17	1																
6	P2	1	CB3	1														
	CB1	1	CB5	1														
	CB4	1	E5	1														
	CB5	3																
	CB6	1																
7	CB2	10	P2	1	CB2	7			CB2	1	CB2	1	E17	1	CB2	1		
			CB2	8					CB3	1					CB3	1		
8	P3	1	CB2	4	CB2	5	CB3	1	CB2	3	CB3	3	CB3	1				
	CB2	11	CB3	1	CB3	2							CB5	1				
	CB3	1			CB5	1												
9	CA7	1	P2	1	P3	1			CB1	1								
	CB2	5	CB2	1	CB2	2			CB3	1								
	CB3	1			E23	1												
	CB5	2																
	E21	1																
10	CB2	3	CB2	3	CB2	3	P1	1	P2	1	P2	1	CB3	5	P2	2		
	CB3	6	CB3	4	CB3	4			CB2	5	P3	1						
	CB5	1	CB4	1	E25	1			CB3	2	CB2	1						
	E15	1			E33	1			CB4	1	CB3	2						
					P2	1												
11	CB2	13																

Continued on following page

TABLE 15—*Continued*

Sewage sample	Number and types[a] of viruses isolated on cell lines																	
	BGM		RD		MA104		L-132		Primary African green monkey		Primary rhesus monkey		HEp-2		Vero		HeLa	
	Type	No.	Type	No.	Type	No.	Type	No.	Type	No.	Type	No.	Type	No.	Type	No.	Type	No.
12	CB3	1																
	CB5	1																
13			P3	2														
14	CB3	1					CB3	1			CB2	1						
											CB3	1						
15	CB3	1																
	E24	1																
16	P3	2	P3	1	P3	2			P1	1	P3	4			CB3	1		
	CB3	3	CB2	3	CB2	1			P3	1					CB5	1		
	CB4	2			CB3	1			CB3	1								
	E12	1			E7	1			E7	1								
					E20	1												
17	P2	1	CB2	1	CB3	1			P2	3	CB1	1	CB3	1	P2	1	CB3	1
	P3	2							P3	4	CB2	1						
	CB2	2																
	CB3	3																
	CB5	1																
	E2	1																
	E12	1																
	E17	1																
18	P3	2	P3	1	CB1	1	P3	3	P3	1								
	CA9	1	CB2	3	CB2	6	CB2	3	CB2	1								
	CB2	10			CB3	4	CB3	1	CB3	1								
					E2	1												
19	P3	1	CB2	2	P3	1	CB5	1	CB5	1	CB2	2	CB5	1				
	CB2	2	CB3	2	CB2	2	E5	3			CB3	1						
	CB3	5			CB3	3					CB5	1						
	CB4	2			CB5	1												
	CB5	2																
	E25	1																
20	CB2	3	P1	1					P1	1	P1	1						
	CB4	1	CB2	1														
	E13	1																
21																		
22	CB1	1	CB3	3			CB3	2					CB3	3			CB3	1
	CB3	2	CB4	1			E5	1										
	CB4	2																
	E15	1																
23	CB3	5	CB3	4									CB3	3				
	CB4	3																
	CB5	2																
	E11	1																
24	CB1	1	CB3	1			E5	1										
	CB2	2	CB4	2			E17	1										
	CB4	7																
	E11	1																
25	CB1	1	CB3	1	P3	2			P3	2	CB1	1	CB3	1	CB4	2		
	CB4	4	CB4	2	CB3	5			E5	2	CB4	1			E5	1		
	E15	2			CB4	4			E7	3	E5	1			E7	1		
	E21	1			E7	2					E15	2			E17	1		
											E31	1						

Continued on following page

TABLE 15—*Continued*

Sewage sample	Number and types[a] of viruses isolated on cell lines																	
	BGM		RD		MA104		L-132		Primary African green monkey		Primary rhesus monkey		HEp-2		Vero		HeLa	
	Type	No.	Type	No.	Type	No.	Type	No.	Type	No.	Type	No.	Type	No.	Type	No.	Type	No.
26	P3	3	CB2	1	CB3	1	E5	4	P1	2	P2	1	CB5	1	P1	1		
	CB1	1	CB4	1	CB4	1			P2	1	E1	1			P2	1		
	CB2	1	CB5	1	E7	1			P3	1	E15	2			P3	1		
	CB3	1							CB5	1								
	CB4	2							E7	1								
	CB5	1																
	E11	1																
	E15	1																
	E19	1																
27	CB3	1	CB3	1	CB4	1	P1	1	P1	1	CB4	1	P2	1				
	CB4	6	CB5	2			CB4	1	CB5	1	E7	3	CB3	1				
	CB5	3	E15	1			E7	1	E7	5								
	E11	3																
28	P3	2	CA16	1	CB3	2			CB3	1	P3	1						
	CB3	8	CB3	3					CB5	1	CB3	1						
29	P1	7	P1	2					P2	2	P1	4			P1	1		
			P2	2							P2	1						
30	CB3	4	CB3	2									CB3	1				
31	P3	1																
	CB2	1																
	CB3	1																
	CB5	1																
	E11	2																
	E15	1																
32	E15	2			CB4	1							CB3	2				
33																		
34	P2	1	P3	1	P3	2			P3	1	E7	1	E11	1				
	E15	1	E7	1														
35	P2	1																
36	P2	5	P1	1					P2	2	P3	3			P2	1		
	P3	5	CB4	1							CB4	1						
37	P3	4	P2	3	P2	4			P3	9	P2	1	P3	2	P3	2		
			P3	4	P3	2			CB2	1								
					CB4	1			E7	1								

[a] P, Poliovirus type; CA, coxsackievirus type A; CB, coxsackievirus type B; E, echovirus type.

[b] Viruses listed under sewage sample 2b were isolations made from a portion of sample 2a neutralized with coxsackie B3 antiserum before inoculation.

types as well as, or better than, the RD cells and do not tend toward single virus type isolations as previously reported (32). It should also be noted that when sewage sample number 2 (Table 15) was pretreated with coxsackievirus B3 antiserum before inoculation into BGM cell cultures, far more (both in type and number) echoviruses were isolated than previously on the L-132 cell line. This latter development indicates that samples with large numbers of viruses, especially when one type appears dominant, should be treated with appropriate antisera to determine whether other virus types are being masked.

Based on the data collected, the following procedures and media are recommended for the cultivation of BGM cells when monitoring environmental samples for indigenous viruses. (i) The growth medium for both stock and test cultures should consist of a mixture of 50% MEM (Eagle) with Hanks salts, L-glutamine, and nonessential amino acids and 50% L-15 medium (Leibovitz) with L-glutamine. (ii) This mixture should be supplemented with 667 mg of $NaHCO_3$ per liter and 10% dialyzed fetal calf serum. (iii) Test cultures should be planted in vessels of like composition used to grow stocks (i.e., glass to glass or plastic to plastic), with a cell concentration of 1.4×10^5 cells per cm^2 of surface area, and should be used at age 3 to 4 days. (iv) Once the cell cultures are ready for virus testing, they should be washed with Earle basic salt solution without serum just before inoculation. (v) At least 80 min should be allowed for virus infection at 25°C. (vi) The overlay medium should consist of medium 199 with Earle salts, supplemented with HEPES buffer, either GG-free newborn calf serum or dialyzed fetal calf serum,

$NaHCO_3$, $MgCl_2$, neutral red, and milk. (vii) This should then be mixed with equal parts of any of the following agars: GIBCO bacteriological, Oxoid technical no. 3, Difco flake, GIBCO purified, Inolex bacteriological, Difco technical bacteriological, or Difco purified.

It is important that laboratories follow a standardized cell line system so that results of environmental virus monitoring are comparable. By following the BGM procedures outlined, laboratories can eliminate variables in monitoring for waterborne viruses and at the same time increase the sensitivity of the analyses.

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