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Comparison of Cell Cultures for Rapid Isolation of Enteroviruses

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Cell culture isolation is still the most reliable method for the detection of enteroviruses from clinical specimens. Rapid diagnosis of enterovirus infection affects patient management. To increase yield and enhance the rapidity of enterovirus isolation in cell cultures, we used Buffalo green monkey kidney (BGM) cells and subpassages of primary human embryonic kidney (HEK) cells in addition to the human diploid fibroblast (MRC-5) cells and primary cynomolgus or rhesus monkey kidney (MK) cells routinely used for enterovirus culturing. Growth characteristics of enteroviruses from 421 specimens were studied. All specimens were cultured in MRC-5, MK, and BGM cells, and 204 of these specimens were also cultured in HEK cells. Forty-two percent of the enteroviruses became positive within 3 days, and 85% did so within 7 days. MRC-5 cells provided the highest yield of enteroviruses overall and were the best cell type for the recovery of poliovirus and echovirus. MK cells provided the second best yield but were more useful than MRC-5 cells for coxsackievirus. BGM cells supported the growth of additional isolates of coxsackievirus and enhanced the speed of isolation. HEK cells supported the growth of additional isolates of both coxsackievirus and echovirus, but subculturing was always required for definite enterovirus cytopathic effects. The recovery rate increased 11% when two additional cell lines were used. The use of two tubes of MK cells significantly increased the yield of all enterovirus types. We conclude that the use of multiple appropriate cell lines increases yield and enhances the rapidity of enterovirus isolation.

The detection of enteroviruses in clinical specimens currently relies on cell culturing. Although it was previously believed that isolation in cell cultures was slow and expensive, several recent studies have proved that many types of enteroviruses which cause diseases common in infants and children can be isolated rather rapidly at a cost comparable to that of bacterial culturing (1, 2, 19). Rapid isolation of enteroviruses affects patient management, and the detection of an enterovirus as a cause of central nervous system disease improves the accuracy of prognosis and aids in infection control (1, 19).

It has been shown that no single cell culture system is suitable for the recovery of all of the enteroviruses commonly recovered from wastewater (14, 16, 18). Data on the detection of enteroviruses in clinical specimens also suggest that increased yield and enhanced speed of enterovirus isolation can be obtained by inoculation into several cell types (2, 19).

To increase yield and enhance the rapidity of enterovirus isolation from clinical specimens, the Clinical Virology Laboratory of the University of Texas Medical Branch, Galveston, has added Buffalo green monkey kidney (BGM) and human embryonic kidney (HEK) cells to the repertoire of routine cell cultures for enteroviruses. This report compares data on the growth of each group of enteroviruses in different cell types.

MATERIALS AND METHODS

Specimens. Between October 1982 and October 1987, 3,369 clinical specimens were submitted to the Clinical Virology Laboratory for enterovirus isolation. The specimens were from cerebrospinal fluid (CSF), serum, throat swabs or combined throat swabs and nasal washes, rectal swabs or stools, urine, and biopsies. A total of 696 specimens were positive for an enterovirus. For the purpose of studying growth characteristics of each virus type in different cell types, we included only 421 specimens which were inoculated in at least three cell types (human diploid fibroblast [MRC-5], primary cynomolgus or rhesus monkey kidney [MK], and BGM cells) and yielded a typeable enterovirus (poliovirus, coxsackievirus, or echovirus). In addition to the three cell types used, 204 of the 421 specimens were also set up in one tube of HEK cells as the fourth cell line. For each specimen, the type of enterovirus isolated, the number of tubes of each cell line in which the specimen was inoculated, growth, and the time required for growth in each cell line were documented.

Cell cultures. Tube cultures of primary cynomolgus or rhesus MK cells and MRC-5 cells were obtained from M. A. Bioproducts, Inc., Walkersville, Md., Earl Clay Laboratories, Novato, Calif., or Viromed Laboratories, Inc., Minneapolis, Minn. Primary cell lines were used up to passage 5. MRC-5 cells were used between passages 23 and 35. BGM cells, originally provided by Marilyn A. Menegus, were propagated in our laboratory and used between passages 23 and 187. Primary HEK cells were originally obtained from Flow Laboratories, Inc., McLean, Va., and subpassages of HEK cells were then prepared in our laboratory up to passage 114. In most cases, a clinical specimen was set up in two tubes each of MRC-5, MK, and BGM cells (October 1982 to December 1986). Beginning in January 1987, one tube of BGM cells was replaced by one tube of HEK cells. However, if available cell cultures were limited, only one tube of each cell line was used or the specimen was not inoculated in all cell lines.

Specimen collection and processing. Throat and rectal specimens were collected with swabs and placed in 2 ml of viral transport medium (veal infusion broth with 0.1% gelatin and antibiotics). Nasal wash specimens were obtained by the method described by Hall and Douglas (4). When throat swab and nasal wash specimens were collected from the same patient at the same time, the specimens were combined before viral culturing and designated in this study as throat

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TABLE 1. Enterovirus serotypes isolated from 421 specimens

Virus (*n*)	Serotype	No. of strains
Poliovirus (67)	1	10
	2	35
	3	22
Coxsackievirus (87)	A9	12
	B1	1
	B2	26
	B3	13
	B4	21
	B5	14
Echovirus (267)	1	4
	2	9
	3	4
	4	50
	5	10
	6	19
	7	34
	9	34
	11	60
	14	5
	15	2
	17	3
	18	3
	20	2
	21	2
	22	3
	25	4
	27	2
	30	8
	31	9

swab specimens. CSF, blood, urine, and stool specimens were collected in sterile containers and transported to the laboratory without placement in viral transport medium. Respiratory tract, urine, and fecal specimens were vigorously vortexed and clarified by centrifugation or filtration in 0.45-μm-pore disposable filters before cell culture inoculation. CSF and serum were inoculated directly into cell cultures. Cell cultures were inoculated with 0.2 ml of specimen or the inoculum was divided equally among tubes if smaller quantities were available. All cultures were incubated at 36°C in the stationary position and observed for cytopathic effects (CPE) daily (except Sunday) for 10 days. Specimens were reported to be positive when a characteristic enteroviral CPE was detected (7, 11). In some cases, a characteristic enteroviral CPE could not be reported until the supernatant fluids were subcultured in various cell lines. In these cases, the original cell line which demonstrated the questionable CPE was reported as the cell line in which the virus grew, but the total number of days required included the total duration until a definite CPE could be reported. Final typing of the viruses was done by neutralization by the Medical Virology Branch, Bureau of Laboratories, Texas Department of Health, Austin. When several clinical specimens collected from the same patient on the same day were positive for an enterovirus, only the most important specimen, such as CSF or serum, was typed; other specimens from that patient were considered to have the same virus type, and each separate isolate was considered and included in the study.

Statistical evaluation. Comparative data were examined for significant patterns. The chi-square analysis was used in the statistical calculations. A P value of less than 0.05 was considered statistically significant.

RESULTS

The 421 studied specimens included CSF (n = 101), serum (n = 15), throat swab (n = 96), rectal swab (n = 203), nasal wash (n = 4), and colon biopsy (n = 2). The isolated enteroviruses included polioviruses (n = 67), coxsackieviruses (n = 87), and echoviruses (n = 267). The serotypes of all of the enteroviruses isolated are shown in Table 1. The most common serotypes were echovirus 11, 4, 7, and 9 and poliovirus 2.

The 421 specimens were separated into two groups based on the number of cell culture tubes used for each specimen (Table 2). Group 1 consisted of 151 (36%) specimens which were set up in equal numbers of tubes (one or two each) of MRC-5, MK, and BGM cells. Group 2 consisted of 270 (64%) specimens which were set up in unequal numbers of tubes of the three cell lines, mostly two tubes of MRC-5, one or two tubes of MK, and one tube of BGM cells. The proportions of specimen type were similar in both groups. Overall, a characteristic enteroviral CPE was first observed in one of the cell lines in 42% of all specimens within 3 days and in 85% within 7 days (data not shown). Table 2 shows the growth characteristics of enteroviruses in each cell line. MRC-5 was the most useful cell line for growing enteroviruses. MRC-5 or MK cells alone would have failed to detect 30 to 43% of the enteroviruses which could be detected by culturing in two to three cell lines. Although BGM cells gave the lowest recovery rate, an extra 4% of specimens were positive only in this cell line. BGM cells also provided the most rapidly detectable CPE; almost half of the enteroviruses detected in BGM cells were positive by day 3. Of 151 group 1 specimens, 5 grew strains of enteroviruses only in BGM cells. The specimens were typed as coxsackieviruses B3 (n = 2) and B4 (n = 1) and poliovirus 2 (n = 2). Overall, BGM was the only cell line capable of growing enteroviruses from 16 specimens. These were typed as coxsackieviruses A9 (n = 1), B2 (n = 1), B3 (n = 2) and B4 (n = 6), echovirus 7 (n = 2) and 11 (n = 1), and poliovirus 2 (n = 3).

TABLE 2. Growth characteristics of enteroviruses in cell lines

Group	Growth (% of total) in:											
	MRC-5 cells				MK cells				BGM cells			
	Total positive	Only cell line positive	Positive by day:		Total positive	Only cell line positive	Positive by day:		Total positive	Only cell line positive	Positive by day:	
			3	7			3	7			3	7
1 (n = 151)	70	32	39	83	58	18	33	83	18	3	44	85
2 (n = 270)	71	29	42	95	56	16	39	88	17	4	52	87
Total (n = 421)	70	30	41	91	57	17	37	86	17	4	49	86

TABLE 3. Growth of enterovirus groups in cultures of three cell lines[a]

Group	Growth (% of total) in:											
	MRC-5 cells				MK cells				BGM cells			
	Total positive	Only cell line positive	Positive by day:		Total positive	Only cell line positive	Positive by day:		Total positive	Only cell line positive	Positive by day:	
			3	7			3	7			3	7
Poliovirus (n = 27)	78	37	48	90	56	11	53	93	19	7	60	80
Coxsackievirus (n = 28)[b]	39	18	9	63	61	25	47	88	29	11	50	63
Echovirus (n = 96)	77	35	41	84	58	18	23	79	15	0	36	100

[a] Data are from group 1 (n = 151 specimens).
[b] Including 2 coxsackievirus A9 and 26 coxsackievirus B strains. For coxsackievirus B strains only, percent total positive in BGM cells was 31 and percent only cell line positive was 12.

The growth of each type of enterovirus in different cell lines is compared in Table 3. All of these specimens were cultured in an equal number of tubes of each cell line. MRC-5 cells provided the best recovery rate for poliovirus and echovirus, whereas MK cells provided the best recovery rate for coxsackievirus. BGM cells detected more coxsackievirus than poliovirus and echovirus but enhanced the rapidity of detection of all types of enterovirus. This cell line also detected 7% of poliovirus and 11% of coxsackievirus strains that other cell lines, including HEK, failed to detect.

A total of 204 specimens were cultured in one tube of HEK cells; 69 (34%) were positive. In general, viruses grown in HEK cells rapidly destroyed the monolayer, and a characteristic enterovirus CPE appeared only when the supernatant fluids were subcultured in HEK cells and/or various other cell types. In 14 specimens (7%) the diagnosis of an enterovirus could only be made by culturing in HEK cells; 2 of these specimens grew directly in HEK cells (echoviruses 6 and 9). In 12 specimens CPE only appeared in HEK and/or other cell lines after the virus was passed from HEK cells. These enteroviruses were coxsackieviruses B3 (n = 1), B4 (n = 1), and B5 (n = 4) and echoviruses 4 (n = 2), 9 (n = 1), 11 (n = 2), and 25 (n = 1). Enteroviral CPE were observed within 3 days in 25% of specimens and within 7 days in 85% of specimens cultured in HEK cells.

The rate of observable CPE for each specimen type was compared (data not shown). The median days for observable CPE were day 4 for CSF, throat swab, and rectal swab specimens and day 5 for serum. The percentages of observable CPE by day 3 were 48% for rectal swabs, 43% for throat swabs, 33% for CSF, and 27% for serum. By day 7, similar percentages of all types of specimens were positive (83 to 88%).

Because MRC-5 and MK are the cell lines most diagnostic laboratories use to grow enteroviruses, we compared the growth of each type of enterovirus in these cell lines. Of 421 studied specimens, 296 were cultured in two tubes each of MRC-5 and MK cells (Table 4); MRC-5 was a better cell line than MK for growing echovirus, whereas MK was better for coxsackievirus. A total of 30% of poliovirus, 6% of coxsackievirus, and 44% of echovirus strains grew in MRC-5 cells but not in MK cells. In contrast, 19% of poliovirus, 53% of coxsackievirus, and 16% of echovirus strains grew only in MK cells.

To determine if the use of two tubes of each cell line (instead of one) would increase the recovery rate or the rapidity of virus isolation, we compared the growth characteristics of enteroviruses which grew from specimens inoculated in one versus two tubes of each cell line (Table 5). When two tubes of MK and BGM cells were used, the recovery rate (percent positive) of enteroviruses significantly increased over that obtained when one tube of each was used. For MK cells, the use of two tubes also significantly increased the recovery of the viruses which did not grow in other cell lines (only positive in MK cells). However, the use of two tubes of BGM cells did not yield more viruses which did not grow in cell lines other than BGM. In other words, although the use of two tubes of BGM cells yielded more isolates of enteroviruses, the viruses which were missed when only one BGM tube was used grew in other cell lines. Overall, two tubes of any cell line did not significantly increase the speed of recovery (percent positive by days 3 and 7) of enteroviruses.

DISCUSSION

Our findings agree with data from previous studies (1, 2, 5) showing that enteroviruses can be isolated from clinical specimens relatively rapidly. Forty-two percent of the enteroviruses in this study were positive within 3 days, and 85%

TABLE 4. Growth of enteroviruses in MRC-5 and MK cells[a]

Virus	Growth (% of total) in:							
	MRC-5				MK cells			
	Total positive	Only cell line positive	Positive by day:		Total positive	Only cell line positive	Positive by day:	
			3	7			3	7
Poliovirus (n = 53)	75	30[b]	48	93	65	19[b]	59	91
Coxsackievirus (n = 66)	27	6	11	78	74[c]	53[c]	41[c]	88
Echovirus (n = 177)	80[c]	44[c]	43[c]	90	52	16	26	84

[a] A total of 296 specimens were cultured in two tubes each of MRC-5 and MK cells.
[b] May also be positive in BGM or HEK cells.
[c] Significantly higher number compared with the other cell type ($P < 0.02$).

TABLE 5. Growth of enteroviruses from specimens cultured in one versus two tubes

Cell line and number of tubes	No. of specimens[a]	%			
		Total positive	Only cell line positive	Positive by day: 3	7
MRC-5					
One	45	71	29	47	94
Two	376	70	35	41	91
MK					
One	111	48	11	34	81
Two	310	60[b]	34[b]	37	87
BGM					
One	274	15	4	45	85
Two	147	22[b]	4	55	88

[a] Number of specimens inoculated in one or two tubes of each cell line; total number for each cell line, 421.

[b] Significantly different from results for the one-tube group of the same cell line ($P < 0.05$).

were positive within 7 days. Because the rapidity of enterovirus isolation affects patient management (1, 19), every effort should be made by clinical laboratories to enhance the rapidity of virus isolation. One method is to use multiple cell lines appropriate for the isolation of enteroviruses. It is not uncommon for a viral culture specimen to be inoculated in three cell types. Cell types which have been used commonly for enteroviruses include primary MK, diploid fibroblast, human fetal kidney, HEK, and HEp-2 cells (6, 8, 10, 15, 17). A recent study (2) showed that the use of BGM and human rhabdomyosarcoma cells in addition to a traditional culture in primary MK and human diploid fibroblast cells increased the rate of recovery of enteroviruses from clinical specimens by 18%; the speed of isolation was also significantly enhanced. We found that BGM and HEK cells together increased our recovery rate by 11%, and BGM cells also enhanced the speed of isolation. The use of MK or MRC-5 cells alone for enterovirus isolation would result in the failure to detect 30 to 43% of enteroviruses.

It was a common belief that inoculation of a viral specimen into two or more tubes of each cell line would increase the recovery rate (13, 17). However, if several cell lines are to be used, inoculation into two tubes of each cell line will make viral culturing cumbersome and expensive. Our study showed that although MRC-5 was the most useful cell line for detecting an enterovirus, the use of two tubes of MRC-5 cells did not increase the speed or rate of recovery of enteroviruses. In contrast, the use of two tubes of MK cells significantly increased the recovery of enteroviruses, especially the strains that did not grow in any other cell lines. Although the use of 2 tubes of BGM cells increased the rate of recovery, the extra strains detected were also positive in other cell lines. These results suggest that only in the case of MK cells does the use of two tubes of cells help increase the rate of recovery of enteroviruses. This exception may reflect the nature of the primary MK cell lines and/or the quality of the MK cell lines received from commercial sources.

BGM cells have been demonstrated to enhance the growth of group B coxsackieviruses (3, 12). Because of a relatively small number of group B coxsackievirus infections in our community during the study period, the rate of recovery of enteroviruses by this cell line alone was slightly lower than the rate previously reported (2).

We initially introduced HEK cells into our laboratory to enhance the recovery of respiratory viruses (9), although others have recognized the usefulness of this cell line for enterovirus isolation (8, 11). We found that the use of HEK cells enhanced the isolation of many serotypes of enterovirus. However, it was difficult to detect characteristic CPE of enteroviruses in HEK cells, and subculturing was always required. It is possible that the further experience of technicians in recognizing characteristic enteroviral CPE in this cell line will make HEK more useful in both increasing yield and enhancing the rapidity of enterovirus recovery.

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LITERATURE CITED

1. **Chonmaitree, T., M. A. Menegus, and K. R. Powell.** 1982. The clinical relevance of 'CSF viral culture': a two-year experience with aseptic meningitis in Rochester, N.Y. J. Am. Med. Assoc. **247**:1843–1847.
2. **Dagan, R., and M. A. Menegus.** 1986. A combination of four cell types for rapid detection of enteroviruses in clinical specimens. J. Med. Virol. **19**:219–228.
3. **Dahling, D. R., G. Berg, and D. Berman.** 1974. BGM, a continuous cell line more sensitive than primary rhesus and African green kidney cells for the recovery of viruses from water. Health Lab. Sci. **11**:275–282.
4. **Hall, C. B., and R. G. Douglas, Jr.** 1975. Clinically useful method for the isolation of respiratory syncytial virus. J. Infect. Dis. **131**:1–5.
5. **Herrmann, E. C.** 1971. Efforts toward a more useful viral diagnostic laboratory. Am. J. Clin. Pathol. **56**:681–686.
6. **Herrmann, E. C., Jr., D. A. Person, and T. F. Smith.** 1972. Experience in laboratory diagnosis of enterovirus infections in routine medical practice. Mayo Clin. Proc. **47**:577–586.
7. **Hsiung, G. D., and C. K. Y. Fong.** 1982. Picornaviridae, p. 89–109. *In* G. D. Hsiung (ed.), Diagnostic virology, 3rd ed. Yale University Press, New Haven, Conn.
8. **Kaplan, M. H., S. W. Klein, J. McPhee, and R. G. Harper.** 1983. Group B coxsackievirus infections in infants younger than three months of age: a serious childhood illness. Rev. Infect. Dis. **5**: 1019–1032.
9. **Kasel, J. A.** 1979. Adenoviruses, p. 229–255. *In* E. H. Lennette and N. J. Schmidt (ed.), Diagnostic procedures for viral, rickettsial and chlamydial infections, 5th ed. American Public Health Association, Washington, D.C.
10. **Lake, A. M., B. A. Lauer, J. C. Clark, R. L. Wesenberg, and K. McIntosh.** 1976. Enterovirus infections in neonates. J. Pediatr. **89**:787–791.
11. **Melnick, J. L., H. A. Wenner, and C. A. Phillips.** 1979. Enteroviruses, p. 471–534. *In* E. H. Lennette and N. J. Schmidt (ed.), Diagnostic procedures for viral, rickettsial and chlamydial infections, 5th ed. American Public Health Association, Washington, D.C.
12. **Menegus, M. A., and G. E. Hollick.** 1982. Increased efficiency of group B coxsackievirus isolation from clinical specimens by use of BGM cells. J. Clin. Microbiol. **15**:945–948.
13. **Nelson, D., H. Hiemstra, T. Minor, and D. D'Alessio.** 1979. Non-polio enterovirus activity in Wisconsin based on a 20-year experience in a diagnostic virology laboratory. Am. J. Epidemiol. **109**:352–361.

14. **Schmidt, N. J., H. H. Ho, J. L. Riggs, and E. H. Lennette.** 1978. Comparative sensitivity of various cell culture systems for isolation of viruses from wastewater and fecal samples. Appl. Environ. Microbiol. **36**:480–486.
15. **Schmidt, N. J., E. H. Lennette, and H. H. Ho.** 1974. An apparently new enterovirus isolated from patients with disease of the central nervous system. J. Infect. Dis. **129**:304–309.
16. **Sigel, M. M., D. F. Rippe, A. R. Beasley, and M. Dorsey, Jr.** 1976. Systems for detecting viruses and viral activity, p. 139–164. *In* G. Berg, H. L. Bodily, E. H. Lennette, J. L. Melnick, and T. G. Metcalf (ed.), Viruses in water. American Public Health Association, Washington, D.C.
17. **Singer, J. I., P. R. Maur, J. P. Riley, and P. B. Smith.** 1980. Management of central nervous system infections during an epidemic of enteroviral aseptic meningitis. J. Pediatr. **96**:559–563.
18. **Sobsey, M. D.** 1976. Methods for detecting enteric viruses in water and wastewater, p. 89–127. *In* G. Berg, H. L. Bodily, E. H. Lennette, J. L. Melnick, and T. G. Metcalf (ed.), Viruses in water. American Public Health Association, Washington, D.C.
19. **Wildin, S., and T. Chonmaitree.** 1987. The importance of the virology laboratory in the diagnosis and management of viral meningitis. Am. J. Dis. Child. **141**:454–457.