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Rapid Shell Vial Culture Technique for Detection of Enteroviruses and Adenoviruses in Fecal Specimens: Comparison with Conventional Virus Isolation Method

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Detection of enteroviruses and adenoviruses mainly in fecal specimens by rapid culture with inoculation onto cell monolayers in flat-bottom tubes by centrifugation and immunofluorescence staining with genus-specific monoclonal antibodies was compared with that by the conventional virus isolation procedure. For both conventional culture and shell vial culture human lung fibroblast cells and tertiary monkey kidney cells were used. For enterovirus detection, 979 clinical specimens (916 stool specimens, 56 cerebrospinal fluid specimens, and 7 nasopharyngeal swabs) were used. Conventional culture detected 74 enterovirus isolates. A cytopathic effect compatible with the presence of an enterovirus after 3 days of incubation occurred in 25 of the 74 (34%) specimens that eventually became positive. The detection rate for enteroviruses by rapid cell culture after 2 to 3 days of incubation was 42 of 74 (57%). The genus-specific enterovirus monoclonal antibody did not react with strains of echovirus types 22 and 23 or enterovirus type 71. Rapid cell culture for the detection of adenoviruses was performed with 567 clinical specimens (536 stool specimens, 25 cerebrospinal fluid specimens, and 6 miscellaneous specimens), in which 42 adenoviruses were found by conventional culture. Nine of the 42 (21%) adenovirus isolates were detected by conventional culture within 3 days after inoculation, whereas 21 (50%) were found by rapid cell culture within 2 to 3 days. Only two of the nine specimens found to be positive for the enteric adenovirus type 41 by conventional culture as well by a type-specific enzyme-linked immunosorbent assay (ELISA) tested positive by rapid cell culture. In conclusion, the rapid shell vial assay allows the early detection and identification of enteroviruses and adenoviruses in clinical specimens but is markedly less sensitive than the conventional isolation procedure according to the eventual results of the conventional isolation procedure. Conventional cell culture remains a prerequisite for serotyping of enteroviral isolates. On the basis of the results for adenovirus type 41, the rapid detection of adenoviruses was not considered to be useful for the detection of clinically relevant adenoviruses in fecal samples.

At present, the diagnosis of enterovirus and adenovirus infections is usually carried out by virus isolation in tube cultures inoculated with throat swabs, stools, cerebrospinal fluid, ocular swabs, urine, or vesicle fluids (5, 9, 10, 13, 21). Of the more recently developed methods, the use of nucleic acid amplification techniques for the direct detection of enteroviruses and adenoviruses in clinical specimens is available only in laboratories highly specialized for the diagnosis of viral infections (7). On the other hand, rapid techniques with short-term culture and immunofluorescence for the detection of, for example, respiratory viruses in clinical specimens are widely used (2, 6, 11, 12, 15). Application of this approach for the examination of fecal specimens for adenoviruses and enteroviruses has been reported less often (17, 19, 20). In the present study we assessed the applicability of the rapid detection of enteroviruses and adenoviruses in clinical specimens (mainly stool samples) using centrifugation after inoculation and testing with fluorescent genus-specific monoclonal antibodies (MAbs) after a fixed short time in comparison to that of the conventional virus isolation procedure in tubes based on the appearance of a cytopathic effect (CPE).

MATERIALS AND METHODS

Clinical specimens and reference viruses. From January 1994 through September 1995 clinical specimens sent for virus isolation to the Regional Laboratory of Public Health in Amsterdam, The Netherlands, were tested for enteroviruses by both conventional culture in tubes and rapid culture. A total of 916 consecutive stool specimens, 56 cerebrospinal fluid samples, and 7 nasopharyngeal swabs were included in the comparative study for the rapid detection of enteroviruses. Furthermore, 34 previously isolated and typed enterovirus strains that had been stored at −70°C were used to evaluate the range of serotypes reactive with the MAbs used in the shell vial test. From January 1994 through December 1994, 536 stool specimens, 25 cerebrospinal fluid samples, and 6 nasopharyngeal swab specimens were examined for adenovirus by rapid cell culture. In addition, 15 stored adenovirus isolates were tested by the rapid technique.

Fecal samples and cerebrospinal fluid specimens were collected and stored at 4°C in vials before being transported as soon as possible to the laboratory at ambient temperature. The nasopharyngeal swab specimens were transported in virus transport medium containing Eagle minimum essential medium (MEM) in Hanks balanced salt solution (BSS) with antibiotics (penicillin, 20,000 U/ml; streptomycin 20,000 μl/ml). It took approximately 1 to 2 days before the specimens arrived in the laboratory, where they were processed on the day of receipt for both the conventional culture and the rapid culture methods in shell vials and afterward were stored at −20°C. Repeat inoculation was performed only when toxic effects to the cells were found. The isolated strains were kept frozen at −70°C.

Pretreatment of the specimens. Approximately 2 to 3 g of feces was suspended in 10 ml of Eagle MEM in Hanks BSS with 5% gelatin and shaken vigorously. After centrifugation at 700 × *g* for 15 min at 25°C, the supernatants were filtered (pore diameter, 0.45 μm). Cerebrospinal fluid and nasopharyngeal swab specimens were inoculated onto the cells without pretreatment.

Conventional virus isolation in tubes and serotyping of isolates. Monolayers of tertiary cynomolgus monkey kidney (t-MK) cells (National Institute of Public Health and the Environment [RIVM], Bilthoven, The Netherlands) and human embryonic lung fibroblast diploid cells were grown in conventional cell culture tubes. The diploid cells were made in-house from fetal lung tissue in 1984; the cells were used at between the 9th and the 15th passages. Prior to specimen inoculation, Optimem 1 (Gibco) maintenance medium with 2% fetal calf serum (FCS) was removed from the cells. A volume of 0.4 ml of the specimen was inoculated in duplicate onto monolayers of t-MK cells and human diploid cells. The tubes were incubated at 36 to 37°C after the addition of maintenance medium Eagle MEM in Hanks and Earle BSS (1:1) (Gibco) with vancomycin

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(0.1 mg/ml), streptomycin (0.1 mg/ml), and 3% FCS. The tubes were examined for a CPE on the next day and twice a week for 3 weeks. When a CPE indicating the presence of enterovirus or rhinovirus was observed, preliminary identification of isolates was performed by hematoxylin-eosin staining of subcultures. Infectivity tests after exposure to low pH were done to distinguish between enterovirus and rhinovirus. Typing of the enteroviruses was performed with chloroform-treated isolates by neutralization tests with antiserum pools obtained from RIVM for the identification of poliovirus type 1, 2, or 3, echoviruses, and coxsackie B virus types 1 to 6 (10). Adenovirus typing was performed as described previously (3, 4) with rabbit antisera against adenovirus types 1, 2, 3, 5, and 7 obtained from RIVM. If these tests failed, isolates were typed in the Laboratory of Virology of RIVM with more extended panels of antisera.

Rapid culture in shell vials. t-MK cells and human diploid cells were grown on coverslips in flat-bottom tubes. Optimem 1 (Gibco) maintenance medium with 2% FCS was aspirated, and 0.4 ml of the filtered sample was inoculated in duplicate onto t-MK cells and human diploid cells. The flat-bottom tubes were centrifuged at 700 × *g* for 40 min at 37°C, and then 1 ml of Eagle MEM in Hanks and Earle BSS with streptomycin (0.1 mg/ml), vancomycin (0.1 mg/ml), and 3% FCS was added. Depending on the day of the week, methanol fixation was carried out 2 to 3 days and 5 to 7 days after inoculation.

Enterovirus detection by immunofluorescence. After fixation, 25 μl of MAb (DAKO-Enterovirus, 5-D8/1 [DAKO, Glostrup, Denmark]) at a 1:20 dilution was added to each of the coverslips, which were incubated at 37°C for half an hour. Then, the cells were washed with phosphate-buffered saline (PBS) and air dried. A total of 25 μl of fluorescein isothiocyanate-conjugated rabbit anti-mouse immunoglobulin (DAKO), diluted 1:40 was added, and the coverslips were incubated for 30 min at 37°C. Again, the cell monolayers were washed with PBS. A buffered glycerol mounting medium was then added. The optimal dilutions of primary antibody and conjugates were determined by checkerboard titration. The enterovirus MAb was tested for cross-reactivity with five clinical isolates of rhinovirus. The slides were read in a fluorescence microscope (Zeiss IM), and a monolayer was scored positive if it contained at least two cells with a specific cytoplasmic fluorescence.

Adenovirus detection by immunofluorescence. After fixation, 25 μl of murine MAb (Imagen Adenovirus reagent, fluorescein isothiocyanate conjugated [DAKO]) at a 1:2 dilution was added to each of the coverslips, and these were incubated at 37°C for half an hour. Then, the cells were washed with PBS and air dried. A buffered glycerol mounting medium was then added. The optimal dilutions of primary antibody were determined by checkerboard titration. The slides were read as described above by looking for specific nuclear and/or cytoplasmic fluorescence.

Adenovirus detection by ELISA. For the detection of adenovirus types 40 and 41 in fecal specimens, we used an enzyme-linked immunosorbent assay (ELISA) developed at the Laboratory of Virology of RIVM (4). This ELISA is based on the use of type-specific peroxidase-labelled MAbs and includes a genus-specific MAb.

RESULTS

Comparison of the sensitivities of conventional virus isolation and rapid culture assays. (i) Enteroviruses. The number of enteroviruses isolated by conventional culture was 74, with the following distribution (Table 1). In three fecal specimens poliovirus type 1, 2, or 3 (all vaccine strains) were detected. We found coxsackie A virus in 8 specimens, coxsackie B viruses in 19 specimens, and echoviruses of various types in 34 samples. Five strains of human enterovirus type 71 were isolated, and in five specimens the enterovirus found could not be typed with the available antisera. Furthermore, 60 adenovirus strains were detected, 47 in diploid cells and 38 in t-MK cells; 3 of the strains could not be typed (data not shown). In addition, two herpes simplex viruses type 1 and four nonidentified viruses were found. By the rapid technique 55 of 74 (74%) of the enteroviruses detected by the virus isolation method were found, and this technique did not detect enteroviruses in conventional virus isolation method-negative specimens. All three polioviruses, 5 of the 8 (63%) coxsackie A viruses, 17 of the 19 (89%) coxsackie B viruses, 28 of the 34 (82%) echoviruses, none of the 5 (0%) human enteroviruses type 71, and 2 of the 5 (40%) not typed enteroviruses were found by rapid detection with the group-specific MAb. For seven specimens the rapid assay result was difficult to interpret due to aspecific staining or extensive detachment of the cell monolayer and the specimens were considered to be negative. The distribution of the conventional virus isolation results for these seven samples was as follows: two echoviruses type 7, 1 echovirus type 15, and one herpes simplex virus type 1 (no cells in shell vial culture); the virus in one sample was not typed; and no virus was isolated from two samples (no cells).

TABLE 1. Results for 979 specimens tested for enteroviruses by the conventional cell culture technique in tubes according to cell type and rapid cell culture technique[a]

Virus and type	No. of specimens positive			
	Conventional culture technique			Rapid cell culture technique for enterovirus
	t-MK cells	Diploid cells	Total	
Poliovirus[b]	3	3	3	3
CAV 7	2	2	2	0
CAV 9	5	5	6	5
CBV 2	6	4	6	6
CBV 3	12	2	12	10
CBV 5	1	1	1	1
EV 2	1		1	1
EV 5		4	4	4
EV 6	1	1	1	1
EV 7	4	3	4	2
EV 9	1	1	1	1
EV 11	3	3	3	3
EV 15	1		1	0
EV 20	4	4	4	4
EV 21	3	1	3	3
EV 22	2		2	0
EV 25	6	4	6	5
EV 26	1	1	1	1
EV 29	2	2	2	2
EV 30	1	1	1	1
Ent 71	5	5	5	0
Ent NT	4	5	5	2
Total	68	52	74	55

[a] The specimens were obtained in 1994 and 1995. Abbreviations: CAV, coxsackie A virus; CBV, coxsackie B virus; diploid cells, human diploid lung fibroblasts; Ent, enterovirus; EV, echovirus; HSV, herpes simplex virus; NT, not typeable.
[b] All strains were vaccine-like strains.

(ii) Adenoviruses. A total of 567 specimens submitted in 1994 were used to evaluate the rapid technique for the detection of adenoviruses. Forty typeable and two (in our hands) nontypeable adenoviruses were recovered by conventional cell culture; 26 (62%) of these were also detected by the rapid culture technique (Table 2). Only two of the nine adenovirus type 41 strains that were isolated by the conventional cell culture procedure were detected by the rapid assay.

Results of the adenovirus ELISA. A total of 23 of the 916 (2.5%) fecal specimens collected during the study period (1994 and 1995) were positive for adenoviruses by the genus-specific adenovirus ELISA. In two specimens adenovirus type 40 was found, and 10 specimens were positive for adenovirus 41. The other ELISA-positive specimens contained nonenteric adenovirus types. All specimens positive for adenovirus type 40 or 41 by ELISA were also positive for adenovirus type 40 or 41 by the conventional virus isolation procedure.

Comparison of the rapidity of conventional and shell vial culture assays. The proportions of specimens positive for enterovirus by conventional culture after 2 to 3 days of incubation were 34% (25 of 74) and 81% (60 of 74) after 5 to 7 days. Staining of the coverslips at the second or third day after inoculation resulted in a detection rate of 57% (42 of 74) for the specimens that were finally proven to contain enterovirus. At the second fixation after 5 to 7 days of incubation, this rate was 74% (55 of 74).

TABLE 2. Results of rapid cell culture for adenoviruses versus conventional cell culture in tubes for 567 clinical specimens[a]

Virus and type	No. of specimens positive	
	Rapid adenovirus cell culture technique	Conventional cell culture technique
AV 1	3	5
AV 2	5	8
AV 3	2	2
AV 5	1	1
AV 7	7	10
AV 8		1
AV 9	1	1
AV 27	1	1
AV 40	2	2
AV 41	2	9
AV NT	2	2
Total	26	42

[a] The specimens were obtained in 1994. Abbreviations: AV, adenovirus; NT, not typeable.

With regard to adenovirus isolation, 21% (9 of 42) and 57% (24 of 42) of the samples showed a CPE by conventional cell cultures 2 to 3 days and after 7 days after inoculation, respectively. By the rapid culture technique, 50% (21 of 42) of the adenoviruses were detected by testing after 2 to 3 days and 62% (26 of 42) were detected by testing after 5 to 7 days.

Influence of cell type on the rates of detection of enteroviruses and adenoviruses by the conventional culture procedure. By the conventional cell culture procedure 68 of 74 (92%) of the enteroviral isolates were detected on t-MK cells after a mean of 7 days. The human diploid cells also yielded 52 of 74 (69%) enteroviral isolates after a mean of 7 days. During the study period from January 1994 through September 1995 a total of 38 of 60 (63%) of the adenoviruses was isolated from the t-MK cells after a mean of 7 days and 47 of 60 (78%) adenoviruses were recovered from the diploid cells after a mean of 11 days of inoculation. Although adenovirus types 40 and 41 are known to be fastidious (3), our conventional virus isolation technique was also able to detect all these viruses in the samples which scored positive in our MAb-based ELISA. Eight of the nine strains of adenovirus type 41 were isolated only on human diploid cells.

With respect to the shell vial assay, we did not observe any influence of the cell type used.

Type-specific reactivities of the enterovirus- and adenovirus-specific MAbs. Thirty-five enteroviral isolates stored at −70°C and belonging to 28 different serotypes were tested by both the conventional virus isolation procedure and the rapid technique. Furthermore, five strains of clinical isolates of rhinovirus were tested, and they all proved to be negative by the rapid technique. The results broken down by enteroviral type are presented in Table 3. The corresponding conventional virus cultures were all positive. The test with the enterovirus-specific MAb scored negative for echovirus types 22, 23, and 25 and some strains of echovirus 1 and 3. Fifteen clinical adenovirus isolates that belonged to types 1, 2, 3, 4, 5, 7, 8, 9, 10, 11, 15, 17, 19, 20, and 21 and that had been stored at −70°C were examined and tested positive by both techniques (data not shown).

DISCUSSION

The main finding of the present study is the rates of detection of 57 and 50% for enterovirus and adenovirus, respectively, by the rapid cell culture technique after 2 to 3 days of incubation. For 34 and 21% of the isolates, a CPE compatible with the presence of an enterovirus or adenovirus, respectively, was observed after 3 days of conventional cell culture, whereas the necessary serological confirmation would take at least another 7 days. The isolation rates after 7 days of inoculation were 74 and 81%, respectively. Thus, the advantage of the rapid cell culture technique is the greater proportion of enteroviruses and adenovirus that can be detected within 2 to 3 days of culture.

Nevertheless, use of the conventional cell culture technique in tubes cannot be discarded and will continue to be needed to be carried out. First, it detects appreciably more isolates of enterovirus and adenovirus, including those with which the MAbs used in this study do not react. Second, it is a prerequisite for the serotyping of the detected isolates.

In the present study we made observations of the specificity and/or sensitivity of the MAbs used in the rapid culture assays. Clinical samples yielding coxsackie A virus types 7 and 9, coxsackie B virus type 3, and echovirus types 7, 15, and 25 by the conventional culture procedure were in some or all cases negative by the rapid culture procedure (Table 1). This however was not due to a lack of reactivity of the MAb that we used, MAb 5-D8/1, because this MAb did react with the viruses that were grown from these same clinical specimens by the conventional culture procedure. Trabelsi et al. (17) also reported that MAb 5-D8/1 reacted with the enterovirus types mentioned above. Probably, the negative results were due to the presence of low amounts of virus in the samples which induced adequate growth in the culture used for the conventional culture procedure but failed to produce a sufficient

TABLE 3. Results of the rapid culture technique for enteroviruses with isolates that had been stored at −70°C

Enterovirus and virus type	Result
CBV 1	Pos
CBV 2	Pos
CBV 3	Pos
CBV 4	Pos
CBV 5	Pos
EV 1	Var
EV 2	Pos
EV 3	Var
EV 4	Pos
EV 5	Pos
EV 6	Pos
EV 7	Pos
EV 9	Pos
EV 11	Pos
EV 13	Pos
EV 14	Pos
EV 15	Pos
EV 16	Pos
EV 17	Pos
EV 18	Pos
EV 21	Pos
EV 22	Neg
EV 23	Neg
EV 25	Neg
EV 27	Pos
EV 29	Pos
EV 30	Pos
EV 33	Pos

[a] Abbreviations: EV, echovirus; CBV, coxsackie B virus; Pos, positive; Neg, negative; Var, variable, the virus preparation was not reproducibly positive by the rapid culture technique, but the corresponding cultures tested by conventional culture technique were all positive.

amount of MAb-reactive antigen in the culture used for the rapid culture procedure.

Another observation was that MAb 5-D8/1 did not react in the culture used for the rapid culture procedure or with the strain isolated from the corresponding culture used for the conventional culture procedure in the case of echovirus types 22 and 23, enterovirus type 71, and some nontypeable enteroviruses. In contrast to our findings, the reactivity of MAb 5-D8/1 with echovirus type 22 was reported earlier by Yousef et al. (19, 20). This reactivity with echovirus type 22, however, could not be confirmed by Samuelson et al. (14), who analyzed the recognition site of this MAb. Samuelson et al. (14) also reported that enterovirus type 71 was not recognized by the MAb. The nonreactivity of echovirus type 22 and enterovirus type 71 with the MAb may be associated with the fact that the RNAs of both viruses deviate significantly from the RNAs of the other members of the enterovirus group (8, 9, 16).

When testing stored isolates of various enterovirus types by the rapid culture procedure to assess the specificity of MAb 5-D8/1, it is possible that the same phenomenon was observed. Isolates of echovirus types 1 and 3 were not reproducibly positive by the rapid culture assay, whereas isolates of echovirus types 22, 23, and 25 were reproducibly negative by this assay. Again, this discrepancy between the two types of culture techniques could be explained by the presence of low titers of virus in the stored preparations. In view of the observations described above, however, the negative results for echovirus type 22 should be ascribed to a lack of reactivity of the MAb with this virus type.

As with some enteroviruses, the rapid culture technique yielded negative results for a large percentage of clinical samples containing adenoviruses (Table 2). In this case the genus-specific antiadenovirus MAb which we used was reported to react with all adenovirus types in the ELISA (4). In agreement with this reported broad specificity, in the present study the MAb proved to be reactive with all isolates that were grown from these specimens by the conventional culture procedure. This indicates that the negative results obtained by the rapid culture assay were due to low concentrations of adenovirus in the samples concerned.

Enteric adenovirus types 40 and 41 are associated with the occurrence of diarrhea and are reported to be fastidious in cell culture (1, 3, 18). Yet, in the present study all specimens positive for adenovirus type 40 or adenovirus type 41 by ELISA were also positive by the conventional virus isolation procedure. In contrast, seven of the nine specimens containing adenovirus type 41 were not found to be positive by the rapid culture technique. The rapid test proved to be poorly sensitive for adenovirus type 41. The discrepant results could be explained by the reasons mentioned above.

One can draw the conclusion that the rapid technique in shell vials with centrifugation after inoculation and detection with an MAb enables the early detection of all enteroviruses and adenoviruses with exception of important enterovirus strains and enteric adenoviruses in clinical specimens. The conventional cell culture procedure remains valuable because it detects more enteroviruses and adenoviruses and allows serotyping.

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