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Specific Detection of Enteroviruses in Clinical Samples by Molecular Hybridization Using Poliovirus Subgenomic Riboprobes

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Enteroviruses were specifically detected in crude clinical specimens or in cell cultures in which the viruses were amplified by dot hybridization by using poliovirus type 1-derived, subgenomic radiolabeled cRNA probes (riboprobes). The sensitivity of this test varied from 2.5 to 33%, when clinical specimens without cell culture were examined, and was about 85% in cell culture lysates. The specificity of the test was 90 to 100%. The riboprobe corresponding to the 5′-noncoding sequence specifically detected the majority of enteroviruses (56 of 57 tested); the riboprobe derived from the VP1 capsid region hybridized with the three poliovirus serotypes and with some coxsackieviruses type A and with echovirus type 7. Echovirus 22 did not hybridize with any riboprobe. In stool specimens, nasal aspirates, and cerebrospinal fluids from patients with meningitis, only one type of virus was identified in different clinical samples from the same patient by the seroneutralization test. Hybridization allowed the detection of enteroviral RNAs easily in stool specimens and nasal aspirates but with a low efficiency in cerebrospinal fluids without amplification of the viruses in cell cultures.

There are a large number of enteroviruses, some of which cause infections in humans with various severities. Poliomyelitis, which is induced by polioviruses, has been almost eradicated in industrialized countries through efficient vaccination programs. Other enteroviruses, such as echoviruses, coxsackieviruses, and human enteroviruses 68 to 71, still cause important medical problems. Their presence is correlated with a variety of illnesses, including aseptic meningitis, herpangina, epidemic myalgia, hand-foot-and-mouth disease, rashes, myocarditis or pericarditis, and hemorrhagic conjunctivitis.

Common diagnoses of enteroviruses are based on virus isolation during the acute phase of the illness in permissive cells or animals and their identification by seroneutralization; these tests are tedious and expensive. Tests to determine rising titers of enterovirus antibodies in serum are of low sensitivity (neutralization test) and low specificity (cross-reactions within different groups of enteroviruses). A high level of antibodies can persist for many years and is not a good indicator of recent infection. Therefore, a rapid test for the detection of these viruses is necessary.

Detection of various viral RNAs by molecular hybridization with radiolabeled cDNA or cRNA probes has been reported recently (3–8, 11–13). We have developed a sensitive and specific test for the detection of enteroviruses by using subgenomic cRNA transcripts of poliovirus type 1 synthesized in vitro (riboprobes) (2). The riboprobe corresponding to the 5′-noncoding region is a probe that hybridizes with the majority of enteroviruses but not with other DNA or RNA viruses or bacteria, and hence, it is specific for enteroviruses. The VP1 riboprobe is a transcript of the part of the sequence that codes for capsid protein VP1; it hybridizes specifically with all three serotypes of polioviruses and, to a lesser extent, with some coxsackieviruses type A and echovirus type 7. This probe can be considered a specific probe for discriminating between polioviruses and other enteroviruses in epidemiologic studies.

We present here the results of a study in which enterovirus was detected in clinical specimens and in lysates of cells infected with these clinical samples by molecular hybridization with 5′-noncoding and VP1 riboprobes.

MATERIALS AND METHODS

Viruses. Poliovirus types 1, 2, and 3; coxsackieviruses; echoviruses; and human enteroviruses 68 to 71 were either isolated from clinical samples in the Virus Laboratory of the University Hospital (Caen, France) and in the National Laboratory of Health (Lyon, France) or were reference strains from the Center for Enteroviruses, World Health Organization (Lyon, France). Titers of viral strains were determined on monolayer cultures of MRC5 cells, buffalo green monkey cells, or human rhabdosarcoma cells in microdilution plaque samples and are expressed as 50% tissue culture infective doses ($TCID_{50}$s) per milliliter.

Clinical specimens. Stool specimens (258 specimens), cerebrospinal fluid (CSF) (333 specimens), and throat swabs and nasal aspirates (67 aspirates) were mainly obtained from children who were hospitalized with meningitis in the pediatric unit of the University Hospital of Caen and from epidemiologic studies made by the Center for Enteroviruses, World Health Organization, at different intervals.

All specimens except those of CSF were suspended in 5 ml of transport medium (Eagle minimal essential medium supplemented with 5 mg of bovine serum albumin per ml, 4.76 mg of HEPES [*N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid], 1,500 U of penicillin per ml, and 1 mg of streptomycin per ml) and mixed. One part was used for infection of permissive MRC5, buffalo green monkey, or rhabdosarcoma cells to constitute the cell lysates. After 1 h of adsorption at 37°C, the cells were overlayed with Eagle minimal essential medium supplemented with 2% fetal bovine serum and incubated at 37°C in the presence of 5% CO_2. In some cases a cytopathic effect appeared 72 h after

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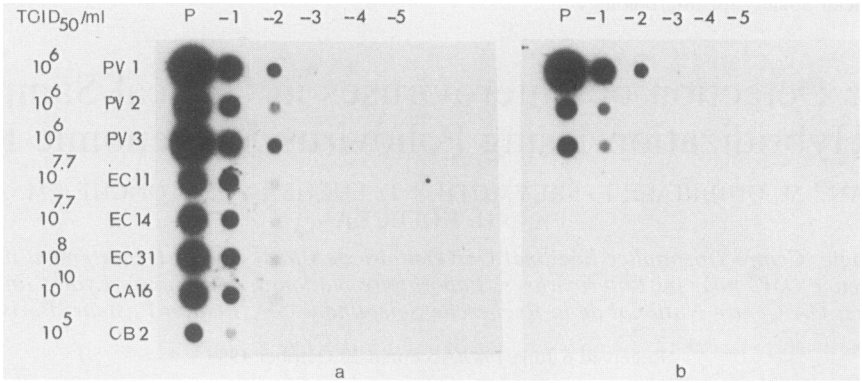

FIG. 1. Sensitivities of 5'-noncoding and VP1 riboprobes in the detection of various enteroviruses. P, cell culture lysate supernatants and their serial dilutions (10^{-1} to 10^{-5}, indicated as -1 to -5, respectively). (a) Filter hybridized with the 5'-noncoding radiolabeled riboprobe. (b) Filter hybridized with the VP1 radiolabeled riboprobe. Abbreviations: PV-1, PV-2, and PV-3, poliovirus serotypes 1, 2, and 3, respectively; EC11, EC14, and EC31, echoviruses 11, 14, and 31, respectively; CA16 and CB2, coxsackieviruses A16 and B2.

infection, but sometimes two to three cell passages were required to observe the cytopathic effect.

To analyze the samples by molecular hybridization, the total cell culture was frozen and thawed three times and clarified by low-speed centrifugation (2,500 × *g* for 15 min), and the supernatants were recovered and incubated with proteinase K (100 μg/ml) for 1 h at 37°C.

The other part of the clinical specimen was stored at −70°C until it was required for hybridization and was then thawed; 200 μl of the sample was incubated directly with proteinase K (100 μg/ml) for 1 h at 37°C.

Portions of the CSF samples were collected directly in the flasks containing proteinase K and stored at −70°C. For hybridization tests, the CSF samples were thawed and incubated for 1 h at 37°C.

Riboprobes. The radiolabeled transcripts of PV1 subgenomic cDNA inserted into the riboprobe vectors (Gemini; Promega Biotech) were used as probes in molecular hybridizations with viral RNA (2). The 5'-noncoding riboprobe was a cRNA with a negative polarity corresponding to nucleotides 221 to 670 of the 5'-noncoding region. This sequence was very conserved among the majority of enteroviruses, and hence, it can be considered as a specific probe in general, but with a wide spectrum for the detection of various enteroviruses (5). It did not hybridize with other viruses (adenoviruses, rotaviruses, hepatitis A virus) or bacterial RNAs or rRNAs. The VP1 riboprobe was a cRNA transcript from nucleotides 3064 to 3417, which was the sequence that codes for the capsid protein VP1.

Dot hybridization. Serial dilutions (10-fold) of reference strains, clinical specimens, and lysates of infected cells were prepared in 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) or in an equal volume of a 3:2 (vol:vol) mixture of 20× SSC–37% formaldehyde and heated at 60°C for 15 min. Portions of 200 μl were applied to a nylon (Biodyne Pall) or a nitrocellulose (Schleicher & Schuell, Inc., Keene, N.H.) membrane under suction by using a 96-well dot blot apparatus (Manifold; Bethesda Research Laboratories, Gaithersburg, Md.). The filters were baked for 2 h at 80°C. Prehybridization of the filters (3 h) and hybridization with radiolabeled probes (10^6 cpm/ml, 16 h) were carried out at 42°C in 50% formamide–3× Denhardt solution (1× Denhardt solution is 0.02% bovine serum albumin, 0.02% Ficoll, and 0.02% polyvinylpyrrolidone)–5× SSC–0.005 M sodium phosphate buffer (pH 7.2)–0.001 M EDTA–100 μg of yeast RNA per ml in sealed plastic bags. The filters were then washed at 65°C successively in 2× SSC, 1× SSC, 0.5× SSC, and 0.1× SSC, always in the presence of 0.1% sodium dodecyl sulfate, for 30 min each time. The air-dried filters were exposed to X-ray film with an intensifying screen at −70°C for 16 to 48 h (1).

RESULTS

Sensitivity of molecular hybridization test with different enteroviruses by using 5'-noncoding and VP1 riboprobes. Poliovirus types 1, 2, and 3; echoviruses 11, 14, and 31; and coxsackieviruses A16 and B2 with known titers ($TCID_{50}$s per milliliter) were diluted 1:1 in a 3:2 mixture of 20× SSC–37% formaldehyde and heated for 15 min at 60°C; 200 μl of this solution or 200 μl of serial dilutions (10^{-1} to 10^{-5}) were spotted onto filters and hybridized in parallel with 5'-noncoding and VP1 radiolabeled riboprobes. The 5'-noncoding riboprobe detected all tested enteroviruses (Fig. 1a), and the VP1 riboprobe detected specifically polioviruses of all three serotypes (Fig. 1b). Poliovirus type 1 gave positive hybridization signals at $TCID_{50}$s of 10 to 10^2 per spot with both riboprobes, whereas other enteroviruses hybridized only at higher titers. Poliovirus types 2 and 3 hybridized to a lesser extent with the VP1 riboprobe, showing fewer sequence similarities in the VP1 region than with poliovirus type 1.

Specificities of 5'-noncoding and VP1 riboprobes for enteroviruses. Supernatants of cell cultures infected with different viruses isolated from clinical samples were treated as described in Materials and Methods; 200 μl of each supernatant was spotted in duplicate onto two filters and hybridized with the 5'-noncoding and VP1 riboprobes. Figure 2a shows the hybridization results obtained with the 5'-noncoding riboprobe; poliovirus type 1, 2, and 3 Sabin and wild-type strains; coxsackieviruses A9, A16, A17, A24, B1, B2, B3, B5, and B6; and echoviruses 3, 4, 6, and 30; human enterovirus 68 hybridized with this probe with various signals, whereas adenovirus type 2, adenovirus type 5, rotavirus, and rotavirus were negative with this probe. Coxsackievirus B6 and echovirus 3 gave hybridization signals after longer exposure times (data not shown). The VP1 probe hybridized only with the three poliovirus types (Sabin or wild-type strains) (Fig. 2b, row A, columns 1 to 6). Other viruses were not detected by this probe. In other experiments, the other viruses that were isolated from nasal and throat aspirates and other clinical samples (mainly human rhinoviruses,

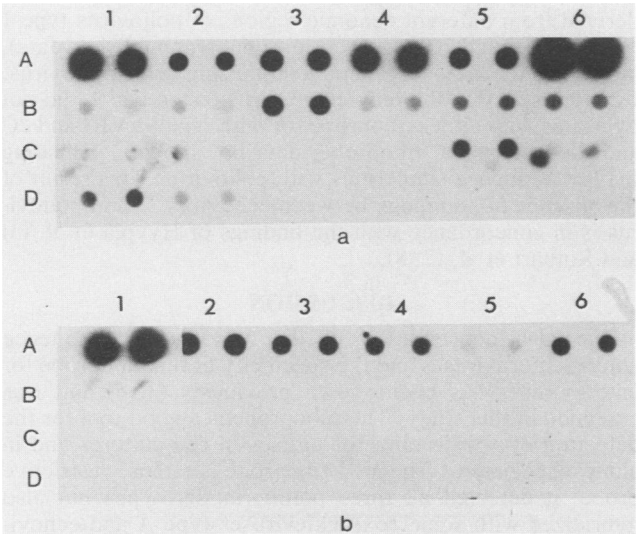

FIG. 2. Specificities of 5′-noncoding and VP1 riboprobes for enteroviruses. (a) Filter hybridized with the 5′-noncoding radiolabeled riboprobe. (b) Filter hybridized with the VP1 radiolabeled riboprobe. Columns 1 to 6 in row A contained the following: poliovirus type 1 Sabin strain, poliovirus type 1 wild type, poliovirus type 2 Sabin strain, poliovirus type 2 wild type, poliovirus type 3 Sabin strain, and poliovirus type 3 wild type, respectively. Columns 1 to 6 in row B contained the following coxsackieviruses: A9, A16, A17, A24, B1, and B2, respectively. Columns 1 to 6 in row C contained the following: coxsackieviruses B3, B5, and B6 and echoviruses 3, 4, and 6, respectively. Columns 1 to 6 in row D contained the following: echovirus 30, enterovirus 68, adenovirus 2, adenovirus 5, rotavirus, and rotavirus, respectively. All samples were dotted in duplicate.

respiratory syncytial viruses, adenoviruses, and cytomegalovirus) were tested by the same test. Only some human rhinoviruses (types 9 and 31 and an unidentified rhinovirus) were positive, with the 5′-noncoding riboprobe showing high sequence similarities in the 5′-noncoding region with poliovirus; the other viruses that were tested were negative with this probe. None of these viruses hybridized with the VP1 riboprobe (data not shown).

Detection of enteroviruses in clinical samples. Clinical samples (stool specimens) (Fig. 3a) and lysates of the cell cultures infected with the same samples (Fig. 3b) were analyzed for the presence of enteroviruses by molecular hybridization with the 5′-noncoding riboprobe. The viruses that multiplied in cell cultures were identified by seroneutralization.

Figure 3 shows 31 samples in which 17 samples were already screened as positive without cell culture (those in Fig. 3a in row A, columns 1, 4, 5, 6, 7, and 8; row B, columns 4 and 7; row C, columns 1, 2, 3, 4, and 8; and row D, columns 1, 3, and 5), indicating the presence of enteroviruses in 55% of the specimens that were analyzed. The same samples and 10 other samples were detected as positive when they were assayed in the cell culture supernatants. The seroneutralization test revealed the presence of unidentified echovirus, echovirus 31, echovirus 27, coxsackievirus B, and unidentified enteroviruses.

Viruses in CSF, nasal aspirates, and stool specimens from patients with meningitis. The clinical symptoms of aseptic meningitis are often related to the presence of enteroviruses. The question arises as to whether the presence of one specific virus is predominant or whether more than one virus is present in different clinical samples (CSF, nasal aspirates, throat swabs, stool and urine specimens, etc.) from the same patient.

We therefore searched for enteroviruses in CSF, stool specimens, and nasal aspirates from 10 children hospitalized with meningitis symptoms. In different samples from the same patient that screened positive by hybridization, only one virus was identified by the seroneutralization test. This indicated that this virus was probably the causative agent of the illness. By the hybridization test of cultured samples with the 5′-noncoding probe, 20 of 22 viral isolates were found to be positive for the presence of virus. Of these 22 samples, 11 were already screened as positive without cell culture. Most of these viruses were detected in stool specimens (six specimens) and in nasal aspirates (four aspirates) but only one was detected in a CSF sample.

Detection of enteroviruses in large numbers of clinical specimens. More than 600 clinical samples collected during 2 years (333 CSF samples, 67 throat or nasal aspirates, and 258 stool specimens) and their respective cell lysates were analyzed by molecular hybridization for the presence of enteroviruses (Table 1).

By using the 5′-noncoding riboprobe in the hybridization test, the sensitivity of virus detection was 2.5 to 33% in clinical samples without amplification of viruses in cell cultures; most of these viruses were found in stool specimens (33%), fewer were found in throat and nasal aspirates (18%), and very few were found in CSF samples (2.5%). When positive cell cultures were examined, 75% positive

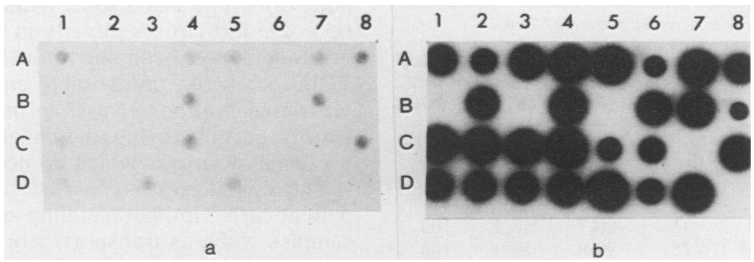

FIG. 3. Detection of enteroviruses in clinical samples. (a) Supernatants of stool specimens hybridized with the 5′-noncoding riboprobe. (b) Supernatants of cell lysates infected with stool specimen supernatants and hybridized with the 5′-noncoding riboprobe. Row A, columns 1, 2, 4, and 6, echoviruses (unidentified); columns 3 and 8, coxsackievirus B; columns 5 and 7, echovirus 31. Row B, columns 1 and 8, echovirus (unidentified); columns 2, 6, and 7, coxsackievirus B; columns 3 and 4, enterovirus unidentified; column 5, echovirus 30. Row C, columns 1, 3, and 6, coxsackievirus B; columns 2 and 7, echovirus (unidentified); columns 4 and 8, enterovirus (unidentified); column 5, echovirus 27. Row D, columns 1 and 8, coxsackievirus B3; columns 2, 3, 4, and 5, coxsackievirus B; column 6, enterovirus (unidentified); column 7, echovirus (unidentified).

TABLE 1. Detection of enterovirus in a large number of clinical specimens

Riboprobe and specimen[a]	No. of specimens/total no. of specimens tested with the indicated cell culture result[b]:					
	CSF (333)		Stool specimen (258)		Throat or nasal aspirate (67)	
	+	−	+	−	+	−
5′-Noncoding						
Do	1/40	2/293	47/140	11/118	6/33	0/34
Dx	21/24	1/180	93/124	1/86	22/27	0/14
VP1						
Do	0/29	1/180	29/127	6/87	3/20	0/14
Dx	1/14	0/128	23/124	1/86	1/27	0/14

[a] Do, Clinical specimens; Dx, lysates of cells infected with clinical specimens.
[b] Numbers in parentheses are the total numbers of specimens.

samples were found in stool specimens, 81.4% were found in throat and nasal aspirates, and 87.5% were found in CSF samples (Table 2). The specificity of the 5′-noncoding riboprobe was 90.7% (clinical specimens) and 98.8% (lysates of cells infected with clinical specimens) for CSF and 100% (in clinical specimens and lysates of cells infected with clinical specimens) for nasal aspirates, respectively. On the other hand, positive hybridization signals that varied between 1 and 9.3% were obtained in samples that were found to be negative by the cell culture method, indicating the presence of viruses which did not replicate in cells. Alternatively, this could have been due to some nonspecific hybridization. These results were obtained with both samples that were treated with SSC and formaldehyde and those that were not; since this treatment was systematically applied, the sensitivity of viral detection was improved.

Enteroviruses detected by poliovirus riboprobes. Of 57 different enteroviruses that hybridized with the 5′-noncoding riboprobe, 56 showed a high level of sequence conservation in this genomic part among the different enteroviruses.

The VP1 riboprobe detected all three poliovirus serotypes (wild or attenuated strains); and coxsackieviruses A6, A11, A13, A17, and A21 and echovirus 7 gave significant and repeated hybridization signals with this riboprobe. All of these viruses also hybridized with riboprobes that were derived from different genomic regions of poliovirus type 1 (regions VP3 and 2C and the 3′ terminus) (unpublished data), indicating their close genetic relationship with poliovirus. Echovirus 22 was the only enterovirus tested which did not hybridize with either riboprobe (or with regions VP3 and 2C and the 3′-terminal riboprobe; data not shown), indicating the low sequence similarities with poliovirus. This result of the absence of homology between echovirus 22 and poliovirus is in concordance with the findings of Hyypiä et al. (3) and Rotbart et al. (7, 8).

TABLE 2. Sensitivity and specificity of the riboprobes used in this study

Riboprobe and specimen[a]	% Specimens with the indicated sensitivity and specificity[b]:					
	CSF (333)		Stool specimens (258)		Throat or nasal aspirates (67)	
	Sensitivity	Specificity	Sensitivity	Specificity	Sensitivity	Specificity
5′-Noncoding						
Do	2.5	99.3	33	90.7	18.1	100
Dx	87.5	99.5	75	98.8	81.4	100
VP1						
Do		99.5		83.6		91.9
Dx		99.5		88.6		97.6

[a] Do, Clinical specimens; Dx, lysates of cells infected with clinical specimens.
[b] Numbers in parentheses are the total numbers of specimens.

DISCUSSION

The high conservation of the 5′-noncoding sequence among enteroviruses and the specificity of this riboprobe for enteroviruses has been shown previously (3, 5) and was extended in this study. This riboprobe is a good tool for the detection of a wide range of viruses in cell cultures and in clinical specimens. The VP1 riboprobe is a more restrictive probe; it detected all three poliovirus serotypes but also hybridized with some coxsackieviruses type A and echovirus 7. The detection of viruses by molecular hybridization depends on the amount of viral genomes present in the sample, their accessibility for hybridization, and their sequence similarity with the probe (1).

Poliovirus types 1, 2, and 3 can be detected at $TCID_{50}$s of 10^2 to 10^3/ml ($TCID_{50}$s of 10 to 10^{-2} per spot) with radiolabeled VP1 or 5′-noncoding riboprobes (2). Other enteroviruses at the same titer were detected with the 5′-noncoding riboprobe with a lower sensitivity, depending on the sequence similarity with poliovirus type 1.

The use of poliovirus riboprobes in hybridization tests allows the specific detection of a wide spectrum of enteroviruses but does not differentiate between the various serotypes. When it is necessary to identify the viral serotype for epidemiological purposes, classical neutralization or immunological tests with monoclonal antibodies or hybridization with oligonucleotides of specific sequences must be used.

When various clinical specimens were examined for the presence of enteroviruses, the most confident results were obtained by analyzing stool specimens. In more than 30% of the specimens shown to be positive after culture, the viruses were detected in crude supernatants without cell culture. Stool specimens are certainly the reservoir of enteroviruses and provide good environmental conditions for their survival. In nasal aspirates and throat swabs, early detection of viruses by hybridization can be obtained. However, we cannot exclude the possibility that there was cross-hybridization with human rhinoviruses in such specimens; the 5′-noncoding sequence was conserved and similar between poliovirus type 1 and some human rhinoviruses. The detection of enteroviruses directly in CSF was the most difficult. This could have been due to the low titer of virus (10^1 to 10^2 $TCID_{50}$s/ml) (9), unfavorable environmental conditions for virus preservation (RNases, proteases, immunoglobulins, or factors accompanying the inflammatory reaction), or the presence of viruses which do not grow in cell cultures.

The rapid diagnosis of viruses in CSF is very important for patient care. Optimal handling and processing conditions of samples, such as transport, storage, and treatment of CSF specimens with proteinase K, enhances the value conferred by the sensitivity and the early application of the hybridization method. Further research and development of more rapid and sensitive techniques, such as amplification of the hybridization target by the polymerase chain reaction (10) after synthesis of a cDNA copy, can enhance the chance of detecting viruses at early stages of illness.

The hybridization test with riboprobes that was used to test for the presence of enteroviruses in clinical samples was relatively rapid and specific. When used for screening large numbers of samples, it reduces the need for laborious and expensive cell cultures.

The radiolabeled probes can be replaced by biotinylated riboprobes; however, they have a lower sensitivity (10 to 100 times), depending on the sequence similarities between poliovirus and other enteroviruses.

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