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Detection of Enteroviruses by Spot Hybridization

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A cloned partial cDNA copy of the coxsackievirus B3 genome was used for detecting enteroviruses in infected cells by employing a nucleic acid hybridization procedure. Cells infected with coxsackieviruses A and B, echovirus, and poliovirus gave positive hybridization signals, whereas cells infected with nonrelated viruses did not.

Most routine diagnostic tests for enterovirus infections are based on virus isolation or occasionally on increases in enterovirus antibody titers between acute and convalescent serum samples collected at 2-week intervals. These methods are time consuming and therefore are unable to allow a definite diagnosis during the acute phase of the illness.

Nucleic acid hybridization techniques have recently been applied successfully to rapid detection of several DNA (1–3, 8, 9) and RNA (5) viruses and viroids (6). In nucleic acid hybridization tests, virus-specific nucleic acids are bound to the solid phase and then detected by radioactively labeled DNA probes. The reaction is highly specific and sensitive, and the use of recombinant DNA technology makes it possible to produce probes in essentially unlimited quantities.

There are numerous related enterovirus serotypes, thus providing a particularly challenging problem in clinical virology. Serological methods are either laborious (neutralization tests) or inadequate (complement fixation tests). We have evaluated the possibility of using a cloned cDNA probe for the detection of enterovirus isolates in cell cultures and directly in clinical specimens. The probe used for hybridization consisted of a cDNA clone prepared with coxsackievirus B3 (strain Nancy) RNA as the template. The cDNA was inserted into the *Escherichia coli* pBR322 plasmid and was propagated in *E. coli* HB101. The cloning procedure and a detailed characterization of the cDNA clone will be described elsewhere (Stålhandske and Pettersson, manuscript in preparation). Sequencing studies of the cDNA show that the insert consists of ca. 4,300 base pairs, representing the 3′ end of the viral RNA (Stålhandske and Pettersson, unpublished data). The purified probe was first used for detection of enterovirus RNA sequences in infected cells. To evaluate the specificity of the probe, tube cultures of LLC-MK_2 cells were infected with either coxsackieviruses A9, B2, B3, and B4, echovirus 17, or poliovirus type 3. The study also included enterovirus isolates from the routine specimen collection maintained at the University of Turku, Turku, Finland. Typing of the isolates was based on neutralization tests with specific antisera (LBM pools; World Health Organization, Copenhagen). Herpes simplex virus type 1, adenovirus type 2, and measles virus were grown in Vero cells, and Nebraska calf diarrhea virus was propagated in LLC-MK_2 cells; these viruses were included in the study as controls for specificity.

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Infected cells were collected when cytopathic effects were observed in 50% of them, and they were stored at −70°C until assayed. When used in the test, ca. 10^6 cells were thawed and treated with proteinase K (E. Merck AG, Darmstadt, Federal Republic of Germany) at a concentration of 0.1 mg/ml in phosphate-buffered saline. After incubation for 1 h at 37°C, the specimens were diluted with a stock solution of 20× SSC (1× SSC = 0.15 M NaCl plus 0.015 M sodium citrate) to a final concentration of 6× SSC with or without previous phenol extraction. Each sample was then divided into two equal parts, which were spotted separately onto nitrocellulose filters (BA 85, Schleicher & Schuell Co., Dassel, Federal Republic of Germany) with a filter manifold. The nitrocellulose sheets were baked for 2 h at 80°C and prehybridized for 2 h at 42°C in 50% formamide–50 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) (pH 7.4)–3× SSC–1× Denhardt solution (4)–0.1% sodium dodecyl sulfate–250 μg of yeast RNA per ml–100 μg of herring sperm DNA per ml, denatured by boiling at an alkaline pH. The DNA probe was labeled with ^{32}P by using nick translation (7), denatured, and added to the hybridization solution. The filters were hybridized overnight at 42°C with 10^6 cpm of labeled probe per ml (specific activity, 10^7 cpm/μg). After hybridization the filters were washed three times in 2× SSC with 0.5% sodium dodecyl sulfate at 42°C, and the results were visualized by autoradiography overnight.

In the first part of the study, the results were compared after spotting of specimens with or without prior phenol extraction. The results (Fig. 1) indicate that phenol extraction did not significantly improve the sensitivity of the test. Cells infected with coxsackieviruses A9, B2, B3, and B4 and echovirus 17 gave positive hybridization of different intensities (Fig. 1). Cells infected with poliovirus type 3 were also positive but yielded a weaker hybridization signal. Cells infected with herpes simplex type 1, adenovirus type 2, Nebraska calf diarrhea virus, and measles virus did not yield positive hybridization signals even after longer exposures in autoradiography (Fig. 2).

To evaluate whether the hybridization assay could detect wild strains of enteroviruses, 10 coded stool specimens, including enterovirus-positive and enterovirus-negative samples, were inoculated in LLC-MK_2 cells and tested by hybridization. Seven of the eight specimens positive by enterovirus isolation were positive in the spot hybridization test, and the degree of positivity was dependent on the stage of infection at which the cells were collected (Fig. 3 and Table 1). All the specimens negative by virus isolation

TABLE 1. Comparison of virus isolation and spot hybridization in detection of enteroviruses

Patient no. and day of collection[b]	Virus isolation		Spot hybridization	
	Virus	Cytopathic effect (intensity)	Spot no.[c]	Intensity
1	Negative			
1–3		−	1	−
4–7		−	3	−
2	B4			
1–3		++	2	++
4–7		+++	10	+
3	B4			
1–3		−	4	−
4–7		++	13	+
4	Negative			
1–3		−	11	−
4–7		−	12	−
5	B4			
1–3		−	14	−
4–7		++	5	−
6	E17			
1–3		±	15	+
4–7		+++	16	++
7	B4			
1–3		+++	6	+++
4–7		+++	7	+
8	B2			
1–3		+++	17	+++
4–7		+++	20	+
9	B3			
1–3		++	8	++
4–7		+++	18	+
10	E30			
1–3		−	9	−
4–7		++	19	+

[a] LLC-MK_2 cells were inoculated with stool specimens, and the presence of enteroviruses was evaluated at two points by both of the tests.

[b] Cells were collected 1 to 3 and 4 to 7 days after infection.

[c] See Fig. 3.

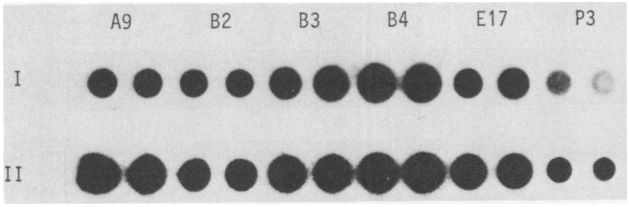

FIG. 1. Spot hybridization detection of enterovirus RNA from infected cells. Cells infected with coxsackieviruses A9, B2, B3, and B4, echovirus 17, or poliovirus type 3 were spotted in duplicate on nitrocellulose filters, and viral sequences were detected by nucleic acid hybridization with the coxsackievirus B3 probe. Positive hybridization was revealed by autoradiography. Each spot contains nucleic acids from 0.5×10^6 infected cells. Row I: Samples were spotted on a nitrocellulose filter immediately after proteinase K treatment. Row II: Samples were treated as in row I but were extracted with phenol before spotting on a nitrocellulose filter.

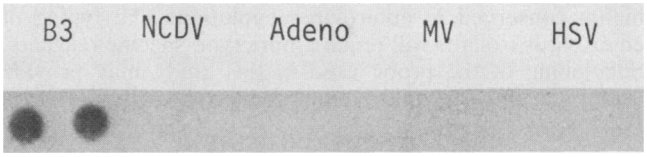

FIG. 2. Specificity of the hybridization reaction. Nucleic acids from cells infected with coxsackievirus B3, Nebraska calf diarrhea virus (NCDV), adenovirus type 2 (Adeno), measles virus (MV), or herpes simplex virus type 1 (HSV) were spotted on a nitrocellulose filter. The filter was hybridized with the coxsackievirus B3 probe. Nucleic acids from 0.5×10^6 infected cells were spotted in each case.

yielded negative signals in the hybridization assay (Fig. 3 and Table 1).

Eight enterovirus isolation-positive and eight enterovirus isolation-negative stool specimens were also directly tested for the presence of enterovirus RNA sequences. Only one of the isolation-positive samples gave a positive reaction by the hybridization method; none of the enterovirus-negative samples yielded a positive hybridization assay reaction.

Problems encountered in laboratory diagnosis of enterovirus infections are the time required for the performance of standard tests and, more critically, the lack of a group-specific reagent to detect the infectious agents. In this paper we describe a method which is capable of detecting the presence of enteroviruses in cell cultures used for routine virus isolation. The method is rapid, and results are available 24 h after beginning the hybridization test. The assay, as performed, appears to be specific for enteroviruses, and the sensitivity is sufficient for the detection of viral nucleic acid sequences from ca. 5,000 infected cells. The sensitivity is, however, not yet sufficient for the detection of viral RNA sequences directly in stool specimens. This may be due to the low amounts of virus in the samples or the presence of RNase activity in stool specimens, or both.

The results indicate that the probe used for hybridization contains sequences which are common to the enteroviruses. This is not a surprising finding since the probe contains the entire gene for the enterovirus replicase, which is likely to be

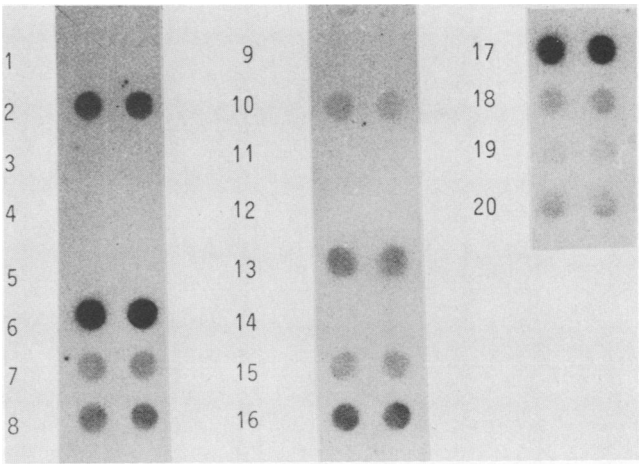

FIG. 3. Spot hybridization detection of enterovirus RNA in cells infected with coded stool specimens. The cells were collected at different stages of infection and spotted in duplicate on a nitrocellulose filter. The filter was hybridized with coxsackievirus B3 probe. Each specimen was assayed in parallel by virus isolation. The numbers of the spots correspond to the numbers in Table 1.

highly conserved in enterovirus evolution. The typing of enterovirus isolates will require more type-specific reagents. Subcloning of the probe used in this study may provide useful reagents for rapid typing of enterovirus isolates.

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