Nucleic Acid Detection Systems for Enteroviruses
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INTRODUCTION

The enteroviruses (EVs) are among the most common and most important viral pathogens of humans (22, 67). The paralytic potential of the polioviruses, prototypic EVs, was recognized as early as the 14th century B.C. in Egyptian art (61). Summer epidemics of poliomyelitis devastated the United States through the 1950s. Since the introduction of poliovirus vaccines in the late 1950s and early 1960s, much of the developed world is now essentially free of poliovirus infections. Developing countries, where vaccines are often either unavailable or ineffective, continue to struggle with these pathogens (6). It has been estimated by the World Health Organization that 4 of every 1,000 school-age children in developing countries contract paralytic disease due to poliovirus infections.

Control of poliovirus infections in much of the world has focused attention on the nonpolio EVs, which include the coxsackieviruses, echoviruses, and newer numbered EVs (Table 1). In the United States alone, the nonpolio EVs are estimated to cause 5 to 10 million symptomatic infections annually (95). In temperate climates, these infections occur during the summer and fall months (70, 95); young children are the most common victims, as both the incidence and severity of nonpolio EV infections vary inversely with the patient's age (70, 71, 95). In addition to the actual diseases that EVs cause, the EV ability to mimic other pathogens is of great concern. Distinguishing EV infections from those due to common bacteria and herpes simplex viruses is often difficult on clinical grounds alone (22). Hence, unnecessary treatment for herpes simplex virus infections is frequently instituted during EV infections.

The EVs have a special place in the history of modern virology. Polioviruses were the first viruses to be successfully propagated in cell culture—a monumental accomplishment which led directly to the development of poliovirus vaccines (36). J. F. Enders and his colleagues were awarded the Nobel Prize for this work, which, in addition to its contribution to vaccine development, has to this day remained the "gold standard" method for all viral diagnosis. The availability of this cell culture technique rapidly led to the identification of new viruses, including the coxsackieviruses and echoviruses (33). Once again, within the past decade, polioviruses have played a significant role in advancing the field of virology. The resolution of the atomic structure of poliovirus type 1 (44) and a similar achievement with rhinovirus 2 (78), a fellow member of the picornavirus family, have elucidated intricate details of virion surface structures, including host cell receptor-binding sites and potential antiviral drug-binding sites.

What follows is a brief discussion of the clinical significance of the nonpolio EVs and a more comprehensive review of the current state of the art in diagnostic methods available for these pathogens. The emphasis of the latter will be on nucleic acid-based detection systems, since that is the area of my personal research endeavors.

CLINICAL SIGNIFICANCE

Acute Infections

The EVs are responsible for a wide array of clinical diseases affecting all major body systems (Table 2). It is important to note that no disease is uniquely associated with any specific EV serotype and that no serotype is uniquely associated with any one disease (22, 67). This is true even of paralytic poliomyelitis, which has been associated with numerous nonpolio EV serotypes (40, 66, 104). For that
reason, it is sufficient to speak of diseases "that EVs cause" and to diagnose "an EV" in the laboratory without necessarily specifying or identifying the particular serotype. Certain clinical syndromes are indeed more likely to be caused by one or a few serotypes (see below), but significant overlap exists among the serotypes and the diseases they cause.

The most common clinical manifestation of EV infection is a nonspecific febrile illness, with or without a rash. This so-called viral syndrome is the most common cause of fever among children. When accompanied, as it often is, by upper respiratory symptoms, the "summer cold" is indistinguishable from the same illness caused by rhinoviruses (fellow picornaviruses) in the winter months. By far the most vexing clinical EV syndrome that the physician encounters is aseptic meningitis (106). The EVs are the most common cause of meningitis in the United States. In young infants with the disease, clinical criteria to distinguish EV meningitis from that due to bacteria and herpes simplex virus are unreliable. As a result, even though EV meningitis is generally benign in outcome and no specific therapy is indicated (or available; see below), thousands of children are hospitalized and treated with unnecessary antibiotics and antithrombin medications annually because of the fear that a case of meningitis is not due to an EV (24). Additional acute clinical EV syndromes of significance include encephalitis, poliomyelitis (particularly due to the polioviruses), myocarditis (particularly due to the coxsackievirus B group) (56), hemorrhagic conjunctivitis (particularly due to serotypes coxsackievirus A24 and EV70) (18, 68), hand-foot-mouth syndrome, Bornholm disease (pleurodynia), and overwhelming neonatal sepsis (particularly due to the echoviruses and coxsackieviruses B) (55, 69). The last syndrome is thought to be due to perinatal transmission, either transplacentally or during birth, from mother to infant. Despite the name, enteric disease is not a prominent manifestation of EV infection, although diarrhea and vomiting may be significant manifestations of certain outbreaks of "summer flu" due to the EVs. EV72 (hepatitis A) is unique in that it is the only EV serotype which almost exclusively causes hepatitis; more-common EV syndromes have not been associated with EV72. For purposes of the ensuing discussion, EV72 will not be considered with the other EVs, since substantial data suggest that, genetically, EV72 is only minimally related to the other EV serotypes (25); reclassification of hepatitis A as a non-EV picornavirus would probably be appropriate. Mild hepatitis, almost always in association with other more significant findings, is common during infection with many EV serotypes.

### Chronic Diseases

In addition to being the causes of well-recognized acute diseases, EVs have been implicated in several chronic illnesses including juvenile onset diabetes mellitus (8, 111), chronic fatigue syndrome (5), dermatomyositis and polymyositis (13, 77), congenital hydrocephalus (38), and amyotrophic lateral sclerosis (37). Evidence for these associations has been largely from serology or from nucleic acid hybridization studies (see below); definitive proof is lacking, and confirmatory studies remain to be done.

Persistent EV infections occur in agammaglobulinemic patients; manifestations almost always include meningoencephalitis (64). Half of all patients with persistent EV meningoencephalitis have concomitant dermatomyositis or polymyositis. These observations confirm the important role of antibody in EV clearance, an unusual phenomenon, because most other viruses are contained largely by cell-mediated immunity. Recently, a syndrome of late-onset muscular atrophy and pain has been reported in individuals who suffered paralytic poliomyelitis 20 to 40 years previously (32, 52); evidence for persistent or latent infection in these individuals has not been found.

### DIAGNOSIS

#### Need for a Rapid Diagnostic Test

There are numerous compelling reasons for seeking a rapid diagnostic assay for the EVs. The diverse spectra of clinical manifestations frequently present the physician with a confusing and lengthy differential diagnosis. The infant with high fever, rash, and/or meningitis cannot be assumed to have EV infection; empiric therapeutic maneuvers for multiple potential pathogens are almost always undertaken. This approach results in significant personal and financial hardships on the patient and family (24). The annual cost to

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**TABLE 1. EV serotypes**

<table>
<thead>
<tr>
<th>Subgroup</th>
<th>Serotypes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Poliovirus</td>
<td>1-3</td>
</tr>
<tr>
<td>Coxsackieviruses A</td>
<td>1-22, 24*</td>
</tr>
<tr>
<td>Coxsackieviruses B</td>
<td>1-16</td>
</tr>
<tr>
<td>Echovirus</td>
<td>1-9, 11-27, 29-31*</td>
</tr>
<tr>
<td>Numbered EVs</td>
<td>68-72</td>
</tr>
</tbody>
</table>

* Coxackievirus A23 and echoviruses 10 and 28 have been reclassified.

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**TABLE 2. Clinical syndromes caused by EVs**

<table>
<thead>
<tr>
<th>Organ system</th>
<th>Disease</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neurologic</td>
<td>Aseptic meningitis*</td>
</tr>
<tr>
<td></td>
<td>Encephalitis*</td>
</tr>
<tr>
<td></td>
<td>Poliomyelitis*</td>
</tr>
<tr>
<td></td>
<td>Transverse myelitis</td>
</tr>
<tr>
<td></td>
<td>Guillain-Barré syndrome</td>
</tr>
<tr>
<td></td>
<td>Cerebellar ataxia</td>
</tr>
<tr>
<td>Respiratory</td>
<td>Common cold*</td>
</tr>
<tr>
<td></td>
<td>Stomatitis, herpangina, hand-foot-mouth syndrome*</td>
</tr>
<tr>
<td></td>
<td>Pharyngitis, tonsillitis, rhinitis*</td>
</tr>
<tr>
<td></td>
<td>Pleurodynia (Bornholm disease)</td>
</tr>
<tr>
<td></td>
<td>Croup, bronchitis, bronchiolitis</td>
</tr>
<tr>
<td></td>
<td>Pneumonia</td>
</tr>
<tr>
<td></td>
<td>Parotitis</td>
</tr>
<tr>
<td>Gastrointestinal</td>
<td>Gastroenteritis*</td>
</tr>
<tr>
<td></td>
<td>Hepatitis*</td>
</tr>
<tr>
<td></td>
<td>Mesenteric adenitis</td>
</tr>
<tr>
<td></td>
<td>Peritonitis</td>
</tr>
<tr>
<td></td>
<td>Pancreatitis (&quot;diabetes mellitus&quot;)</td>
</tr>
<tr>
<td>Cardiovascular</td>
<td>Myocarditis*</td>
</tr>
<tr>
<td></td>
<td>Pericarditis</td>
</tr>
<tr>
<td></td>
<td>Endocarditislike valve lesions</td>
</tr>
<tr>
<td></td>
<td>?Myocardial infarction</td>
</tr>
<tr>
<td>Miscellaneous</td>
<td>Febrile, exanthematous illness*</td>
</tr>
<tr>
<td></td>
<td>Neonatal sepsis*</td>
</tr>
</tbody>
</table>

* Most common and/or most important manifestations.
# Due to serotypes other than EV72 (hepatitis A).
society for tens of thousands of unnecessary hospital days and treatments is substantial. Furthermore, although most EV illness is benign, certain diseases are severe enough to warrant specific antiviral therapy were such available. Promising anti-EV compounds have been developed (53, 63, 65, 74, 108) but remain untested in humans, at least in part because a diagnostic test to identify infected patients rapidly is not now available. Finally, as noted above, numerous diseases have been circumstantially linked to the EVs without definitive proof. A clear association for the EVs with these and, potentially, other illnesses of unknown etiology would facilitate our understanding and treatment of such conditions.

Implied in this discussion has been the inadequacy of current diagnostic modalities. The following section reviews that issue.

Current Diagnostic “Gold Standards”

Since its inception more than 40 years ago, cell culture continues to be the mainstay of EV diagnosis, despite well-recognized limitations. The technique is labor intensive and requires a high level of expertise. As many as 25 to 35% of EV serotypes, particularly the coxsackievirus A group, do not grow at all in cell culture (24, 58, 105). The latter can be identified by sucking mouse inoculation, a technique too cumbersome to be widely available. The serotypes that do grow in cell culture may do so slowly. Reported mean isolation times for EVs from cerebrospinal fluid (CSF) range from 3.7 to 8.2 days (24, 50). Cultures from other sites may become positive sooner, but meningitis and encephalitis cause the greatest clinical consternation because their presentations are often indistinguishable from those of bacterial and herpes simplex virus central nervous system infections. The use of multiple cell lines may improve the yield or the rapidity with which an EV is isolated (23, 31) at the cost of increasing the labor and resources required.

Serologic testing has had only a limited role in EV diagnosis. The best-studied clinical application has been with coxsackievirus B immunoglobulin M assays, which take advantage of the shared antigen among the six coxsackievirus B serotypes and the early appearance of the immunoglobulin M class of antibodies (9, 34, 62). Except for the three poliovirus serotypes, other EVs do not appear to share a single antigen which can be exploited for serologic studies. If the specific serotype of an infecting EV is known or suspected, e.g., in community-wide outbreaks, confirmatory immunoglobulin G serology can be performed to document a rise in antibody titer from the acute to the convalescent phase of infection, thus providing information useful for epidemiologic purposes but of little benefit to the individual patient. In the usual scenario, when a patient presents with meningitis or other acute manifestations of illness and an EV is suspected, serologic testing for non-coxsackievirus B, nonpoliovirus serotypes is not an option; in fact, not even the coxsackievirus B immunoglobulin M method is widely available outside of the research laboratory setting.

Antigen Detection

The absence of a widely shared antigen has hampered the development of immunoassays for the EVs much as it has restricted the use of serologic assays (42, 109, 110). The greatest success has been with assays limited to a particular subgroup of EV serotypes, e.g., the coxsackievirus B serotypes, which share a common antigen (109). Recent reports of shared VP3-2C antigens among many EV serotypes (76) and of a monoclonal antibody that cross-reacts with the VP1 capsid protein of multiple serotypes (113) are promising, but further testing is required to determine the clinical relevance of those observations.

Nucleic Acid Hybridization

The earliest efforts at RNA-RNA hybridization for the EVs were designed to explore the genetic similarity among seemingly distantly related serotypes (112). Although the predicted homologies from those studies were somewhat inaccurate because of limitations in the available molecular technology, the conclusion that EVs share sequences across a broad range of serotypes has been borne out. Biological clues certainly supported that hypothesis: EVs share their seasonality (70, 95), overlap in their clinical manifestations (22, 67), and are virtually identical by physicochemical criteria (91), a fact responsible for their classification as EVs. The EVs also have in common their fecal-oral route of transmission (from which the name enteroviruses evolved) and their appearance by electron microscopy. Early and very limited sequence comparisons confirmed genomic regions of great similarity among certain serotypes (43), as did heteroduplex mapping techniques using electron microscopy (30).

The subsequent evolution of nucleic acid-based detection systems for the EVs is a testimony to the ongoing emergence of ever more advanced molecular technology and the availability of this technology to the clinical microbiologist. The landmark technology that has facilitated the development of today’s promising EV detection systems includes molecular cloning, transcription vectors, automated DNA synthesizers, and the polymerase chain reaction (PCR). CDNA probes. Early hybridization studies using viral RNA labeled during replication within cells (112) or cDNA labeled directly during reverse transcription (102) identified homologies among a number of the EVs. With the advent of molecular cloning techniques, segments of several EVs were successfully cloned into plasmid vectors, permitting the development of well-defined cDNA probes for the further study of genomic similarities among the serotypes. The first such studies, utilizing cloned and well-characterized regions of coxsackievirus B3 and poliovirus type 1 (48, 89), were reported in 1984. These investigations, and similar ones over the ensuing 5 years (16, 21, 35, 47, 54, 73, 90, 101, 114) (Table 3), provided valuable data regarding conserved sequences well in advance of the actual sequencing of specific EV serotypes. Furthermore, this approach identified genomic regions that were broadly shared across most, if not all, serotypes and could therefore become potential generic or pan-EV probes for clinical diagnosis. Probes derived from the 5′ noncoding region of the EV genome and others which included the proteinase- and polymerase-coding regions in the 3′ half of the genome appeared to be the most broadly cross-reactive (Table 3).

Although promising in cross-hybridization studies with target EVs grown in cell culture, the application of these cDNA probes to the direct testing of clinical specimens proved to be problematic. Initially, CSF reconstruction experiments were performed in which various biologic variables that occur in clinical specimens were introduced, along with virus, into normal CSF to determine the impact of those variables on viral detection by hybridization (90). The sensitivity of this assay, under conditions optimized for CSF, was between 10² and 10⁵ 50% tissue
<table>
<thead>
<tr>
<th>SSCP probe primer sequences and probe nucleic acids and nucleotides</th>
<th>CB probe most cross-reaction</th>
<th>Other probes and sequence-specific CB probe</th>
<th>Appropriate hybridization temperature</th>
<th>Target frequency of probes and probes not closed</th>
<th>Target specificity</th>
<th>Target sequence(s)</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5'-TAG CTG CTC ACT GCC ATT CAC CTA GAC ACC GGT TTA CAA-3'</td>
<td>CB probe most cross-reaction</td>
<td>Other probes and sequence-specific CB probe</td>
<td>Appropriate hybridization temperature</td>
<td>Target frequency of probes and probes not closed</td>
<td>Target specificity</td>
<td>Target sequence(s)</td>
<td>Reference(s)</td>
</tr>
</tbody>
</table>

**TABLE 3** Nucleic acid hybridization studies demonstrating sequence relationship among EVs.
TABLE 4. Nucleic acid-based detection of EVs directly* from clinical samples

<table>
<thead>
<tr>
<th>Investigator(s) (reference)</th>
<th>Yr</th>
<th>Assay(s)</th>
<th>Probesa</th>
<th>Specimen(s)</th>
<th>Sensitivity vs culture (%)</th>
<th>Specificity vs culture (%)</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hyytiä et al. (48)</td>
<td>1984</td>
<td>Dot blot</td>
<td>CB3 cDNA</td>
<td>Stool</td>
<td>12.5</td>
<td>100</td>
<td>Sensitivity was 45% for PVs and 0% for nonpolio EVs 2 false-positive hybrids may actually have been false-negative cultures</td>
</tr>
<tr>
<td>Cova et al. (28)</td>
<td>1988</td>
<td>Dot blot</td>
<td>PV1 RNA</td>
<td>Stool</td>
<td>26</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>Rotbart (79)</td>
<td>1989</td>
<td>Dot blot</td>
<td>PV1 and CB3 cDNA</td>
<td>CSF</td>
<td>33</td>
<td>85 93</td>
<td></td>
</tr>
<tr>
<td>Petitjean et al. (75)</td>
<td>1990</td>
<td>Dot blot</td>
<td>PV1 RNA</td>
<td>Stool</td>
<td>33</td>
<td>91</td>
<td>Some false-positive hybrids may actually have been false-negative cultures</td>
</tr>
<tr>
<td></td>
<td>1990</td>
<td>PCR and DOT blot</td>
<td>Oligomer</td>
<td>CSF</td>
<td>100</td>
<td>NA' 100</td>
<td>Patient with persistent infection despite negative cultures</td>
</tr>
<tr>
<td>Rotbart (81)</td>
<td>1990</td>
<td>PCR and DOT blot</td>
<td>Oligomer</td>
<td>CSF</td>
<td>100</td>
<td>64 100</td>
<td>All false-positive PCR hybrids were likely actually false-negative cultures</td>
</tr>
</tbody>
</table>

* Hybridization performed directly on sample without prior passage in cell culture.

| Abbreviations: CB, coxsackievirus B; PV, poliovirus.
| NA', Not applicable. |

culture infective doses of added virus. The highest sensitivity was with serotypes very homologous to the probes; the lowest sensitivity was with more distantly related serotypes. The first trial of cDNA probes directly on clinical CSF specimens (Table 4) (79), however, resulted in only 33% sensitivity and 85% specificity compared with cell culture; 2 of 10 samples from patients with clinical aseptic meningitis (likely due to EV infection) that did not grow a virus in cell culture were positive by the hybridization assay. Thus, it appeared that overall the new technique was less sensitive than culture but for occasional individual specimens might be more sensitive. Very limited data are available regarding the titer of EVs in infected CSF, however; the range is thought to be between 10^3 and 10^5 infectious virions (107). Those clinical specimens with higher titers of EVs, particularly serotypes closely related to the probe sequences, are likely those that were successfully detected with the cDNA probes. Serotypes that do not grow in cell culture (e.g., certain coxsackieviruses A), if present at a high enough titer and if genetically homologous enough to the probe, probably constitute those occasional samples from patients with clinical aseptic meningitis that were culture negative and hybridization positive. Although this initial trial was somewhat encouraging, the need for improved sensitivity is paramount in advancing the clinical utility of such an assay. Others, using EV cDNA probes directly on clinical stool specimens, have found similar inadequate sensitivity when the method is compared with cell culture (Table 4) (48).

We identified three possible strategies to enhance the sensitivity of nucleic acid hybridization detection of the EVs: development of more-sensitive probes, improvement of target retention before and during the hybridization procedure, and in vitro amplification of target sequences before hybridization. Each of these approaches, but particularly the last, has contributed to the progress toward a clinically useful assay.

RNA probes. The commercial availability in 1984 of transcription vectors (39) made it possible to prepare RNA probes from the cDNA sequences described above. There are several theoretical advantages of single-stranded RNA probes over cDNA probes: RNA-RNA hybrids have greater affinity than do DNA-RNA hybrids, there are no vector sequences present to cause nonspecific hybridization, and there is no self-annealing among probe strands. A further advantage is the ability to separately produce labeled RNA transcripts of both senses. Cloned cDNA fragments of poliovirus 1, coxsackievirus B3, and echovirus 9 were excised from their original vectors, by restriction enzyme digestion and recloned into transcription vectors (83). Negative-sense, radioactive RNA transcripts were generated by linearizing the new recombinants with a restriction enzyme chosen to result in a positive-strand DNA template and then reacting the DNA with promoter-specific polymerases in the presence of ^3H- or ^32P-labeled UTP. We learned that the resultant RNA probe in the crude transcription mix performed as well as cleaner (DNase-treated and/or phenolchloroform-extracted) preparations. Hybridization optimization experiments also revealed the ideal temperature and duration of hybridization and defined the role of formamide concentrations in the ultimate signal/noise ratio.

These RNA probes were then tested in three types of experiments (83). First, in a comparative trial against its cDNA progenitor, the RNA probe derived from poliovirus 1 was shown to be 10- to 100-fold more sensitive in detecting serial dilutions of poliovirus 1 and coxsackievirus A9. Next, the three RNA probes were individually tested against 23 different serotypes of animal and human picornaviruses. Each probe demonstrated significant hybridization with many of the target viruses, but no probe detected all serotypes. Finally, the three probes were used in combination in a hybridization reaction to 16 human EVs. All 16 were detected (Table 3). These 16 EVs included two serotypes that do not grow in tissue culture (coxsackieviruses A4 and A17), several that grow only sluggishly, and three not previously detectable with nick-translated cDNA probes (echovirus 22 and coxsackieviruses A4 and A17). A similar approach taken by others also resulted in probes more sensitive than comparable cDNA sequences (28). An RNA probe comprising 450 nucleotides of the 5' noncoding region of poliovirus detected all 12 EV serotypes tested (Table 3).
(28), whereas an RNA probe derived from the coding region for the VP1 protein of poliovirus was poliovirus specific (28).

To determine the analytic sensitivity of this assay, we prepared and quantitated positive-sense RNA that was absolutely complementary to the coxsackievirus B3-derived negative-sense RNA probe by using the same transcription vector construct but oppositely oriented primer and polymerase. This positive-sense RNA was used as target to test the probe. Under optimized solid-phase hybridization conditions, 10 pg of target RNA was shown to be the lower limit of sensitivity (88). The clinical sensitivity of hybridization with RNA probes, while slightly improved over the results with cDNA reagents, is still too low to be of practical use (Table 4) (28, 75). In one study, sensitivity (versus culture) of an RNA probe derived from poliovirus was 2.5% for CSF specimens, 18.1% for throat or nasal aspirates, and 33% for stool specimens (75).

Improvement of target retention prior to and during hybridization. To determine the extent of loss of target EV RNA during the course of our solid-phase hybridization assay, we separately prepared 3H-labeled EV virions and 3H-labeled EV RNA. Added separately to CSF, target molecules could be monitored for retention through the hybridization procedure by scintigraphy (88). Free RNA added to CSF was sufficiently degraded by RNases within 15 s of contact with the body fluid to result in loss of >90% of counts during filtration through all commercially available membranes tested. Much of the remaining RNA was lost during subsequent hybridization incubations and washes. RNase inhibitors were protective, but only if added to the CSF prior to addition of the RNA. In contrast, radiolabeled virions survived exposure to CSF and the hybridization and wash procedures, with >65% retention of counts on membrane filters. We determined that formaldehyde added to the CSF-virion specimen protects the RNA during the subsequent heating step in which viral capsids are presumably opened (allowing access of probes to target RNA during hybridization).

The ability of free RNA in CSF makes the preservation of intact virions of utmost importance. Addition of formaldehyde or another RNase inhibitor immediately after collection of the specimen from the patient protects any RNA that becomes exposed by viral capsid opening during transport to the laboratory as well as during the subsequent hybridization protocol. Unfortunately, free EV RNA or partially exposed RNA (e.g., defective particles) in the specimen collected from the patient cannot be expected to remain viable for hybridization, regardless of technique. The preservation of intact RNA within virions is also of paramount importance for the enzymatic amplification approach discussed below.

Amplification of target sequences prior to hybridization. Despite optimization of hybridization methods, development of more-sensitive probes, and attempts at reducing target loss during hybridization, clinical applicability was still restricted by the low titer of EVs in certain specimens. In this laboratory, we have employed two strategies for increasing the number of target RNA molecules available for hybridization with EV probes: biologic and enzymatic. In the biologic amplification approach, 10² 50% tissue culture infective doses of EVs (below the level of sensitivity of direct hybridization) were inoculated onto LLC-MK2 tissue culture cells and allowed to incubate for various lengths of time (87). Medium was decanted, and membrane filters were applied directly to the infected monolayer. The entire cell sheet was peeled off the culture dish onto the membrane, which was then fixed and hybridized with cDNA probes.

Although cytopathic effect was not noted until after 60 h of incubation in cell culture, the same inoculum of virus was detected with this "monolayer blot" technique in 36 h by scintillography or in 42 h by autoradiography (87). Further enhancement of these methods may permit even briefer incubations in tissue culture before hybridization. We had hoped that EV serotypes that do not produce cytopathic effect in tissue culture might still replicate their RNA, allowing detection with monolayer blot hybridization. Our attempts with several such coxsackievirus A serotypes, however, were unsuccessful, resulting in no detectable hybridization signals (unpublished data).

The enzymatic amplification of single genes or short sequences of DNA by repeated cycling of polymerase chain elongation reactions has recently been reported (72, 94). Critical to the success of this technique is the ability to specifically bind (hybridize) short oligomeric sequences to regions of the target genome; these oligomers serve as primers for the elongation of complementary strands and as probes for detection of the amplified product. Our first efforts in this area, therefore, were directed at demonstrating oligomeric hybridization to a variety of EV serotypes. With the availability of rapid synthetic methods for producing and cloning DNA oligomers (50, 51), we designed EV oligomeric probes to test their spectrum of reactivity and, in the hope that a nonisotopic system might be developed, to facilitate the ultimate adaptation to the diagnostic laboratory (85). Two 22-mer sequences were chosen on the basis of sequence information then available for the few EV serotypes that had been fully sequenced. These oligomers were labeled either with 32P in a standard kinase end-labeling reaction or with alkaline phosphatase added during the synthetic process by means of a carbon chain linker arm chemically attached to one of the nucleotides. Cross-reactivity of the probes with multiple EV serotypes was observed (Table 3), confirming that short-sequence probes with broad diagnostic potential can be chosen and that a colorimetric assay using this technology is feasible (85).

Sensitivity of the nonisotopic probes, however, was 10-fold lower than with the same oligomeric sequence labeled with 32P and 100- to 1,000-fold lower than with RNA probes. Much as cDNA (and therefore RNA) probes can be chosen to be either broadly cross-reactive or serotype specific, serotype-specific oligomers with potential for use in a checkerboard-type analysis to determine the serotype of a particular isolate can also be designed (Table 3) (4). It is unlikely, however, that the need to know the particular serotype of an infecting EV will arise often enough in clinical practice to require routine use of large panels of serotype-specific reagents. Thus, while the sensitivity of oligomeric EV sequences used directly as probes is low, their potential as primers for enzymatic amplification is great because of the broad range of EV serotypes that do bind the oligomeric molecules.

By using a computer-assisted analysis of the genomic RNA sequences of the six EV serotypes that had been fully sequenced at the time (poliovirus types 1 to 3 and coxsackieviruses B1, B3, and B4) (49, 51, 57, 99), we identified three 20- to 25-base regions of absolute (100%) sequence conservation that define a 154-base segment within the 5' noncoding region of the viral genome (80, 82). The 5' end was chosen because of its demonstrated conservation among many serotypes in the cDNA and RNA probe studies noted above (Table 3). The two flanking sequences were chosen as primers for PCR, and the middle sequence, which did not overlap with either of the primers, was used as a probe.
Since the selection of those oligomers, three additional EVs have been fully sequenced (coxackieviruses A9 and A21 and EV70) (19, 45, 98); all three are also 100% homologous to the primers and probe initially chosen. These three oligomeric strands were synthesized as single-stranded DNA by using an automated synthesizer. The downstream primer and the probe were synthesized in the antisense orientation to genomic viral RNA, and the upstream primer was synthesized in the sense orientation to genomic RNA. Eleven EV serotypes were separately added to saline or CSF, and EV RNA was extracted and precipitated. A first strand of cDNA was generated by reverse transcription with the downstream primer. PCR was then performed using both upstream and downstream primers, with 25 repeated cycles of denaturation, annealing, and extension. Successful RNA amplification with the described primers was achieved for all 11 EV serotypes tested. Bands 154 bases long, a distance that spanned both primers and the intervening sequence, were seen after the first 25 PCR cycles for all EV serotypes except coxackieviruses 2 and 22, both of which were detectable following an additional 25 cycles. Hybridization with the 32P-labeled oligomeric probe confirmed the gel results. In contrast, the same procedure with the same primers and probe applied to herpes simplex virus, varicella-zoster virus, and respiratory syncytial virus resulted neither in 154-base bands by electrophoresis nor in positive hybridization (80, 82). Two laboratories have recently reported similar success using different primer and probe combinations, also within the 5' noncoding region of the EV genome (20, 46). In one of those reports, a single pair of primers amplified the RNA of eight of nine EV serotypes tested and six of six rhinovirus serotypes tested; discrimination between EVs and rhinoviruses was made in the subsequent hybridization step by using genus-specific probes, although some cross-reactivity of the EV probe was seen with rhinovirus serotypes (46). In the second study, 40 of 41 EV serotypes were amplified by using a single primer pair (46). Echovirus 22 is the only EV serotype thus far tested by PCR analysis that has consistently been difficult to amplify with primers that amplify many other EV serotypes (20, 46, 82); this serotype is known to be atypical of the EVs in many respects and, like EV72 (hepatitis A), may require reclassification (26).

The first two attempts at clinical application of this method have been extremely promising (Table 4). EV RNA has been shown to be persistently present in the CSF of a child with agammaglobulinemia, even during periods of virus culture negativity (86). The patient, like others with agammaglobulinemia and EV meningoencephalitis (64), had waxing and waning symptoms of infection despite periods of virus culture negativity. Negative cultures have been presumed to be due to virus neutralization by aggressive gamma globulin replacement therapy; however, this PCR application was the first definitive proof that virus does persist throughout both culture-positive and culture-negative intervals. In a second clinical application, and one most germane to the practicing physician, CSF specimens collected from children undergoing lumbar puncture for suspected meningitis were studied prospectively for EV infection by PCR (81). Of 12 patients with the clinical diagnosis of aseptic meningitis, all were positive for EV RNA by PCR; one child with the clinical diagnosis of viral encephalitis was also positive. Four children with bacterial meningitis and three with miscellaneous noninfectious illnesses were all negative for EVs by PCR. That study found PCR to be more sensitive than and equally specific to concurrently performed cell cultures for the diagnosis of EV meningitis. Studies such as this will neces-

sitate calculation of true sensitivity and specificity on the basis of a new "gold standard" that must include supplemental data in addition to viral culture results (Table 4). In the absence of practical and reliable serologic tests or immunoassays, clinical stigmata of EV infections may help to corroborate PCR findings (81).

**PATHOGENESIS**

Much of what is assumed to be the pathogenesis of EV infections has been derived from experiments done more than 40 years ago involving poliovirus inoculations into nonhuman primates (10, 11). The methodology at the time included careful histologic observations and cell culture. Similar approaches as well as immunoassay techniques have more recently been applied to the study of specific nonpolio EVs (56, 60). Nucleic acid probes used for in situ hybridization offer advantageous usages over immunoassay and cell culture for pathogenesis studies while preserving the tissues for concomitant histologic evaluation. In situ hybridization allows detection of latent viral nucleic acid (where no replication is occurring) and can localize viral genomes within cells (e.g., to nuclear versus cytoplasmic compartments). Furthermore, in the case of the EVs, probes broadly reactive across many serotypes can detect viral infection in patients in whom the infecting serotype is unknown (in much the same way that such detection can occur in body fluids, as noted above, by solid-phase hybridization).

In Vitro Studies

The potential use of nucleic acid probes for EV in situ hybridization has been studied in a coxackievirus B3 cell culture infection (84). Cytospin preparations of infected and uninfected LLC-MK2 monkey kidney cells were hybridized, using an in situ technique, with coxackievirus B3-derived RNA probes for both sense and antisense target RNA. During productive infection, both RNA moieties were detected, in an approximate ratio of 40 sense EV RNA molecules to 1 antisense molecule, an observation consistent with quantitation by nonhybridization methods (7). Uninfected cells demonstrated no hybridization with either orientation of probe. The ability to detect viral RNA of both sense and antisense configurations may in itself have important applications to the study of pathogenesis. There is evidence, for example, that the ability to generate the antisense molecule may influence the establishment of persistent infection by the EVs (see below) (17).

Animal Models Using Animal Picornaviruses

Nucleic acid-based studies of EV pathogenesis have been performed in animal model systems as well as on human tissue specimens. The earliest applications involved a model of demyelinating central nervous system infection caused by Theiler's murine encephalomyelitis virus (TMEV), a naturally occurring murine picornavirus (59). This same pathogen constituted an important early model of human paralytic poliomyelitis, as the early phase of murine infection with TMEV results in an acute polioencephalomyelitis. It has since been observed that mice that survive the early disease develop a later-onset demyelinating encephalopathy that resembles human multiple sclerosis (59). In situ hybridization experiments with cDNA probes in this model system during the early 1980s represent some of the earliest ad-
vances in the development of nucleic acid-based pathogenesis research (14, 15, 96, 97). During the acute polioencephalomyelitis phase of infection, neurons distributed widely in the brain were shown to be infected; white matter was relatively spared during this early phase (96). The clinical symptoms were demonstrated to be secondary to neuronal lysis by virus rather than to hypoxia. Specific regions of the central nervous system were most affected, including the pyramidal layer of the hippocampus and the anterior horn cells of the spinal cord. Other areas, such as the molecular layer and dentate gyrus of the hippocampus and the entire cerebellum, were unaffected. In contrast, when surviving animals were studied during the late demyelinating phase of their infection, viral RNA was consistently detected in glial cells of the white matter (15, 97). The investigators propose that the pathogenesis of the late demyelinating phase of this infection involves persistent white-matter infection, the host’s inflammatory response, and the ability of circulating antibodies to limit the further spread of virus. Most recently, RNA probes for antisense viral RNA have demonstrated that a block at the level of synthesis of the viral antisense RNA (a critical step in replication of EVs) may explain the ability of TMEV to persist throughout the late (demyelinating) phases of disease (17). Endothelial cells have also been shown by in situ hybridization to be targets of TMEV central nervous system infection, implying a possible additional route for EVs to cross the blood brain barrier (115).

Recently, in situ hybridization of TMEV has been used in a murine model of gestational EV infection (2). As noted above, human infection with EVs during pregnancy has been associated with both teratogenesis and a syndrome of overwhelming neonatal sepsis, the former likely due to infection early in gestation and the latter due to infection near term or during delivery. Pregnant mice were experimentally infected with TMEV in early, middle, and late gestation and sacrificed before delivery. Fetuses and placentas were cultured for TMEV and studied by in situ hybridization using a TMEV-derived single-stranded RNA probe. Late-gestation infections uniformly resulted in infection of the murine placentas, but fetal infection never occurred (2). In situ hybridization revealed a functional barrier to infection at the level of the spongiosphoblast and labyrinth interface. The labyrinthine layer, that region of the placenta that lies closest to the fetus and in which maternal-fetal vascular exchange occurs, is entirely spared of TMEV RNA in late-gestation infection. In contrast, early-gestation infection results in both placental and fetal infection (3). In situ hybridization of early-gestation infection demonstrates dramatic virus infection of the labyrinthine placenta and often massive fetal infection. Tissue tropisms within the fetus could readily be determined and included heart, brain, liver, and lung, analogous to the target tissues most affected in human gestational EV infections. Serially timed sacrifices following early-gestation TMEV infection have revealed that giant cells near the lacunae containing maternal blood and surrounding the yolk cavity and embryo are the principal target cells of infection (1). These giant cells are known to be phagocytic, and the hypothesis raised by this study is that the functional maturity of these cells determines whether the placenta permits (early-gestation) or prevents (late-gestation) TMEV infection of the fetus. Culture and in situ hybridization of middle-gestation TMEV infection reveal an intermediate pattern of fetal infection and placental distribution of viral RNA. A similar nucleic acid-based approach to pathogenesis is currently being applied in the same model system to study the effect of various exogenous factors on the integrity of the placental-fetal barrier (unpublished data).

Using another murine picornavirus, encephalomyocarditis virus (EMCV), investigators have developed a model of chronic inflammatory myopathy (29). EMCV induces an acute infection in mice followed by resolution over several weeks. Virus is culturable from muscle during the period of histologically documented inflammation; as the inflammation resolves, EMCV can no longer be recovered. In situ hybridization localized the virus to the myocytes themselves, commonly in areas of inflammation. Occasionally EMCV RNA was detected where there was no inflammation, and, conversely, areas of inflammation existed where no viral RNA could be shown. EMCV could be detected by in situ hybridization beyond the period of cultivability of virus and beyond the time when extracted muscle tissue could be shown by dot blot hybridization to contain viral RNA. Similar viral persistence was observed in heart and brain, tissues known to be target organs for EMCV. Again, in situ hybridization demonstrated viral RNA beyond the period of viral cultivability.

Animal Models Using Human EV Serotypes

Several in situ hybridization model systems using human EVs in animals have been reported. In the first, a cDNA probe derived from coxsackievirus B3 was used to study myocardial coxsackievirus infection in athymic mice (54). Virus was found to be distributed randomly and multifocally in the hearts of infected animals, with evidence for cell-to-cell spread seen in the regions of histologic lesions; infection was transmural.

Coxsackievirus B3-induced myocarditis in mice was also studied by another group of investigators using in situ as well as dot blot hybridization methods (114). By the former technique, viral RNA was detected with a biotin-labeled cDNA probe in muscle fibers but not in the lymphocytes participating in the inflammatory reaction. When coxsackievirus B1 was used, murine skeletal-muscle myositis was induced and was also studied by the same two hybridization techniques (114). Once again, hybridization signal was confined to myocytes. In situ hybridization results were confirmed by a dot blot assay on extracted RNA using a hybridization index method described below.

Coxsackievirus B3 has also been used to induce and study pancreatitis in a murine model (103). In situ hybridization using biotin or 32P-labeled cDNA probes derived from coxsackievirus B3 revealed viral RNA within necrotic acinar cells of the exocrine pancreas; cells within the islets of Langerhans were never involved in the histologic inflammation or shown to be infected by in situ hybridization. The initial inflammation and necrosis of the pancreas was followed by brief recovery and subsequent recrudescence inflammation. This second phase of pancreatitis was not associated with recovery of virus by cell culture or with detectable RNA by in situ hybridization. Myocarditis also developed, according to histologic criteria, and coxsackievirus B3 could be recovered from heart tissue, but in situ hybridization was not sensitive enough to detect viral RNA in those tissues (103).

Finally, in a primate animal model using a human EV, monkeys were intraspinally inoculated with virulent or attenuated strains of poliovirus type 1 (27). Virulent strains produced more-intense and more widely distributed inflammatory lesions in the central nervous system than attenuated strains; in situ hybridization revealed poliovirus-killed neu-
rons. Small neural cells in the spinal cord were found to contain viral RNA after infection with virulent strains but not after infection with attenuated poliovirus infection. Quantitation of autoradiographic grains per cell, obtained by in situ hybridization, revealed no differences between strains or between neurons from paralyzed versus clinically asymptomatic infected monkeys (27). These investigators reached the important conclusion that motor neuron death in poliomyelitis is the result of direct viral infection, multiplication, and lysis of neurons rather than of inflammation in response to the infection as others had hypothesized.

Studies of Human Tissues

Nucleic acid hybridization techniques for the EVs have also been applied to human tissues in an effort to make disease associations and study pathogenesis (Table 5). In one approach, RNA has been extracted from biopsy specimens of muscle tissues and studied by dot blot hybridization using a coxsackievirus B2-derived cDNA probe (5, 12, 13). Results were expressed as an index of virus-specific versus cellular-RNA-specific hybridization signal strengths, calculated by densitometry quantitation of the autoradiograms obtained separately with two probes, coxsackievirus B2 and β-tubulin. The latter is said to be a marker for total cellular RNA, and therefore its use is a way to standardize the extraction procedure from specimen to specimen. These studies associated EV infection with 9 of 17 patients with myocarditis and dilated cardiomyopathy (12), 5 of 9 patients with polymyositis or dermatomyositis (13), and 20 of 96 patients with chronic fatigue syndrome (5). Unlike in situ hybridization, this method cannot localize viral RNA to cell type or to compartments within cells (Table 5).

EV detection by in situ hybridization in human tissues has also been accomplished by a few investigators (Table 5). The paucity of these reports probably reflects the rarity of obtaining human tissue specimens during acute infection, the unlikelihood of EV persistence in tissues beyond the acute infection, and/or the relative insensitivity of the technique for low levels of viral RNA, with the last factor probably a result of both limitations in hybridization technology and the liability of free (nonencapsidated) intracellular RNA. A single patient with neurologic deterioration accompanying Staphylococcus aureus sepsis had a rise in antibody titer to coxsackievirus B5 and in situ hybridization studies of brain at autopsy which were positive for EV RNA (41). The data were presented in letter-to-the-editor format, so hybridization details were not provided except that the probe was biotin-labeled cDNA derived from coxsackievirus B4. Hybridization revealed signal in the meninges and perivascular areas; pictures demonstrating those findings were not published. Confounding the conclusion that the patient had fulminant and fatal coxsackievirus B5 meningoencephalitis in addition to documented staphylococcal sepsis was the absence of any abnormal CSF findings as well as negative viral cultures and antibody tests on the CSF. The same probe was used to study autopsy heart specimens from patients suspected, either because of serologic conversion or actual viral isolation, of having coxsackievirus infection at the time of death (35). Six of 13 such patients had positive cardiac in situ hybridization assays, with probe distributed in two different and seemingly mutually exclusive patterns, either perivascularly or within cardiac myocytes. In our experience, only a single patient (of approximately 10 studied to date) with suspected EV myocarditis has been found positive by in situ hybridization using coxsackievirus-de-
rived EV probes (unpublished data). In a third in situ hybridization study of human tissues for EV infection, muscle biopsy specimens from 33 patients were blindly studied for EV RNA (77). When an RNA probe derived from TMEV was used, three of five specimens from patients with adult-onset dermatomyositis were positive; 15 patients with other types of inflammatory myopathy, 9 patients with noninflammatory myopathy, and 4 patients with no muscle disease were all negative by in situ hybridization. Positive hybridization was mostly limited to macrophages within the interstitial connective tissue of muscle bundles. Control probes, consisting of a combination of RNA probes derived from poliovirus type 1 and coxsackievirus B3, produced negative results with all 33 specimens, leading the investigators to suggest that a novel picornavirus, genomically more similar to TMEV than to human EV serotypes, may be etiologically responsible for some cases of dermatomyositis.

The novelty of nucleic acid-based testing for tissue diagnosis of human EV infections necessarily engenders skepticism in interpreting results. This is true primarily because those cases in which these newer techniques are applied usually involve patients in whom conventional virologic techniques have failed to establish EV causality. That failure could be due to the timing of tissue sampling (perhaps beyond the acute-infection stage, when viral isolation might have been expected), to infection by serotypes that do not readily grow in cell culture, or to the inability of serology to definitively prove causation of a particular syndrome by an agent (versus coincidental infection with the agent near the time of disease onset). Controls are thus of utmost importance in interpreting such studies. Table 5 summarizes the controls used in the hybridization studies of human tissues described above.

CONCLUSIONS

During the past 6 years, great strides have been made toward the adaptation of nucleic acid-based detection systems to clinical EV diseases. Inherent shortcomings in hybridization techniques, primarily due to inadequate sensitivity, make direct hybridization of clinical samples unfeasible. Recently introduced nucleic acid amplification methods appear to have the potential for overcoming the inadequacies of direct hybridization. Critical to the preliminary success of enzymatic amplification techniques for the EVs is substantial groundwork, including the demonstration of genomic-sequence conservation across a broad range of EV serotypes; determination of regions of greatest conservation; demonstration that oligomeric sequences could hybridize to many EV serotypes as potential primers and probes; and, finally, the sequencing of enough EV serotypes from which specific primer and probe reagents could be constructed.

In situ hybridization studies using the same probe reagents employed for diagnostic testing promise to lend new insights into the pathogenesis of EV infections. Once again, particularly with chronic or postinfectious diseases, sensitivity will likely be the limiting variable. It is probably reasonable to assume that a PCR-based assay, or similar amplification scheme, will someday be applied in situ, with the same dramatic impact on the field of viral pathogenesis that PCR has had on the field of viral diagnostics.

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