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Nonisotopic Oligomeric Probes for the Human Enteroviruses

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Oligonucleotide probes were prepared from highly conserved regions of human enteroviral genomes. These reagents were labeled with either ^{32}P or alkaline phosphatase and were successfully used in blot assays to detect a wide variety of human enteroviruses, proving the potential utility of oligomeric sequences as pan-enteroviral probes. The availability of nonisotopic probes will ultimately make a hybridization assay for enteroviruses easier, shorter, and more adaptable for routine diagnostic laboratories.

The human enteroviruses (EVs) are responsible for 10 million to 30 million infections in the United States annually, ranging in severity from asymptomatic to fatal. While infections of the central nervous system and the heart are of greatest clinical concern, these viruses are multisystemic in their tropisms and virulences. The clinical diagnosis of EV infection is complicated by the nonspecific nature of many of the syndromes, which often make them indistinguishable from diseases caused by bacteria or other viruses. Tissue culture continues to be the mainstay of the laboratory diagnosis of EVs, despite its suboptimal sensitivity and the inherent delays in obtaining results (1). The broad antigenic diversity among the nearly 70 serotypes has made immunoassays impractical (2, 16, 17). For these reasons, we have previously developed nucleic acid probes for the EVs and have demonstrated that a single probe, or a combination of two probes derived from one or two serotypes, can detect a wide representation of additional serotypes in hybridization assays (6, 8, 10). In this study we refined this technology in two important respects. First, oligomeric sequences, which were used as radioactively labeled probes, successfully detected multiple serotypes of EVs, much as did our larger cDNA and RNA probes. Second, enzyme labeling of these oligomers permitted their use in a colorimetric assay, thereby potentially facilitating the use of this hybridization technique in routine diagnostic laboratories.

Two oligomeric sequences, each of which was 22 nucleotides long, were chosen from genomic regions of high conservation based on published EV sequences. The L4 sequence dCGGACACCCAAAGTAGTCGGTT is complementary to bases downstream from nucleotide 535 of coxsackievirus B3 (13). The L5 sequence dCACACTGAC CTGCTCTGGTTGG is complementary to bases downstream from base 421 of the protease gene of echovirus 9 (14). The L4 probe is also perfectly complementary to published sequences of polioviruses 1, 2, and 3 and to coxsackieviruses B1 and B3, with a 1-base mismatch to coxsackievirus B4. The L5 probe has a 1-base mismatch with polioviruses 1 and 3, a 3-base mismatch with poliovirus 2 and coxsackieviruses B1 and 3, and a 5-base mismatch with coxsackievirus B4. This echovirus-derived sequence was chosen with the hope that it is more homologous with other echoviruses and coxsackieviruses A, the sequences of which are not available. Protected linker arm nucleoside 3′-phosphoramidite was prepared by the method of Ruth et al. (12) and was incorporated directly into the oligonucleotide during probe synthesis (11) with a DNA synthesizer (model 380A; Applied Biosystems). ^{32}P labeling of the oligomers was done by standard kinase end labeling (4), followed by purification with C-18 Sep-pak cartridges (Waters Associates, Inc., Milford, Mass.). The specific activity was 8×10^3 cpm/fmol of DNA. Alternatively, the oligomers were covalently coupled to alkaline phosphatase as described previously (3).

Test blots were prepared by adding 10^5 or 10^6 50% tissue culture infective doses ($TCID_{50}$s) of various EVs (assuming a particle-to-infectivity ratio of 100:1 for the EVs, this represents 10^7 to 10^8 RNA molecules and 50 to 500 pg of RNA) to 100 μl of phosphate-buffered saline, followed by treatment with salt, formaldehyde, and heat, as reported previously (9). Control specimens, which consisted of herpes simplex virus, varicella-zoster virus, respiratory syncytial virus, or phosphate-buffered saline to which no virus was added, were treated identically. EV and control mixtures were then brought to 50 mM in NaOH and applied to nylon membrane filters (GeneScreenPlus; Dupont, NEN Research Products, Boston, Mass.) through a slot blot apparatus (Schleicher & Schuell, Inc., Keene, N.H.). Initially, replicate blots were prepared with only EVs and a saline control to determine the spectrum of reactivity with these oligomeric probes (Fig. 1). Blots were prehybridized in a solution of 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate [pH 7.0]), 1% sodium dodecyl sulfate (SDS), and 0.5% bovine serum albumin at 50°C for 15 min. Conditions for hybridization and washing procedures were based on those that were determined previously to produce an optimal signal/noise ratio (data not shown). Hybridization was with 5 nM probe in fresh prehybridization buffer, which was incubated for 30 min at 55°C (^{32}P-labeled probes) or 50°C (enzyme-labeled probes) with shaking. ^{32}P-labeled hybridized blots were washed 3 times (15 min each time) in a mixture of 1× SSC and 1% SDS at 40°C. Enzyme-hybridized blots were washed twice at 45°C in 1× SSC–1% SDS and twice in 1× SSC–1% Triton X-100, for 5 min each time, followed by two final washes in 1× SSC alone at room temperature. Radioactive blots were exposed to XAR-5 film (Eastman Kodak Co., Rochester, N.Y.) with Cronex Lightning Plus (E. I. du Pont de Nemours & Co., Inc., Wilmington, Del.) intensifying screens in sealed cassettes for 18 h.

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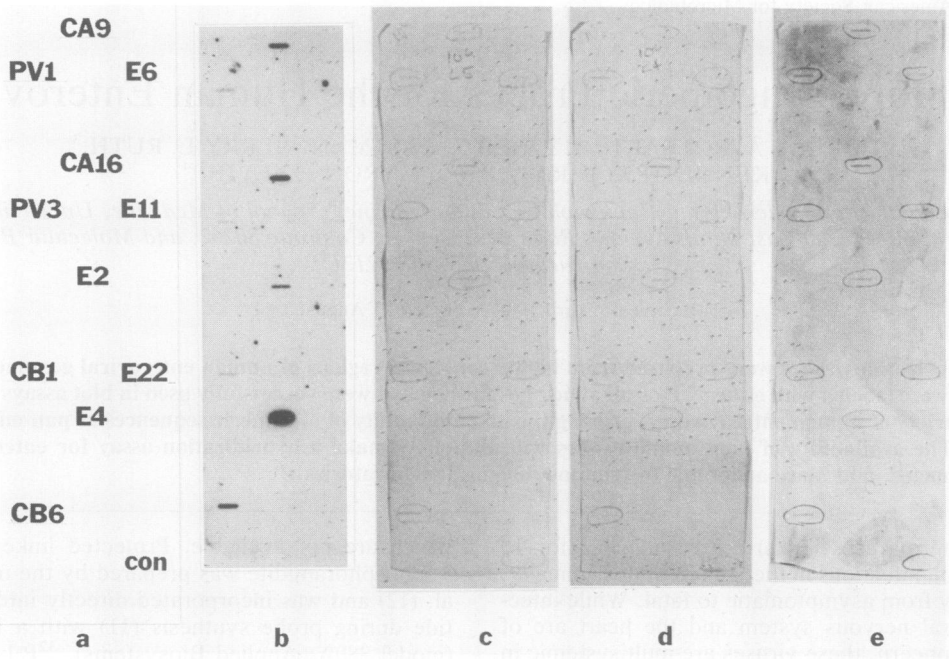

FIG. 1. (a) Pattern of application of 11 EV serotypes and saline control to replicate filter blots (b through e). Abbreviations: PV, poliovirus; C, coxsackievirus; E, echovirus; con, phosphate-buffered saline control. (b) Hybridization of ^{32}P-labeled L5 oligomer to 10^5 $TCID_{50}$s of each serotype and control. (c) Hybridization of enzyme-labeled L5 oligomer to 10^6 $TCID_{50}$s of viruses and control. (d) Hybridization of the enzyme-labeled L4 oligomer to 10^6 $TCID_{50}$s of viruses and control. (e) Hybridization of a combination of enzyme-labeled L4 and L5 oligomeric probes to 10^6 $TCID_{50}$s of viruses and control.

The enzyme-labeled blots were developed in a sealed plastic bag for 1 to 2 h in the dark at 44°C in a buffer (100 μl of buffer per cm^2 of blot) composed of 0.1 M Tris hydrochloride (pH 8.5), 0.1 M NaCl, 0.05 M $MgCl_2$, and 0.1 mM $ZnCl_2$. To each 10 ml of that buffer, 44 μl of Nitro Blue Tetrazolium (75 mg/ml in 70% dimethylformamide) and 33 μl of 5-bromo-4-chloro-3-indolyl phosphate (50 mg/ml in 70% dimethylformamide) were added sequentially. To then confirm the specificity of the hybridization reactions, a second set of replicate blots was prepared. These contained two EVs which were consistently positive in the initial experiments (coxsackievirus B6 and echovirus 4), the three non-EV viral controls, and saline (Fig. 2).

The results obtained with the ^{32}P-labeled oligomers are typified in Fig. 1b, in which the L5 probe was hybridized to 11 different EV serotypes ($TCID_{50}$, 10^5) representing each of the major subgroups of these pathogens. The strongest hybridization signals with this oligomer were seen with echoviruses 2 and 4 and coxsackieviruses A9, A16, and B6. These findings were nearly identical to those obtained with the enzyme-labeled L5 oligomer (Figure 1c), except that the sensitivity of the latter was slightly reduced, with the resultant signals being more obvious at 10^6 $TCID_{50}$. The L4 probe also detected a number of the EV serotypes, but with a somewhat different pattern than the L5 oligomer (Fig. 1d). Finally, the combination of the two enzyme-labeled oligomers gave the broadest spectrum of hybridization with the EV serotypes tested (Fig. 1e). The L4 and L5 probe combination resulted in no significant cross-reaction with non-EV controls (Fig. 2).

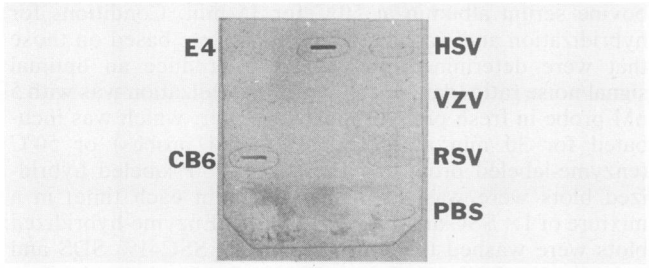

FIG. 2. Hybridization with a combination of enzyme-labeled L4 and L5 oligomeric probes to 10^6 $TCID_{50}$ of EVs or non-EV controls (varicella-zoster virus titer, 5×10^5). Abbreviations: C, coxsackievirus; E, echovirus; HSV, herpes simplex virus; VZV, varicella-zoster virus; RSV, respiratory syncytial virus; PBS, phosphate-buffered saline (no virus added).

Nucleic acid probes derived from EVs hold promise as diagnostic tools for these pathogens. Two obstacles have limited their applicability in hybridization techniques: low sensitivity and the requirement for radioactive reagents. This report begins to address directly both of these concerns. Despite our best efforts at the optimization of probes and hybridization conditions, the limit of sensitivity of our hybridization assays was 10^2 to 10^5 $TCID_{50}$ of target viruses (6, 8, 10). Only the RNA of intact virions can be detected, as free or partially exposed RNA is immediately degraded by endogenous nucleases (7). Clinical specimens of, for example, cerebrospinal fluid contain 10^1 to 10^3 titratable virions (15), explaining the 33% sensitivity (compared with tissue culture) that we have observed in clinical trials with radioactive cDNA and RNA probes (H. A. Rotbart, *in* B. Semler and E. Ehrenfeld, ed., *Molecular Aspects of Picornavirus Infection and Detection*, in press). The expected sensitivity in the clinical application of the enzyme-labeled oligomers reported here is even lower. However, a recently described DNA amplification technique called the polymerase chain reaction offers promise in just this type of situation (5).

Oligomeric DNA primers are annealed to a target strand of nucleic acid which can then be replicated 10^5-fold in a few hours in vitro by using repeated chain elongations. The present report provides evidence that such oligomeric sequences can be chosen to detect multiple serotypes of the EVs generically, making improved sensitivity via target amplification a possibility for the future.

Furthermore, our successful use of nonisotopic labeling in these studies paves the way for adaptation of the method to routine diagnostic laboratories. We envision the ultimate processing of a clinical specimen for EVs to include amplification with carefully chosen oligomeric primer sequences, followed by detection of the amplified segments with yet another oligomer, this one being labeled with enzyme for a rapid colorimetric hybridization assay. Additionally, nonisotopic EV probes should be readily adaptable to in situ hybridizations on fixed paraffin-embedded or fresh-frozen tissue sections. Those studies are under way in our laboratory.

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