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Simultaneous Detection of Infectious Human Echoviruses and Adenoviruses by an *In Situ* Nuclease-Resistant Molecular Beacon-Based Assay

Daniela Dunams,[a] Payal Sarkar,[a] Wilfred Chen,[b] and Marylynn V. Yates[a]

Department of Environmental Sciences, University of California, Riverside, California, USA,[a] and Department of Chemical Engineering, University of Delaware, Newark, Delaware, USA[b]

A multiplex methodology using two nuclease-resistant molecular beacons that target specific genomic regions of adenovirus 2 and echovirus 17 during simultaneous infection in A549 cells is presented. Using fluorescence microscopy, visualization of enteroviral and adenoviral replication was possible within 3 h postinfection.

Cell culture and PCR are commonly used to detect human viruses in environmental media. However, both methods suffer from deficiencies: replication of some viruses in cell culture may require several days to weeks to reach maximum production and the subsequent visualization of cytopathic effects (CPE) (1), some viruses do not produce visible CPE, and others, such as wild-type hepatitis A virus and noroviruses, are difficult to propagate in the laboratory. Detection of different viruses requires the use of different cell lines. Molecular methods are more rapid but do not distinguish between infective and noninfective virus particles (6, 7, 9). This research was undertaken to develop a method that could simultaneously detect multiple infective viruses in one cell line, in a rapid, sensitive, and quantitative manner.

A molecular beacon (MB) is a single-stranded fluorescently labeled oligonucleotide probe with a stem-loop structure (15). A reporter fluorophore is attached to the 5′-arm terminus, and a quencher is attached to the 3′-arm terminus, forming a nonfluorescent complex by fluorescence resonance energy transfer (FRET). When the loop region of the MB hybridizes to a complementary nucleotide sequence, a spontaneous conformational change occurs, resulting in fluorescence of the fluorophore due to the increased distance to the quencher (10).

We designed two MBs targeting specific regions of the adenovirus and enterovirus genomes. These viruses were selected because of their high prevalence in the environment, unrelated genomic structures, and the differences in their replication cycles. The goal was to utilize MB technology to rapidly, simultaneously detect infection of enteroviruses and adenoviruses, at low concentrations, in a single cell.

A549 cells were seeded in 12-well plates (Corning, Corning, NY) and grown to 90% confluence. After washing of the cells with phosphate-buffered saline (PBS), 240 μl adenovirus 2 (prepared in Dulbecco's PBS [Sigma-Aldrich, St. Louis, MO]) adsorbed onto the cell monolayer for 1 h at 37°C in a 5% CO_2 atmosphere; the plates were rocked gently by hand every 15 min. The inoculum was aspirated to remove the unbound virus particles, and then 2.5 ml of SeaKem (Lonza Rockland Inc., Rockland, ME) overlay was added to each well. The overlay consisted of a 1:1 mixture of 1% SeaKem agar and 2× Dulbecco's modified Eagle's medium (DMEM; Gibco, Grand Island, NY) containing 2% fetal bovine serum (FBS) (HyClone; Thermo Scientific), 1 M $MgCl_2$, 100 units/ml penicillin (HyClone), 100 units/ml streptomycin (HyClone), and 10 mM sodium pyruvate (HyClone). After 6 days of incubation at 37°C in a 5% CO_2 atmosphere, the overlay was removed and the cells were treated with 1 ml 0.4% crystal violet-0.1% phenol-12% ethanol solution for 1 h. The excess solution was aspirated, cells were washed twice with 1× PBS, and plaques were counted.

Echovirus 2 was propagated in BGMK cells in 12-well plates using the method of the U.S. Environmental Protection Agency (USEPA) (16). The inoculum was replaced with 3 ml agarose overlay (1:1, 2% agarose and maintenance medium [2× autoclavable minimum essential medium {AMEM} with 2% FBS]). After 2 to 3 days of incubation at 37°C in a 5% CO_2 atmosphere, the overlay was removed and the cells were treated with 0.8% crystal violet-3.7% formaldehyde solution for 5 h. The plaques were then counted.

The adenovirus MB probe (5′-6-carboxyfluorescein [FAM]-CGTGCGCGGAGCGGCTCGGAGGAGAACGC/thiol-dA/CG-Dabcyl-3′; stem portions are underlined) was designed to complementarily target the 5′ untranslated region (UTR) of the E1A gene. The enterovirus probe (5′-Alexa Fluor 647-GCCGGTGATTAGCCGCATTCAGGGGACC/thiol-dG/GC-Dabcyl-3′) was designed to target a conserved 5′ UTR found in all enterovirus genomes. GenBank (www.ncbi.nlm.nih.gov/GenBank/) was used to select the enterovirus and adenovirus sequences for the design of the MB probes. Mfold (mfold.rna.albany.edu/) and IDT SciTools were used to predict the thermodynamic properties and secondary structures of the probes. For greater nuclease resistance, the MB backbone was constructed with sulfur (substituting for nonbridging oxygen) and a 2′-sugar deoxy with a 2′-O-methyl group (TIB Molbiol, Adelphia, NJ) (2, 11, 12, 14). For intracellular delivery, a thiol-maleimide bridge was formed on the quencher end to facilitate the conjugation of TAT peptide (American Peptide, Vista, CA) (12, 13).

The fluorophores were selected to ensure that their excitation and emission bands did not cause spectral overlap. The adenovirus MB was labeled at the 5′ end with 6-FAM (excitation wave-

Received 22 June 2011 Accepted 10 December 2011
Published ahead of print 22 December 2011
Address correspondence to Marylynn V. Yates, marylynn.yates@ucr.edu.
Supplemental material for this article may be found at http://aem.asm.org/.
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doi:10.1128/AEM.05937-11

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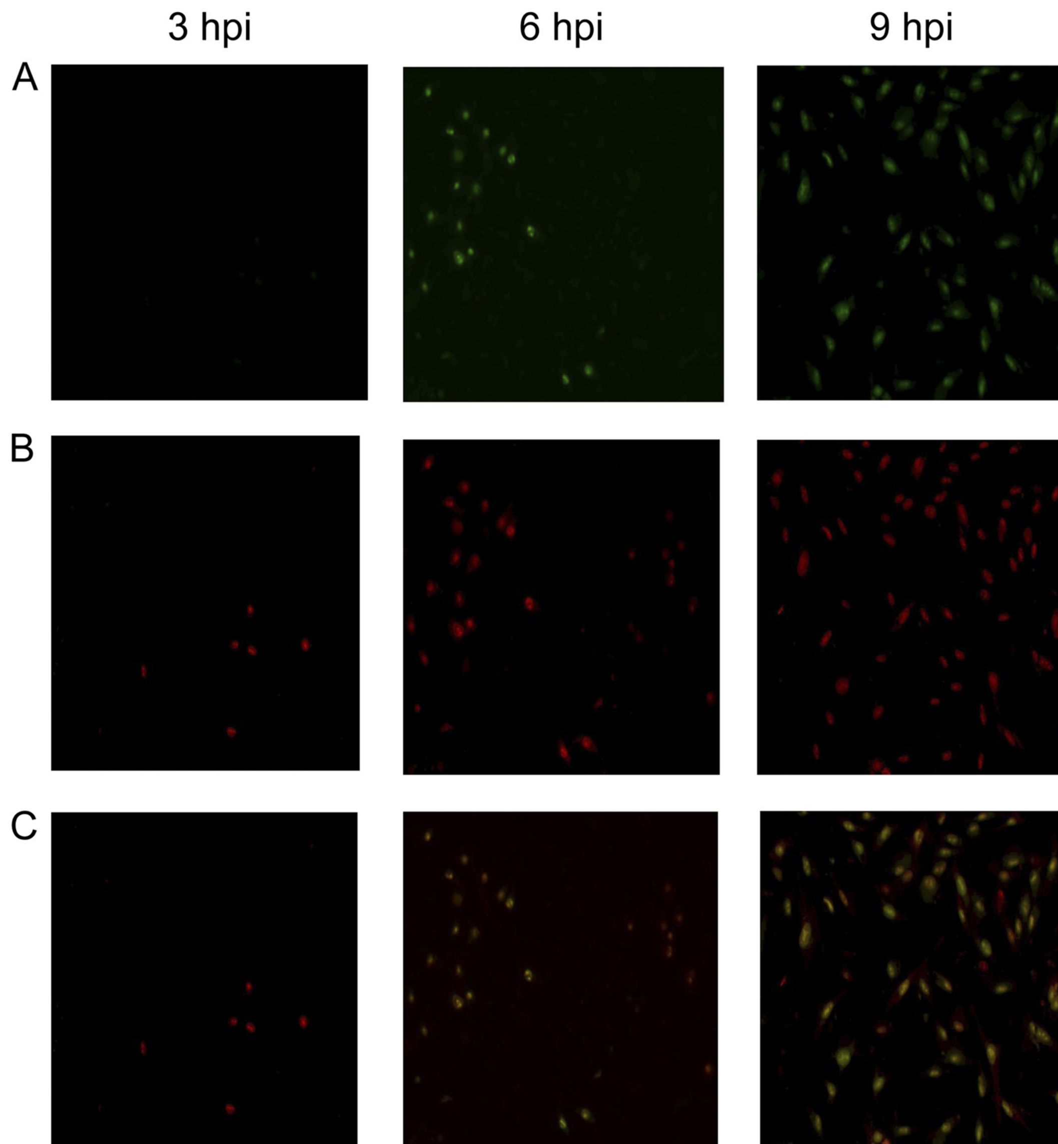

FIG 1 Simultaneous detection of adenovirus 2 and echovirus 17. Visualization of infected (multiplicity of infection of 0.1 PFU/cell) cells using an adenovirus filter set (A), using an echovirus filter set (B), and with fluorescent images merged (C).

length, 495 nm; emission wavelength, 520 nm). The enterovirus MB was labeled at the 5′ end with Alexa Fluor 647 (excitation wavelength, 650 nm; emission wavelength, 665 nm). Dabcyl was the quencher for both MB probes.

Complementary oligonucleotides (IDT, Coralville, IA) were designed for both viruses to verify efficient peptide-conjugated MB-mediated intracellular delivery. The oligonucleotides were complementary to the MB loop regions. Both MB probes were ordered from TIB Molbiol. For peptide conjugation, 150 μM TAT peptide was mixed with 100 μM MB probe and stored at 4°C overnight in the dark. One-hundred-microliter aliquots of the peptide-linked MB were stored at −20°C. As MB probes are light sensitive, experiments were performed in near-darkness.

The Olympus IX71 inverted microscope with a reflected fluorescence system (Olympus America, Inc., San Diego, CA) was used to take fluorescence and phase-contrast images. Image-Pro Plus analysis software (Media Cybernetics, Inc., Bethesda, MD) was used for image analysis. The 6-FAM-labeled fluorescent probe was detected using a D480/30-nm exciter and a D535/40-nm emitter filter set, and the Alexa Fluor 647-labeled fluorescent probe was detected using an ET 640/30-nm exciter and an ET 690/50-nm emitter (Chroma Technology, Bellows Falls, VT). Images were obtained using a ProgRes MF^{scan} monochrome charge-coupled device (CCD) camera (Jenoptik, Rochester, NY).

TABLE 1 Comparison of detection methods

Detection limit (PFU/ml)	Duration (hpi)			
	MB-based assay		Plaque assay	
	Adenovirus	Echovirus	Adenovirus	Echovirus
1	7	5	168	48
10	4.5	3	168	48
100	2	2	168	48

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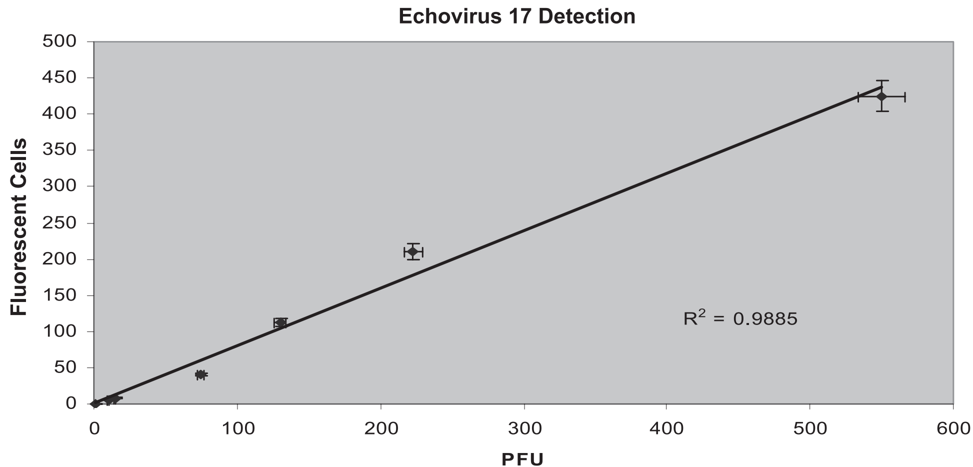

FIG 2 Correlation between the number of echovirus PFU and fluorescent cells at 5 h postinfection; slope = 0.80.

A549 cells were seeded into an 8-well Lab-Tek chambered coverglass (Fisher Scientific) and allowed to grow for 18 h at 37°C in a 5% CO_2 atmosphere to 95% confluence. The F-12 growth medium was removed, and cell monolayers were washed with 1× Leibovitz L-15 medium (Gibco, Grand Island, NY). Equal amounts of 2 μM adenovirus and enterovirus probe were gently mixed, and 100 μl of the probe mixture was applied to the cells for 45 min at 37°C in a 5% CO_2 atmosphere. After inoculum removal, the cells were washed once with 1× Leibovitz L-15 medium and then infected with predetermined dilutions of adenovirus 2 and echovirus 17 in 1× Leibovitz L-15. The first image (time = 0 min) was taken after the addition of viruses to the cell monolayer. The chamber well was kept on the microscope stage at room temperature for the duration of the experiment. Fluorescence images were taken at 30-min intervals.

The specificity of the MB probes was demonstrated by lack of recognition of the nontarget genome or by the observation of oligonucleotide (see Fig. S1 and Fig. S2 in the supplemental material).

The MB-based fluorescence assay detected echovirus 17 at 3 h postinfection (hpi); adenovirus 2 infection was observed slightly later (Fig. 1). The MB-based assay detected 1 PFU of echovirus and adenovirus very rapidly: 5 and 7 h, respectively, were required, an improvement over the time required to detect these viruses using plaque assay (Table 1).

The number of viruses enumerated using the plaque assay versus the number of fluorescent cells detected using the MB-based assay is shown in Fig. 2 and 3 for echovirus 17 and adenovirus 2, respectively. The correlations obtained ($r^2 > 0.98$) suggest that the MB-based assay can be used quantitatively.

A multiplex assay requires highly specific probes and a very low rate of false positives. Each MB probe was tested against the other virus and its oligonucleotide, and no recognition was observed. For all specificity tests, the incubation periods were either 9 or 12 h to determine the stability of the internalized probes.

MBs have been used for the detection of viral infection in living cells because the MB probe can report the presence of a single infective particle (18, 19). In this study, 1 PFU of adenovirus could be detected at 7 h postinfection (hpi), but 5 hpi were required for echovirus detection. This is likely because uncoating of the echovirus genome takes place in the cytoplasm while adenovirus ge-

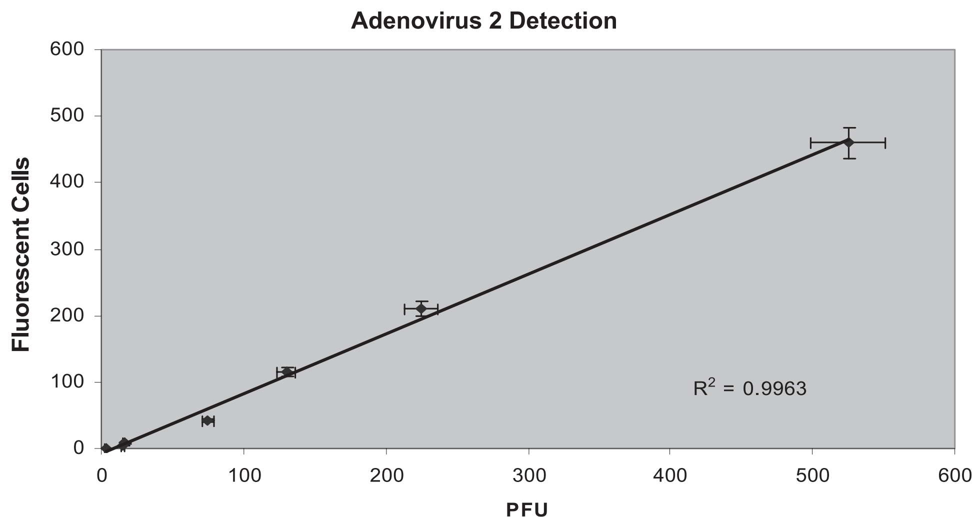

FIG 3 Correlation between the number of adenovirus PFU and fluorescent cells at 7 h postinfection; slope = 0.88.

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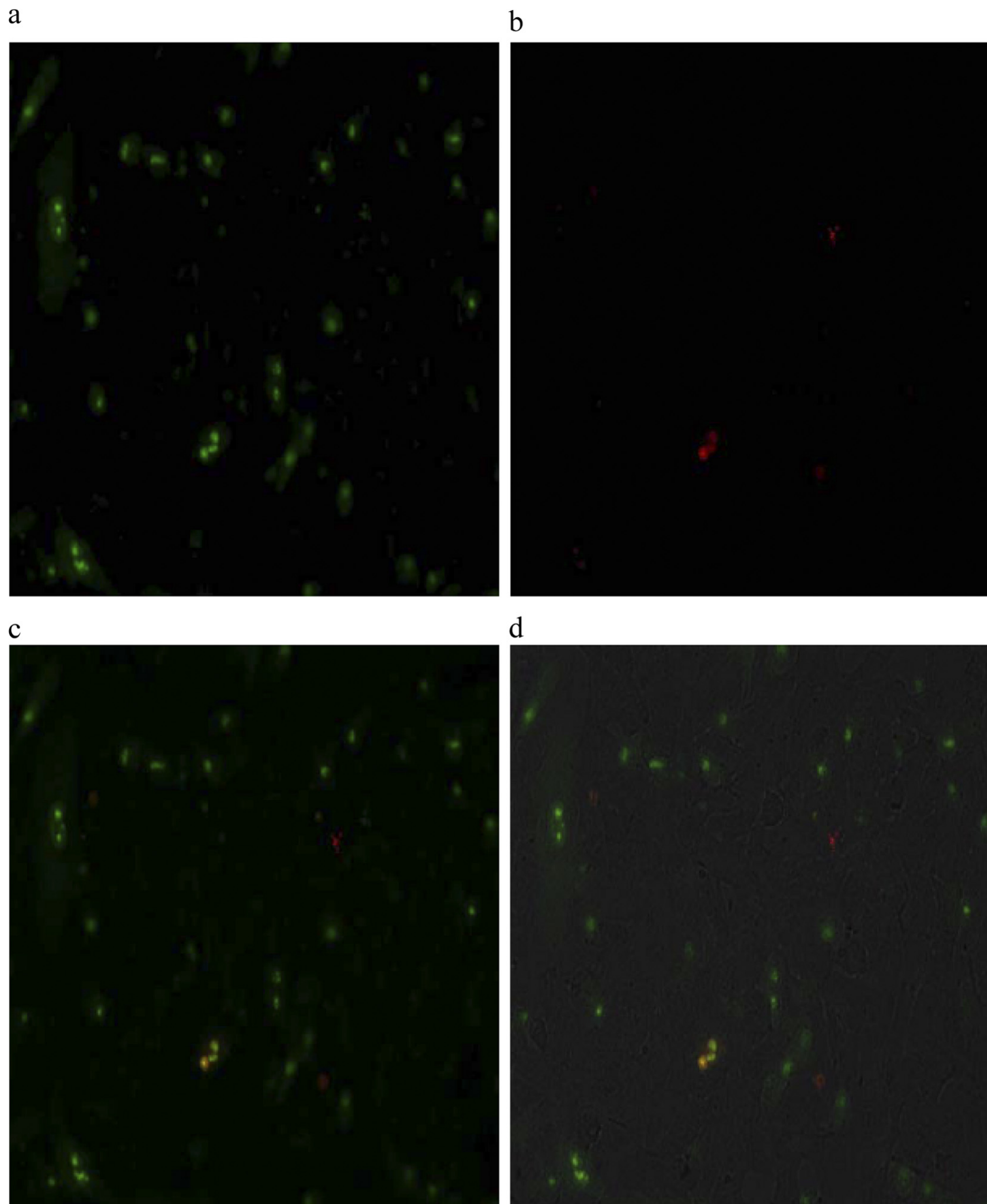

FIG 4 Simultaneous detection of adenovirus 2 and echovirus 17 at 4 h postinfection. Visualization of cells infected with a high concentration of adenovirus 2 (multiplicity of infection of 1 PFU/cell) and a lower concentration of echovirus 17 (multiplicity of infection of 0.1 PFU/cell) using an adenovirus filter set (a), using an echovirus filter set (b), with fluorescent images merged (c), and with fluorescent images merged with phase contrast (d).

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nome uncoating takes place in the nucleus. Due to the increased time required for the virus to travel to and enter the nucleus, it takes longer for the MB to detect the target. This hypothesis is supported by the results of experiments in which 10-fold-higher concentrations of adenoviruses than of enteroviruses were used. After 2 h, adenovirus infection was detected, but echovirus 17 was not detected until 4 h postinfection (Fig. 4).

While some cells were infected by only adenovirus or echovirus, the majority appear to be infected by both viruses (Fig. 1). There are many reports of multiple viruses infecting a single cell simultaneously (3, 4, 8, 17), and with some viruses this event is favored because reassortment of parental gene products increases the fitness of virus progeny (4). Also, coinfection is important in the life cycle of many RNA viruses (5).

One of the advantages of using the MB technology is real-time quantification of viral infectivity at low concentrations. A linear correlation between PFU and the number of fluorescent cells infected with echovirus 17 or adenovirus 2 was found (Fig. 2 and 3). These graphs provide evidence that the MB-based assay provides comparable information on the number of infective viruses present but does so in a much more rapid fashion than the standard plaque assay (Table 1).

ACKNOWLEDGMENTS

This work was supported by EPA STAR grant RD83300801 and NSF grant CBET0755775.

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