Real-Time Nucleic Acid Sequence-Based Amplification Using Molecular Beacons for Detection of Enterovirus RNA in Clinical Specimens

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The detection of enteroviruses (EV) in clinical specimens, particularly spinal fluid, by molecular amplification techniques has important advantages over cell culture, namely, greater sensitivity and faster results. The impact on clinical decision-making as well as overall health care costs can be substantial (11, 14). Although reverse transcription-PCR (RT-PCR) is the most commonly used test, nucleic acid sequence-based amplification (NASBA) using the Nuclisens Basic Kit has proved of equal or greater sensitivity for detection of enteroviruses (2, 6, 7).

In the Nuclisens Basic Kit, amplified RNA products are detected by hybridization using electrochemiluminescently (ECL) labeled probes, a highly sensitive methodology. Recently, real-time RT-PCR using TaqMan chemistries has been applied to further shorten both technical hands-on time and time to result (3, 10). In this report, we compare the Nuclisens EasyQ Enterovirus Test, which utilizes real-time molecular beacons as probes (NASBA-beacon), to our standard NASBA with ECL probes (NASBA-ECL) for detection of EV in clinical specimens.

MATERIALS AND METHODS

Samples and patients. Samples submitted to the Clinical Virology Laboratory at Yale New Haven Hospital (YNHH) for EV diagnosis were used. For the retrospective study, RNA extracts from 53 samples stored at −70°C for 1 to 3 months were used. For the prospective study, 107 samples submitted for EV diagnosis from mid-May through August 2004 were tested within 0 to 3 days by NASBA with ECL detection as the standard clinical test. The RNA extracts were then retested by real-time NASBA-beacon (Nuclisens EasyQ Enterovirus Test) within 0 to 10 days. Extracts not tested the same day were stored at −70°C.

The retrospective study included 53 cerebrospinal fluid (CSF) samples from 53 patients. The prospective study included 107 samples from 107 patients: 80 CSF and 27 stool samples. The stool samples were submitted as part of an epidemiologic investigation of an outbreak of meningitis, nausea, and vomiting in a church youth group returning from Mexico. Spinal fluid and stool samples were submitted in sterile containers. Patients’ ages ranged from 5 days to 93 years, and 34 percent were over 18 years of age.

Virus isolation. Viral cultures on EV were performed at YNHH as previously described (7). Depending on the month and the amount of sample available, 1 to 5 cell systems in roller tubes were inoculated. The Connecticut State Department of Health Virology Laboratory cultured stool samples from the church group in primary monkey kidney, A549, and human fibroblasts as part of an epidemiologic investigation.

Sample processing. CSF samples were vortexed. A 10% suspension of stool (wt/vol) in phosphate-buffered saline with antibiotics was vortexed and then centrifuged at 2,000 × g for 15 min, and the clarified supernatant was used for testing. Aliquots of samples were then added to lysis buffer, as described below. If testing was delayed, specimens in lysis buffer were frozen at −70°C.

Nucleic acid isolation. RNA was isolated with reagents provided in the Nuclisens kit. Brieﬂy, 100 μl of a stool sample or 200 μl of CSF, together with an internal system control (2,000 copies for the retrospective study and 4,000 copies for the prospective study), was added to 1.0 ml of prewarmed Nuclisens lysis buffer. In addition, 100 μl of Base Matrix (Boston Biomedica, Inc.) was added to facilitate extraction and recovery of RNA. Silica particles (50 μl of suspension) were added and allowed to bind the nucleic acids. After centrifugation, the silica pellet was washed twice with NASBA wash buffer, twice with 70% ethanol, and once with acetone. The silica pellet was then dried and the nucleic acids eluted at 56°C in 50 μl of elution buffer. For both NASBA methods, 5 μl of extracted nucleic acid was used for amplification.

Nuclisens Basic Kit NASBA-ECL. The Nuclisens Basic Kit (bioMerieux, Durham, NC) was used as previously described (7). Primers were directed to the 5′ noncoding region of the EV genome (poliovirus type 1, strain Sabin 1). Specimen results with wild-type (WT) EV signals of ≥50,000 ECL units were considered positive for EV RNA, regardless of the internal system control (IC) signal. Specimens with WT EV signals of <450 ECL units and IC signals of ≥50,000 ECL units were considered negative for EV RNA and not inhibitory. Specimens with WT signals of <450 and IC signals of <50,000 ECL units were considered inhibitory to amplification. Thus, these results were deemed “invalid,” and the extraction and amplification were repeated. WT signals with 450 to 649 ECL units were considered indeterminate. Note that the internal system control was the same for both Nuclisens kits but was designated IC in the Basic kit and SC in the EasyQ kit.

Nuclisens EasyQ Enterovirus NASBA (NASBA-beacon). Lyophilized enzymes and reagents in the EasyQ Enterovirus kit were reconstituted as directed, and a working reagent with primers and probes was prepared to a final KCl concentration of 90 mM. To each well of a test strip, 5 μl of the extracted control or patient sample was added, followed by 10 μl of the working reagent/primer-

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The characteristics and resolution of the five initially discrepant samples, one from the retrospective study and four from the prospective study, are given in Table 1. When the discrepant samples were retested by NASBA-beacon, two tested positive and thus were in agreement with NASBA-ECL. One retested indeterminate, and two retested negative; all three of these were determined to be true EV positives. Of note, all four initially discrepant CSF samples had low ECL values of <10,000 (Table 1). In total, 7 of 37 positive CSF samples had ECL values of <10,000 by NASBA-ECL. The two samples with the lowest ECL values repeatedly tested negative by NASBA-beacon. The discrepant stool sample had higher NASBA-ECL values yet repeatedly tested indeterminate by NASBA-beacon. It was confirmed as EV positive by nested RT-PCR at the CDC and was sequenced as coxsackievirus A1. One NASBA-ECL-negative CSF sample was initially NASBA-beacon indeterminate but retested as NASBA-beacon negative and had a normal CSF profile and a negative EV culture.

The final results for NASBA-ECL and NASBA-beacon are given in Table 2. Results for CSF and stool samples were similar and were combined. The kappa coefficient, testing agreement between NASBA-ECL and NASBA-beacon, was 0.9579. The two tests were not significantly different in sensitivity ($P = 0.2482$ by McNemar’s test).

### RESULTS

#### Comparison of enterovirus NASBA-ECL and NASBA-beacon results

In the retrospective study of 53 spinal fluid samples, 20 samples tested positive by NASBA-ECL. NASBA-beacon initially called 19 positive and 1 indeterminate. In the prospective study of 107 samples, NASBA-ECL detected 35 positives. Of these 35, NASBA-beacon initially called 31 positive, 3 indeterminate, and 1 negative.

### TABLE 1. Characteristics of initially discrepant EV-positive samples

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Age</th>
<th>Sample</th>
<th>NASBA-ECL* Result by:</th>
<th>NASBA-beacon Result by:</th>
<th>CSF profile</th>
<th>Culture result</th>
<th>Clinical findings</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Initial</td>
<td>Repeat</td>
<td>Initial</td>
<td>Repeat</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>6 yr</td>
<td>CSF</td>
<td>7,959</td>
<td>6,691</td>
<td>Indeterminate</td>
<td>Positive</td>
<td>Abnormal</td>
</tr>
<tr>
<td>2</td>
<td>10 wk</td>
<td>CSF</td>
<td>3,701</td>
<td>Not done</td>
<td>Indeterminate</td>
<td>Positive</td>
<td>Normal</td>
</tr>
<tr>
<td>3</td>
<td>15 yr</td>
<td>Stool</td>
<td>1,179,369</td>
<td>26,863</td>
<td>Indeterminate</td>
<td>Indeterminate</td>
<td>Not done</td>
</tr>
<tr>
<td>4</td>
<td>17 yr</td>
<td>CSF</td>
<td>Invalid</td>
<td>1,271</td>
<td>Indeterminate</td>
<td>Negative</td>
<td>Abnormal</td>
</tr>
<tr>
<td>5</td>
<td>9 wk</td>
<td>CSF</td>
<td>2,937</td>
<td>651</td>
<td>Negative</td>
<td>Negative</td>
<td>Abnormal</td>
</tr>
</tbody>
</table>

* Expressed in ECL units, with $\geq 650$ considered positive.
* The stool sample was also positive for EV by nested RT-PCR at CDC; the amplicon sequenced as coxsackievirus A1.
* CSF profile: 940 nucleated cells, 13% granulocytes, 72% lymphocytes, 4% monocytes; protein, 103 mg/dl; glucose, 64 mg/dl; sample collected on day 5 of illness.

#### Table 2. Comparison of NASBA-ECL and real-time NASBA-beacon for detection of EV in CSF and stool specimens

<table>
<thead>
<tr>
<th>Result by NASBA-beacon</th>
<th>NASBA-ECL result</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td>52</td>
<td>52</td>
</tr>
<tr>
<td>Indeterminate</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Negative</td>
<td>2</td>
<td>105</td>
</tr>
<tr>
<td>Total</td>
<td>55</td>
<td>105</td>
</tr>
</tbody>
</table>

* Sensitivity of NASBA-ECL, 100%. Sensitivity of NASBA-beacon, 94.5% overall: 35 of 37 (94.5%) EV-positive CSF samples and 17 of 18 (94.4%) EV-positive stool samples. The specificity of both assays was 100%. NASBA-ECL and NASBA-beacon were not significantly different ($P = 0.2482$ by McNemar’s test).
Table 3, both NASBA methods were significantly more sensitive than culture (P < 0.0001 by McNemar’s test). Cell culture detected only 59% of positive CSF samples and 22.2% of positive stools tested. Time to isolation ranged from 1 to 7 days, with a median of 3 days. The low sensitivity for stool culture was surprising, since stools contain higher concentrations of virus than CSF and larger sample volumes are available for culture inoculation than is usual for CSF. However, the CDC identified the EV in the majority of positive stool samples as coxsackievirus A1, a serotype that grows poorly in cell culture. No samples were culture positive and NASBA-ECL negative. However, one sample that was positive by culture and NASBA-ECL was negative by NASBA-beacon (Table 1, patient 5).

Identification of EV types detected. As part of an epidemiological investigation, typing was performed by the CDC for 20 EV-positive samples, 18 stool and 2 spinal fluid samples. For the 18 positive stool samples, 15 EV were typed as coxsackievirus A1 and 3 were typed as echovirus 30. Two CSF samples from the same outbreak were typed as echovirus 30. Nasba-ECL detected all 20 positive samples. Nasba-beacon detected all but one: a stool sample that tested as EV indeterminate rather than positive. The 35 other EV-positive spinal fluid samples in this study were not typed.

Characteristics of EV-positive patients. Patients with positive spinal fluid samples ranged in age from 1 week to 50 years: 15 (41.6%) were less than 10 weeks of age, 8 (22.2%) were between 1 and 10 years, 8 (22.2%) were between 10 and 18 years, and 5 (13.9%) were between 23 and 50. One patient’s age was not available. Stool samples were collected from a church youth group and their chaperones. Of patients with positive stool samples, 17 were 13 to 17 years old and 1 was 33 years old.

CSF profiles were available for 35 of 37 EV-positive samples. As previously noted, normal protein levels or cell counts were a frequent finding in EV-positive spinal fluid samples, occurring in 45.7% of cases (5). Two infants, 6 weeks and 10 weeks of age, had both normal protein levels and normal cell counts, and two infants had normal cell counts only. Twelve patients (32.3%) had normal protein levels but elevated cell counts.

Discussion

NASBA with detection by ECL-labeled probes is a highly sensitive and specific amplification method for enteroviruses and other RNA targets (2, 6, 8, 15). In this study, we compared NASBA-ECL with real-time NASBA using molecular beacons and found excellent agreement. Virus isolation, in contrast, was significantly less sensitive than both molecular methods for both CSF and stool samples, detecting only 42.5% of positive samples tested.

The real-time NASBA-beacon assay had a sensitivity of 94.5% compared to NASBA-ECL. While not statistically significant, this difference was consistent with the data reported by Ginocchio et al. (Clinical Virology Symposium, 2003) and by Lenciotti and Kerst for West Nile virus (4). In the latter study, real-time NASBA-beacon and TaqMan assays were similar in sensitivity, but both were slightly less sensitive than NASBA-ECL (4).

In this study, two CSF samples with values of <3,000 ECL units were NASBA-beacon negative after repeat testing. The frequency of such low-positive samples will depend on sample handling and transport, the number of CSF samples tested, the time of sample collection in the course of disease, and variations in primer detection efficiency for the circulating enterovirus types. In our laboratory, EV-positive samples with values of <3,000 ECL units occurred for 3.3% of positive CSF samples in 2003 and 6.7% in 2004.

Use of the miniMag semiautomated Nucliens extractor to concentrate the nucleic acid extracts in a 25-μl elution volume, instead of the 50-μl elution volume obtained by manual Nucliens extraction, could increase the sensitivity of NASBA-beacon for low-positive samples (F. Zhang, R. Manji, and C. C. Ginocchio, presented at the Clinical Virology Symposium, Clearwater Beach, FL, April 2004). However, this strategy has not yet been proven in a prospective study of clinical samples.

The cutoff for indeterminate results for EV NASBA-beacon was not clearly defined when we initiated our study. As previously suggested by Ginocchio and colleagues, we adopted the value for the negative control plus 5% as the indeterminate cutoff. Consequently, two indeterminates by NASBA-beacon were designated true positives after repeat testing. An additional true positive was repeatedly in the indeterminate range. The cutoff for a positive result was taken as the value for the negative control plus 20%, as recommended by the manufacturer. No false positives were detected.

The real-time NASBA-beacon assay had important advantages over NASBA-ECL, namely, fewer steps prior to amplification and no postamplification hybridization and detection steps. Together, these resulted in a reduction of 90 min in assay time and a labor savings of approximately 25 min in hands-on time per run. In addition, the real-time beacon format eliminated opening of the reaction tube postamplification and thus reduced the risk of amplicon contamination. Technologists found the NASBA-beacon procedure much more user-friendly and easier to learn and perform than NASBA-ECL.

A significant advantage of both NASBA assays is the use of an internal control that is spiked into the patient sample prior to nucleic acid extraction and monitors both extraction and amplification. This internal control, called IC in the NASBA-ECL assay and SC in the NASBA-beacon assay, is the same in...
vitro RNA and is amplified in a multiplex assay by the same primers. Most published real-time TaqMan assays for enteroviruses lack such controls.

Of note, the reagent cost and equipment rental for the real-time NASBA-beacon assay were significantly greater than those for NASBA-ECL. To compensate for the expense of renting real-time beacon equipment, it would be advantageous to bring other real-time NASBA assays in-house; a number are becoming available (1, 9, 12, 13). For laboratories that have a real-time PCR instrument, it is apparently possible to perform NASBA-beacon in a TaqMan instrument (4).

In conclusion, the availability of a real-time NASBA-beacon assay for EV diagnosis is an important advance, providing highly sensitive results in as little as 4 h, in a user-friendly format that can be implemented by laboratories with little molecular experience. Most importantly, it provides the opportunity to positively impact patient management.

ACKNOWLEDGMENTS

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REFERENCES


