JOURNAL OF CLINICAL MICROBIOLOGY, July 2005, p. 3136–3139 Vol. 43, No. 7
0095-1137/05/$08.00+0 doi:10.1128/JCM.43.7.3136–3139.2005

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

Marie L. Landry,[1,2]* Robin Garner,[2] and David Ferguson[1,2]

Department of Laboratory Medicine, Yale University School of Medicine, New Haven, Connecticut 06520,[1] and Clinical Virology Laboratory, Yale New Haven Hospital, New Haven, Connecticut 06504[2]

Received 16 February 2005/Returned for modification 28 March 2005/Accepted 6 April 2005

Real-time nucleic acid sequence-based amplification (NASBA) using molecular beacon technology (NASBA-beacon) was compared to standard NASBA with postamplification hybridization using electrochemiluminescently labeled probes (NASBA-ECL) for detection of enteroviruses (EV) in 133 cerebrospinal fluid and 27 stool samples. NASBA-ECL and NASBA-beacon were similar in sensitivity, detecting 55 (100%) and 52 (94.5%) EV-positive samples, respectively. There were no false positives. Both NASBA assays were significantly more sensitive than culture. Real-time NASBA-beacon reagents and equipment rental were more expensive than those for NASBA-ECL; however, time to result was shortened by 1.5 h, hands-on time was reduced by 25 min, and the assay was much simpler for technologists to learn and perform.

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 electrochemiluminscently (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 8 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 CSF 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 ≥650 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-

* Corresponding author. Mailing address: Department of Laboratory Medicine, P.O. Box 208035, Yale University School of Medicine, New Haven, CT 06520-8035. Phone: (203) 688-3475. Fax: (203) 688-8177. E-mail: marie.landry@yale.edu.

TABLE 1. Characteristics of initially discrepant EV-positive samples

Patient no.	Age	Sample	Result by: NASBA-ECL[a] Initial	NASBA-ECL[a] Repeat	NASBA-beacon Initial	NASBA-beacon Repeat	CSF profile	Culture result	Clinical findings
1	6 yr	CSF	7,959	6,691	Indeterminate	Positive	Abnormal	Negative	Aseptic meningitis
2	10 wk	CSF	3,701	Not done	Indeterminate	Positive	Normal	Negative	Fever, irritability
3	15 yr	Stool	1,179,369	26,863	Indeterminate	Indeterminate	Not done	Negative[b]	Fever, cough, rhinorrhea, vomiting, dizziness
4	17 yr	CSF	Invalid	1,271	Indeterminate	Negative	Abnormal[c]	Negative	Fever, headache, neck stiffness, photophobia
5	9 wk	CSF	2,937	651	Negative	Negative	Abnormal	EV isolated from CSF and rectal swab	Fever, vomiting, loose stools

[a] Expressed in ECL units, with ≥650 considered positive.
[b] The stool sample was also positive for EV by nested RT-PCR at CDC; the amplicon sequenced as coxsackievirus A1.
[c] 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.

probe mixture. The strips were placed in the EasyQ incubator and incubated for 2 min at 65°C, followed by 2 min at 41°C. During this incubation, 5 μl of reconstituted enzyme reagent was pipetted to the strip caps. After the incubations were completed, the strips were centrifuged and the enzyme pulse-spun to the bottoms of the wells. Strips were then returned to the EasyQ reader and amplification begun. After 150 min, the reaction was terminated and the data were analyzed.

For patient samples, the WT EV-positive cutoff was determined by multiplying the real-time fluorescent unit (RFU) value of the negative control by 1.2. The WT EV-negative cutoff was determined by multiplying the negative control by 1.05 (C. C. Ginocchio, F. Zhang, and P. Sillekens, presented at the Clinical Virology Symposium, Clearwater Beach, FL, April 2003). Patient specimens with RFU values greater than the negative cutoff but less than the positive cutoff were considered indeterminate for EV and were retested, after reextraction if the original sample remained, or by reamplification of the extract.

The cutoff for the internal system control (SC) spiked into each patient sample was determined by multiplying the RFU value of the SC spiked into the negative control by 0.8. EV-negative patient specimens had to have an SC RFU value greater than or equal to this value to be considered valid. If the SC RFU value of an EV-negative patient specimen was less than this value, the test was repeated. EV-positive specimens could have an SC either above or below the SC cutoff and be considered acceptably amplified.

The negative control for the retrospective study was negative CSF, and that for the prospective study was nuclease-free water, extracted in parallel with the clinical samples. The mean and range of values of extracted negative CSF and water were similar (data not shown).

Nested enterovirus RT-PCR. Stool samples from the church youth group outbreak were also tested by nested RT-PCR at the Centers for Disease Control and Prevention (CDC).

Resolution of discrepancies. Samples from which enterovirus was isolated were considered true positives. Samples positive by both NASBA-ECL and NASBA-beacon but negative or not tested by culture were also considered true positives. A sample positive by either molecular test, but for which culture was either negative or not done was considered a true positive if either (i) EV was detected by culture and/or both molecular assays in another sample from the patient, (ii) the clinical course and CSF profile were typical for aseptic meningitis, and Gram stain and bacterial cultures were negative, or (iii) the patient had a febrile illness compatible with enterovirus infection and had a positive RT-PCR in addition to a positive NASBA 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.

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 of virus isolation. In total, 113 of the 160 samples were cultured, including 40 of 55 true positives. As shown in

TABLE 2. Comparison of NASBA-ECL and real-time NASBA-beacon for detection of EV in CSF and stool specimens[a]

Result by NASBA-beacon	NASBA-ECL result: Positive	NASBA-ECL result: Negative	Total
Positive	52	0	52
Indeterminate	1	0	1
Negative	2	105	107
Total	55	105	160

[a] 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. Comparison of two NASBA methods and cell culture for detection of EV-positive samples

Method	No. (%) of true EV positives detected by the indicated method in:		
	CSF (n = 86)	Stool (n = 27)	All samples (n = 113)
NASBA-ECL	22	18	40 (100)
NASBA-beacon	21	17[a]	38 (95.0)
Cell culture	13	4[b]	17 (42.5)[c]

[a] One NASBA-ECL positive, culture-negative stool sample was indeterminate by real-time NASBA-beacon.
[b] The predominant EV type identified was coxsackievirus A1, which grows poorly in cell culture.
[c] Both NASBA-ECL and NASBA-beacon were more sensitive than culture ($P < 0.0001$ 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 Lanciotti 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 Nuclisens extractor to concentrate the nucleic acid extracts in a 25-μl elution volume, instead of the 50-μl elution volume obtained by manual Nuclisens 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

This work was supported in part by bioMerieux, Durham, NC.

We gratefully acknowledge the expertise of the staffs of the YNHH Clinical Virology Laboratory, the Connecticut DPH Virology Laboratory, and the CDC Enterovirus Laboratory, as well as the data collection and organizational skills of Geraldine Russo.

REFERENCES

1. **Chantratita, W., W. Pongtanapisit, W. Piroj, C. Srichunrasmi, and S. Seesuai.** 2004. Development and comparison of the real-time amplification based methods—NASBA-Beacon, RT-PCR TaqMan and RT-PCR hybridization probe assays—for the qualitative detection of SARS coronavirus. Southeast Asian J. Trop. Med. Public Health **35:**623–629.
2. **Fox, J. D., S. Han, A. Samuelson, Y. Zhang, M. L. Neale, and D. Westmoreland.** 2002. Development and evaluation of nucleic acid sequence based amplification (NASBA) for diagnosis of enterovirus infections using the NucliSens Basic Kit. J. Clin. Virol. **24:**117–130.
3. **Lai, K. K., L. Cook, S. Wendt, L. Corey, and K. R. Jerome.** 2003. Evaluation of real-time PCR versus PCR with liquid-phase hybridization for detection of enterovirus RNA in cerebrospinal fluid. J. Clin. Microbiol. **41:**3133–3141.
4. **Lanciotti, R. S., and A. J. Kerst.** 2001. Nucleic acid sequence-based amplification assays for rapid detection of West Nile and St. Louis encephalitis viruses. J. Clin. Microbiol. **39:**4506–4513.
5. **Landry, M. L.** 2005. Frequency of normal cerebrospinal fluid protein level and leukocyte count in enterovirus meningitis. J. Clin. Virol. **32:**73–74.
6. **Landry, M. L., R. Garner, and D. Ferguson.** 2003. Comparison of the NucliSens Basic kit (nucleic acid sequence-based amplification) and the Argene Biosoft Enterovirus consensus reverse transcription-PCR assays for rapid detection of enterovirus RNA in clinical specimens. J. Clin. Microbiol. **41:** 5006–5010.
7. **Landry, M. L., R. Garner, and D. Ferguson.** 2003. Rapid enterovirus RNA detection in clinical specimens by using nucleic acid sequence-based amplification. J. Clin. Microbiol. **41:**346–350.
8. **Moore, C., E. M. Clark, C. I. Gallimore, S. A. Corden, J. J. Gray, and D. Westmoreland.** 2004. Evaluation of a broadly reactive nucleic acid sequence based amplification assay for the detection of noroviruses in faecal material. J. Clin. Virol. **29:**290–296.
9. **Moore, C., S. Hibbitts, N. Owen, S. A. Corden, G. Harrison, J. Fox, C. Gelder, and D. Westmoreland.** 2004. Development and evaluation of a real-time nucleic acid sequence based amplification assay for rapid detection of influenza A. J. Med. Virol. **74:**619–628.
10. **Nijhuis, M., N. van Maarseveen, R. Schuurman, S. Verkuijlen, M. de Vos, K. Hendriksen, and A. M. van Loon.** 2002. Rapid and sensitive routine detection of all members of the genus enterovirus in different clinical specimens by real-time PCR. J. Clin. Microbiol. **40:**3666–3670.
11. **Robinson, C. C., M. Willis, A. Meagher, K. E. Gieseker, H. Rotbart, and M. P. Glode.** 2002. Impact of rapid polymerase chain reaction results on management of pediatric patients with enteroviral meningitis. Pediatr. Infect. Dis. J. **21:**283–286.
12. **Schneider, P., L. Wolters, G. Schoone, H. Schallig, P. Sillekens, R. Hermsen, and R. Sauerwein.** 2005. Real-time nucleic acid sequence-based amplification is more convenient than real-time PCR for quantification of *Plasmodium falciparum*. J. Clin. Microbiol. **43:**402–405.
13. **Stevens, W., T. Wiggill, P. Horsfield, L. Coetzee, and L. E. Scott.** 2005. Evaluation of the NucliSens EasyQ assay in HIV-1-infected individuals in South Africa. J. Virol. Methods **124:**105–110.
14. **Tattevin, P., S. Minjolle, C. Arvieux, V. Clayessen, R. Colimon, J. Bouget, and C. Michelet.** 2002. Benefits of management strategy adjustments during an outbreak of enterovirus meningitis in adults. Scand. J. Infect. Dis. **34:** 359–361.
15. **Yoo, J. H., J. H. Choi, S. M. Choi, D. G. Lee, W. S. Shin, W. S. Min, and C. C. Kim.** 2005. Application of nucleic acid sequence-based amplification for diagnosis of and monitoring the clinical course of invasive aspergillosis in patients with hematologic diseases. Clin. Infect. Dis. **40:**392–398.