JOURNAL OF CLINICAL MICROBIOLOGY, Jan. 2003, p. 346–350 Vol. 41, No. 1
0095-1137/03/$08.00+0 DOI: 10.1128/JCM.41.1.346–350.2003

Rapid Enterovirus RNA Detection in Clinical Specimens by Using Nucleic Acid Sequence-Based Amplification

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Received 7 June 2002/Returned for modification 8 August 2002/Accepted 17 October 2002

Enterovirus (EV) detection by nucleic acid sequence-based amplification was compared with EV isolation in cell culture. The NucliSens Basic kit (bioMerieux) was utilized for RNA detection. For virus isolation, samples were inoculated into MRC-5, primary rhesus monkey kidney, A549, rhabdomyosarcoma, and/or Buffalo green monkey kidney cells.

Enteroviruses (EV) are a diverse group of small, nonenveloped RNA viruses that are transmitted largely by the fecal-oral route, that replicate in high titer in the enteric tract, and that are carried by the blood to target organs. EV meningitis is a common infection in the United States and usually has a benign outcome. Nevertheless, it leads to a large number of hospitalizations of both children and adults. In addition to meningitis, other EV neurological manifestations include encephalitis, poliomyelitis, Guillain-Barré syndrome, transverse myelitis, cerebellar ataxia, peripheral neuritis, and Reye's syndrome. Other serious EV infections that can lead to hospitalization include those causing neonatal sepsis, myocarditis, and pericarditis and those in immunocompromised hosts. The ability to rapidly differentiate EV infections from bacterial illness can reduce hospitalization time, antimicrobial usage, and diagnostic tests (9), as well as reduce the anxiety of patients and families. Furthermore, specific antiviral agents are becoming available (11).

Currently over 60 distinct EV serotypes, which differ antigenically and in their abilities to grow in various cell cultures and suckling mice, are recognized. The mainstay of EV diagnosis in diagnostic laboratories has been isolation of virus in cell culture. However, many coxsackievirus group A serotypes grow poorly or not at all in cell culture and require suckling mouse inoculation (4). Successful isolation of multiple EV serotypes increases with the number of cell lines inoculated (2, 4, 5, 7). Identification of cytopathic effect (CPE) due to an EV necessitates subpassage in culture, staining of infected cultures with pools of EV monoclonal antibodies, and/or neutralization of infectivity with type-specific antiserum. Time to detection of CPE is usually 2 to 7 days.

In contrast, nucleic acid amplification techniques can provide results within 1 day, can detect serotypes that grow poorly in cell culture, and can significantly alter the medical care offered to patients (9, 10). The target for amplification is within the highly conserved 5′ untranslated region in order to detect the broadest spectrum of EV serotypes. Most publications have focused on reverse transcription PCR (RT-PCR), including a commercial Amplicor kit from Roche Molecular Systems (8–10, 12, 14). However, the Amplicor kit is no longer available, and clinical laboratories must find other alternatives.

Recently, nucleic acid sequence-based amplification (NASBA) has been used for rapid diagnosis of EV (3; F. Zhang, C. C. Ginocchio, A. Malhotra, and C. Chakrabarti, Abstr. 18th Annu. Clin. Virol. Symp., abstr. T5, 2002). In this study, we have compared NASBA with isolation in cell culture for detection of EV in a variety of clinical samples.

MATERIALS AND METHODS

Samples. Samples submitted to the Clinical Virology Laboratory at Yale New Haven Hospital (YNHH) for EV culture from July to December of 2001 were tested by both virus isolation and EV NASBA. In total, 172 samples from 142 patients were tested by both methods: 102 cerebrospinal fluids (CSF), 15 nasopharyngeal (NP) swabs, 14 throat swabs, 13 rectal swabs, 19 stool specimens, 4 mouth swabs, and 1 each of the following: brain biopsy sample, oral lesion swab, skin lesion swab, urine sample, and serum sample. Swabs and tissue were submitted in viral transport medium (VTM) (M4; MicroTest, Inc., Lilburn, Ga.). Spinal fluids, stools, and urine were submitted in sterile containers. Patients ranged in age from 10 days to 81 years old, and 30% of patients were over 18 years of age. The normal range for CSF cell counts at YNHH was <6 nucleated cells/μl, all lymphocytes, and that for CSF protein levels was 15 to 45 mg/dl. However for neonates, cell counts of up to 42 nucleated cells/μl and protein levels of over 100 mg/dl have been reported.

Isolation. One tube each of five cell systems, primary rhesus monkey kidney (RhMK), human embryonic lung fibroblast (MRC-5), human epidermoid tumor (A549), rhabdomyosarcoma (RD), and Buffalo green monkey kidney (BGM) cells (Viromed Laboratories, Minneapolis, Minn.; Diagnostic Hybrids, Athens, Ohio), was inoculated with 0.1 to 0.2 ml of sample when sample volume permitted. Culture tubes were incubated at 35°C in a roller drum for 14 days. Cultures showing CPE were passaged to fresh culture tubes and were identified by immunofluorescence by using EV monoclonal antibody pools (Chemicon International, Temecula, Calif.).

NASBA procedure. The NucliSens Basic kit (bioMerieux, Durham, N.C.) was utilized for RNA detection, and the EV NASBA protocol (Zhang et al., Abstr. 18th Annu. Clin. Virol. Symp.), available on the NucliSens website, was followed.

(i) Sample processing. CSF samples and NP and rectal swabs in VTM were vortexed. For stool samples, a 10% (wt/vol) suspension was prepared in VTM, frozen at −70°C, thawed rapidly, vortexed, and centrifuged at 2,000 × *g* for 15 min and the clarified supernatant was used. If testing was delayed, the specimens were frozen at −70°C.

(ii) IC. An EV-specific internal control (IC), provided by bioMerieux and consisting of cloned EV sequences modified to contain a 20-bp fragment of potato leafroll virus, was added to each specimen in order to evaluate RNA extraction and amplification efficiency. The IC sequence is also available on the company website. The IC was coamplified with the test sample by using a single pan-EV-specific primer pair. The single-stranded RNA products were differentiated and detected by hybridization with either wild-type (WT) EV or IC

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TABLE 1. Comparison of EV NASBA and culture results by sample type

Sample type	Total no. tested	No. of samples:						Total no. (%):	
		NASBA$^+$ culture$^+$	NASBA$^+$ culture$^-$	NASBA$^-$ culture$^+$	NASBA$^-$ culture$^-$	NASBA invalid[a] culture$^+$	NASBA invalid[a] culture$^-$	NASBA invalid[a]	Positive by NASBA or culture
CSF	102	21	10	0	69	0	2	2 (2)	31 (30)
NP or throat	29	5	0	3[b]	13	0	8	8 (28)	8 (28)
Rectal or stool	32	8	0	0	19	2	3	5 (16)	10 (31)
Other[c]	9	0	0	0	8	0	1	1 (11)	0
Total	172	34	10	3[b]	109	2	14	16 (9)	49 (29)

[a] Samples testing invalid by NASBA were reextracted and reamplified at least once before the final result was obtained.
[b] These culture isolates were ultimately identified as parechovirus and not enterovirus.
[c] Four mouth swabs and one each brain biopsy sample, oral lesion swab, skin lesion swab, urine sample, and serum sample.

capture probes, followed by hybridization with a generic ruthenium-labeled detection probe that is provided in the NucliSens Basic kit. Extensive work by others (C. C. Ginocchio et al., unpublished), as well as our laboratory (data not shown), showed that in the absence of inhibitors EV NASBA consistently detected 10 copies of EV RNA per amplification reaction. To detect inhibition, 200 copies of IC per amplification reaction were included.

(iii) Nucleic acid isolation. RNA was isolated from specimens by a modified Boom extraction (1). Briefly, 100 (stools) or 200 μl (swabs and CSF) of specimen and 20 μl of IC (2,000 copies) were 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 for 10 min at room temperature. After centrifugation, the silica pellet was washed five times, twice with NASBA wash buffer, twice with 70% ethanol, and once with acetone. The silica pellet was then dried at 56°C. Elution buffer (50 μl) was added, the pellet was resuspended, and nucleic acids were eluted by incubation at 56°C. After centrifugation, 5 μl of the extracted nucleic acid was taken for amplification.

(iv) Primers and probes. Primers and target-specific probes were purchased from Operon Technologies, Inc. (Alameda, Calif.). Primers were directed to the 5′ noncoding region of the EV genome (poliovirus type 1, strain Sabin 1) and modified for use in the NASBA system. The primers used were P1.3 (5′-AATTCTAATACGACTCACTATAGGGCACCGGATGGCCAATCCA-3′ [the underlined portion indicates the overhang portion encoding the T7 RNA polymerase promoter, as required in NASBA]) and P2.2 (5′-GATGCAAGGTCGCATATGAGGGTGTGAAGAGCCTATTGAG-3′ [the underlined sequence is complementary to the generic ECL detection probe sequence]). The probes used were a WT EV-specific capture probe (5′-biotin label-CTCCGGCCCCTGAATGCGGGCTAAT-3′ and an IC-specific capture probe (5′-biotin label-GCAAAGTATCATCCCTCCAG-3′).

Both the EV- and IC-specific capture probes were coupled to streptavidin-coated paramagnetic beads according to the manufacturer's instructions. A generic electrochemiluminescent (ECL) detection probe, provided in the Basic kit, consisted of a ruthenium-labeled DNA oligonucleotide homologous to the 5′ end of the P2 primer.

(v) Amplification. NASBA amplification was performed in a 20-μl reaction mixture that contained 5 μl of extracted nucleic acid, 5 μl of enzyme solution (avian myeloblastosis virus reverse transcriptase, *E. coli* RNase H, T7 RNA polymerase), 70 mM KCl, 0.2 μM primers, and the following nucleotides: 2.0 mM ATP, CTP, and UTP; 1.5 mM GTP; 0.5 mM ITP; and 1.0 mM dATP, dCTP, dGTP, and dTTP. The amplification was allowed to proceed for 2.5 h at 41°C in a circulating water bath.

(vi) Detection. The general NASBA detection principle was as follows. An aliquot of the amplification reaction mixture was added to a hybridization solution containing both the capture probe-saturated paramagnetic beads and the ruthenium-labeled ECL oligonucleotide. After incubation, the paramagnetic beads carrying the hybridized amplicon-ECL probe complexes were captured on the surface of an electrode by means of a magnet. Voltage applied to this electrode triggered the ECL reaction. The light emitted by the hybridized ruthenium-labeled probes was proportional to the amount of amplicons generated in the amplification reaction. An ECL signal above the cutoff level indicated the presence of target (i.e., EV) RNA. The ECL signal for the IC RNA was used to determine the validity of the assay run.

Specifically, EV amplification products were diluted 1:10 and then detected by hybridization in two separate reaction mixtures containing two different probes, one specific for the WT EV product and the second specific for the IC product; both reaction mixtures contained the generic ruthenium-labeled ECL detection probe. Hybridization was carried out at 41°C in a circulating water bath for 30 min. Results were obtained by analysis of the ECL hybridization products using the NASBA QR system and the NucliSens Basic kit software program (13).

(vii) Interpretation. Prior to this study, the ECL positive cutoff value was set at three or more times the highest ECL value for a negative sample, after multiple runs of both negative CSF samples and CSF samples containing non-EV viral isolates (data not shown). The indeterminate zone was established at an ECL value of more than two times but less than three times the highest negative ECL value. The minimum IC signal was based on the repeated ability to detect EV RNA down to 10 copies when IC was greater than 19,000 ECL counts. To be conservative, the IC cutoff was set at 2.5 times the minimum value, or 50,000 ECL counts.

ECL readings were evaluated for both the WT EV ECL signal and the IC signal. Specimens with WT signals ≥650 ECL units were considered positive for EV RNA, regardless of the IC signal. Specimens with WT signals <450 ECL counts and IC signals ≥50,000 ECL counts were considered negative for EV RNA and not inhibitory. Specimens with WT signals <450 ECL units but whose IC signal was <50,000 ECL units were considered inhibitory to amplification. When a NASBA result could not be obtained due to inhibition of the amplification reaction, the sample was reextracted and retested. If a valid result could not be obtained despite retesting, the final NASBA result was classified as "invalid." WT signals of 450 to 649 ECL counts were considered indeterminate, and the specimens were retested (Zhang et al., Abstr. 18th Annu. Clin. Virol. Symp.).

Parechovirus RT-PCR. Three cell culture harvests from NP samples that were NASBA negative yet designated as EV by growth in culture were tested by a parechovirus RT-PCR at the Centers for Disease Control and Prevention, courtesy of Mark Pallansch and Steven Oberste.

Resolution of discrepancies. In cell culture, typical CPE confirmed by subculture and immunofluorescence was considered a true positive. In addition, virus isolates obtained from NASBA-negative or invalid samples were tested by EV NASBA. A NASBA-positive, culture-negative sample was considered a true positive in one of the following circumstances: (i) EV was detected by culture and NASBA in another sample from that patient or (ii) the CSF profile was typical for aseptic meningitis with elevated nucleated cells and protein, normal glucose, a negative Gram stain, and negative bacterial cultures; the clinical illness was compatible; and the NASBA result was repeated and confirmed.

RESULTS

Comparison of EV NASBA and culture results. Of the 172 samples tested by both methods, 46 were positive for EV and 3 were positive for parechoviruses, including 31 CSF samples, 8 NP or throat samples, and 10 rectal or stool samples (Table 1). Of the 46 EV-positive samples, NASBA detected 44 (96%) and culture detected 36 (78%). All 31 positive CSF samples were detected by NASBA. However, 10 of the NASBA-positive CSF samples were culture negative. Five culture-positive samples were NASBA negative or invalid. Three NASBA-negative isolates with EV-like CPE, from throat or NP samples, were identified as parechovirus by RT-PCR at the Cen-

ters for Disease Control and Prevention. EV were also isolated from two NASBA-invalid stool samples. The culture isolates from these two stool specimens were successfully amplified by NASBA. One of these two patients also had an EV-positive CSF sample by both culture and NASBA.

The frequency of invalid results due to inhibition of the NASBA reaction was troublesome. The initial NASBA test result was invalid for 31 samples, including 9% of CSF samples, 22% of rectal and stool samples, and 45% of throat and NP samples. After reextraction and reamplification, NASBA results for 16 of 172 samples remained invalid: 2% of CSF samples, 16% of rectal or stool samples, and 28% of NP or throat samples.

NASBA results were reported within 24 h for 61% of samples and within 48 h for 73%. NASBA was not done on weekends, so samples arriving after 9 a.m. Friday were not reported until Monday afternoon. The necessity to repeat NASBA testing for 18% of samples due to invalid results also lengthened time to reporting.

The average time to reporting positive cultures was 6 days (range, 2 to 14 days). Only 50% of positives were reported by day 5, and 90% were reported by day 11. For the 133 negative cultures, the time to reporting was 14 days.

Validity and ease of interpretation of NASBA results. As recommended by C. Ginocchio in collaboration with the manufacturer (Zhang et al., Abstr. 18th Annu. Clin. Virol. Symp.), a positive EV result was determined by a WT ECL of ≥650 ECL counts. For a valid negative result, an IC ECL value of ≥50,000 ECL counts was required. Of 172 samples tested, final NASBA results were 44 positive, 112 negative, and 16 invalid.

For all 44 positive samples, the WT ECL was >1,000 ECL counts, and for 41 samples (93%), the WT ECL was greater than 2,000 ECL counts. The range of positive values was 1,072 to 1,358,000 ECL counts, with a median of 44,000 ECL counts. In contrast, 93 of the 112 negative samples had WT ECL values of 1 ECL count, and all had values of less than 50 ECL counts. The highest negative WT ECL result was 47 ECL counts, and a rhinovirus was subsequently isolated from this sample. Thus, differentiating positives from negatives was clear-cut. There were no indeterminates in this study.

For samples called NASBA negative, IC ECL values were >100,000 ECL counts in 95% of cases, with a median ECL value of >500,000 ECL counts.

However, one CSF sample negative by these criteria (WT ECL = 1 ECL count; IC ECL = 57,000 ECL counts) was from a child with aseptic meningitis for whom EV was detected by both NASBA and culture in throat and stool. The patient's CSF had not originally been submitted to the Clinical Virology Laboratory for testing but was obtained from the Chemistry Laboratory refrigerator 3 days after collection. The culture of this CSF sample was subsequently negative. However, due to the EV-positive throat and stool from this patient and an IC ECL value just above the cutoff, the CSF was retested by NASBA three times. The second result was identical to the first, but on the third attempt, the WT ECL was 1,027 ECL counts and the IC ECL was 314,665 ECL counts. For the purpose of the study, the first valid negative result was taken. It may be that the IC value of 57,000 ECL counts indicated some degree of inhibition, since when an IC ECL of >300,000 ECL counts was obtained, a positive WT ECL signal was detected. However, it is also possible that the false-negative results were due to sampling error in a borderline sample.

For the 47 invalid results, 31 invalid on initial testing and 16 still invalid after repeat testing, 43 (92%) had IC ECL values of <30,000 ECL counts and 32 (68%) had IC ECL values of <10,000 ECL counts. Four of 47, however, had IC ECL values of 46,021 to 49,898 ECL counts, close to the 50,000-ECL-count cutoff. Two of these four borderline IC results were on a single CSF sample, which, on being tested a third time, had an IC value of 555,513 ECL units but remained EV negative.

Of note, three rhinoviruses, one parainfluenza virus 1, and one herpes simplex virus type 1 were isolated from samples that tested negative by NASBA.

Use and performance of different cell cultures for EV isolation. While the goal was to inoculate five cell lines during the peak season (June through October) when EV was suspected, the actual number of cell lines inoculated was determined by the sample volume available. In November and December, only three cell lines (MRC-5, RhMK, and A549) were routinely inoculated. Of the 39 samples (31 CSF, 8 NP or throat, 10 rectal or stool) from which EV or parechovirus was isolated, 18 were inoculated into all five cell lines, 11 were inoculated into four cell lines, 7 were inoculated into three cell lines, and 3 were inoculated into two cell lines. In contrast, for the 10 NASBA-positive but culture-negative CSF samples, only 1 was inoculated into all five cell types and 5 were inoculated into only one or two cell types. Insufficient sample volume was exclusively a problem with CSF.

As shown in Table 2, the utility of a particular cell line varied. Each cell line inoculated was the first to show CPE for some of the samples, and each cell line was also the only cell line to show CPE for some samples. Of note, for 22 of the 39 culture-positive samples, only one cell line was positive. Another surprising finding during the study season was the utility of A549 cells. A549 cells were the least frequently inoculated (25 of the 39 positive samples); however, they had the highest positive rate (17 of 25 or 68%). In contrast, BGM and RD cells, added during peak EV season specifically to enhance EV recovery, had the lowest positive rates during the study period. Nevertheless, they were the only cell lines positive in 4 of 39 cases. The three parechoviruses were recovered in BGM and/or A549 cells.

Patient characteristics and spinal fluid profiles. The ages of positive patients were as follows: 61% were 1 year old or younger, 90% were 18 years old or younger, and 10% were over 18 years old.

The 31 EV-positive CSF samples had a median of 218 nucleated cells/μl (range, 10 to 1,650 nucleated cells/μl) and a median CSF protein level of 53 mg/dl (range, 26 to 122 mg/dl). Nine of the EV-positive CSF samples had protein values in the normal range.

The 71 EV-negative CSF samples had a median of 2 nucleated cells/μl (range, 0 to 930 nucleated cells/μl) and a median CSF protein level of 38 mg/dl (range, 8 to 295 mg/dl). There were 31 EV-negative CSF samples that had an elevated cell count or protein level or both. Nine were from neonates, and the CSF values fell within a range acceptable for neonates. Of the remaining 22, 9 had mildly elevated protein levels only and 4 had elevated cell counts of 12, 14, 20, and 52 nucleated cells/μl. The last of these was from a child with a history of

TABLE 2. Use and performance of different cell cultures for samples detected by culture and/or NASBA

Cell culture used for EV isolation	Culture-positive samples (n = 39)[a]				No. (%) of culture-negative, NASBA-positive CSF samples (n = 10) inoculated into cell line[d]
	Total no. (%) of positive samples inoculated into cell line	No. (%) EV positive	No. (%) of times first positive cell line	No. (%) of times only positive cell line	
MRC-5[b]	39 (100)	15 (38)	6 (15)	3 (8)	9 (90)
RhMK[b]	39 (100)	22 (51)	14 (36)	9 (23)	8 (80)
A549[b]	25 (64)	17 (68)	11 (44)	6 (15)	3 (30)
BGM[c]	30 (77)	7 (23)	7 (23)	2 (7)	3 (30)
RD[c]	28 (72)	9 (32)	5 (18)	2 (7)	3 (30)

[a] Thirty-six EV and 3 parechoviruses were isolated; parechoviruses were isolated in A549 and/or BGM cells.
[b] Cell cultures used year-round.
[c] Cell cultures used only during peak EV season (June to October).
[d] Only one sample was inoculated into all five cell types; two samples each were inoculated into two, three, or four cell types; three samples were inoculated into only one cell type.

fever and severe headache 2 weeks prior to coming to the emergency department. While the diagnosis was presumed viral meningitis, the CSF was obtained late in the course of illness. Nine samples had both elevated cell counts and protein levels. The five patients with the highest CSF cell counts (78 to 930 nucleated cells/μl) and protein levels (63 to 295 mg/dl) were diagnosed with varicella-zoster myelitis, EV meningitis (isolated from throat and stool and detected by NASBA on repeat amplification), central nervous system Lyme disease, aspergillosis involving the brain, and *Haemophilus influenzae* meningitis. One additional patient had encephalitis of uncertain etiology after an extensive work-up. Two immunocompromised hosts with mildly abnormal CSF profiles had multiple negative cultures and no clear etiology. One patient had a clinical course compatible with viral or Lyme meningitis but had an incomplete laboratory work-up.

DISCUSSION

EV NASBA performed very well during the study period, detecting all 31 positive CSF samples, including 10 CSF samples that were culture negative. No false-positive EV NASBA results were observed, and one sample containing parainfluenza virus 1, one containing herpes simplex virus type 1, and three containing rhinovirus tested negative by NASBA. EV NASBA failed to detect parechovirus in three upper respiratory tract samples that were isolated in culture. Failure to detect parechovirus 1 and 2 (formerly echovirus 22 and 23) is a problem common to all molecular assays using broadly reactive EV primers from the 5′ untranslated region (10). Parechoviruses have recently been reclassified as a separate genus and are no longer considered EV (6). However, since they share clinical, epidemiologic, and cell culture characteristics with EV, the failure of EV molecular tests to detect parechovirus is a disadvantage.

A major reason for false-negative CSF cultures in this study appeared to be inadequate CSF for comprehensive culture. Due to different growth patterns and multiple circulating EV types, five different cell cultures were inoculated during the peak season. However, in actual practice the 0.5 to 1.0 ml of CSF needed to inoculate a spectrum of cell types is often not available. In contrast, 0.1 to 0.2 ml of sample is sufficient for molecular testing. Over one-half of the positive isolates in this study grew in only one of the inoculated cultures, emphasizing the need to use multiple cell lines to maximize recovery. RhMK, MRC-5, RD, and BGM cells are recognized to be useful in the isolation of a spectrum of EV (2, 4). RD and BGM have been reported to increase the isolation rate and reduce time to recovery for coxsackievirus group A and echoviruses (RD cells) and for coxsackievirus group B (BGM cells) (2). A549 cells are primarily useful for the isolation of adenovirus, herpes simplex virus, and varicella-zoster virus, and few papers have evaluated their role in the isolation of EV (5, 7). Thus, the EV isolation rate for A549 during the test period was unexpected, as were the relatively modest results obtained with RD and BGM cells.

The primary reason that NASBA was implemented in our laboratory instead of RT-PCR was the shorter time to result for NASBA (6.5 h) compared to that for the commercially available EV RT-PCR kit (9 h). Thus NASBA could be completed the same day for samples received by 9 or 10 a.m. Since patients are usually discharged by 48 h, a result in the first 24 h of hospitalization will have the greatest impact on treatment and will reduce unnecessary tests. With EV NASBA, 61% of results were reported within 24 h and 73% were reported within 48 h. Longer delays were due to lack of test availability from Friday after 9 a.m. until Monday morning and the need to repeat testing on 18% of the samples in which amplification was inhibited.

Only one of 39 positive cultures was reported within 48 h, and only 50% were reported by day 5. Culture results for 133 negatives and one positive (78% of all samples) were not reported until day 14. Many laboratories discard samples at day 10 of observation rather than day 14. Had we read cultures for only 10 days, we would have missed 6 (15%) of our culture positives, conferring an even greater advantage to EV NASBA results.

Previous investigators who have devoted substantial effort to validate of the sensitivity and specificity of EV NASBA have used different primer pairs, one set derived from coxsackievirus A24 (3) and one set from poliovirus 1 (Zhang et al., Abstr. 18th Annu. Clin. Virol. Symp.). In this study we have used the poliovirus 1 primers and the IC recommended by Zhang et al. (Abstr. 18th Annu. Clin. Virol. Symp.) and provided on the company's website. The objective readout of ECL units and the clear differentiation of positive from negative results made

interpretation clear-cut. The availability of the IC was critical in detecting failed amplification, since NASBA was invalid on initial testing for 18%, and on final testing for 9%, of samples. Failure of amplification by NASBA was most troublesome for throat and NP samples, which resulted in repeat testing, increased costs, and delayed reporting. The reason for the high frequency of inhibition for throats and stools in particular was not clear. Dilution of samples 1:10 prior to extraction did not consistently help. With freezing and thawing of samples, a reduction in inhibition was noted. The role of base matrix or VTM in inhibition of amplification is under investigation. A parallel study of RT-PCR in our laboratory using the same RNA extracts found that PCR was less susceptible to inhibition (D. Ferguson, R. Garner, and M. L. Landry, Abstr. 18th Annu. Clin. Virol. Symp., abstr. T8, 2002). It is possible that use of three enzymes in the EV NASBA, instead of one or two in RT-PCR, may make NASBA more susceptible to inhibitors. A recent publication on EV NASBA did not report invalid results, but an IC was not used, and thus inhibition of amplification may not have been recognized (3). It is also possible that the invalid rate could be reduced with an automatic extraction procedure. Last, perhaps an IC ECL cutoff of 50,000 ECL counts is too conservative. However, reducing the IC ECL cutoff to 30,000 ECL counts would have changed the initial invalid rate from 18 to 16%, not a major difference.

EV NASBA failed to detect a low-positive CSF that was received by the Clinical Virology Laboratory 3 days after collection. This sample was also culture negative; hence it was not classified as a false negative. However, since a positive result was anticipated based on EV detection in the throat and stool of the patient, the sample was reextracted and retested to yield a low-positive result on the third attempt. Delayed processing or improper sample storage may have contributed to this result, which highlights a general concern that RNA and infectious virus may be lost when samples are not processed promptly, despite holding at 4°C or even storage at −70°C. Validation of the EV NASBA in our laboratory involved retesting known EV culture-positive clinical specimens stored at −70°C from previous seasons. While NASBA detected more positives than culture after storage at −70°C, EV could not be detected by either NASBA or culture in a number of samples (data not shown). This was surprising since EV are considered to be stable for weeks at 4°C and for years at −70°C. However, similar problems with archival samples have been reported by another group (14). While loss of virus is more likely associated with multiple freeze-thaw cycles, suboptimal handling could contribute to falsely negative results in samples such as CSF that may contain low titers of virus.

In conclusion, EV NASBA was significantly more sensitive and more rapid than culture in detecting EV in clinical specimens. The NucliSens Basic kit facilitated the implementation of molecular testing for EV in our laboratory, and the short assay time for NASBA allowed same-day reporting.

ACKNOWLEDGMENTS

We gratefully acknowledge Christine Ginocchio of North Shore University Hospital for providing invaluable help and advice, Mark Pallansch and Steve Oberste of the CDC for identifying the parechoviruses, the virology laboratory staff for their expert technical work, and Maria Hernandez for her careful assembly of the laboratory data.

ADDENDUM IN PROOF

In contrast to results obtained in 2001, in 2002 invalid NASBA results were obtained for only 3 of 134 (2%) of CSF samples upon initial testing. Furthermore, all three CSF samples with initially invalid results had valid results upon repeat NASBA testing. Since only four non-CSF samples were tested by NASBA in 2002, insufficient data were obtained for analysis. The reason for the reduction in the rate of invalid results for CSF samples between the years 2001 and 2002 is not clear. However, the explanation may be increased expertise with the manual Nuclisens extraction procedure.

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