

Detection of enterovirus RNA in cerebrospinal fluid (CSF) using NucliSens EasyQ Enterovirus assay

S.E. Capaul*, M. Gorgievski-Hrisoho¹

Institute for Infectious Diseases, University of Bern, Friedbühlstrasse 51, CH-3010 Bern, Switzerland

Accepted 20 August 2004

Abstract

Rapid detection of enterovirus (EV) infections is essential in the management of aseptic meningitis. Molecular approaches have opened the way to such rapid, but also specific and sensitive, diagnostic tests. The aim of this study was to compare the performance of the CE marked NucliSens EasyQ Enterovirus assay with an in-house two-step RT-PCR assay using cerebrospinal fluid (CSF) and throat swab samples. In addition, specificity was tested with clinical isolates positive for viruses with clinical importance in CSF samples.

For nucleic acid extraction, the NucliSens miniMAG and NucliSens magnetic extraction reagents were used. Subsequently real-time nucleic acid sequence-based amplification (NASBA) RNA amplification was performed using NucliSens EasyQ basic kit reagents and NucliSens EasyQ Enterovirus reagents. An EV-specific internal homologous control (IC) RNA was used to monitor the entire NucliSens EasyQ procedure at the individual sample level. No IC but an external inhibition control was available for the RT-PCR method. For the NucliSens EasyQ procedure, amplification and real-time detection reactions were carried out in the NucliSens EasyQ analyzer. The real-time NASBA enterovirus detection was based on NASBA amplification and real-time molecular beacon technology. Data were analyzed using the manufacturer's software on the NucliSens EasyQ analyzer. For the in-house assay, RT-PCR amplicons were detected using agarose gel analysis.

The analysis of clinical samples positive for HSV-1, HSV-2, adenovirus, CMV, VZV, mumps and rhinovirus were all negative by NucliSens EasyQ Enterovirus assay. Three rhinovirus samples were, however, strongly positive in RT-PCR.

A total of 141 clinical samples were retrospectively tested, including 126 cerebrospinal fluid (CSF) samples and 15 throat swabs. The 91 CSF samples were negative by both methods, 31 CSF samples and 14 throat swab samples were positive by both methods. The four CSF samples were positive by RT-PCR only. One throat swab sample was negative in NucliSens EasyQ but positive in RT-PCR.

The sensitivity and specificity of both methods seem to be more or less comparable. However, the in-house RT-PCR assay appears to amplify some rhinovirus strains and should therefore not be used for throat swab samples. NucliSens EasyQ Enterovirus assay gave more invalid results than the in-house RT-PCR, which is obvious taken into account the difference in quality control between the CE marked NucliSens EasyQ Enterovirus assay and the in-house enterovirus assay. The NucliSens EasyQ procedure can be completed within 5 h versus 9.5 h for the RT-PCR. NucliSens EasyQ Enterovirus assay showed to be a standardized, rapid, specific, sensitive and reliable procedure for the detection of enterovirus RNA.

© 2004 Elsevier B.V. All rights reserved.

Keywords: Enterovirus; Molecular diagnosis; RT-PCR; NASBA; NucliSens EasyQ

Abbreviations: CMV, cytomegalovirus; CSF, cerebrospinal fluid; ECL, electrochemiluminescence; f, female; HSV-1, herpes simplex virus type 1; HSV-2, herpes simplex virus type 2; EV, enterovirus; IC, internal homologous control; m, male; NASBA, nucleic acid sequence based amplification; PCR, polymerase chain reaction; RNA, ribonucleic acid; RT, reverse transcriptase; RT-PCR, reverse-transcribed polymerase chain reaction; TCID₅₀, tissue culture infective dose 50%; WT, wildtype

* Corresponding author. Present address: Lochmühleweg 10A, 4950 Huttwil, Switzerland.

E-mail addresses: capaul.frischknecht@freesurf.ch (S.E. Capaul), meri.gorgievski@ifik.unibe.ch (M. Gorgievski-Hrisoho).

¹ Tel.: +41 31 632 35 62.

1. Introduction

Enteroviruses (EV) are important pathogens with different manifestation. EV are small RNA viruses that are transmitted by the rectal–oral route, that replicate in high titer in the enteric tract, and that are carried by the blood to target organs (Landry et al., 2003a). EV consists of more than 60 different serotypes. While most infections either are asymptomatic or result in minor illnesses, aseptic meningitis, neonatal sepsis, myocarditis or pericarditis lead to a large number of hospitalizations. Enteroviruses are the most important viruses causing aseptic meningitis (Gorgievski-Hrisoho et al., 1998). The ability to rapidly differentiate EV infections from bacterial illness can reduce hospitalization time, antimicrobial usage and diagnostic tests.

The mainstay of the enterovirus diagnosis is the virus isolation in cell culture. However, the turnaround time for cell culture is usually 5–7 days for positive results and 10 days for negative reports (Gorgievski-Hrisoho et al., 1998). It is also known that some of the EV serotypes, especially coxsackievirus group A do not grow well or not at all in tissue culture (Hsiung, 1994; Landry et al., 2003a). In contrast, sensitive nucleic acid amplification techniques can provide results within 1 or 2 days, can detect serotypes that grow poorly in cell culture, and can thus significantly alter the medical care offered to patients. Many procedures that bases on reverse-transcribed polymerase chain reaction (RT-PCR) have been described in the past (Glimaker et al., 1993; Rotbart, 1990; Zoll et al., 1992). Because the EV group is genetically so divergent all the published amplification methods are targeted within the highly conserved 5′ non-coding region in order to detect the broadest spectrum of EV serotypes (Lai et al., 2003)

The nucleic acid sequence-based amplification (NASBA; bioMérieux, Boxtel, The Netherlands) is targeted at RNA. It makes use of the simultaneous enzymatic activities of avian myeloblastosis virus reverse transcriptase, RNase H, and T7 RNA polymerase under isothermal conditions (Compton, 1991). New CE marked NucliSens EasyQ Enterovirus assay working on the NucliSens EasyQ analyzer are also targeted within the 5′ non-coding region of the enterovirus genome and were designed in order to obtain broad serotype specificity in combination with a high sensitivity. The aim of this study was to compare the performance and convenience of the CE marked Nuclisens EasyQ Enterovirus assay with an in-house two-step RT-PCR assay using CSF and throat swab samples. In addition, specificity of both methods was tested with clinical isolates positive for viruses with clinical importance in CSF samples and with genetically closely related rhinovirus group.

2. Materials and methods

2.1. Samples

The 126 CSF samples including 35 enterovirus positive and 91 enterovirus negative samples were included retro-

spectively to this study. The samples were collected during a time period from June 2002 to August 2003. The samples were submitted to the university hospital laboratory for enterovirus analysis from patients with clinical description of aseptic meningitis. Additional 15 enterovirus positive throat swab samples were included to this comparison study. The samples were originated from 93 male and 48 female patients. The 52 samples originated from adults over 18 years, and 89 from children under 18 years old. All the samples were original analyzed with the in-house enterovirus RT-PCR assay. The 71 samples originated from Bern and the rest from different university and local hospitals in Switzerland. All the samples were stored at -70°C . Local ethical approval guidelines were followed for use of clinical material and access to diagnostic results.

2.2. Nucleic acid extraction

The nucleic acid extraction of CSF and throat swab samples was performed with the NucliSens miniMAG and the NucliSens magnetic extraction reagents. The extraction was done according manufacturer's instructions (bioMérieux, Geneva, Switzerland). The sample volume was 200 μl and the elution volume 25 μl . An EV-specific internal homologous control (IC) RNA was included to each sample during the extraction. This nucleic acid extract was used for both amplification methods.

2.3. Real-time NASBA enterovirus amplification and detection

NucliSens EasyQ (real-time NASBA) enterovirus RNA amplification was performed using NucliSens EasyQ basic kit reagents (containing generic reagents like enzymes and nucleotides for real-time NASBA) and NucliSens EasyQ Enterovirus reagents (containing enterovirus specific amplification primers and molecular beacons, enterovirus internal control RNA and Basematrix) according to the manufacturer's instructions (bioMérieux, Geneva, Switzerland). The input volume was 5 μl nucleic acid extract. An EV-specific internal homologous control RNA was measured to monitor the entire NucliSens EasyQ procedure at the individual sample level. Amplification reactions were carried out in the NucliSens EasyQ analyzer. The real-time NASBA enterovirus detection was based on molecular beacons. Two different molecular beacons were used, one specific for the WT enterovirus amplicon and one for the internal control amplicon. The use of two fluorescent dyes (6-FAM for WT, and 6-ROX for the internal control) allows the synthesis of target and internal control RNA to be followed individually. Data were analyzed using the manufacturer's NucliSens EasyQ director software on the NucliSens EasyQ analyzer.

Table 1
Comparison of EV real-time NASBA and EV RT-PCR

	NASBA EV assay	
	Positive	Negative
EV RT-PCR positive	31 CSF; 14 throat swab	4 CSF; 1 throat swab
EV RT-PCR negative	0 CSF; 0 throat swab	91 CSF; 0 throat swab

2.4. RT-PCR for amplification of enterovirus RNA

An in-house RT-PCR for enterovirus detection is based to a work of Rotbart (1990). The RT reaction was carried out with reverse primer MD90 (5'-ATTGTCACCATAAGCAGCCA) and OmniScript reverse transcriptase reagents according to manufacturer's instructions (Qiagen, Basel, Switzerland). RT-reaction was performed with 12 µl nucleic acid eluat and 8 µl RT reagents. The following PCR amplification was carried out with forward primer MD91 (5'-CCTCCGGCCCCTGAATGCGGCTAAT) and reverse primer MD90. The 10 µl of RT-product and 90 µl PCR reagents were performed to the amplification step consisting of 40 cycles with following program: 95 °C for 1 min, 60 °C for 1 min, and 72 °C for 1 min. The amplicons were detected using agarose gel analysis. For every sample we performed an external universal inhibition control in real-time PCR using TaqMan Exogenous IPC reagents (Applied Biosystems, Rotkreuz, Switzerland).

2.5. Specificity studies

The specificity of NucliSens EasyQ Enterovirus assay and of the enterovirus in-house RT-PCR was tested with clinical isolates positive for viruses with clinical importance in CSF samples and with genetically very similar rhinoviruses. The definitive identification of the viruses was originally confirmed with monoclonal antibodies. Three HSV-1, three HSV-2, three adenovirus, three CMV, three VZV isolates, nine Rhinovirus and two mumps type strains, Jeryl Lynn and Enders, were extracted as upon described and amplified parallel with NucliSens EasyQ Enterovirus assay and with in-house RT-PCR.

3. Results

3.1. Comparison of EV real-time NASBA and EV RT-PCR results

The 126 CSF samples and 15 throat swab samples were extracted with NucliSens miniMAG and parallel analyzed with EV real-time NASBA and EV in-house RT-PCR. As shown in Table 1 91 CSF samples were negative by both methods. The 31 CSF samples and 14 throat swab samples were positive by both methods. The four CSF samples and one throat swab sample were positive by RT-PCR only. All

Table 2
Patients with positive and discrepant results

Sample identification	Age	Sex	Sample	NASBA EV	RT-PCR
Be004	13	f	CSF	+	+
Be008	<1	m	CSF	+	+
Be012	<1	f	CSF	+	+
Be016	14	m	CSF	–	+
Be018	6	f	CSF	+	+
Be019	5	m	CSF	+	+
Be023	5	m	CSF	+	+
Be027	15	m	CSF	+	+
Be030	10	m	CSF	+	+
Be034	16	m	CSF	+	+
Be038	8	m	CSF	+	+
Be042	1	m	Throat swab	+	+
Be045	1	m	CSF	+	+
Be049	4	f	CSF	+	+
Be053	36	f	CSF	–	+
Be057	1	m	CSF	+	+
Be060	1	m	CSF	+	+
Be064	1	m	CSF	+	+
Be068	5	f	CSF	+	+
Be071	<1	m	Throat swab	+	+
Be075	9	m	Throat swab	+	+
Be079	7	m	CSF	+	+
Be082	<1	f	CSF	+	+
Be083	<1	f	CSF	+	+
Be087	5	m	CSF	+	+
Be090	9	m	CSF	+	+
Be094	6	f	CSF	+	+
Be099	<1	m	CSF	+	+
Be105	8	m	CSF	+	+
Be106	7	m	CSF	+	+
Be110	9	m	CSF	+	+
Be114	<1	m	CSF	+	+
Be118	6	m	CSF	+	+
Be120	<1	f	CSF	–	+
Be121	6	m	CSF	+	+
Be126	7	m	Throat swab	+	+
Be130	<1	m	Throat swab	+	+
Be136	<1	m	Throat swab	+	+
Be139	<1	m	Throat swab	+	+
Be148	<1	m	Throat swab	+	+
Be151	3	f	Throat swab	+	+
Be155	9	m	Throat swab	+	+
Be159	5	m	Throat swab	+	+
Be163	<1	f	Throat swab	+	+
Be166	4	m	Throat swab	+	+
Be170	5	m	CSF	–	+
Be172	5	f	CSF	+	+
Be175	7	m	CSF	+	+
Be177	<1	f	Throat swab	–	+
Be179	<1	f	Throat swab	+	+

Age in years: <1, less than 1-year-old. Sex: f, female; m, male. Sample: CSF, cerebrospinal fluid. NASBA EV, RT-PCR: +, positive; –, negative.

the positive and discrepant samples with patient age, sex and results of both analysis can be seen in Table 2.

3.2. Specificity testing

HSV-1, HSV-2, adenovirus, CMV and VZV isolates, three of each, were all negative by EV NASBA and by EV RT-PCR. Two mumps type strains, Jeryl Lynn and Enders, were also

negative by both methods. All nine rhinovirus isolates were negative by real-time NASBA method but three were strongly positive in RT-PCR.

3.3. Patient characteristic

The 45 samples were positive with both methods. The 41 (45) out of these originated from children under 10 years old. The 4 (45) positive samples originated from children between 10 and 18 years old. None of the positive sample (with both methods positive) originated from adults over 18 years.

3.4. Comparison of the performance of the EV real-time NASBA and EV RT-PCR assays

The NucliSens EasyQ Enterovirus assay can be completed within 5 h versus 9.5 h for the RT-PCR divided in two working days. The enterovirus specific internal control provides control at the individual sample level. For the in-house RT-PCR method only an external inhibition control was available which does not control the extraction step. The real-time NASBA results were analyzed using the manufacturer's NucliSens EasyQ director software on the NucliSens EasyQ analyzer and were classified as positive, negative or invalid in case the internal control was not detected or the signal was too weak. Compared to the subjective reading of the positive and weak positive bands after RT-PCR in agarose gel the NASBA results were easy and clear to interpret. Some invalid samples (data not shown) were found with NASBA method which were not invalid with the universal inhibition control in real-time PCR. NucliSens EasyQ is a closed tube format, which minimizes the risk of amplicon contamination and is therefore more secure than the RT-PCR where the tubes has to be opened after PCR reaction.

4. Discussion

The results in this comparative study showed that the sensitivity and specificity of the NucliSens EasyQ Enterovirus assay and the in-house two-step RT-PCR assay were more or less comparable. The 141 clinical samples were retrospectively tested, including 126 CSF samples and 15 throat swabs. The four CSF samples and one throat swab sample were positive by RT-PCR only. The discrepant CSF samples were probably samples with very low viral load and the throat swab sample cannot be excluded from rhinovirus group (see specificity analysis described below). The specificity analysis with clinical isolates positive for viruses with clinical importance in CSF samples (HSV-1, HSV-2, Adeno, CMV, VZV and mumps) and with rhinoviruses showed no cross-reaction by NucliSens EasyQ Enterovirus assay. However in this study it could be shown that our in-house RT-PCR appear to amplify some rhinovirus strains and should therefore not be used for throat swab samples. It is known that the enteroviruses are genetically very similar to the rhinoviruses,

and thus, cross-reactivity is common between these viruses (Lai et al., 2003).

The purpose of this study was further to compare the performance and user convenience of NucliSens EasyQ Enterovirus assay with that of the in-house two-step RT-PCR assay. The study showed that the performance and the user convenience of NucliSens EasyQ Enterovirus assay was better than that of the RT-PCR. First of all NucliSens EasyQ Enterovirus assay could be completed in the same day within 5 h versus 9.5 h for the RT-PCR divided in two working days. This is important since a rapid and accurate diagnosis of enterovirus infection is essential for the appropriate clinical management of patients with aseptic meningitis. For nucleic acid extraction the new NucliSens miniMAG platform was used in combination with NucliSens magnetic extraction reagents. This is the next generation "Boom" (Boom et al., 1990) technology based on magnetic silica extraction (Van Deursen et al., 2004). The NucliSens EasyQ is a closed system, which comprises the real-time NASBA amplification step with automatic analysis of the data. No RT-reaction and separate detection step is necessary. So it is easier and requires less technical work from laboratory personal. The PCR contamination risk can be avoided in NucliSens EasyQ because the tubes with amplification product never have to be opened. The NASBA results were analyzed using the manufacturer's NucliSens EasyQ director software on the NucliSens EasyQ analyzer and were clear to interpret compared to the subjective reading of the positive and weak positive bands after RT-PCR in agarose gel. An EV-specific internal homologous control RNA provides control over the entire NucliSens EasyQ procedure including the extraction. For the in-house RT-PCR only an external inhibition control was available which does not control the extraction step. During the evaluation some of the samples were invalid in the NucliSens EasyQ procedure (data not shown) that were not invalid with the universal inhibition control in real-time PCR. The observation is not surprising taken into consideration the different level of quality control between the two assays. The NucliSens EasyQ assay uses a homologous internal control added before extraction and hence safeguarding the whole process from extraction to end result, while the in-house RT-PCR assay only uses an inhibition control added after extraction and hence only checking inhibition of the RT-PCR reaction. A more indepth analysis of this phenomenon will be performed at a later stage.

It was interesting that all the samples (45) positive in both methods originated from children and none from adults over 18 years. The 41 (45) were isolated from children even younger than 10 years old.

The current results confirm previous experiences of the sensitivity of both molecular assays. Participation of the enterovirus external quality control QCMD 2003 showed for both assays a score of 100% (data not shown). Virus titer of 0.3 TCID₅₀/ml could be detected by both assays, testing positive for RT-PCR and equivocal for NucliSens EasyQ Enterovirus assay. Other studies on the NASBA based detection of enteroviruses verify the findings in this study about the

sensitivity and user convenience of the enterovirus NASBA assay. In this context, it has to be pointed out that the primer and probe sequences in the current CE marked NucliSens EasyQ Enterovirus assay have been further optimized and the detection technique been upgraded from ECL-based end-point detection to real-time detection with molecular beacons. Hence, the whole amplification and detection including data analysis is much faster and easier in the NucliSens EasyQ Enterovirus assay than in the first (ECL-based) NASBA assays (Leone et al., 1998). An earlier study actually reports on the development and evaluation of such an assay, i.e. NASBA amplification using NucliSens basic kit and enterovirus detection based on ECL (Fox et al., 2002). Furthermore, the performance of the NASBA technique was compared to different in-house prepared primers and probes, combined with various detection methods. It was concluded that the NASBA assay was as sensitive as the more laborious in-house RT-PCR assays and that NASBA was a suitable alternative to RT-PCR for sensitive amplification and detection of enterovirus sequences in a range of clinical specimens. This conclusion was further substantiated by two other studies (Landry et al., 2003a, 2003b). In the first one the enterovirus NASBA assay with NucliSens basic kit was compared to the Argene Biosoft Enterovirus Consensus Reverse Transcription-PCR assay (Enterovirus Consensus RT-PCR kit; Argene Biosoft) and virus isolation using 82 different clinical samples. It was concluded that the enterovirus NASBA assay and the Argene Biosoft RT-PCR had comparable sensitivities and that both molecular methods were more sensitive than culture. In the second study (Landry et al., 2003b), 172 different clinical samples were tested in the enterovirus NASBA assay and in cell culture. The NASBA detection with 96% was more sensitive than virus isolation in cell culture with 78%.

In conclusion, the CE marked NucliSens EasyQ Enterovirus assay together with the NucliSens EasyQ analyzer offers a rapid, specific, sensitive and reliable alternative procedure for the detection of enterovirus RNA. Because of its high level of standardization and quality control it offers an attractive alternative for the various in-house assays currently being used for enterovirus detection.

Acknowledgments

We would like to thank Laurent Kaiser, M.D., Division of Infectious Diseases, Virology, University Hospitals of Geneva, Switzerland, for providing us rhinovirus isolates

for the specificity analysis. We thank all members of the molecular biology team for their excellent technical support in this study. We thank Pierre van Aarle and Carmelo Martinez (bioMérieux) for providing NucliSens EasyQ Enterovirus Kits and for their invaluable help and advice in the NASBA technique.

References

- Boom R, Sol CJA, Salimans MMM, Jansen CL, Wertheim van Dillen PME, van der Noordaa J. Rapid and simple method for purification of nucleic acids. *J Clin Microbiol* 1990;28:495–503.
- Compton J. Nucleic acid sequence-based amplification. *Nature* 7 March 1991;350(6313):91–2.
- Fox JD, Han S, Samuelson A, Zhang Y, Neale ML, Westmoreland D. Development and evaluation of nucleic acid sequence based amplification (NASBA) for diagnosis of enterovirus infections using the NucliSens Basic Kit. *J Clin Virol* 2002;24:117–30.
- Glimaker M, Johansson B, Olcen P, Ehrnst A, Forsgren M. Detection of enteroviral RNA by polymerase chain reaction in cerebrospinal fluid from patients with aseptic meningitis. *Scand J Infect Dis* 1993;25:547–57.
- Gorgievski-Hrisoho M, Schumacher JD, Vilimonovic N, Germann D, Matter L. Detection by PCR of enteroviruses in cerebrospinal fluid during a summer outbreak of aseptic meningitis in Switzerland. *J Clin Microbiol* 1998;36:2408–12.
- Hsiung GD. Picornaviridae. In: Hsiung GD, Fong CKY, Landry ML, editors. *Hsiung's diagnostic virology*. 4th ed. New Haven, CT: Yale University Press; 1994. p. 119–40.
- Lai KKY, Cook L, Wendt S, Corey L, Jerome KR. Evaluation of real-time PCR versus PCR with liquid-phase hybridization for detection of enterovirus RNA in cerebrospinal fluid. *J Clin Microbiol* 2003;41:3133–41.
- Landry ML, Garner R, Ferguson D. Rapid enterovirus RNA detection in clinical specimens by using nucleic acid sequence-based amplification. *J Clin Microbiol* 2003a;41:346–50.
- Landry ML, Garner R, Ferguson D. Comparison of the NucliSens basic kit (nucleic acid sequence-based amplification) and the Argene Biosoft enterovirus consensus reverse transcription-PCR assay for rapid detection of enterovirus RNA in clinical specimens. *J Clin Microbiol* 2003b;41:5006–10.
- Leone G, van Schijndel H, van Gemen B, Kramer FR, Schoen CD. Molecular beacon probes combined with amplification by NASBA enable homogeneous, real-time detection of RNA. *Nucleic Acids Res* 1998;26:2150–6.
- Rotbart HA. Enzymatic RNA amplification of the enteroviruses. *J Clin Microbiol* 1990;28:438–42.
- Van Deursen P, Verhoeven A, de Bie P, Grinsven A, Jacobs M, van de Wiel P. Nucleic acid isolation using NucliSens miniMAG instrument. *Clin Microbiol Infect* 2004;10(Suppl 3):832.
- Zoll GJ, Melchers WJ, Kopecka H, Jambroes G, Van der Poel HJA, Galama JMD. General primer-mediated polymerase chain reaction for detection of enteroviruses: application for diagnostic routine and persistent infections. *J Clin Microbiol* 1992;30:160–5.