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Evaluation of a commercially available reverse transcription-PCR enzyme immunoassay (Enterovirus Consensus kit) for the diagnosis of enterovirus central nervous system infections

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ABSTRACT

A commercial reverse transcription (RT)-PCR amplification method was compared with culture for the diagnosis of enterovirus meningitis. In total, 99 cerebrospinal fluid (CSF) specimens were examined with the Enterovirus Consensus kit and shell vial culture. RT-PCR allowed the amplification of enterovirus cDNA and its detection in a microtitre plate by hybridisation. Clinical information and CSF analysis were used to resolve the discrepancy in results. The detection limit of the RT-PCR assay was determined with the Third European Union Concerted Action Enterovirus Proficiency Panel. There were 34 true-positive CSF specimens. Of these, RT-PCR detected 33 (sensitivity 97%), while culture detected 19 (sensitivity 54.5%). RT-PCR failed to detect one culture-positive specimen that contained inhibitors. When samples from the Third European Union Concerted Action Enterovirus Proficiency Panel were tested, the RT-PCR method gave identical results to those expected. The Enterovirus Consensus kit was rapid and statistically more sensitive than culture ($p < 0.01$) for the detection of enteroviruses in CSF, and may offer considerable benefits in the clinical management of patients with enterovirus meningitis.

Keywords Enterovirus, enzyme immunoassay, meningitis, RT-PCR

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INTRODUCTION

Enteroviruses are important pathogens with a wide range of clinical manifestations. Most infections are mild or asymptomatic, but enteroviruses may also result in serious or even fatal disease [1]. They are the most commonly identified cause of virus meningitis in the paediatric population, accounting for 80–92% of all cases in which a causative agent is identified [2].

Specific diagnosis of enterovirus meningitis has many advantages in patient management [3]. Traditionally, enteroviruses have been detected by isolation in cell culture, with their identity established by neutralisation of infectivity with serotype-specific antisera. Although virus isolation has been regarded as the reference method for detecting enterovirus infection, cell culture procedures are poorly standardised, require multiple cell lines, and are labour-intensive and time-consuming; also, many of the viruses in this diverse group do not grow well in tissue culture [4]. Recent developments in molecular detection technology mean that diagnosis of enterovirus infections is achieved increasingly by non-culture-based methods, particularly nucleic acid amplification methods such as reverse transcription (RT)-PCR [5]. Several clinical studies have demonstrated the importance of the increased sensitivity and rapid turn-around time of RT-PCR and other molecular approaches for enterovirus detection [6,7].

Unnecessary investigations and empirical therapy with antibacterial and anti-herpes simplex virus agents can be avoided by rapid and specific diagnosis of enterovirus meningitis, thus reducing the duration of hospital stay [8,9]. Since the first report of the successful clinical application of molecular methods to enterovirus detection [10], many procedures have been described, including 'in-house' assays [11–15] and commercially

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available kits [16–19]. Various endpoint detection or quantification systems, such as colourimetric microwell hybridisation assays [14,16–20] and a real-time RT-PCR method based on TaqMan technology [21], have been described in order to achieve the sensitivity required for optimal clinical utility. The present study evaluated a new commercial assay (Enterovirus Consensus kit) in comparison with virus culture for the diagnosis of enterovirus central nervous system infections in children and adults. The sensitivity and specificity were determined in relation to clinical information and cerebrospinal fluid (CSF) analysis.

MATERIALS AND METHODS

Patients and CSF specimens

In total, 99 consecutive CSF specimens from 99 patients, aged 2–71 years, were collected in sterile containers and sent to the Microbiology Department for diagnostic evaluation. The patients were admitted between April 2001 and December 2002 to the University Hospital of Ioannina as a result of acute neurological illness, with clinical signs that resulted in the treating physician requesting a lumbar puncture. Clinical information was obtained from the request form and from the physician. None of the patients was immunosuppressed, and none had been receiving antibiotics.

Quantitative and qualitative cytological CSF analysis, and direct examination of CSF by Gram's stain for bacteria, by acid-fast stain for mycobacteria, and by India ink stain for *Cryptococcus*, were performed systematically. Culture of CSF on appropriate media (i.e., blood agar, MacConkey agar, chocolate agar, *Haemophilus* isolation agar, Sabouraud's dextrose agar, thioglyconate on broth and Löwenstein-Jensen egg medium) for bacteria and fungi was performed simultaneously.

CSF analysis for enterovirus isolation and genome detection was performed immediately (during laboratory working hours), or the CSF was stored at −80°C and processed within 24 h.

Enterovirus cell culture

CSF was cultured directly on two different cell lines (buffalo green monkey kidney and human embryonic rhabdomyosarcoma cells) (Vircell, Granada, Spain) with the standard shell vial culture procedure [22]. In brief, shell vials were inoculated with 200 μL of CSF and centrifuged at 700 *g* for 45 min at room temperature. After addition of maintenance medium (Eagle's minimum essential medium containing fetal bovine serum 2% v/v), the shell vials were incubated at 37°C for 48 h. The coverslips were removed from the vials and attached to a microscope slide, which was then incubated with 25 μL of monoclonal antibody (Vircell) directed against the VP1 enterovirus capsid protein for 30 min at 37°C [23]. After washing, each slide was incubated with 25 μL of fluorescein-labelled anti-mouse immunoglobulin for 30 min at 37°C. The presence of typical fluorescent inclusions confirmed the presence of virus.

RT-PCR assay

The Enterovirus Consensus kit (Argene Biosoft, Varilhes, France) was used according to the manufacturer's instructions, with one exception, mentioned below.

Specimen preparation

Virus RNA was extracted from 140 μL of CSF with the QIAamp Viral RNA Blood Mini kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. Elution was performed with an RNase-free buffer; total virus RNA was stored at −80°C until required for RT-PCR.

Amplification

Detection of 64 serotypes (polioviruses 1–3, coxsackieviruses A1–A22, A24 and B1–B6, echoviruses 1–9, 11–21, 24–27, 29–33, and enteroviruses 68–71) was performed by a single amplification with the consensus stair primer system. The primers (Penter-1 and -2) were selected from within the 5′ non-coding region of the enterovirus genome and were 85% identical to known enterovirus RNA sequences, thereby allowing amplification of a sequence with a 3′ mutation with good efficiency [24,25]. A 10-μL aliquot of the extracted material was added to 40 μL of a master mix prepared using buffers and enzymes provided by the manufacturer. RT of target RNA and amplification of cDNA was done in a single-tube reaction following the amplification program described by the manufacturer. The RT-PCR was carried out in a PTC 200 thermocycler (MJ Research, Reno, NV, USA). A positive control, consisting of a plasmid with a coxsackie B4 virus sequence provided by the manufacturer, was used as an amplification-positive sample. The absence of inhibitors was demonstrated by addition of this positive control (included in the inhibition control pre-mix) to a duplicate sample after RNA extraction and RT. The amplified cDNA products were analysed with electrophoresis on agarose 2% w/v gels in the presence of ethidium bromide (final concentration in gel and buffer of 0.5 μg/mL) and visualised by UV illumination. The size of the amplified fragment, located in the non-coding conserved portion of the enterovirus genome, was 425 bp. This step, not included in the manufacturer's recommendations, was preliminary to the hybridisation detection step, and enabled possible contamination or problems with the amplification (e.g., a positive band with the negative control) to be detected.

Detection

Following chemical denaturation, the single-stranded PCR products obtained from samples and internal controls were hybridised separately with two different biotinylated probes and detected with a streptavidin–peroxidase system in a microtitre plate. Results were scored as positive when the optical density value at 450/650 nm was above a cut-off value calculated for each serial assay according to the recommendations of the manufacturer.

The total time required for the Enterovirus Consensus kit was *c.* 7 h, comprising 1 h for RNA extraction, 3 h for RT-PCR, and 3 h for the detection step. To validate the assay, each RT-PCR analysis included four types of control: (1) a positive control of RNA extracted from poliovirus oral trivalent vaccine (Sabin); (2) an inhibition control; (3) an amplification-negative

control of sterile distilled water; and (4) a negative control for detection, which was tested in duplicate during the detection step.

External quality control

To determine the sensitivity and specificity of the enterovirus detection method with different enterovirus serotypes, the laboratory participated in the external quality assessment programme established by the members of the European Union (EU) Quality Concerted Action (QCCA) of Nucleic Acid Amplification in Diagnostic Virology (University Medical Centre, Utrecht, The Netherlands). The third enterovirus panel, which was shipped by the EU-QCCA Working Party on Enteroviruses, consisted of 13 coded samples: three samples of a dilution series of echovirus 11 (25.2, 252 and 25 100 × 50% tissue culture infective doses ($TCID_{50}$)/mL of the original virus stock), three samples of a ten-fold dilution series of coxsackievirus A9 (4, 0.4 and 0.04 $TCID_{50}$/mL), one sample of echovirus 6 (20 000 $TCID_{50}$/mL), one sample of coxsackievirus B5 (317 $TCID_{50}$/mL), one sample of enterovirus 71 (56 $TCID_{50}$/mL), two samples with no virus, and two samples containing the 'Amorced Enterovirus RNA' preparation (10^4 and 10^7 RNA copies/mL) (Table 1). Samples were tested blind, and the results were compared with the expected results, based upon those obtained by three reference laboratories, with a total of four different molecular methods, before distribution of the panel.

Data interpretation

RT-PCR results were compared with those of virus culture. In the case of discrepancies, the RT-PCR was repeated and clinical information and the results of CSF analysis were obtained. Patients were defined as having an enterovirus central nervous system infection on the basis of the following criteria: clinical evidence consistent with meningitis/encephalitis (i.e., fever, headache, vomiting, nuchal rigidity, photophobia, abdominal pain, irritability, seizures, alterations of consciousness) and the absence of another detectable pathogen, and either (1) detection of an enterovirus genome in CSF virus culture, or (2) positive CSF enterovirus culture without a positive RT-PCR.

Table 1. European Union Concerted Action Enterovirus Proficiency Panel

Code	Enterovirus serotype	Dilution[a]	Virus titre[b]	Expected results
EV-C01	COX A9	10^{-8}	0.036	Negative
EV-C02	COX A9	10^{-7}	0.36	Positive
EV-C03	COX A9	10^{-6}	3.6	Positive
EV-C04	No virus			Negative
EV-C05	ECHO 6	10^{-4}	20 000	Positive
EV-C06	ECHO 11	10^{-6}	25.2	Positive
EV-C07	COX A5	10^{-5}	317	Positive
EV-C08	ENTERO 71	10^{-5}	56.4	Positive
EV-C09	ECHO 11	10^{-3}	25 200	Positive
EV-C10	No virus			Negative
EV-C11	ECHO 11	10^{-5}	252	Positive
EV-C12	Control RNA		10^{4c}	Positive
EV-C13	Control RNA		10^{7c}	Positive

[a]Dilution of original stock.
[b]Titre of original virus stock before inactivation and freeze–drying ($TCID_{50}$/mL: echovirus 6, 2.0×10^8; echovirus 11, 2.5×10^7; enterovirus 71, 5.6×10^6; coxsackievirus A9, 3.6×10^6; coxsackievirus B5, 3.2×10^7).
[c]RNA copies/mL.

Sensitivity, specificity and positive and negative predictive values were calculated. Statistical significance was calculated with chi-square tests and Yates' correction, with p values < 0.05 being regarded as significant.

RESULTS

CSF was collected from 79 children (aged 2–13 years) and 20 adults (aged 18–71 years) with acute neurological illness for whom virus aseptic meningitis/encephalitis was suspected. Quantitative and qualitative cytological examinations of CSF revealed white cell counts (WCCs) of 5–1210/mm^3, with a predominance of lymphocytes (n = 87), normal glucose values, and protein values of 15–217 mg/dL. The percentages of lymphocytes and neutrophils were, respectively, 7–100% (median 68%) and 2–90% (median 34%). The results of bacteriological and fungal investigations were negative in all cases. The duration of hospitalisation was short (3–9 days; mean 6.3 days).

Table 2 summarises the results obtained with virus culture and the Enterovirus Consensus kit. Of the 99 patients, samples from 65 (65.5%) were negative by both RT-PCR and culture, while samples from 18 patients were positive by both techniques. There was an 83.8% correlation between the two assays.

Samples from 33 (33.3%) patients were positive by RT-PCR and 19 (19.1%) by culture. Sixteen discrepant results were obtained: RT-PCR was positive for 15 culture-negative specimens, while one culture-positive specimen was negative by RT-PCR. Very few enterovirus cultures were performed from throat or rectal samples, so it was not possible to use culture results from these sites to resolve discrepancies between CSF cultures and RT-PCR. However, according to the criteria listed above to resolve discrepancies, the sensitivity, specificity and positive and negative predictive values for the Enterovirus Consensus kit were 97%, 100%, 100% and 98.5%, respectively. The corresponding figures for virus culture were 57.5%, 100%, 100% and 82.5%, respectively. The Enterovirus Consensus kit detected significantly more patients ($p < 0.01$) with enterovirus meningitis/encephalitis than did culture.

Of the 33 patients who yielded RT-PCR-positive samples, 31 were children aged 2–12 years (median 7 years). On admission, the clinical symptoms of these children were: fever (87.1%); vomiting (64.5%); headache (58%);

Table 2. Comparison of the results of the Enterovirus Consensus RT-PCR kit with virus culture for 99 cerebrospinal fluid specimens

	Specimen numbers						Predictive value	
Method	True-positive	True-negative	False-positive	False-negative	Sensitivity (%)	Specificity (%)	Positive (%)	Negative (%)
PCR	33	65	0	1	97.0	100.0	100.0	98.5
Culture	19	66	0	14	57.5	100.0	100.0	82.5

nuchal rigidity (35.5%); anorexia (22.6%); abdominal pain (12.9%); photophobia (12.9%); cough (9.7%); malaise (9.7%); drowsiness (6.4%); rash (6.4%); and nausea, otitis, rhinitis, pharyngitis and movement disorders (3.2% each). WCCs were in the range 15–1202/mm^2 (median 62/mm^3), with a predominance of lymphocytes in 25 CSFs. Six CSFs had a relative percentage of neutrophils of 60–90%, with WCCs of 20–1202/mm^3. CSF protein values were normal in 24 specimens. Sixteen of 31 specimens were culture-positive, with WCCs of 20–1202/mm^3.

CSF from one child contained RT-PCR inhibitors (inhibition control result < 0.8 optical density units and specimen result negative with the enterovirus probe control), but yielded a positive culture result. This patient (a female aged 6 years) had signs and symptoms consistent with aseptic meningitis (fever, headache and malaise), but had a WCC of only 12 cells/mm^3, with a predominance (85%) of lymphocytes. The original CSF specimen was re-extracted and retested, and was again negative by RT-PCR. This result was therefore classified as a false-negative.

Two patients from the adult group yielded positive RT-PCR samples: one patient, aged 71 years with a clinical history of diabetes mellitus, was admitted with fever, drowsiness and nuchal rigidity; the second, aged 18 years, was admitted with fever, nuchal rigidity and photophobia. CSF parameters were normal for the first patient (WCC of 5/mm^3), while CSF from the second patient had a WCC of 380/mm^3 (90% lymphocytes) and normal protein and glucose levels. These two patients also yielded enterovirus-positive samples.

Of the 65 patients who yielded RT-PCR- and culture-negative samples, 47 were children and 18 were adults. On admission, these patients had signs and symptoms of meningeal inflammation with, in addition to headache, fever and nuchal rigidity, neurological manifestations that included irritability (13.8%), alterations of consciousness (7.7%) and seizures (6%). The WCCs were 5–1210/mm^2 (median 96/mm^3), with a predominance of lymphocytes and mononuclear cells in 59 CSFs. Six CSFs had 75–90% neutrophils, and WCCs of 40–980/mm^3. CSF protein values were normal for 52 specimens.

When samples from the Third QCCA Enterovirus proficiency panel were tested by RT-PCR, the results obtained were as expected. For coxsackievirus A9, 1 $TCID_{50}$ (vial 3) and 0.1 $TCID_{50}$ (vial 2) were detected, but 0.01 $TCID_{50}$ (vial 1) was not detected. Both samples containing coxsackievirus B5 (100 $TCID_{50}$; vial 7), as well as the only sample containing echovirus 6 (10000 $TCID_{50}$; vial 5), were positive. For echovirus 11, the samples containing 10 000 $TCID_{50}$ (vial 9), 100 $TCID_{50}$ (vial 11) and 10 $TCID_{50}$ (vial 6) were positive. The only sample containing enterovirus 71 (10 $TCID_{50}$; vial 8) was also positive (Table 1).

DISCUSSION

The 99 patients in this prospective study formed a homogeneous group with symptoms of meningitis/encephalitis and an absence of detectable bacterial pathogens in CSF. The RT-PCR method detected all enterovirus serotypes, except echoviruses 22 and 23, which are thought to be genetically distinct picornaviruses and have been reclassified recently as parechoviruses 1 and 2, and which are isolated rarely from CSF samples [1,25–27].

The primers (Penter-1 and -2) used in the Enterovirus Consensus kit form a 'stair' primer system. The principle of stair primers is that a classical pair of primers is replaced with a set of equimolar oligonucleotides of variable length, in which the 5′-terminal sequences are unchanged (with a constant length of amplified fragment), but the 3′ end is displaced base by base [28]. The advantages of stair primers are their recognition of all the known enterovirus serotypes [24,25] and the efficient amplification that results in the presence of mutations which affect the 3′ extremity. This is important for quantitative PCR and the

avoidance of non-specific priming [28]. Detection of the amplified product with a specific 5′-biotinylated probe is not entirely specific, because of cross-reaction with rhinovirus 3 [25,29]. This was not a problem in the present study, since rhinovirus is found only in respiratory samples, and 60 clinical specimens analysed in parallel with the Enterovirus Consensus kit and PCR techniques described by Zoll *et al.* [15] and Rotbart *et al.* [18] yielded similar results, with a sensitivity for the Enterovirus Consensus kit that was equivalent to that obtained by other techniques described previously for clinical samples [24,25]. Moreover, Penter primers amplified all 64 serotypes of enteroviruses, including both prototype and field strains [25], while the primers described by Zoll *et al.* [15] did not recognise coxsackieviruses A11, A17 and A24 and echovirus 16, and the Rotbart primers did not always recognise echoviruses 1 and 5 [30].

A specific virological diagnosis of enterovirus meningitis depends on isolation of the virus from CSF in tissue culture, although the sensitivity for enterovirus serotypes is only 65–75%, mainly because of the inability of certain serotypes (coxsackie A viruses) to grow in cell culture [2,31] and the low titres of enterovirus in CSF [32]. In the present study, shell vial culture was used as an alternative to conventional virus isolation. This assay has the important features of being more rapid, sensitive and specific [33]. The sensitivity of shell vial culture was 57.5%, similar to that reported by Taggart *et al.* [34] (50%) and Carroll *et al.* [16] (55.8%), and to the sensitivity of conventional cell culture [19–21,35].

Many of the drawbacks related to culture are overcome by amplification assays. The commercial RT-PCR assay evaluated in the present study had a sensitivity compared to culture of 97%, which was in accordance with the sensitivity of other similar methods [18,19,36], and a specificity of 100%, which was slightly better than reported previously [18–20]. These figures were also similar to those reported for different amplification methods, such as the Roche Amplicor EV test [16], a real-time RT-PCR assay [21,35] and nucleic acid sequence-based amplification methodology [17,37]. As reported previously [16,30,36,38], there was a statistically significant improvement in the detection of enterovirus central nervous system disease with PCR compared to culture, and a very good correlation (80–85.9%) between the two assays [16,34]. Recently, Buck *et al.* [39] compared a newly described shell vial assay, in which a mixture of human colon carcinoma and genetically engineered buffalo green monkey kidney cells (Super E-mix) was used, with two commercially available RT-PCR assays (one of which was the Enterovirus Consensus kit) and conventional cell culture for the diagnosis of enterovirus meningitis. Even though the Super E-Mix procedure had a greater sensitivity than conventional cell culture (i.e., 76% vs. 51%), the Enterovirus Consensus kit appeared to offer more sensitive detection of enterovirus in CSF [39]. Inclusion of the two dilution series of coxsackievirus A9 and echovirus 11 from the EU-QCCA Proficiency Panel provided an opportunity in the present study to evaluate the detection limit of the assay, which was found to be similar to that obtained with a real-time fluorescence PCR assay [40].

Diagnosis of aseptic meningitis with RT-PCR requires not only a sensitive method, but also a technique that can monitor problems associated with PCR inhibitors [16,21,41]. Although the exact nature of inhibition is unknown [16,21], it could be explained by the presence of a high level of RNase activity or by *Taq* DNA polymerase inhibitors that were co-purified with RNA [36]. The Enterovirus Consensus kit contains a plasmid control that allows the detection of amplification inhibitors in the specimen. Another advantage of the kit was that the method was relatively easy to perform, with the entire assay completed in < 8 h, compared to 48–72 h for shell vials. It has been suggested [42] that PCR assays for enterovirus detection should be introduced as routine tests, as an early positive PCR result could conserve considerable healthcare resources [6,16,43]. The format of the kit is such that the reagents can be used in a flexible way, so that laboratories in which small numbers of samples are tested on a regular basis can make use of the methodology.

The clinical diagnosis of aseptic meningitis depends on routine examination of CSF [44], and it is therefore appropriate to evaluate the RT-PCR assay against an observation of pleocytosis without any other cause [2]. In previous reports describing PCR assays, the proportion of meningitis cases without pleocytosis was low (3.2–7%) [38,45]. Moreover, in some studies, the absence of pleocytosis could be used to discourage RT-PCR testing of all patients [45], or of older children (aged > 2 years) and adults [19]. The

present study examined only five CSF specimens without pleocytosis (WCC ≤ 5/mm^3), and one was positive. Consequently, it seems that cytological examination of CSF may not be of great value in ruling out virus meningitis or in deciding whether to perform RT-PCR for enteroviruses. Indeed, irrespective of patient age and cytological results, RT-PCR assay may be of value in all cases of suspected enterovirus meningitis [42].

Among the patients with negative RT-PCR results, clinical manifestations and CSF analysis indicated that they suffered from a virus central nervous system infection. The results were sufficient to rule out enterovirus infection, but specific tests (i.e., culture and PCR) for other neurotropic viruses were not performed. However, epidemiological studies have indicated that a specific virus pathogen is identified in 60% of aseptic meningitis patients when consistent conventional diagnostic methodologies are used, compared with 80–85% of cases if conventional laboratory methods and enterovirus PCR are combined [2,31].

In conclusion, the Enterovirus Consensus RT-PCR kit demonstrated superior sensitivity and speed compared with virus culture, excellent specificity and positive predictive value (100% each), a clear delineation of positive samples, minimal amplification inhibition, and a high negative predictive value (98.5%). Importantly, the kit recognised all known serotypes of enteroviruses, and provided an inhibition control to monitor the amplification efficiency. The kit was suitable for use in the routine diagnostic laboratory, and the implementation of such methodology would have a considerable impact on patient care by reducing the duration of hospitalisation, inappropriate use of antibiotic therapy, and unnecessary investigations.

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