Detection of Enterovirus RNA in cerebrospinal fluid: comparison of two molecular assays

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ABSTRACT

Enterovirus (EV) and Human Parechovirus (HPeV) are a major cause of infection in childhood. A rapid diagnostic test may improve the management of patients with EV and HPeV infection.

The aim of this study is to evaluate the performance of the GeneXpert enterovirus assay (GXEA) for detection of EV RNA compared to a user-developed reverse-transcriptase (RT) quantitative real-time PCR (qPCR) in routine clinical practice. Also a RT-qPCR assay for detection of HPeV RNA in different clinical samples was developed and evaluated. Cerebrospinal fluid (CSF) from 232 patients suspected for meningitis was collected and tested for EV and HPeV using RT-qPCR assays. In parallel an aliquot of the samples was tested using the GXEA and viral culture.

EV RNA was detected in 22 (19.0%) and 28 (24.1%) of 116 samples using GXEA and RT-qPCR assay, respectively. EV was isolated from 10 of 116 (8.6%) samples by viral culture. GXEA had a sensitivity, specificity, positive predictive value and negative predictive value of 82.1%, 100%, 100% and 96.2%, respectively.

In this study, molecular assays were superior to viral culture in detecting EV RNA in CSF. GXEA showed a high specificity but a lower sensitivity for the detection of EV RNA compared to the RT-qPCR assay.
INTRODUCTION

Human non-polio Enterovirus (EV) is a major cause of infection in children, with 10 to 30 million infections annually, especially in neonates and young infants.\(^1\)\(^-\)^\(^5\) Recently, Human Parechovirus (HPeV) types 1–18 have been described and show epidemiological and clinical characteristics similar to EV.\(^2\) The clinical spectrum of EV and HPeV infections varies from nonspecific febrile illness to severe systemic illnesses involving the central nervous system (CNS).\(^2\)\(^,\)\(^6\)\(^-\)\(^8\) EV infections are the most common cause of aseptic meningitis and account for 80 to 90% of all cases of CNS infections for which a possible causative agent is identified.\(^9\) EV infections are associated with significant morbidity and mortality in neonates, particularly ante- or perinatally infections. EV infections have also been associated with severe neurodevelopmental sequelae.\(^6\)\(^,\)\(^10\)\(^,\)\(^11\)

Viral culture used to be the “gold standard” for the diagnosis of EV infection in different clinical specimens such as feces, throat swabs and cerebrospinal fluid (CSF). However, viral culture takes 4–8 days and the diagnosis is often too late to influence clinical decision making.\(^12\) Sensitivity of viral culture is relatively low (53–75%) and some EV serotypes grow poorly.\(^12\)\(^-\)\(^17\) A rapid diagnostic test may improve the management of patients with EV infections. Rapid diagnostic tests include nucleic acid amplification technology, such as reverse-transcriptase (RT) quantitative real-time PCR (qPCR) and nucleic acid sequence-based amplification (NASBA).\(^9\)\(^,\)\(^18\)\(^-\)\(^23\) The advantages of RT-qPCR are the small amounts of samples required and its rapidity (7–24 hours), high sensitivity and specificity (almost 100%).\(^6\)\(^,\)\(^12\)\(^,\)\(^17\)\(^,\)\(^24\)\(^,\)\(^25\) Nowadays traditional viral culture has therefore been replaced by nucleic acid amplification tests as the gold standard for detection of EV in CSF.

The GeneXpert enterovirus assay (GXEA) is designed as an integrated system combining specimen processing, EV amplification and detection in a disposable cartridge which takes 2.5 hours to detect EV in CSF.\(^26\)\(^-\)\(^28\)

The aim of this study is to evaluate the performance of the GXEA for the detection of EV RNA compared to a user-developed RT-qPCR in routine clinical practice. In addition, a RT-qPCR assay for detection of HPeV RNA in different clinical samples was developed and evaluated.
MATERIAL AND METHODS

Clinical specimens
CSF of 232 patients suspected of meningitis was collected from May 2007 till December 2009 and tested for the presence of EV and HPeV RNA, using RT-qPCR assays based on the 5’ UTR region of the EV and HPeV genome, respectively. In parallel, an aliquot of the samples was tested using the GXEA. Of 116 samples enough material was still available to perform a viral culture on tertiary Cynomolgus monkey kidney (tMK) cells. Samples were stored at 4°C before being used within 16 hours of receipt.

Viral RNA isolation
An aliquot of the samples (200 µl) was used to extract viral RNA using the MagNA Pure LC Total Nucleic Acid Isolation Kit (Roche Diagnostics, Basel, Switzerland) as described previously. Each sample was eluted in 50 µl buffer. All samples had been spiked before extraction with an internal control virus (phocine distemper virus) to monitor for efficient extraction and amplification, as described previously.

RT-qPCR
The isolated viral RNA was reverse transcribed using MultiScribe RT and random hexamers (both from Applied Biosystems, Foster City, CA). Detection of EV was performed using a RT-qPCR assay as previously described. In addition, an in-house RT-qPCR assay was developed for the detection of HPeV using Primer Express (Applied Biosystems). Conserved target regions were identified using BLAST (www.ncbi.nlm.nih.gov/blast). Sequences of the primers and probes used are summarized in Table 4.1. Potential cross-reactivity of the HPeV-specific assay with other related picornaviruses was excluded by using the RT-qPCR assay on samples known to contain RNA of Coxsackievirus A (A2, A9, A13, A14, A16, A24), Coxsackievirus B (B1, B3, B4, B5), Echovirus (1–7, 9, 12, 14, 15, 17, 19–21, 24–27, 29, 31, 32), Enterovirus 71, Poliovirus 1, Poliovirus 3 and/or Rhinoviruses. Real-time PCR procedures were performed as described previously.

GeneXpert
The GXEA was performed according to the manufacturer’s instructions (Cepheid, Sunnyvale, CA, USA) within 16 hours of receipt of the samples as previously described. In short,
### Table 4.1 Primer and probes used for real-time PCR detection

<table>
<thead>
<tr>
<th>Virus</th>
<th>Target</th>
<th>Forward primer (s) (5’–3’)</th>
<th>Reverse primer (s) (5’–3’)</th>
<th>Probe(s)a</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enterovirus</td>
<td>5’ UTR</td>
<td>TCC TCC GGC CCC TGA</td>
<td>AAT TGT CAC CAT AAG CAG CCA</td>
<td>6FAM-CGG ACC GA CTA CTT TGG GTG ACC GT</td>
<td>31</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>GAT TGT CAC CAT AAG CAG CCA</td>
<td>6FAM-CGG ACC GA CTA CTT TGG GTG TCC GT</td>
<td></td>
</tr>
<tr>
<td>HPeV</td>
<td>5’ UTR</td>
<td>TGC AAA CAC TAG TT GCA TGC CC</td>
<td>TCA GAT CCA TAG TGY CAC TT GG TTA CCT</td>
<td>6FAM-CGA AGG ATG CCC AGA AGG TAC CCG</td>
<td>This study</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>TCA GAT CCA CAG TGT CTC TT GG TTA CCT</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a FAM = 6-carboxyfluorescein.
140 µl of CSF was added to the GeneXpert cartridge and then processed automatically for the different steps of sample preparation and amplification. Results were available within 2.5 hours.

**Viral culture**

Viral culture was performed on confluent layers of tMK cells. After inoculation of 0.25 ml of clinical specimen and absorption to the cells for 1 hour, 1 ml of culture medium was added and cells, maintained at 37°C on roller drums, were examined daily during 14 days for a cytopathic effect. Typing of the virus isolates was carried out by neutralization or complement fixation with intersecting antiserum pools by standard procedures.

**RESULTS**

A RT-qPCR specific for the detection of HPeV was developed (Table 4.1). As both EV and HPeV belong to the picornavirus family, the specificity of the RT-qPCR was tested for cross-reactivity with samples known to contain picornavirus RNA. No detection of other picornaviruses was observed (data not shown). Results of the user-developed RT-qPCR assay were taken as the gold standard.

A total of 232 patients were included in this study. The mean age was 28.8 years (range 0–84.9 years). There were more males than females (ratio 1.3:1).

EV was isolated from 10 of 116 (8.6%) samples with viral culture. EV RNA was detected in 22 (19.0%) and 28 (24.1%) of these samples using the GXEA and RT-qPCR assay, respectively (Table 4.2). Two viral culture positive samples were negative for both molecular assays.

All 232 samples were tested with the GXEA and RT-qPCR assays. EV RNA was detected in 32 (13.8%) and 40 (17.2%) of these samples, respectively (Table 4.3).

<table>
<thead>
<tr>
<th>Table 4.2 Results of GeneXpert and RT-qPCR versus viral culture</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
</tr>
<tr>
<td>Viral culture pos</td>
</tr>
<tr>
<td>Viral culture neg</td>
</tr>
<tr>
<td>Total</td>
</tr>
</tbody>
</table>

Pos = positive; Neg = negative.
Fifteen (6.5%) of the 232 samples tested with the GXEA gave an invalid result. No invalid results were reported using the RT-qPCR assay. In 8 patients (20%) EV RNA was detected with the RT-qPCR assay. They would have been missed if only a GXEA was performed. In 4 of these 8 patients EV RNA was also detected by RT-qPCR assay in feces and throat swabs of the same subject, making the possibility of a false positive result, generated by this assay, unlikely. Unfortunately, no additional material of the other 4 patients was available.

The GXEA had a higher sensitivity and specificity than viral culture (Table 4.4).

### Table 4.3 Results of RT-qPCR versus GeneXpert

<table>
<thead>
<tr>
<th></th>
<th>RT-qPCR positive</th>
<th>RT-qPCR negative</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>GXEA positive</td>
<td>32</td>
<td>0</td>
<td>32</td>
</tr>
<tr>
<td>GXEA negative</td>
<td>7</td>
<td>178</td>
<td>185</td>
</tr>
<tr>
<td>GXEA invalid</td>
<td>1</td>
<td>14</td>
<td>15</td>
</tr>
<tr>
<td>Total</td>
<td>40</td>
<td>192</td>
<td>232</td>
</tr>
</tbody>
</table>

### Table 4.4 Sensitivity of GeneXpert versus viral culture

<table>
<thead>
<tr>
<th></th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>Positive predictive value</th>
<th>Negative predictive value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Viral culture</td>
<td>28.6% (8/28)</td>
<td>97.7% (86/88)</td>
<td>80.0% (8/10)</td>
<td>81.1% (86/106)</td>
</tr>
<tr>
<td>GeneXpert</td>
<td>82.1% (32/39)</td>
<td>100% (178/178)</td>
<td>100% (32/32)</td>
<td>96.2% (178/185)</td>
</tr>
</tbody>
</table>

*Gold standard = RT-qPCR.*

### Table 4.5 Results of HPeV positive patients

<table>
<thead>
<tr>
<th>Patient</th>
<th>RT-qPCR PCR</th>
<th>GeneXpert</th>
<th>Viral culture</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Positive</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td>2</td>
<td>Positive</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td>3</td>
<td>Positive</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td>4</td>
<td>Positive</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td>5</td>
<td>Positive</td>
<td>Positive</td>
<td>Positive</td>
</tr>
<tr>
<td>6</td>
<td>Positive</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td>7</td>
<td>Positive</td>
<td>Positive</td>
<td>Not performed</td>
</tr>
<tr>
<td>8</td>
<td>Positive</td>
<td>Positive</td>
<td>Negative</td>
</tr>
<tr>
<td>Total positive</td>
<td>8</td>
<td>3</td>
<td>1</td>
</tr>
</tbody>
</table>
HPeV RNA was detected in 8 of 232 (3.4%) samples by the HPeV RT-qPCR assay (Table 4.5) and in three of these samples an EV was also detected with both the GXEA and RT-qPCR assays. The number of coinfection in this study is comparable to other studies. Patients with a HPeV infection were missed when only a GXEA was performed, as GXEA is not yet available for the detection of HPeV RNA.

**DISCUSSION**

It is important for clinicians to have a rapid test result for EV and HPeV infection. This results in an adequate management of patients, reduction in hospitalization rate, prevention of outbreaks, rapid exclusion of other infectious diseases and reduction in the rate of unnecessary (antibiotic) drug usage. The molecular assays described in this study are more rapid than viral culture, which requires a couple of days to weeks. The GXEA and RT-qPCR assays generated results within 3 and 24 hours, respectively. If necessary, the turnaround time of the RT-qPCR assay can even be reduced to less than 5 hours, close to the turnaround time of direct-immunofluorescence assays.

This retrospective study confirmed the findings of others that molecular assays are more sensitive than viral culture in diagnosing EV infection. Vuorinen et al. performed a conventional not real time RT-PCR and viral culture on 591 samples (301 CSF, 61 feces and 229 respiratory airway samples). In 6% of the samples both a positive RT-PCR and viral culture was detected, in 15% only a positive RT-PCR was detected and in 1% only a positive viral culture. Yerly et al. compared RT-qPCR and viral culture in the detection of EV in the CSF of 38 patients during a summer outbreak of aseptic meningitis. In 34% a viral culture was positive and in 66% a RT-qPCR was positive.

In this study, RT-qPCR assay performed better than GXEA in detecting EV infection. It is unlikely that this observation was due to a higher rate of false positive results of RT-qPCR assay. This is supported by the detection of EV RNA in other samples (feces, throat swabs) of individuals with GXEA negative and RT-qPCR positive CSF samples. Therefore, the GXEA appears to have a lower sensitivity for the detection of EV RNA compared to the RT-qPCR assay. This is similar to the findings of Kost et al. who reported a sensitivity of 97.1% for the GXEA, which was lower than the sensitivity of molecular assays used in a reference laboratory. In contrast, Marlowe et al. compared the GXEA with the NASBA and a user-developed RT-qPCR assay. The sensitivities of these three tests were 100%, 88% and 100%
respectively. The lack of difference in sensitivity between the RT-qPCR and the GXEA found in their study may be explained by the use of a one-step RT-qPCR. During the validation of the EV RT-qPCR used in the present study, the two-step EV RT-qPCR appeared to be more sensitive than the one-step RT-qPCR.

Among the disadvantages of the GXEA encountered in the present study was that the GXEA has only been validated for CSF specimens, not for feces, blood and throat specimens. The means that a laboratory needs to use alternative assays to test for the presence of EV in such specimens. A higher rate of invalid results was found using the GXEA than the RT-qPCR assay. In this study, 7.4% of the samples gave an invalid result with the GXEA. This is comparable to the percentage reported in other studies.\textsuperscript{27,28,34} Such invalid results may be caused by blood rests or cells in the CSF sample.

It is not always feasible to obtain the minimum amount of CSF (at least 140 μl) required to run a GXEA, especially in young children and infants. All of the isolated RNA and DNA was subsequently used for the GXEA. None was left to test for the presence for other viruses such as herpes simplex and varicella zoster, also known to cause meningitis.

Finally, like EV, HPeV infection can present as a sepsis-like illness, especially in very young children. Because it is difficult to differentiate viral from bacterial infection based on initial clinical symptoms, antibiotics are usually initiated in young children. As GXEA is not yet available for the detection of HPeV RNA, additional diagnostic tests are required to exclude HPeV as the causative agent of the infection. The home-brew RT-qPCR assays, in contrast, detect both EV and HPeV and may therefore be preferred above the GXEA.

Despite the mentioned disadvantages of the GXEA, its ease of use, random access capability and minimal hands-on time still make it a valuable tool for diagnosing EV meningitis in clinical settings.

**CONCLUSION**

In this study population molecular assays have been demonstrated to be more sensitive than viral culture. GXEA demonstrated a higher specificity but lower sensitivity than the home-brew RT-qPCR for the detection of EV RNA.
Acknowledgments

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REFERENCES


