GeneXpert Enterovirus assay: one-year experience in a routine laboratory setting and evaluation on three proficiency panels

Running title: GeneXpert Enterovirus in routine laboratory setting

Katja Seme¹, Tina Močilnik¹, Kristina Fujs Komloš¹, Ana Doplihar¹,
David H. Persing², Mario Poljak¹*

Institute of Microbiology and Immunology, Faculty of Medicine, University of Ljubljana,
Slovenia¹,
Cepheid, Sunnyvale, CA²

*Corresponding author. Prof. Mario Poljak, MD, PhD. Mailing address: Institute of Microbiology and Immunology, Faculty of Medicine, Zaloška 4, 1000 Ljubljana, Slovenia. Phone: +386 1 543 7453. Fax: +386 1 543 7418. E-mail: mario.poljak@mf.uni-lj.si
Abstract

Prospective unblinded comparative evaluation of three assays for detection of enteroviral RNA performed on 83 positive and 79 negative cerebrospinal fluid samples showed initial and resolved sensitivity of 90.4% and 98.8% for Cepheid GeneXpert Enterovirus Assay, 94.0% and 97.6 % for Argene Enterovirus Consensus Kit and 100% and 100% for in-house real-time PCR, respectively. Initial and resolved specificities for all three assays were 100%.
Enteroviruses comprise a large group of immunologically distinct serotypes of viruses belonging to Picornaviridae family (18). Infection with enteroviruses is associated with protean clinical manifestations ranging from asymptomatic or mild febrile illness to severe and potentially fatal syndromes including paralysis, aseptic meningitis, encephalitis, myocarditis, neonatal systemic infection (1). Enteroviruses are the most common cause of aseptic meningitis in both children and adults, and may cause up to 90% of cases of aseptic meningitis for which an etiology is identified (1, 14, 18, 19). Rapid detection and characterization of enteroviral meningitis is essential for making decisions for patient management and treatment (5, 15, 17, 20). Providing the enterovirus test results on a daily basis can have a substantial health care impact and was shown to be highly cost effective (9, 12, 17). Since conventional diagnosis of enteroviral meningitis by cell culture from cerebrospinal fluid (CSF) is time-consuming, expensive and lacks sensitivity, some commercial and several in-house PCR protocols as well as nucleic acid sequence-based amplification (NASBA) assays have been developed during the last 15 years in order to improve enteroviral detection from CSF (2-4, 6, 7, 11, 13, 16, 20-22, 24-26). The most widely used PCR-based assays for routine diagnosis of enteroviral meningitis are those detecting PCR-products in microtiter wells (6, 21, 22) and those employing real-time PCR technology (2, 3, 13, 24-26). However, all of these approaches require specially trained laboratory staff, thus limiting the ability of “real-time” technology to deliver STAT patient results which would be most useful for making patient management decisions.

The latest development in the field of molecular diagnosis of enteroviral meningitis is a fully automated real-time multiplex, reverse transcription-PCR assay GeneXpert Enterovirus Assay (GXEA) (Cepheid, Sunnyvale, CA)(10). GXEA is at present the only FDA-approved assay for qualitative detection of enterovirus RNA in CSF. It combines automated nucleic acid sample preparation, amplification and real-time detection of enteroviral RNA in a disposable, macro/microfluidic cartridge using the GeneXpert Dx system instrument. To date, only one evaluation of GXEA has been published in peer reviewed journals. A multicenter beta trial on 102 CSF samples obtained from patients with suspected meningitis (34
enterovirus positive) showed that GXEA had a sensitivity and specificity of 97.1% and 100%, respectively and that it is suitable for rapid, on-demand testing (10). In analytical studies, the GXEA detected 63 enterovirus serotypes tested, with limits of detection ranging from 0.0002 to 200 TCID$_{50}$/ml or LD$_{50}$/ml. It showed no cross-reactivity with 24 different microorganisms known to cause meningitis-like symptoms and tolerated influence of interfering substances on test performance (10).

We prospectively evaluated GXEA over a one-year period and compared it with two other PCR-based assays for detection of enteroviral RNA in CSF. When assessing a new molecular diagnostic assay in our laboratory, the candidate assay is usually first evaluated on proficiency panels like the Quality Control for Molecular Diagnostics (QCMD) panel. If the candidate assay shows successful performance on QCMD panels it is then evaluated prospectively in routine settings in comparison with the existing diagnostic methods for a certain period of time (usually 6-12 months).

The GXEA was first evaluated on three Enterovirus QCMD panels from years 1999/2000, 2002 and 2004 consisting of 35 panel samples in total. A plasma sample containing Coxsackievirus A9 (QCMD 2002) was excluded from analyses since GXEA is recommended for testing CSF only. All 8 enterovirus negative panel samples tested GXEA negative while enterovirus RNA was detected using GXEA in 25 out of 26 enterovirus positive panel samples. Enterovirus positive samples consisted of different tenfold dilution series of Poliovirus 2, Coxsackieviruses A9, A16 and B5, Ehoviruses 6, 9 and 11 and Enterovirus 71 in concentrations ranging from 0.03 to 25,000 TCID$_{50}$/ml. Detailed composition of three Enterovirus QCMD panels included in the study is available on the QCMD home page (http://www.qcmd.org/). A single false-negative GXEA result was obtained when testing panel sample EV-B07 from 1999/2000 QCMD panel containing 0.036 TCID$_{50}$/ml of Coxsackievirus A9. This result was not unexpected since this particular sample tested enterovirus negative in all three QCMD reference laboratories and was reported enterovirus negative by 40 (81.6%) out of 49 participating laboratories (23).
Following the successful performance of the GXEA on QCMD panels the assay was implemented in daily routine testing of CSF samples. GXEA was evaluated in parallel with an in-house real-time PCR assay, as described previously (8). Briefly, after RNA isolation from 140 µl of CSF using QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany), 5 µl of RNA was amplified using previously described primers targeting 120 bp of 5’-non coding region and LightCycler RNA Master Hybridization Probes Kit (Roche Applied Science, Mannheim, Germany) on Light Cycler 2.0 Instrument (Roche Applied Science). Thus each CSF sample was tested for the presence of enterovirus RNA using GXEA and in-house real-time PCR on the same day. Since the in-house real-time PCR assay had been implemented in daily routine only 7 months before starting of GXEA evaluation and due to lack of an internal control in the in-house real-time PCR, all CSF samples were additionally tested once weekly using Enterovirus Consensus Kit (Argene SA, Varilhes, France), following the manufacturer’s instructions, as described previously (6). Briefly, 10 µl of RNA previously extracted for the in-house real-time PCR and stored for a maximum of one week at -70°C was amplified using one-step reverse-transcription PCR and primers targeting a 425 bp region of the 5’-non coding region followed by detection of PCR products in a microtiter plate using a biotinylated probe. The in house real-time PCR and Argene assay were performed by two technicians with substantial experience in molecular diagnostics while GXEA testing was performed by 8 operators, mainly without any experience in molecular diagnostics.

A total of 162 CSF samples were tested prospectively for the presence of enterovirus RNA using three different PCR assays from July 15, 2006 until July 14, 2007. Fifteen of 162 samples had to be retested due to different performance problems of the initial run. Seven samples were retested using GXEA due to an initial invalid run because of internal control failure while 11 samples (3 of them retested using GXEA) were retested using Argene assay due to initial negative internal control results (8 samples) or indeterminate enterovirus results (3 samples). The enteroviral RNA status of all 15 samples with GXEA and Argene assay initial performance problems was resolved successfully after repeated testing. As shown in Table 1, 149 (91.9%) of 162 samples initially tested concordantly with all three assays. The
initial sensitivity and specificity of GXEA, Argene, and in-house real-time PCR are shown in Table 2. All samples with discordant results were retested using all three assays. Of 5 initially Argene negative samples, 3 samples became positive on repeat while 2 samples remained Argene enterovirus negative in repeated testing (Table 1). The latter two samples which tested repeatedly negative using the Argene assay but repeatedly GXEA and in-house RT-PCR positive were finally considered to be false negative results for the Argene assay. In both patients these results were supported by clinical diagnoses of enteroviral meningitis and their CSF samples tested negative for HSV DNA, CMV DNA, VZV DNA, EBV DNA, HHV-6 DNA; these samples were also IgM and RNA negative for tick-borne encephalitis virus. Of 8 initially GXEA negative samples, 7 tested enterovirus positive while one sample remained GXEA negative upon repeat (Table 1). The latter sample, which tested repeatedly GXEA negative but repeatedly Argene assay and real-time PCR positive was finally considered to be a GXEA false negative. This result was supported by clinical diagnosis of enteroviral meningitis, and CSF and blood samples tested negative for all previously listed viruses. The most surprising finding in this evaluation were 7 initially GXEA negative samples which all became highly positive upon repeat testing. Although these 7 samples were initially reported as enterovirus negative by the GeneXpert Dx instrument, we noticed after the first such discrepant result (due to non-blinded nature of our comparative evaluation) that the end-point fluorescence in these samples showed some evidence of enteroviral amplification signal ranging from 3 to 15. In contrast, results from concordant enterovirus negative specimens and negative controls invariably showed enterovirus-specific end-point fluorescence values at zero. The GXEA incorporates a co-amplified control for detection of inhibition; this comprises an "Armoured RNA" in-process control which is added at low levels to the sample before it is extracted, in order to control for efficiency of both extraction and amplification. Lower than expected end-point values could come from PCR inhibitors or from operator error (pipetting errors, incomplete or incorrect reagent dispensing into the GeneXpert cartridge). PCR inhibitor(s) extremely labile to freeze-thawing might be more likely, since repeat testing after a freeze-thaw cycle when freezing lasted at least one hour resolved all the discrepant
samples with end-point fluorescence above zero but below the threshold for a positive result. In contrast, repeat testing of these samples on the same day or without a freeze-thaw cycle or sample dilution 1:10 resulted again in end-point fluorescence above zero but below the threshold for a positive result. According to our results, checking for end-point fluorescence values above zero in all enterovirus negative samples should be used as an indication for the need for a repeat test. In our experience, this additional check took less than a minute and could be performed reliably by individuals without a strong background in molecular diagnostics. Further evaluation of GXEA results with lower than expected end-point values from other parts of the world (if recognized) could be important, since this phenomenon might be due to different regional practices of lumbar puncture or other factors.

After repeat testing, the results of the three assays agreed in 159 (98.1%) of 162 samples (Table 1). The resolved sensitivity and specificity of GXEA, Argene, and in-house real-time PCR are shown in Table 2. As described in detail earlier, simple manual checks of all “enterovirus negative” GXEA results significantly improved sensitivity of the GXEA from 90.4% to 98.8% (Table 2). Therefore, we plan to monitor all GXEA negative samples prospectively to determine if this parameter remains useful for the determination of the need for repeat testing.

In conclusion, GXEA – the only FDA-approved assay for detection of enteroviral RNA in CSF is an important new tool for the diagnosis of enteroviral meningitis. It delivers enterovirus PCR results on a STAT basis, and meets a critical patient need for definitive diagnostic results in the evaluation of meningitis. Our study, the first to be published on a series of European patients, confirms previously described levels of sensitivity and specificity of the GXEA (10). In our experience, further improvements in the performance of GXEA were obtained by manually checking enterovirus end-point fluorescence values. For infrequent results for which enterovirus end-point values are above zero but below the GXEA threshold for a positive result, we suggest repeating the test after a short freeze-thaw cycle. After one year experience with GXEA in a routine laboratory setting we consider GXEA to be an
excellent system for routine or “on demand” testing for enteroviral meningitis due to its complete automation and rapid-result capability.
REFERENCES


hospitalization and antibiotic use for infants 90 days of age or younger. Pediatrics 120:489-496.


TABLE 1. Results of enterovirus RNA initial and resolved testing using GeneXpert Enterovirus Assay (GXEA), in-house real-time PCR and Argene Enterovirus Consensus Kit on 162 CSF samples.

<table>
<thead>
<tr>
<th>Testing result</th>
<th>No. of samples</th>
<th>Initial result</th>
<th>No. of samples</th>
<th>Resolved result</th>
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<tbody>
<tr>
<td>GXEA pos./ real-time PCR pos./Argene pos.</td>
<td>70</td>
<td></td>
<td>80</td>
<td></td>
</tr>
<tr>
<td>GXEA neg./real-time PCR neg./Argene neg.</td>
<td>79</td>
<td></td>
<td>79</td>
<td></td>
</tr>
<tr>
<td>GXEA pos./ real-time PCR pos./Argene neg.</td>
<td>5</td>
<td></td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>GXEA neg./ real-time PCR pos./Argene pos.</td>
<td>8</td>
<td></td>
<td>1</td>
<td></td>
</tr>
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</table>
TABLE 2. Initial and resolved sensitivity and specificity of the GeneXpert Enterovirus Assay (GXEA), in-house real-time PCR and Argene Enterovirus Consensus Kit for the detection of enterovirus RNA in 162 CSF samples.

<table>
<thead>
<tr>
<th>Testing</th>
<th>GXEA</th>
<th>Real-time PCR</th>
<th>Argene Assay</th>
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<tbody>
<tr>
<td></td>
<td>Sensitivity</td>
<td>Specificity</td>
<td>Sensitivity</td>
</tr>
<tr>
<td>Initial</td>
<td>90.4%</td>
<td>100%</td>
<td>100%</td>
</tr>
<tr>
<td>Resolved</td>
<td>98.8%</td>
<td>100%</td>
<td>100%</td>
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