Prevalence and Management of Invalid GeneXpert Enterovirus Results Obtained with Cerebrospinal Fluid Samples: a 2-Year Study[▽]

Susan E. Sefers,[1] Anna K. Raymer,[1] Jessica T. Kilby,[1] David H. Persing,[3,4] and Yi-Wei Tang[1,2]*

Departments of Pathology[1] and Medicine,[2] Vanderbilt University Medical Center, Nashville, Tennessee; Cepheid, Sunnyvale, California[3]; and Department of Pathology, Stanford University School of Medicine, Palo Alto, California[4]

A total of 525 cerebrospinal fluid (CSF) samples submitted during the 2007 and 2008 enteroviral seasons were included in a study to determine the prevalence of and potential risk factors for invalid Cepheid GeneXpert enterovirus assay (GXEA) results, as well as possible solutions for the problem. The invalid GXEA results were reported for 43 (8.2%) specimens and correlated with increased visibility of red blood cells ($P < 0.0001$) but not with CSF xanthochromia and clotting. Invalid GXEA result rates were markedly diminished by 82.1% and 96.0% and test sensitivities were minimally decreased by 1.7% and 3.6% when these specimens were tested at a 1:5 dilution and after a freeze-thaw cycle, respectively.

Enterovirus (EnV) meningitis can be difficult to distinguish from disease caused by other etiologic agents when patients present with nonspecific pathogenic symptoms and signs, such as fever, headache and stiff neck, and pleocytosis in cerebrospinal fluid (CSF) (4, 8, 9). Nucleic acid amplification-based procedures for the detection of EnV RNA in CSF have replaced cell culture as the test of choice (10, 11, 14). The GeneXpert enterovirus assay (GXEA; Cepheid, Sunnyvale, CA) is designed as an integrated system combining specimen processing, EnV amplification, and detection in a disposable cartridge which takes 2.5 h to detect EnV from CSF (6, 7, 12). It is designed for on-demand testing, such that "stat" PCR results can be returned to the emergency room physicians in time for patient management decisions to be made in real time. The system includes an internal control that provides a means to detect amplification inhibitors. When the internal control does not amplify, the presence of an amplification inhibitor is assumed and the result is reported as "invalid." Invalid results, if and when they occur, could delay patient management in the emergency setting.

We performed a 2-year prospective study to determine the prevalence of invalid GXEA results and explore the potential risk factors related to the occurrence of invalid results. We also validated two alternative procedures to minimize the occurrence of these invalid results for EnV detection in CSF.

(This study was presented in part at the 25th Annual Meeting of the Pan American Society for Clinical Virology, Daytona Beach, FL, 19 to 22 April, 2009.)

Clinical samples. CSF specimens submitted to Vanderbilt University Medical Center between 1 April 2007 and 20 September 2008 for detection of EnV by PCR were collected consecutively. Specimens with enough leftover volume and still unfrozen after the completion of diagnostic testing were included in the study.

Determination of CSF characteristics. Each CSF sample was visually examined for visible red blood cells (RBCs), clotting, and the presence of xanthochromia. RBC visibility was graded semiquantitatively on a scale using rankings of clear, 1+, 2+, 3+, and 4+. These grades were based on specimen colors and turbidities which roughly correspond to CSF RBC counts of $<200/\mu l$ (clear), 200 to $5,000/\mu l$ (1+), 5,000 to $75,000/\mu l$ (2+), 75,000 to $150,000/\mu l$ (3+), and $>150,000/\mu l$ (4+).

GXEA. The GXEA kit was purchased from Cepheid (Sunnyvale, CA). The information from each kit lot was recorded. CSF samples were tested using the GXEA as previously described (6, 7). In brief, 140 μl of CSF was added to the GeneXpert cartridge and then processed automatically for the different steps of sample preparation and amplification. After the diagnostic procedure was completed, the following two additional procedures were performed within 48 h on unfrozen CSF specimens with enough leftover volume prior to the routine GXEA procedure: (i) a 1:5 dilution, in which a CSF specimen was diluted once with saline in a 1:5 ratio; and/or (ii) a freeze-thaw cycle, in which a CSF specimen was quickly frozen in dry ice and thawed in a 37°C water bath one time.

During the study period, a total of 525 CSF specimens were submitted to the diagnostic laboratories. The samples spanned two EnV seasons, with 301 collected in 2007 and 224 collected in 2008. The patients' ages ranged from less than 1 day old to 74 years old, with an average age of 8 years. The ratio of males to females was 0.56:0.44. Among the 525 CSF samples enrolled in this study, 95 were positive for EnV by the GXEA, giving a positive rate of 18.1%. Invalid GXEA results were reported for 43 (8.2%) specimens during the 2-year study period. The invalid-result rate was 9.6% in 2007 and 6.3% in 2008, with no significant change in the invalid-result rate from 2007 to 2008 ($\chi^2 = 1.96$, $P > 0.05$). Included in the evaluation were GXEA kits with seven different lot numbers purchased during the 2-year study period, and invalid results were evenly distributed among the seven lots.

* Corresponding author. Mailing address: Molecular Infectious Diseases Laboratory, Vanderbilt University Hospital, 4605 TVC, Nashville, TN 37232-5310. Phone: (615) 322-2035. Fax: (615) 343-8420. E-mail: yiwei.tang@vanderbilt.edu.

[▽] Published ahead of print on 15 July 2009.

TABLE 1. Invalid GXEA results correlate to levels of visible RBCs in CSF specimens

Visible RBC ranking	No. of specimens	No. (%) of specimens with invalid result	Invalid-result odds ratio[a]	No. (%) of EnV-positive specimens[b]	EnV-positive odds ratio
None	388	19 (4.9)	1.00	81 (20.9)	1.00
1+	57	3 (5.3)	1.07	8 (14.0)	0.67
2+	35	5 (14.3)	2.92	8 (22.9)	1.10
3+	26	8 (38.8)	6.28	6 (23.1)	1.11
4+	19	8 (42.1)	8.60	3 (15.8)	0.76

[a] χ^2 trend = 38.01, $P < 0.0001$.
[b] Includes those specimens that previously tested invalid.

Assuming that the built-in internal control was functioning correctly to detect inhibitory or interfering substances, we next assessed potential factors associated with the occurrence of invalid results. Invalid results correlated with visible RBCs in the CSF specimens tested (Table 1). Of the 525 CSF samples tested, 388 samples were graded as clear, of which only 19 (4.9%) samples gave invalid results. The invalid-result rate but not the EnV-positive rate correlated with RBC cellularity (χ^2 trend = 38.01, $P < 0.0001$). In comparison to the results for clear specimens, CSF specimens with 1+, 2+, 3+, and 4+ RBC cellularity gave odds ratios at a 1.07-, 2.92-, 6.28-, and 8.60-fold likelihood, respectively, of encountering invalid results (Table 1). Visible RBC clots were only seen in six samples and were not related to the occurrence of invalid results (data not shown). Xanthochromia was noted in 44 CSF specimens of which 2 (4.5%) tested invalid; however, no statistical significance was discovered ($\chi^2 = 0.85$, $P > 0.05$) in comparison to the 41 (8.5%) invalid findings in the 481 nonxanthochromic samples. These data indicated that invalid GXEA results were correlated with increased RBC cellularities of the CSF specimens tested.

CSF specimens with sufficient volume that were still unfrozen were retested to pursue potential solutions for minimizing the occurrence of invalid results. Twenty-eight CSF specimens were tested again after a 1:5 dilution. All but five diluted samples gave valid results, thus diminishing the invalid-result rate by 82.1% (Table 2). Twenty-five samples were subjected to a one-time freeze-thaw cycle before retesting, as reported recently (12), and all invalid results were resolved except for one sample, reducing the invalid-result rate by 96.0%. Taken together, we found that a one-time freeze-thaw cycle or a 1:5 dilution of the tested specimen markedly diminished the invalid-result rate, with no statistical difference indicated between the results for the two procedures (Fisher's exact test, $P > 0.05$).

We next studied whether the two procedures influenced

diagnostic sensitivities. Among all positive specimens with enough leftover volume, retesting after a 1:5 dilution and a one-time freeze-thaw cycle achieved sensitivities of 98.3% and 96.4%, respectively (Table 2). There was no statistical difference in sensitivity between the two procedures (Fisher's exact test, $P > 0.05$). In addition, there were three and four specimens with originally invalid results that became positive after the dilution and the freeze-thaw cycle, respectively. Together, these data indicated that using a 1:5 dilution or one-time freeze-thaw cycle on CSF specimens with RBC cellularity remarkably diminished invalid test results without significantly decreasing the test's sensitivity.

This study presents our 2-year data on EnV detection by the Cepheid GXEA. Invalid results occurred at a rate of 8.2% when original, untreated CSF specimens were used. These results were in the range of the results of other published studies, which have ranged from 6.7% to 16% (7, 12), and show that the internal control is functioning correctly to detect inhibition. We found that invalid results were highly correlated to the level of RBC cellularity, i.e., "bloody tap." Importantly, we also found that one of two simple procedures carried out prior to testing, a 1:5 dilution or a one-time freeze-thaw cycle, remarkably diminished the frequency of invalid results without significantly decreasing the test's diagnostic sensitivity. This information may be useful for laboratories performing on-demand testing for EnVs in CSF, especially for those supporting emergency room management of meningitis cases. One limitation of this study is that the results from the GXEA were not compared with a reference method; therefore, the possibility of false-negative results, as previously indicated (12), was not addressed. However, a review of our data on negative specimens failed to show the "low positive but below cutoff" results reported by Seme et al. in any of our reported negative specimens with valid internal control results. Thus, in our experience, a false-negative result due to partial inhibition that is undetected by the internal control seems unlikely, but it is intriguing that Seme et al. showed a potential benefit in incorporating a freeze-thaw step to address this issue in their laboratory (12).

Bloody taps are not uncommon in the evaluation of pediatric or adult meningitis, so rendering these specimens evaluable would potentially be useful, especially if results need to be returned quickly. The Cepheid product insert claims that there is no inhibition in CSF samples with RBCs up to 2.5% blood (vol/vol). In our study, when 3+ or higher (>75,000/μl) RBC cellularities in CSF specimens were visualized, the invalid GXEA results increased significantly, with an odds ratio over 3. There are several potential explanations for the RBC cellularity-related invalid results. First, components of RBCs are

TABLE 2. A 1:5 dilution of fresh specimens or a one-time freeze-thaw of specimens diminishes invalid results without decreasing test sensitivity

Method for diminishing invalid results	No. of invalid specimens treated	No. invalid after use of method	% Diminishment of invalid-result rate[a]	No. of positive specimens treated	No. positive after use of method	% Sensitivity after use of method[b]
1:5 dilution	28	5	82.1	58	57	98.3
Freeze-thaw	25	1	96.0	55	53	96.4

[a] There was no significant difference in rates of diminishment of invalid results for dilution and freeze-thawing; Fisher's exact test, $P > 0.05$.
[b] There was no significant difference in sensitivity of GXEA after dilution and freeze-thawing; Fisher's exact test, $P > 0.05$.

known to inhibit in vitro nucleic acid amplification. Heme is a well-known amplification inhibitor (1, 2, 15). In our study, however, a freeze-thaw cycle prior to testing significantly diminished invalid-result frequencies. Since heme is present in these samples, it is difficult to determine whether heme played a significant role as an inhibitor in the GXEA test system. It has been reported that several commonly used one-step RNA-targeted enzymes, such as r*Tth*, are usually not affected by heme (3).

A second explanation could be that the high level of RBCs in CSF affects the lysis of cells and virions, resulting in incomplete release of viral nucleic acids. This could be manipulated by adjustment of the volume of the CSF specimen. The additional dilution or freeze-thaw cycle can improve cell and virion lysis and viral nucleic acid release, thereby diminishing invalid-result frequencies (12). The last explanation is that, in the GXEA test cycle, the sample is pumped from one chamber to another within the microfluidic device. High levels of RBCs, with or without clot formation, could block the movement of liquids between test chambers. Both of the two explanations are supported by the fact that either a 1:5 dilution or a one-time freeze-thaw cycle remarkably diminishes the frequency of invalid results.

There have been numerous studies of specimen treatments or added reagents that act as amplification facilitators and also of possible solutions for amplification inhibitors (1, 3, 5, 13). Adding additional chemicals or steps to the lysis procedure of the GXEA could potentially enhance cell and virus lysis, thereby diminishing invalid results, but would constitute "off-label" use and require validation as performed here. In our routine diagnostic services, any CSF specimens with visible RBCs are automatically diluted to 1:5 prior to testing to keep the test turnaround time rapid without significantly decreasing test sensitivity.

REFERENCES

1. **Abu Al-Soud, W., and P. Radstrom.** 2000. Effects of amplification facilitators on diagnostic PCR in the presence of blood, feces, and meat. J. Clin. Microbiol. **38:**4463–4470.
2. **Akane, A., K. Matsubara, H. Nakamura, S. Takahashi, and K. Kimura.** 1994. Identification of the heme compound copurified with deoxyribonucleic acid (DNA) from bloodstains, a major inhibitor of polymerase chain reaction (PCR) amplification. J. Forensic Sci. **39:**362–372.
3. **Al-Soud, W. A., and P. Radstrom.** 2001. Purification and characterization of PCR-inhibitory components in blood cells. J. Clin. Microbiol. **39:**485–493.
4. **Archimbaud, C., M. Chambon, J. L. Bailly, I. Petit, C. Henquell, A. Mirand, B. Aublet-Cuvelier, S. Ughetto, J. Beytout, P. Clavelou, A. Labbe, P. Philippe, J. Schmidt, C. Regagnon, O. Traore, and H. Peigue-Lafeuille.** 2009. Impact of rapid enterovirus molecular diagnosis on the management of infants, children, and adults with aseptic meningitis. J. Med. Virol. **81:**42–48.
5. **Boddinghaus, B., T. A. Wichelhaus, V. Brade, and T. Bittner.** 2001. Removal of PCR inhibitors by silica membranes: evaluating the Amplicor *Mycobacterium tuberculosis* kit. J. Clin. Microbiol. **39:**3750–3752.
6. **Kost, C. B., B. Rogers, M. S. Oberste, C. Robinson, B. L. Eaves, K. Leos, S. Danielson, M. Satya, F. Weir, and F. S. Nolte.** 2007. Multicenter beta trial of the GeneXpert enterovirus assay. J. Clin. Microbiol. **45:**1081–1086.
7. **Marlowe, E. M., S. M. Novak, J. J. Dunn, A. Smith, J. Cumpio, E. Makalintal, D. Barnes, and R. J. Burchette.** 2008. Performance of the Gene-Xpert enterovirus assay for detection of enteroviral RNA in cerebrospinal fluid. J. Clin. Virol. **43:**110–113.
8. **Mulford, W. S., R. S. Buller, M. Q. Arens, and G. A. Storch.** 2004. Correlation of cerebrospinal fluid (CSF) cell counts and elevated CSF protein levels with enterovirus reverse transcription-PCR results in pediatric and adult patients. J. Clin. Microbiol. **42:**4199–4203.
9. **Ramers, C., G. Billman, M. Hartin, S. Ho, and M. H. Sawyer.** 2000. Impact of a diagnostic cerebrospinal fluid enterovirus polymerase chain reaction test on patient management. JAMA **283:**2680–2685.
10. **Rotbart, H. A., J. P. Kinsella, and R. L. Wasserman.** 1990. Persistent enterovirus infection in culture-negative meningoencephalitis: demonstration by enzymatic RNA amplification. J. Infect. Dis. **161:**787–791.
11. **Schlesinger, Y., M. H. Sawyer, and G. A. Storch.** 1994. Enteroviral meningitis in infancy: potential role for polymerase chain reaction in patient management. Pediatrics **94:**157–162.
12. **Seme, K., T. Mocilnik, K. F. Komlos, A. Doplihar, D. H. Persing, and M. Poljak.** 2008. GeneXpert enterovirus assay: one-year experience in a routine laboratory setting and evaluation on three proficiency panels. J. Clin. Microbiol. **46:**1510–1513.
13. **Shafer, R. W., D. J. Levee, M. A. Winters, K. L. Richmond, D. Huang, and T. C. Merigan.** 1997. Comparison of QIAamp HCV kit spin columns, silica beads, and phenol-chloroform for recovering human immunodeficiency virus type 1 RNA from plasma. J. Clin. Microbiol. **35:**520–522.
14. **Smalling, T. W., S. E. Sefers, H. Li, and Y. W. Tang.** 2002. Molecular approaches to detecting herpes simplex virus and enteroviruses in the central nervous system. J. Clin. Microbiol. **40:**2317–2322.
15. **Staudinger, R., N. G. Abraham, R. D. Levere, and A. Kappas.** 1996. Inhibition of human immunodeficiency virus-1 reverse transcriptase by heme and synthetic heme analogs. Proc. Assoc. Am. Physicians **108:**47–54.