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Evaluation of a Rapid and Completely Automated Real-Time Reverse Transcriptase PCR Assay for Diagnosis of Enteroviral Meningitis[▽]

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Nucleic acid amplification tests (NAATs) for enterovirus RNA in cerebrospinal fluid (CSF) have emerged as the new gold standard for diagnosis of enteroviral meningitis, and their use can improve the management and decrease the costs for caring for children with enteroviral meningitis. The Xpert EV assay (Cepheid, Sunnyvale, CA) is a rapid, fully automated real-time PCR test for the detection of enterovirus RNA that was approved by the U.S. Food and Drug Administration for *in vitro* diagnostic use in March 2007. In this multicenter trial we established the clinical performance characteristics of the Xpert EV assay in patients presenting with meningitis symptoms relative to clinical truth. Clinical truth for enteroviral meningitis was defined as clinical evidence of meningitis, the absence of another detectable pathogen in CSF, and detection of enterovirus in CSF either by two reference NAATs or by viral culture. A total of 199 prospectively and 235 retrospectively collected specimens were eligible for inclusion in this study. The overall prevalence of enteroviral meningitis was 26.04%. The Xpert EV assay had a sensitivity of 94.69% (90% confidence interval [CI] = 89.79 to 97.66%), specificity of 100% (90% CI = 99.07 to 100%), positive predictive value of 100%, negative predictive value of 98.17, and an accuracy of 98.62% relative to clinical truth. The Xpert EV assay demonstrated a high degree of accuracy for diagnosis of enteroviral meningitis. The simplicity and on-demand capability of the Xpert EV assay should prove to be a valuable adjunct to the evaluation of suspected meningitis cases.

Enteroviruses are responsible for a wide variety of diseases in children and adults, including nonspecific febrile illnesses, neonatal sepsis, meningitis, and encephalitis (16). These infections are very common, with an estimated 5 to 10 million symptomatic infections occurring annually in the United States, primarily in infants and young children (21). Enteroviruses may cause up to 90% of aseptic meningitis cases for which a specific etiology is identified. Occurring mainly in the summer and fall, enteroviral meningitis leads to a large number of hospitalizations of both children and adults.

Enteroviral meningitis may be difficult to differentiate from partially treated bacterial meningitis because the cerebrospinal fluid (CSF) pleocytosis may have an early predominance of neutrophils. Many patients with enteroviral meningitis are hospitalized and treated with parenteral antibiotics until the clinical picture improves and the bacterial cultures of blood and CSF are negative after 48 h of incubation, or viral cultures or nucleic acid tests for enterovirus are positive. If patients have been previously treated with antibiotics early in the course of illness, then CSF bacterial cultures may be unreliable and longer empirical antibiotic courses may be necessary.

CSF viral cultures lack sensitivity, rarely provide results in a clinically relevant time frame, and require high-level technical expertise to perform. Nucleic acid amplification tests (NAATs) for enterovirus RNA in CSF have emerged as the new gold standard for diagnosis of enteroviral meningitis (13–15). These tests have better sensitivity than culture, the results can be available within hours of specimen collection, and the costs are similar to viral cultures (12). Currently, a wide variety of user-developed NAATs with different performance characteristics are used by diagnostic laboratories. However, the technical complexity of most NAATs requires that they are run in batches and restricts their availability to high-complexity laboratories with appropriate expertise in nucleic acid diagnostics.

Significant healthcare resources are utilized in diagnosing and caring for infants and young children with enteroviral infections. Several investigators have demonstrated that early diagnoses provided by NAATs can improve the management and decrease the costs of caring for children with enteroviral meningitis by decreasing length of hospital stay, hospital charges, and antibiotic use (3, 6, 10, 11, 19). Despite the enhanced performance characteristics and potential for improving the paradigms for diagnosis and patient management, until recently there was no U.S. Food and Drug Administration (FDA)-approved test for enterovirus RNA detection. On 16 March 2007 the FDA cleared the first NAAT for detection of EV RNA, the Xpert EV assay (Cepheid, Sunnyvale, CA).

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The Xpert EV assay is a qualitative *in vitro* diagnostic test designed to detect enterovirus RNA in CSF specimens by reverse transcription-PCR (RT-PCR). The results from this test are intended for use as an aid, in conjunction with other laboratory and clinical evidence, in the diagnosis of enteroviral meningitis. Unlike other NAATs, the Xpert EV assay is designed to be run on-demand, so results are available in a time frame suitable for making emergent medical management decisions.

In this multicenter evaluation, we determined the performance characteristics of the Xpert EV assay for the detection of enterovirus RNA in CSF specimens from patients presenting with meningitis symptoms. The sensitivity and specificity of the Xpert EV assay were established relative to clinical truth. The clinical trial data described here served as the basis for the Xpert EV Assay 510(k) application to the FDA (no. K061062).

MATERIALS AND METHODS

Study design. This was a multicenter trial to assess the clinical performance of the Xpert EV assay on CSF specimens obtained by lumbar puncture from patients with signs and symptoms of meningitis as part of their routine evaluation. Excess CSF was tested by the Xpert EV assay at the trial sites, and by two laboratory-developed NAATs at the Centers for Disease Control and Prevention (CDC), including (i) an assay that uses Mariani/SmartCycler (SC) EV primers and probes (SC assay) (4) and (ii) a VP1 RT-seminested-PCR assay (VP1 assay) developed at the Centers for Disease Control and Prevention (CDC) as previously described (9). If available, the results of the laboratory-developed reverse transcription-PCR (RT-PCR) assays that were in routine diagnostic use at the enrolling sites were also recorded. Viral culture was performed on a subset of specimens, either if ordered by the physician at the enrolling site or if sufficient excess CSF was available to be analyzed at a central laboratory. The performance of the Xpert EV assay was calculated by comparison to clinical truth. Clinical truth was defined as clinical and laboratory evidence consistent with meningitis, which included: (i) fever, headache, vomiting, nuchal rigidity, photophobia, abdominal pain, irritability, seizures, and/or alterations of consciousness (2); (ii) absence of another detectable pathogen in CSF (Gram stains and bacterial cultures were done on all CSF specimens); and (iii) detection of enterovirus RNA in CSF in both reference NAATs or a positive CSF enterovirus culture.

All subjects with conflicting evidence regarding diagnosis of enteroviral meningitis were presented to a panel of three board-certified pediatric infectious disease specialists, who determined their diagnosis based on available information, but without knowledge of the Xpert EV Assay results. The primary endpoint for the study was clinical specificity and the secondary endpoint was clinical sensitivity relative to clinical truth.

Investigational sites. Enrollment was performed at five sites that prospectively collected specimens and one site that retrospectively collected specimens. The enrollment sites were Gainesville, FL; Dallas, TX, Denver, CO; Atlanta, GA; Milwaukee, WI; and Nashville, TN. The study was performed with the approval or exemption of the local institutional review boards (IRBs) at each investigational site.

Eligibility criteria. All of the inclusion and none of the exclusion criteria were met in order for the patient to be enrolled in the study. The inclusion criteria were that CSF was obtained as part of an evaluation for meningitis and an enterovirus NAAT and/or viral culture was ordered on the CSF by a physician, a sufficient excess volume of CSF was available ($\geq$0.5 ml for prospective and $\geq$0.7 ml for retrospective specimens), and informed consent was provided by the patient or legal guardian and assent for minors was obtained if required by the reviewing IRB. Exclusion criteria were that CSF had been centrifuged and that the Xpert EV assay and the two reference NAATs were not performed within the same freeze-thaw cycle of the specimen.

Specimens. CSF specimens were collected by lumbar puncture according to standard procedures from subjects with suspected meningitis. Aliquots were prepared for testing with the Xpert EV assay and the two reference laboratory NAATs from all specimens, and an aliquot was also prepared for viral culture if a sufficient volume of CSF remained. All aliquots were frozen and stored at −70°C prior to testing with the Xpert EV assay or shipping to the central laboratories on dry ice.

NAATs. The GeneXpert Dx system consists of an instrument, a personal computer, and disposable cartridges that perform fully automated and completely integrated sample processing and real-time multiplex PCR detection of nucleic acid sequences. The Xpert EV assay includes reagents, primers, and probes for the simultaneous detection of enterovirus RNA and an internal sample processing control in 2.5 h as previously described (4). The EV primers and probe used in this assay bind to the 5′ nontranslated region of the genome and their sequences have been described previously (4).

Prospectively collected samples were tested with locally developed NAATs and the Xpert EV assay at five of the enrolling sites and the reference VP-1 and SC EV assays at the CDC. All retrospectively collected specimens were tested with the Xpert EV assay at one of the enrolling sites (Emory University) and the two reference NAATs at the CDC. The amplicons from the VP-1 assay were isolated and sequenced to identify the enterovirus serotypes detected in clinical samples as previously described (9). The VP-1 and SC assays were chosen as reference methods because of their superior analytical sensitivity and ability to amplify all enterovirus serotypes. All of the NAATs used in the present study except the VP-1 assay target the 5′ nontranslated region of the enterovirus genome. The seminested VP-1 assays targets the genome sequences that encode the immunodominant capsid protein.

Only the results of the SC and VP-1 assays were used in defining clinical truth. The locally developed NAATs performed at each enrollment site were the standard of care used at that site but were not used to define cases, since subsets of CSF samples were tested with each assay and the assays were not uniform in design or performance characteristics. The Xpert EV assay, the two reference NAATs and viral culture, if it was not the laboratory's routine method for detecting enterovirus, were performed within the same sample freeze-thaw cycle. The results from Xpert EV assay and reference NAATs were not used in patient management decisions.

Viral cultures. Viral cultures were performed on all samples of sufficient volume. Cultures were performed either locally at enrollment sites or at a central reference laboratory (Tricore Laboratories, Albuquerque, NM). A variety of cell lines and detection methods were used at the enrolling sites. The reference laboratory used shell vial culture with Super E-mix cells (Diagnostic Hybrids, Athens, OH) and fluorescent antibody staining (Chemicon, Temecula, CA) for detection and identification of enteroviruses (1).

Statistics. The results of the Xpert EV assay were compared to clinical truth for calculation of the sensitivity, specificity, positive predictive value, negative predictive value, accuracy, and prevalence. The clinical trial was conducted over six sites, four lots of reagents, two patient categories (retrospective and prospective samples), both patient genders, two categories of CSF red blood cell counts (RBC $<$ 1,000/mm^3 and $\geq$ 1,000/mm^3), and three age groups (neonates, $\leq$2 months; children, $>$2 months to 17 years; and adults, $\geq$18 years). The data homogeneity and poolability across categories were determined with the Fisher exact test, and groups with P values $\geq$0.025 were considered poolable. Each two by two table statistic was treated as a binomial response, and the exact binomial confidence intervals were determined. Lower 95% confidence intervals (CI) for the overall statistics were computed by using 90% two-sided confidence intervals. The study size was set to have a statistical power of $>$90%. All calculations were made using SAS system version 9.1 (Cary, NC).

RESULTS

A total of 475 CSF specimens were collected for this trial, including 225 prospective and 250 retrospective specimens (Fig. 1). Of the 225 prospective specimens, 199 were evaluable and included in the analysis. These samples were collected between July and December 2005. Of the 250 retrospective specimens, 235 were evaluable and included in the analysis. These samples were collected between January 2004 and November 2005. The 434 enrolled patients included 155 (35.7%) neonates, 227 (52.3%) children, and 52 (12%) adults, 242 (55.8%) of which were males and 192 (44.2%) were females.

The prevalence, sensitivity, specificity, positive predictive value, negative predictive value, and accuracy were calculated separately by site, patient age, patient type, patient gender, reagent lot, and CSF RBC count. Since the Fisher exact test P value was $\geq$0.025 for all statistics, the data were considered poolable among the various factors (data not shown). The

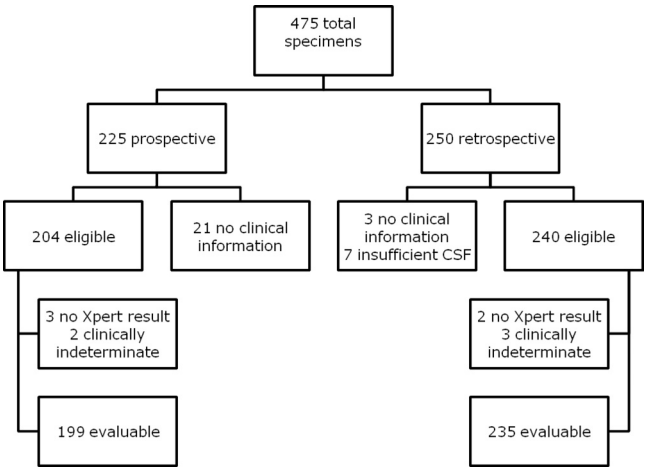

FIG. 1. Accountability of specimens collected for Xpert EV assay clinical trial. A total of 475 CSF specimens were collected, including 225 prospective and 250 retrospective specimens. Of the 225 prospectively collected specimens, 199 were evaluable and included in the analysis. The 26 ineligible specimens included 21 with no clinical information, 3 with no Xpert EV results, and 2 for which the diagnosis could not be determined by the medical review panel (clinically indeterminate). Of the 250 retrospectively collected specimens, 235 were evaluable and included in the analysis. The 15 ineligible samples included 7 with insufficient CSF volume, 3 with no clinical information, 2 with no Xpert EV results, and 3 that were clinically indeterminate.

site-specific and overall performance characteristics of the Xpert EV assay relative to clinical truth are shown in Table 1. Overall, the prevalence of enterovirus meningitis was 26.04% and the Xpert EV assay had a sensitivity of 94.69% (90% CI = 89.79 to 97.66%), specificity of 100% (90% CI = 99.07 to 100%), positive predictive value of 100%, negative predictive value of 98.17, and an accuracy of 98.62%.

There were 6 cases of enteroviral meningitis with false-negative Xpert EV tests at two of the test sites. Both reference RT-PCR tests were positive in four of these cases (two, echovirus 6; one, echovirus 18; and one, enterovirus 71). In the remaining two cases enterovirus RNA was detected only in the SC assay. Enterovirus cultures were negative in all six, and CSF pleocytosis was present in two of the Xpert EV false-negative samples.

Enterovirus culture was performed in 320 of the 434 (73.7%) cases enrolled in the study. Viral cultures were performed for 57 specimens at the clinical trial sites and for 263 specimens at the reference laboratory. One specimen had an indeterminate culture result, and one specimen had cultures performed both at the site and the reference laboratory that were discrepant. These specimens were omitted from the data analysis. Table 2 shows the viral culture results versus clinical truth for the remaining 318 specimens. The sensitivity of culture was only 35.5% for diagnosis of enteroviral meningitis, but it was 100% specific.

TABLE 2. CSF viral culture results in patients with or without enteroviral meningitis (n = 318)

Enterovirus culture result	No. of samples tested for enteroviral meningitis (clinical truth)[a]	
	Positive	Negative
Positive	27	0
Negative	49	242

[a] Clinical truth was defined as clinical and laboratory evidence consistent with meningitis, which included (i) fever, headache, vomiting, nuchal rigidity, photophobia, abdominal pain, irritability, seizures, and/or alterations of consciousness; (ii) absence of another detectable pathogen in the CSF; and (iii) detection of enterovirus RNA in CSF in both reference NAATs or a positive CSF enterovirus culture.

Laboratory-developed RT-PCR assays were the standard of care at five of the six clinical trial sites. Table 3 compares the results of the laboratory-developed and Xpert EV assay results on the 416 samples for which the results of both assays were available. Overall, the agreement between the composite laboratory developed and Xpert EV assay results was 95.7%. All of the 14 Xpert EV-positive samples that were negative by the laboratory-developed tests were true positives after determination of clinical truth. There were four Xpert EV-negative, laboratory-developed assay-positive samples, only two of which were considered true positives by clinical truth. The increased diagnostic yield with the Xpert EV assay approached but did not reach statistical significance (McNemar's test, P = 0.052).

The frequency distribution of enterovirus serotypes identified in 100 samples by sequencing of the VP1 amplicon is shown in Fig. 2. A total of 16 different serotypes were identified with coxsackievirus B5, echovirus 6, echovirus 18, and echovirus 30 comprising 75% of the positive samples. Enterovirus 71, an important cause of viral encephalitis, was detected in three cases.

TABLE 1. Site-specific and overall performance characteristics of the Xpert EV assay for diagnosis of enteroviral meningitis (n = 434)

Site	No. of samples[a]					% [b]					
	Total	TP	FP	TN	FN	Prevalence	Sensitivity	Specificity	PPV	NPV	Accuracy
1	15	2	0	13	0	13.33	100	100	100	100	100
2	34	8	0	24	2	29.41	80	100	100	92.31	94.12
3	44	3	0	41	0	6.82	100	100	100	100	100
4	84	27	0	57	0	32.14	100	100	100	100	100
5	22	6	0	16	0	27.27	100	100	100	100	100
6	235	61	0	170	4	27.66	93.85	100	100	97.70	98.30
Total	434	107	0	321	6	26.04	94.69	100	100	98.17	98.62

[a] TP, true positive; FP, false positive; TN, true negative; FN, false negative.
[b] PPV, positive predictive value; NPV, negative predictive value.

TABLE 3. Comparison of Xpert EV assay and laboratory-developed RT-PCR assay (LDA) results by site

Site	Xpert EV assay/LDA results (no. of samples):				Total no. of samples
	Positive/positive	Positive/negative	Negative/positive	Negative/negative	
2	8	0	1	25	34
3	3	0	0	41	44
4	23	4	1	56	84
5	6	0	0	16	22
6	50	10	2	170	232
Total	90	14[a]	4[b]	308	416

[a] All of the 14 Xpert EV assay-positive/LDA-negative samples were from patients with EV meningitis as determined by clinical truth.
[b] Two of the four Xpert EV assay-negative/LDA-positive samples were from patients with EV meningitis as determined by clinical truth.

DISCUSSION

NAATs for detection of enterovirus RNA have replaced viral culture and become the method of choice for the diagnosis of enteroviral meningitis at many centers for a number of compelling reasons. Currently, a wide variety of user-developed and two FDA-cleared NAATs with different performance characteristics are used by diagnostic laboratories. The NucliSens EasyQ enterovirus assay, which uses nucleic acid sequence based amplification to detect enterovirus RNA in the CSF, was approved by the FDA in June 2008. The technical complexity of most NAATs requires that they are run in daily batches, and this restricts their availability to laboratories with appropriate expertise in nucleic acid diagnostics. In facilities lacking the appropriate expertise, the tests are often sent to central or regional laboratories, leading to further delays imposed by specimen transport, thereby limiting the clinical utility of the results. In addition, user-developed tests lack standardization, and poor interlaboratory reproducibility of enterovirus NAAT results were observed in a multicenter proficiency testing study (22). The availability and acceptance by clinical laboratories of an FDA-cleared assay with well-defined performance characteristics will contribute to optimization and standardization of molecular diagnostics for enteroviral meningitis.

The analytical sensitivity and specificity of the Xpert EV assay relative to other NAATs for detection of enterovirus RNA in CSF have been previously published (4, 5, 18). The sensitivity ranged from 97.1 to 100%, and the specificity was 100% in these studies. In this multicenter study we established the clinical performance characteristics of the Xpert EV assay relative to a definition of enteroviral meningitis that included clinical evidence of meningitis, the absence of another detectable pathogen in CSF, and either detection of enterovirus in CSF by two reference NAATs or by viral culture. Using this definition of clinical truth we found the Xpert EV assay to have a sensitivity of 94.69% and a specificity of 100%.

We also compared the results of the various laboratory-developed NAATs used at the clinical trial sites as the standards of care with the Xpert EV results and found an overall agreement in 95.9% of cases. However, the Xpert EV Assay detected ca. 10% more cases of EV meningitis than the individual laboratory-developed tests.

In the present study only 35.5% of samples from patients with enteroviral meningitis were positive by culture. Although a variety of conventional viral culture methods were used at the clinical trial sites, the majority (82.2%) of the cultures were performed at a reference laboratory that used state-of-the art shell vial cultures using a mixture of sensitive cell lines and pan-enterovirus antibody for detection and identification of enteroviruses (1).

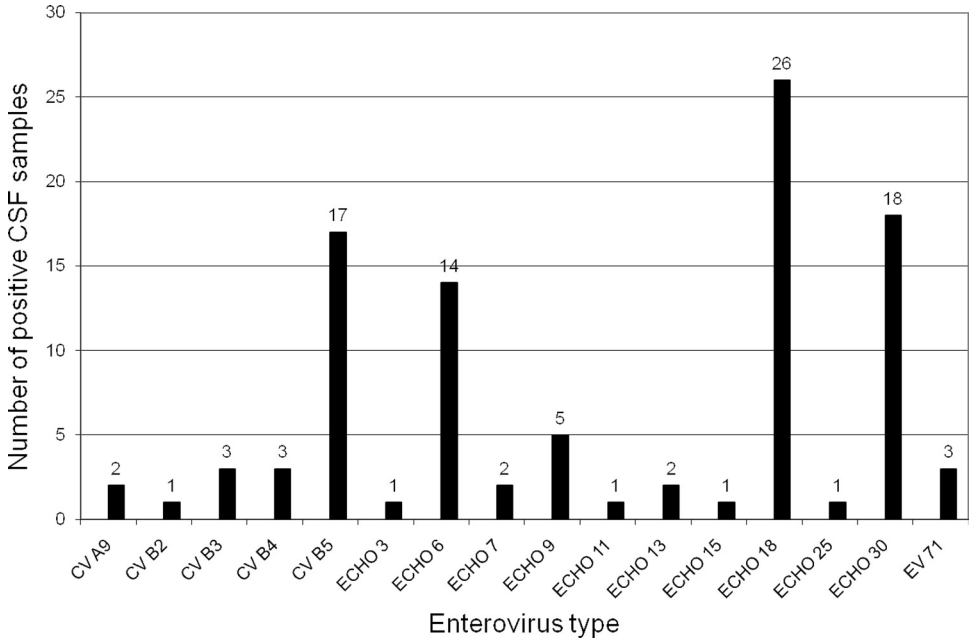

FIG. 2. Frequency distribution of enterovirus serotypes detected in CSF as determined by nucleic acid sequence analysis of VP1 amplicons. CV, coxsackievirus; ECHO, echovirus; and EV, enterovirus.

False-positive results due to target and amplicon cross-contamination are a significant concern with many NAATs. Since the Xpert EV assay cartridge is completely self-contained and performs all assay steps including sample preparation, false-positive results due to cross-contamination should not occur. As anticipated, no false-positive Xpert EV assay results occurred in the present study.

The on-board positive control in the Xpert EV assay ensures that the sample is adequately prepared, the critical reagents are functioning properly, and the sample is free of interfering substances, thus reducing the risk of false-negative test results due to operator error or interfering substances. In the present study the on-board control failed on the first attempt in only 13 of 434 (3%) tests performed on evaluable patients, indicating that the assay is robust and not prone to operator error. All but one gave a valid result on the second attempt. The actual rate of invalid results may be higher in routine practice. Sefers et al. (17) reported an 8.2% invalid rate over a 2-year period of routine use. These authors found that either a 1:5 sample dilution of CSF in saline, or freezing and thawing the undiluted CSF markedly reduced the invalid rate with minimal impact on the sensitivity of the assay. All samples in the present study were subjected to one freeze-thaw cycle before testing with the Xpert EV assay.

The primers and probes used in the Xpert EV assay detect all of the recognized enterovirus serotypes, including polioviruses, and do not cross-react with other microorganisms known to cause meningitis-like symptoms (4). The present study included patients infected with 16 different serotypes, which confirmed the inclusivity of the assay. However, the Xpert EV assay will not detect the closely related human parechoviruses, which like enteroviruses can also cause aseptic meningitis, acute flaccid paralysis, and encephalitis (20). Of note, three samples were found to contain enterovirus 71 that has the unique ability among the nonpolio enteroviruses to cause brain stem encephalitis and paralysis. Enterovirus 71 has caused widespread outbreaks of disease in Eastern Europe and Southeast Asia and smaller outbreaks in the North America, Europe, and Australia (7). NAATs are particularly important for detection of this agent since it is difficult to recover from CSF by culture. In the present study all three patients with enterovirus 71 detected in their CSF had a clinical course more consistent with meningitis rather than encephalitis. Only one of the 113 patients with enterovirus detected in the CSF had seizures and/or altered consciousness, suggestive of encephalitis. Coxsackievirus B5, one of the limited number of enterovirus serotypes associated with encephalitis, was detected in this patient.

A child with a positive EV NAAT result could potentially have bacterial meningitis due to either a false-positive NAAT result or a bacterial coinfection. Although rare coinfections of the CSF by bacteria and EVs have been reported (23), a recent, retrospective, cohort study of children presenting to emergency departments in 20 participating hospitals showed that among 735 children with a positive EV NAAT result none had bacterial meningitis (0%; 95% CI = 0 to 0.5%) (8). In our study population we found six cases of bacterial meningitis, two caused by *Escherichia coli*, two caused by *Streptococcus agalactiae*, and one each caused by *Neisseria meningitidis* and *S. pneumoniae.* All six cases were negative for EV RNA by the NAATs. In mixed infections the clinical presentations are severe enough that the early identification of EV from the CSF is unlikely to deter clinicians from use of antibiotics. Consequently, the detection of EV RNA in CSF from a patient with a clinically compatible illness is sufficient to establish the EV as the cause of the CNS disease.

The benefits of the Xpert EV assay are the ability to obtain reliable results rapidly, within several hours of spinal fluid collection, and to decentralize the testing process so that rapid turnaround time for results can be realized within the medical decision making process. The Xpert EV assay is a fully automated NAAT that can deliver reliable molecular diagnostic results for enteroviral meningitis on demand without the need of specially trained laboratory staff, batching, or dedicated molecular diagnostic facilities. The results of rapid, on-demand testing for enteroviral meningitis could affect the decision to admit patients with suspected meningitis to hospital. Theoretically, patients with positive tests could be managed more conservatively than those with negative tests, without the need for hospitalization, intravenous antibiotics, and attendant nosocomial risks. Since enteroviral meningitis accounts for a large number of hospitalizations of children and adults, this strategy has the potential to simultaneously save significant healthcare resources and provide the most appropriate patient management.

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