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Multicenter Evaluation of a Commercially Available PCR Assay for Diagnosing Enterovirus Infection in a Panel of Cerebrospinal Fluid Specimens

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Thirteen laboratories participated in blind tests of a panel of 20 coded cerebrospinal fluid specimens (7 uninfected samples, 3 samples infected with 1 50% tissue culture infective dose [$TCID_{50}$]/0.1 ml [nonenterovirus strains], and 10 samples infected with 10, 1, or 0.1 $TCID_{50}$/0.1 ml [three different enterovirus serotypes]) on the Amplicor enterovirus PCR assay (Roche Diagnostic Systems). The panel was also evaluated by in-house PCR (two nested-PCR and three one-step PCR assays) or tissue culture (eight laboratories). The viral load was shown to influence greatly the sensitivity of the assay. The average sensitivity of the Amplicor test ranged from 67 to 98% for viral titers of 1 to 10 $TCID_{50}$/0.1 ml, respectively; titers of 0.1 $TCID_{50}$/0.1 ml resulted in a sensitivity of only 16%. The overall specificity of the Amplicor test was 98%. The Amplicor assay compared favorably to the five in-house PCR tests (no significant difference in either sensitivity or specificity) and was much more sensitive than tissue culture ($P < 0.001$), even for high viral loads. It was easy to perform, rapid (about 6 h), well-standardized, and appeared to be suitable for the diagnosis of enterovirus meningitis on a routine basis in laboratories trained in molecular biology techniques.

There are 67 serotypes of human enteroviruses that are responsible for infections ranging from asymptomatic to fatal infections. They cause most of the cases of viral meningitis and are involved in encephalitis, especially in neonates (3, 6, 13, 14). They are responsible for chronic infection of the central nervous system in agammaglobulinemic patients (12). The conventional diagnosis of enterovirus infections of the central nervous system relies on the recovery of virus from the cerebrospinal fluid (CSF) or from a peripheral site (e.g., throat, stools) by cell culture. However, this technique is not very sensitive, especially for CSF specimens (due to low viral burden) and takes an average of 6 to 7 days to detect viral growth.

Molecular methods have recently been introduced for diagnosing enterovirus infection (4, 7, 18, 24). Enzymatic amplification of viral cDNA synthesized by reverse transcription (RT)-PCR is a sensitive method for the detection of enterovirus directly from CSF specimens (8, 10, 16, 17, 19–21). Primers with the sequence of a conserved part of the 5′ untranslated region of the enterovirus genomes make it possible to detect most of the enterovirus serotypes, with the exception of echoviruses 22 and 23 (EV-22 and EV-23), which are very different from other members of the enterovirus genus (5).

One of the major disadvantages of many PCR assays is the risk of false-positive results because of the carryover of previously amplified DNA, especially when a nested technique is used to increase the sensitivity of the test. A combined RT-PCR assay for enterovirus RNA has been developed by Roche Diagnostic Systems (Amplicor enterovirus) (21). This assay uses a single thermostable enzyme (recombinant *Thermus thermophilus*) and buffer conditions in a single reaction tube. The combination of these factors plus the use of dUTP in place of dTTP efficiently prevents false-positive amplification (11). The amplified products are also detected by a simple, microwell colorimetric assay, which increases the sensitivity of the test while avoiding the use of a two-step PCR, even when viral loads are low.

The present study evaluates the ability of the Amplicor assay to detect enteroviruses in CSF specimens on a multicenter basis. A panel of 20 CSF specimens, some of which had been infected artificially with different loads of enterovirus or other viruses, were subjected to blind tests by 13 laboratories trained in the molecular diagnosis of viral infections. The results of the

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TABLE 1. Primers from the 5′ untranslated region of the enterovirus genome used in the PCR tests

Test and/or laboratory	Primer	
	Direction[a]	Sequence
Amplicor test (one-step PCR)	1	5′-biotinyl-GGCCCCTGAATGCGGCTAAT
	2	5′-biotinyl-CAATTGTCACCATAAGCAGCCA
Laboratory 1 (seminested PCR)	Outer and inner 1	5′-CAAGCACTTCTGTTTCCCCGG
	Outer 2	5′-ATTGTCACCATAAGCAGCCA
	Inner 2	5′-CTTGCGCGTTACGAC
Laboratory 2 (one-step PCR)	1	5′-AAGCACTTCTGTTTCC
	2	5′-CATTCAGGGGCCGCAGGA
Laboratory 3 (one-step PCR)	1	5′-CAAGCACTTCTGTTTCCCCGG
	2	5′-ACGGACACCCAAAGTAGTCG
Laboratory 4 (seminested PCR)	Outer 1	5′-CAAGCACTTCTGTTTCCCCGG
	Inner 1	5′-TCCTCCGGCCCCTGAATGCG
	Outer and inner 2	5′-ATTGTCACCATAAGCAGCCA
Laboratory 5 (one-step PCR)	1	5′-TCCTCCGGCCCCTGAATGCG
	2	5′-ATTGTCACCATAAGCAGCCA

[a] 1 and 2 correspond to downstream and upstream directions, respectively.

study are reported and compared with those obtained in some of the participating laboratories with cell culture and in-house PCR.

MATERIALS AND METHODS

Laboratories and techniques. Thirteen laboratories from teaching hospitals (12 in France and 1 in Argentina) took part in the study; a code number (1 to 13) was assigned to each laboratory. A panel of 20 CSF specimens was tested using the Amplicor enterovirus kit (Produits Roche, Neuilly-sur-Seine, France) in all 13 laboratories. The CSF panel was also tested by in-house PCR and cell culture in four laboratories (laboratories 1 to 4), by in-house PCR in one laboratory (laboratory 5), and by cell culture in four laboratories (laboratories 6 to 9).

CSF samples. A panel of 20 coded CSF samples was prepared on 17 July 1995 by the French National Reference Center for Enteroviruses (Lyon, France). A large volume of CSF was obtained from a patient with a CSF ventriculoperitoneal shunt; it showed neither chemical nor cytologic abnormalities and tested negative for enterovirus by cell culture and PCR. All through this report, viral infectivities will be expressed as 50% tissue culture infective dose per 100 ml and abbreviated $TCID_{50}$. Seven of the samples were not infected (undiluted CSF), 3 were infected with one $TCID_{50}$ of a nonenterovirus virus (herpes simplex virus type 1, adenovirus, and mumps virus), and 10 samples were infected with enterovirus strains (coxsackievirus B3 [Nancy strain], EV-11 [Gregory strain], and poliovirus type 3 [Sabin strain]). The specimens infected with enteroviruses contained various viral loads, as determined by titration on BGM cells: 0.1 $TCID_{50}$ (one sample for each enterovirus serotype), 1 $TCID_{50}$ (one sample for coxsackievirus B3 and poliovirus type 3; two samples for EV-11, one of which had been contaminated by erythrocytes), and 10 $TCID_{50}$ (one sample for each serotype). The samples were immediately frozen at −80°C and sent by mail in dry ice to the participating laboratories. On reception, the samples were stored at −80°C until tested. Blind experiments were done in all laboratories.

Amplicor enterovirus assay. The Amplicor tests were performed between July and August 1995. Except for two laboratories, none of the participating laboratories had any experience with the Amplicor enterovirus kit. The Amplicor enterovirus test was used according to the manufacturer's instructions except for minor changes introduced in two laboratories, which will be discussed later. Briefly, 100 μl of a CSF specimen was mixed with 400 μl of lysis solution containing guanidium thiocyanate, glycogen, and dithiothreitol in Tris buffer and incubated for 10 min at room temperature. Five hundred microliters of isopropanol was then added, and the mixture was centrifuged for 10 min at 16,000 × *g*. The resulting pellet was washed with 750 μl of 70% ethanol and resuspended in 200 μl of bicine buffer containing manganese acetate and potassium acetate. A 50-μl aliquot of this mixture was added to an equal volume of master mixture containing uracil-*N*-glycosylase (AmpErase; Roche), biotinylated primers (see Table 1), deoxynucleotide triphosphate (with dUTP in place of dTTP), and recombinant *Thermus thermophilus* enzyme (Roche) in a bicine buffer. Amplification was performed in a thermal cycler (PCR system 9600 or 2400; Perkin-Elmer, Saint Quentin en Yvelines, France). An initial step of RT at 60°C for 30 min was followed by 35 cycles of amplification. Each amplification cycle consisted of denaturation (94°C, 70 s for the first cycle and 10 s for the next 34 cycles), annealing (58°C, 10 s), and extension (72°C, 10 s). The PCR products were detected using an enterovirus-specific probe (5′-GAAACACGGACACCCAAA GTA) coated on the microwell plates. The biotin-labeled PCR products chemically denatured to form single strands were hybridized to the probe and detected by an avidin-horseradish peroxidase system. Results were scored as positive if the optical density at 450 nm (OD_{450}) was >0.34.

The laboratories were instructed to extract each CSF specimen twice and test each extracted sample in duplicate, giving four PCR results for each sample.

In-house PCR. Five laboratories also tested the CSF panel with an in-house PCR. The primers used in these experiments had sequences in the 5′ untranslated region of the enterovirus genome (Table 1). RNA was extracted from 100 μl of CSF by all laboratories except for laboratory 5, which used 50 μl. Some of these techniques have been described elsewhere (2, 10, 24), and only the main features of each test are summarized below.

Laboratory 1 used an RT-seminested PCR test (in seminested PCR, one primer is common to both the outer and inner amplified fragments). Proteinase K (Sigma, Saint Quentin Fallavier, France) was used before RNA extraction and avian myeloblastosis virus (AMV) enzyme (Promega, Charbonnières, France) for RT. Two series of 30 amplification cycles were performed, and PCR products were analyzed by agarose gel electrophoresis.

Laboratory 2 used RNAzol B (Bioprobe Systems, Montreuil-sous-Bois, France) for RNA extraction and Moloney murine leukemia virus enzyme (Gibco-BRL Life Technologies, Erigny, France) for RT. Thirty-five amplification cycles were performed, and the PCR products were identified by slot blot hybridization onto a nylon membrane using a specific biotinylated probe (5′-biotinyl-GGCCGCC AACGCAGCC) and alkaline phosphatase-labeled streptavidin (Dako, Copenhagen, Denmark).

Laboratory 3 used RNAzol B for RNA extraction and AMV enzyme for RT. The PCR products were analyzed after one set of 35 amplification cycles by hybridization using a biotinylated probe (5′-biotinyl-TTAGCCGCATTCAGGG GCCGGAGG) and the Gen-Eti-K detection system (Sorin, Antony, France).

Laboratory 4 used a RT-seminested PCR test. RNAzol B was used for RNA extraction, and Moloney murine leukemia virus enzyme was used for RT. Two series of 30 and 25 amplification cycles were performed successively, and PCR products were detected by agarose gel electrophoresis.

Laboratory 5 used RNA Now (Biogentex/Ozyme, Montigny le Bretonneux, France) for RNA extraction and AMV enzyme (Promega) for RT. One set of 40 amplification cycles was done, and PCR products were analyzed by Southern blot hybridization using a digoxin-labeled oligonucleotide (5′-AAACACGGACACC CAAAGTA).

Cell culture. Eight laboratories analyzed the CSF panel by cell culture by standard procedures for enterovirus culture. In each laboratory, 200 μl of each CSF sample was inoculated in duplicate onto at least two cell lines: human embryonic lung fibroblasts and an immortalized cell line susceptible to enteroviruses (Hep2, KB, Vero, or BGM). A blind passage was systematically done before a sample was interpreted negative. The results of cell culture were recorded blindly with respect to those of PCR test(s).

TABLE 2. Overall results of the Amplicor assay on a panel of 20 CSF specimens in 13 laboratories

CSF sample code no.	Virus	$TCID_{50}$/100 μl	Total no. of samples	Amplicor PCR-positive samples[a]		
				%	$OD_{250} > 2$	$0.35 < OD_{250} < 2$
16	Coxsackievirus B3	10	51	100	46	5
9	Poliovirus type 3	10	50	98.0	44	6
18	EV-11	10	49	96.1	46	3
4	Coxsackievirus B3	1	36	70.6	14	22
10	EV-11	1	35	68.6	12	23
5	Poliovirus type 3	1	33	64.7	6	27
8	EV-11[b]	1	32	62.7	8	24
15	Poliovirus type 3	0.1	10	19.6	1	9
11	EV-11	0.1	8	15.7	0	8
17	Coxsackievirus B3	0.1	6	11.8	0	6
1	Herpes simplex virus	1	0	0	0	0
12	Adenovirus	1	1	1.9	0	1
19	Mumps virus	1	2	3.9	0	2
2			0	0	0	0
3			0	0	0	0
6			0	0	0	0
7			1	1.9	0	1
13			1	1.9	0	1
14			2	3.9	0	2
20			3	5.9	2[c]	1

[a] Percentage or number of positive samples among 51 replicates in 13 laboratories.
[b] Sample containing blood.
[c] Two PCR results from the same extraction in one laboratory.

RESULTS

Multicenter evaluation of the enterovirus Amplicor assay. The CSF panel was processed as described in Materials and Methods in 12 of the 13 laboratories, resulting in 4 PCR tests for one CSF sample and for each laboratory. Laboratory 13 performed two tests on the first extraction and only one test on the second extraction. A total of 1,020 PCR results were therefore available. Table 2 illustrates the overall results of the Amplicor assay on the CSF panel in the 13 laboratories. The average sensitivity for enterovirus genome detection was 61% (standard deviation [SD], 4.3%). Except for laboratory 11, whose test had a low sensitivity (37.5%), the sensitivities were 50 to 77.5%. The sensitivity varied greatly according to the viral load: it was 98% for 10 $TCID_{50}$ (SD, 0.8%), 67% for 1 $TCID_{50}$ (SD, 3.3%), and 16% for 0.1 $TCID_{50}$ (SD, 1.6%). As shown in Table 2, there was a good correlation between the viral load and the mean value, expressed as OD units, of each sample. The rates of EV-11 detection in clear and hemorrhagic CSF specimens were the same (Table 2).

The average specificity for enterovirus detection was 98% (three false-positive results in CSF specimens containing nonenterovirus isolates and seven such results in uninfected specimens [Table 2]). Only one false-positive result was obtained in a single laboratory during the first PCR of the first extraction; all the other false-positive results were obtained with replicates, especially during the second extraction, suggesting that the risk of carryover is not totally eliminated (as illustrated, for instance, by sample 20 in Table 2).

Table 3 shows the results for the panel of 20 CSF specimens, both for the first PCR of the first extraction (13 laboratories) and for the four replicates (12 laboratories). Except for one replicate in a single laboratory, all specimens containing 10 $TCID_{50}$ of enterovirus gave positive results. The main interlaboratory differences in sensitivity occurred with samples containing 1 $TCID_{50}$ of enterovirus (25 to 100%). The sensitivity was very low with a load of 0.1 $TCID_{50}$ for all laboratories (0 to 33%).

Comparison of the Amplicor assay and in-house PCR. The Amplicor and in-house PCR tests were done in 5 of the 13 laboratories. Single tests were run for in-house PCR. The overall sensitivities were 62% for the Amplicor technique and 68% for in-house PCR tests. The sensitivities of the five in-house PCR tests were not different (data not shown). Those of the various PCR techniques were the same for the three viral loads (Table 4). The specificity was 100% in all tests.

Comparison of the Amplicor assay and cell culture. Comparison of the Amplicor assay and cell culture was done in 8 of the 13 laboratories. The overall sensitivity of the Amplicor technique was 56% versus 30% for cell culture ($P < 0.001$ by the chi-square test). The sensitivities of the two techniques varied greatly with the viral load, but the Amplicor test was always more sensitive than cell culture (Table 5). Detection by cell culture varied greatly, depending on the virus serotype. For instance, at a viral load of 10 $TCID_{50}$, EV-11 was recovered in all cases, coxsackievirus B3 was detected by six of eight laboratories, and poliovirus type 3 was detected by only one laboratory. The same concentrations of all three serotypes were detected in all laboratories by the Amplicor assay. The specificity was 100% for PCR and cell culture in all laboratories (Table 5).

DISCUSSION

Over the past few years, PCR has become the technique of choice for detecting viral genomes. It is especially suitable for

TABLE 3. Results of the multicentric quality control study in 13 different laboratories using the Amplicor assay

Virus samples and inoculum ($TCID_{50}$/100 μl)	First amplification of the first extraction			Two extractions and two amplifications per extraction		
	No. of samples per laboratory[a]	No. of positive samples		No. of tests per laboratory[b]	No. of positive tests	
		Mean (%)	Range		Mean (%)	Range
Enterovirus						
10	3	2.85 (94.9)	2–3	12	11.76 (98)	11–12
1	4	2.62 (65.4)	1–4	16	10.67 (66.7)	4–16
0.1	3	0.38 (12.8)	0–2	12	1.88 (15.7)	0–4
Nonenterovirus						
1	3	0.07 (2.5)	0–1	12	0.23 (2)	0–2
0	7	0	0	28	0.55 (2)	0–3

[a] 13 laboratories.
[b] 12 laboratories.

TABLE 4. Comparison of the Amplicor assay and in-house PCR tests in five laboratories on a panel of 20 CSF samples

Virus samples and inoculum ($TCID_{50}$/100 μl)	Amplicor PCR (first amplification of the first extraction)			In-house PCR		
	No. of samples per laboratory	No. of positive samples		No. of samples per laboratory	Mean no. (%) of positive samples	No. of positive samples (range)
		Mean (%)	Range			
Enterovirus						
10	3	3 (100)	3	3	3 (100)[a]	3
1	4	3 (75)	2–4	4	3 (75)[a]	1–4
0.1	3	0.25 (8.3)	0–1	3	0.8 (26.6)[a]	0–3
Nonenterovirus						
1	3	0	0	3	0	0
0	7	0	0	7	0	0

[a] Differences between results obtained with for the mean number of positive samples by Amplicor assays and in-house PCR tests were nonsignificant by the chi-square test.

clinical specimens likely to contain a low viral burden. However, its great sensitivity and the number of parameters that can interfere with the enzyme reactions involved in RT and amplification make the technique difficult to standardize, so that it may be less than perfectly reproducible between laboratories as shown with the hepatitis C virus (9, 23). The present study was therefore carried out to evaluate a commercially available PCR assay designed to detect enterovirus RNA in CSF specimens using a multicenter quality control study involving 13 laboratories.

There were only small overall differences in the sensitivities of tests done by the different laboratories. The main factor affecting the sensitivity of the test was the viral load. The sensitivities of enterovirus PCR assays reported so far varied greatly, depending on technical considerations, including the number of cycles and amplification steps (single-step or nested-PCR assays) and the way amplified products were detected (gel electrophoresis or hybridization). The best assays achieved a sensitivity of 0.1 to 0.01 PFU when tested on dilutions of enterovirus in cell culture medium (8, 15, 16). However, such sensitivities are rarely reached with clinical specimens, partly because of the presence of inhibitors and nucleic acid-degrading compounds responsible for false-negative results. The data from the five laboratories which compared in-house PCR tests to the Amplicor test show that all the tests on the CSF panel had a similar sensitivity of about 1 $TCID_{50}$, despite the fact that the Amplicor assay is a single-step test. The risk of losing the RNA pellet during sample extraction could be minimized by performing this step at 4°C instead of room temperature; this change may help improve the performance of the Amplicor assay. Moreover, in samples containing low viral doses (1 and almost 0.1 $TCID_{50}$), the variability in sensitivity observed between the different measures is related partly to the Poisson distribution of the viral particles within the different aliquots of a same specimen. For exquisitively sensitive techniques such as PCR, these variations—that cannot be reduced by any means—may represent an important factor of interlaboratory variability.

The Amplicor assay was much more sensitive than cell culture, even for samples containing 10 $TCID_{50}$ of enterovirus. Similar results have been obtained in other studies which compared the recovery of enterovirus by cell culture and PCR in CSF and other clinical specimens (1, 10, 15–17, 19–21). Hence, the use of PCR techniques can improve the diagnosis of enterovirus infections considerably. In a recent study comparing the Amplicor test to viral culture in CSF specimens from 38 patients with aseptic meningitis, the rates of enterovirus detection were 66 and 34%, respectively, by the two tests (22). The excellent results reported with the Amplicor test in these clinical studies emphasize that biologically occurring titers observed in CSF specimens from patients with enteroviral aseptic meningitis are well within the detection limits of the assay.

The overall specificity of the Amplicor assay was 98%. It should be noted that 11 of the participating laboratories had never used the Amplicor enterovirus kit before the study, and the two remaining laboratories had tested it only once. However, the occurrence of some false-positive results shows that care must be taken to avoid contamination, despite the facts

TABLE 5. Comparison of the Amplicor assay and cell culture in eight laboratories on a panel of 20 CSF samples

Virus samples and inoculum ($TCID_{50}$/100 μl)	First amplification of the first extraction			Cell culture			Significance level[a]
	No. of samples per laboratory	No. of positive samples		No. of samples per laboratory	Mean no. (%) of positive samples	No. of positive samples (range)	
		Mean (%)	Range				
Enterovirus							
10	3	3 (100)	3	3	1.88 (62.5)	1–2	$P < 0.01$
1	4	2.38 (59.4)	1–4	4	1 (25)	0–2	$P < 0.05$
0.1	3	0.25 (8.3)	0–1	3	0.12 (4.2)	0–1	NS
Nonenterovirus							
1	3	0	0	3	0	0	NS
0	7	0	0	7	0	0	NS

[a] *P* values were determined for the results (mean number of positive samples) obtained by Amplicor assay and cell culture by the chi-square test with Yate's correction for small effects. NS, nonsignificant.

that the test is performed in a single tube to which no reagents are added between the RT and amplification steps and that a uracil-*N*-glycosidase protocol is used to prevent the carryover of recently amplified DNA. One potential source of false-positive results is the enzyme-linked immunosorbent assay-like format of the Amplicor test. Standardized washing procedures should be used to avoid such problems.

Despite the need to further improve the performance of the kit, the Amplicor assay appears to be a suitable tool for the clinical laboratory setting; it compares favorably with in-house PCR tests and is easier to perform, both in terms of practicability and rapidity (about 6 h for a complete test). It is also much more sensitive than cell culture with CSF samples. Last but not least, the Amplicor enterovirus assay produced results that were nearly identical from all the participating laboratories. Standardized procedures in enterovirus PCR are urgently needed for large-scale evaluation of the implications of this family of viruses in different clinical situations. Semiroutine use of this commercial PCR assay may be a great help in clarifying the role of enteroviruses in acute and chronic infections of the central nervous system. Testing of other clinical specimens in addition to the CSF will also be of great interest.

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