DNA Probe Array for the Simultaneous Identification of Herpesviruses, Enteroviruses, and Flaviviruses

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Viral infections of the central nervous system (CNS) are caused by a variety of viruses, namely, herpesviruses, enteroviruses, and flaviviruses. The similar clinical signs provoked by these viruses make the diagnosis difficult. We report on the simultaneous detection of these major CNS pathogens using amplification by PCR and detection of amplified products using DNA microarray technology. Consensus primers were used for the amplification of all members of each genus. Sequences specific for the identification of each virus species were selected from the sequence alignments of each target gene and were synthesized on a high-density microarray. The amplified products were pooled, labeled, and cleaved, followed by hybridization on a single array. This method was successfully used to identify herpesviruses, namely, herpes simplex virus type 1 (HSV-1), HSV-2, and cytomegalovirus; all serotypes of human enteroviruses; and five flaviviruses (West Nile virus, dengue viruses, and Langat virus). This approach, which used highly conserved consensus primers for amplification and specific sequences for identification, would be extremely useful for the detection of variants and would probably help solve some unexplained cases of encephalitis. The analytical sensitivity of the method was shown to be 500 genome equivalents ml⁻¹ for HSV-1, 0.3 50% tissue culture infectious doses (TCID₅₀s) ml⁻¹ for the enterovirus coxsackievirus A9, and 200 TCID₅₀s ml⁻¹ for West Nile virus. The clinical sensitivity of this method must now be evaluated.

Viral infections of the central nervous system (CNS), such as encephalitis, meningitis, and meningoencephalitis, are due to a wide range of viruses mostly belonging to the genera *Herpesvirus*, *Enterovirus*, and *Flavivirus*. Human herpesviruses, herpes simplex virus type 1 (HSV-1), HSV-2, cytomegalovirus (CMV), Epstein-Barr virus (EBV), varicella-zoster virus (VZV), and human herpesvirus 6 (HHV-6) have a DNA genome and are important causes of encephalitis, myelitis, and meningitis (17, 40). The most serious disease caused is herpes simplex virus encephalitis (10, 40). HSV-1 is believed to be responsible for 95% of cases of this disease, and HSV-2 is responsible for the remaining 5% (3). The high rates of morbidity and mortality from herpes simplex virus encephalitis that occur without early antiviral therapy emphasize the need for the development of a rapid and accurate diagnostic method (39), especially as virus-specific therapy with acyclovir, ganciclovir, and foscarnet is available.

Between 80 and 92% of aseptic meningitis cases with an identified etiologic agent are currently attributed to human enteroviruses (29). These viruses belong to the *Enterovirus* genus of the *Picornaviridae* family, which includes 64 nonpoliovirus serotypes and 3 poliovirus serotypes. Other clinical manifestations due to these single-stranded positive-sense RNA viruses are varied and include acute respiratory illness; meningoencephalitis; myocarditis; hand, foot, and mouth dis-

ease; neonatal multiorgan failure; and acute flaccid paralysis (24).

The genus *Flavivirus* of the family *Flaviviridae* comprises over 70 single-stranded positive-sense RNA viruses that are transmitted by arthropods. Of the 40 flaviviruses associated with human illness, tick-borne encephalitis virus (TBE), West Nile encephalitis virus (WN), Japanese encephalitis virus (JE), Murray Valley encephalitis virus (MV), and St. Louis encephalitis virus (SLE) cause infections of the CNS. These viruses used to have restricted niches but are now spreading across the world, as demonstrated, for example, by the West Nile virus outbreak in New York City (2), which subsequently spread to the whole of the United States and Canada.

Nucleic acid amplification techniques, and especially PCR, have revolutionized the diagnosis of CNS viral infections (10, 11). While virus isolation is efficient for the diagnosis of meningitis due to enteroviruses, this method is insensitive for cases due to herpes simplex virus and arboviruses (9). Furthermore, virus isolation takes time and requires costly facilities and trained personnel. Many different amplification strategies that mostly use virus-specific primers in a series of independent PCRs or consensus primers have been reported. Human herpesviruses have also been detected by multiplex PCR (5, 13, 25, 34).

The use of consensus PCR primers that exploit sequence homology between related viruses has been reported for all three families of viruses. The amplified product is then identified by DNA sequencing, hybridization with specific probes (1) or restriction enzyme analysis (34). All known human herpesviruses were amplified in two assays (16) and in one assay (30, 34) by using primers localized in the DNA polymerase

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FIG. 1. 2-L tiling strategy. To determine if the reference sequence of a virus is present in the amplification product, two probes are used for each nucleotide of the sequence. These probes, usually 20-mer oligonucleotides, have the same sequence at all except one position, where one probe has the expected base (match probe) and the other one has one of the three other bases (mismatch probe). If the sequence is present, the fluorescence intensity of a majority of match probes will be higher than that of the corresponding mismatch probes.

gene. Enterovirus RNA has been detected with primers localized within the highly conserved 5' noncoding region (NCR) (18). Many universal flavivirus assays targeting the NS5 region have been published (21, 26, 31, 33), with virus-specific PCRs available for JE and WN (8, 33) and for SLE (21, 22).

Different CNS infections result in similar neurological symptoms, which make diagnosis difficult. While there are numerous publications on the separate molecular detection of each of these virus families, only a few authors have reported on the simultaneous detection of herpesviruses and enteroviruses (7, 27, 28), and no method for the detection of all three families has yet been published. Flaviviruses could explain some of the undetermined etiology of neurological infections, as reported for encephalitis (32 to 75%) (12, 14, 19, 32).

This paper describes a diagnostic tool that uses a DNA probe array for the simultaneous detection of viruses from three different families whose members cause CNS diseases that sometimes have similar clinical manifestations and its analytical validation with prototype species from the three families. The approach that we report here first uses broadly reactive primers for each family and then uses the DNA probe array technology to differentiate between virus species within each family.

MATERIALS AND METHODS

Virus. Virus cultures were obtained from different sources: HSV-1 strain MacIntyre, HSV-2 strain G2, and Rhinovirus strains RH16 and RH18 were from the American Type Culture Collection (ATCC; Manassas, Va.); and CMV strain AD169 was from bioMérieux (Marcy l'Etoile, France). Enterovirus serotypes were kindly provided by H. G. M. Niesters (Erasmus MC, Rotterdam, The Netherlands) and B. Lina (Université Claude Bernard, Lyon, France). The concentrations of nonpoliovirus enterovirus serotypes were determined by H. G. M.

Niesters, as described by van Doornum et al. (37). The flaviviruses West Nile virus strain Eg101, Langat virus strain Langat, dengue virus serotype 2 (DEN-2) strain NGC, and DEN-4 (strain YUNH) were obtained from Pasteur Institute, Paris, France, while dengue virus serotype 1 strain Hawaii was obtained from Pasteur Institute, Dakar, Senegal; these viruses were propagated in Vero cells.

Nucleic acid extraction. Viral nucleic acids were extracted by use of a DNA Blood mini kit (QIAGEN, Courtaboeuf, France) for DNA viruses and a QiAmp Viral RNA mini kit (QIAGEN) for RNA viruses, according to the manufacturer's instructions. Briefly, for DNA viruses, 100 μ l of sample was mixed with 100 μ l of phosphate-buffered saline and 20 μ l of proteinase K. Lysis was then performed at 56°C for 10 min with 200 μ l of lysis buffer. After addition of an equal volume of absolute ethanol, the mixture was loaded onto a spin column, which was then successively washed with 500 μ l of wash buffers provided with the kit. The purified nucleic acids were then eluted with 100 μ l of the elution buffer provided with the kit. The protocol was similar for RNA viruses, except that lysis was done at room temperature for 10 min.

Primers. For the herpesviruses, the DNA polymerase gene was targeted. A pair of consensus primers that allowed the amplification of three herpesviruses (HSV-1, HSV-2, and CMV) were modified from the primers of Johnson et al. (16), HVF (GTGTTGGACTTTGCCAGCCT) and HVR (GTCCGTGTCCCC GTAGATGA). A pair of primers specific to the 5' NCR of enteroviruses, ENTB (GGTACCTTTGTRCGCCTG) and ENTG (CCAAAGTAGTCGGTTCCG) (P. Renaud, E. Guillot, C. Mabilat, C. Vachon, B. Lacroix, G. Vernet, M.-A. Charvieu, P. Laffaire, April 2004, France, patent number WO0202811), that allow the amplification of a region of 500 bp were used. Typing of enteroviruses was done by amplification of part of the 2A and the VP1 genes, as described by Caro et al. (6) Flaviviruses were amplified with a pair of primers specific to the NS5 region, cFD2 (GTGTCCAGCCGGCGGTGTCATCAGC) and MA (CATGTGGGAARAGRGARRAG) (31).

RT-PCR. Reverse transcription (RT)-PCR was performed with a Titan one-tube RT-PCR system (Roche Molecular Biochemicals, Mannheim, Germany). Amplification of the three virus families was achieved in three separate reaction tubes, each of which contained primers specific for one family. DNA was added to the herpesvirus tube, and RNA was added to the enterovirus and flavivirus tubes. Each reaction contained 1 $\times$ reaction buffer, 1.5 mM MgCl₂, 5 U RNase inhibitor, 5 U avian myeloblastosis virus enzyme mix and Expand High Fidelity enzyme blend, 0.2 mM deoxynucleoside triphosphates dNTPs, 5 mM dithiothreitol, 27.5 μ l of template, and molecular-grade water to a volume of 50 μ l. The

TABLE 1. Number of regions and total length of sequence used for the identification of viruses by using the DNA microarray

Pathogen	Target gene	No. (%) of probes on array	Regions of target gene analyzed by chip		Amplicon length (nt) ^b
			No. of regions	Total length of sequence analyzed (nt) ^c	
Herpesvirus	DNA polymerase	4,774 (11.7)			
HSV-1			5	173	535
HSV-2			6	251	535
CMV			7	92	600
EBV			1	38	NA
VZV			12	399	NA
HHV-6			6	140	NA
Enterovirus	5' NCR	1,608 (3.96)	1	22	500
	VP1	10,261 (25.3)	3–8	21–277	NA
Flavivirus	NS5 region	9,700 (23.9)			
WN			3	48	250
TBE			3	48	250
JE			3	58	250
SLE			3	31	250
MV			3	48	250
Polyomavirus	T antigen	1,240 (3.06)			
JC			2	116	NA
BK			2	148	NA
Paramyxovirus	Nucleoprotein	3,682 (9.07)			
Mumps virus			15	215	NA
Measles virus			7	133	NA
<i>Toxoplasma gondii</i>	B1	502 (1.23)	1	251	NA

^a nt, nucleotides.^b Length of amplicon is given only for the validated viruses: herpesviruses, enteroviruses, and flaviviruses. NA, not applicable.^c The number of regions and length of sequence used for the typing of enteroviruses varied between the serotypes.

concentrations of primers were as follows: 0.2 μ M for herpesvirus consensus primers and enterovirus primers and 0.15 μ M for flaviviruses primers. The same PCR cycling conditions were used for the amplification of DNA and RNA viruses. RT-PCR started with a reverse transcription step at 50°C for 30 min, followed by denaturation at 94°C for 5 min and then 40 cycles each consisting of 30 s denaturation at 94°C, 1 min annealing at 59°C with a touchdown of 0.1°C per cycle, and 1 min extension at 68°C. A final extension of 7 min at 68°C was done. PCR was performed in a GeneAmp System 9700 thermocycler. Typing of enteroviruses was done as described by Caro et al. (6), except that a single-step RT-PCR was performed with the cycling conditions described above with an extension time of 90 s.

PCR product quantitation. Amplicons were quantified with an Agilent 2100 bioanalyzer and a DNA 1000 LabProbe array (Agilent Technologies, Massy, France), according to the manufacturer's instructions. Quantitation was performed with 1 μ l of amplification products.

Labeling and cleavage of PCR products. Forty microliters of each of the three PCR products was mixed and first biotin labeled in a mixture containing 2.5 μ l of RNase-free and DNase-free water (Sigma, St. Louis, Mo.) and 75 μ l of 0.1 M *meta*-biotinphenylmethyldiazomethyl (bioMerieux) at 95°C for 25 min in a dry bath (4). DNA fragmentation was then performed by incubation of the reaction mixture with 36 mM HCl at 95°C for 5 min in a final volume of 250 μ l. Fragmented labeled DNA was purified with a QIAquick 8 PCR purification kit, according to the manufacturer's protocol (QIAGEN), except for the PB buffer, which was replaced by PN buffer provided by the same company.

DNA probe array design. Sequence alignments of the target gene used for the detection of each virus family were constructed with the CLUSTALW program (version 1.4) (35) and included all available sequences of the relevant virus strains. This allowed the selection of probes necessary for the identification of each virus species in the case of herpesviruses and flaviviruses and the genus in the case of enteroviruses. This selection was performed in four steps. First, the amplified portions of targeted viruses sequences were chopped into probes of identical lengths that overlapped by 1 base. Then, probes that were not shared by a high enough proportion of strains from targeted viruses (typically 90%) were discarded. Probes which cross-hybridized with nonrelated viruses likely to be

found in clinical samples were also eliminated. Finally, probes which did not cross-hybridize with related viruses within the same family were selected. In this way, species-specific sequences were obtained for herpesviruses and flaviviruses, and both genus-specific sequences and serotype-specific sequences (for typing purposes) were selected for enteroviruses. The repertoire of selected probes was then synthesized on the array by using a 2-L array tiling strategy, which allows the detection of a probe in a sample (36). This strategy consists of the synthesis of two probes of length (20-mers), one perfectly complementary to the original probe, with the other containing a mismatch at a specified position (typically, position 12 for 20-mers) (Fig. 1). The instability of probe-target mismatches relative to the stability of perfect matches is used to discriminate differences between nucleic acid targets through the identification of the probe which gives the highest fluorescence intensity (the match probe). If the signature sequence is present in the specimen, the fluorescence intensity of a majority of match probes will be higher than that of the corresponding mismatch probes. A total of 40,588 different probes have been synthesized on the final array of 0.8 by 0.8 cm with cells of 20 by 20 μ m. Table 1 shows the number of probes representing the viruses validated in this study (herpesviruses, flaviviruses, and enteroviruses) and other viruses that have not yet been validated.

DNA probe array hybridization and analysis. Labeled and cleaved amplicons were denatured at 95°C for 10 min in a 500- μ l final volume containing 10 $\times$ SSPE (1 $\times$ SSPE is 0.18 M NaCl, 10 mM NaH₂PO₄, and 1 mM EDTA [pH 7.7]), 0.1% Triton X-100, 0.06% antifoam, and 0.09% sodium azide. Hybridization was done at 45°C for 45 min on an Affymetrix GeneChip Fluidics Station 400. After the probe array was washed with 6 $\times$ SSPE, 0.005% Triton X-100, 0.001% antifoam, and 0.03% sodium azide and with 4 $\times$ SSPE, 0.005% Triton X-100, 0.001% antifoam, and 0.03% sodium azide, the probe array was stained with 500 μ l buffer containing 0.01 mg streptavidin-RPE (Dako, Glostrup, Denmark), 0.5 mg bovine serum albumin, and Tris-Cl (pH 7.2) at 35°C for 25 min. After the array was washed, the fluorescent signal emitted by the target bound to the probes was detected by a GeneArray scanner at a wavelength of 570 nm and with a pixel resolution of 3 μ m. The highest signals came from the probes that best matched the target viral sequence. Probe array fluorescence intensities, nucleotide base call, sequence determinations, and reports were generated by functions available

FIG. 2. Amplification of HSV-1 (lane 2), HSV-2 (lane 3), CMV (lane 4), West Nile virus (lane 5), and poliovirus 1 (lane 6). Lane 1, molecular weight markers. The PCR products were separated by using an Agilent 2100 bioanalyzer.

on the GeneChip 3.2 software. The ratio between the median intensity obtained for the sequence tiled and the median background intensity ($I_{t/b}$) was considered a criterion for identification. The background intensity was calculated as the mean for 16 probes in representative regions of the array. The percent base right score was determined by the percent homology between the experimentally derived sequence and the reference sequence tiled on the array. This criterion was used in addition to $I_{t/b}$ to differentiate between the two closely related herpesviruses HSV-1 and HSV-2.

RESULTS

RT-PCR. PCR products of the expected size were generated for each target (535 bp for HSV-1 and HSV-2, 600 bp for CMV, 500 bp for enteroviruses, and 250 bp for flaviviruses) (Fig. 2). DNA and RNA viruses were amplified by using the same amplification kit and the same cycling conditions to simplify the diagnostic procedure. No deleterious effect of the reverse transcription step was observed on herpesvirus amplification (data not shown).

DNA probe array hybridization and analysis. Specific identification of each virus was achieved on the array by using several regions within the sequence amplified for herpesviruses and flaviviruses. The number of regions per amplicon varied between the viruses (Table 1). A single signature sequence was used for enterovirus genus identification. Serotyping could be achieved by using VP1-specific probes, which were also present on the array.

The threshold for identification was determined to be an $I_{t/b}$ of 1. This was done by analyzing data from arrays hybridized with negative cerebrospinal fluid (CSF) from patients with cranial trauma (three arrays) and with water (two arrays). The mean $I_{t/b}$ of all herpesviruses, enteroviruses, and flaviviruses probes was calculated from the five arrays to be 0.33 (0.20 to

TABLE 2. $I_{t/b}$ s obtained with closely related species on the microarray after hybridization of a particular virus (HSV-1, HSV-2, or West Nile virus) on the microarray

Virus hybridized on microarray	Species (% base right score with closely related species represented on microarray)	$I_{t/b}$
HSV-1	HSV-1 (97.1)	54.4
	HSV-2 (75.6)	10.2
	CMV	0.51
	VZV	0.40
	EBV	0.80
	HHV-6	0.50
HSV-2	HSV-2 (96.3)	49.4
	HSV-1 (73.1)	7.3
	CMV	0.45
	VZV	0.30
	EBV	0.90
	HHV-6	0.40
WN	WN (100)	26.9
	JE	0.56
	TBE	0.80
	SLE	0.50
	MV	0.40
	DEN-1	0.50
	DEN-2	0.56
	DEN-3	0.55
	DEN-4	0.46

0.80). The threshold was given as the mean ratio added to three times the standard deviation (0.06), which gave a value of 0.5. It was, however, reasonable to use a value of 1 and not below, as it means that the median intensity of the sequence tiled is at least equal to and not below the background intensity. When a particular virus was hybridized on the array, the $I_{t/b}$ s obtained for closely related viruses were similar to those obtained for nonspecific hybridization (Table 2). This shows that the sequences represented on the array are virus specific. It should be noted that an additional criterion, the percent base right score, was used to differentiate between HSV-1 and HSV-2 (see below).

Moreover, when a single DNA probe array was hybridized with equal amounts of amplicons obtained from the three targets HSV-1, human poliovirus type 1 (PV-1), and WN, each target was correctly identified with an $I_{t/b}$'s of 1.90 for HSV-1, 4.57 for WN, and 2.03 for PV-1 (Table 3). This suggests that the hybridization of each type of amplicon is an independent event and that several nonrelated sequences can be correctly identified on the same probe array.

Herpesviruses. HSV-1, HSV-2, and CMV were all amplified with the herpesvirus consensus primer pair and were distinguished by the probes on the probe array. Culture supernatants of HSV-1 and HSV-2 and of CMV patient samples which were characterized by real-time PCR (38) were tested. All samples of the three viruses were correctly identified (Table 4). HSV-1 and HSV-2, being very closely related, were subjected to the two criteria ratio and percent base right score. Both viruses were first identified as the herpes simplex type by using the $I_{t/b}$ threshold of 1, and then the two viruses were differentiated by using the percent base right score. The threshold for the score was calculated from the 16 HSV-1 and HSV-2 samples from Table 4 as the mean percent base right score (72.98%) ob-

TABLE 3. Detection of HSV-1, PV-1, and WN hybridized on a single DNA microarray

Probes represented on the microarray	$I_{t/b}$	Detection
Flaviviruses		
DEN-1	0.58	—
DEN-2	0.33	—
DEN-3	0.35	—
DEN-4	0.30	—
JE	0.34	—
Langat	0.30	—
MV	0.30	—
SLE	0.35	—
WN	4.57	+
TBE	0.60	—
Herpesviruses		
HSV-1	1.90	+
HSV-2	0.70	—
VZV	0.30	—
EBV	0.30	—
CMV	0.40	—
HHV-6	0.42	—
Enteroviruses	2.03	+

tained with heterologous probes for the samples added to three times the standard deviation (4.3%), which gave a value of 85.88%. A score above the threshold of 86% allows the identification of the herpes simplex virus present. A score below the threshold means that a herpes simplex type is

TABLE 5. Sensitivity of HSV-1 and West Nile virus detection by DNA microarray and comparison with detection by use of the Agilent array

Virus	Titer ^a	Detection by DNA microarray		Detection by Agilent array (no. of copies of amplicon/reaction)
		Ratio $I_{t/b}$	Detection	
HSV-1	5,000	7.2	+	4.92e + 10
	500	2.1	+	No detection
	50	0.3	—	No detection
WN	1,995.26	19.5	+	8.0e + 10
	199.52	4.5	+	No detection
	19.95	0.4	—	No detection

^a The titers are in TCID₅₀ ml⁻¹ for West Nile virus and in Geq ml⁻¹ for HSV-1.

present but that it cannot be further identified. Table 4 shows that 15 of 16 samples can thus be correctly identified by using this threshold, suggesting that 93.75% of HSV-1 and HSV-2 samples can be correctly differentiated.

The analytical sensitivity of this method was evaluated for HSV-1 to be 500 genome equivalents (Geq)/ml of CSF spiked with virus (Table 5). This was done by using virus material from various sources (virus culture quantified by electron microscopy and virus culture quantified by the TaqMan assay). Similar results were obtained for HSV-2 (data not shown).

The dynamic range of detection was tested by hybridizing the probe array with nucleic acids extracted from CSF samples

TABLE 4. Detection of HSV-1 and HSV-2 (virus cultures from patient samples) and of CMV (patient samples) with average base right score and $I_{t/b}$

Virus and sample type	Sample reference	Quantification by real-time PCR (Geq/ml) ^a	Result with corresponding sequence on the microarray		Result with other closely related sequences on the microarray ^b	
			% Base right score	$I_{t/b}$	% Base right score	$I_{t/b}$
HSV-1, culture	01/242	1.69e + 07	95.4	47.2	75	9.3
	01/297	2.43e + 07	96.5	41.6	73.5	7.8
	01/342	7.82e + 07	96.5	47.7	71.7	10.9
	01/583	3.10e + 06	96.5	60.3	76.5	13.2
	01/594	8.43e + 04	93.5	22.1	68.9	5.2
	01/694	7.56e + 07	97.1	54.3	75.6	10.2
	01/697	1.69e + 07	96.5	53	74.8	11.3
	01/704	2.59e + 07	95.4	58.2	75.3	11.7
HSV-2, culture	01/245	5.61e + 06	94.5	38.7	70.8	7.4
	01/401	1.4e + 08	98.4	54.8	70.2	7.4
	01/479	2.27e + 07	94.9	69.3	72.3	12.5
	01/539	2.80e + 04	84.9	6.2	61.8	2.5
	01/580	3.32e + 07	97.9	84.3	73.7	10.7
	01/582	4.95e + 05	93.9	27.1	71.2	4.9
	01/732	1.82e + 04	91.8	10	83.2	2.6
	01/733	1.37e + 08	99.6	49.2	73.1	7.3
CMV						
Plasma	03/6051	5.22e + 05	100	7	NA ^c	NA
Plasma	03/5857	1.39e + 04	100	13.4	NA	NA
Plasma	03/5268	7.94e + 04	99.7	13.7	NA	NA
Plasma	03/4081	1.50e + 06	99.7	29.3	NA	NA
Urine	03/1230	1.03e + 04	94.3	8.5	NA	NA
Plasma	03/3896	1.18e + 04	95.6	1.1	NA	NA

^a Quantification was performed by H. G. M. Niesters (Erasmus Hospital, Rotterdam, The Netherlands).

^b The closely related sequences were HSV-2 for HSV-1 and HSV-1 for HSV-2.

^c NA, not applicable.

TABLE 6. Dynamic range of detection of HSV-2 by DNA microarray

HSV-2 concn in sample before extraction (no. of genomes ml ⁻¹)	No. of amplicon copies hybridized on chip (detection by Agilent array)	% Base right score with probes for the following:			<i>I_{tb}</i> with probes for the following:		
		HSV-2	HSV-1	VZV	HSV-2	HSV-1	VZV
10 ⁸	2.92e + 12	100	84.4	45.6	84.3	5.4	0.4
10 ⁶	3.01e + 11	98.9	80.9	41.8	39.4	3.2	0.4
10 ³	8.90e + 10	96.8	74.6	48.1	3.7	0.6	0.4

containing 10⁸, 10⁶, and 10³ genomes ml⁻¹ of HSV-2 (Table 6). These three concentrations of virus were correctly identified by the DNA microarray. This shows that large amounts of material on the array do not affect the base right scores and that no major saturation effects hinder detection.

Enteroviruses. Fifty-six of 61 nonpoliovirus serotypes and 3 poliovirus serotypes of enteroviruses were successfully identified as enteroviruses by the enterovirus genus-specific signature sequence of 22 nucleotides present on the array (Table 7). Five enteroviruses (CV-A1, CV-A5, CV-A14, CV-A19, and CVA-22) were not tested due to the nonavailability of the viruses. No cross-reaction with rhinoviruses RH16 and RH18 was observed during either amplification or hybridization, showing that the primers are specific to the enterovirus genus but still amplify human parechovirus enterovirus type 1 (HPeV-1) and HPeV-2.

The analytical sensitivity was determined with the Quality Control for Molecular Diagnostics (QCMD) Enterovirus Proficiency Panel 2003 (Table 8), since no sample quantified in genome equivalents/ml was available. The sensitivity limit is 0.3 50% tissue culture infectious doses (TCID₅₀s) ml⁻¹ for coxsackievirus A9. The panel also confirmed that rhinovirus RH16 was indeed not detected by our protocol. Parechovirus 1 was not detected from this panel. However, it is interesting that parechoviruses 1 and 2 (previously echoviruses 22 and 23, respectively) were successfully amplified and detected by the probe array when they were tested at high titers (Table 7), while no sequence of these two viruses was included in the probe array design.

Flaviviruses. Probes specific for a large number of flaviviruses were represented on the probe array. WN, DEN-1, DEN-2, and DEN-4, which may be considered representatives of mosquito-borne flaviviruses, and Langat virus, which is a tick-borne virus, were chosen for this initial validation. RNA extracted from these five viruses was correctly amplified by the flavivirus group primers, and the viruses were typed to the species level by the probes selected for these flaviviruses. No cross-hybridization between the different viruses was observed (Table 9).

The sensitivity of West Nile virus detection was 200 TCID₅₀s ml⁻¹ (Table 5). This method of combining amplification with flavivirus group primers and hybridization on a microarray is more sensitive than that involving the use of the group primers only (10⁵ TCID₅₀s ml⁻¹) and is as sensitive as the seminested method with an internal primer, both reported by Scaramozzino et al. (31). While hybridization of this sample on the array gave a base right score of 100% and an *I_{tb}* of 4.5, detection by the Agilent array was negative.

All amplicons were quantified by using Agilent probe arrays prior to hybridization on the DNA probe array. It is interesting

that detection by the DNA probe array was 10-fold more sensitive than that by the Agilent array, as illustrated in Table 5 for HSV-1 and WN.

DISCUSSION

This paper describes the specific detection of viruses from three major families that cause CNS diseases, human herpesviruses (HSV-1, HSV-2, and CMV), human enteroviruses (59 of 64 existing serotypes), and five flaviviruses, by using a consensus approach. The detection of these three families from a single sample has not yet been reported, with most papers describing the multiplex detection of herpesviruses and enteroviruses. Such simultaneous investigation of several potential agents is very appropriate to CNS diseases, as very often, small volumes of CSF are available for molecular biology diagnosis and the clinical symptoms provoked by these viruses are not clear-cut. Flaviviruses are pathogens of the CNS with a growing importance in both industrialized and developing countries and should be considered in the panel of viruses to be searched for. Despite extensive testing, the majority of encephalitis cases remain unexplained (14).

The importance of high-density microarrays in microbiology has been demonstrated in *Mycobacterium* species identification and antibiotic resistance determination (36), in multilocus typing of *Staphylococcus aureus* (39), and genotyping of human immunodeficiency virus (15, 20). Oligonucleotide probes, synthesized on the microarray, have been designed to determine every single nucleotide of target sequences in the virus present in a sample being tested. The assay established the percent homology between the microarray signature sequences that are specific to a virus or a subtype and the corresponding sequences of the virus being analyzed. For reliable detection, several regions of the target gene were used for the identification of each virus except enteroviruses, where only one region of 22 nucleotides was found to be a consensus sequence among all strains of all serotypes for which sequences are available. Maximum sensitivity was provided by the use of several overlapping probes for each target sequence. Specificity has also been optimized through the careful selection of probe sequences by excluding sequences that could cross-hybridize with nucleic acids generated by closely related viruses (exclusion perimeter). The assay has allowed the use of consensus primers, which reduces or eliminates the need for multiplexing for the specific identification of each species. In this paper, 3 herpesviruses, 59 serotypes of enteroviruses, and 5 flaviviruses have been detected by using consensus primers.

The concept of the microarray-based reagent allows flexibility in the detection of more viruses than those mentioned here. A single probe array can be synthesized with a large number of

TABLE 7. Detection of 59 enterovirus serotypes and $I_{t/b}$ s obtained with heterologous FV and HV probes

Enterovirus serotype ^a	$I_{t/b}$ for homologous and heterologous probes ^b		
	EV	FV	HV
E-1	43.03	0.36 (0.30–0.45)	0.44 (0.30–0.58)
E-2	57.61	0.33 (0.30–0.45)	0.45 (0.40–0.50)
E-3	14.33	0.50 (0.20–0.80)	0.61 (0.40–0.85)
E-4	12.86	0.56 (0.30–0.80)	0.65 (0.40–0.94)
E-5	8.26	0.53 (0.20–0.80)	0.72 (0.40–0.92)
E-6	22.52	0.39 (0.30–0.50)	0.58 (0.40–0.78)
E-7	12	0.51 (0.30–0.80)	0.67 (0.40–0.89)
E-9	10.4	0.66 (0.30–0.90)	0.63 (0.42–0.82)
E-11	7.09	0.57 (0.30–0.86)	0.63 (0.40–0.80)
E-12	5.85	0.50 (0.30–0.70)	0.57 (0.40–0.73)
E-13	10.7	0.41 (0.20–0.60)	0.63 (0.40–0.90)
E-14	54.48	0.40 (0.30–0.60)	0.50 (0.40–0.80)
E-15	60.66	0.40 (0.20–0.70)	0.49 (0.40–0.68)
E-16	1.06	0.60 (0.33–0.90)	0.49 (0.20–0.75)
E-17	25.41	0.34 (0.20–0.45)	0.54 (0.30–0.88)
E-18	28.47	0.60 (0.30–0.90)	0.45 (0.35–0.57)
E-19	30.81	0.63 (0.30–0.90)	0.48 (0.32–0.68)
E-20	44.73	0.29 (0.20–0.37)	0.39 (0.30–0.50)
E-21	11.57	0.51 (0.20–0.80)	0.64 (0.35–0.93)
E-24	63.35	0.39 (0.30–0.57)	0.63 (0.40–0.90)
E-25	13.73	0.47 (0.35–0.83)	0.59 (0.40–0.80)
E-26	9.27	0.50 (0.20–0.64)	0.57 (0.40–0.87)
E-27	5.52	0.38 (0.30–0.53)	0.63 (0.40–0.90)
E-29	24.05	0.40 (0.30–0.60)	0.63 (0.32–0.83)
E-30	58.96	0.36 (0.20–0.55)	0.46 (0.38–0.58)
E-31	10.06	0.50 (0.20–0.70)	0.54 (0.36–0.80)
E-32	50.68	0.36 (0.29–0.60)	0.51 (0.40–0.68)
E-33	44.91	0.33 (0.20–0.50)	0.43 (0.34–0.50)
CV-A2	46.34	0.43 (0.30–0.80)	0.66 (0.40–0.90)
CV-A3	18.03	0.41 (0.30–0.60)	0.59 (0.25–0.90)
CV-A4	85.12	0.46 (0.20–0.90)	0.74 (0.40–0.90)
CV-A6	42.82	0.38 (0.20–0.55)	0.62 (0.40–0.88)
CV-A7	65.34	0.34 (0.20–0.45)	0.51 (0.30–0.70)
CV-A8	76.69	0.44 (0.30–0.65)	0.59 (0.35–0.90)
CV-A9	35.42	0.29 (0.20–0.35)	0.38 (0.30–0.40)
CV-A10	28.04	0.45 (0.30–0.70)	0.73 (0.40–0.90)
CV-A11	79.64	0.38 (0.30–0.50)	0.47 (0.40–0.60)
CV-A12	54.11	0.51 (0.30–0.90)	0.61 (0.40–0.88)
CV-A13	25.26	0.38 (0.30–0.55)	0.43 (0.37–0.51)
CV-A15	94.92	0.40 (0.30–0.58)	0.43 (0.30–0.58)
CV-A16	66.51	0.34 (0.25–0.45)	0.52 (0.30–0.75)
CV-A17	93.59	0.33 (0.20–0.47)	0.55 (0.38–0.78)
CV-A18	83.86	0.38 (0.30–0.72)	0.62 (0.40–0.90)
CV-A20	76.87	0.36 (0.30–0.53)	0.56 (0.40–0.78)
CV-A21	85.62	0.39 (0.30–0.58)	0.55 (0.36–0.87)
CV-A24	70.72	0.34 (0.20–0.52)	0.64 (0.40–0.87)
CV-B1	6.27	0.44 (0.30–0.60)	0.70 (0.56–0.90)
CV-B2	8.76	0.44 (0.30–0.85)	0.60 (0.40–0.87)
CV-B3	14.58	0.71 (0.45–0.93)	0.52 (0.45–0.62)
CV-B4	22.68	0.47 (0.30–0.93)	0.64 (0.40–0.87)
CV-B5	24.85	0.49 (0.30–0.72)	0.57 (0.40–0.81)
CV-B6	50.05	0.39 (0.29–0.62)	0.66 (0.40–0.90)
EV-68	65.38	0.35 (0.30–0.53)	0.44 (0.30–0.58)
EV-69	31.98	0.49 (0.30–0.93)	0.60 (0.35–0.90)
EV-70	82.74	0.36 (0.28–0.58)	0.42 (0.30–0.50)
EV-71	58.56	0.37 (0.30–0.53)	0.43 (0.30–0.50)
PV-1	4.51	0.38 (0.30–0.45)	0.46 (0.40–0.50)
PV-2	28.66	0.37 (0.29–0.63)	0.45 (0.30–0.50)
PV-3	32.42	0.32 (0.27–0.38)	0.40 (0.32–0.50)
Parechovirus 1	6.48	0.65 (0.30–0.90)	0.79 (0.40–0.90)
Parechovirus 2	24.02	0.54 (0.30–0.90)	0.71 (0.40–0.90)
RH16	0.3	0.50 (0.20–0.70)	0.46 (0.30–0.60)
RH18	0.4	0.45 (0.20–0.60)	0.50 (0.23–0.60)

^a E, echovirus; CV-A, coxsackievirus A; CV-B, coxsackievirus B; EV, enterovirus; RH, rhinovirus.

^b Minimum and maximum values are provided in parentheses.

signature sequences from many viruses of interest. For instance, other pathogens that infect the CNS, especially in immunocompromised patients (polyomaviruses [JC and BK], measles, EBV, mumps virus, and *Toxoplasma gondii*), can be amplified in different combinations, depending on the clinical history of the patient, and can be hybridized on the same microarray. Furthermore, the multiplex approach can also be used to reduce the consumption of precious CSF. Such flexibility would have considerable impact on the cost of such a diagnostic tool.

We suggest that this tool be used in the following manner: nucleic acid is extracted from a sample of 200 μ l of CSF and used for three amplification tubes. The amplicons are pooled at the step of purification of labeled DNA. Finally, the amplicons resulting from the three tubes are hybridized on a single probe array so that the clinician can know whether the patient is infected with one of these viruses. Mixed infections are rare but can be detected by this method, where hybridization of more than one type of amplicon is possible. Amplicons resulting from WN, PV-1, and HSV-1 have been successfully identified after their simultaneous hybridization on a single array in this study. The availability of an efficient system for the extraction of both RNA and DNA would further reduce the volume of CSF required.

The analytical sensitivity of this method for HSV-1, HSV-2, and CMV detection is 500 Geq ml^{-1} . This respects the limit suggested by Linde et al. (23) of 10 to 20 genomes μl^{-1} for HSV-1 detection in CSF. The sensitivities for enterovirus and flavivirus detection were 0.3 TCID₅₀s ml^{-1} and 200 TCID₅₀s ml^{-1} , respectively. Detection by this assay was, as expected, more sensitive than agarose gel detection and also than Agilent array detection. Samples that were probably not quantified by the Agilent array because they had a DNA content lower than the limit of 0.5 ng μl^{-1} were successfully identified by the assay. This can be explained by the larger amounts of amplicons analyzed by the DNA microarray than by the Agilent array.

The sequence selected for the DNA array as well as the threshold for $I_{t/b}$ determined from negative controls allowed the specific detection of HSV-1, HSV-2, CMV, 59 human enteroviruses, and 5 flaviviruses. No cross-hybridization between viruses of the same family was observed. The two closely related herpesviruses HSV-1 and HSV-2 were identified as herpes simplex type by using the ratio threshold and were then differentiated by using the additional criterion of percent base right score. The threshold for this score was determined by using the percent base right scores for 16 samples of HSV-1 and HSV-2. Primers specific to enteroviruses did not allow the amplification of two strains of rhinoviruses but amplified, probably less efficiently than enteroviruses, parechoviruses 1 and 2. All serotypes of enteroviruses were successfully detected by using a genus-specific sequence. The array also allows the typing of these serotypes by using the VP1 gene. As an example, coxsackieviruses B6 and A17, PV-1, and PV-3 were typed and were correctly identified (data not shown). The typing of four serotypes was given as an example in this paper. The amplicons resulting from the identification primers (5' NCR) and typing primers (2A and VP1) can be pooled and hybridized on the same probe array. Such an approach would confirm

TABLE 8. Analysis of QCMD Enterovirus Proficiency Panel 2003 by PCR followed by chip analysis

Sample	Identification	Titer (TCID ₅₀ ml ⁻¹)	Result expected by QCMD	Result obtained	
				<i>I_{tb}</i>	Detection
EV03-07	Coxsackievirus A9	30	+	2.2	+
EV03-10	Coxsackievirus A9	3	+	1.4	+
EV03-05	Coxsackievirus A9	0.3	+	1	+
EV03-04	Coxsackievirus A9	0.03	±	0.4	—
EV03-08	Coxsackievirus B5	32	+	1.2	+
EV03-12	Coxsackievirus B5	300	+	2	+
EV03-06	Echovirus 11	250	+	1.2	+
EV03-11	Echovirus 11	25	+	1	+
EV03-01	Rhinovirus 16	3	—	0.3	—
EV03-02	Negative		—	0.3	—
EV03-03	Negative		—	0.3	—
EV03-09	Parechovirus 1	30,000	—	0.3	—

the presence of an enterovirus in the case of the emergence of a new serotype.

A few viruses from the *Flaviviridae* family that may be considered representatives of mosquito-borne and tick-borne viruses were tested and successfully typed on the array. Any cross-hybridization was well below the threshold determined. The use of the highly conserved NS5 region was very appropriate for the selection of consensus primers but also allowed distinction of the different flaviviruses. Three serotypes of dengue virus were tested and could be differentiated by using this region of the flavivirus genome. The use of genus-specific consensus primers and the presence of specific probes on the array would allow the detection of any of the flaviviruses in the case of obscure nonspecific symptoms. Also, this assay can be used for the diagnosis of hemorrhagic fevers. The sensitivity was evaluated with West Nile virus only due to the limited availability of virus samples. However, the lack of cross-reaction between the probes specific for each flavivirus shows that the

microarray is very promising for the detection of members of this virus family, which contains many emerging and reemerging members.

The data in this paper show that in the case of herpesviruses and flaviviruses, virus-specific sequences from highly conserved regions of the genome can be obtained. This approach would allow the detection of variants, as the primers would always cover a highly conserved region. The analytical sensitivity of the assay has been evaluated by using selected virus species which are major concerns for public health. Specimens collected in various geographical areas from patients in different clinical settings must now be tested to evaluate the clinical sensitivity of the assay and to confirm its pertinence as a clinical diagnostic tool. We believe that this assay will be useful in explaining the vast numbers of cases of encephalitis with unknown etiologies. Moreover, with the emergence of new pathogens, the determination of causal agents of CNS diseases is becoming increasingly important. This DNA probe array tech-

TABLE 9. Detection of five members of the flavivirus genus by DNA microarray

Probe	Ratio				
	DEN-1 (Hawaii) (10 ^{5.4} TCID ₅₀ ml ⁻¹)	DEN-2 (NGC) (10 ^{7.1} TCID ₅₀ ml ⁻¹)	DEN-4 (YUNH) (10 ⁷ TCID ₅₀ ml ⁻¹)	Langat (Langat) (10 ⁷ TCID ₅₀ ml ⁻¹)	WN (Eg101) (10 ^{7.3} TCID ₅₀ ml ⁻¹)
Flaviviruses					
DEN-1	23.63^a	0.50	0.41	0.45	0.41
DEN-2	0.75	45.26	0.90	0.53	0.56
DEN-3	0.50	0.50	0.95	0.45	0.55
DEN-4	0.80	0.56	98.53	0.50	0.46
JE	0.89	0.82	0.64	0.36	0.56
Langat	0.30	0.30	0.40	30	0.40
MV	0.45	0.50	0.30	0.30	0.40
SLE	0.85	0.65	0.84	0.50	0.50
WN	0.90	0.86	0.51	0.40	26.95
TBE	0.82	0.60	0.82	0.75	0.75
Herpesviruses					
HSV-1	0.80	0.58	0.78	0.50	0.88
HSV-2	0.60	0.50	0.60	0.40	0.70
VZV	0.30	0.40	0.30	0.40	0.32
EBV	0.50	0.50	0.60	0.40	0.90
CMV	0.46	0.43	0.46	0.42	0.58
HHV-6	0.58	0.50	0.52	0.46	0.62
Enteroviruses	0.33	0.39	0.33	0.39	0.42

^a Boldface indicates an *I_{tb}* result above the threshold.

nique, which provides exhaustive typing capabilities for CNS viruses, is especially designed for reference centers, which will be able to use it to confirm infections and to further characterize virus isolates. In that respect, it complements rather than replaces easy-to-use, low-cost reagents that are essential for field detection of these viruses, especially in less developed countries.

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