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Typing of Enteroviruses by Use of Microwell Oligonucleotide Arrays▽†

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We have developed a straightforward assay for the rapid typing of enteroviruses using oligonucleotide arrays in microtiter wells. The viral nucleic acids are concomitantly amplified and labeled during reverse transcription-PCR, and unpurified PCR products are used for hybridization. DNA strands are separated by alkaline denaturation, and hybridization is started by neutralization. The microarray hybridization reactions and the subsequent washes are performed in standard 96-well microtiter plates, which makes the method easily adaptable to high-throughput analysis. We describe here the assay principle and its potential in clinical laboratory use by correctly identifying 10 different enterovirus reference strains. Furthermore, we explore the detection of unknown sequence variants using serotype consensus oligonucleotide probes. With just two consensus probes for the coxsackievirus A9 (CVA9) serotype, we detected 23 out of 25 highly diverse CVA9 isolates. Overall, the assay involves several features aiming at ease of performance, robustness, and applicability to large-scale studies.

Enteroviruses belong to the *Picornaviridae* family of small, nonenveloped RNA viruses. There are currently more than 100 recognized human enterovirus types, and the number is constantly increasing (24; N. J. Knowles, Picornavirus Home Page [http://www.picornaviridae.com/]). Clinical manifestations of enterovirus infections range from rash, respiratory symptoms, myocarditis, and severe illness in newborns to central nervous system (CNS) infections, such as acute flaccid paralysis, meningitis, and encephalitis (1, 2, 5, 13, 25; P. Huttunen et al., submitted for publication). Enteroviruses are the main cause of aseptic meningitis with known etiology; the proportion of cases ranges from 80 to 92% (21). Both respiratory and CNS infections caused by enteroviruses require medical consultation and hospitalization and are often treated with antibiotics because of lack of rapid and sensitive early detection of the pathogen. The prevalence and clinical significance of enteroviruses is further emphasized by multiple severe outbreaks in recent years, including massive epidemics of hand, foot, and mouth disease in Asia, mainly caused by enterovirus 71 (EV71) (6; CDC, outbreak notice, 2008). In light of the poliovirus eradication campaign and the chance of new outbreaks via enterovirus recombination (8), it is important to develop rapid means for the diagnosis and large-scale monitoring of enteroviruses and their serotypes circulating in the population. Moreover, antiviral drugs against enteroviruses are under development, and their availability will significantly increase the need for rapid, sensitive, and specific diagnostic tests.

The diagnosis of enterovirus infections has classically been based on isolation of the virus followed by neutralization typing using an antiserum panel. The procedure is slow and laborious, since more than one cycle of cell culture propagation may be required. Therefore, the results are not available at the acute phase of the infection, when they would be necessary for differential diagnosis and in order to avoid unnecessary antibiotic treatment. During the past 10 years, reverse transcription-PCR (RT-PCR) has largely replaced or complemented enterovirus isolation in diagnostic laboratories. The assays are usually based on the amplification of conserved regions in the 5′ noncoding region of the genome, but although the tests are highly sensitive and recognize all enteroviruses, they do not provide information on the virus types associated with the illnesses. This would be needed for epidemiological purposes and for specific identification of polioviruses during the eradication campaign. Recently, it has been shown that the sequence of the VP1 capsid protein correlates well with the serotype concept (19) and with the current classification of enteroviruses into A, B, C, and D species by phylogenetic criteria (24). It has also become possible to amplify the VP1 coding region directly from clinical samples for typing (18), but further identification requires sequence analysis of the amplicons, which is not a routine procedure in diagnostic laboratories.

New developments in microarray techniques have made them applicable for specific identification of microbial sequences in clinical samples using approaches that are becoming applicable in daily laboratory diagnosis of infections (17). These methods have several advantages over the current analysis systems: even in simple arrays, hundreds of individual sequences can be detected by oligonucleotide probes; the assay principles are versatile; allowing their usage in clinical laboratories; and the system also makes high-throughput analysis possible. We describe here a highly specific test system based on direct labeling of the sequence of interest in the PCR followed by use of the amplification products for typing by oligonucleotide arrays. The whole procedure is carried out in microtiter wells, making it applicable to current laboratory practice. We exemplified the assay concept with 10 important

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TABLE 1. Oligonucleotide probes used in the array

Array position	Virus serotype	Oligonucleotide sequence (5′→3′)
1	CVA16	CAAACGGTGAGCTAGTACC
2	CVA21	CTTGAGTCATTCTTTGG
3	CVB3	CAAGTACTCAACAGCCCTC
4	CVB4	GAATCAAACAACCTGAAGCG
5	E11	TTTTCCACACAACCAACACTGAC
6	E30	ATATCGCCCACTATGCCAC
7	EV70	GTGTGTATGCGATCATTTGAG
8	EV71	TTTTTTCTTAACTCACATAGCACAGC
9	PV1	CGTGACCATTATGACCGTG
10	CVA9	GACCACTGATAAGCATGTTAAC
11	CVA9_C1	TTTTCAAGACATGCCAGTG
12	CVA9_C2	TTTTTGGCCAATCAACACC
13	CVA9_C3	TTTTTTTGTGGCGTGGC
14	CVA9_C4	TTTTACAAATGGTTCAGATGC
C	Positive control	TTTTGCAACTCTGCAGCGGA

enterovirus prototype strains and then demonstrated that diverse isolates of the same serotype could be typed with a small set of serotype consensus probes. With further bioinformatic efforts to design probes detecting a broader set of viruses, and with the development of routine-use array imagers, this assay system offers an efficient and rapid alternative to the methods currently used for the specific identification of enterovirus types.

MATERIALS AND METHODS

Virus samples. Virus samples were originally obtained from different sources and cultivated in cell cultures for isolation of viral RNA. Of the virus prototype strains, coxsackievirus A16 (CVA16; strain G-10), CVB3 (strain Nancy), EV70 (strain J670/71), and EV71 (strain BrCr) were from WHO, and CVA21 (Kuykendall), CVA9 (Griggs), CVB4 (JVB), E11 (Gregory), and E30 (Bastianni) were obtained from the ATCC. Partial cDNA for poliovirus 1 (PV1), including the VP1 coding region, was kindly provided by Merja Roivainen (NPHI, Helsinki, Finland). Clinical CVA9 isolates used in this study have been described previously (23) or originated from laboratory collections.

RT-PCR, Cy5 labeling, and sequencing. RT-PCR was performed essentially as described previously (18) except that the 3′-end primer (AN88) was labeled with Cy5 (indocarbocyanin; Eurogentec, Seraing, Belgium). The sensitivity of the PCR performed using primers AN89 and Cy5-AN88 was similar to that of an unlabeled reaction (data not shown). The resulting labeled amplicons were used unpurified in hybridizations or were gel purified for sequencing. The amplicons were sequenced in both directions using primers AN232 and AN233 (18). The partial VP1 sequences of the CVA9 isolates have been deposited in the GenBank sequence database (see below).

Oligonucleotide microarrays. Oligonucleotide probes for the enterovirus prototype strains were designed using published VP1 sequences, aiming at a melting temperature around 60°C and a length of approximately 20 nucleotides (Table 1). The CVA9 serotype consensus probes were designed to be as short as possible, with a melting temperature of at least 50°C and a GC content less that 60%. The probes were immobilized at their 5′ ends; to improve the availability for hybridization, some of the oligonucleotides had four extra T nucleotides as spacers at their 5′ ends (CVA9 consensus probes and the E11 and EV71 probes). Microarrays in 96-well microtiter plates were prepared at the VTT microarray service using proprietary 5′-end immobilization chemistry. The array plates are available from the service on request (http://www.vtt.fi/biochipservice).

Hybridization, scanning, and image analysis. Each PCR mixture was diluted 1:10 in denaturation solution (50 mM NaOH), and after incubation for 5 min at 37°C, 25 μl was transferred to an array well containing 25 μl of neutralization solution (0.9 M NaCl, 0.09 M trisodium citrate, 0.02 M citric acid, 0.3% Tween 20, and 1 nM Cy5-labeled oligonucleotide complementary to the control spots in the array). The plate was incubated in a plate shaker (600 rpm) at 37 for 1 h, washed at room temperature (~22°C) with TBST (20 mM Tris-HCl [pH 8.0], 150 mM NaCl, 0.1% Tween 20) in a standard plate washer, and dried by centrifuging upside down at 500 × *g* for 1 min. The plate was scanned through the bottom with the Tecan LS400 confocal laser scanner at 10-μm resolution using the 633-nm laser and a 670/25 emission filter. Image analysis, spot detection, and quantification were performed with ArrayPro software (Media Cybernetics). For each spot, a net signal value was obtained by subtracting the local mean background around the spot from the mean pixel intensity within the spot; the median of three replicate spots was used for the subsequent analysis.

Thermodynamic prediction for probe-target hybrids. The predicted free energy change of probe-target hybrid formation at 37°C (in kilocalories per mole) was calculated based on the probe and target sequences on the DINAMelt server at http://dinamelt.bioinfo.rpi.edu/ (16). The calculations were performed using the two-state hybridization model with the following parameters: temperature, 37°C; total strand concentration, 2e-09 M; $[Na^+]$, 0.61 M; $[Mg^{2+}]$, 0 M.

Nucleotide sequence accession numbers. The partial VP1 sequences of the CVA9 isolates and the Griggs reference strain used in this study were deposited in the GenBank database and have the following accession numbers (with the isolate identification numbers in parentheses): EU735076 (3745), EU735077 (4394), EU735078 (6326), EU735079 (6397), EU735080 (8216), EU735081 (8693), EU735082 (10043), EU735083 (11299), EU735084 (33725), EU735085 (38050), EU735086 (45072), EU735087 (52440), EU735088 (54523), EU735089 (55690), EU735090 (56297), EU735091 (64104), EU735092 (64166), EU735093 (67603), EU735094 (71478), EU735095 (84562), EU735096 (88794), EU735097 (89914), EU735098 (90935), EU735099 (106464), and EU735100 (Griggs strain).

RESULTS

Assay concept. Our approach to enterovirus genotyping was based on the hybridization of amplified and labeled copies of viral nucleic acids onto oligonucleotide arrays in the wells of 96-well microtitration plates. The overall workflow of the assay is shown in Fig. 1A. After viral RNA isolation and reverse transcription, we amplified a portion of the VP1 coat protein coding region by seminested PCR using broad-specificity degenerate primers that have previously been shown to recognize all the known 64 prototype strains and 22 proposed new enterovirus types, as well as many recent enterovirus isolates (18). Direct labeling of the amplicon with a Cy5-containing primer during the PCR circumvented a separate labeling step. As the labeled primer (AN88) and the resulting Cy5-labeled strands corresponded to the antisense strand of the viral genome, the oligonucleotide probes corresponded to the sense strand. The DNA strands of the PCR product were separated by alkaline denaturation, followed by neutralization in the array wells, resulting in the immediate start of the hybridization reaction. This was more convenient and reliable than heat denaturation and cooling, because there was no concern about premature strand reannealing. Identification of virus types was based on quantification of fluorescent signal intensities on the spots representing the specific probes in the array (Fig. 1).

Typing enterovirus prototype strains. To evaluate the specificity of the assay, we tested 10 clinically significant enterovirus serotypes representing the four species: CVA16 and EV71 (*Human enterovirus A* [HEV-A]); CVA9, CVB3, CVB4, E11, and E30 (HEV-B); CVA21 and PV1 (HEV-C); and EV70 (HEV-D). The viral nucleic acid samples were amplified and labeled with Cy5 using the seminested PCR protocol, and amplicons of the expected size (~300 bp) were visualized by agarose gel electrophoresis (see Fig. S1 in the supplemental material). Aliquots of the reaction mixtures were hybridized with the serotype-specific probe set in the microwell arrays (Fig. 2A; Table 2). All samples gave clear signals in the spots containing the cognate probes, while the signals with the other strain-specific probes remained at the background level. Thus, the assay unambiguously distinguished 10 different enterovirus reference strains using specific oligonucleotide probes.

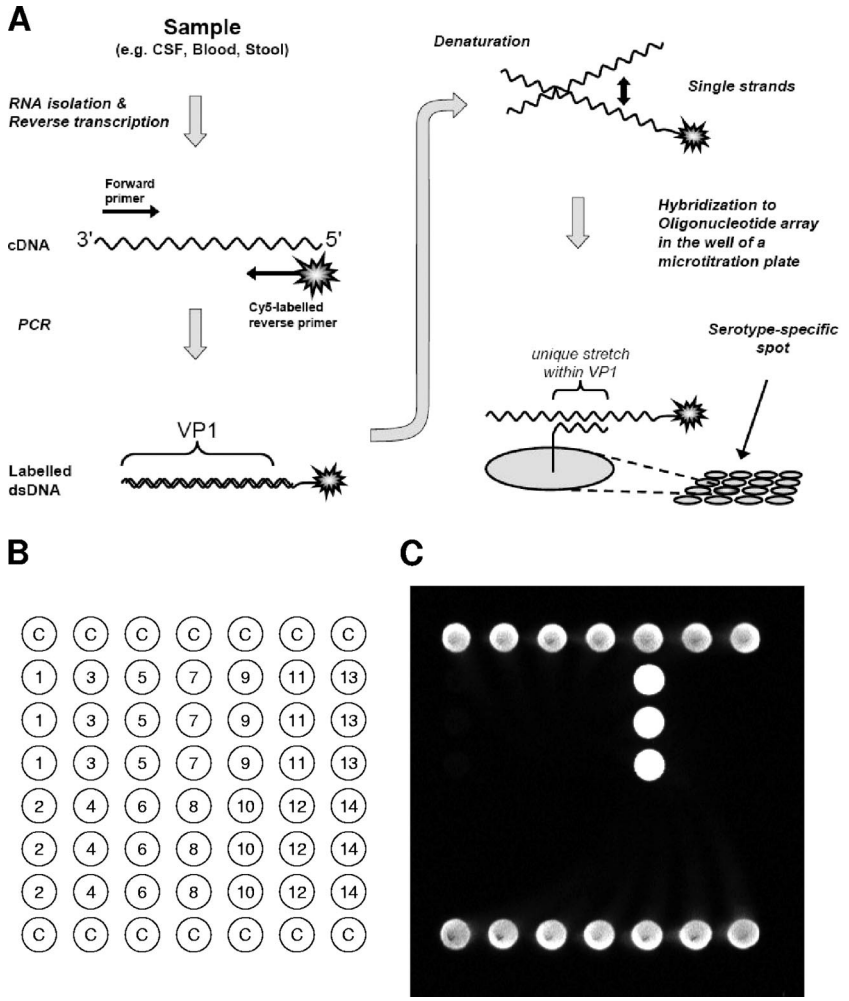

FIG. 1. Principle of the assay. (A) Viral RNA is isolated from the clinical sample and reverse transcribed to cDNA using a generic primer recognizing the VP1 coding region. The cDNA is then PCR amplified with a primer pair that anneals to highly conserved sequences in the VP1 region. The reverse primer contains a Cy5 label; thus, the PCR product will be directly labeled in the reaction. There is no purification or separate labeling step after the PCR amplification. The crude PCR mix is first denatured by NaOH and then added to the array wells containing neutralization solution. The labeled strand hybridizes specifically to the serotype-specific oligonucleotides, spotted onto the solid phase. To detect the fluorescent spots, the arrays are finally imaged with a confocal laser scanner. (B) Array map. The symbol C denotes control spots that are used to aid in the image analysis in positioning the grid. Spot numbers 1 to 14 correspond to the oligonucleotides listed in Table 1. (C) Image of a scanned array hybridized with a PV1 sample. The PV1-specific oligonucleotides were spotted in the positions numbered 9.

The E11 sample additionally hybridized to the CVA9_C1 probe (i.e., one of the CVA9 consensus probes [see below]), albeit at a much lower intensity than that with which it hybridized to the E11 cognate probe (net signal, 1,104 versus 1,4676, respectively). This cross-hybridization can be explained by partial sequence identity between the CVA9_C1 probe and the E11 sequence. There is an identical 12-nucleotide stretch (C AAGACATGCCA) in the sequences, with a predicted free energy change of hybridization at 37°C of −14.1 kcal/mol.

Linearity of the hybridization signals. Aiming at a simple and rapid protocol, we applied a fixed volume (2.5 μl) of each crude PCR mixture to the hybridization reaction mixtures; i.e., the amount was not adjusted to compensate for different yields in the individual seminested PCRs. To estimate the sensitivity of the assay in detecting low-yield targets, we assayed a series of amplicon dilutions made from purified and quantified CVB3 and CVB4 PCR products (Fig. 3). The hybridization signal was linearly dependent on the amount of target, and even 2 ng/well was easily detected. In comparison, the amplicon yield in the RT-PCRs from cell lysates was usually more than 100 ng per 2.5-μl reaction volume when estimated on an ethidium bromide-stained agarose gel.

Detection of clinical CVA9 isolates with serotype consensus probes. We next explored the possibility of designing generic serotype consensus probes for the detection of all or most isolates of a given serotype. We tested this concept with a collection of 25 CVA9 samples that included the Griggs prototype strain and 24 geographically diverse isolates, previously typed by neutralization tests (23), but whose genome sequences at the VP1 PCR amplicon region were unknown. Using available GenBank database sequences covering the CVA9 VP1 amplified region, we looked for conserved sites in this serotype and designed four consensus probes (Table 1). Together, the set of probes recognized 93% (50 of 54) of the

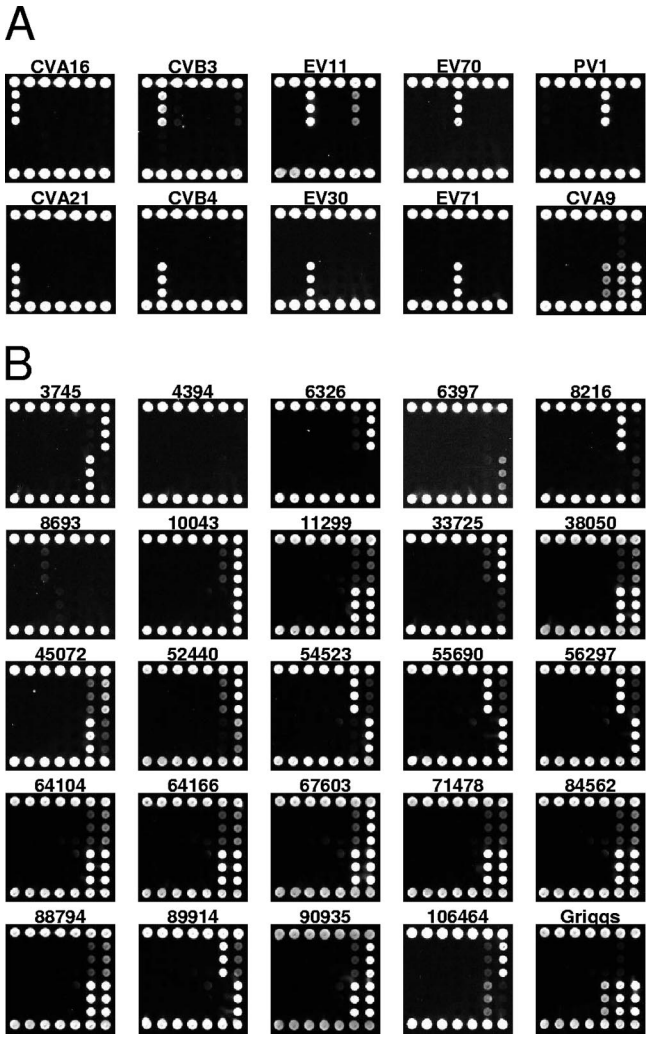

FIG. 2. Images of well arrays hybridized with enterovirus samples. Sample names are given above each image. (A) Panel of 10 prototype strains. (B) Twenty-four CVA9 isolates and the Griggs prototype strain. Note that the array contains five probes for CVA9, of which the probe in position 10 is specific for the Griggs prototype strain, while the probes in positions 11 through 14 are consensus probes designed to hybridize to a broad range of different CVA9 isolates.

CVA9 VP1 sequences in GenBank such that at least one of the probes had a complete match with its target. We then assayed the collection of 25 CVA9 samples with arrays containing the 4 CVA9 consensus probes in addition to the 10 previously described strain-specific probes. Overall, 23 of the 25 samples were clearly detectable (i.e., giving a signal above 100) with at least one of the consensus probes (Fig. 2B; Table 3). As a demonstration of the hybridization specificity, the CVA9 samples gave negligible signals with the 10 individual enterovirus strain-specific probes. Interestingly, the strain-specific CVA9 probe detected only the Griggs reference strain but did not recognize any of the tested CVA9 isolates. In contrast, the individual consensus probes recognized 13 to 20 different CVA9 samples (Table 3). Due to this redundancy of the consensus probes, the same overall result (23 CVA9 samples recognized out of 25) would also have been achieved using just the two consensus probes C3 and C4.

Mismatch hybridization behavior of the CVA9 consensus probes. We determined the sequences of the 25 CVA9 VP1 PCR products and compared each with the four consensus probe sequences. The resulting 100 pairwise probe-target comparisons consisted of 41 perfect matches and 28 single-base, 13 two-base, 15 three-base, and 3 four-base mismatches, respectively (see Table S1 in the supplemental material). While the hybridization signals generally decreased with an increasing number of mismatches, some probe-target pairs tolerated even three mismatches, still giving detectable signal (see Table S1 in the supplemental material). Thus, the number of mismatches per se was insufficient to predict the hybridization performance. However, by comparing the hybridization signals with the calculated thermodynamic strengths of the hybrids (Fig. 4), we could derive a useful empirical rule for the behavior of the partially matched hybrids in our setup. At the assay conditions used (37°C, 0.61 M Na^+), a predicted ΔG of approximately −12 kcal/mol or lower was required for detection, and a value lower than −16 kcal/mol was required for strongly positive signals (i.e., signals over 2,000).

DISCUSSION

Array-based platforms have previously been used for the genotyping of enteroviruses, for example, in the genomic anal-

TABLE 2. Individual probe signals obtained with 10 enterovirus reference strains

Sample serotype	Signal with the indicated probe													
	Prototype strain specific[a]										CVA9 consensus			
	CVA9	CVA16	CVA21	CVB3	CVB4	E11	E30	EV70	EV71	PV1	CVA9_C1	CVA9_C2	CVA9_C3	CVA9_C4
CVA9	**879**[b]	0	0	0	0	0	0	0	0	0	78	1,314	0	11,146
CVA16	0	**3,761**	0	0	0	0	18	0	0	0	10	0	18	3
CVA21	0	0	**3,141**	0	0	0	0	0	0	0	2	0	0	0
CVB3	0	0	0	**1,689**	38	26	0	0	0	0	0	0	105	0
CVB4	0	0	0	0	**4,693**	1	0	0	0	0	1	0	20	0
E11	0	0	0	0	0	**14,676**	0	0	1	0	1,104	0	1	3
E30	0	0	0	0	0	0	**1,119**	0	0	0	0	0	0	0
EV70	0	0	0	0	0	4	0	**1,094**	0	0	0	0	0	0
EV71	0	0	0	0	0	7	0	0	**2,634**	0	0	0	12	0
PV1	0	33	0	0	0	0	0	0	0	**7,323**	0	0	0	0

[a] Each probe signal represents the median net signal of three replicate spots in the same well.
[b] Boldface signal values on the diagonal represent the cognate probe-sample pairs.

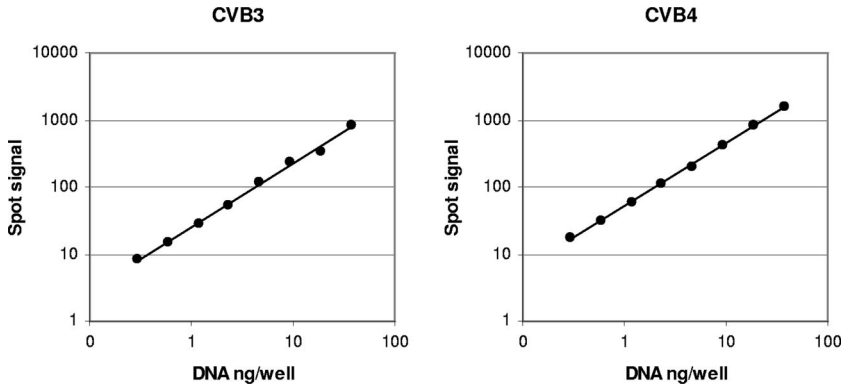

FIG. 3. Linearity of hybridization signals in response to the amount of DNA target. For this experiment, the double-stranded RT-PCR products of CVB3 and CVB4 virus samples were purified with a PCR purification kit (Qiagen), quantitated by absorbance at 280 nm, and serially diluted for the hybridization assay. The plotted signals are median net signals calculated from three replicate spots.

ysis of vaccine-derived poliovirus strains (4, 12) and in the detection and differentiation of enterovirus 71 and coxsackievirus A16 strains (3). Those methods mainly employ technological solutions originally developed for microarray-based gene expression studies, e.g., printing arrays on glass slides and labeling amplicons by polymerase-based incorporation of labeled nucleotides.

Our approach here introduces several features that make the method easy to perform and amenable to high-throughput testing. We labeled the amplification product concomitantly with the PCR, obviating a separate labeling step. Use of a 5′-Cy5-labeled primer did not decrease PCR efficiency and resulted in adequate signal intensity on the array spots. Further, we directly hybridized the unpurified PCR products with the arrays in the wells, and although the reaction mixtures contained as much as 2 μM free labeled primers, we did not detect any increased background hybridization signal in comparison to the purified PCR products (data not shown). Strands were separated by diluting the PCR product in an alkaline solution, followed by neutralization in the array wells only at the beginning of the hybridization step. This protocol ensured complete strand separation until the initiation of the hybridization. Strand reannealing kinetics during the hybridization reaction at 37°C appeared to be sufficiently slow to avoid competition with the probe hybridization. We did not apply asymmetric PCR (in order to generate excess free la-

TABLE 3. Individual probe signals obtained with CVA9 strains

Virus strain	Signal with the indicated probe													
	Prototype strain specific										CVA9 consensus			
	CVA9	CVA16	CVA21	CVB3	CVB4	E11	E30	EV70	EV71	PV1	CVA9_C1	CVA9_C2	CVA9_C3	CVA9_C4
3745	0	0	0	0	0	1	1	0	0	0	24	1,331	2,033	10
4394	0	0	0	0	0	0	0	0	0	0	2	6	2	0
6326	0	0	0	0	0	1	3	1	0	0	152	0	4,154	1
6397	0	0	0	0	0	0	0	0	0	0	4	7	0	161
8216	0	0	0	0	0	0	0	0	0	0	4,735	12	28	175
8693	0	0	0	0	0	79	0	0	29	0	2	5	0	4
10043	0	0	0	0	0	0	0	0	0	0	265	3	4,199	6,428
11299	0	0	0	0	0	0	0	0	0	0	420	4,380	924	7,869
33725	0	0	0	0	0	0	0	0	0	0	286	0	2,933	229
38050	0	0	0	0	0	0	0	0	0	0	445	7,279	1,577	9,577
45072	0	0	0	0	0	0	0	0	0	0	324	1,542	896	208
52440	0	0	0	0	0	0	0	0	0	0	627	63	3,517	1,224
54523	0	0	0	0	0	0	0	0	0	0	5,706	71	315	4,160
55690	0	0	0	0	0	0	0	0	0	0	7,634	7	478	6,636
56297	0	0	0	0	0	0	0	1	0	0	8,863	4	501	7,851
64104	0	0	0	0	0	0	0	0	0	0	551	7,285	1,483	8,900
64166	0	0	0	0	0	0	0	0	0	0	670	8,182	1,446	10,379
67603	0	0	0	0	0	8	0	0	0	0	750	7,461	4,889	14,791
71478	17	0	0	0	0	0	0	0	0	0	452	7,093	751	10,087
84562	0	0	0	0	0	0	0	0	0	0	552	5,422	1,378	11,440
88794	0	0	0	0	0	0	0	0	0	0	486	6,700	1,460	12,228
89914	0	0	0	0	0	0	0	0	0	0	4,749	6	762	8,128
90935	9	0	0	0	0	0	0	0	0	0	664	6,796	4,524	12,604
106464	0	0	0	0	0	0	0	0	0	0	102	368	1,040	40
Griggs	879	0	0	0	0	0	0	0	0	0	78	1,314	0	11,146

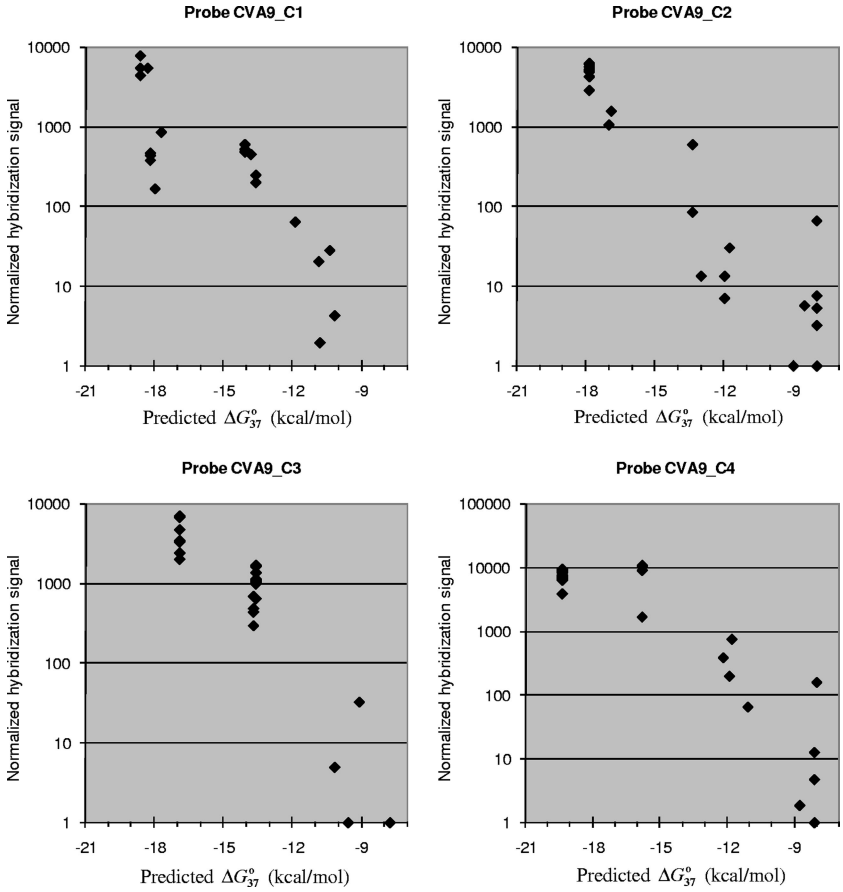

FIG. 4. Effects of free energy of probe-target hybrids on signal level. A total of 100 probe-target pairs between the 4 CVA9 consensus probes and the 25 sequenced CVA9 VP1 regions were analyzed. The thermodynamic calculations were performed on the DINAMelt server at http://dinamelt.bioinfo.rpi.edu/ (16) as described in Materials and Methods. The signals are normalized to the amount of target present in the hybridization (based on quantification of the PCR products on an ethidium bromide-stained agarose gel; see Fig. S1 in the supplemental material). Detailed data on the individual probe-target hybrids are presented in Table S1 in the supplemental material. ΔG°_{37}, free energy change of probe-target hybrid formation at 37°C.

beled strands available for hybridization), because the required adjustments to primer concentrations would have decreased the amplification efficiency in the PCR with degenerate primers. Performing the hybridization reactions with microarrays printed in 96-well microtiter plates facilitated liquid handling by allowing the use of standard multichannel pipettes, plate shakers, and washers. Therefore, the method described is easily adaptable to the simultaneous analysis of 96 PCR products at a time.

There is considerable nucleotide sequence variation in the enterovirus VP1 coding region, even between different isolates of the same serotype. As a general rule, strains with at least 75% nucleotide identity are considered to represent the same serotype (19). In most cases, it is not possible to design a single probe that would perfectly match all known sequence variants within the serotype, and, in fact, in previous studies more than 10,000 enterovirus-specific probes were used to identify all 64 serotypes (10). While it is possible to increase the number of probes to identify all serotypes, this may not be cost-effective. Longer oligonucleotide probes would allow more mismatches without a significant decrease in the hybridization signal, but they could also display more cross-reactivity between serotypes. Alternatively, one could design a moderate number of short probes against the most conserved sequences within the serotype. We tested the latter approach for the identification of diverse CVA9 samples isolated over a time span of nearly 50 years (23). Without prior sequence information on the 24 isolates, we designed consensus probes based on 54 CVA9 VP1 sequences available in GenBank. The four probes designed recognized 93% of the database sequences such that at least one of the probes had a perfect match. In the hybridization tests, the probe set recognized 23 of 25 (92%) clinical CVA9 isolates as positive, thus reflecting the coverage of CVA9 sequences in GenBank. There was redundancy in the probe set; we would have identified the same 23 isolates as positive using just the two consensus probes CVA9_C3 and CVA9_C4. Moreover, any other pair of two consensus probes would have recognized at least 21 of the 25 samples as CVA9 positive. Thus, in practice, two optimally designed consensus probes may suffice to identify 90% of the virus isolates within a diverse serotype group such as CVA9.

A probe design strategy that is based on relatively short consensus sequences must also take into account the cross-hybridization behavior of the probes. For example, a 12-base

identical sequence stretch was sufficient to cause a cross-reactive signal between the E11 sample and the CVA9_C1 consensus probe (see Results and Table 2). Therefore, an ability to reliably predict the hybridization performance of partially matching probe-target pairs would help in designing more-robust consensus probes (as well as in avoiding unwanted cross-reactivities with other serotypes). Since the destabilizing effect of mismatches is known to vary considerably depending on the oligonucleotide position, the sequence context, and the mismatched nucleotides themselves (for a review, see reference 22, we used the calculated thermodynamic strengths of the probe-target hybrids to derive practical rules for designing functional probes. In the case of the sequence similarity between E11 and the CVA9_C1 consensus probe, the calculated free energy change at 37°C of −14.1 kcal/mol clearly predicted the modest cross-reactivity observed.

On the other hand, even a perfect probe-target sequence match does not always guarantee a strong hybridization: in individual targets the DNA secondary structure can significantly interfere with the probe binding. Although this effect can be anticipated to some extent by secondary-structure prediction algorithms (9, 20), the random occurrence of point mutations in the vicinity of the conserved probe binding sites makes this difficult. It will still be safe to include some redundancy in the probe sets, as was done here. In conclusion, the analysis of the mismatch hybridization behavior of the consensus probes in this study will now give useful practical guidelines for the future bioinformatic design of consensus probes for the other enterovirus serotypes.

Based on these initial proof-of-principle tests, the prospect of extending the serotype consensus probe strategy to all human enteroviruses does not seem unrealistic. Assuming an average need for two consensus probes to identify each serotype, one would need an array of approximately 200 probes to cover all 100 serotypes currently recognized. The arrays in microtiter wells could easily accommodate the required number of spots. In addition, this collection could include probes detecting the conserved 5′ noncoding region for group-specific identification (rhinovirus-enterovirus distinction) (7, 14) and sequences from the genes encoding nonstructural proteins to monitor recombinations. We are currently exploring the feasibility of such a strategy in our assay platform.

The arrays-in-wells approach developed for rapid identification and typing of enteroviruses will be applicable in various studies. As demonstrated here, the assay can specifically detect clinically significant representatives from all four enterovirus species. Efficient typing of enteroviruses would be important in replacing the cumbersome neutralization assay currently used. Hybridization-based typing can provide rapid availability of results that are clinically relevant, for example, in the differential diagnosis of CNS infections (11; Huttunen et al., submitted). The seminested RT-PCR amplification can potentially amplify targets directly from clinical specimens, as demonstrated by Nix et al. (18), or could be performed on early-stage cell cultures, unlike the neutralization tests, which often require two to three rounds of cell culture. The time frame of the procedure (4 h from sample preparation to scanning of the hybridized array) would reduce the cost of hospitalization and decrease the unnecessary use of antibiotics. The ability to rapidly type even hundreds of samples in parallel in the 96-well format can also provide valuable information on pathogens circulating in the population for epidemiological purposes, for understanding of the pathogenetic mechanisms behind the illnesses, and for evaluation of the need for vaccines and the development of antiviral compounds. Moreover, there is a need to develop screening methods for emerging enteroviruses. A similar assay could be rather easily designed for rhinoviruses and parechoviruses, which include pathogens closely resembling enteroviruses in terms of molecular properties, diversity, and outcome of infection. The approach is also amenable to multiplexing to include other significant viruses causing infections of the CNS, such as herpesviruses and flaviviruses. (10, 15).

In summary, the arrays-in-wells approach provides rapid and sensitive identification of clinically significant enteroviruses. The approach has several advantages, including efficient serotype-specific consensus probes, direct labeling of the amplicons, a convenient hybridization procedure, and minimal hands-on time.

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