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Combining Multiplex Reverse Transcription-PCR and a Diagnostic Microarray To Detect and Differentiate Enterovirus 71 and Coxsackievirus A16

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Cluster A enteroviruses, including enterovirus 71 (EV71) and coxsackievirus A16 (CA16), are known to cause hand-foot-and-mouth disease (HFMD). Despite the close genetic relationship between these two viruses, EV71 is generally known to be a more perpetuating pathogen involved in severe clinical manifestations and deaths. While the serotyping of enteroviruses is mostly done by conventional immunological methods, many clinical isolates remain unclassifiable due to the limited number of antibodies against enterovirus surface proteins. Array-based assays are able to detect several serotypes with high accuracy. We combined an enterovirus microarray with multiplex reverse transcription-PCR to try to develop a method of sensitively and accurately detecting and differentiating EV71 and CA16. In an effort to design serotype-specific probes for detection of the virus, we first did an elaborate bioinformatic analysis of the sequence database derived from different enterovirus serotypes. We then constructed a microarray using 60-mer degenerate oligonucleotide probes covalently bound to array slides. Using this enterovirus microarray to study 144 clinical specimens from patients infected with HFMD or suspected to have HFMD, we found that it had a diagnostic accuracy of 92.0% for EV71 and 95.8% for CA16. Diagnostic accuracy for other enteroviruses (non-EV71 or -CA16) was 92.0%. All specimens were analyzed in parallel by real-time PCR and subsequently confirmed by neutralization tests. This highly sensitive array-based assay may become a useful alternative in clinical diagnostics of EV71 and CA16.

Enteroviruses belong to the RNA virus family *Picornaviridae* and include polioviruses, type A coxsackieviruses (CA), type B coxsackieviruses (CB), echoviruses (ECV), and enterovirus types 68 to 71 (EV68-71) (25, 31). The Asia-Pacific region has had large epidemics of enterovirus infections for over 10 years. There has been a significant increase in epidemics of EV71, a member of the human enterovirus A species, known to be the cause of hand-foot-and-mouth disease (HFMD) (5, 8). Most people infected by this serotype are asymptomatic or have mild symptoms, but some may develop severe neurologic diseases or die, especially young children (17).

We currently lack a reliable method of diagnosing enteroviruses early. Presently, the identification and serotyping of enteroviruses are based on conventional immunological methods, including immunohistochemical detection (40), indirect immunofluorescence assay (IFA) (20, 30), and neutralization testing (24, 30). These methods are very useful in diagnosis from biopsy specimens taken from patients who have been infected for a while or from samples isolated from cultures of infected material. However, they are not very sensitive in detecting the virus in specimens with low titers of the virus, limiting their ability to diagnose the infection early.

Progress in molecular biology is making direct assaying of low-copy viral DNA or RNA sequences from clinical samples increasingly possible. In fact, reverse transcription-PCR (RT-PCR) has been used to diagnose enterovirus infections early (13, 29), and DNA sequencing has been used for serotyping (7, 14, 35). However, genomic information for many enterovirus serotypes is limited, and there is a high genetic heterogeneity among different strains, so misdiagnosis might be frequent (27). Therefore, we are still left with a need for a rapid and accurate means of diagnosing HFMD.

DNA microarray technology could make this possible. It could be designed to simultaneously detect multiple virus targets by hybridizing to many virus-specific nucleotide probes. It has, in fact, been successfully used to develop diagnostic microarrays for hepatitis C virus (10), severe acute respiratory syndrome coronavirus (SARS-CoV) (15, 18), influenza virus (16), and other viruses (4). In an effort to develop such a microarray-based method of sensitively and accurately detecting multiple serotypes of the enteroviruses in clinical samples, we combined multiplex RT-PCR (MRT-PCR) and a diagnostic microarray, a strategy we had used previously to develop a SARS diagnostic microarray (15). The microarray we developed was found to be sensitive and accurate. Although it will not replace established techniques, it may be used to improve

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TABLE 1. Candidate probes and primers used for EV microarray analysis

Probe or primer	Domain	Amplicon (bp)	Sequence (5′–3′)
Probe[a]			
pan-EV	5′ UTR		TGTCGTAACGGGCAACTCTGCAGCGGAACCGACTACTTTGGGTGTCCGTGTTTCCTTTTA
EV71-1	VP1		TCACCTGCGAGCGCCTATCAATGGTTTTATGACGGGTATCCCACATTCGGTGAACACAAA
EV71-2	VP1		ATCTATTCAAAGCCAACCCAAATTATGCTGGTAATTCTATTAAACCAACTGGTGCCAGTC
CA16-1	VP2		CTGAGTATGTGCTCGGCACTATCGCAGGAGGGACCGGGAATGAGAATTCTCATCCTCCCT
CA16-2	VP2		CCTTTTGACTCAGCTCTCAACCACTGCAACTTTGGTCTACTGGTCGTCCCGGTAGTACCA
RTC			GGTCCGAAGGTTTCACCATCACTGGCTCGCCTCTCAACGATTTCCGTAAACTTTGGCCCC
HC			AGCATTCCGAGTAACTCCTCAACCTGGAGTTCCACCTGAAGAAGCAGGGGCCGCGGTAGC
NC			TGGCTGTAGTTGTGACCAACTCCGCGAACCCTTGATGCAGTCTGCGGATGCATCAACGTT
Primer[b]			
panEV-s		185	GTGTGAAGAGTCTATTGAGC
panEV-a			ATTGTCACCATAAGCAGCCA
EV71-s		421	ACGAACCCCTCAGTTTTTGT
EV71-a			TTAACCACCCTAAAGTTGCC
CA16-s		483	ATGCGAGTAAATTCCACCAG
CA16-a			ACACCATCATCAGTAGTGAG
RTC-s		701	CCCTAAAGACGATCTCCTCT
RTC-a			TTAACCACGAGCGAGCCAGG
HC-s		141	GTAAGTCCATCGGTCCATAC
HC-a			GAGTACCAAACCAAGGATAC
NC-s		183	AGAAACACAGTCTGTACCGT
NC-a			AAGCCCTGTAGACGACATCA

[a] Abbreviations for probes: pan-EV, pan-enterovirus probe; EV71-1 and EV71-2, enterovirus 71 probes; CA16-1 and CA16-2, coxsackievirus A16 probes; RTC, RT-PCR control probe from the arabidopsis *GA4* gene; HC, hybridization control probe from the plant chloroplast *RbcL* gene; NC, negative-control probe from SARS-CoV.

[b] Abbreviations for primers: pan-EV, panenterovirus primer; EV71, enterovirus 71 primer; CA16, coxsackievirus A16 primer; RTC, RT-PCR control primer from the arabidopsis *GA4* gene; HC, hybridization control primer from the plant chloroplast *RbcL* gene; NC, negative-control primer from SARS-CoV. s, sense strand; a, anti-sense strand.

clinical diagnosis of enterovirus infections in clinical and scientific studies.

MATERIALS AND METHODS

Specimen collection. Throat swab specimens were collected from patients suspected to have HFMD and sent to the Center for Disease Control, Taiwan, and the Clinical Virology Laboratory, Chang Gung Memorial Hospital, Tao-Yuan, Taiwan, and identified by viral culture (32), neutralization testing (24), and IFA (33). All of the specimens were inoculated into RD cells (ATCC no. CCL-136), MRC-5 cells (ATCC no. CCL-171), and Vero cells (ATCC no. CCL-81) and cultured in MEM medium supplemented with 10% fetal bovine serum (HyClone) at 37°C. When more than 50% of the cell monolayer exhibited the cytopathic effect, the cells were subjected to neutralization testing and IFA, using a pan-enterovirus antibody (Chemicon) for positive or negative enteroviruses and serotype-specific antibodies (Chemicon) for serotypes of enteroviruses. Neutralization testing was conducted with RD cells, following the standard procedure for typing enteroviruses.

Positive control. The virus isolates of EV71 (TW/2272/98; GenBank accession no. AF119795) (33) and CA16 obtained from clinical specimens and confirmed by neutralization testing that were used as standard virus controls in this study were propagated individually in RD cells and cultured in MEM medium supplemented with 10% fetal bovine serum at 37°C.

For testing the specificity of probes, in addition to these two main serotypes, we have collected 11 serotypes of other enteroviruses derived from the clinical isolates, including EV68, CA10, CA24, CB1-6, ECV9, and ECV30. Except the first two, which were confirmed by RT-PCR and direct sequencing, the serotypes were verified by neutralization testing.

Standard viral RNA. For quantification of the viral RNA load in the samples, a standard RNA was generated by in vitro transcription from a DNA construct which contains the 5′ untranslated region (UTR). The in vitro-synthesized RNA was quantified by spectrophotometry, and the quantity was expressed as a relative copy number, determined by the equation [(μg of RNA/μl)/(molecular weight)] × Avogadro's number = viral copy number/μl (11, 28). To further validate the predicted concentration, we conducted real-time RT-PCR to determine the relative copy number of standard RNA. Based on the copy number of standard RNA, we then quantified the viral titer relative to that of this standard RNA by real-time RT-PCR analysis.

Viral RNA extraction. Viral RNA was isolated from 200 μl of viral transport medium or a suspension of culture medium using the QIAamp Viral RNA Mini kit (QIAGEN) according to the manufacturer's instructions and was eluted in 50 μl of RNase-free water. The eluted RNA was stored at −70°C until needed.

MRT-PCR. To reverse transcribe RNA to cDNA, 2 μl of extracted viral RNA, 1 μl of control RNA template (10^4 copies), and 10 pmol of each primer pair, including panEV-s/a, EV71-s/a, CA16-s/a, RTC-s/a, and NC-s/a (Table 1), were adjusted to a final solution of 10 μl. The mixture was incubated at 70°C for 5 min and snap-cooled on ice before being added to 10 μl of reaction mixture containing 4 μl of Moloney murine leukemia virus (M-MLV) reverse transcriptase reaction buffer (5×; Promega), 1 μl of deoxynucleoside triphosphate (10 mM each) mix, 0.5 μl of M-MLV reverse transcriptase (100 U; Promega), 0.5 μl of RNasin RNase inhibitor (20 U; Promega), and 4 μl of RNase-free water for reverse transcription at 42°C for 30 min.

PCR was conducted by using 20 μl of cDNA with 80 μl of reaction mixture containing 10 μl of PCR buffer [10×; 200 mM Tris-HCl (pH 8.8), 20 mM $MgSO_4$, 100 mM KCl, 100 mM $(NH_4)_2SO_4$, 1% Triton X-100, 1 mg of bovine serum albumin/ml], 1 μl of *Taq* DNA polymerase, 1 μl of deoxynucleoside triphosphate (10 mM each) mix, and 68 μl of distilled water under the following conditions: initial denaturation at 95°C for 2 min, followed by 40 cycles at 95°C for 15 s, 55°C for 15 s, and 72°C for 30 s, and a final extension at 72°C for 10 min. The amplicons were purified with the QIAquick PCR purification kit (QIAGEN) and eluted in 50 μl of distilled water.

To reduce the risk of carryover contamination, sample preparation and PCR amplification were performed in different rooms with separated air-conditioning, using different sets of the pipette system. To create a positive control that ensures that each MRT-PCR is working, we included a primer set (RTC-s/a) and the corresponding RNA template from the arabidopsis *GA4* gene for MRT-PCR. The specificity of the control PCR has been tested by using the DNA or RNA extracted from clinical samples or culture cell lines that were free of enterovirus infection.

Fluorescent dye labeling. To label cDNA targets with fluorescent dye for array hybridization, 50 μl of DNA products which were obtained by MRT-PCR amplification were added to 10 μl of the reverse primer mixture, including panEV-a, EV71-a, CA16-a, RTC-a, and NC-a (10 pmol for each primer, as shown in Table 1), denatured at 95°C for 3 min, and cooled on ice. Forty microliters of labeling reaction mixture (10 μl of Klenow fragment buffer [10×; Takara], 0.5 μl of Klenow fragment [2 U; Takara], 1 μl of dUTP mix [0.5 mM dTTP, 1 mM dATP,

TABLE 2. Degenerate probes used for EV microarray analysis

Probe[a]	Domain	Sequence (5′–3′)[b]	No. of degenerate sites[b]
pan-EV*	5′ UTR	TGTCGTAA**Y**G**S**GCAA**S**TC**Y**G**YR**GCGGAACCGACTACTTTGGGTGTCCGTGTTTC**M**TTTTA	7
EV71-1*	VP1	TCACC**Y**GCGAGCGC**Y**TA**Y**CA**R**TGGTTTTA**Y**GACGGGTA**Y**CCCAC**R**TT**Y**GGTGAACACAAA	8
EV71-2*	VP1	ATCTATT**Y**AAAGC**Y**AA**Y**CCAAATTATGC**Y**GG**Y**AATTCTAT**Y**AAACC**R**ACTGGTGC**Y**AGTC	8
CA16-1*	VP2	CTGA**R**TATGTGCTCGGC**W**CTATCGCAGGAGGGACCGGGAA**Y**GA**R**AATTCTCATCCTCCCT	4
CA16-2*	VP2	CC**M**TTTGA**Y**TCAGCTCT**Y**AACCACTGCAA**Y**TTTGGTCTACTGGTC**R**TCCCGGTAGTACCA	5

[a] Abbreviations: pan-EV*, degenerate pan-enterovirus probe; EV71-1* and EV71-2*, degenerate enterovirus 71 probes; CA16-1* and CA16-2*, degenerate coxsackievirus A16 probes.
[b] The degenerate sites in each probe are represented by characters in boldface type.

1 mM dCTP, and 1 mM dGTP], 0.5 μl of Cy5-dUTP [1 mM; Amersham Biosciences], and 28 μl of distilled water) was added to the solution and incubated at 37°C for 30 min. The labeled cDNA probes were purified with the QIAquick PCR purification kit (QIAGEN) and then dried in an Eppendorf Vacufuge concentrator.

Microarray printing. All synthesized probes (Tables 1 and 2) were resuspended in 50% dimethyl sulfoxide to a final concentration of 500 ng/μl and spotted onto Corning Ultra-GAPS slides in a triplicate format. The microarray slides were stored at room temperature in the dark until needed.

Hybridization and data analysis. Microarray slides were first blocked in prehybridization solution (25% formamide, 5× SSC, 0.1% sodium dodecyl sulfate [SDS], and 1% bovine serum albumin) at 42°C for 45 min. At the same time, the Cy5-labeled target cDNA was resolved in 6 μl of hybridization solution (25% formamide, 5× SSC, 0.1% SDS, 0.5 mg of oligonucleotide/ml, 0.5 mg of yeast tRNA/ml, and 0.5 mg of salmon sperm DNA/ml). After the sample was mixed with 1 μl of the Cy5-labeled hybridization control cDNA (10^4 copies), the solution was denatured at 95°C for 3 min and then cooled to room temperature. The heat-denatured target cDNA was hybridized to microarrays at 42°C for 1 h. The arrays were washed sequentially with a primary solution (2× SSC and 0.1% SDS) at 42°C for 10 min, a second solution (0.1× SSC and 0.1% SDS) at room temperature for 10 min, and a final solution (0.1× SSC) at room temperature for 10 s. The arrays were then immediately rinsed with 100% ethanol alcohol and dried with compressed air. The array signals were obtained using a GenePix 4000B scanner and analyzed using GenePix Pro software (Axon Instruments).

RESULTS

Enterovirus sequence database and bioinformatics analyses for the design of enterovirus diagnostic microarrays. To ensure the sensitivity of array-based detection of pathogens in clinical samples, appropriately designed probes and primers are essential. To optimally design unique oligonucleotide probes for the detection of specific serotypes of viruses, all probe candidates were put through four screening phases (phases I to IV). For a start, we created an EV71 genome sequence database which included all the available nucleotide sequences, including seven full-length EV71 genomes (AF119795, AF119796, AF136379, AF176044, AF304457, AF304458, and AF304459) and partial sequences from Taiwanese isolates at NCBI. Additionally, we included partial sequences of EV71 genomes which were sequenced in our labs in the database, including 74 sequences of the 5′ UTR, 43 sequences of VP4, 21 sequences of VP2, 7 sequences of VP3, 94 sequences of VP1, 17 sequences of 2A, and 7 sequences of 2B. We then performed multiple sequence alignment using the CLUSTAL W Multiple Sequence Alignment program (version 1.83) at EMBL-EBI (http://www.ebi.ac.uk/clustalw/) (36). For each aligned position, we computed an entropy according to the formula $\Sigma P_i \times \log(P_i)$, where i is the observed probability of each nucleotide, A, U, C, or G. For a probe length of 60 nucleotides, we summed the total entropy over all consecutive 60-nucleotide segments and ranked them according to the entropy sum. A probe with lower absolute entropy was considered to be better conserved than those with higher absolute entropy over this specific genomic segment among all EV71 strains. A number of candidates with lower entropy sums had been chosen first and were found to be highly conserved within the EV71 genome through the phase I screening. Then these candidates had to pass the phase II screening, at which time they were scanned through the human coding sequences (CDS) in our database, using BLAST version 2.2.5 (1). Any of the probes that survived the phase II screening and that had no more than 15 consecutive nucleotides identical to any of the human CDS were further searched against a collection of CDS from nonenterovirus pathogens through the phase III screening. Probes that had less than 15 consecutive bases identical to any of the nonenterovirus CDS were saved. Finally, the phase IV screening was performed. In this phase, the probes were compared with all available non-EV71 RNA sequences. Those with no more than 15 identical consecutive nucleotides were saved for laboratory validation. We used the same approach when designing CA16-specific probes. To reduce the difficulty of MRT-PCR, we chose two adjacent virus probes positioned within 500 bp of the genomic sequence, a position at which two target sequences were efficiently amplified by a single PCR primer pair. We also designed enterovirus-specific probe candidates to detect most enteroviruses other than EV71 and CA16.

For array hybridization and detection of virus during the early stage of infection, while the viral load in clinical specimens is still low, it is necessary to amplify the copy number of virus-specific sequences. For amplification of viral genes, specific primer sets for the enteroviruses, EV71, and the CA16 serotypes were designed, using Vector NTI suite 6.0. Common optimality criteria, like secondary structures, dimers, hairpins, melting temperatures, and free-energy changes (ΔG^o) between any two primers were taken into consideration when selecting an optimized primer set for MRT-PCR.

Screening and optimization of primers and probes for detection of EV71 and CA16. To select effective primer pairs from the predicted candidates, we tested each individual PCR primer pair in a separate reaction for each serotype. Standard viral RNAs of EV71 and CA16 were produced through in vitro transcription assay with their 5′ UTR fragments and adjusted to a tenfold serial dilution, ranging from 10^0 to 10^4 copies/μl, as templates for RT-PCR analysis. Quantification of all virus samples was based on these standard templates. Screening measures for each primer pair, for example, PCR specificity, efficiency, and yield, were used to screen for the competent

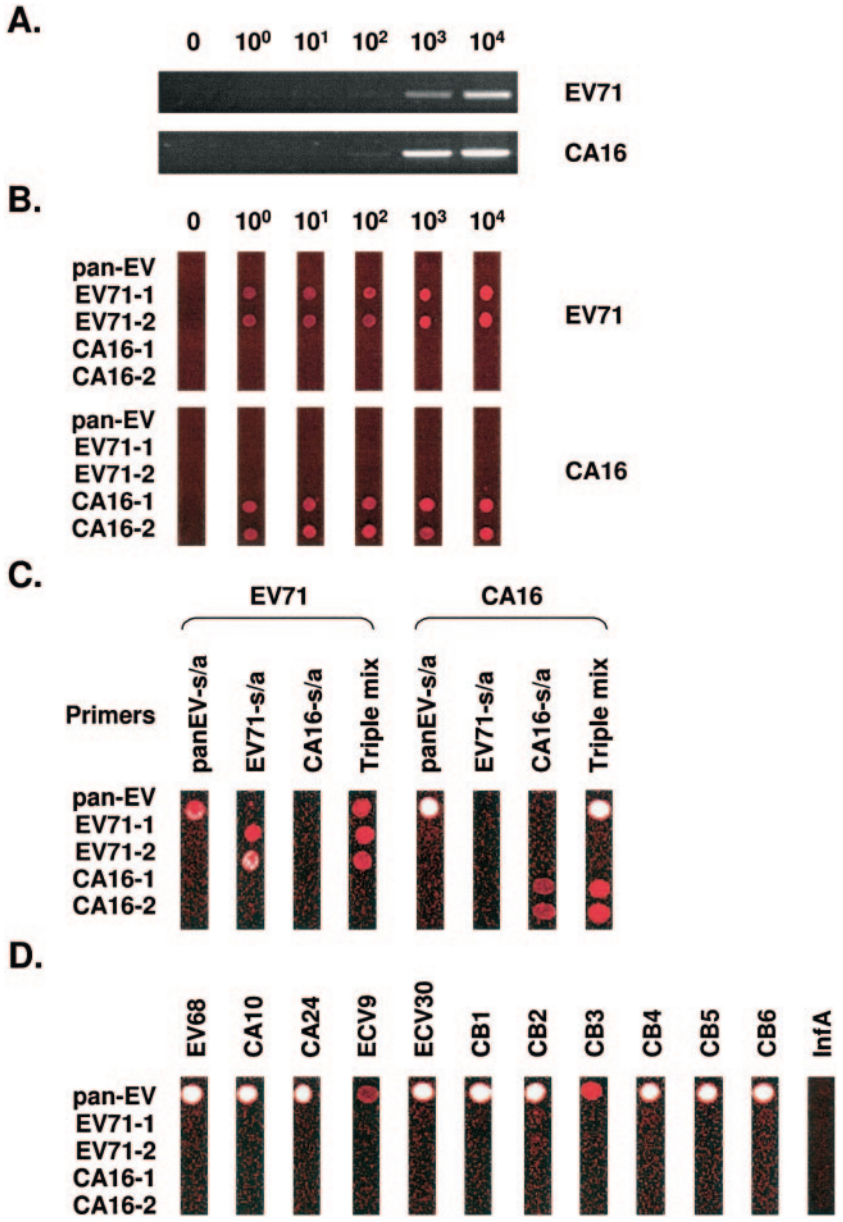

FIG. 1. Sensitivity and specificity of array probes. (A) Results of agarose gel electrophoresis of RT-PCR products of EV71 and CA16, using serial dilutions of enteroviruses as templates. The upper numbers ranging from 0 to 10^4 represent copies of viral RNA per PCR. (B) Cy5-labeled RT-PCR amplicons of EV71 and CA16 were detected by specific probes. (C) MRT-PCR products of EV71 and CA16 were hybridized to pan-EV, EV71, and CA16 probes on a gene chip. The target copy number for EV71 and CA16 was 10^4 copies per reaction. The triple mix contained three primer sets, including panEV-s/a, EV71-s/a, and CA16-s/a. (D) Array hybridization with MRT-PCR products of 11 other enteroviruses and influenza A virus (InfA).

primer pairs for EV71 and CA16 separately, before each pair was subjected to MRT-PCR assay. The final serotype-specific primer sets were selected and listed in Table 1, and their amplicons are shown in Fig. 1A. As assessed by agarose gel analysis, the sensitivities of the panEV-s/a primers for EV71 and CA16 were 10^2 and 10^1 copies, respectively. However, according to PCR/microarray analysis, the sensitivities of pan-EV primers and probes for EV71 and CA16 both fell into a single-digit range for virus level (data not shown). Similar results were also observed for the two virus-specific primers and probes used for detection of EV71 and CA16 (Fig. 1A and B). The array-based assay was generally able to detect a theoretical single copy of starting RNA, whereas gel electrophoresis was less efficient in detecting low titers of the virus, suggesting that the diagnostic microarray is efficient in detecting early or low titers of the enterovirus in specimens.

After the validated primers had passed the first-run screening, we tested their applicability for MRT-PCR assay. An appropriate combination of PCR primer pairs and titers would allow for more efficient and specific PCR amplification. Therefore, the final combination of primer pairs for MRT-PCR was repeatedly tested by mixing two serotype-specific primer pairs

(EV71-s/a and CA16-s/a) together with the panEV-s/a primers to determine the optimal conditions for the MRT-PCR assay. Because agarose gel electrophoresis is much less sensitive than array detection, the MRT-PCR products were directly labeled with Cy5-dUTP fluorescent dye for hybridization with the prototype array. We found that the MRT-PCR amplicons of both EV71 and CA16 could be specifically detected by their virus-specific probes with no cross hybridization to other probes noted (Fig. 1C), suggesting that serotype-specific primers and probes provide accurate detection.

To further assess the analytical specificity of the assay, RNAs from 11 enterovirus serotypes from the clinical isolates, including EV68, CA10, CA24, CB1-6, ECV9, and ECV30, were tested by MRT-PCR, using primer pairs panEV-s/a, EV71-s/a, and CA16-s/a (Fig. 1D). The amplicons were then Cy5 labeled and hybridized to the array. As shown in Fig. 1D, the pan-EV probe effectively detected all of the 11 serotypes but none of the nonenteroviruses, e.g., dengue virus type 2, influenza A virus, rhinoviruses 2 and 14, and SARS-CoV. Moreover, since rhinoviruses also belong to the family of *Picornaviridae*, several previous studies have shown that cross recognition of rhinoviruses might occur in the diagnosis of enteroviruses by RT-PCR (12, 26, 37). To test whether pan-EV primers and probes also recognize rhinoviruses, we first conducted sequence alignment analysis between rhinoviruses and pan-EV primers. The result revealed that at least six mismatch sites were present in the rhinoviruses (data not shown). In agreement with this, both RT-PCR and array assays also detected no specific signal by the pan-EV primers and probes (data not shown), suggesting that the common 5′-UTR region of enteroviruses could be specifically detected by this array-based method. This screening allowed us to choose three virus-specific primer sets and five virus-specific probes for the detection of EV71, CA16, and other enteroviruses.

Application of degenerate probes for detection of EV71 and CA16. However, the existing target sequence in the clinical isolates might evolve over time (6, 34), and thus the existing target sequence in the clinical specimens might not be in agreement with that in the database. To reduce the possibility of inconsistent clinical diagnosis, we sequenced 59 clinical isolates bidirectionally from primer binding sites that were amplified by primer set panEV-s/a, 12 by primer set EV71-s/a, and 19 by primer set CA16-s/a. Comparing the sequenced information with the probe sequences, we identified mismatches in the current probe sequences (data not shown). We then redesigned each array probe by replacing the mismatched nucleotides with degenerate nucleotides. The number of degenerate nucleotides in a single probe (denoted by an asterisk [*]), however, was limited to eight sites to reduce the chance of cross hybridization with nonspecific targets. The degenerate probes used in the final diagnostic microarray are listed in Table 2, in which the degenerate sites in each probe are represented by characters in boldface type. In addition to the aforementioned virus-specific probes, three control probes were also designed for RT-PCR (RTC), hybridization (HC), and negative-control (NC) assays (Table 1). In particular, the negative-control assay was designed to rule out the possibility of a false-positive result, which may result from nonspecific PCR and/or hybridization in the array-based assay.

These degenerate probes were spotted onto microarray slides in a triplicate format. To evaluate the efficacy of these probes, target sequences were amplified by RT-PCR with their specific primer pairs and subsequently labeled by Cy5-dUTP priming with their anti-sense primers for array hybridization. The results of the array suggest that the virus-specific amplicons were specifically detected by pan-EV*, EV71*, and CA16* degenerate probes without nonspecific cross hybridization to the control probes (Fig. 2A). The specificity of the three control probes, namely RTC, HC, and NC, for RT-PCR, hybridization, and negative controls, respectively, was also validated by hybridization with PCR products amplified by specific primers (Fig. 2B). Finally, we used clinical specimens to test

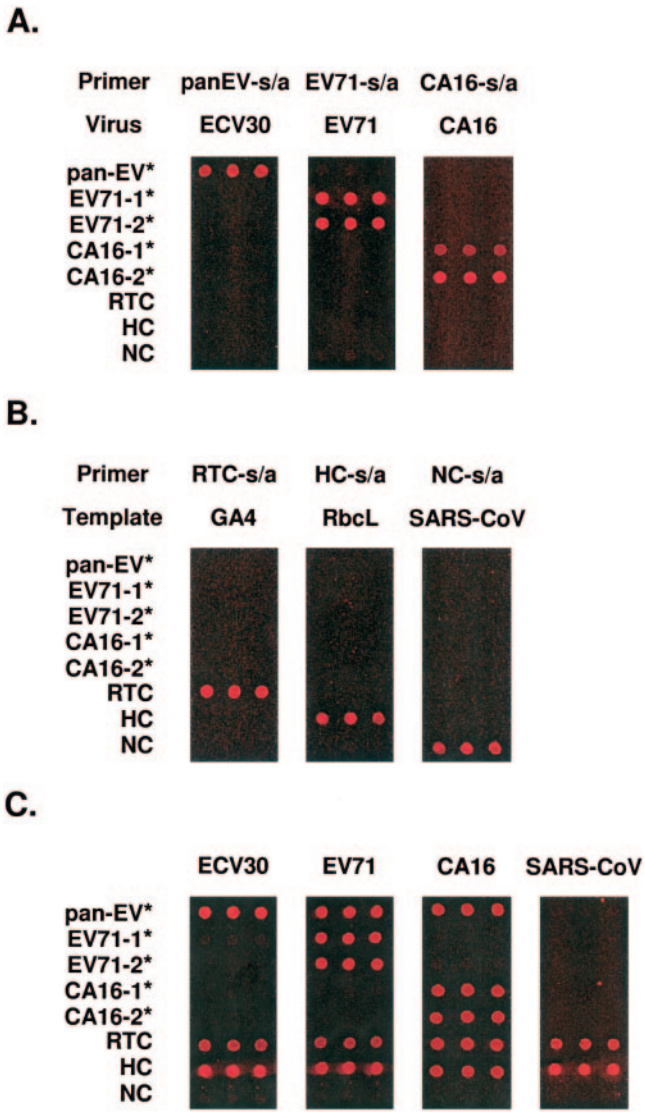

FIG. 2. Combining MRT-PCR and microarray for clinical detection of EV71 and CA16 in specimens. (A) The hybridization specificity of degenerate probes was validated by ECV30, EV71, and CA16 specimens. (B) Control assays were performed by using *GA4*, *RbcL*, and SARS-CoV RNAs for RT-PCR, hybridization, and negative-control experiments, respectively. (C) A diagnostic hybridization assay for detecting pan-enteroviruses, EV71, and CA16. The entire array detection procedure was confirmed by the positive (RTC and HC)- and negative (NC)-control probes.

TABLE 3. Results of 144 diagnoses from HFMD-related specimens by array-based assay

Serotype[a]	No. of samples	No. of positive diagnoses with indicated probe[b]				Detection rate (%)
		pan-EV*	EV71*	CA16*	Control[c]	
Enterovirus 71	25	24	23		1	92.0 (23/25)
Coxsackievirus A16	24	23		23	1	95.8 (23/24)
Coxsackievirus A9	2	2				100.0 (2/2)
Coxsackievirus A24	1	1				100.0 (1/1)
Coxsackievirus B4	1				1	0 (0/1)
Coxsackievirus B5	1				1	0 (0/1)
Echovirus 4	4	4				100.0 (4/4)
Echovirus 6	5	5				100.0 (5/5)
Echovirus 9	1	1				100.0 (1/1)
Echovirus 11	2	2				100.0 (2/2)
Echovirus 30	5	5				100.0 (5/5)
Poliovirus 2	2	2				100.0 (2/2)
Poliovirus 3	1				1	0 (0/1)
Other enteroviruses	49	46			3	93.9 (46/49)
EV-negative specimens	21				21	100.0 (21/21)
Total	144	115	23	23	29	93.8 (135/144)

[a] The serotypes were determined by neutralization testing.
[b] Abbreviations for probes: pan-EV*, degenerate pan-enterovirus probe; EV71*, degenerate enterovirus 71 probes (EV71-1* and EV71-2*); CA16*, degenerate coxsackievirus A16 probes (CA16-1* and CA16-2*).
[c] Two control probes, RTC and HC, were used. Note that some serotypes were detected only by the control probes.

the array. Three virus-specific primer sets, one RT-PCR primer control set, and one NC control primer set were mixed for MRT-PCR. The Cy5-labeled HC was used as a hybridization control, which was added into each MRT-PCR product for microarray analysis. As shown in Fig. 2C, ECV30 was detected only by the pan-EV* probe, not by the EV71* or CA16* probes (left panel). EV71 and CA16 were detected by their specific degenerate probes (two middle panels). In contrast, for the SARS-CoV specimen, while we did not detect a hybridization signal for the pan-EV*, EV71*, or CA16* probe, we did observe signals for the RTC and HC control probes. Together, these array results suggest that the enterovirus diagnostic microarray was able to detect EV71 and CA16 in clinical specimens.

The diagnostic results for 144 clinical specimens. To assess the diagnostic accuracy for clinical samples, we obtained a total of 144 clinical specimens for the array-based assay. In parallel, all of these specimens were analyzed by real-time PCR and subsequently confirmed by neutralization testing as the gold standard for diagnosis. As shown in Table 3, the sensitivity of the array was 92.0% for EV71 and 95.8% for CA16. Two clinical EV71 samples were not correctly detected by the EV71-specific probes, though one of these two was successfully identified by the pan-EV* probe. Since both the RTC and HC control probes in the array displayed clear signals, we concluded that the negative results were not due to the failure of reverse transcription or hybridization procedures. Only one of the CA16 specimens was not detected by virus-specific probes. For those missed specimens, we conducted a real-time RT-PCR assay with another set of specific primers to confirm the results (data not shown). The sensitivity of the assay for non-EV71, -CA16 enteroviruses was 91.9% (68/74). Overall, the array-based diagnostic sensitivity was 93.8% for the 144 clinical specimens.

DISCUSSION

Because EV71 infection may cause fatal brainstem encephalitis, neurogenic shock, and neurogenic pulmonary edema (22, 39), early diagnosis from clinical specimens allows for the selection of therapeutic strategies aimed to prevent such adverse outcomes. We combined the advantages of MRT-PCR with microarray analysis to develop a means of sensitively and accurately detecting enterovirus serotypes in clinical samples so that the time-consuming cell culture amplification of enterovirus might be circumvented. Synthesized DNA probes for microarray analysis range from 20 to 70 nucleotides, and long oligonucleotide probes display higher sensitivity in microarray hybridization than short ones (2, 19). Even so, because the genetic heterogeneity of pathogenic viruses makes it difficult to uncover a conserved continuous, long genomic region for the purpose of designing long oligonucleotide probes, short oligonucleotide probes are more often used in diagnostic microarrays (19). We used serotype-specific oligonucleotide probes carrying polymorphic sites to overcome the problem of genetic heterogeneity in the same serotype. Using 144 clinical samples from patients suspected of having HFMD, we were able to achieve 93.8% diagnostic accuracy with this microarray. High-throughput identification of serotypes requires a high analytic efficiency. The array-based method we developed was found to be both relatively undemanding and efficient. The entire assay processes could be completed within 6 h, starting from RNA preparation through final array image analysis, thereby making it an applicable alternative for screening of enterovirus in the laboratory.

Nevertheless, the most positive feature of this diagnostic microarray is its capacity and flexibility in implementing many virus-specific probes onto a diagnostic array to allow for the simultaneous detection of multiple pathogens with the same trace amount of sample in a single experiment. These advan-

tages would make such a diagnostic tool very useful in the early determination of numerous pathogens present in a clinical specimen. However, probe design requires elaborate bioinformatic analysis of sequence information derived from various strains or serotypes. The accessibility of genome sequence information is, therefore, a major restricting factor for the design of suitable pathogen-specific probes. For those pathologically common but less-sequenced serotypes, such as CA10, ECV6, EV68, and EV70, the limited sequence information is rarely sufficient for meaningful bioinformatic analysis of probe design compared to that of the more frequently sequenced ones, e.g., CA9, CA23, CB1-6, ECV30, and EV71. Large-scale, genome-wide sequencing of a variety of strains for those less-sequenced serotypes may be a straightforward way to resolve this issue, though it would be very costly and time-consuming. For a more practical approach, sequencing efforts may be aimed only at the conserved genomic regions, such as VP1, VP2, and VP4 (3, 6, 9, 21, 23, 38). This would reduce the need for sequencing work and facilitate the procedure. Based on the findings of this study, we believe that a PCR/microarray hybridization assay capable of detecting a number of enterovirus serotypes and confirming EV71 and CA16 infections can be built.

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