

Utility of RT-PCR-based Dot-blot Hybridization for Detecting and Genotyping Echoviruses

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We attempted to detect and identify virus types quickly by improving an RT-PCR-based dot-blot hybridization test for echoviruses, important human pathogens mainly causing aseptic meningitis. This test was applied to reference viruses of seven echovirus serotypes prevalent in Korea (E6, 7, 9, 11, 13, 25, and 30) and seventy isolates of echovirus isolated in Korea between 2002 and 2004. The primers for target DNA and hybridization probes (25mer, 50mer, and 70mer) were designed within the VP1 region of the echovirus. In RT-PCR, a nonradioactive digoxigenin-DNA labeling mix was added instead of dNTP to initiate PCR. The PCR product was then hybridized against 25mer, 50mer, and 70mer probe DNA spotted on nylon membranes and the reaction was observed. To investigate the optimal conditions for hybridization, various concentrations of target DNA (0.1, 1, 10, and 100 ng/μl), size of probe DNA (25mer, 50mer, and 70mer), concentrations of probe DNA (10~50 pM), and reaction time were included. In the test zone, the optimal condition in terms of time and cost was a reaction time of 1 h with 10 ng/μl target DNA concentration and 10 pM of a 50mer probe. We found 100% diagnosis of the serotypes for seven reference echoviruses and 90% (63/70) sensitivity for clinical isolates. Also, tests with this probe for reactivity with seven reference echoviruses by using DNA chips showed that diagnostic identification was possible without other serotype cross-reactivity. Therefore, efficiency analysis of probe and target DNA on clinical specimens by using dot-blot analysis indicated that this system can be applied to the prestages of the DNA chip and that the dot blot analysis itself can be used in applications to develop a tool for diagnosing specific viral serotypes.

Key Words: Echovirus; DNA probe; RT-PCR; Dot-blot hybridization

INTRODUCTION

Echoviruses belong to the *enterovirus* genus of the *Picornaviridae* family. More than 70 serotypes of enteroviruses have been identified, and among them, echoviruses accounts for 28 serotypes. Major clinical symptoms caused by echo-

viruses range from subclinical infections and colds to fatal conditions such as aseptic meningitis and encephalitis (11). Serotype identification is very important in analyzing pathogens that may cause outbreaks, monitoring the prevalence of enteroviruses, and predicting and confirming diseases. For some enteroviruses, each viral serotype is connected to a specific disease (2,4,6,9,17). For diseases caused by enteroviruses, developing convenient methods for serotype identification will allow researchers to study the relationship between a particular enterovirus, its serotypes, specific symptoms, and the severity of disease.

The serotype analysis used currently is a cell culture and neutralization test that generally requires 2~3 weeks (7,8,

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12), and the results of virus detection tests do not provide appropriate immediate assistance in treating patients. Among the genetic analysis of viral diagnosis, DNA chip technology provides prompt diagnosis of a variety of viruses, but this method is very costly. In contrast, the dot-blot method can detect serotypes simply and quickly, and can reduce the cost.

This study aimed to develop an RT-PCR-based dot-blot hybridization method to diagnose viruses and to identify virus types in the shortest time. To diagnose echoviruses, we used reference strains of the seven common major serotypes isolated in Korea (echovirus 6, 7, 9, 11, 13, 25, and 30) (5). We also used 70 echovirus isolates by detecting the sensitivity level for the potential of this method as a diagnostic tool. To determine optimal conditions for hybridization, we varied the required conditions, such as the length and concentration of the probe, and the temperature and duration of the hybridization reaction. By evaluating the sensitivity and specificity of this method and comparing it with clinical isolates of echoviruses, we examined the applicability of this dot-blot method for diagnosis and a screening test of DNA chips.

MATERIALS AND METHODS

1. Viral strains

The reference strains of echovirus 6 (strain D'Amori), 7 (strain Wallace), 9 (strain Barty), 11 (strain Gregory), 13 (strain Del Carmen), 25 (strain JV-4), and 30 (strain Bastianni) were obtained from The American Type Culture Collection (ATCC). Reference viruses were propagated in human rhabdomyosarcoma (RD) cells in Dulbecco essential medium (DMEM) supplemented with 10% fetal bovine serum at 37°C in 5% CO₂. Viral stocks were stored at -70°C.

2. Clinical specimens

Seventy echovirus-positive stool samples obtained from patients with aseptic meningitis in Korea from 2002 to 2004 were used. All the samples were tested for viral isolation and identification. The crude stool extracts were inoculated in RD monolayer cultures and screened for the presence of echoviruses using a neutralization assay. To

determine the sensitivity of the dot-blot assay, 70 strains representing the seven different echovirus serotypes were used (twenty-five of echovirus 6, nine of echovirus 7, five of echovirus 9, three of echovirus 11, eight of echovirus 13, five of echovirus 25, and fifteen of echovirus 30).

3. Extraction of viral RNA

Viral RNA was extracted from a 10% stool suspension with TRIzol reagent (Gibco, BRL, Grand Island, NY, USA) according to the manufacturer's instructions. After precipitation with isopropanol for 10 min at room temperature, the RNA pellet was washed with 75% ethanol, air dried, and dissolved in 20 µl of diethyl pyrocarbonate (DEPC, Sigma Chemical Co., St. Louis, MO, USA)-treated water. RNA was either used directly in the RT-PCR or stored at -70°C.

4. Detection of echoviruses by RT-PCR

First-strand synthesis was carried out on the viral RNA using SuperScript II (Invitrogen, Carlsbad, CA, USA) and type-common reverse primer (222R, 5'-ATTGTCACCAT-AAGCAG CCA-3'). The reaction mixtures contained 5 µl of 5× first-strand buffer (250 mM Tris-HCl, pH 8.3, 375 mM KCl, and 250 mM MgCl₂), 2 µl of 0.1 M dithiothreitol, 1 µl of 10 pM of 222R primer, 1 µl of 25 mM deoxynucleoside triphosphates, viral RNA, and DEPC-treated water to yield a final volume of 25 µl. The mixtures were denatured at 95°C for 2 min and cooled on ice for 1 min, and 1 µl of RNase inhibitor and 1 µl of SuperScript II reverse transcriptase were added. The tubes were incubated for 1 h at 42°C.

The amplification was carried out in a 50 µl reaction mixture containing 1.25 mM MgCl₂, 1× PCR buffer, 0.2 mM nonradioactive digoxigenin (DIG)-DNA labeling mix (Roche Applied Science, Mannheim, Germany), 1 µM of reverse primer (222R) and forward primer (292F, 5'-CCC-TGAATGCGGC TAATCC-3'), and 2.5 U of Taq DNA polymerase. The reaction was run in a thermocycler and under the following conditions: 94°C for 3 min, 94°C for 30 s and 42°C for 30 s, 35 cycles of 68°C for 30 s, and a final extension step at 72°C for 10 min. The PCR products

were analyzed on 1% agarose gels. Samples were purified using a Bioneer PCR purification kit (Bioneer, Daejeon, Korea) for dot-blot hybridization.

5. Design of genotype-specific oligonucleotide

The RT-PCR was carried out using the primers mentioned above and amplified a 373 base pair (bp) region from the VP1 gene of the echoviruses. To facilitate the design of a type-specific probe for each of the seven echoviruses, the nucleotide sequences of the VP1 region of the known echoviruses were aligned. The probes were located in a variable region in the VP1 region, and the probe sizes were 22~34mers, 50~52mers, and 68~70mers (Table 1).

6. Dot blot hybridization

Probes were transferred to the nylon membrane, and the membrane was air dried and UV cross-linked for 1 min at 120,000 $\mu\text{J}/\text{cm}^2$ to immobilize the probes. The nylon membrane was prehybridized with freshly prepared hybridization solution ($2\times$ SSC containing 0.1% SDS) at 50°C for 30 min. The target DIG-labeled PCR product was denatured at 99°C for 10 min, and chilled on ice for 5 min before the addition of the DIG-labeled target (1 $\mu\text{l}/\text{ml}$) in the hybridization solution. Hybridization was performed at 50°C for 1~12 h. The membrane was washed twice in $2\times$ SSC and 0.1% SDS for 5 min at room temperature and twice in $0.1\times$ SSC and 0.1% SDS for 15 min at 42°C. The DIG-labeled hybrids were detected by enzyme-linked immunosorbent assay using anti-DIG Fab fragments conjugated to alkaline phosphatase. After hybridization, the DIG-DNA probes were revealed using an enzyme-catalyzed color reaction with 5-bromo, 4-chloro, 3-indolyl phosphate and nitroblue tetrazolium (Roche Diagnostics GmbH, Mannheim, Germany), producing a purple precipitate.

7. Determination of the optimum probe and target DNA concentration and hybridization time for the dot-blot assay

The oligonucleotide probes were serially diluted to determine the optimal concentrations for the method; the concentrations of the specific probes were 10^{-3} , 10^{-2} , 10^{-1} , 1, and

10 $\text{pM}/\mu\text{l}$ for 70mer and 10, 25, 50, 100, and 500 $\text{pM}/\mu\text{l}$ for 25mer. These were applied on the same membrane strip to determine the optimal probe concentration. The dot-blot experiments were also carried out using different hybridization times (1, 2, 5, and 12 h) to determine the optimal hybridization time.

8. Specificity for the detection of amplified PCR

The specificity of detection of each echovirus of the type-specific probes was tested. DIG-labeled PCR products of the reference and clinical echoviruses were tested by dot-blot hybridization with the oligonucleotide probe of each echovirus.

9. DNA chip hybridization

For DNA chip hybridization, the same oligonucleotide probe (70mer) was used as in the dot-blot hybridization. The oligonucleotide probe was spotted on the GeneSlide (Tokyo Kohan Co. Ltd., Tokyo, Japan) using an OmiGrid 100 microarray spotter. The RT-PCR for the microarray samples was performed as described above in section 2.4, except that this method used the Cy5-labeling reaction (Amersham Biosciences, Uppsala, Sweden). Cy5-labeled DNA was mixed with hybridization buffer (7.5 μl of $20\times$ SSC, 0.5 μl of 10% SDS, 2.5 μl of $50\times$ Denhardt's solution, and 2.5 μl of $10\times$ human Cot1 genomic DNA) and then incubated at 95°C for 5 min. Hybridization was carried out on a slide at 60°C for 14~16 h at high humidity. Arrays were washed by immersion into $2\times$ SSC~0.2% SDS twice for 10 min and $0.05\times$ SSC twice for 5 min, and dried by centrifugation (650 rpm, 2 min). Slides were scanned on an Agilent microarray scanner to detect Cy5 fluorescence.

RESULTS

1. Evaluation of the effectiveness of the type-specific probe

Specificity test was conducted to assess whether reference strains of echoviruses could react specifically with each probe. For the probe DNA, 10 pM each of 70mer E6, E7, E9, E11, E13, E25, or E30 was used. For the target DNA,

Table 1. Type-specific 70mer, 50mer, & 25mer oligonucleotides of echoviruses

Target specificity	Nucleotide sequence	Length	GenBank accession no.
Echo6	AAACCCAGGAYACGACTCCCGACA	24	AF081321
	GACACGACTCCCGACAAGATGATTGATAGCTGGATTATCAATACCAAACA	50	
	CAAAACCCGGGACACGACTCCCGACAAGATGTATGATAGCTGGATTATCAATACCAAACAAGTGGCGCAG	70	
Echo7	CTACACCAAGGACCAAGACAATGTTAAT	28	AY302559
	CTACACCAAGGACCAAGACAATGTTAATAGGTACATGTCGTGGACAATAA	50	
	CTACACCAAGGACCAAGACAATGTTAATAGGTACATGTCGTGGACAATAAATGCCAGAAGAATGGTGCAA	70	
Echo9	GGTAGACCCTGAGAGCACAGATCGC	25	X92886
	GGTGACCCTGAGAGCACAGATCGCTTTGATGCATGGGAGATAAGCGTGCG	50	
	TGAAGCAAGGGGTGACCCTGAGAGCACAGATCGCTTTGATGCATGGGAGATAAGCGTGCGTGACATGGTT	70	
Echo11	TTAYATGGGAGAATACCACACAACCAAC	28	AF295498
	CCACACAACCAACACTGACCAGACAAAATTATTTGCCTCATGGACTATTA	52	
	GCCTCATGGACTATTAGTGACGACGCATGGTTCAAATGAGACGCAAGCTAGAGATCTTCACTTACGTCC	68	
Echo13	GCAGATCGCYAAGTACGCTAGT	22	AY302539
	TGACACTCACGGGGATGCAGCCGACGCAAAGTACGCCAGTTGGACGATAA	50	
	TGACACTCACGGGGATGCAGCCGACGCAAAGTACGCCAGTTGGACGATAACCACCCGAAAAGCTGCACAG	69	
Echo25	ACGTGTATGGGACAAAAGAGAATGGTGACATCAA	34	AY302549
	TGGGACAAAAGAGAATGGTGACATCAAGCGCTTCACCAACTGGAGAATAA	51	
	TGGGACAAAAGAGAATGGTGACATCAAGCGCTTCACCAACTGGAGAATAAACACACGTCAGGTCGTGCA	69	
Echo30	CAGTAYGCIACAGAGAAGGCTAA	23	AF081340
	TGCCACAGAAAAGGCTAATGATGATTTGGACAGATACACTAACTGGGAGA	50	
	TGCCACAGAAAAGGCTAATGATGATTTGGACAGATACACTAACTGGGAGATCACAACTAGGCAGGTGGCA	69	

the PCR product from each of the seven echoviruses was used (Fig. 1). The hybridization was performed for 1 h at 50°C. Specific probes of each virus serotype reacted only with the corresponding serotype, and no cross-reactivity was observed between serotypes (Fig. 2). E6 exhibited low sensitivity because a spot was observed only in the membrane. The echovirus did not react with a coxsackievirus B1 probe, and coxsackievirus B1 DNA did not react with the echovirus probe, indicating specificity (data not shown).

2. Determination of optimal conditions for dot-blot hybridization

We used probes of 25mer and 70mer to evaluate the effect of probe length. For target DNA, the standard virus E11 probe (100 ng/μl) was used. As shown in Fig. 3A, a reaction was evident up to 10 pM for the 25mer probe. For

the 70mer probe, a coloring reaction, although vague, was observed up to 10^{-3} pM. At a given concentration, the sensitivity was greater in the 70mer probe than in the 25mer probe. At the different concentrations of E11 probe (70mer), the target DNA (100 ng/ml) of echovirus 11 was diluted by 10^{-1} at the start of hybridization (Fig. 3B). In the target DNA, a reaction was observed up to 1 ng/μl. With echo 11 (10 ng/μl) as the target DNA, the probe concentration of 25mer, 50mer, and 70mer was 50 pM, 10 pM, and 1 pM, respectively (Fig. 3C). Varying the hybridization time from 1 h to 12 h had no significant effect on the reactions. These data show that the reactivity greater than average was obtained with minimum cost and time by using a target DNA concentration of 10 ng/μl and 10 pM of the 50mer probe with a reaction time of 1 h.

3. Specificity and sensitivity when used with clinical specimens

To evaluate the sensitivity of the dot-blot analysis for viral identification and typing, clinical isolates of 70 echovirus positive specimens were used as test targets. The specific probe of each virus serotype reacted only with its corresponding isolate serotype (Table 2). The 70mer probe detected 100% of echoviruses 9, 11, 13, and 25 among the clinical isolates. In contrast, the sensitivity levels were 80% for echovirus 6 isolates, 89% for echovirus 7 isolates, and

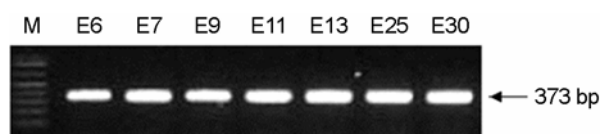


Figure 1. Amplification of seven echovirus reference strains by VP1 RT-PCR assays. Lane 1, 1 kb plus DNA Ladder (Invitrogen, Carlsbad, CA, USA); lane 2, echovirus 6; lane 3, echovirus 7; lane 4, echovirus 9; lane 5, echovirus 11; lane 6, echovirus 13; lane 7, echovirus 25; lane 8, echovirus 30. The primers 292F and 222R were used for PCR, and 2.5 μl of each PCR product was analyzed by electrophoresis on a 1% agarose gel. The 373 bp RT-PCR products are indicated by the arrow.

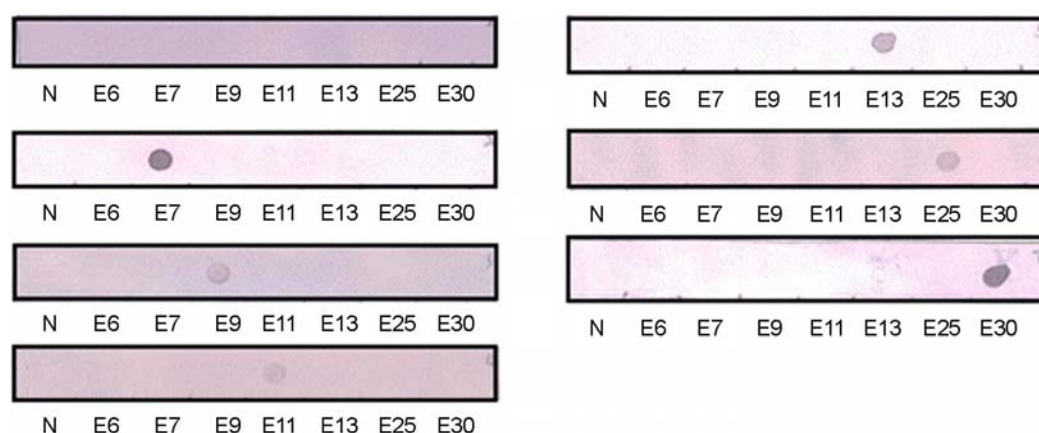


Figure 2. Specificity of echovirus species-specific probes by dot-blot hybridization. For the probe DNA, 10 pM each of 70mer from E6, E7, E9, E11, E13, E25, and E30 was spotted on nylon membranes. Hybridization was performed with the PCR fragments of each virus amplified by 292F and 222R primers. Dot 1, dH₂O; dot 2, echovirus 6; dot 3, echovirus 7; dot 4, echovirus 9; dot 5, echovirus 11; dot 6, echovirus 13; dot 7, echovirus 25; dot 8, echovirus 30.

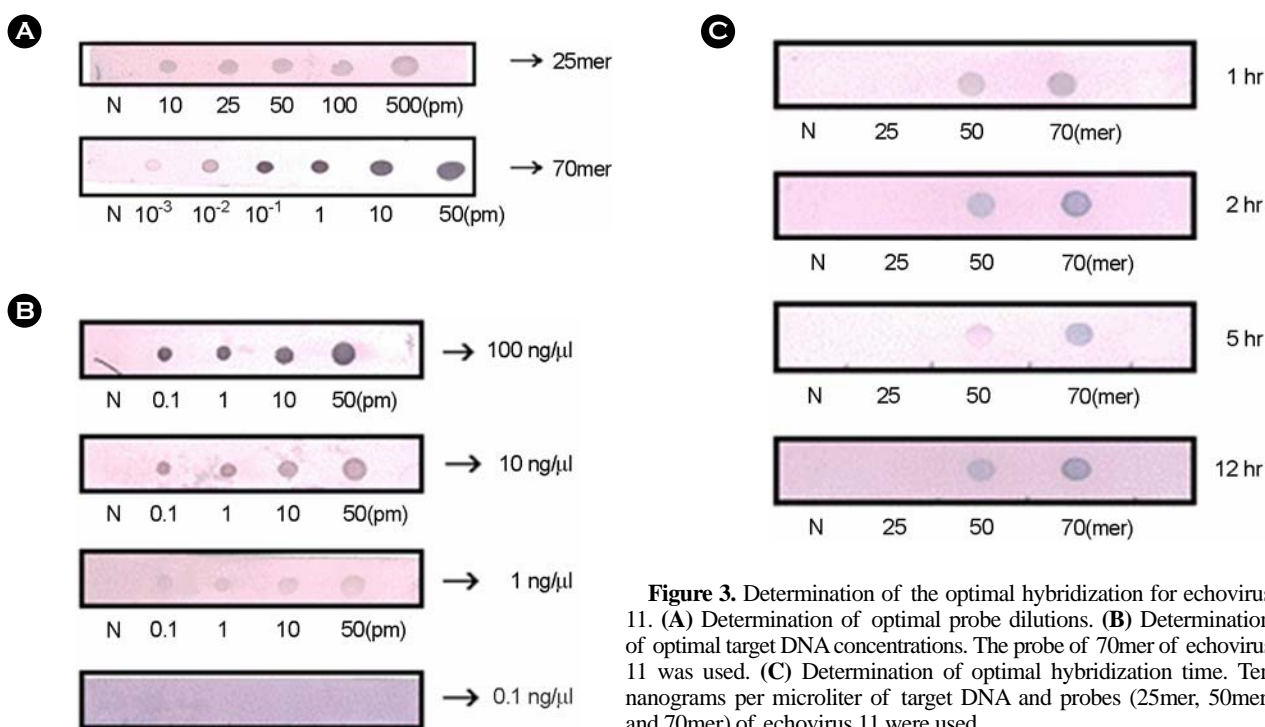


Figure 3. Determination of the optimal hybridization for echovirus 11. (A) Determination of optimal probe dilutions. (B) Determination of optimal target DNA concentrations. The probe of 70mer of echovirus 11 was used. (C) Determination of optimal hybridization time. Ten nanograms per microliter of target DNA and probes (25mer, 50mer, and 70mer) of echovirus 11 were used.

Table 2. Virus detection in clinical samples in Korea during 2002 ~2004

	70mer	50mer
Echo6	20/25	19/25
Echo7	8/9	8/9
Echo9	5/5	5/5
Echo11	3/3	3/3
Echo13	8/8	8/8
Echo25	5/5	4/5
Echo30	14/15	14/15

93% for echovirus 30 isolates. Serotypes of the 70 clinical isolates were determined from the results of the neutralization test. The results of dot-blot analysis using the 70mer probe indicated that 63 of the 70 clinical isolates reacted only with its corresponding serotype and that the remaining seven did not display any reaction at all, giving a sensitivity of 90%. For each serotype, the sensitivity was about 80~100%, but for echoviruses 9, 11, and 13, both the 50mer and 70mer probes detected 100%. The lowest detection rates in our experiments were observed for the 70mer (80% or 20/25) and 50mer (76% or 19/25) probes for echovirus 6.

Interestingly, none of the 70 specimens displayed cross-reactivity in our dot-blot test. None of the coxsackievirus serotypes (B1, B3, and B5), which belong to the enterovirus family, reacted with the echovirus probe.

4. Echovirus type identification by DNA chip

Results of the DNA chip test using standard echoviruses are shown in Fig. 4. All seven echoviruses produced positive results with the corresponding probe, and none displayed any cross-reactivity with other serotype. The reproducibility was confirmed after repeating the echovirus identification test three times with DNA chips using standard viruses.

DISCUSSION

Among over 70 different serotypes of enteroviruses, including polioviruses, coxsackieviruses, echoviruses, and other new enterovirus types, echoviruses show the most varied serotypes (1~9, 11~27, 29~34). The enterovirus genome structure is a single-stranded positive-sense RNA virus, having about 7,500 nucleotides, that codes a 5'-noncoding region, structural proteins (VP1, VP2, VP3, and

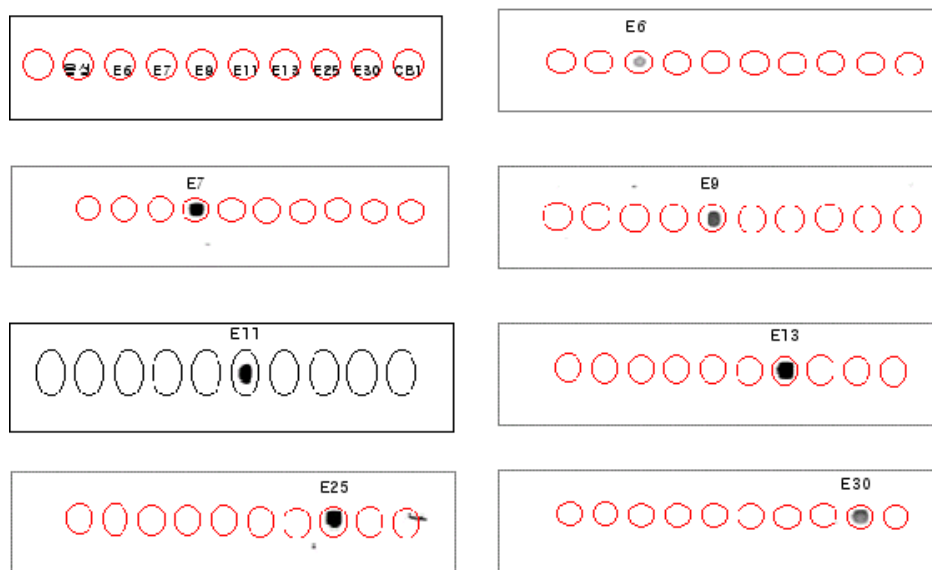


Figure 4. Detection of echoviruses by DNA chip. Microarrays were constructed that included oligonucleotide probes to detect echovirus 6, 7, 9, 11, 13, 25, and 30 DNA. Dot 1 & 2, dH₂O; dot 2, echovirus 6; dot 3, echovirus 7; dot 4, echovirus 9; dot 5, echovirus 11; dot 6, echovirus 13; dot 7, echovirus 25; dot 8, echovirus 30; dot 9, coxsackievirus B1.

VP4), nonstructural proteins (2A, 2B, 2C, 3A, 3C, and 3D), a 3'-noncoding region, and polyadenylation. The VP1 region is known as the coding region of the neutralizing antigenic motif (10,13,14).

The serotyping and sequence data are useful for monitoring outbreaks and for studying the molecular epidemiology of outbreaks. However, the neutralization test to differentiate serotypes is very time consuming and labor intensive. Therefore, we attempted to diagnose viruses by using RT-PCR-based dot-blot hybridization test. From thorough review of sequencing data (1,3,15,16), we were able to design the simple RT-PCR and dot-blot test to differentiate echovirus serotypes.

The serotype identification testing by dot-blot and neutralization antibody tests using 70 clinical specimens indicated that none of the 70 specimens displayed cross-reactivity between serotypes, suggesting the potential usefulness of serotype identification by RT-PCR followed by dot-blot analysis. However, seven (10%) of the 70 specimens did not produce any reaction at all, and five (20%) of the 25 specimens of echovirus 6 did not produce any reaction, giving a low overall sensitivity level of 80%.

This result may be explained by the following reasons. First, the PCR product and hybridization rate can differ

quantitatively because of diversity in the nucleotide sequences of clinical specimens. Investigating the nucleotide sequences of 26 specimens of the 70 clinical specimens indicated a similarity of 79.3~100% with each probe (data not shown). The nucleotide homology of five clinical echovirus 6 samples was 79.3% with the 70mer echovirus 6 probe showing the lowest identity. Second, in the case of echovirus 6, the reactive signal was comparatively very weak in the tests for both clinical isolates and standard viruses. We assume that the hybridization rate for the PCR product used to detect echovirus 6 created in this study was lower than that of the other serotypes.

The efficiency of this dot-blot method was verified using screening test for the development of DNA chips in this study. Our results proved the effectiveness of the dot-blot method for estimating the reaction of chips. Because the chips are so expensive to manufacture, dot-blot tests may be a good alternative in preliminary studies.

By applying the probe created in this study to BLAST program, we found no matches through all the sequences currently listed in GenBank except for each of the corresponding serotypes of echovirus. In addition, in the 70 clinical specimens found to be echoviruses that we tested, no cross-reactivity was displayed between serotypes. However,

further tests are required on potential cross-reactions with other enteroviruses or serotypes in addition to the echoviruses investigated, because this study has conducted only on seven echovirus specimens. Although we found no cross-reaction with coxsackievirus B1, additional testing of other echovirus serotypes, a variety of enteroviruses, and diverse specimens is required.

This study suggests a simple method to test echoviruses and perform serotyping using RT-PCR and dot-blot methods. Use of this method for the diagnosis of clinical specimens and identification of serotypes requires additional tests to increase the sensitivity. With such testing, in the future, serotyping can be completed at the same time as the clinical specimens are diagnosed to obtain a wealth of information on outbreaks and transmission patterns of echoviruses quickly and economically.

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