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Optimization of a Diagnostic DNA Chip for Fish Rhabdovirus

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A DNA chip that rapidly and accurately detects the viral genes in rhabdovirus-infected fish was developed. The N, M1, and G proteins of three rhabdovirus strains, infectious hematopoietic necrosis virus (IHNV), viral hemorrhagic septicemia virus (VHSV), and flounder rhabdovirus (HIRRV), were selected for use as probes. The sequences of the corresponding genes were obtained, and probes were prepared by PCR using specific primer sets. The specificity of the probes was confirmed by cross PCR. The prepared probes were spotted on poly-L-lysine- or aminosilane-coated glass slides and hybridized with target DNA under several different conditions in order to determine the optimal hybridization temperature, glass-slide coating, and target cDNA concentration.

Key words: Rhabdovirus, Diagnostic DNA chip, Probes

Introduction

Among fish rhabdoviruses, viral hemorrhagic septicemia virus (VHSV), infectious hematopoietic necrosis virus (IHNV), and flounder rhabdovirus (HIRRV) cause diseases with high mortality among salmonids (Oh, 1999) and sea trout in large-scale nurseries (Miller et al., 1998). Rhabdovirus is predominantly detected in the fins, skeletal muscle, and internal organs of infected fish. Infected fish have pale gills, and the gall bladder is pale yellow (Bruchhof et al., 1995; Schutze et al., 1999). Histological changes include pyknosis and necrosis of hematopoietic tissue (Wolf, 1988).

At present, the best way to control viral disease is avoidance of virus introduction through rapid detection. DNA chip technology is an ideal approach to the extensive, parallel identification of nucleic acids for the analysis of gene expression. Simultaneous analysis for the presence of multiple markers makes it possible in one experiment to determine the complete genetic profile of a single strain or to distinguish one strain from a very large collection of possible alternatives. Thus, DNA chip technology is potentially useful for the screening of multiple microbial isolates in a diagnostic assay (Bowtell and Sambrook, 2003).

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In this study, we developed a DNA chip to detect and identify rhabdovirus in infected fish. The probes were based on three of the five structural proteins of rhabdovirus: nucleotide capsid protein (N), matrix protein (M1), and the envelope glycoprotein (G).

Materials and Methods

Culture of viruses

Chinook salmon embryo (CHSE-214) cells and carp EPC cells were grown at 18°C in Eagle's minimum essential medium (MEM) with 10% fetal bovine serum (FBS) and 1% antibiotic (Gibco BRL, USA). The temperature for virus propagation was 15°C. Viral cytopathic effects (CPE) were observed by phase-contrast microscopy.

Fabrication of the DNA chip

The N, G, and M1 proteins were selected as probes for the DNA chip, and the sequences of their genes were obtained through a GenBank search. The probes were synthesized from total RNA of rhabdovirus-infected cells by reverse transcription (RT)-PCR. RT of total RNA was accomplished using an RT premix kit (Bioneer, Korea). PCR was carried out in 20 μl of a commercially prepared PCR mixture containing 1 U Taq polymerase, dNTPs (0.25 mM each of ATP, TTP, GTP, and CTP), 10 mM Tris-Cl, 40 mM KCl, and

1.5 mM $MgCl_2$. Specific primers (10 pmol) for each of the viruses (Table 1) and several concentrations of the target cDNA (see below) were added, and the PCR was carried out as follows: pre-reaction at 94°C for 5 min; 30 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, extension at 72°C for 30 s; and post-extension at 72°C for 7 min (Kim, 2003; Katagiri et al., 1997). PCR products were visualized by 1% agarose gel electrophoresis.

Spotting of probes

The probes were suspended in 50% dimethylsulfoxide (DMSO), at a concentration of 200 ng/μL, and then spotted on glass slides coated with aminosilane (Nuricell, Korea) or poly-L-lysine (Sigma, USA). The slides were developed using a genomics microarrayer system (MGII TAS microarrayer; BioRobotics, UK). The humidity for the spotting reaction was adjusted to 60-65%.

Isolation of total RNA from rhabdovirus-infected cell lines

Virus-infected cell lines were homogenized in 1 μL of TRIzol reagent (Invitrogen, USA) per 10^5-10^6 cells. The mixtures were allowed to stand at room temperature for 5 min. Two hundred microliters of chloroform were then added to each, and the mixtures were vigorously shaken for 15 s. The samples were again allowed to stand at room temperature and were then centrifuged for 15 min at 12,000×*g*. The aqueous phases were transferred to new tubes, 0.5 μL of isopropanol was added to each, and the samples were mixed by inverting. The samples were again centrifuged for 15 min at 12,000×*g*, and the supernatants were discarded. The RNA pellets were dried for 10 min under vacuum and dissolved in RNase-free water (0.1% DEPC-treated water). The RNA/DNA absorbance ratio (A_{260}/A_{280}) was measured using a spectrophotometer (Perkin Elmer, USA), and RNA degradation was checked by 1% agarose gel electrophoresis. For electrophoresis, total RNA was denatured at 65°C for 15 min, and 3 μg were loaded into each well.

Preparation of target cDNA by reverse transcription

For the RT reaction, the reaction mixture (20 μL), consisting of total RNA (50-100 μg), oligo(dT)18, M-MLV transcriptase (100 U/μL), Cy5-dUTP (0.1 mM), and dNTPs (0.5 mM of dATP, dGTP, and dCTP with 0.1 mM dTTP), was incubated at 42°C for 1.5 h (Bowtell and Sambrook, 2003; Tran et al., 2002). The samples were then incubated with 2 μL 0.5 M EDTA at 98°C for 3 min followed by 5 μL 1 N NaOH at 37°C for 10 min to remove extra RNA. The products were neutralized by the addition of 10 μL 1 M Tris-Cl buffer (pH 8.0), and unincorporated dyes and free nucleotides were removed by filtering through Micro Bio Spin 30 columns (BioRad, USA).

Hybridization and scanning

Labeled cDNAs were mixed with hybridization solution (5× SSC/0.2% SDS), denatured at 100°C for 2 min, and then applied to the prepared DNA chip. Target cDNA was hybridized in a hybridization cassette (Telechem International Inc., USA) to prevent drying. After hybridization (described in Results and Discussion), the slides were washed twice with 2× SSC/0.2% SDS for 10 min, once with 0.2× SSC for 10 min, and finally once with 0.1% SSC for 5 min. Hybridized slides were dried by centrifugation at 1,000×*g* for 1 min and visualized using a DNA chip scanner (ScanArray; Perkin Elmer, USA) (Schena, 2003).

Results and Discussion

Design of the DNA microarray

The specific primer sets used to prepare the probes for the rhabdovirus DNA chip were designed considering the GC content, T_m, and length of the structural genes of the virus (Table 1). Total RNA extracted from the viruses was subjected to RT-PCR in order to obtain DNA probes, and PCR was carried out using specific primer sets for each virus. Typical ethidium bromide-stained agarose gels of the amplification products are shown in Fig. 1. A cross-PCR assay was used to determine primer specificity (Fig. 2). In the PCR for IHNV, about 600 bp each of the N, M1, and G genes were detected, whereas there were no bands corresponding to VHSV and HIRRV (Fig. 2A). Likewise, the amplification of VHSV by PCR was specific for the gene and did not yield products corresponding to IHNV and HIRRV, as determined by agarose gel electrophoresis (Fig. 2B). PCR cross-reaction was also not observed for HIRRV (Fig. 2C).

Determination of hybridization temperature

The effect of different hybridization temperatures on the specificity of the signal was analyzed. Fig. 3 show DNA chips of IHNV, VHSV, and HIRRV that were hybridized with RNA from virus-infected fish overnight at 62°C and 65°C on an aminosilane-coated glass slide. At 62°C (Fig. 3A), N, M1, G, and β-actin were accurately and strongly detected. However, only VHSV M1 was detected at 65°C (Fig. 3B) and no signal observed for HIRRV (Fig. 3B(1)). Hybridiza-

Table 1. Specific primer sets used in amplification of probes

Virus strain	Code protein		Primer	Products (bp)
IHNV	N	Up	5′-aatgtccttgaggttgtgac-3′	575
		Down	5′-ccatcgtcatacctatcgtt-3′	
	G	Up	5′-gatagagaaggcgcttgtaa-3′	594
		Down	5′-tcaagacattcctctctgct-3′	
	M1	Up	5′-agaaggcgaagacatactga-3′	598
		Down	5′-acttccctcgtattcatcct-3′	
HIRRV	N	Up	5′-ccaacaaagcactaggaatc-3′	575
		Down	5′-gatggacttcatgcagagat-3′	
	G	Up	5′-atcccctgtttacataccct-3′	575
		Down	5′-ggaggtcttgtccacaaata-3′	
	M1	Up	5′-tgatattcccaagaatgctc-3′	571
		Down	5′-gattgccatgttcttgactt-3′	
VHSV	N	Up	5′-gcactgtccgtacttctctc-3′	567
		Down	5′-aagagattccatgcacagat-3′	
	G	Up	5′-ggtcgagaacatctcaacat-3′	585
		Down	5′-ttaagcgttctgaggtagg-3′	
	M1	Up	5′-cagatcagctctctttcgt-3′	582
		Down	5′-tgtccctactccaacttgtc-3′	
β-actin		Up	5′-actacctcagaagatcctg-3′	564
		Down	5′-ttgctgatccacatctgctg-3′	

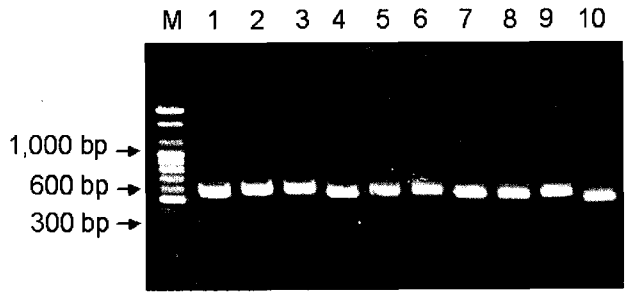

Fig. 1. cDNA probes prepared from IHNV, VHSV and HIRRV by RT-PCR using viral structural gene specific primers. Marker (M), 100 bp DNA ladder (Bioneer, Korea). Lanes 1-3: IHNV. 1, N-gene; 2, M1-gene; 3, G-gene. Lanes 4-6: VHSV. 4, N-gene; 5, M1-gene; 6, G-gene. Lanes 7-9: HIRRV. 7, N-gene; 8, M1-gene; 9, G-gene. Lane 10: β-actin.

tion at 65°C resulted in poor DNA chip performance owing to low signal intensities and high background. By contrast, hybridization at 62°C resulted in strong signals from all four probes. Therefore, 62°C was chosen as the optimal hybridization temperature.

Effect of the coating materials for the slide glass

The performances of different coating agents applied to the slide glass were also tested. Poly-L-lysine has been shown to be an inexpensive and simple coating agent for glass slides used in microarray analysis. However, poly-L-lysine coated slides often bind dust and other substances present in a room, and

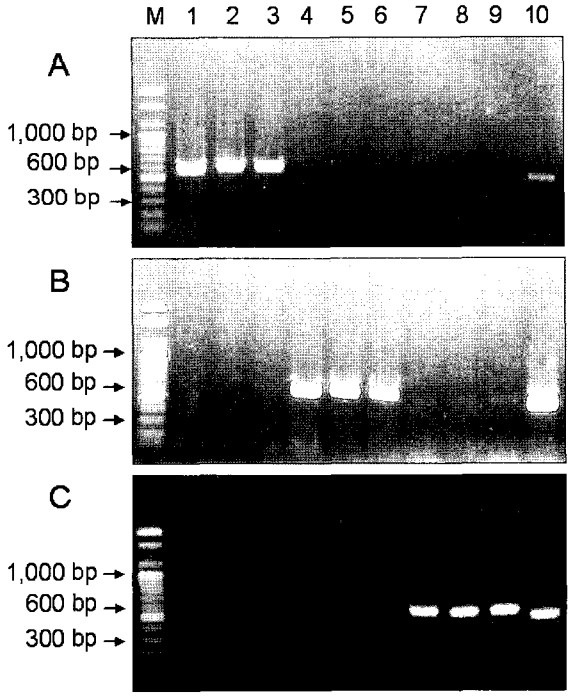

Fig. 2. Identification of specificity of IHNV, HIRRV and VHSV cDNA probes by cross PCR reaction with specific primer sets. A, B, and C represent gel images for IHNV, VHSV, and HIRRV as template, restively. Marker (M), 100 bp DNA ladder. Lane 1, IHNV N primer; 2, IHNV M1 primer; 3, IHNV G primer; 4, VHSV N primer; 5, VHSV M1 primer; 6, VHSV G primer; 7, HIRRV N primer; 8, HIRRV M1 primer; 9, HIRRV G primer; 10, β-actin primer.

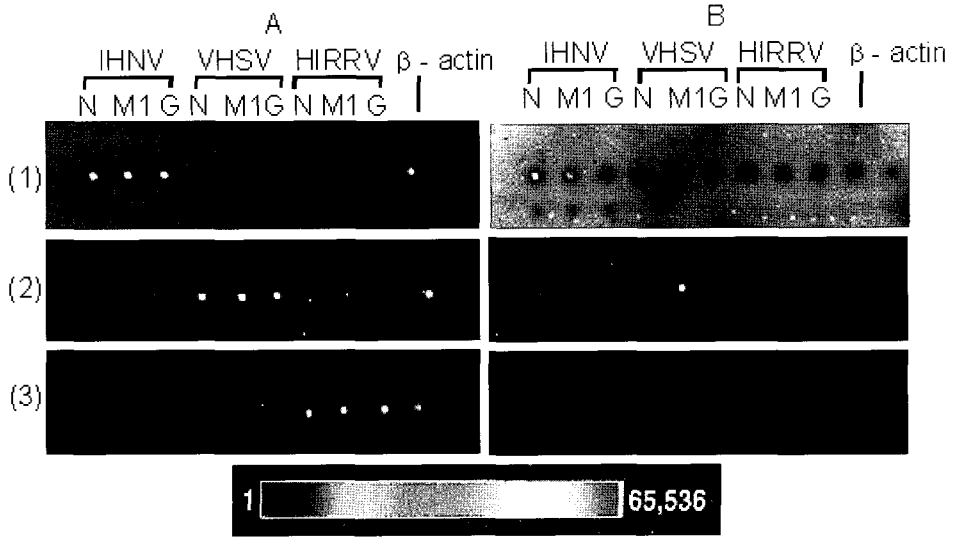

Fig. 3. Effect of hybridization temperature on probe spotting hybridization temperature was 62℃ (A) and 65℃ (B). A, 62℃, (1), IHNV, (2), VHSV, (3), HIRRV; B, 65℃ (1), IHNV, (2), VHSV, (3), HIRRV. The chips were prepared on the aminosilane coated slide glasses using Genomics microarrayer. The color bar represents the intensity of signal.

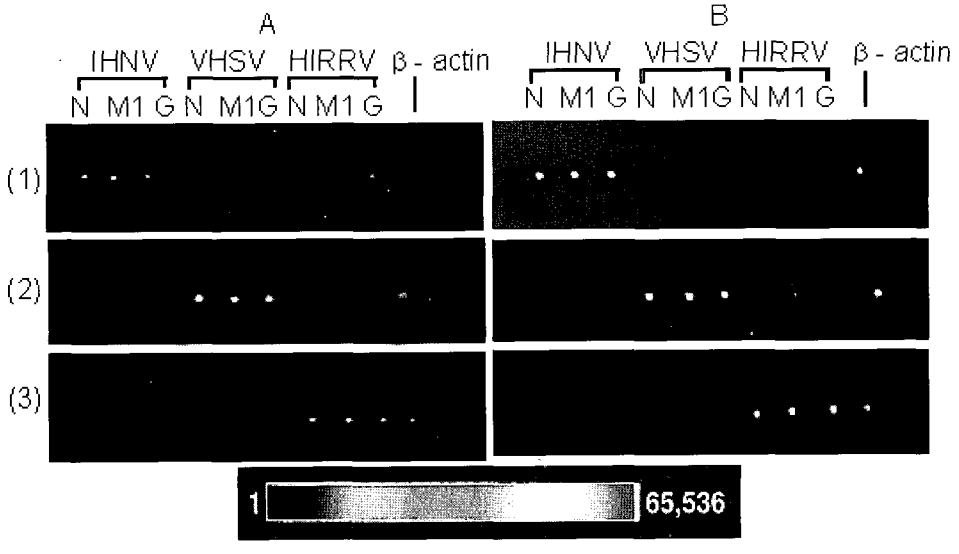

Fig. 4. Effect on the coated materials of slide glass. The spotting was performed using Genomics microarrayer. A, poly-L-lysine coated slide glass (hybridization temperature was 65℃), (1), IHNV, (2), VHSV, (3), HIRRV; B, aminosilane coated slide glass (hybridization temperature was 62℃) (1), IHNV, (2), VHSV, (3), HIRRV. The color bar represents the intensity of signal.

DNA on the glass slide sometimes detaches during hybridization (Bowtell and Sambrook, 2003; Zammatteo et al., 2000). Nonetheless, clear signals were obtained using DNA chips prepared on poly-L-lysine-coated slides (Fig. 4A). It should be noted that the DNA chip for IHNV showed lower signal intensity than that obtained with the chips for the other viruses, although this might be attributable to the fact that the expression of IHNV structural proteins is relatively lower.

To increase the interaction between the glass slide and the probes, the slides were coated with aminosilane (Nuricell, Korea) (Fig. 4B), which has been shown to be a better matrix than poly-L-lysine for DNA chips. Probes bind to aminosilane through electrostatic interactions between the positively charged

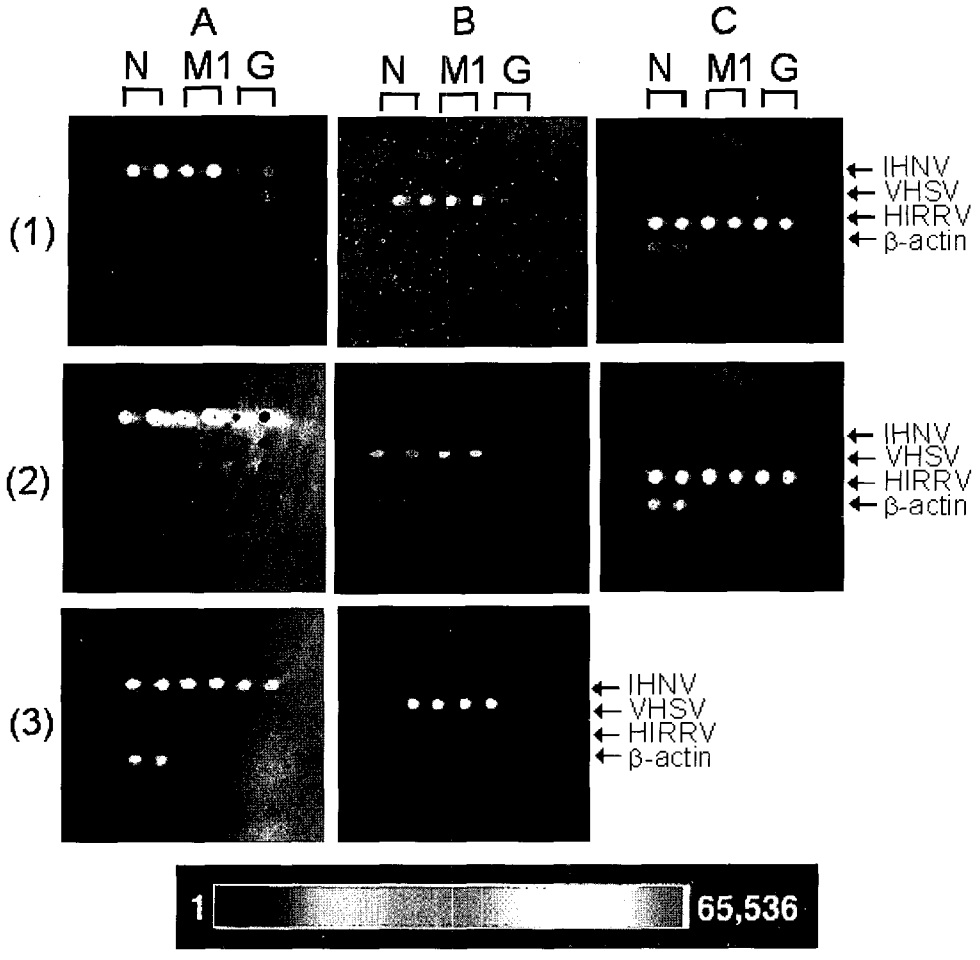

Fig. 5. Effect of total RNA concentration of hybridization. The chips were prepared on aminosilane coated slide using MG II TAS microarrayer. A, IHNV, (1) 40 μg, (2) 80 μg, (3) 100 μg; B, VHSV, (1) 40 μg, (2) 50 μg, (3) 60 μg; C, HIRRV, (1) 40 μg, (2) 50 μg. The color bar represents the intensity of signal.

amine groups of silane and the negatively charged phosphodiester backbone of the DNA. DNA chips on aminosilane-coated glass slides showed correct signals and low background (Fig. 4). In addition, the sensitivity was higher with the aminosilane coating than with poly-L-lysine. Therefore, aminosilane-coated glass slides were used as substrates for the DNA chip probes.

Determination of total RNA concentration

Fig. 5 shows the effect of total RNA concentration on the sensitivity of the DNA chip. When 40 μg of total RNA was used for the IHNV DNA chip, only the N and M1 genes were detected (Fig. 5A); signals were obtained for the other two genes, G and β-actin, when the total RNA concentration was increased to 100 μg. Similarly, for the VHSV DNA chip, G protein and β-actin were not detected when 40 μg of total RNA was used (Fig. 5B), whereas signals were readily detected for the N and M1 genes at total RNA concentrations between 40 and 100 μg. All HIRRV genes on the DNA chip hybridized when 50 μg of total RNA was used. Increasing the concentration of total RNA from 40 to 100 μg produced increased specificity only for IHNV; no significant concentration-dependent differences were observed for VHSV and HIRRV.

Acknowledgments

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