

A Diagnostic Microarray for Subtyping and Pathotyping Avian Influenza Virus

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Abstract

The ability to readily identify new and potentially pandemic strains of influenza virus will allow a more rapid response by health care officials to reduce the spread and human impact of the disease. We report a low-density microarray for the rapid detection and identification of potential pandemic influenza A virus subtypes H5, H7, and H9 in the present study. Subtype- and pathotype-specific probes were designed to target the hemagglutinin (HA) gene segment. The system consisted of RT-PCR using universal primers for the HA gene. RT-PCR products were hybridized against a microarray consisting of short probes 31-41 nucleotides in length. Cy3-labeled DNA targets were generated with a Cy3-labeled primer. Reference strains (n=46) of different avian influenza subtypes (H1-H15, except H16), and 34 unidentified field strains of avian influenza virus (AIV) from wild bird habitats and live bird markets was used for validation. All referenced subtypes belonged to H5, H7, and H9 and unidentified field strains were correctly typed by their HA-subtypes and pathotypes. These included 3 highly pathogenic H5N1 avian influenza viruses which had caused South Korean outbreaks in 2003, 2005, and 2008. In addition, there were no cross-hybridization reactions with other poultry respiratory virus-

es, suggesting that the microarray was specific for influenza A viruses. The developed microarray was capable of subtyping and pathotyping the potential pandemic influenza A virus subtypes H5, H7, and H9.

Keywords: Avian influenza, Microarray analysis, Hemagglutinin

Introduction

Influenza viruses have segmented, negative-sense, single-strand RNA genomes and are members of the family Orthomyxoviridae. Influenza A viruses are divided into subtypes based on the antigenic relationships of the surface glycoproteins, hemagglutinin (HA, H1-H16) and neuraminidase (NA, N1-N9). All influenza A subtypes have been isolated from avian species. The H5 and H7 viruses have resulted in highly pathogenic avian influenza (HPAI) in susceptible species¹.

The H1, H2, and H3 influenza A virus HA subtypes have become established in the human population, and the H5, H7, and H9 AIV subtypes have resulted in increasing numbers of cases in the human host in recent years. The HPAI H5 and H7 viruses have resulted in continuous outbreaks with both a devastating economic loss in the poultry industry and a severe threat to human health with high mortality². Avian influenza virus (AIV) subtype H9N2 has been isolated from terrestrial poultry worldwide and has caused repeated human infections in Asia since 1998³⁻⁶. These significant problems for the poultry industry and the increased risk of AIV subtype H5, H7, and H9 transmission to humans highlighted the need for a highly sensitive, accurate, and rapid diagnostic tool.

The diagnostic tools for AIV identification typically require virus isolation, culture, and molecular characterization by sequencing, results in 3 to 7 days to culture the virus, and can test only a few samples simultaneously⁷. This is considered the gold standard for virus identification and characterization⁸. Among current AIV detection methods, real-time reverse transcription polymerase chain reaction (RRT-PCR) assays can detect a broad range of influenza A virus subtypes using a single primer set targeting a short portion of the conserved matrix (M) gene segment⁹. Subtype specific RRT-PCR methods for AIV diagnosis are

currently available to AIV subtypes¹⁰. Although AIV RRT-PCR-based assays are rapid and sensitive, the PCR assay capability is limited and cannot be easily expanded to simultaneous AIV subtyping and pathotyping in a multiplex fashion.

The microarray is capable of conducting thousands of analyses. Although RRT-PCR is very sensitive for AIV detection, microarrays are a useful adjunct to AIV subtyping methods¹¹⁻¹⁹. Commercial influenza virus microarray systems are available, but they are expensive with minimal applicability to large-scale AIV surveillance.

We describe a rapid and inexpensive approach for the detection of potential pandemic AIV subtypes H5, H7, and H9 using diagnostic microarrays. This microarray is designed to detect highly pathogenic AI and to provide a detailed analysis of possible AIV pathogenicity using simultaneous cleavage site sequences of the HA gene. We validated this microarray with unknown AIV isolates in wild bird habitats and live bird markets (LBM) during the 2007-2008 winter seasons.

Results

Primers for RT-PCR

The universal primer set for full length amplification of the HA gene was used for RT-PCR. Primers were specific to the conserved ends of the HA gene and generated 1.7 kb full-length PCR amplicons. The best results were achieved with annealing temperatures of 57°C with normal PCR conditions. The infectious bronchitis virus (IBV) strain (Massachusetts) and the Newcastle disease virus strain (La Sota) were amplified with primers used in this study to validate the specificity of the RT-PCR amplification. Amplicons were not visualized on gel electrophoresis, suggesting that the primers were specific for AIV.

Hybridization

H5 subtype samples (n=6) revealed specific patterns in H5 candidate probes. However, 2 HPAI H5N1 samples displayed cross-reactivity with H9-43 probes. All H5N1 samples displayed specific hybridization patterns in probes for HPAI discrimination. None of the LPAI samples displayed the signal pattern of HPAI probes. Although the H5-5 probe displayed signal intensity, a single H7N8 sample was completely discriminated and didn't display cross-reactivity with other subtype probes. H9 subtype samples (n=6) displayed a signal pattern in H9 candidate probes. The 2 candidate probes H9-43 and H5-5 displayed cross-reactivity in non-target subtypes. The other probes

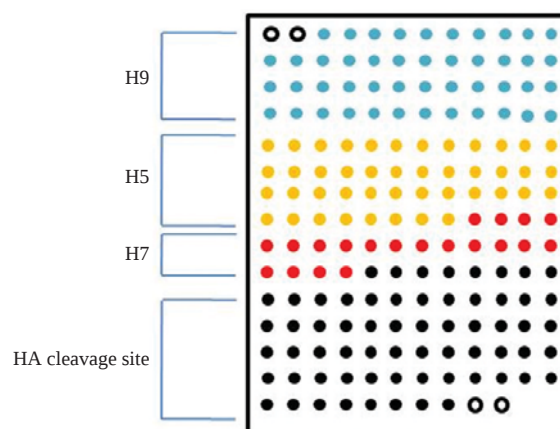


Figure 1. Layout of diagnostic microarrays for AIV subtyping and pathotyping. Microarray layout for 78 probes of HA and M gene with position markers (open circle). Among 78 probes, 22 probes for identification of the H5 subtype (yellow dot), 10 probes for the H7 subtype (red dot), 23 probes for the H9 subtype (blue dot), and 32 probes for pathotyping (black dot) were spotted on silylated slides in duplicate, and 4 blocks were located on the microarray slides.

demonstrated subtype-specific signal patterns. All H5, H7, and H9 HA subtypes and pathotypes were correctly determined among 46 AIV isolates. Each subtypes reacted with subtype-specific probes, and HP H5N1 displayed positive reactions with HPAI-specific probes. No signals could be observed for the LPAI. These findings suggested that distinct patterns of hybridization were obtained for each of the subtypes and pathotypes (Figure 2).

Validation with Field Samples

The results suggested that the specific signal intensities observed in the images of this microarray could be used as a diagnostic tool for the subtyping and pathotyping of unknown AIV strains. We applied this diagnostic microarray to unknown AIV isolates from Korean wild bird habitats and LBMs during 2007-2008. A total of 32 unknown AIV isolates were examined. Wild bird habitats yielded low pathogenic H5 subtypes (n=3), a single low pathogenic H7 subtypes (n=1) and an H9 subtype (n=1). Live bird markets (LBM) yielded 12 H9N2 subtypes. Sequencing of the HA gene identified all determined H5, H7, and H9 subtypes. Low pathogenicity samples were identical to sequencing results except a single isolate A/Chicken/Korea/LBM444/08 (H9N2) (Table 3). The other identified subtypes included H1N2 (2), H2N8 (1), H3N8 (1), H4N8 (3), H6N1 (1), H10N4 (1), and H11N2 (2) in wild bird habitats, and H3N2 (2), H4 (1), and H6 (2) in LBM.

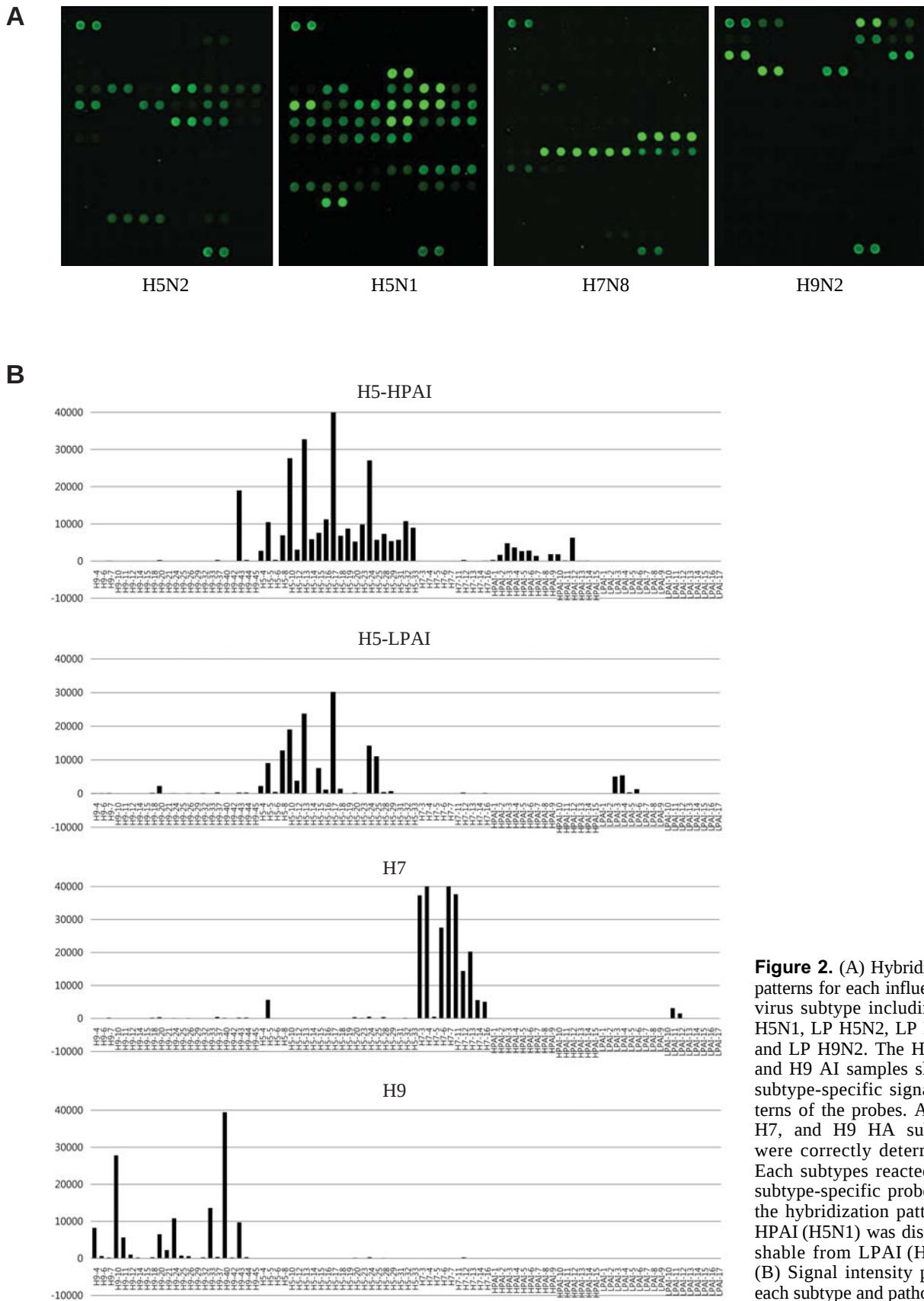


Figure 2. (A) Hybridization patterns for each influenza A virus subtype including HP H5N1, LP H5N2, LP H7N2, and LP H9N2. The H5, H7, and H9 AI samples showed subtype-specific signal patterns of the probes. All H5, H7, and H9 HA subtypes were correctly determined. Each subtypes reacted with subtype-specific probes and the hybridization pattern of HPAI (H5N1) was distinguishable from LPAI (H5N2). (B) Signal intensity plot of each subtype and pathotype.

Table 1. Influenza A viruses used in the study.

No.	Virus	Pathotype (Clade)
1	A/Wild bird/Kr/7-1/05 (H1N1)	LPAI
2	A/NWS/(H1N1)	LPAI
3	A/Wild bird/Kr/4-5/04 (H2Nx)	LPAI
4	A/Duck/Kr/LBM1354/06 (H3N2)	LPAI
5	A/Wild bird/Kr/4-47/04 (H3N8)	LPAI
6	A/Wild bird/Kr/6-D1/05 (H3N8)	LPAI
7	A/Wild bird/Kr/8-G50/05 (H3N8)	LPAI
8	A/Duck/Kr/LBM182/07 (H3N8)	LPAI
9	A/Mallard/Kr/LBM347/07 (H3N8)	LPAI
10	A/Wild bird/Kr/4-27/04 (H4Nx)	LPAI
11	A/Wild bird/Kr/7-D10/05 (H4Nx)	LPAI
12	A/Wild bird/Kr/8-G32/05 (H4N2)	LPAI
13	A/Ck/Kr/Es/03 (H5N1) ^a	HPAI (2.5)
14	A/Ck/Kr/Is/06 (H5N1) ^b	HPAI (2.2)
15	A/Ck/Kr/Gimje/08 (H5N1) ^c	HPAI (2.3.2)
16	A/Wild bird/Kr/AG63/06 (H5N2)	LPAI
17	A/Wild bird/Kr/8-G9/05 (H5N2)	LPAI
18	A/Wild bird/Kr/EG16/06 (H5N2)	LPAI
19	A/Wild bird/Kr/8-G26/05 (H6N1)	LPAI
20	A/Wild bird/Kr/10BD62/06 (H6N1)	LPAI
21	A/Duck/Kr/LBM399/07 (H6N1)	LPAI
22	A/Wild bird/Kr/1-58/04 (H6N5)	LPAI
23	A/Wild bird/Kr/HDR16/03 (H7N2)	LPAI
24	A/Dk/Kr/BC10/07 (H7N3)	LPAI
25	A/Wild bird/Kr/GG1/07 (H7N7)	LPAI
26	A/Wild bird/Kr/GH171/07 (H7N7)	LPAI
27	A/Wild bird/Kr/5-80/05 (H7N8)	LPAI
28	A/Wild bird/Kr/ESD24/04 (H8N4)	LPAI
29	A/Wild bird/Kr/GG82/04 (H8N4)	LPAI
30	A/Duck/Kr/LBM186/07 (H9N2)	LPAI
31	A/Silky fowl/Kr/LBM194/07 (H9N2)	LPAI
32	A/Chicken/Kr/LBM267/07 (H9N2)	LPAI
33	A/Silky fowl/Kr/LBM342/07 (H9N2)	LPAI
34	A/Chicken/Kr/LBM371/07 (H9N2)	LPAI
35	A/Chicken/Kr/LBM398/07 (H9N2)	LPAI
36	A/CK/Kr/01310/01 (H9N2)	LPAI
37	A/Silky fowl/Kr/LBM446/07 (H9N2)	LPAI
38	A/Wild bird/Kr/B-D72/06 (H10N2)	LPAI
39	A/Wild bird/Kr/CSM26/05 (H10N4)	LPAI
40	A/Wild bird/Kr/GS67/05 (H10N4)	LPAI
41	A/Wild bird/Kr/NDG54/05 (H11N3)	LPAI
42	A/Dk/Kr/ES13/04 (H12N1)	LPAI
43	A/Wild bird/Kr/A-G53/06 (H12N5)	LPAI
44	A/Gull/Maryland/704/77 (H13N6)	LPAI
45	A/mallard/Gurjev/263/82 (H14N5)	LPAI
46	A/Shearwater/w.Aust/2576/79 (H15N9)	LPAI

^aSequence of HA₀ cleavage site was PQREXKRKRGLF^bSequence of HA₀ cleavage site was PQGERRRKRGLF^cSequence of HA₀ cleavage site was PQRERRRKRGLF

Discussion and Conclusions

Wild waterfowl (predominantly of the order Anseriformes and Charadriiformes) are AIV reservoirs²⁰. However, little is known about the dynamics of HPAI

in wild birds, and the importance of wild birds in the spread of HPAI remains uncertain. Bird-to-bird and bird-to-mammal transmission in LBM could emerge as the focus of AIV in new hosts, with possibly evolving low pathogenic avian influenza (LPAI) into HPAI viruses²¹. Many HPAI and LPAI outbreaks have been reported in terrestrial poultry, and LPAI could become highly pathogenic after circulation in poultry flocks². Therefore, AIV surveillance in wild bird habitats, live bird markets, and poultry farms provide important epidemiological information of potential pandemic AIV strains circulating in wild birds as well as in domestic poultry.

Microarrays designed to subtype influenza viruses have been previously demonstrated by several groups¹²⁻¹⁴. These groups designed specific probes in a microarray for analysis of the influenza A viruses H1N1, H3N2, H1N2, and H5N1 and the human influenza B viruses, but were not specifically designed for AIV. Han *et al.*⁶ recently developed a microarray for subtyping of all influenza A viruses on one chip. However, these groups were not designed for pathotyping AIV subtypes H5 and H7.

This diagnostic approach enabled rapid detection and characterization of AIV isolates. Early detection and rapid response to HPAI can reduce the economic losses of influenza outbreaks. The entire diagnostic microarray assay can be completed in fewer than 5 hours, and can be performed using direct labeling of RT-PCR products with a single fluorophore per target molecule without the need for a second target amplification step. The use of one-step RT-PCR with this microarray could minimize the time and risk of contamination.

Combimatrix Corporation (Mukilteo, WA) announced a commercial influenza virus microarray system. They developed the microarray for subtyping the 15 HA and 9 NA subtypes. However, the cost per chip (\$700) is prohibitive for application to national influenza virus surveillance. The cost of the microarray in the present study is below \$15 per sample. This is cost effective for large-scale surveillance, and the assay demonstrated a high specificity and sensitivity. This finding demonstrated that AIV simultaneous detection, subtyping, and pathotyping could be simple and inexpensive.

All reference strains in this study were correctly hybridized with respect to their HA-subtypes and pathotypes. Pathogens important for the differential diagnosis of AIV were included in the study (NDV strain La Sota and IBV strain Massachusetts) and tested negative. All AIV isolates used in a validation study were LPAI strains, and 3 AIV H5 subtypes, a single H7 subtype, and a single H9 subtype were detected in

Table 2. Probes for subtyping and pathotyping the AIV subtypes H5, H7, and H9.

Subtype probe	Sequence	Pathotype probe	Sequence
H5-4	5'-NH2-T(15)AGTGTAGCTGGATGGCT(C/T/A)C-3'	HPAI-1	5'-NH2-T(15)AGAAGAAAAAAGAGGACTATTT-3'
H5-5	5'-NH2-T(15)TTGACAAAATGAACACTCA(G/A)TT-3'	HPAI-2	5'-NH2-T(15)GAAGAAGAAAAAAGAGGACTATTT-3'
H5-6	5'-NH2-T(15)ATTTGCATTGGTTACCATGC-3'	HPAI-3	5'-NH2-T(15)RAGAAAAAAGAGGACTATTT-3'
H5-8	5'-NH2-T(15)AC(T/C)CAAAAGGCAATAGATGG-3'	HPAI-4	5'-NH2-T(15)AGAAGAAAAAAGAGGACTTTTT-3'
H5-10	5'-NH2-T(15)CA(G/A)CTTAGGGATAATGCAA-3'	HPAI-5	5'-NH2-T(15)AAGAAGAAAAAAGAGGATTATTT-3'
H5-12	5'-NH2-T(15)TACAAAATTGTCAAGA(A/C)AGG-3'	HPAI-6	5'-NH2-T(15)GAAGAAAAAAGAGGGGCTATTT-3'
H5-13	5'-NH2-T(15)AAACACCTATTGAGC(A/G)GAAT-3'	HPAI-7	5'-NH2-T(15)CAGAAGAAAAAAGAGAGGTCTATTT-3'
H5-14	5'-NH2-T(15)ACAC(C/T)TATTGAGCAGAATAAA-3'	HPAI-8	5'-NH2-T(15)AAGAAAAACAAGAGGCCTATTT-3'
H5-15	5'-NH2-T(15)TACAAAATTGTCAAGAAAGG(G/A)-3'	HPAI-9	5'-NH2-T(15)AGAAGAAAGAAAGAGGACTGTTT-3'
H5-16	5'-NH2-T(15)C(C/T)GTTGGAAGGGAATTTAA-3'	HPAI-10	5'-NH2-T(15)AGAAGAAAGAAAAAGAGGACTATTT-3'
H5-17	5'-NH2-T(15)A(C/T)GGTTGTTTCGAGTTCTATCA-3'	HPAI-11	5'-NH2-T(15)AAAGAAGAAGAAAAAGAGGATTATTT-3'
H5-18	5'-NH2-T(15)AG(T/C)GATCAGATTGTCATTGG-3'	HPAI-12	5'-NH2-T(15)GAAGAAGAAGAAAGAGAGGACTATTT-3'
H5-19	5'-NH2-T(15)AAACACCTATTGAGC(A/G)GAAT-3'	HPAI-13	5'-NH2-T(15)AGGAGGAGGAGAGGCCTATTT-3'
H5-20	5'-NH2-T(15)GGGGCGATAAACTCTAG(T/C)AT-3'	HPAI-14	5'-NH2-T(15)CGCGTGAGGAGAGGCCTATTT-3'
H5-23	5'-NH2-T(15)T(A/G)AAACACCTATTGAGCAGAATA-3'	HPAI-15	5'-NH2-T(15)CGTGTGAGGAGAGGCCTATTT-3'
H5-24	5'-NH2-T(15)GGCAGGGAATGGTAGATGG-3'	LPAI-1	5'-NH2-T(15)CAAAAAGAAACAAGAGGCTTATTT-3'
H5-25	5'-NH2-T(15)CTGGTTCTCATGGAAAA(T/C)GA-3'	LPAI-2	5'-NH2-T(15)AAAGAGAAAAAAGAGGCCTATTT-3'
H5-28	5'-NH2-T(15)GGGCGATAAACTCTAGTATGCC-3'	LPAI-3	5'-NH2-T(15)CAAAGAGAAACAAGAGGACTATTC-3'
H5-29	5'-NH2-T(15)ATAAACTCTAGTATGCCATTCCACA-3'	LPAI-4	5'-NH2-T(15)TCAAAGAGAAACAAGAGGACTATTT-3'
H5-31	5'-NH2-T(15)TC(A/T)ACACTAAACCAGAGATT-3'	LPAI-5	5'-NH2-T(15)AAAGAGAAACAAGAGGCCTATTT-3'
H5-32	5'-NH2-T(15)TGAAACACCTATTGAGC(A/G)GA-3'	LPAI-6	5'-NH2-T(15)CAAAGAGAAACAAGAGGCTTATTT-3'
H5-33	5'-NH2-T(15)C(A/T)ACACTAAACCAGAGATTG-3'	LPAI-7	5'-NH2-T(15)CAGATGACCAGAGGCCTTTTT-3'
H7-3	5'-NH2-T(15)CTTCGGGGCATCATGTTT-3'	LPAI-8	5'-NH2-T(15)CCAAAGAAAAAGAGGCCTTTTT-3'
H7-4	5'-NH2-T(15)ATGC(G/A)GTGCACTATTTGTATATA-3'	LPAI-9	5'-NH2-T(15)CCAAAGCCAAGAGGCCTTTTT-3'
H7-5	5'-NH2-T(15)CATCAAAAATGCACAAGG(G/A)G-3'	LPAI-10	5'-NH2-T(15)CCAAAGCCCAGAGGCCTTTTT-3'
H7-6	5'-NH2-T(15)AATGCTGAAGAAGATGG(C/G/A)AC-3'	LPAI-11	5'-NH2-T(15)CCAAAGGGAAGAGGCCTATTT-3'
H7-7	5'-NH2-T(15)AAGAATGGAACATGC(G/A)GT-3'	LPAI-12	5'-NH2-T(15)CCAAAGGGAAGAGGCCTATTT-3'
H7-11	5'-NH2-T(15)AAATGAACACTCAAATCCTGGTA-3'	LPAI-13	5'-NH2-T(15)CCCAAGACCAGAGGCCTTTTT-3'
H7-12	5'-NH2-T(15)TGGCGATCATTCCGAC-3'	LPAI-14	5'-NH2-T(15)CCCAAGACCAGAGGTCTTTTT-3'
H7-13	5'-NH2-T(15)A(T/C)GCAACTGAAACGGTGG-3'	LPAI-15	5'-NH2-T(15)CCCAAGACCAGGGGCCTCTTT-3'
H7-14	5'-NH2-T(15)CAATGTGACCAATTCTAGAA-3'	LPAI-16	5'-NH2-T(15)CCCAAGACCAGGGGCCTTTTT-3'
H7-16	5'-NH2-T(15)G(A/G)AGAAGCTCTGAGGCAAAT-3'	LPAI-17	5'-NH2-T(15)CCCAAGGCCAGAGGCCTTTTT-3'
H9-4	5'-NH2-T(15)CCA(G/A)TCAACAACTCCACAGA-3'	PM ^a	5'-NH2-T(15)CATCCCCCTGGGACTGGAGT-3'
H9-6	5'-NH2-T(15)T(G/A)CTCCACACAGAGCACA-3'		
H9-7	5'-NH2-T(15)TGATAGGGCCAAGGCC-3'		
H9-10	5'-NH2-T(15)C(G/T/A)ACTGTGCGCTCATCTCT-3'		
H9-11	5'-NH2-T(15)C(A/C)ACAACTCCACAGAACTGT-3'		
H9-12	5'-NH2-T(15)(G/A)TGTGCAACAAATCTGGGA-3'		
H9-14	5'-NH2-T(15)CT(G/A)TTCAAGACGCCCAATA-3'		
H9-15	5'-NH2-T(15)CGCCCAATACACAAATAAT(A/C)G-3'		
H9-18	5'-NH2-T(15)(C/A/T)GGCTACCAATCAACAAAC-3'		
H9-20	5'-NH2-T(15)GG(A/G/T)GCCATAGCTGGATTCA-3'		
H9-21	5'-NH2-T(15)TTCCAGCATTCAAATGA(T/C)CA-3'		
H9-24	5'-NH2-T(15)CAGAAAATAGAAGGGGT(C/G/A)AA-3'		
H9-25	5'-NH2-T(15)AATAGAAGGGGTCAA(G/A)CTGG-3'		
H9-26	5'-NH2-T(15)TTCT(T/C)TTCATGTGGGGCATA-3'		
H9-29	5'-NH2-T(15)CGCCCAATACACAAATAATAG(G/A)-3'		
H9-32	5'-NH2-T(15)AAAATAGAAGGGGTCAA(G/A)CT-3'		
H9-33	5'-NH2-T(15)ATTTATTGACTGTGCGCTC-3'		
H9-37	5'-NH2-T(15)GGAAGAGAATGGTCCTA(C/T)AT-3'		
H9-40	5'-NH2-T(15)ACTGTGCGCTCATCTCTTGT-3'		
H9-42	5'-NH2-T(15)ATGTGGGGCATAAATCA(C/T)C-3'		
H9-43	5'-NH2-T(15)ATAGAGGGAGGTTGGTCAGG-3'		
H9-44	5'-NH2-T(15)GGCAATTGACAAAATAAC(A/G)TC-3'		
H9-45	5'-NH2-T(15)A(T/C)CATGAATTCAGCGAGGTT-3'		

^aPM: Position Marker, non-homologous Cy3 labeled probe

Table 3. Subtyping and pathotyping of field samples using microarray.

Location	Number (%) of AIV positive/samples	Virus	HA ^a	RRT-PCR ^b	Microarray	Sequencing ^c
Wild bird habitats	16 (0.86)/1866	A/Wild bird/Kr/17-28/07 (H1N2)	+	+	—	H1
		A/Wild bird/Kr/20-38-1/07 (H1N2)	+	+	—	H1
		A/Wild bird/Kr/12-C-3/06 (H2N8)	+	+	—	H2
		A/Wild bird/Kr/15-71-3/07 (H3N8)	+	+	—	H3
		A/Wild bird/Kr/17-7/07 (H4Nx)	+	+	—	H4
		A/Wild bird/Kr/21-107/08 (H4Nx)	+	+	—	H4
		A/Wild bird/Kr/11-DG28/06 (H4N8)	+	+	—	H4
		A/Wild bird/Kr/12-AG17/06 (H5N2)	+	+	H5 LPAI	H5 LPAI
		A/Wild bird/Kr/17-14/07 (H5N2)	+	+	H5 LPAI	H5 LPAI
		A/Wild bird/Kr/19-74/07 (H5N2)	+	+	H5 LPAI	H5 LPAI
		A/Wild bird/Kr/17-75/07 (H6N1)	+	+	—	H6
		A/Wild bird/Kr/21-9/08 (H7Nx)	+	+	H7 LPAI	H7 LPAI
		A/Wild bird/Kr/20-36/07 (H9N2)	+	+	H9	H9
		A/Wild bird/Kr/11-AD1/07 (H10N4)	+	+	—	H10
		A/Wild bird/Kr/18-32/07 (H11Nx)	+	+	—	H11
		A/Wild bird/Kr/12-B16/06 (H11N2)	+	+	—	H11
Live bird market	18 (6.52)/276	A/Mallard/Kr/LBM397/07 (H3N2)	+	+	—	H3
		A/Duck/Kr/LBM176/08 (H3N2)	+	+	—	H3
		A/Mallard/Kr/LBM188/08 (H4Nx)	+	+	—	H4
		A/Duck/Kr/LBM436/08 (H6Nx)	+	+	—	H6
		A/Mallard/Kr/LBM1674/07 (H6N2)	+	+	—	H6
		A/Silkyfowl/Kr/LBM1260/06 (H9N2)	+	+	H9	H9
		A/Chicken/Kr/LBM618/07 (H9N2)	+	+	H9	H9
		A/Duck/Kr/LBM1621/07 (H9N2)	+	+	H9	H9
		A/Chicken/Kr/LBM1627/07 (H9N2)	+	+	H9	H9
		A/Silkyfowl/Kr/LBM1632/07 (H9N2)	+	+	H9	H9
		A/Duck/Kr/LBM1673/07 (H9N2)	+	+	H9	H9
		A/Duck/Kr/LBM1705/07 (H9N2)	+	+	H9	H9
		A/Chicken/Kr/LBM178/08 (H9N2)	+	+	H9	H9
		A/Chicken/Kr/LBM190/08 (H9N2)	+	+	H9	H9
		A/Chicken/Kr/LBM410/08 (H9N2)	+	+	H9	H9
		A/Chicken/Kr/LBM414/08 (H9N2)	+	+	H9	H9
		A/Chicken/Kr/LBM438/08 (H9N2)	+	+	H9	H9
		A/Chicken/Kr/LBM444/08 (H9N2)	+	+	—	H9
Total	34 (1.59)/2142					

^aAll samples were tested for hemagglutinin activity using erythrocytes of chicken.

^bAll samples were detected by real-time RT-PCR using M-specific probe⁹.

^cNucleotide sequence of the HA gene of field isolates were determined to validate HA subtypes and pathotypes.

wild bird habitats. Additionally, 12 H9N2 subtypes were detected in LBM using microarrays. All H5, H7, and H9 HA subtypes and pathotypes were correctly determined, and were identical to sequencing results except for a single isolate A/Chicken/Korea/LBM444/08 (H9N2). Fecal samples are the best source of virus isolation for LPAI. However, the detection of AIV genomes in fecal samples presents a particular challenge because of the great number of PCR inhibitors. Further, gene amplification and hybridization of recent isolates could fail or display low efficiency due to mutations on primer or probe binding sites. These results demonstrated that the continuous updates of the probes for newly emerged AIV will be required.

This microarray will hopefully be expanded to cover new pandemic candidate AIV strains, including the AIV subtype H3N2 which was recently outbreak in canines²² and AIV subtype H6 which has caused infections in poultry in north America and south east Asia²³⁻²⁶. Further study is also required to analyze the neuraminidase gene for exploration of oseltamivir-resistant genes and subtyping.

In conclusion, we developed a diagnostic microarray for the simultaneous and inexpensive rapid detection of AIV subtypes H5, H7, and H9. This diagnostic microarray could provide enormous potential for the rapid subtyping and pathotyping of AIV subtypes H5, H7, and H9 in national surveillance programs.

Materials and Methods

Viral RNA Samples

Viral isolates representative of all known influenza A virus HA subtypes except H16 were tested. A total of 46 viruses were used in this study. Viruses were originated from the College of Veterinary Medicine, Konkuk University (n=31), and from the National Veterinary Research and Quarantine Service (n=15, including 3 HP H5N1 strains). These are described in Table 1. A total of 34 viruses isolated from wild bird habitats (n=16) and LBM (n=18) were used for validation during AIV surveillance studies in 2006-2008. Specificity tests were conducted with Newcastle Disease Virus (NDV) strain La Sota and Infectious Bronchitis Virus (IBV) strain Massachusetts.

Probe Design and Selection

We analyzed all HA and M gene sequences from the Genbank Database (Influenza virus resource database) for subtyping and pathotyping: (<http://www.ncbi.nlm.nih.gov/genomes/FLU/FLU.html>). A total of 392 sequences were selected from the database and were aligned with sequences of isolates. A total of 87 probes were selected using in-house programs for the selection of subtype-specific candidate probes. We selected 22 probes for the identification of H5 subtypes, 10 probes for H7 subtypes, 23 probes for H9 subtypes, and 32 probes for AIV pathotyping. The 39 oligonucleotides used for subtyping were degenerative probes.

To identify H5, H7 and H9 subtype among all subtypes, probes (n=55) were printed on silyated slide glasses, and 32 probes were spotted on the slide glass for AIV pathotyping. Probes were specific for HPAI (n=15) and were selected to identify LPAI (n=17). All candidate probes were described in Table 2. The subtype-specific candidate probes had greater than 2 oligonucleotides difference compared to other subtype sequences. The oligonucleotide microarray layout was described in Figure 1. The length of the candidate probes was from 31 bp to 41 bp for optimization of hybridization temperatures.

Fabrication of Microarray

The 5' amine-modified probes (n=87) were synthesized by Bioneer Inc. (Daejeon, KOREA). Probes were linked poly T (15mer) at the 5' end. All probes were re-suspended in spotting solution at 50 μ M (3X SSC, 1.5 M Betaine), transferred in 384-well plates for spotting, and then spotted on silyated slides (Cell Associate, USA) using a microarrayer (Cartesian Technology, USA). Slides were incubated in a humidity cham-

ber at 25°C and 70% humidity for 16 hours after spotting. Spotted slides were washed with 0.1% Sodium Dodecyl Sulfate (SDS) for 5 min and were washed twice in double-distilled water. Finally, the spotted slides were incubated in 250 mL of sodium borohydride solution (NaBH₄ 0.625 g, PBS 187.5 mL, ethanol 62.5 mL) for 5 min and were washed twice in double-distilled water with gentle agitation. Spotted slides were centrifuged in 800 r.p.m. and stored at room temperature.

PCR for Dye-labeling

Viral genomic RNA was extracted using an RNeasy mini kit (Qiagen, CA). A two-step multiplex RT-PCR for the HA and M genes was performed using the universal primer set for the full length amplification of the HA and M genes²⁷. cDNA was synthesized using the M-MLV reverse transcription kit (Invitrogen, USA) and the synthesized cDNA was amplified in a 25 μ L reaction mixture containing 6.5 μ L of sterile ddH₂O, 12.5 μ L of 2X DyeMix (Enzymomics, France), 1 μ L of HA gene primers (10 pmol), 1.5 μ L of M gene primers and 1 μ L of template cDNA. The reaction occurred under the following cycling conditions, including denaturation for 5 min at 95°C, 35 cycles of 60 s at 94°C, 60 s at 57°C, 90 s at 72°C, and a final extension of 10 min at 72°C. The reverse HA and M gene primers were labeled Cy3 at the 5' end (Bioneer, Korea).

Microarray Hybridization

The oligonucleotide microarray was covered by a CoverWell perfusion chamber (Grace-Bio Labs, USA) to hybridize the Cy3-labeled PCR product. PCR products were denatured for 3 min at 99°C, and 10 μ L of denatured PCR product was mixed with 90 μ L of hybridization buffer (3X SSC, 0.3% Sarcosyl). The mixture was hybridized onto the oligonucleotide arrays in a hybridization oven (FINEPCR, Korea) for 1 h at 50°C. The oligonucleotide arrays were washed in 1X SSC, 0.1% SDS, and 1X SSC for 5 min after hybridization and in 0.1X SSC for 1 min at room temperature.

Scanning and Image Analysis

Hybridization signals were detected using the microarray scanner Genepix 4000B (Axon instrument, USA) at PMT gain 500, laser power 100%. Fluorescence intensity was analyzed after scanning using the Genepix 4.1 software (Axon instrument, USA). The local background intensity was subtracted from median values of each spot.

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