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Detection and Identification of Avian Influenza Virus by cDNA Microarray

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Abstract

Influenza A viruses consisting of all known 16 HA and 9 NA subtypes have been isolated from birds. We have created a diagnostic avian cDNA microarray containing probes corresponding to the highly conserved matrix (M) gene, and selected hemagglutinin (HA), and neuraminidase (NA) subtypes of AIV. cDNA RT-PCR products from the HA, NA, and M genes of various avian influenza isolates and subtypes were used to create an avian influenza virus (AIV) cDNA microarray. The microarray was evaluated against a panel of AIV isolates in order to appraise its application in AIV detection and identification. Utilizing the M gene as a pan-influenza marker, all 10 samples were identified as being strains of type A influenza. The array was able to correctly HA- and NA-subtype subtype 7 out of 10 test samples. This included correctly identifying, subtyping, and determining the geographic origin of all of the H5 subtypes and the two H7 samples of U.S. origin.

Keywords: Influenza A; Avian Influenza; Microarray; H5; H7; Virus Identification; Hemagglutinin; Neuraminidase; Matrix

Introduction

Influenza viruses are enveloped, single-stranded RNA viruses belonging to the *Orthomyxoviridae* family [1]. The viral genome is composed of eight gene segments of negative sense single-stranded RNA. The viral envelope is coated with surface projections, comprised predominantly of the hemagglutinin (HA) and neuraminidase (NA) proteins, which are also major antigenic determinants. The HA and NA proteins are antigenically variable with 16 distinct hemagglutinin and 9 distinct neuraminidase subtypes that have all been identified in wild birds [2]. Avian Influenza Virus (AIV) is a contagious viral pathogen affecting many species of birds throughout the world. Clinical signs of Avian Influenza (AI) vary from mild respiratory distress to death in chickens, while wild birds are generally asymptomatic [3,4]. The highly pathogenic H5N1 subtype has been associated with the infection of humans in Southeast Asia and Africa who have come in direct contact with infected birds [5,6]. These concerns persist as human infections with an avian H7N9 virus were first reported in 2013 [7].

The advent of the quantitative real time reverse transcriptase-PCR assay (RRT-PCR) revolutionized AI diagnosis by increasing the sensitivity, specificity, and speed of analysis compared to traditional diagnostic methods [8-13]. RRT-PCR tests are designed in regions of high sequence conservation, and produce only plus/minus results, but phylogenetic sequence analysis has demonstrated multiple sublineages, particularly in the HA subtypes. This may lead to a lack of discrimination between H5 sequences, based on pathogenicity or geographical origin [14]. RRT-PCR, in general, is also limited because it relies on specific primers, which may fail when the corresponding viral sequences mutate [14,15].

Microarray technology has been applied to a wide variety of applications including gene expression studies, drug discovery, analyses of evolutionary relationships, and detection of nucleotide mutations. Microarrays have an unparalleled ability to analyze thousands of nucleic acid sequences simultaneously and because of this, DNA microarrays can be used as a diagnostic tool to detect a wide range of bacterial and viral pathogens [16-18]. Oligonucleotide and cDNA microarrays have been successfully used to detect and identify influenza viruses [14,19-27]. Several groups have also reported the development of microarray-based technologies for detecting and typing avian influenza virus [28-30]. These microarray-based methods for avian influenza virus detection have been shown to be as sensitive as real-time RT-PCR and they can differentiate between different HA subtypes and pathotypes [29,31]. As more influenza sequence information becomes publicly available, microarrays can become more comprehensive in their representation of circulating strains. Because of the genetic diversity observed in strains of AIV, and the ready availability of HA sequence data; the microarray is an ideal tool for AIV detection and identification.

Both RRT-PCR and traditional microarray technologies are somewhat limited in their abilities to detect pathogens which do not have sequences in common with the amplifying primers or with the sequences placed on the array. This limits the use of these technologies in identifying new and emerging pathogens [32,33]. Advances in microarray fabrication techniques have dramatically increased the number of features that can be spotted on an array. This has led to the development of resequencing microarrays. By spotting degenerate probes on the array, these platforms sequence pathogens by direct hybridization. One such microarray, RPM-Flu v.3.1 can detect all known human and avian influenza serotypes as well as 84 additional viral and bacterial respiratory pathogens [34].

We have created a cDNA microarray containing probes representing the matrix (M), HA, and NA genes of AIV. The AIV cDNA microarray was evaluated against a panel of AIV isolates in order to appraise its application in AIV detection and identification. This AI cDNA microarray is capable of identifying and pathotyping currently circulating AI strains.

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Materials and Methods

RT-PCR and microarray probes

PCR primers were designed by evaluating multiple sequence alignments of the matrix, hemagglutinin, and neuraminidase genes using DNASTAR MegAlign software (DNASTAR; Madison, WI). Regions of identity among strains within the same HA type were used to design PCR primers for HA gene amplification. PCR primers (Sigma Aldrich –Sigma Genosys; St. Lois, MO) were resuspended in DEPC water at a concentration of 100 µM. The primer pairs used to amplify M, HA and NA gene segments are represented in Table 1. Gene segments were amplified in a One-Step RT-PCR reaction (Qiagen; Valencia, CA). PCR reactions were carried out in a 50 µL volume (50°C for 30 min, 95°C for 15 min, and 40 cycles of 94°C for 1 min, 60°C for 2 min, and 72°C for 2 min, followed by a final extension for 10 min at 72°C). AIV PCR products were purified using the Qiagen QIAQuick PCR Purification Kit (Qiagen; Valencia, CA). Amplification of the desired gene segment was confirmed by agarose gel electrophoresis. Amplified PCR products were resuspended in 10 µL of 0.01% SDS, 3X SSC (spotting solution), and stored in Genetix 384-well, V-bottom plates (Genetix; Boston, MA).

Microarray printing and processing

Each genetic element on the avian influenza virus cDNA microarray was spotted in duplicate in each of four subarrays, yielding a total of eight spots for each element. Silanated amine glass microarray slides (CEL Associates; Pearland, TX) were placed inside an OmniGrid Accent robotic spotter (Genomic Solutions, Gene Machines, Ann Arbor, MI) and spotting was conducted at room temperature and 65% humidity. After spotting, the slides were left to dry at room temperature and humidity for 30 min and then UV cross linked in a Stratagene Stratalinker 2400 (Stratagene; La Jolla, CA) at 400 mJ. Slides were blocked at 55°C in a solution of 1% BSA, 3.5X SSC, and 10% SDS for 20 min followed by three washes in ddH_2O. Slides were then boiled for 3 min to denature the double-stranded cDNA, followed by a cold-fix in 100% ice-cold ethanol for 5 sec. Slide quality was confirmed by staining with SYBR Green II dye (Invitrogen; Carlsbad, CA).

Sample preparation

AIV RNA was isolated from allantoic fluid via Trizol® extraction, resuspended in 50 µL of DEPC water, and stored at -80°C. The Ambion Amino Allyl Message Amp II aRNA Amplification Kit (Ambion; Austin, TX) was used to create indirectly-labeled aRNA (amplified RNA) from the starting viral RNA. A modified T7 Oligo(dT) Primer (5'-AAACGACGGCCAGTGAATTGTAATA CGACTCACTATAGGCGCAGCAAAAGCAGG-3') containing a T7 promoter sequence and the AIV Uni3 primer [10] was used instead of the proprietary kit primer in order to prime reverse transcription and first strand cDNA synthesis. Eleven µL of viral RNA in DEPC water was used as starting material. Two rounds of amplification were performed according to the manufacturer's protocol. Dye coupling was completed using AlexaFluor 555 (Invitrogen, Molecular Probes; Carlsbad, CA). Labeled aRNA was purified and dye incorporation efficiency was validated using a NanoDrop® ND-1000 UV-Vis Spectrophotometer (NanoDrop Technologies; Wilmington, DE).

Hybridization and analysis

10 µg of labeled aRNA target was concentrated by evaporation to 6 µL, incubated at 95°C for 1 min and resuspended in 29 µL of preheated (65°C for 3 min) Telechem UniHyb Hybridization buffer (Telechem; Sunnyvale, CA). The 35 µL total volume was hybridized to the microarray slide under a Nunc mSeries LifterSlip (Nunc Brand; Rochester, NY). Hybridization was carried out for 3 hr in a 50°C water bath. After hybridization, unbound probe was removed by washing with 0.5X SSC, 0.01% SDS for 5 sec followed by one wash with 0.2X SSC, 0.2% SDS for 15 min. This was followed by two washes in 0.2X SSC for 1 min. each and finally three washes with ddH_2O. Slides were dried via centrifugation and placed in an arrayWoRxe Biochip Reader (Applied Precision; Issaquah, WA) for scanning and analysis of fluorescent intensities.

Files of scanned fluorescent intensities were transferred from the arrayWoRxe Biochip Reader to SoftWoRx Tracker (Applied Precision; Issaquah, WA) for data analysis from which values for spot mean intensity, background mean intensity, and background standard deviation were determined. Background intensities were determined using the SoftWoRx Tracker cell method. On each slide, spot intensities were normalized to that slide's mean background spot intensity. Elements on the array were considered positive for hybridization if ≥ 75% (6/8) of the spots on the array had spot-

Strain	Subtype	Gene	Forward Primer	Reverse Primer	Amplicon Length
PekingDuck/Singapore/645/97	H5N3	HA5	CAGATTTGCATTGGTTACCATGC	GATTTCACRTATTTGGGGCATTC	920
		NA3	AGCAAAAGCAGGTGCGAG[3]	CGATCCAGGTTCATTGTC	1385
Ck/PA/13609/93	H5N2	M	AGCAAAAGCAGG[1]	GACGATCAAGAATCCACAATA	849
		HA5	TCTGCATTGGTTATCATGC	TATTGCTCCAAATAGGCCTC	990
		NA2	ATGAATCCAAATCAGAAGATAATAAC	CCATCAGGCCATGAGCCTG	1310
Ck/Puebla/8624-602/94	H5N2	HA5	AGCCAAAAGCAGGGGT[3]	AGTAGAAACAAGGGTG[2]	995
		NA2	ATGAATCCAAATCAGAAGATAATAAC	CCATCAGGCCATGAGCCTG	1323
Tk/WI/68	H5N8	HA5	GGTTATCATGCAAACAATTC	TATTGCTCCAAACAGACCTC	982
Ck/DE/HOBO/03	H7N2	M	AGCAAAAGCAGG[1]	GACGATCAAGAATCCACAATA	848
Ck/NY/13142-5/94	H7N2	HA7	AGCCAAAAGCAGGGGA[3]	AGTAGAAACAAGGGTG[2]	1726
Tk/OR/71	H7N3	HA7	AGCCAAAAGCAGGGGA[3]	AGTAGAAACAAGGGTG[2]	1726
		NA3	AGCAAAAGCAGGTGCGAG[3]	CGATCCAGGTTCATTGTC	1383
Ck/Korea/96006/96	H9N2	M	AGCAAAAGCAGG[1]	GACGATCAAGAATCCACAATA	849
		HA9	AGCCAAAAGCAGGGGA/T[3]	AGTAGAAACAAGGGTG[2]	1727
		NA2	ATGAATCCAAATCAGAAGATAATAAC	CCATCAGGCCATGAGCCTG	1382

[1]Uni3' (AGCAAAAGCAGG), [10]
[2]Derivative of Uni5' (AGTAGAAACAAG), [10]
[3]Extended derivative of Uni3'

Table 1: Primers used for the amplification of avian influenza genes (HA, NA, and M).

normalized intensity values that were >2.5X the mean background intensity. Some experiments resulted in more than one element on the array being determined positive for hybridization. In those cases, a one-way ANOVA analysis ($p<0.01$) on the average spot normalized intensity values was performed to determine which microarray element exhibited the highest spot-normalized intensity.

Results

Microarray design

The M, HA, and NA genes from various strains of AIV were amplified using the primers listed in Table 1. Primers were derived from alignments of 24 M, 137 NA1, 418 N2, 156 N3, 114 H5, 157 H7, and 81 H9 AIV gene sequences. An example of one such alignment is the HA5 alignment depicted in Figure 1. Based on this alignment, five H5 clades roughly corresponding to three geographic regions were assigned. The four H5 hemagglutinin elements on the microarray represent four of these five designated clades. CK/PA/13609/93 (H5N2) is contained within the USA1 clade, CK/Puebla/8624-602/94 (H5N2) belongs to the Mexico and Central America clade, TK/WI/68 (H5N8) is contained within the USA3 clade, and Peking Duck/Singapore/645/97 (H5N3) belongs to the Eurasia clade. The USA2 (wildfowl) clade is not represented on the array.

The avian influenza microarray was designed as illustrated in Figure 2 and contains elements for three HA subtypes (H5, H7, H9), three NA subtypes (N1, N2, N3), M, and the Newcastle disease virus fusion (F) gene, as a negative control. The matrix genes from three different HA subtypes of AIV (CK/PA/13609/93 (H5N2), CK/DE/HOBO/03 (H7N2), and CK/Korea/96006/96 (H9N2)) are present on the microarray to identify Type A influenza regardless of HA or NA subtype [35]. PCR products from the 16 unique genetic elements were spotted in duplicate to create a 32 spot sub-array. Four duplicate sub-arrays are created on each slide resulting in 8 "spots" for each genetic element.

AIV panel

All elements on the array were evaluated and confirmed for their specificity and utility with homologous hybridizations using aRNA from the same strain used to make that element on the array (data not shown). The ability of the microarray to HA- and NA-subtype AIV was determined by the analysis of 10 blind RNA samples. Each viral RNA sample was amplified, indirectly labeled with fluorescent dye and hybridized to the AI microarray as described in Materials and Methods. The mean fluorescent intensity values of each spot and its mean background intensity value was determined and spots with mean intensity values <2.5X the mean background intensity values were eliminated from analysis and considered negative for hybridization. Elements on the array were considered positive if ≥ 75% of the eight spots on the array passed this analysis. Hybridizations that resulted in more than one HA element being scored as positive were further analyzed by performing a one-way ANOVA analysis on the average spot normalized intensity values.

Use of the microarray is depicted in Figure 3 for sample B. All 24 M gene elements (100%) had positive hybridization signals (>2.5X mean background intensity) identifying the sample as a type A influenza. Similarly, the N1 genetic element (and none of the N2 and N3 elements) had positive hybridization signals (>2.5X mean background intensity) allowing the sample to be neuraminidase sub-typed as N1. For only one HA element (CK/NY/13142-5/94– H7) did 75% of the spots exhibit positive hybridization signals (>2.5X mean background intensity) allowing sample B to be sub-typed as H7. The identification of sample B as an H7N1 type A AIV was determined to be correct when the code for the blind samples were read (Table 2, Softbill/IL/33445-136/92 H7N1).

The results for a second example, sample C, is show in Figure 4A and 4B. Again, all 24 M gene elements (100%) had positive hybridization signals identifying the sample as a type A influenza and 100% of the N1 gene elements had positive hybridization signals allowing it to be neuraminidase sub-typed as N1. The only HA elements to pass the criteria for hybridization were those elements representing the H5 subtype. However, two H5 elements passed the hybridization screening criteria (CK/PA/13609/93 and Peking Duck/Singapore/645/97) with the Peking Duck/Singapore/645/97 H5 spot normalized intensity being 3-fold more intense (Figure 4B). Statistical analysis (ANOVA) confirmed that the hybridization signal to the H5 element from Peking Duck/Singapore/645/97 was significantly higher ($p<0.0013$) than the CK/PA/13609/93 H5 signal, indicating that the H5 gene from sample C was most likely related to the Eurasian H5 phylogenetic clade. This analysis was found to be correct as sample C was coded for an H5N1 type A influenza (CK/Hong Kong/220/97).

A more complex example was provided by sample D, Figure 4C and 4D. All 24 matrix gene elements (100%) had positive signals indicating that the sample was a type A influenza. In this example, 100% of the N2 gene elements had positive hybridization signals allowing it to be neuraminidase sub-typed as an N2 virus. However, in this instance, five HA elements passed the formal screening criteria for hybridization, three elements representing the H5 subtype and two elements representing the H7 subtype. The spot normalized intensity value for the H5 element representing the Mexico and Central American clade (CK/Puebla/8624-602/94) was determined to be significantly higher ($p<9.16$ E-11) than the other four spot normalized intensity values, indicating that the H5 gene from sample D was most likely related to the Mexico and Central America H5 phylogenetic clade. The microarray analysis that sample D of the blind panel was an H5N2 type A influenza originating in Mexico or Central America was confirmed (Table 2, H5N2 Ck/Puebla/8624-602/94).

A summary of the microarray analysis for the panel of AIV isolates is summarized in Table 2. The AI cDNA microarray correctly identified 100% (10/10) of the unknown isolates correctly as type A influenza viruses. Correct HA and NA subtyping was achieved for 70% (7/10) of the isolates. In one instance, sample A, the HA subtyping was incorrect as there was no element corresponding to the correct HA subtype (H1) on the microarray. Similarly, for sample F no NA subtype was determined as there was no element corresponding to the correct NA subtype on the array (N7). In two instances, samples F and H, correct H7 subtypes were not identified as the only two H7 elements present on the microarray are representative of strains isolated in the United States.

With only one exception, the HA sequences of the samples used to evaluate the microarray are known. A comparative sequence analysis (Clustal W version 1.83) analysis was performed on each of these hemagglutinin gene sequences with respect to the hemagglutinin gene sequences present on the microarray in order to evaluate the ability of the microarray to detect and hybridize to heterologous sequences [36]. The comparisons are illustrated in Table 3. The analysis showed that of the isolates correctly HA sub-typed by the microarray, there was 78-100% homology between the unknown and the correct HA elements on the microarray. When sequence homology was < 78%, the microarray was unable to correctly identify the HA subtype.

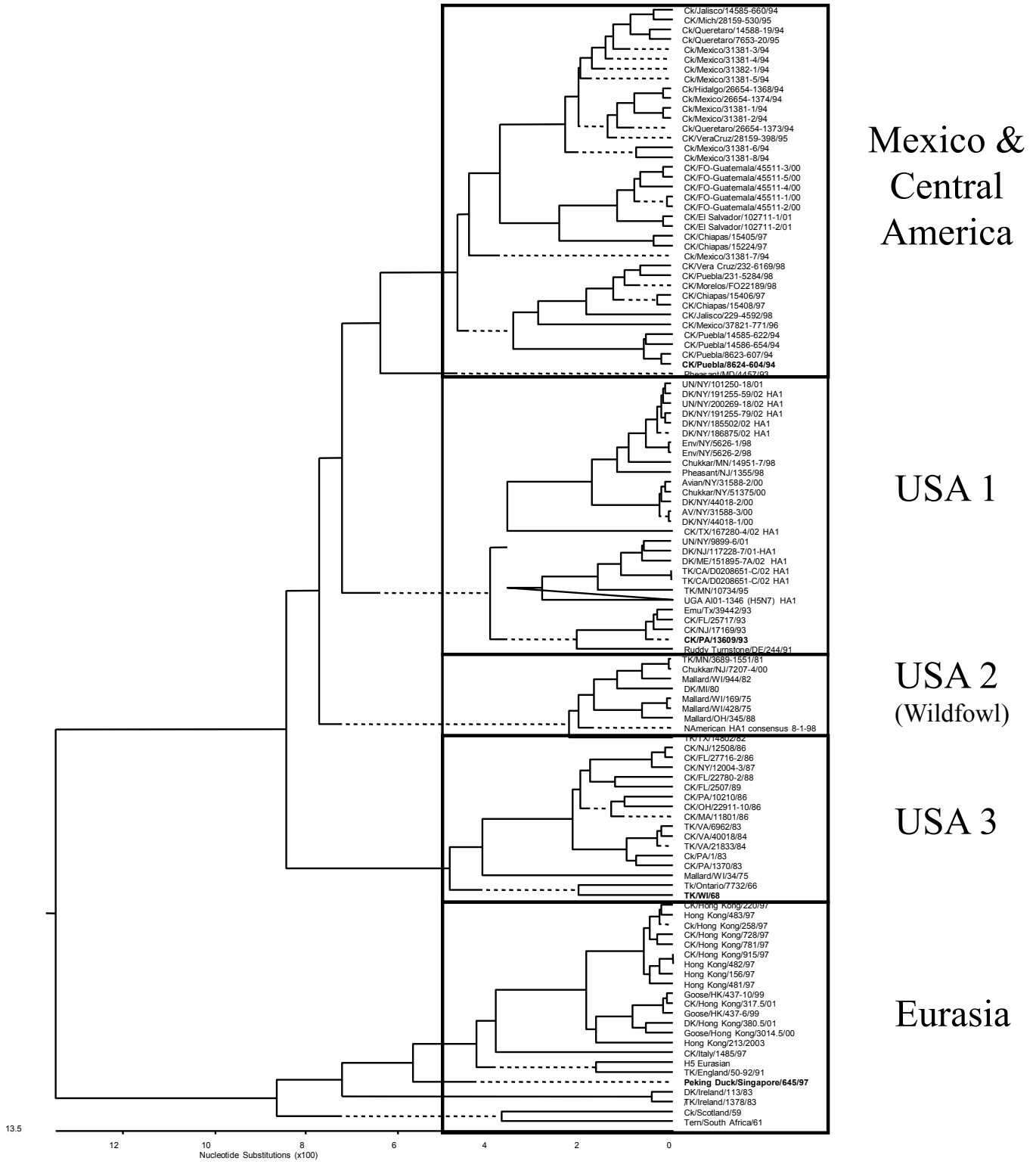

Figure 1: Phylogenetic analysis of 114 AIV HA5 genes.HA5 gene sequences were aligned using DNASTAR MegAlign software (DNASTAR; Madison, WI) and organized into five clades which generally correspond to distinct geographic regions or species (USA2 – wildfowl).

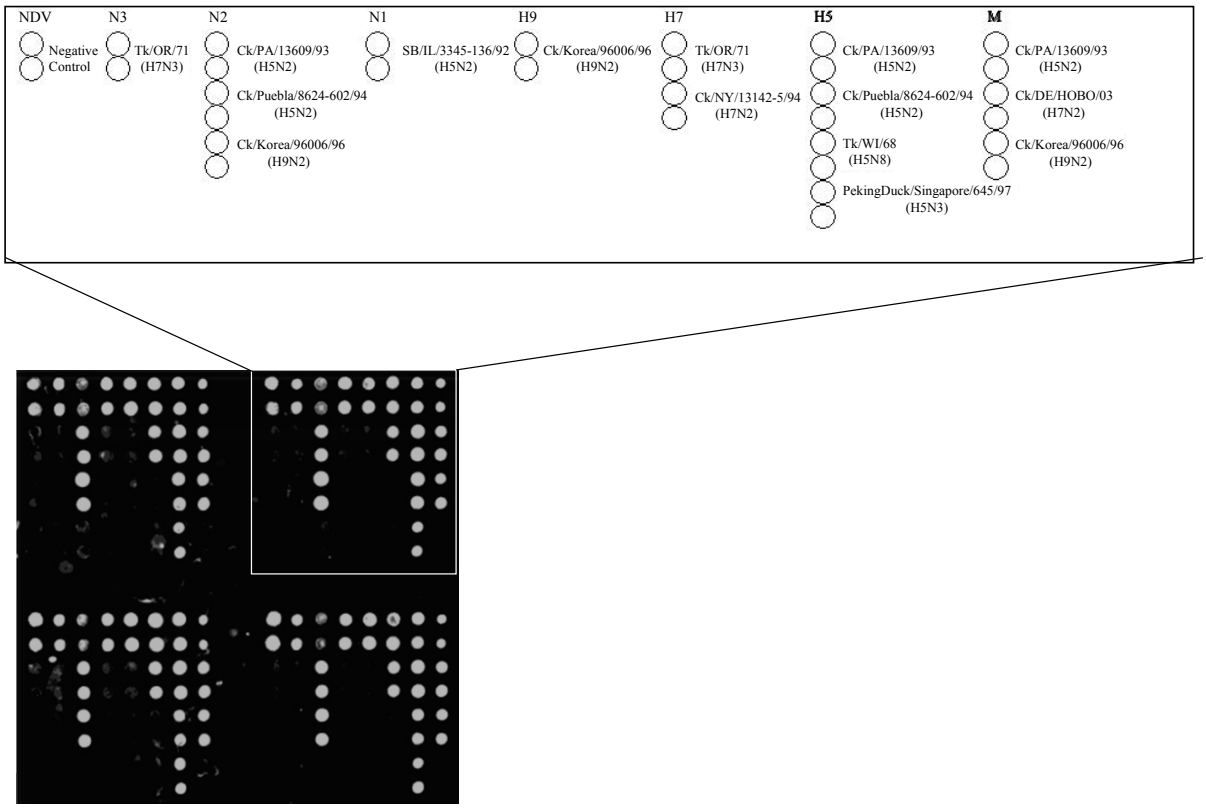

Figure 2: Design of the avian influenza cDNA microarray. Image of a spotted microarray containing four identical sub-arrays stained with SYBR Green II dye (Invitrogen; Carlsbad, CA).Each sub-array contains elements (2 spots/element) for three HA subtypes (HA5, HA7, HA9), three NA subtypes (NA1, NA2, NA3), M, and the Newcastle disease virus F gene, as a negative control. The genetic source of each genetic element is indicated.There are 32 spots per sub-array and 4 sub-arrays on each slide for a total of 128 spots, representing 16 unique genetic elements.

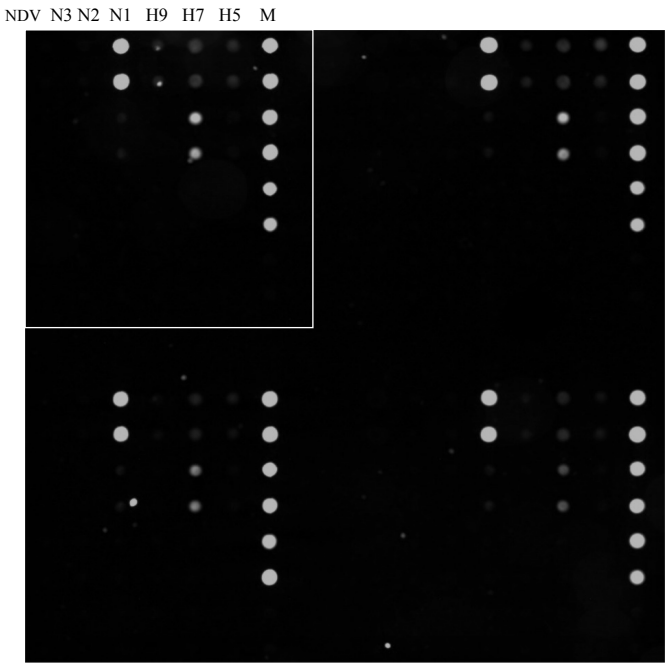

Figure 3: Microarray analysis of test sample B.Image of a microarray slide hybridized to fluorescently-labeled RNA amplified from test sample B as described in Materials and Methods.Elements corresponding to specific genetic elements is spotted in columns (Figure 2) and is labeled for one of the four sub-arrays present on the slide.

Discussion

New diagnostic capabilities are needed to address the number of genetically unique influenza virus strains circulating in both avian and mammalian species, the potential for interspecies transmission, and the global dissemination of certain AIV isolates. The time limitations of virus isolation in embryonating eggs (3 days – 2 weeks) is an unacceptable delay given the rapid spread of certain AIV isolates, and the limited subtype information available from RRT-PCR severely restricts important information from being obtained in a single experiment. Several investigators have developed microarrays for the identification and typing of avian influenza [28-31,33]. These platforms have been designed to be robust and inclusive. By contrast, the low-density AIV cDNA microarray described in this paper is avian specific and is focused on the most significant circulating avian influenza subtypes: H5, H7, and H9, and N1, N2, and N3. These hemagglutinin subtypes were selected because H5 and H7 avian influenza viruses are currently the only HA subtypes known to be capable of mutating from low pathogenicity to high pathogenicity. H9 is the most prevalent circulating hemagglutinin subtype in the avian and has also demonstrated the capability for avian-to-human transmission [37].

The low density cDNA microarray described here can detect, identify, HA and NA subtype, and in the case of the HA5 subtype, phylogenetically/geographically characterize avian influenza isolates. A blind panel of ten avian influenza isolates, A-J, was tested against our array to validate its detection and subtyping ability (Table 2). Utilizing the M gene as a pan-influenza marker, all 10 samples were identified as being type A influenza viruses. The array was able to correctly HA- and NA-subtype subtype 7/10 test samples. Due to the number and

Unknown Sample, Strain, and Subtype		Microarray Result
A - Dk/NJ/7717-70/95	H1N1	H7 N1
B - SB/IL/33445-136/92	H7N1	H7 N1
C - Ck/HongKong/220/97	H5N1	H5 Eurasia N1
D - Ck/Puebla/8624-602/94	H5N2	H5 Mexico N2
E - Ck/NJ/12220/97	H9N2	H9N2
F - Ck/VIC/85	H7N7	Type A
G - Dk/Singapore/97	H5N3	H5 Eurasia N3
H - Ck/PAK/1369-CR2/95	H7N3	H_N3
I - Ck/Scotland/59	H5N1	H5 Eurasia N1
J - Ck/NJ/294508-12/04	H7N2	H7 N2

Table 2: Results from panel of AIV isolates. Subtype and strain designation as indicated in key after determination of microarray result.

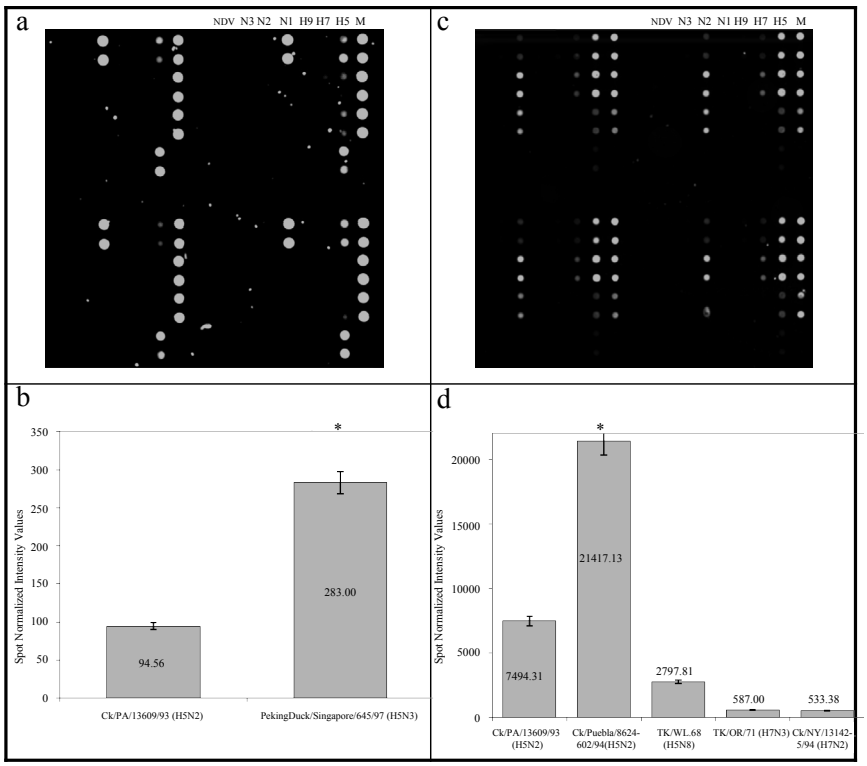

Figure 4: Microarray analysis of test samples C and D.Image of microarray slides hybridized to fluorescently-labeled amplified RNA derived from test sample C (Panel A) and test sample D (Panel C).The organization of the genetic elements on the slide is indicated.Panel B shows the mean spot normalized intensity values for the two HA5 elements which produced positive hybridization signals to sample C.Panel D shows the mean spot normalized intensity values for the five genetic elements (3 HA5 and 2 HA7) which produced positive hybridization signals when hybridized to sample D.Intensity values were compared using one-way ANOVA analysis to the highest value.

	Unknown Panel									
	A	B	C	D	E	F	G	H	I	J
Microarray Element	Dk/NJ/7717-70/95 (H1N1)	SB/IL/33445-136/92 (H7N1) *	Ck/HongKong/220/97 (H5N1)	Ck/Puebla/8624-602/94 (H5N2)	Ck/NJ/12220/97 (H9N2)	Ck/VIC/85 (H7N7)	Dk/Singapore/97 (H7N3)	Ck/PAK/1369-CR2/95 (H7N3)	Ck/Scotland/59 (H5N1)	Ck/NJ/294508-12/04 (H7N2)
PekingDuck/Singapore/645/97 (H5N3)	62	-	93	80	57	35	100	54	88	10
Ck/PA/13609/93 (H5N2)	60	-	79	91	57	36	82	32	80	18
Ck/Puebla/8624-602/94 (H5N2)	60	-	78	100	57	36	80	32	78	27
Tk/WI/68 (H5N8)	61	-	79	88	57	32	80	32	82	28
Ck/NY/13142-5/94 (H7N2)	37	-	36	29	40	75	26	76	34	95
Tk/OR/71 (H7N3)	37	-	35	27	39	75	25	76	30	87
Ck/Korea/96006/96 (H9N2)	57	-	58	54	87	37	57	36	56	35

Table 3: Homology between hemagglutinin gene elements present on the microarray and hemagglutinin gene sequences from the test panel. Clustal W (version 1.83) sequence analysis of test panel hemagglutinin gene sequences and hemagglutinin gene sequences present on the microarray. Only partial sequence (445 bp) was available for the HA gene from sample B (*) precluding analysis.

genetic diversity of the H5 genetic elements on the array, we were able to correctly identify the geographic origin of all four unknown H5 samples, including the Eurasian H5N1 strain CK/Hong Kong/220/97. These results demonstrate the capability of identifying AIV strains and gives further insight into the epidemiology of H5 strains. Therefore, this technique, as validated here, can identify type A influenza via the conserved matrix gene, differentiate between the H5, H7, and H9 hemagglutinin subtypes, and also differentiate between the N1, N2, and N3 neuraminidase subtypes of avian influenza.

The limitations of the current version of this cDNA microarray relate to the limited number of elements present in the system. Three samples were incorrectly or incompletely identified. In one case we were unable to subtype the NA gene due to the absence of the appropriate NA subtype on the array (N7). Similarly, the lack of an H1 element prevented the HA subtyping of one sample.

The array successfully identified both H7 samples (B and J) which were determined to have originated in the United States. A phylogenetic analysis of 157 H7 sequences revealed considerable sequence diversity within this subtype (data not shown). The two incorrectly identified H7 samples (F and H) were of non-U.S. origin and no elements representing non-U.S. clades of H7 are present on the array. Although HA positive hybridization signals can be generated between sequences exhibiting as little as 27% sequence identity, correct HA subtype identification required 78% sequence homology (Table 3). The F and H H7 sequences exhibited 75-76% sequence identity to the sequences of the HA elements present on the microarray, below the required nucleotide identity needed to yield a statistically significant hybridization signal.

The AIV cDNA microarray can potentially be applied to clinical diagnostic samples, but the sensitivity of the array to detect low viral concentrations in various clinical samples (nasal or oral/pharyngeal swabs, serum, lung tissue, and blood) has yet to be determined. Genetic elements from other viral and/or bacterial pathogens could also be added to the array to increase the diagnostic range of the test [27,36]. To begin development of an avian respiratory pathogen specific microarray, the addition of Newcastle disease virus (NDV) elements would provide the greatest benefit. In the field, velogenic NDV (the most virulent form of NDV) and HPAI are often indistinguishable due to the severity and variability of their clinical signs.

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