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Development of a DNA microarray-based multiplex assay of avian influenza virus subtypes H5, H7, H9, N1, and N2

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Summary. – Outbreaks of highly pathogenic avian influenza have caused considerable economic losses in the poultry industry and have also resulted in human deaths since 2004. Rapid subtyping of highly pathogenic avian influenza viruses(HPAIVs) in clinical specimens is a prerequisite of prompt control of disease and prevention of its spreading. In this study, we describe development of a DNA microarray-based detection and subtyping of HPAIVs in field samples. DNA copies of matrix (M) protein genes for the H5, H7, and H9 subtypes of hemagglutinin (HA) and the N1 and N2 subtypes of neuraminidase (NA) were prepared by RT-PCR and specific primers and then spotted onto aldehyde slides to form DNA microarrays. The HPAIV samples to be tested were subjected to total RNA isolation, RT-PCR with universal primers and Cy3 labeling, and the obtained double-stranded DNAs (targets) were finally hybridized with DNA microarrays (probes). A fluorescent spot on the microarray, detected by scanning indicated positive hybridization, i.e. the involved subtype. The assay was specific as various heterologous viruses or HPAIVs of other subtypes tested were negative. No cross-hybridization among different subtypes could be detected. The assay was more sensitive than RT-PCR and chicken embryo inoculation and could be also used for field samples. Summing up, the assay has proved useful for simultaneous detection and differentiation of main epidemic HPAIV subtypes.

Keywords: DNA microarray; avian influenza virus; subtyping

Introduction

Avian influenza is a highly contagious viral disease affecting the respiratory, digestive, and/or nervous systems of many species of birds. It is caused by influenza A virus of the family *Orthomyxoviridae*. Based on the two types of viral surface glycoproteins, avian influenza viruses (AIVs) are subdivided into 16 HA and 9 neuraminidase (NA) subtypes (Fouchier *et al.,* 2005). Alternatively, AIV strains are classified as either low pathogenic (LPAI) or highly pathogenic (HPAI), on the basis of specific molecular genetics and pathogenesis. Most AIVs are LPAI viruses, with most common subtype H9N2, which are usually associated with mild disease in poultry. In contrast, HPAI viruses with most common subtypes being H5N1 and H7N1, have caused major poultry outbreaks and severe economic losses (Abdelwhab *et al.,* 2012). Over the past decade HPAI and LPAI viruses have been found to be associated with human infections (Peiris *et al.,* 2007). Human cases of AI (H5N1) have now been reported in Asia, Africa, the Pacific, Europe, Near East, Indonesia, and Vietnam (Peiris *et al.,* 2004). Rapid and sensitive diagnostic tools for the identification of influenza viruses are crucial for

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Abbreviations: AIV = avian influenza virus; HPAIV = highly pathogenic AIV; LPAIV = low pathogenic AIV; HA = hemagglutinin; NA = neuraminidase; M = matrix; IBDV = infectious bursal disease virus; NDV = Newcastle disease virus; IBV = infectious bronchitis virus

early detection, epidemiologic investigations, and timely responses to an influenza pandemic threat.

Currently, viral culture paired with serological HA typing is the standard method for detecting and typing influenza A viruses (Alexander, 2008). These procedures are time-consuming and can take days or even weeks to provide specific results. Over the last decade nucleic acid testing of AIV has been widely implemented by using target amplification methods such as RT-PCR, real-time RT-PCR, nucleic acid sequence-based amplification and loop-mediated isothermal amplification (Bao *et al.,* 2012). These technologies allow for rapid diagnosis of AIV, in particular the subtyping of the H5 subtypes (Vernet, 2007). These methods use universal primers and probes to detect all subtypes of influenza A, and specific primers and probes to distinguish between HA and NA gene subtypes in a simple, single tube assay format following RNA isolation (Ivshina AV *et al.,* 2004)

DNA microarrays have been proven to be powerful tools for virus identification and subtyping. For example, the high-density oligonucleotide microarray was able to identify foodborne RNA viruses including hepatitis A virus and coxsackievirus (A and B). The FluChip microarray has been documented for detection of H1N1, H3N2, and H5N1 strains (Mehlmann*et al.,* 2006). More recently, a low-density microarray has been described to be a useful diagnostic tool for H5N1 virus (Zhao *et al.,* 2010). However, all of these microarray based assays require multiple, specific primer sets and two or more enzymatic amplification steps of influenza viral RNA prior to hybridization. The multiple steps involved in these assays make them complicated, time consuming and susceptible to contaminations, and may produce false negative results due to the presence of gene mutations, PCR inhibitors and RNA degradation. In this study, development of a DNA microarray-based assay of HPAIVs of subtypes H5, H7, H9, N1, and N2 in field samples is described. The assay has proved specific and more sensitive than RT-PCR and chicken embryo inoculation.

Materials and Methods

Viruses. A/goose/Guangdong/1/96 (H5N1), A/African/starling/983/7 (H7N1), A/chicken/Heibei/1/2002 (H7N2), and A/turkey/ Wisconsin/1/66 (H9N2) were maintained in authors' laboratory. These viruses were inoculated into embryonated chicken eggs and were characterized by HA inhibition (HAI) and neuraminidase inhibition test. Viral propagation and viral RNA extraction were performed in the biosafety level 3 (BSL-3) laboratory. Other avian viruses were used for the specificity tests of the microarray in this experiment, including the Newcastle disease virus (NDV), infectious bronchitis virus (IBV), infectious laryngotracheitis virus (ILV), and infectious bursal disease virus (IBDV). These viruses were kindly provided by the avian disease research groups at the Harbin Veterinary Research Institute, Chinese Academy of Agricultural Sciences, Harbin, China. AI, ND and IB viruses were propagated in 9-day-old embryonated chicken eggs for 3 days at 37°C. IBDV was propagated in chicken embryonic fibroblast cells.

DNA microarray preparation. Primers based on conserved sequences of the matrix (M) protein gene of AIV; the HA gene of AIV subtypes H5, H7, and H9; and the NA gene of subtypes N1 and N2 were designed in this experiment. A primer pair of chicken GAPDH gene was designed as the positive control. All of the primers were derived from the alignments and analyses of the nucleotide sequences retrieved from the considerable GenBank data, and conducted by the MegAlign program (DNASTAR, USA). The sequences of primers are listed in Table 2.

Viral RNA was extracted from infected allantoic fluid using the Trizol LS Reagent (Invitrogen). RT-PCR was performed using the SuperScript one-step RT-PCR kit (Promega). PCR-amplified viral cDNAs were subsequently cloned using a Topo TA cloning kit (Invitrogen). Recombinant plasmids were purified from lacZ mutant bacteria and sequenced as described previously (Willer *et al.,* 1999). The recombinant plasmids were used for PCR. The PCR products were purified using MicroSpin S-400 columns (Amersham/Pharmacia Biotech), precipitated with ethanol, and re-suspended in 30 µl of spotting solution (3× SSC, 1.5 mol/l betaine). The concentration of purified PCR products was determined and adjusted to ~300 ng/µl. Finally, the DNA was transferred into Genetix 384-well plate for spotting purposes.

Aldehyde slides were spotted using a biochip array at room temperature and 65% relative humidity. Printed arrays were air dried for a few minutes at 50 to 60°C and stored overnight at room temperature over desiccant. The arrays were rehydrated for 10 sec at 65°C, dried briefly at 80°C on a heating block, and then UV cross-linked in a UVP CL-1000 Ultraviolet Crosslinker at 65 mJ. Subsequently, the arrays were washed and treated with sodium borohydride (Sengupta *et al*., 2003) and stored in a dark, humidity-free environment (Li *et al*., 2001).

HPAIV sample preparation. Total RNA was extracted from infected allantoic fluid or the respective organism by homogenizing cells using the TRIzol LS reagent. Reverse transcription was performed according to the method of Lai and Chambers (Lai et al., 1995). PCR was carried out in a 50-µl volume containing 5 U of Taq DNA polymerase (Takara); 0.5 mmol/l each dATP, dCTP, and dGTP; 0.2 mmol/l dTTP (Sigma); 0.1 mmol/l Cy-3 labeled dUTP (Amersham); 1× thermophilic DNA polymerase buffer (Promega); 2 mmol/l $MgCl_2$; and 4 µg of the uni12 and uni13 universal primers (Offringa et al., 2000). The primer extension reaction consisted of 95°C for 2 min, followed by 30 cycles of 94°C for 1 min, 40°C for 2 min, and 72°C for 3 min, with a final extension step of 72°C for 10 min. The PCR products were purified with the QIAquick PCR purification kit (Qiagen) and resuspended in 10 µl of 1x hybridization buffer containing 0.1 mol/l of Cy3-labeled internal positive control probe.

Fig. 1

AIV diagnostic DNA microarray layout
Legend: Negative (N) and positive (G) controls, M gene (M), H5, H7, H9, 1xN1, 2xN1, 3xN1, and 1xN2, 2xN2, and 3xN2.

Hybridization. The hybridization procedures were conducted according to the manual and are briefly described as follows. 5 µl of each of the fluorescent PCR products and a hybridization buffer (50% formamide, 10× SSC, 0.2% SDS) was denatured at 95°C for 4 min, cooled in an ice bath for 2~3 min, and then applied to the arrays and overlaid with 18×18-mm^2 coverslips. Microarray hybridization was performed in a humid chamber for 8 hrs at 42°C. After hybridization, the slides were washed consecutively with 2× SSC plus 0.2% sodium dodecyl sulfate (42°C, 5 min), 500 µl 2× SSC (30°C, 5 min) and 500 µl 0.2× SSC (30°C, 5 min). Hybridized slides were scanned with a VersArray ChipReader system (Bio-rad). The microarray images were captured at a laser power of 100 and a PMT gain of 80 and analyzed with ScanArray software Genpixpro4.0.

Determination of sensitivity and specificity. A serial dilution of the H5 subtype AIV allantoic fluid (3.6x10^7 EID50/ml) was analyzed using the microarray assay. The limit of detection was defined as the lowest concentration at which a positive signal could be detected. PCR fragments for H5, N1, and M genes from the H5N1 strain (A/goose/Guangdong/1/96) were loaded onto the sub-array separately and simultaneously to determine whether any cross-hybridization occurred. Specificity of the multiplexed microarray assays was evaluated by testing cross-reactivity with RNA extracted from 15 subtypes of influenza A virus and other viral pathogens such as IBDV, IBV, and NDV.

Typing and subtyping influenza viruses by RT-PCR and array hybridization. Examinations of HA subtype 1 in 15 and 30 experimentally infected samples and 21 field samples was performed by RT-PCR and microarray.

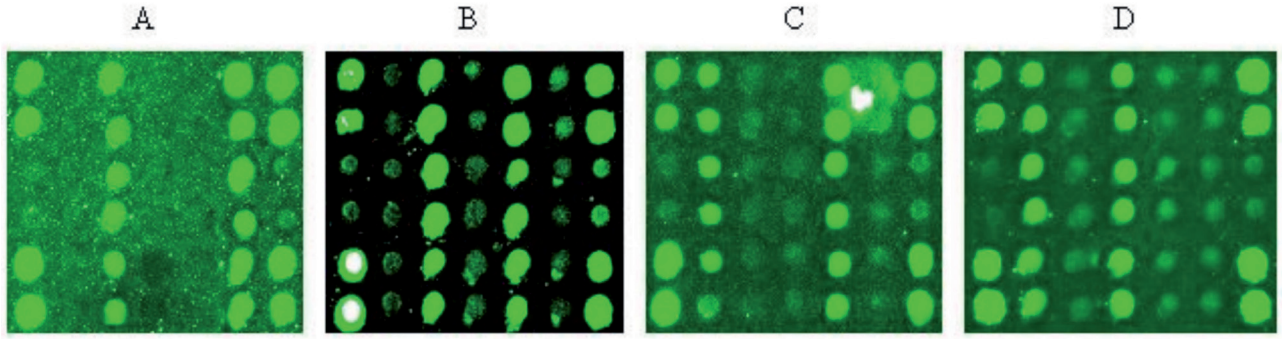

Fig. 2

Specificity of the DNA microarray assay
Results with the subtype H5N1 (A), H7N1 (B), H7N2 (C), and H9N2 (D).

Table 1. Detection of AIVs using various diagnostic methods

Samples	Positive / total		
	Egg inoculation	RT-PCR	DNA microarray
Field samples			
Cloacal swabs, 24 hr	4/10	5/10	4/10
Cloacal swabs 48 hr	6/6	6/6	6/6
Cloacal swabs 72 hr	4/4	4/4	4/4
Tissue samples	10/10	10/10	10/10
Total %	24/30 (80.0%)	23/30 (76.6%)	24/30 (80.0%)

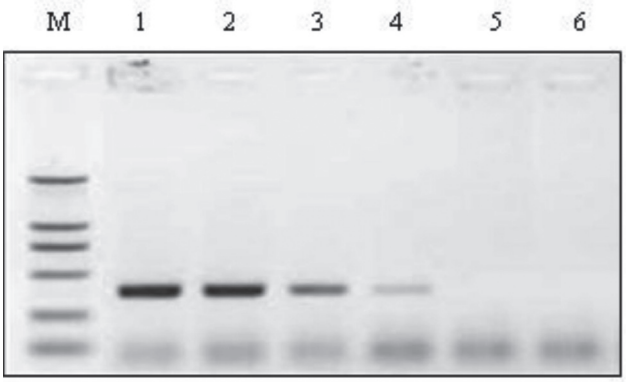

Fig. 3

Sensitivity of RT-PCR
10^{-1} to 10^{-5} dilutions of a H5N1 AIV sample (lanes 1~5), DNA size marker (lane M), and negative control (lane 6).

Results

DNA microarray preparation

Eleven separate fragments of genes for AIV HA, NA, M, and GAPDH from chicken were cloned. The recombinant plasmids were re-amplified by PCR and the PCR products were spotted onto aldehyde slides. The DNA microarray layout is shown in Fig. 1.

Specificity and sensitivity of the assay

The specificity of the microarray-based assay was tested with RNAs extracted from 15 HA and 9 NA subtypes of influenza virus and other avian viruses such as Newcastle disease (ND) and infectious bursal disease (IBD). The results showed that the microarray has good specificity for detection and differentiation of main epidemic HPAIV subtypes. Other avian viruses were also tested to be negative with the DNA microarray. A selection of the resulting fluorescence images is shown in Fig. 2.

The sensitivity of the microarray-based assay was tested with 10 serial dilutions of H5N1 subtype AIV allantoic fluid. A comparison with the RT-PCR results revealed that for RNA at 10^{-4}-fold dilutions, the microarray produces a higher fluorescent intensity whereas the RT-PCR array cannot provide a clear result (Fig. 3 and 4).

Subtyping of HPAIVs in field samples

To further assess the utility of the array in diagnostics, we tested 30 infected samples and 21 field samples. For the cloacal swabs and tissue samples, the microarray test had a 100% coincidence rate with chicken embryo inoculation and 96.7% coincidence rate with RT-PCR. Of the 21 field samples tested, 14 were AIV positive using the microarray assay. This was in agreement with the RT-PCR and chicken embryo inoculation results (Table 1).

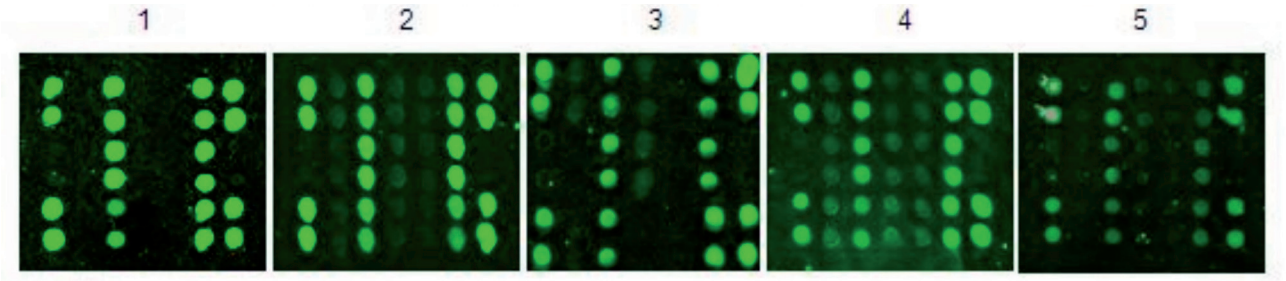

Fig. 4

Sensitivity of the DNA microarray assay
10^{-1} to 10^{-5} dilutions of a H5N1 AIV sample (chips 1~5).

Discussion

The gold standard for detecting AIV is virus isolation via viral culture in embryonated chicken eggs, followed by subtyping and using the hemagglutinin inhibition assay and NA serology test. Virus isolation is a sensitive and reliable method of AIV identification, having the advantage that the virus can be recovered. On the other hand, the main drawbacks are that it can only detect live viruses and requires greater biosecurity, time, and expense. Immunology methods for testing AIV have low skill requirements, but poor sensitivity and specificity. Antibody detection assay can only indicate whether the sampled animals were infected or vaccinated with AIV, but cannot reflect the current state of infection and demonstrate whether the animal is a virus carrier.

Many molecular approaches for subtyping AIV have been developed in recent years. RT can be combined with enzyme-linked immunosorbent assay with high sensitivity and low requirements for laboratory equipment and operators, but fails to detect multiple subtypes simultaneously (Leijon *et al.,* 2011). Multiplexed real-time RT-PCR combined with hemagglutinin inhibition test and real-time RT-PCR targeting matrix and hemagglutinin genes with separate procedures has the advantages of low cost and high-throughput; however, in dealing with avian influenza, real-time RT-PCR has its restrictions (Spackman *et al.,* 2003). Due to the high sequence homology between subtypes, strain differentiation is not possible. In addition, due to the changing nucleotide composition of AIV, real-time RT-PCR primers must be constantly re-evaluated. Real-time RT-PCR can be multiplexed, but is restricted to detect only two genes per assay and is therefore limited in full subtyping AIV. Usually, real-time RT-PCR must be combined with sequencing in order to confirm its results or to discriminate between them; this is both labor-intensive and time-consuming. Nucleic acid sequence-based amplification has been employed to detect AIV H5 or H7 (Wang *et al.,* 2010). Nucleic acid sequence-based amplification is confined to the detection of RNA and is easily affected by RNAase. Moreover, it is highly sequence specific, which limits its ability to detect a virus that is constantly mutating at the nucleotide level. The use of microarrays to type and subtype human influenza viruses has been recently reported. However, few such reports focus on the detection and subtyping of avian viruses.

Our results demonstrate the ability of a DNA microarray to detect, identify, and subtype various avian influenza isolates. Our method, as validated herein, can identify type

Table 2. Primers used in gene cloning

Primer	Sequence (5'-3')	Acc. No.	PCR product size (bp) and position (nt-nt)
5H (f)	GGAATATGGTAACTGCAACACCA	AF144305	372(876-1247)
5H (r)	AACTGAGTGTTCATTTTGTCAATG		372(1224-1595)
7H(f)	AATGCACAAGGAGGAGGAACT	Z12617	501(1129-1629)
7H(r)	TGACGCCCCGAAGCTAAACCA		501(1609-2109)
9H(f)	TCAACAAACTCCACCGAAACTGT	D90305	732(79-810)
9H(r)	TCCCGTAAGAACATGTCCATACCA		732(787-1518)
1xN1(f)	CTACTTGTCAATGGTGAATG	AF250362	614(1-614)
1xN1(r)	CAGTCGAGTTGAATGCTCCT		614(595-1208)
1xN2(f)	CACAACTGCCTGTTCCATCAT AATGCACTTTCTTTCTGA	AF250362	624(405-1028)
1xN2(r)			624(1008-1631)
1xN3(f)	ACCAAGCAACTGACTCAAACC	AF250362	537(873-1409)
1xN3(r)	TGAATCCAAATCAAAAGATAAT		537(1388-1924)
2xN1(f)	GCAAAAGCAGGAGTGAAAAGATGAA ACAACTTGAGCTGGAC-	J02136	567(2-568)
2xN1(r)	CATGCTA		567(546-1112)
2xN2(f)	TTTGCATCCTTTTTCTCAAGGACA ACGGGCCTATTAGGAGCCTCT-	J02136	617(308-924)
2xN2(r)	TC		617 (904-1520)
2xN3(f)	GCCCATTGTCAGGAAGTGCTCAG	J02136	599(816-1414)
2xN3(r)	ATTGATGTTCGCCCCATCAGG		599(1394-1992)
M(f)	TTCTAACCGAGGTCGAAAC AAGCGTCTACGCTGCAGTCC	AF144306	229(33-261)
M(r)			229 (242-470)
Gapdh(f)	ACCATCTTCCAGGAGCGAGATC	XR_005803	340 (1531-1870)
Gapdh(r)	GCCATCCACAGTCTTCTGAGTG		340(1849-2188)
Uni12	AGCAAAAGCAGG	–	–
Uni13	AGTAGAAACAAGG		

A influenza via the conserved matrix gene, differentiate between the H5, H7, and H9 HA subtypes, and differentiate between the N1 and N2 subtypes of avian influenza.

This method can be applied to clinical situations pending further validation experiments to determine the sensitivity of the array and to increase the number of representative HA and NA (i.e., H1–4, H6, and H8). In addition, other viral and/or bacterial pathogens could be added to the array to increase its diagnostic power and to aid the medical community in differential diagnoses.

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