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DNA microarray-based solid-phase RT-PCR for rapid detection and identification of influenza virus type A and subtypes H5 and H7☆

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Abstract

Endemic of avian influenza virus (AIV) in Asia and epizootics in some European regions have caused considerable public concern on a possible pandemic of AIV. A rapid method for virus detection and effective surveillance in wild avian, poultry production as well as in humans is required. In this article, a DNA microarray-based solid-phase polymerase chain reaction (PCR) approach has been developed for rapid detection of influenza virus type A and for simultaneous identification of pathogenic virus subtypes H5 and H7. This solid-phase RT-PCR method combined reverse-transcription amplification of RNA extract in the liquid phase with sequence-specific nested PCR on the solid phase. A simple ultraviolet cross-linking method was used to immobilize the DNA probes over an unmodified glass surface, which makes solid-phase PCR a convenient possibility for AIV screening. The testing of 33 avian fecal and tracheal swab specimens was completed in less than 2 h with 94% accuracy.

Keywords: Avian influenza virus; Solid-phase RT-PCR; DNA microarray

1. Introduction

Avian influenza virus (AIV) belongs to the virus family Orthomyxoviridae. It is an infectious disease and has caused great economic losses in poultry and started to threaten human life (WHO, 2010). The AIV genome consists of 8 segments of negative-sense single-stranded RNA. Based on antigenic differences in the hemagglutinin (HA) and neuraminidase (NA) proteins, AIV is divided into 16 HA and 9 NA subtypes, and is classified as either highly pathogenic avian influenza virus (HPAIV) or low pathogenic avian influenza virus (LPAIV) based on the ability to cause disease and mortality (Alexander, 2007).

Although virus cultivation combined with hemagglutination inhibition (HI) and neuraminidase inhibition (NI) tests is still the standard for influenza virus detection (Knipe and Howley, 2001), many nucleic acid amplification techniques, such as reverse transcriptase PCR (RT-PCR) (Lee et al., 2001), real-time RT-PCR (Spackman et al., 2002), and real-time NASBA (van Aarle et al., 2006), have been developed for more sensitive and rapid diagnosis.

PCR amplification products can be analyzed in parallel by using high-density oligonucleotide microarrays. Several studies about microarray for AIV identifications have been reported (Gall et al., 2009a; Han et al., 2008; Kessler et al., 2004; Li et al., 2001; Wang et al., 2008). In these studies, the AIV viral RNA was amplified through RT-PCR and the resulting product was hybridized on the spotted probes and detected through fluorescent labeling. Although differentiation of all 16 HA subtypes of AIV has been demonstrated (Gall et al., 2009b), hybridization as a post-PCR process inevitably increased the complexity of the assay.

To enable multiplex amplification and sequence detection done in one step, microarray-based solid-phase PCR was developed (Huber et al., 2001). This approach combined

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liquid-phase PCR with simultaneous nested amplification using oligonucleotide primers attached to a glass slide. The PCR amplicons remained covalently bound to the solid surface and could be identified by fluorescence scanning. This method was further applied for single nucleotide polymorphism detection (Huber et al., 2002; Pemov et al., 2005; Shapero et al., 2001), bacteria identification (Mitterer et al., 2004), and disease genotyping (Khodakov et al., 2008). Solid-phase PCR has shown its advantages of high throughput, ease of operation, and specific detection; however, protocols for attaching probes on the glass surface were complicated and the method has never been applied for a fragile material like viral RNA.

In this article, we extended the strategy and developed a DNA microarray platform where RT-PCR of viral RNA occurred in the liquid solution and nested amplification occurred on the microarray elements with specific oligonucleotide probes for interrogating different influenza types. A very simple ultraviolet (UV) cross-linking immobilization procedure was used to immobilize TC-tagged oligonucleotide probes on a bare glass slide. With this method, AIV can be quickly detected with subtypes of H5 and H7 simultaneously identified. Thirty-three specimens from wild birds and experimentally infected chicken were tested. The microarray-based platform can be a good tool for rapid detection of AIV and could be widely employed for on-site screening of potential AIV carriers in wild and domestic poultry.

2. Materials and methods

2.1. Virus strains

Four inactivated AIV strains, namely, H1N1 A/DK/ALB 35/76, H5N1 A/CK/Scotland/59 06.04.67, H16N3-A/Gull/Denmark/68110/02, and H7N5 A/Chick/Nether/2993–17/03 AV 506/03, were used in this study. Two strains, a Newcastle diseases virus and an infectious bursal disease virus (IBDV), were used to check the specificity of the PCR. All the strains were kindly supplied by the National Veterinary Institute, Technical University of Denmark.

2.2. Sample collection from wild birds for method validation

A total of 33 samples which include cloacal ($n = 24$) and tracheal ($n = 9$) swabs were collected and used to validate the solid-phase RT-PCR method. The samples were originating from a national surveillance program for AIV in wild birds ($n = 20$) and chickens experimentally infected with AIV ($n = 13$). The swabs were dipped in 2 mL phosphate-buffered saline to release the fecal or tracheal materials from the swabs. Nine hundred microliters of the mixture sample was subjected to a centrifugal force of 18,894×g for 5 min (Eppendorf, Hamburg, Germany) to remove debris, and the supernatant was collected for isolating viral RNA.

2.3. Isolation of RNA from AIV virus

Isolation of RNA from AIV virus was performed using the RNeasy Kit (Qiagen, Hilden, Germany) according to the manufacturer's instruction. The RNA was eluted using 50 μL of RNase-free water. The isolated RNA was used as a template for evaluation of the solid-phase RT-PCR.

2.4. Primers and probes

Table 1 shows a list of primers and probes used for amplification. The matrix (M) gene was selected for AIV screening as it has been found to be conserved across all type A influenza viruses (Starick et al., 2000). Primers and probes for H5 and H7 subtypes were targeted at the HA gene of the influenza virus with avian origin. Each forward primer was Cy5 labeled at the 5′ end so that the solid-phase RT-PCR results could be directly visualized on the microarray. The probes (immobilized primers) were modified at the 5′ end with a poly(T)10-poly (C)10 tail to facilitate the attachment to the solid substrate as previously described (Gudnason

Table 1
List of specific primers and probes used in this study

Type or subtype	Target gene	Primers and probes	Sequences (5′–3′)	Amplicon (bp)
A	Matrix	Forward DB-MF (24–47, sense) Reverse DB-MR (101–124, antisense) M gene probe (74-93, sense)	CY5-AGA TGA GTC TTC TAA CCG AGG TCG TGC AAA AAC ATC TTC AAG TCT CTG TTTTTTTTTTCCCCCCCCCC TCA GGC CCC CTC AAA GCC GA	100[a]
H5	HA	Forward DB-H5LH1 (1508-1532, sense) Reverse DB-H5RH1 (1640-1659, antisense) H5 Probe (1613-1636, sense)	CY5-ACA TAT GAC TAC CCA CAR TAT TCA G AGA CCA GCT AYC ATG ATT GC TTTTTTTTTTCCCCCCCCCC TCW ACA GTG GCG AGT TCC CTA GCA	151[b]
H7	HA	Forward DB-LH6H7 (1477-1503, sense) Reverse DB-R4H7 (1586-1608, antisense) H7 Probe (1545-1574, sense)	CY5-GGC CAG TAT TAG AAA CAA CAC CTA TGA GCC CCG AAG CTA AAC CAA AGT AT TTTTTTTTTTCCCCCCCCCC CCG CTG CTT AGT TTG ACT GGG TCA ATC T	131[c]

[a] Based on GenBank accession no. CY015082 (A/chicken/Scotland/1959 [H5N1]).
[b] Based on GenBank accession no. CY015081 (A/chicken/Scotland/1959 [H5N1]).
[c] Based on GenBank accession no. AB438941 (A/chicken/Netherlands/2586/2003 [H7N7]).