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Electronic microarray assays for avian influenza and Newcastle disease virus

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Microarrays are suitable for multiplexed detection and typing of pathogens. Avian influenza virus (AIV) is currently classified into 16 H (hemagglutinin) and 9 N (neuraminidase) subtypes, whereas Newcastle disease virus (NDV) strains differ in virulence and are broadly classified into high and low pathogenicity types. In this study, three assays for detection and typing of poultry viruses were developed on an automated microarray platform: a multiplex assay for simultaneous detection of AIV and detection and pathotyping of NDV, and two separate assays for differentiating all AIV H and N subtypes. The AIV–NDV multiplex assay detected all strains in a 63 virus panel, and accurately typed all high pathogenicity NDV strains tested. A limit of detection of 10^1–10^3 $TCID_{50}$/mL and 200–400 EID_{50}/mL was obtained for NDV and AIV, respectively. The AIV typing assays accurately typed all 41 AIV strains and a limit of detection of 4–200 EID_{50}/mL was obtained. Assay validation showed that the microarray assays were generally comparable to real-time RT-PCR. However, the AIV typing microarray assays detected more positive clinical samples than the AIV matrix real-time RT-PCR, and also provided information regarding the subtype. The AIV–NDV multiplex and AIV H typing microarray assays detected mixed infections and could be useful for detection and typing of AIV and NDV.

1. Introduction

Avian influenza and Newcastle disease viruses have worldwide distribution and are the most economically important viruses of poultry and, in the case of avian influenza, may also be the source of highly pathogenic human strains. Avian influenza virus (AIV) and Newcastle disease virus (NDV) can cause severe respiratory and gastrointestinal diseases, and in the case of NDV also neurological diseases in poultry. Both are reportable diseases, notifiable to federal veterinary agencies and the World Organization for Animal Health (OIE).

Avian influenza viruses (family *Orthomyxoviridae*, genus *Influenza A*) and NDV (family *Paramyxoviridae*, genus *Avulavirus*), also known as avian paramyxovirus type I (APMV-1), are single-stranded, negative-sense RNA viruses with genomes of approximately 13.5 kb and 15.2 kb, respectively. Influenza A viruses are classified into 16 H and 9 N subtypes according to the antigenic characteristics of two surface proteins, the hemagglutinin (HA) and neuraminidase (NA) (e.g. H5N1 AIV). The H5 and H7 subtypes are the most important in poultry as these viruses may be highly pathogenic and cause severe outbreaks. Low pathogenic H5 and H7 strains are also reportable/notifiable because mutations in the furin cleavage site of the HA protein can lead to high virulence. Virulence of NDV is a function of the sequences surrounding the protease cleavage site of the fusion (F) protein (Rott and Klenk, 1988). Sequences with one or two pairs of basic amino acids followed by a phenylalanine correlate with high virulence, while sequences with a single basic amino acid followed by a leucine correlate with low virulence.

Highly virulent AIV causes systemic diseases, whereas low pathogenic strains only affect the upper respiratory tract. For AIV, vaccines are usually derived from inactivated autogenous viruses or viruses of the same H type, whereas for NDV, strains of low pathogenicity are used as live vaccines. The genome of these viruses undergoes frequent mutations which continually leads to emergence of new variants, and this has been problematic for both laboratory diagnosis and vaccine production.

There are a number of detection methods for AIV and NDV but virus isolation and RT (reverse transcription)-PCR, including real-time RT-PCR, are commonly used (Berhane et al., 2012; Wise et al., 2004; Spackman et al., 2002). Virus isolation is labour intensive, and in the case of AIV involves inoculation of fertilized eggs, and takes several days to achieve results. Real-time RT-PCR has the advantage of speed and is often used for monitoring

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outbreaks. However, real-time PCR can fail to detect viral variants with mutations in the probe/primer binding regions and the ability of existing commercial real-time thermal cyclers to multiplex targets is currently limited. For AIV, subtyping has traditionally been done by hemagglutination inhibition and neuraminidase inhibition assays following virus isolation, but these assays are labour-intensive, time-consuming and require microscopic observation. For NDV, conventional methods used for pathotyping involve inoculation of live birds and calculating the intracerebral pathogenicity index (ICPI), the intravenous pathogenicity index (IVPI), and the mean death time (MDT) (Alexander, 1997). As with conventional methods for AIV subtyping, these methods are labour-intensive, time-consuming, and additionally involve testing in "live animal" systems.

Rapid subtyping and pathotyping methods utilizing PCR, sequencing and microarrays such as a multiplex RT-PCR that differentiates between a subset of AIV subtypes (He et al., 2009) and real-time RT-PCRs for NDV pathotyping have recently been developed (Aldous et al., 2001; Farkas et al., 2009; Fuller et al., 2009; Li and Zhang, 2004). However, alternative methods are desirable and needed for multiplex detection of mixed infections in poultry and rapid typing of all 16 H subtypes for surveillance data collection and outbreak management.

Microarrays allow a high degree of multiplexing, are suitable for AIV typing, and several microarrays have been described for this purpose (Gyarmati et al., 2008; Lu et al., 2009; Metzgar et al., 2010; Wang et al., 2008). However, these microarrays either do not differentiate between all 16 H types (Wang et al., 2008), require several manual liquid handling steps (Gyarmati et al., 2008), or are designed primarily for human viruses (Lu et al., 2009). These methods also all rely on passive hybridization, thus increasing the amount of time required to obtain results. One microarray which utilizes rapid electronically driven printing and hybridization for AIV H typing has been described (Gall et al., 2009b).

Three electronic microarray assays were developed in this study for detection and typing of AIV and NDV: two individual assays for H and N typing of AIV, and one multiplex assay for detection of AIV and NDV and for NDV pathotyping. All three assays show promise for diagnostic use in rapid detection and typing of avian influenza and Newcastle disease.

2. Materials and methods

2.1. Samples

A total of 173 samples were obtained from experimental infections of turkeys and chickens for validation of the AIV H and N subtyping assays. Birds (10 per group) were infected with 10^6 EID_{50} viruses of H5, H6, H7 or H9 subtypes via the oronasal route. A high and a low pathogenic strain of each of H5 and H7 subtypes were used for individual groups. Cloacal swabs were taken before, and at various days post-inoculation (DPI) between days 1 and 29, and samples from five birds of each group were pooled into two lots. Oropharyngeal swabs were collected from DPI 3 onwards. For testing of other subtypes in which no clinical samples were available, 40 cloacal and oropharyngeal samples from uninfected chickens were inoculated (spiked) with approximately 10^7 EID_{50}/mL of propagated virus (Table 1). In addition, 50 field negative cloacal and oropharyngeal chicken and turkey samples determined to be avian influenza-negative by real-time RT-PCR (Spackman et al., 2002) were used to determine specificity. Due to depletion of samples used in validation of other tests, 28 AIV-negative field samples were spiked with AIV of H3 ($n = 1$), H5 ($n = 17$), H7 ($n = 9$), and H10 ($n = 1$) subtypes. These were used for validation of the AIV–NDV multiplex assay, along with 40 avian influenza-negative field samples (Table 1). Furthermore, 60 samples were obtained from chickens infected experimentally via the cloacal route with a high pathogenicity NDV strain (APMV-1 Amazon) with a titer of approximately 10^6 EID_{50} for validation of the AIV–NDV multiplex assay (Table 2). Samples were collected on DPI 1–3. In addition, 40 NDV-negative field samples were used for validation. All experimental infection studies conformed to the guidelines of the Canadian Council on Animal Care. Virus isolation, real-time RT-PCR, typing and titer determination of AIV were performed by the National Reference Laboratory (Winnipeg, MB) according to published methods (Berhane et al., 2012). Virus isolation, pathotyping and titer determination of NDV were performed as recommended by OIE terrestrial animal health manual (http://www.oie.int/fileadmin/Home/eng/Health standards/tahm/2.03.14 NEWCASTLE DIS.pdf).

2.2. RNA extraction and PCR

For AIV, 500 μL of allantoic fluid or clinical swab material (for validation of the H and N typing assays) were mixed with 500 μL of RLT buffer and extracted using the RNeasy® Kit (QIAGEN Inc., Mississauga, ON, Canada) using the recommended protocol with a slight modification. Briefly, 1000 μL of the sample in RLT® buffer mixture described above was mixed with 500 μL 70% ethanol before being applied to the RNeasy® column. Final elution was performed with 50 μL nuclease-free water (Spackman and Suarez, 2008). For all NDV and AIV clinical samples used for validation of the AIV–NDV multiplex microarray assay, RNA was extracted from samples using Ambion MagMAX™ Viral RNA Isolation Kit (Applied Biosystems, ON, Canada) according to the manufacturer's instructions.

A multiplex RT-PCR with six primers was developed to amplify simultaneously approximately 280, 332 and 468 bp of the AIV matrix (M), NDV fusion (F) and NDV M genes, respectively (Table 3). All reverse primers were synthesized with the reverse complementary sequence of the Red Universal Reporter Probe at the 5′ end (Huang et al., 2009). Two published primers (Creelan et al., 2002) and four in-house designed primers were used. For AIV H typing, two previously published primers (Hoffmann et al., 2001; Phipps et al., 2004) were modified to amplify an approximately 640 bp fragment in the HA2 region. For AIV N typing, two newly designed and one modified (Alvarez et al., 2008) forward primers were combined with a previously published reverse primer. Primers used in the RT-PCR are listed in Table 3.

All RT-PCRs were performed in 25 μL reactions, and the SuperScript™ III One-Step RT-PCR System with Platinum® *Taq* DNA Polymerase (Invitrogen, Carlsbad, CA) was used. Reverse transcription (RT) was carried out for 30 min at 60 °C and followed by denaturation at 95 °C for 2 min for the AIV H typing assay and the AIV–NDV multiplex assay. RT for the AIV N typing assay was performed as in Alvarez et al. (2008). PCR for the AIV H typing assay consisted of 40 cycles: 94 °C for 60 s, 55 °C for 60 s, 68 °C for 90 s, with a final extension step of 68 °C for 7 min. The 25 μL reaction mixture consisted of 1 μL of RNA, 1 μL of enzyme mix in 1× reaction buffer, and 1 μM of each primer. As described by Alvarez et al. (2008), the AIV N typing assay consisted of 8 cycles of step-down PCR at 94 °C for 30 s, 56 °C for 30 s (decreasing by 2 °C after each cycle until 42 °C) and 68 °C for 90 s. An additional 36 cycles consisted of 94 °C for 30 s, 46 °C for 30 s and 68 °C for 75 s. The reaction mixture used was the same as the AIV H typing assay, except that 1.2× reaction buffer was used. The forward primer 1 was used at 2 μM, whereas the remaining forward primers and the reverse primer were used at 1 μM. The AIV–NDV multiplex PCR consisted of 40 cycles: 94 °C for 30 s, 50 °C for 30 s, 68 °C for 45 s, with a final extension step of 68 °C for 7 min. The reaction mixture consisted of 1 μL of RNA, 1 μL of the enzyme mix in 1.2× reaction buffer,