**ORIGINAL ARTICLE**

# Multiplex one-step real-time PCR assay for rapid simultaneous detection of velogenic and mesogenic Newcastle disease virus and H5-subtype avian influenza virus

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## Abstract

H5 avian influenza virus (AIV) and velogenic Newcastle disease virus (v-NDV) are pathogens listed in the OIE Terrestrial Animal Health Code and are considered key pathogens to be eliminated in poultry production. Molecular techniques for rapid detection of H5 AIV and v-NDV are required to investigate their transmission characteristics and to guide prevention. Traditional virus isolation, using embryonated chicken eggs, is time-consuming and cannot be used as a rapid diagnostic technology. In this study, a multiplex real-time RT-PCR (RRT-PCR) detection method for six H5 AIV clades, three v-NDV subtypes, and one mesogenic NDV subtype was successfully established. The detection limit of our multiplex NDV and H5 AIV RRT-PCR was five copies per reaction for each pathogen, with good linearity and efficiency ($y = -3.194x + 38.427$ for H5 AIV and $y = -3.32x + 38.042$ for NDV). Multiplex PCR showed good intra- and inter-assay reproducibility, with coefficient of variance (CV) less than 1%. Furthermore, using the RRT-PCR method, H5 AIV and NDV detection rates in clinical samples were higher overall than those obtained using the traditional virus isolation method. Therefore, our method provides a promising technique for surveillance of various H5 AIV clades and multiple velogenic and mesogenic NDV subtypes in live-poultry markets.

## Introduction

Influenza viruses, which belong to the family *Orthomyxoviridae* [1], are single-stranded negative-sense RNA viruses. Among the four types of influenza viruses, A, B, C and D, influenza A viruses have the highest antigenic variability.

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There are 18 and 11 influenza virus hemagglutinin (HA) and neuraminidase (NA) subtypes, respectively [2]. Avian influenza viruses (AIVs) can infect a variety of animals, causing severe economic loss to the poultry industry and posing a threat to public health. Among them, the AIV H5 subtype, a highly pathogenic avian influenza virus (HPAIV), has been posing a substantial threat to public health [3], with H5N1 infections reported in 860 people, causing 454 deaths by May 2018 [4]. Therefore, H5 detection methods should focus on testing not only prevalent clades but also other clades as well. This is important in cases of reappearance of non-epidemic strains.

Newcastle disease, an acute infectious disease caused by Newcastle disease virus (NDV), is distributed across at least six continents [5] and affects more than 200 animal species. Like HPAIV, velogenic Newcastle disease virus (v-NDV) is a vital pathogen that is seriously harmful to the poultry industry. v-NDV has been listed in the OIE (Office International Des Epizooties) Terrestrial Animal Health Code along with HPAIV as a vital disease of which they must be informed [6]. Estimation of chicken embryo MDT

(mean death time) is used to distinguish the virulence of NDV isolates. Isolates with MDT <60 h, 61-90 h, and >90 h are characterized as velogenic, mesogenic, and lentogenic, respectively [7]. v-NDV infection often causes disease in poultry through single or multiple virus infections, with high morbidity and mortality.

In poultry, H5 HPAIV, and velogenic and mesogenic NDV infections can lead to respiratory symptoms, other clinical symptoms, and related pathological changes. Therefore, it is necessary to quickly diagnose these virus infections. However, there are difficulties in differential diagnosis when mixed infections with other avian viruses occur. The gold standard method of identifying H5 HPAIV and v-NDV encouraged by OIE is virus isolation using embryonated chicken eggs followed by serological tests and MDT analysis [8]. RRT-PCR (real-time reverse transcription polymerase chain reaction) [9] is a method with high specificity and sensitivity. Multiplex PCR methods for detection of H5 HPAIV and v-NDV have been reported [10, 11]. However, rapid and sensitive high-throughput techniques such as RRT-PCR are more suitable for epidemic surveillance in large numbers of chicken flocks.

In this study, a TaqMan RRT-PCR assay to efficiently and simultaneously detect H5 AIV and velogenic and mesogenic NDVs was successfully established by targeting the H5 HA and NDV F genes. Our results show that this multiplex RRT-PCR method is highly sensitive with good reproducibility and can be used in live-poultry markets for the simultaneous surveillance of multiple H5 AIV clades and multiple velogenic and mesogenic NDV subtypes.

## Materials and methods

### Virus strains

To test the sensitivity and specificity of H5 AIV and NDV multiplex PCR, eight H5 virus strains and seven NDV strains were used for comparison (Tables 1 and 2). The

**Table 1** The H5 AIV isolates used in this study

| Virus strain | Abbreviation | Subtype | Clade |
|---|---|---|---|
| A/chicken/Guangxi/5233/2002 | Gx5233 | H5N1 | 3 |
| A/goose/Jiangsu/650/2003 | JS650 | H5N1 | 2.5 |
| A/duck/Yangzhou/130/2003 | Y130 | H5N1 | 1 |
| A/chicken/Yang zhou/YJD/2014 | YJD | H5N6 | 2.3.4.4 |
| A/duck/Beijing/BJ7/2014 | BJ7 | H5N2 | 7.2 |
| A/goose/Eastern China/0403/2014 | 0403 | H5N1 | 2.3.2.1c |
| A/chichken/Guangdong/ GD1602/2016 | GD1602 | H5N6 | 2.3.4.4 |
| A/chicken/Jiangsu/6285/2016 | Z6285 | H5N1 | 2.3.2.1c |

**Table 2** The NDV isolates used in this study

| Virus strain | Subtype | Accession no. | Host |
|---|---|---|---|
| Mukteswar | Class II, III | JF950509.1 | Chicken |
| Herts/33 | Class II, IV | AY741404.1 | Chicken |
| F48E8 | Class II, IX | FJ436302.1 | Chicken |
| YB-04-15 | Class II, VIId | MK049165 | Chicken |
| LaSota | Class II | JF950510.1 | Chicken |
| LX | Class II, I | KF494201.1 | Chicken |
| YZ-1-07-Ch | Class I | GQ281095.1 | Chicken |

H5 AIVs examined included members of six different clades and three NA subtypes, covering a 14-year time span (Table 3). The NDV strains examined included three velogenic strains (Herts/33, F48E8, and YB-04-15), one mesogenic strain (Muktaswar), and three lentogenic strains (LX, LaSota and YZ-01-07-Ch).

In addition, AIVs of all for the other HA subtypes except for H2, including (A/duck/Shandong/SDd11/2013(H1N1), A/duck/Jiangsu/YZD3/2013(H3N2), A/duck/Anhui/AHd38/2014(H4N6), A/duck/Jiangsu/119/2015(H6N2), A/Chicken/Zhejiang/JX164/2015(H7N9), A/duck/Yangzhou/02/2005(H8N4), A/Chicken/Eastern China/0923/2015(H9N2), A/duck/Jiangsu/XZD53/2014(H10N7), A/duck/Jiangsu/YZD1/2013(H11N9)), infectious bursal disease virus (IBDV, AF092943), infectious bronchitis virus (IBV, EU031525), and Marek's disease virus (MDV, DQ530348), were included as negative controls. H2-subtype avian influenza viruses have not been endemic in eastern China in recent years and have an extremely low isolation rate. As a result, no H2 AIV strains were available for this study.

**Table 3** Sensitivity of the RRT-PCR method

| Strain | | Virus type | $EID_{50}$/0.1ml | Detection limit |
|---|---|---|---|---|
| YB-04-15 | | VIId Class II NDV | $10^{-9}$ | 1.0 $EID_{50}$ |
| Mukteswar | | III Class II NDV | $10^{-8}$ | 0.1 $EID_{50}$ |
| Herts/33 | | IV Class II NDV | $10^{-8}$ | 0.1 $EID_{50}$ |
| F48E8 | | IX Class II NDV | $10^{-8}$ | 1.0 $EID_{50}$ |
| YJD | | Clade 2.3.4.4 H5N6 AIV | $10^{-8}$ | 0.1 $EID_{50}$ |
| | 0403 | Clade 2.3.2.1c H5N1 AIV | $10^{-8.33}$ | 0.021 $EID_{50}$ |
| BJ7 | | Clade 7.2 H5N2 AIV | $10^{-6.625}$ | 0.42 $EID_{50}$ |
| Gx5233 (2002) | | Clade 3 H5N1 AIV | $10^{-7.625}$ | 0.42 $EID_{50}$ |
| JS650 (2003) | | Clade 2.5 H5N1 AIV | $10^{-7.33}$ | 20 $EID_{50}$ |
| Y130 (2002) | | Clade 1 H5N1 AIV | $10^{-9}$ | 10 $EID_{50}$ |

## Animals

Twenty 6-week-old specific-pathogen-free (SPF) chickens immunized with LaSota vaccine (hemagglutination inhibition [HI] titer: 6 $\log_2$) were purchased from Weike Technology Co. (Yangzhou, China). SPF chickens were randomly divided into two groups. All experiments involving live viruses and animals were performed in negative-pressure isolators with HEPA filters in biosafety level 3 animal facilities in accordance with the institutional biosafety manual. Animal suffering was minimized by providing free access to food and water and by frequent, twice a day, monitoring by laboratory staff. A 12-h light/dark cycle was maintained, and the temperature was kept between 20 and 25 °C.

## Multiplex PCR

### Primers and probes

Sequence alignments were made using DNA Star 8.1.3 software, and Primer Express 3.0.1 was used to identify suitable primers and probes in conserved H5 HA and NDV F gene regions. The primers and probes were as follows: HA forward primer, CTTGCGACTGGGCTCAGAAAT; HA reverse primer, TTTGGGTGGATTCTYTGTCTGC; HA probe, VIC-CATTCCYTGCCATCC-MGB; F forward primer, GGTCAATCATAGTCAAGTTGCTCC; F reverse primer, AACCCCAAGAGCTACACTGCC; F probe: FAM-AAGCGTTTYTGTCTCCTTCCTCC-BHQ1. The amplicons obtained using HA and F primer pairs were 178 bp and 210 bp in length, respectively. Alignment results are shown in Fig. 1 and Fig. 2.

## Plasmids

Plasmids pNDV/ZJ1 and H5-QD5HA, which contain the NDV F and H5 HPAIV HA genes, respectively, were constructed and reported previously [12, 13]. The two plasmids were used as quantitative standards for RRT-PCR, and a pNDV/ZJ1 and H5-QD5HA mixture was serially diluted to obtain a series with $5 \times 10^5$, $5 \times 10^4$, $5 \times 10^3$, $5 \times 10^2$, $5 \times 10^1$, and $5 \times 10^0$ copies of each plasmid/5 µL.

## Thermal cycling

Each multiple detection reaction was performed in a 20-µL final volume containing 5 µL of DNA template, 10 µL of 2× HiScript II U+ One Step qRT-PCR Mix (Vazyme Co., Nanjing, China), 1 µL of U+ Enzyme Mix, 0.35 µL of 20 µM HA-FP, 0.35 µL of 20 µM HA-RP, 0.35 µL of 20 µM F-FP, 0.35 µL of 20 µM F-RP, 0.35 µL of 20 µM HA-Probe, 0.30 µL of 20 µM F probe, 1.55 µL of $H_2O$, and 0.4 µL of 50x ROX reference dye. Thermal cycling was performed in an ABI 7500 real-time PCR instrument (Applied Biosystems) with the following conditions: 55 °C for 15 min; 95 °C for 5 min; and 40 cycles of 95 °C for 10 s and 60 °C for 31 s. Machine-collected data were further analyzed using 7500 System SDS 1.2 software.

## Artificially inoculated samples

To mimic clinical v-NDV and H5 AIV coinfection and to reduce the number of laboratory animals used, 20 chickens, separated into two groups of 10, were challenged with $10^6$ $EID_{50}$ of velogenic NDV F48E8 (group A) or $10^6$ $EID_{50}$ of YB0415 (group B) on day 0. On day 8, three birds in each
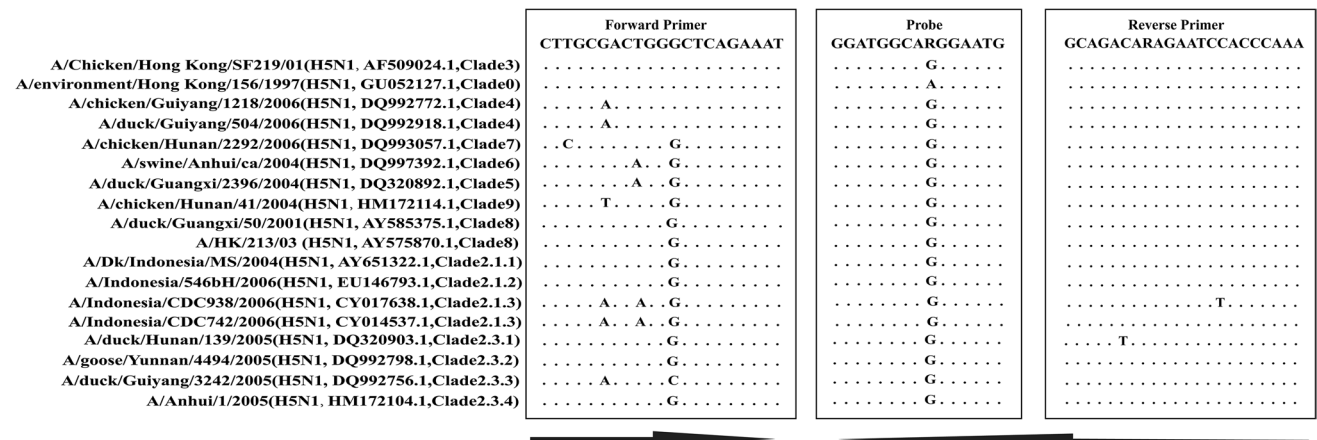


**Fig. 1** Alignment of the probe and primer sequences for H5 AIV. On the left of the figure are the names and accession numbers of H5-AIV strains of various clades. The forward primer, probe, and reverse primer are shown from left to right. As the reverse primer and probe were both on the reverse complementary strand, the arrow at the bottom shows the direction of amplification on the positive DNA strand. The dots indicate nucleotides that are identical in the primers or probe, and capital letters indicate nucleotides that differ

**Fig. 2** Alignment of the probe and primer sequences for v-NDV. On the left of the figure are the abbreviations or accession numbers of the v-NDV strains. The forward primer, probe and reverse primer are shown from left to right. As the reverse primer and probe were both on the reverse complementary strand, the arrow at the bottom shows the direction of amplification on the positive DNA strand. The dots indicate nucleotides that are identical in the primers or probe, and capital letters indicate nucleotides that differ

group were challenged with $10^3$ EID$_{50}$ of H5 AIV GD1602 (group A) or $10^3$ EID$_{50}$ of Z6285 (group B). Only three chickens were challenged with GD1602 or Z6285 per group, using eye drops, because H5 AIV infection without H5 AIV vaccination causes death.

Oropharyngeal and cloacal swab samples were collected from each chicken for determination of virus shedding on days 1, 3, 5, 7, 9, 11, and 13. Dead chickens were excluded from sampling because their stiffness would have made sample collection difficult, and also because the precise time of death was not known, making accurate comparisons of virus isolation and qPCR results difficult. Swab samples were immediately placed into 1 mL of antibiotic-containing PBS (penicillin, 2000 unit/mL; streptomycin, 10 mg/mL; gentamicin, 250 μg/mL; and kanamycin, 250 μg/mL) and stored at −70 °C. A 200-μL sample of this suspension was used for viral RNA extraction followed by RRT-PCR. Another 200-μL volume was used for virus isolation using 10-day-old embryonated SPF eggs. After incubation for 3 days at 35 °C, the presence of NDV and AIV in the eggs was determined by HA-HI assay with positive serum.

## Extraction of RNA from throat and stool specimens

A Easy Pure Viral DNA/RNA Kit (TransGen Co., Beijing, China) was used to extract viral nucleic acids from allantoic fluids or antibiotic-containing PBS. Oropharyngeal or cloacal samples (200 μL) were incubated with proteinase K at 56 °C for 15 min. After centrifugation, anhydrous ethanol was added to the filter column, and the mixture was passed through. After two washes using wash buffer, nucleic acids were eluted in 35 μL of elution buffer and stored at -70 °C for RRT-PCR.

## Results

### Verification of the primers and probes

According to an epidemiological survey of the laboratory and data published in recent years, the current epidemic H5 subtype avian influenza viruses are mainly clade 2.3.4 and clade 2.3.2, so we chose these two clades as the focus of our attention. In order to give consideration to other clades, we also selected some strains of clades 1, 2, 3, and 7, covering a wide range representing the general prevalence of H5. Similarly, the Newcastle disease viruses that we selected included typical velogenic, mesogenic and lentogenic strains.

Two primer-and-probe sets were designed and compared with H5 HA and NDV F gene sequences downloaded from the NCBI GenBank database. The H5 HA probe mostly matched the target gene, with some mispairings occurring in three main sites of the forward primer (Fig. 1). Considering the persistence of H5 AIV antigenic drift and antigenic shift, we believe that the originally designed primers and probe still correspond to the most conserved regions of the H5 HA gene. Moreover, the primer-and-probe set can accurately amplify the HA gene of H5 AIV strains from different years and various clades by RRT-PCR.

The sequences of the NDV F probe and primers were aligned with NDV F gene sequences downloaded from the NCBI GenBank database (Fig. 2). One nucleotide difference was identified in the F probe when compared with the VII subtype v-NDVs, and three sites that differed from the distant F48E8 v-NDV were identified. Moreover, comparison with F48E8 revealed two nucleotide differences in the forward primer.

## Establishment of a one-step RRT-PCR assay for H5 AIV and v-NDV

We further investigated the specificity of the designed primer and probe sets and determined the sensitivity of the assay using strains of different clades or subtypes (Table 3). All three v-NVD strains and one mesogenic strain were examined, and Herts/33, F48E8, YB-04-15 and Muktaswar were successfully detected. However, LX, LaSota, and YZ-01-07-Ch could not be amplified using the F primer and probe sets. The H5 HA probe and primers successfully detected members of various H5 AIV clades, including clade 2.3.4.4, clade 2.3.2.1c, clade 7.2, clade 3, clade 2.5, and clade 1, while the other AIV subtypes, IBDV, IBV, and MDV were not amplified in our RRT-PCR. These results show that our RRT-PCR assay for H5 AIV and NDV is highly specific for members of various H5 AIV clades as well as velogenic and mesogenic NDV.

The sensitivity of the F probe and primer set for NDV isolates of different subtypes was between 0.1 and 1.0 $EID_{50}$. The sensitivity of the H5 HA probe and primer set for the currently prevalent clades, clade 2.3.4.4 and clade 2.3.2.1c, was 0.1 $EID_{50}$ and 0.021 $EID_{50}$, respectively. The sensitivity of the H5 HA probe and primer set to previously prevalent clades, clade 7.2, clade 3, clade 2.5 and clade 1, was between 0.42 and 20 $EID_{50}$.

Using a mixture of pNDV/ZJ1 and H5-QD5HA plasmids as quantitative standards, we determined the sensitivity of our multiplex PCR. Six dilutions of the plasmid mixture (5, 50, 500, 5000, 50,000, and 500,000 copies of each plasmid) were used to obtain standard curves and amplification plots. Amplification plots of the HA and F primer-and-probe sets showed that the detection limit was five copies each for the F and HA genes per PCR reaction (Fig. 3). For standard curves, the HA and F primers were within the allowable limits, with $y = -3.194x + 38.427$ ($R^2 = 0.995$, efficiency $= 105.616\%$) and $y = -3.32x + 38.042$ ($R^2 = 0.999$, efficiency $= 100.072\%$), respectively. The HA primers had slightly higher efficiency than the F primers, which might have resulted from the shorter amplicon length produced by the HA primers. Moreover, when the two target genes were present in the same number of copies, the Ct value of the HA primers was lower than that of the F primers. This may have been due to differences in primer/probe binding efficiency, because the pNDV/ZJ1 plasmid is larger than the H5-QD5HA plasmid.
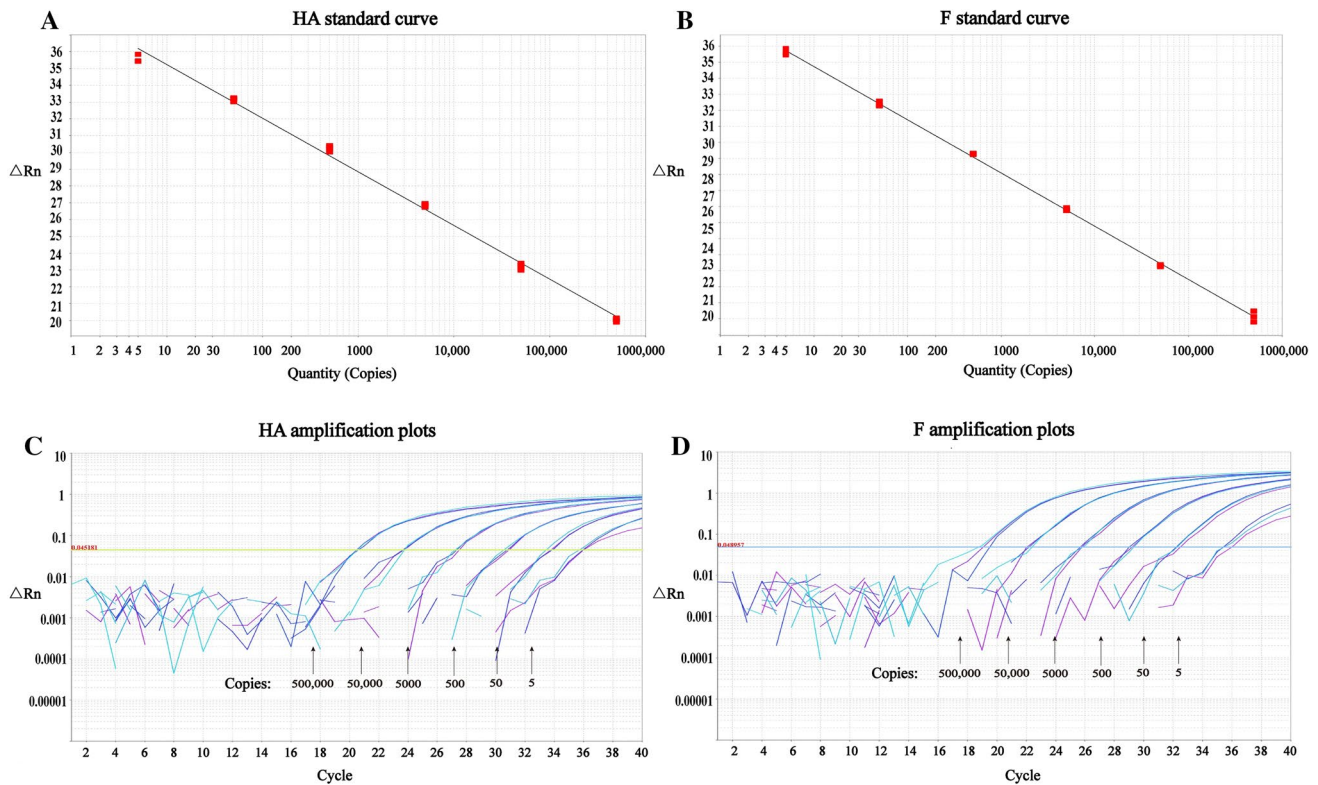


**Fig. 3** Standard curves and amplification plots for multiplex detection. (A) HA standard curves between $5 \times 10^0$ and $5 \times 10^5$ drawn using ABI 7500 software ($y = -3.194x + 38.427$, $R^2 = 0.995$, efficiency $= 105.616\%$). (B) F standard curves between $5 \times 10^0$ and $5 \times 10^5$ drawn using ABI 7500 software ($y = -3.32x + 38.042$, $R^2 = 0.999$, efficiency $= 100.072\%$). (C) and (D) Amplification plots in logarithmic format corresponding to the standard curves in panels A and B. The number of copies of standard plasmids in plasmid mix is indicated

To investigate the reproducibility of our multiplex PCR system, intra- and inter-assay comparisons were performed by testing four dilutions of the plasmid mixture, with $10^5$ to $10^1$ copies of each plasmid per reaction. In intra-assay comparisons, triplicate samples of the same dilution were performed on the same plate, while in the inter-assay comparisons, Ct values of multiplex PCR reactions were determined on days 1, 3, and 5. The coefficient values (CV%) for the intra- and inter-assay comparisons were both < 3% (Tables 4 and 5), showing that this multiplex PCR method has high reproducibility.

## Application of the established RRT-PCR for detection of H5 AIV and v-NDV in clinical samples

No deaths were observed between days 1 and 7 in the F48E8 and YB0415 groups. However, in the GD1602 group, two chickens died on day 11, and another three died on day 13. None of the chickens in the Z6285 group died. Our detection results led to the identification of two major groups. Two more F48E8-virus-positive fecal samples were found using the virus isolation method than by using RRT-PCR, and the false-negative error ratio of RRT-PCR for F48E8 was 2/80 (Fig. 4). However, for the other three strains tested in our artificial inoculation experiment, RRT-PCR had a higher detection rate than the virus isolation method. Specifically, 15 (15/80) and 4 (4/46) more positive samples were detected in the YB0415 and GD1602 groups, respectively, using the RRT-PCR method than by using the traditional isolation method (Fig. 4). For the Z6285 group, there were 17 (17/60) more positive samples detected using RRT-PCR

than detected using virus isolation. The $P$-values of $t$-tests comparing the percentage of samples that were positive by virus isolation and by RRT-PCR for F48E8, YB0415, G1602, and Z6285 were 0.6845, 0.0145, 0.2875, and 0.0009, respectively. The Z6285 H5-AIV and YB0415 v-NDV detection rates in clinical samples were extremely significantly and significantly higher using the RRT-PCR method than those obtained using the traditional virus isolation method. This indicates that the RRT-PCR method has better sensitivity than the traditional virus isolation method, but it might also indicate a higher false-positive rate. To verify the RRT-PCR results, we started a second passage of viruses on eggs for the samples that were positive in RRT-PCR but negative in the HA test (n = 36). After the second passage, 5 of 36 samples showed positive results in the HA experiment, demonstrating the sensitivity of the RRT-PCR method.

## Discussion

In this study, alignments of HA gene sequences from H5 AIV isolates collected from 2012 to 2016 were used to identify conserved regions. These HA-HI regions were used to design primers and probes to detect the prevalent (clade 2.3.2.1c and clade 2.3.4.4) and early clades. Our probes and primers, which were designed in 2013 [12], were still able to detect the clades and subtypes prevalent in 2016, indicating that the regions recognized by the probes and primers had remained highly conserved. Importantly, no HA-positive samples were missed by our RRT-PCR detection method. The alignment of genes of velogenic and mesogenic NDV

**Table 4** Intra-assay comparison of the HA and F probe in multiplex detection of H5 AIV and velogenic NDV

| Probe: | HA | | | | F | | | |
|---|---|---|---|---|---|---|---|---|
| Copy no.: | $5\times10^4$ | $5\times10^3$ | $5\times10^2$ | $5\times10^1$ | $5\times10^4$ | $5\times10^3$ | $5\times10^2$ | $5\times10^1$ |
| 1 | 21.116 | 24.485 | 28.399 | 31.833 | 22.443 | 25.905 | 29.211 | 32.808 |
| 2 | 21.026 | 24.660 | 28.245 | 31.635 | 22.409 | 26.112 | 29.320 | 33.231 |
| 3 | 21.006 | 24.451 | 28.258 | 31.420 | 22.426 | 25.967 | 29.352 | 33.156 |
| Mean | 21.049 | 24.532 | 28.301 | 31.629 | 22.426 | 25.995 | 29.294 | 33.065 |
| SD | 0.059 | 0.112 | 0.085 | 0.207 | 0.017 | 0.106 | 0.074 | 0.226 |
| CV% | 0.28% | 0.46% | 0.30% | 0.65% | 0.08% | 0.41% | 0.25% | 0.68% |

**Table 5** Inter-assay comparison of the HA and F probe in multiplex detection of H5 AIV and velogenic NDV

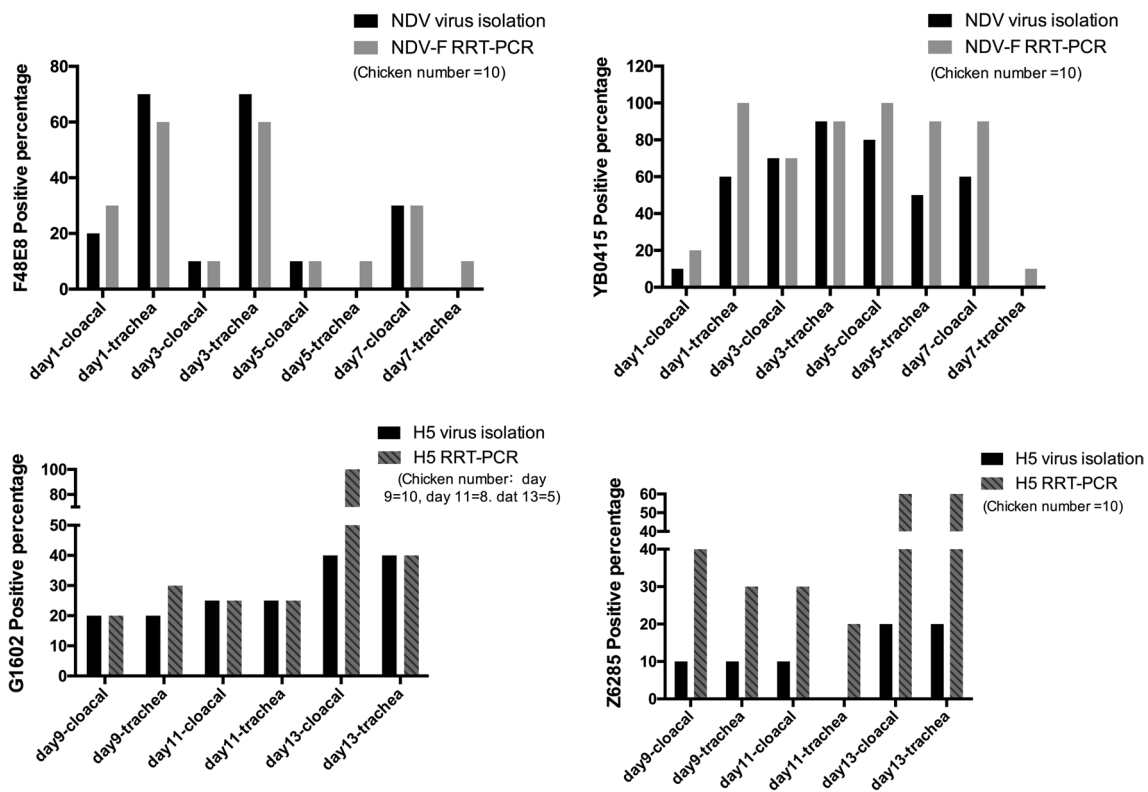| Probe: | HA | | | | F | | | |
|---|---|---|---|---|---|---|---|---|
| Copy no.: | $5\times10^5$ | $5\times10^4$ | $5\times10^2$ | $5\times10^1$ | $5\times10^5$ | $5\times10^4$ | $5\times10^2$ | $5\times10^1$ |
| Day 1 | 19.100 | 22.786 | 29.615 | 33.196 | 19.856 | 23.446 | 30.291 | 33.923 |
| Day 3 | 19.384 | 22.888 | 29.460 | 33.167 | 19.930 | 23.356 | 29.842 | 33.679 |
| Day 5 | 19.207 | 22.659 | 29.232 | 32.949 | 19.930 | 23.095 | 29.777 | 33.742 |
| Mean | 19.230 | 22.778 | 29.436 | 33.104 | 19.905 | 23.299 | 29.970 | 33.781 |
| SD | 0.143 | 0.115 | 0.193 | 0.135 | 0.043 | 0.182 | 0.280 | 0.127 |
| CV% | 0.75% | 0.50% | 0.65% | 0.41% | 0.21% | 0.78% | 0.93% | 0.37% |

**Fig. 4** Comparison of virus isolation and the RRT-PCR method using artificially inoculated samples. Cloacal and tracheal swabs from were collected 10 chickens over a period of 13 days. The percentage of samples that were positive using each detection method is shown. F48E8 and YB0415 samples were collected on days 1, 3, 5 and 7 after virus inoculation on day 0 whereas GD1602 and Z6285 samples were collected on days 9, 11 and 13 after virus inoculation on day 8. No deaths were observed in the Z6285, F48E8 and YB0415 groups. However, in the GD1602 group, two chickens died on day 11, and another three died on day 13. The $P$-values of the $t$-test comparing virus isolation and our RRT-PCR method with F48E8, YB0415, G1602 and Z6285 were 0.6845, 0.0145, 0.2875 and 0.0009, respectively

isolates was done in February 2016, and the probes and primers were designed in March 2016.

Although some multiplex PCR detection methods for v-NDV and H5 HPAIV have been reported, these methods are not suitable for high-throughput detection. RRT-PCR allows the simultaneous analysis of dozens of samples and saves time with respect to sample preparation. The reverse transcription system of the RRT-PCR assay is based on specific primers, which greatly improves the PCR amplification efficiency and reduces the background noise and contamination from reverse transcription products.

In this study, a multiplex RRT-PCR for the detection of H5 AIV and velogenic and mesogenic NDV based on the HA and F genes was successfully established. TaqMan MGB and TaqMan BHQ1 methods were used to detect the HA and F gene, respectively. The BHQ1 and MGB (minor-groove binding) groups are quenching groups that do not produce fluorescence themselves. Compared with TAMARA as a conventional quenching group, the background of qPCR using BHQ1 and MGB was reduced and the sensitivity increased [14]. The TaqMan MGB probe has a minor-groove

binder at the 3' end, which allows the probes to be shortened to as few as 13 bases, enhancing the combination of probes and templates and allowing differences of a single base to be distinguished [15]. To the best of our knowledge, this is the first TaqMan MGB method described for the detection of multiple H5 clades and the first report of an F TaqMan BHQ1 probe for v-NDV detection. Combining this multiplex RRT-PCR approach with virus isolation may provide a fast and reliable means of detecting H5 AIV and v-NDV in clinical samples.

Viruses of subtypes III to X of class II NDVs are mostly velogenic and have a wide host range [16]. The majority of v-NDVs are in class II, and the most prevalent NDV subtype in China is subtype VII, class II. In this study, detection of F48E8 by RRT-PCR in the artificial inoculation experiment was less accurate than the traditional approach, which might have been the result of mismatched sites. When compared with the F probe sequence, F48E8 has the most sequence differences and the greatest genetic distance to subtype VII, as it is a v-NDV of subtype IX, which has rarely been reported to infect poultry in recent years [17]. For the subtype VII

virus YB0415, the misdetection rate was zero (Fig. 4). Considering the epidemiological situation of v-NDV in China, we believe that our multiplex RRT-PCR detection method is suitable for clinical testing of v-NDV.

AIV of the H5N1 subtype, which can cause huge losses to the poultry industry, first emerged in Guangdong in 1996, posing a great threat to public health [18]. Therefore, it is of public-health significance to control H5 HPAIV and to perform early diagnosis using more-sensitive methods. Since 2014, H5 HPAIV clades 2.3.4.4 and 2.3.2.1c have been prevalent throughout China [19]. Furthermore, a recent study has revealed that clade 2.3.4.4 H5N6 has replaced clade 2.3.2.1 H5N1 as the dominant AIV subtype, especially in ducks in southern China [20]. We used one H5N6 strain in our artificial inoculation study. Our results show that our multiplex assay can detect clade 2.3.4.4 H5N6 more accurately than clade 2.3.2.1c H5N1, although the misdetection rates in both clades was zero.

In summary, our multiplex RRT-PCR method for detecting H5 AIVs and velogenic and mesogenic NDV showed high sensitivity, reproducibility, and specificity. Our RRT-PCR approach is fast, highly sensitive, and convenient for screening samples in large volumes for clinical diagnosis. Therefore, it can be applied before traditional virus isolation methods, which can be used subsequently to confirm virus infection. Moreover, it is expected that this multiplex RRT-PCR assay will be applied in the surveillance of clinical samples in both live-poultry markets and breeder farms for the detection of H5 AIV and velogenic and mesogenic NDV.

## Compliance with ethical standards

**Conflict of interest** None of the authors has competing interests to declare.

**Ethical approval** This study was performed in strict concordance with the Guide for the Care and Use of Laboratory Animals of the Ministry of Science and Technology of the People's Republic of China. The protocols for animal experiments were approved by the Jiangsu Administrative Committee for Laboratory Animals (approval number: SYXK-SU-2007-0005), and complied with the guidelines of Jiangsu laboratory animal welfare and ethics of Jiangsu Administrative Committee of Laboratory Animals.

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