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Research article

A multiplex TaqMan real-time RT-PCR assay for the simultaneous detection of H4, H6, and H10 avian influenza viruses

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ABSTRACT

Avian influenza viruses (AIVs) have caused a large number of epidemics in domestic and wild birds, and even posed a health challenge to humans. Highly pathogenic AIVs have attracted the most public attention. However, low pathogenic AIVs, including H4, H6, and H10 subtype AIVs, have spread covertly in domestic poultry, without obvious clinical signs. The emergence of human infections with H6 and H10 AIVs and the evidence of seropositivity of H4 AIV in poultry-exposed individuals indicated that these AIVs sporadically infect humans and could cause a potential pandemic. Therefore, a rapid and sensitive diagnostic method to simultaneously detect Eurasian lineage H4, H6, and H10 subtype AIVs is urgently required. Four singleplex real-time RT-PCR (RRT-PCR) assays were established based on carefully designed primers and probes of the conserved regions of the matrix, H4, H6, and H10 genes and combined into a multiplex RRT-PCR method to simultaneously detect H4, H6, and H10 AIVs in one reaction. The detection limit of the multiplex RRT-PCR method was 1–10 copies per reaction when detecting standard plasmids, and showed no cross-reaction against other subtype AIVs and other common avian viruses. Additionally, this method was suitable to detect the AIVs in samples from different sources, the results of which showed high consistency with virus isolation and a commercial influenza detection kit. In summary, this rapid, convenient, and practical multiplex RRT-PCR method could be applied in laboratory testing and clinical screening to detect AIVs.

1. Introduction

When an influenza virus with a novel hemagglutinin (HA) protein emerges and spreads efficiently in a susceptible population, an influenza virus pandemic might occur. Historically, several influenza pandemics in humans, caused by H1N1, H2N2, and H3N2 subtypes, have caused huge damage to human health, and H1N1 and H3N2 became seasonal influenza A viruses that continue to

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circulate in humans [1]. Cross-species transmission events of other avian influenza viruses (AIVs) are often accompanied by cumulative mutations of the HA protein [2] or reassortment with other avian-, swine-, even human-origin viruses [3]. Sporadic cases of human infection caused by different subtypes of AIVs have been described, such as H5, H7, H6, H9, and H10 subtype AIVs [3–6]. Therefore, various subtypes of AIVs remain a health threat to the public, with the potential to cause a pandemic in humans.

The H6 subtype AIVs are prevalent in birds worldwide and have been one of the most common subtypes detected in domestic birds in live poultry markets (LPMs) in China [7–9]. Importantly, H6 AIVs infect a broader range of host including mice, ferrets, pigs and even humans [10–12]. The first human infection with H6 subtype AIV (H6N1) was reported in May 2013 in China [13]. Additionally, H10 AIVs could spread in mammalian hosts, including harbor seals in Europe and farmed mink in Sweden [14,15]. Cases of human infection with H10N7 AIV in Egypt and Australia, and cases of fatal human infection with H10N8 AIV in China have been described, attracting significant public attention [4,16]. H4 AIVs have spread widely in birds in Asian, North American, and European countries [17,18] and have occasionally been isolated from pigs [19]. Previous seroepidemiological analysis provided evidence that individuals exposed to poultry are at great risk of H4 virus infection [10,20]. A previous study also indicated that H4 AIVs could efficiently replicate in mice without prior adaptation and could transmit between guinea pigs through direct contact [21]. Therefore, regular surveillance of AIVs is important and provides an important basis for the prevention and control of AIVs.

Traditional virus isolation is considered as the gold standard [22], and generic real-time reverse transcriptase PCR (RRT-PCR) technology, which specifically detects matrix (M) genes from influenza viruses, is commonly used in clinical diagnosis [23]. Many multiplex rRT-PCR assays have been generated to differentiate subtypes of influenza viruses, however, the primers and probes of subtyping rRT-PCR assay should be updated timely to adapt to the rapid evolution of the AIV genes. Therefore, in the present study, we established a TaqMan-probe-based multiplex RRT-PCR assay to simultaneously detect H4, H6, and H10 AIVs in one reaction.

2. Methods and materials

2.1. Viruses and samples

As shown in Table S1, a total of 50 wild-type AIVs were used to examine the detection specificity of this multiplex RRT-PCR method. Additionally, five other common respiratory viruses were also used in this research (Table S1).

In addition, 147 samples (47 cloacal swabs and 100 faecal samples) from LPMs in eastern China were collected from 2013 to 2021 and evaluated previously using traditional virus isolation in our laboratory. All the samples were stored at −80 °C until used. Thirty-two nose and/or throat swabs from humans presenting with influenza-like symptoms were harvested and evaluated using a commercial Influenza A and B Virus Real-Time RT-PCR Kit (Liferiver Bio–Tech Corp., Shanghai, China). All specimens were kept at −80 °C. Genomic RNA from the samples was collected using the magnetic bead method (Liferiver Bio–Tech) based on the manufacturer's instructions.

2.2. Designing primers and probes

Considering that the M gene of influenza A viruses was highly conserved and HA genes were the basis of subtyping, we designed the primers and probes targeting M and HA genes to differentiate H4, H6 and H10 subtype influenza viruses. The HA sequences of 286 H4 subtype AIVs were obtained from the GenBank database and aligned using the Molecular Evolutionary Genetics Analysis (MEGA) software version 6.0. Primers and probes targeting the conserved region of H4 HA genes were then designed. The primers and probes targeting the H6 HA genes were validated in a previous study, and the limit of detection (LOD) was 30 copies per reaction when serially diluted H6 plasmids were used as templates [24]. The primers and probes targeting H10 HA genes were modified based on a previous study [25]. Additionally, the specific primers and probes for the M gene were carefully designed in a previous study, with a LOD of 10 copies per reaction [26]. All primers and probes are shown in Table 1 and were synthesised by Tsingke Co., Ltd (Beijing, China).

Table 1
List of the designed primers and probes used in this study.

Target	Name	Sequence	Position	Product	Reference publications
M	*M*-F	5-CTTCTAACCGAGGTCGAAACG-3	7–27	165 bp	[26]
	M-R	5-CTTTAGCCACTCCATGAGAGC-3	121–141		[26]
	M-FAM-P	5-FAM-CCTCAAAGCCGAGATC-MGB-3	57–72		[26]
H4	H4–F	5-GACCCARGGATACAAGGACA-3	1563–1582	95 bp	Newly designed
	H4-R	5-AAATGCAAATCTGGCACC-3	1676–1693		Newly designed
	H4–CY5–P	5-CY5-TTGTGGGCTTGTC-MGB-3	1648–1660		Newly designed
H6	H6–F	5-TYTGGCATAAGTGTGACAATG-3	1454–1474	171 bp	[24]
	H6-R	5-GACTGCTCGATACCGTACTAT A-3	1603–1624		[24]
	H6-VIC-P	5-VIC-GAATCGGTAAAGCT-MGB -3	1555–1568		[24]
H10	H10–F	5-CACAGTACAGAGAAGAAG C-3	1499–1517	76 bp	Newly designed
	H10-R	5-ACAAARCATGAYGCCCCG A-3	1592–1610		[25]
	H10-TR-P	5-Texas Red-TGAMACTCTCTTC-MGB-3	1547–1559		Modified [25]

F, forward; R, reverse; P, probe; FAM, Fluorescein amidite; Cy5, Cyanine 5; VIC, 2′-chloro-7′phenyl-1,4-dichloro-6-carboxy-fluorescein.

2.3. Development of the multiplex RRT-PCR method

To test H4, H6, and H10 AIVs in one reaction, a multiplex RRT-PCR method was designed and developed. The final 20 μL reaction volume comprised 5 μL of 4 × TaqMan Fast Virus One-step Master Mix (Thermo Fisher Scientific, Waltham, MA, USA), 0.5 μL of each of the four forward primers (10 μM), 0.5 μL of each of the four reverse primers (10 μM), 0.5 μL of each of the four probes (10 μM), 1 μL of template, and 8 μL of RNase-free water. The RRT-PCR reaction was carried out in a CFX96 Real-time RT-PCR instrument (C1000 Touch, Bio-Rad, Hercules, CA, USA) with 5 min of reverse transcription at 50 °C, a 20 s denaturation reaction at 95 °C, followed by 40 amplification cycles (95 °C for 3 s, 60 °C for 30 s) [26].

2.4. Analytical performance

The LODs of the assay were assessed using eight 10-fold serial dilutions of quantitative standard plasmids (pHW2000-M, pMD-19 T-H4, pMD-19 T-H6, and pMD-19 T-H10) from 10^7 to 10° copies per μL [26,27]. Each dilution was repeated three times in one assay. In this study, the matrix (M) gene was from the A/duck/Zhejiang/W24/2013(H5N8) strain [28], and the H4, H6, and H10 genes were from A/duck/Zhejiang/77127/2014 (H4N2) [17], A/chicken/Zhejiang/1664/2017 (H6N1) [29] and A/chicken/Zhejiang/2CP2/2014 (H10N7) [30], respectively.

2.5. Analytical specificity

BLASTn analysis was conducted to assess the performance of the designed primers and probes with the following criteria: (1) no mismatch was found within the last five bases at the 3′-end, (2) a maximum of one mismatch within the ten bases at the 3′-end, (3) no more than three mismatches in the primers, and (4) the degenerate primers or probes should be exactly matched with the targets [26,31].

Various subtype AIVs (H1–H7 and H9–H11), influenza B viruses, and other avian pathogens were evaluated to assess the specificity of this multiplex RRT-PCR assay.

Additionally, double-mixed plasmid samples were produced by mixing two target plasmids (pHW2000-M, pMD-19 T-H4, pMD-19 T-H6, and pMD-19 T-H10) in different ratios (10^6, 10^4, and 10^2 copies/μL) per reaction, followed by detection using the multiplex RRT-PCR assay, which was performed to evaluate possible competitive inhibition.

2.6. Diagnostic performance

A total of 147 field samples (Table S2), isolated from chickens, ducks and geese, was prepared and assessed using traditional virus isolation [32–34], including 47 cloacal swabs (4 were H4-positive, 6 were H6-positive, and 4 were H10-positive) and 100 faecal samples (10 were positive for H4, 22 were positive for H6, and 12 were positive for H10). All the samples, in phosphate buffered saline (PBS), were vortexed to mix and left to stand for more than 30 min at room temperature. Subsequently, the samples were aliquoted into sterile tubes and kept at −80 °C until use.

An additional 32 clinical samples were collected from humans between 2016 and 2021, of which 5 were H1N1 influenza virus-positive samples, 5 were influenza H3N2 virus-positive samples, 5 were influenza B virus (IBV)-positive samples, 2 were rhinovirus (RhV)-positive samples, 2 were adenovirus (ADV)-positive samples, 2 were respiratory syncytial virus (RSV)-positive samples, and 5 were SARS-CoV-2-positive samples [26,35,36].

In this study, we also produced spiked samples as follows: H4N2 (A/duck/Zhejiang/77127/2014), H6N1 (A/chicken/Zhejiang/1664/2017), and H10N7 (A/chicken/Zhejiang/2CP2/2014) AIVs were respectively added to throat swabs, cloacal swabs, and faecal samples that were previously tested and found to be negative for influenza virus using the commercial Influenza A and B Virus Real-Time RT-PCR Kit.

In summary, 147 field samples isolated from poultries, 32 clinical samples isolated from humans and 90 spiked samples produced based on the normal poultry samples were included in this study.

When detecting clinical/field samples, four positive controls (10^4 copies/μL DNA plasmids of M, H4, H6 and H10 genes) and a negative control (water) were set to exclude false negative and false positive reactions.

Table 2
Performance parameters of the multiplex rRT-PCR in this study.

Targets	Singleplex rRT-PCR assay			Multiplex rRT-PCR assay		
	Slope	Ordered in origin	LOD	Slope	Ordered in origin	LOD
M	3.308	40.796	1	3.452	41.527	1
H4	3.303	41.251	10	3.458	42.735	10
H6	3.167	40.964	10	3.518	41.561	10
H10	3.325	42.843	10	3.357	42.086	10

3. Results

3.1. Singleplex rRT-PCR assays for the M, H4, H6, and H10 genes

Four plasmids (pHW2000-M, pMD-19 T-H4, pMD-19 T-H6, and pMD-19 T-H10) containing the primers and probe binding sites of the M, H4, H6, and H10 genes were serially 10-fold diluted with Diethyl Pyrocarbonate water and used to detect the sensitivity of the four singleplex RRT-PCR assays. The results of amplification and the standard curves are shown in Fig. S1. As shown in Table 2, the LODs of the singleplex real-time PCR assays to detect H4, H6 and H10 genes was 10 copies per reaction, and 1 copy for M gene (plasmid DNA). The standard curves showed that the singleplex RT-PCR assays were efficient, with high reaction efficiencies of 100.6%, 100.8%, 106.9%, and 99.9%. At the tested concentration range, the squared regression coefficient (R^2) value of the four singleplex assays was $\geq$0.98.

3.2. Multiplex rRT-PCR for simultaneously detecting the M, H4, H6, and H10 genes

Four equimolar target plasmids were mixed at concentrations ranging from 10^7 to 1 copy/μL and tested using the multiplex RRT-PCR to evaluate the potential competitive reaction among the four RRT-PCR reactions. The results showed no significant difference in the mean cycle threshold (Ct) values between the multiplex RRT-PCR method and corresponding singleplex method for M, H4, H6 and H10 gene detection, indicating good consistency between the two assays (Fig. S2). The LOD of the H4, H6 and H10 genes in the multiplex assays were 10 copies per reaction, and the LOD of the M gene was 1 copy (Table 2). Standard curves were constructed in the range of 1 to 10^7 copies of the four targets (M, H4, H6 and H10) with the following equations: $y = -3.452x + 41.527$ (M: efficiency = 94.9%, $R^2 = 0.997$), $y = -3.458x + 42.735$ (H4: efficiency = 94.6%, $R^2 = 0.997$), $y = -3.518x + 41.561$ (H6: efficiency = 92.4%, $R^2 = 0.990$) and $y = -3.357x + 42.086$ (H10: efficiency = 98.5%, $R^2 = 0.988$). The R^2 value of each multiplex reaction was $\geq$0.98 and the efficiency values ranged from 92.4% to 98.5%, which was considered as acceptable for an RRT-PCR assay.

The potential competitive effect of this multiplex RRT-PCR assay was further tested by detecting all possible combinations of 10^2, 10^4, and 10^6 copies of the target plasmids (Fig. S2). Firstly, no false-positive signals were produced when detecting the mixed samples. However, the plasmid at low concentrations were more susceptible to competitive inhibition when detecting mixed samples. In this study, a significant difference was observed when detecting the H10 plasmid at low concentration (10^2 copies/μL) in single sample and mixed sample. No significant differences were observed between the low target concentrations in single positive samples and double positive samples when detecting M, H4 and H6 genes. Similar Ct values were obtained when detecting high-concentration plasmids (10^6 copies/μL) under the interference of other plasmids. Therefore, these results indicated that this multiplex RRT-PCR assay was a promising assay to identify co-infection with different subtypes of AIVs.

3.3. Analytical specificity and repeatability of the multiplex RRT-PCR assay

Firstly, BLASTn analysis was performed to detect the performance of the primers and probes, and the results indicated that no cross-reactivity was observed for these primers and probes with other pathogens and had specificity against their target genes. Meanwhile, the specificity of this multiplex RRT-PCR assay was assessed using different subtypes of influenza viruses (H1–H7 and H9–H11) and other avian respiratory pathogens. All tested influenza A virus subtypes produced positive amplification for the M gene (Fluorescein amidite (FAM) signal), and only H4, H6 and H10 subtype AIVs could produce corresponding positive signals simultaneously. Other pathogens (influenza B, APMV-4, IBV, IBDV, and NDV) did not produce any amplification, indicating that there was no cross-reactivity with other pathogens (Table S1). Compared with reference rRT-PCR analysis, the multiplex RRT-PCR method showed a slightly higher Ct value for the evaluation of M gene of influenza A viruses, but the difference was not significant.

As shown in Table 3, the coefficient of variation (CV) for the multiplex detection of M, H4, H6, and H10 genes was <5% in intra-assays and inter-assays, which suggested high stability of the multiplex RRT-PCR method.

3.4. Clinical performance

Among the 147 field samples, a total of 55 (55/58, 94.83%) positive influenza A viruses were evaluated using the multiplex RRT-

Table 3
The coefficients of variation of intra- and inter-assays.

Plasmid copy number per reaction (copies/μl)	Ct value (Mean ± SD), CV (%)							
	M		H4		H6		H10	
	Intra-	Inter-	Intra-	Inter-	Intra-	Inter-	Intra-	Inter-
Low (10^2)	34.16 ± 0.17, 0.49%	33.99 ± 0.11, 0.31%	35.86 ± 0.23, 0.64%	36.16 ± 0.30, 0.84%	34.66 ± 0.29, 0.85%	35.22 ± 0.39, 1.10%	37.46 ± 0.45, 1.19%	37.68 ± 0.34, 0.91%
Medium (10^4)	26.81 ± 0.30, 1.11%	26.50 ± 0.32, 1.19%	28.29 ± 0.17, 0.59%	28.44 ± 0.39, 1.37%	26.40 ± 0.02, 0.08%	26.75 ± 0.23, 0.85%	30.41 ± 0.12, 0.41%	30.51 ± 0.34, 1.11%
High (10^6)	20.02 ± 0.04, 0.19%	20.55 ± 0.38, 1.87%	22.13 ± 0.06, 0.27%	22.52 ± 0.31, 1.37%	20.52 ± 0.04, 0.22%	21.03 ± 0.28, 1.34%	22.60 ± 0.11, 0.47%	22.94 ± 0.42, 1.82%

PCR assay, including 14 cloacal swabs (14/14, 100%) and 41 (41/44, 93.18%) faecal samples. Additionally, 13 samples were positive for H4 (4 cloacal swabs and 8 faecal samples), 27 were positive for H6 (6 cloacal swabs and 21 faecal samples), 13 were positive for H10 (3 cloacal swabs and 10 faecal samples). Overall, the sensitivity and specificity of this multiplex RRT-PCR compared with traditional virus isolation were 96.95–99.16% and 81.25–96.43%, respectively (Table S3).

For the 32 human specimens, 10 positive amplifications for the M gene (10/10, 100%) were produced. No samples yield positive signals for H4, H6 and H10 genes, including the samples known to be positive for H1N1 and H3N2 influenza A viruses, IBV, RhV, ADV, RSV, and SARS-CoV-2.

To evaluate the interference of different sample types on the multiplex RRT-PCR assay, H4N2, H6N1, and H10N7 AIVs, respectively, were spiked into throat swabs, cloacal swabs, and faecal samples. Ninety spiked samples were finally assessed using the multiplex RRT-PCR assay (Table S4). The results indicated that there was no obvious interference in the activity of this multiplex RRT-PCR when detecting various samples, and high sensitivity and specificity were achieved for the different samples.

4. Discussion

In this study, one singleplex RRT-PCR assay for H4 gene was successfully developed and combined with other three previously designed sets of primers and probes for M, H6 and H10 genes to differentiate H4, H6, and H10 AIVs in a one-step multiplex RRT-PCR method. The specific primers and probes targeting H4 genes were carefully designed and primers and probes of M, H10 and H6 genes were modified according to previous studies to be applied together in a multiplex assay. Additionally, an internal control was often used to achieve detection of samples containing inhibitors in the real-time assay and avoid false negatives. However, a total of four fluorescent dye-labeled probes were designed due to the limited fluorescent channels of the CFX96 Real-time RT-PCR instrument in this study. According to the previous study, another technology, like MNAzyme, could achieve a quintuplex PCR which has shown its superiority in detecting 4 genes and including an internal control [37]. Alternatively, researchers can make appropriate adjustments as needed, such as adding an internal control by reducing the detection of M genes.

It has been reported that serial dilutions of cloned plasmid appear to be more robust and suitable as a standard for RRT-PCR assays compared to RNA run-off transcripts [38]. As described previously, the serially diluted DNA plasmids (pHW2000-M, pHW2000-H4, pHW2000-H6 and pHW2000-H10) were used as quantitative standards to detect the limit of detection (LOD) of the real-time RT-PCR assay [39,40]. The four singleplex RRT-PCR reactions were evaluated as sensitive, with LODs of 1–10 copies per reaction (DNA plasmid) of the M, H4, H6, and H10 genes. Previous studies reported an analytical sensitivity of 7.9×10^3 copies per reaction (RNA) in detecting H4 AIVs [41], $10^{1.5}$ EID_{50} per mL in detecting H6 AIVs [42], and 76 copies (DNA plasmid) of H10 genes [43]. Additionally, there was a microarray method for detection and characterization of influenza A viruses which have been previously generated with detection limit of 1.5×10^1 copies (M) [44] and a designated Riems Influenza A Typing Array version 2 (RITA-2) for subtyping avian influenza virus with high inclusivity and exclusivity [45]. In our previously published studies, the detection limits of RRT-PCR assay were 30 copies per reaction and 10 copies to detect H6 and H10 DNA plasmids, respectively [24,25], indicating the good reproducibility of these singleplex PCR assays. Meanwhile, the one-step multiplex RRT-PCR method combining these four singleplex assays showed similar detection limits when detecting equally mixed amounts of plasmids.

However, a mutual competitive effect was found within the multiplex RRT-PCR assay when detecting low-concentration plasmids (especially the H10 plasmids) mixed with other high-concentration plasmids. This limit has been found in many other multiplex RRT-PCR methods [26,46–48]. Therefore, another singleplex RRT-PCR reaction was needed when a suspicious positive was detected in the multiplex reaction.

Compared with the singleplex methods, the multiplex RRT-PCR assay could obviously reduce reagent costs, the turnaround time for specimens, and the potential for contamination. Undoubtedly, the multiplex RRT-PCR assay was more suitable for preliminary screening of a quantity of specimens in a short time, including field detection, clinical testing, and research. In conclusion, an effective multiplex RRT-PCR diagnostic method was constructed to detect M, H4, H6, and H10 genes in one reaction employing carefully designed primers and probes. Additionally, this multiplex method could be applied to detect various samples from different sources. High sensitivity and specificity were observed in testing H4, H6, and H10 AIVs from the Eurasian lineage, indicating that it is a sensitive, practical, and promising method for laboratory testing and clinical screening of AIVs.

Author contribution statement

Fan Yang: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.
Sijing Yan; Danna Wu: Performed the experiments.
Linwei Zhu; Hangping Yao; Dalu Dong: Performed the experiments; Contributed reagents, materials, analysis tools or data.
Nanping Wu: Analyzed and interpreted the data.
Chunsheng Ye: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data.
Haibo Wu: Conceived and designed the experiments; Wrote the paper.

Data availability statement

Data included in article/supp. Material/referenced in article.

Additional information

No additional information is available for this paper.

Ethics statement

The Animal Ethics Committee of First Affiliated Hospital, School of Medicine, Zhejiang University approved all animal experiment in this study (No. 2019–39).

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2023.e15647.

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