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Development of a duplex real-time PCR method for the detection of influenza C and D viruses

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Abstract

Influenza viruses are major respiratory pathogens known to infect human and a variety of animals and are widely prevalent worldwide. Genome structure of influenza D virus (IDV) is identical to that of influenza C virus (ICV), and phylogenetic analyses suggest that IDV and ICV share a common ancestry and high homology. To date, the prevalence of ICV and IDV in China is unclear, but these viruses represent a potential threat to public health due to cross-species transmission and zoonotic potential. To efficiently monitor ICV and IDV, it is necessary to establish a dual detection method to understand their prevalence and conduct in-depth research. A duplex real-time PCR method for the simultaneous detection of ICV and IDV was developed. TaqMan fluorescent probes and specific primers targeting *NP* gene of ICV and *PB1* gene of IDV were designed. This method exhibited good specificity and sensitivity, and the detection limit reached 1×10^1 copies/μL of plasmid standards of each pathogen. Thirty-one clinical swine samples and 10 clinical cattle samples were analyzed using this method. One positive sample of IDV was detected, and the accuracy of clinical test results was verified by conventional PCR and DNA sequencing. The duplex real-time PCR detection method represents a sensitive and specific tool to detect ICV and IDV. It provides technical support for virus research and clinical diagnosis of ICV and IDV. This information will benefit animal and human health.

Keywords: Influenza C virus, Influenza D virus, Real-time PCR, Multiplex detection

Background

Influenza viruses belong to the family *Orthomyxoviridae*, which are single-stranded, negative-stranded, and segmented RNA viruses. Influenza viruses are divided into four types based on the difference between nucleoprotein (NP) and membrane protein (M): influenza A virus (IAV), influenza B virus (IBV), influenza C virus (ICV), and influenza D virus (IDV) (Hause et al. 2014). ICV was first isolated in 1947 (Taylor 1949) and is known to infect humans (Anton et al. 2011; Calvo et al. 2013; Salez

et al. 2014; Thielen et al. 2018). Studies have shown that ICV was an important cause of upper respiratory diseases in children under the age of six and increased the risk of complications of lower respiratory diseases in children under two years of age (Matsuzaki et al. 2006). ICV is frequently coinfected with other viruses and bacterial pathogens, which makes the causal role of ICV uncertain in these cases (Sederdahl and Williams 2020). In addition to infecting humans, ICV has been detected in pigs, cattle, dogs and dromedary camels (Asha and Kumar 2019; Bailey et al. 2018; Guo et al. 1983; Hause et al. 2013; Zhang et al. 2018). It represents a significant public health concern given its pathogenicity in children.

In 2011, IDV was isolated from pigs with influenza-like illness for the first time in the United States, when it

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was determined to be an influenza C-like virus based on its similar genetic organization to ICV (Hause et al. 2013). After the first isolation of IDV, it was also detected in cattle, horses and sheep (Murakami et al. 2016; Nedland et al. 2018; Zhai et al. 2017). To date, according to pathological, epidemiological and serological studies, cattle are considered to be the primary natural host for IDV (Collin et al. 2015; Ferguson et al. 2015; Hause et al. 2014), and small ruminants may be a potential host for IDV transmission to other animals (Quast et al. 2015). Bovine respiratory disease (BRD) is the most disastrous disease affecting cattle industry in the United States, and studies have also shown that coinfection by other respiratory viruses along with IDV may cause BRD (Collin et al. 2015; Mitra et al. 2016; Ng et al. 2015). It is worth noting that IDV seropositivity was detected in workers who were in contact with cattle closely on farms (White et al. 2016), and studies have shown that IDV could replicate and spread in ferrets and guinea pigs (Sreenivasan et al. 2015; Yen et al. 2007; Zhu et al. 2013). These results indicated that IDV had a high zoonotic potential. Approximately 50% homology was noted between emerging IDV and ICV, and no cross-reactivity was found between antibodies against them (Hause et al. 2014).

At present, the prevalence of ICV and IDV in China is unclear. ICV and IDV have been detected in many countries, it is known that ICV and IDV were spread all over the world, so more comprehensive epidemiological investigations and pathogen-related researches are needed (Asha and Kumar 2019). These viruses also have potential significance in terms of public health due to their cross-species transmission and zoonotic potential. Thus, ICV and IDV monitoring using sensitive and practical detection methods is an urgent issue. We chose to develop a duplex real-time PCR method for ICV and IDV based on the excellent performance of real-time PCR in clinical testing. Compared with existing detection methods, the duplex real-time PCR detection method exhibits higher sensitivity and detection efficiency to realize the rapid and efficient identification and detection of ICV and IDV.

Results

Preparation of plasmid standards

Concentration of recombinant plasmid ranged from 1×10^7 copies/μL to 1×10^1 copies/μL based on 10-fold multiple dilutions. Plasmid standards of each pathogen concentration in the range of 1×10^7 copies/μL ~ 1×10^1 copies/μL were selected for singleplex real-time PCR. The results of the standard curve showed good amplification efficiency and correlation coefficient (ICV $R^2 =$ 0.9927, E value = 96%; IDV $R^2 =$ 0.9966, E value = 109%) (Fig. 1), indicating proper dilution of the plasmid standard.

Optimization of the multiplex reaction system

To obtain the best amplification efficiency, duplex real-time PCR was performed with probes in final concentrations ranging from 100 nM to 400 nM and primers with final concentrations ranging from 400 nM to 1100 nM. Then, the fluorescence intensity and Cq values of different combinations were compared. ICV plasmid standard at a concentration of 1×10^7 copies/μL and IDV plasmid standard at a concentration of 1×10^1 copies/μL were used as templates for reaction. The optimal final concentration of probe was 100 nM, and the optimal primer concentration was 400 nM (Table 1).
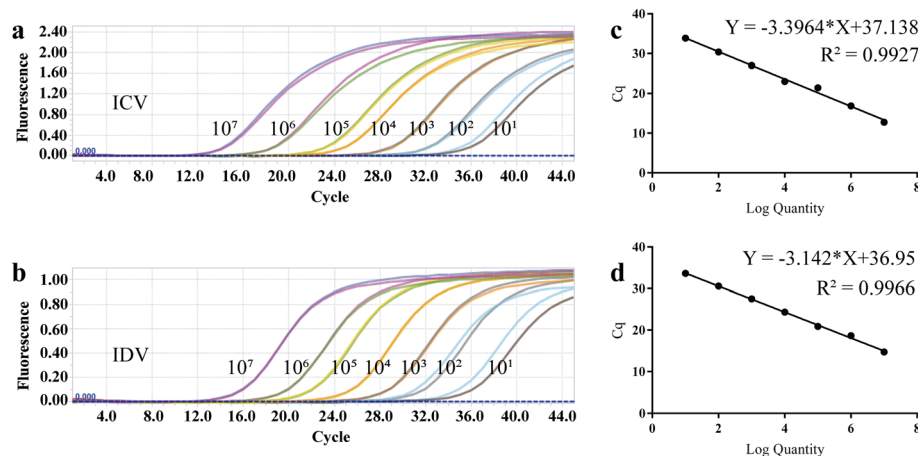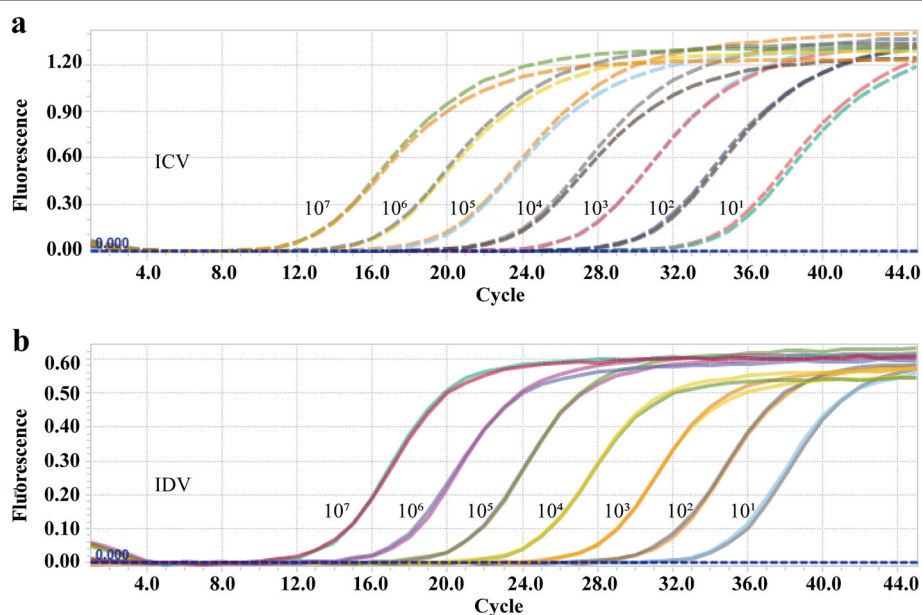

Fig. 1 Preparation of plasmid standards. **a**, **b** Amplification curves of ICV and IDV for each plasmid standard at concentrations ranging from 1×10^7 copies/μL to 1×10^1 copies/μL; **c**, **d** standard curves of plasmid standards of ICV and IDV. All standard curves were generated using GraphPad Prism 6 software

Table 1 ICV and IDV detected by duplex real-time PCR assay using different primer concentrations

Primer concentration (nM)	IDV Cq values	ICV Cq values	Probe concentration (nM)	IDV Cq values	ICV Cq values
400	34.82	12.00	100	34.82	13.28
500	36.44	12.92	150	36.44	12.90
600	38.21	12.73	200	38.21	12.70
700	39.89	12.59	250	39.89	12.67
800	–	12.41	300	–	12.56
900	–	12.38	350	–	12.45
1000	–	12.49	400	–	12.49
1100	–	12.55	450	–	12.63

"-": No signal detected, negative (–), ICV, influenza C virus; IDV, influenza D virus

Fig. 2 Sensitivity of duplex real-time PCR assay. **a**, **b** Amplification curves of plasmid standards of ICV and IDV at concentrations ranging from 1×10^7 copies/μL to 1×10^1 copies/μL detected by duplex real-time PCR

Table 2 Sensitivity test results of duplex real-time PCR assay

Pathogen	Concentration	Repeat times	Positive number	Positive rate
ICV	1×10^2	15	15	100%
	1×10^1	15	15	100%
	1×10^0	15	13	87%
IDV	1×10^2	15	15	100%
	1×10^1	15	15	100%
	1×10^0	15	9	60%

The cutoff line of positivity was automatically decided using a Roche LightCycler® 96 Instrument. *ICV* influenza C virus, *IDV* influenza D virus

Table 3 Specificity test results of duplex real-time PCR

Positive samples	Singleplex conventional PCR detection and DNA sequencing[a]										Duplex real-time PCR detection[b]
	ICV	IDV	JEV	CSFV	IAV	TTsuV1	TTsuV2	PRRSV	PCV2	PCV3	
ICV	+	–	–	–	–	–	–	–	–	–	+
IDV	–	+	–	–	–	–	–	–	–	–	+
JEV	–	–	+	–	–	–	–	–	–	–	–
CSFV	–	–	–	+	–	–	–	–	–	–	–
IAV	–	–	–	–	+	–	–	–	–	–	–
TTsuV1	–	–	–	–	–	+	–	–	–	–	–
TTsuV2	–	–	–	–	–	–	+	–	–	–	–
PRRSV	–	–	–	–	–	–	–	+	–	–	–
PCV2	–	–	–	–	–	–	–	–	+	–	–
PCV3	–	–	–	–	–	–	–	–	–	+	–

[a] These samples were detected by conventional PCR and confirmed by DNA sequencing. [b] Criteria of positivity: Cq ≤ 38, invalid (?); Cq > 38 or no signal detected, negative (–). *PCV2* porcine circovirus type 2, *PCV3* porcine circovirus type 3, *JEV* Japanese encephalitis virus, *CSFV* classical swine fever virus; *IAV* influenza A virus, *TTsuV1* Torque teno sus virus 1, *TTsuV2* Torque teno sus virus 2, *PRRSV* porcine reproductive and respiratory syndrome virus, *ICV* influenza C virus, *IDV* influenza D virus

Effectiveness of duplex real-time PCR assay

In this study, effectiveness of duplex real-time PCR detection method was assessed in terms of sensitivity, specificity and repeatability.

For sensitivity testing, plasmid standards of ICV and IDV at concentrations of 1×10^7 copies/μL to 1×10^1 copies/μL were detected using the optimized system. Results showed that samples with the lowest concentration of 1×10^1 copies/μL could be identified as positive using this method (Fig. 2). Repeated tests showed 100% positivity in plasmid sample with concentration of 1×10^1 copies/μL. However, strong instability in plasmid sample with concentration of 1×10^0 copies/μL was noted, and the positive rate was less than 90%. Therefore, 1×10^1 copies/μL is the detection limit of this duplex real-time PCR detection method (Table 2). Sensitivity test results showed that this method has excellent sensitivity, facilitating the detection of ICV and IDV in clinical samples with low viral concentrations.

To assess the specificity, the optimized method was used to detect ICV and IDV in samples positive for porcine circovirus type 2 (PCV2), porcine circovirus type 3 (PCV3), Japanese encephalitis virus (JEV), classical swine fever virus (CSFV), IAV, Torque teno sus virus 1 (TTsuV1), Torque teno sus virus 2 (TTsuV2) and porcine reproductive and respiratory syndrome virus (PRRS V), RNase-free water was used as a negative control (Table 3). The results showed that the target pathogens were detected, and negative results were obtained for all other pathogens, indicating that this method exhibits good specificity. The viruses selected for specific testing can cause respiratory diseases of the host. Pigs are co-hosts of ICV and IDV. Thus, the possibility of coinfection in clinical samples necessitates the need for an assay that can differentiate between these viruses. The results of specificity test demonstrated that this method has the ability to specifically detect ICV and IDV in clinical samples coinfected with other viruses.

Regarding repeatability testing, three replications were performed in triplicate for the analysis of intra-assay and interassay variability, and the coefficient of variation was calculated. As showed in Table 4, coefficient of variation (CV, %) values of Cq values were all less than 2%,

Table 4 Repeatability results of duplex real-time PCR assay

Plasmid	Concentration	Intra-assay Cq value		Interassay Cq value	
		Mean ± SD[a]	CV%	Mean ± SD	CV%
ICV	1×10^7	13.26 ± 0.26	1.96	13.79 ± 0.26	1.91
	1×10^6	16.65 ± 0.16	0.96	16.86 ± 0.22	1.33
	1×10^5	20.41 ± 0.06	0.29	20.54 ± 0.13	0.65
	1×10^4	24.45 ± 0.08	0.33	24.03 ± 0.24	0.99
	1×10^3	27.51 ± 0.02	0.07	27.55 ± 0.29	1.06
	1×10^2	31.16 ± 0.1	0.32	31.86 ± 0.21	0.67
	1×10^1	34.51 ± 0.06	0.17	34.51 ± 0.39	1.12
IDV	1×10^7	15.03 ± 0.1	0.67	15.42 ± 0.25	1.63
	1×10^6	18.23 ± 0.03	0.16	18.93 ± 0.27	1.43
	1×10^5	21.73 ± 0.18	0.83	21.74 ± 0.15	0.68
	1×10^4	25.03 ± 0.25	1	26.02 ± 0.22	0.84
	1×10^3	28.62 ± 0.32	1.11	29.26 ± 0.26	0.87
	1×10^2	32.03 ± 0.05	0.16	32.14 ± 0.21	0.66
	1×10^1	32.89 ± 0.21	0.64	34.74 ± 0.53	1.52

[a] *SD* Standard Deviation, *CV* coefficient of variation
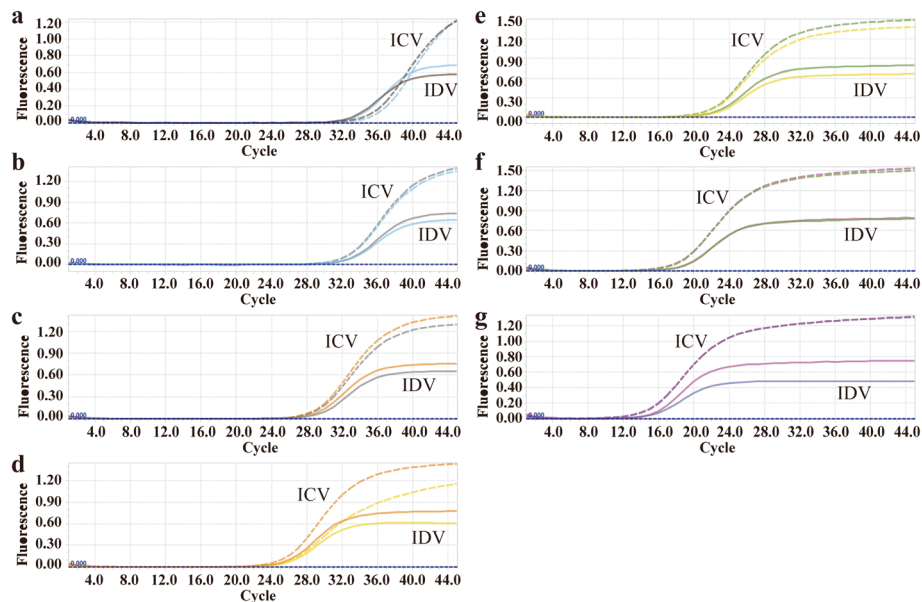
Fig. 3 Coinfection simulation experiments performed with mixed templates at the same concentrations. **a–g** Amplification curves of ICV + IDV at concentrations ranging from 1×10^7 copies/μL to 1×10^1 copies/μL were used in these experiments. Two replicates were included per reaction.

demonstrating that this method is stable and exhibits good repeatability.

Coinfection simulation test

Coinfection simulation experiment was performed using 1×10^7 copies/μL $\sim 1 \times 10^1$ copies/μL plasmid standards at the same concentration. (Fig. 3). The

performance to identify clinical samples with similar concentrations of two viruses was simulated. Another coinfection simulation experiment involved mixing plasmid standards of two pathogens. One standard was present at a concentration of 1×10^7 copies/μL, and the other was present at a concentration of 1×10^1 copies/μL (Fig. 4). The purpose of this
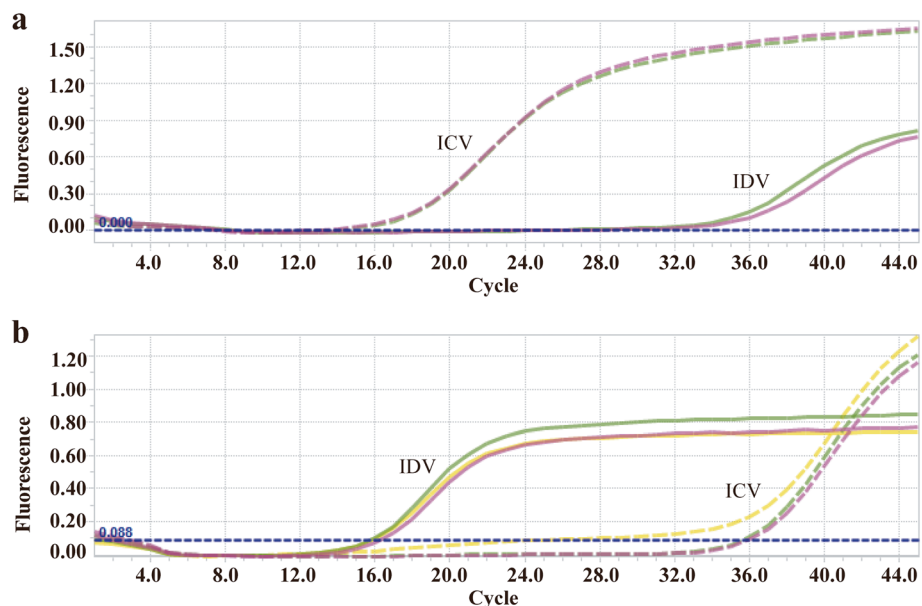
Fig. 4 Coinfection simulation experiments with mixed templates at different concentrations. **a** Amplification curves of ICV plasmid standard at concentrations of 1×10^1 copies/μL + IDV plasmid standard at concentrations of 1×10^7 copies/μL. **b** Amplification curves of ICV plasmid standard at concentrations of 1×10^7 copies/μL + IDV plasmid standard at concentrations of 1×10^1 copies/μL.

experiment was to simulate whether a high concentration of one virus would affect the detection of another virus present at a low concentration, given that the concentrations of two viruses can significantly vary in clinical samples. The two coinfection simulation test results suggested that low concentrations of ICV and IDV could be detected in mixed samples.

Clinical sample detection

The method established in this research was used to assess clinical samples, including 31 nasal swab and lung tissue samples collected from pig farms and 10 nasal swabs and blood samples collected from cattle farms in China, to verify its performance in clinical application. No positive ICV samples were detected, and one positive IDV sample was detected. Conventional PCR primers for *M* gene of ICV and *P42* gene of IDV were designed and synthesized. Then, the conventional PCR was used to assess the clinical samples, the positive samples were underwent DNA sequencing for verification. The positive control samples included lung tissues of pigs with respiratory symptoms that were collected from Guangxi Province in 2019. The detection results obtained using this method were consistent with conventional PCR tests and DNA sequencing, demonstrating that this duplex real-time PCR detection method is effective in clinical detection.

Discussion

There have been five recorded influenza pandemics since 1900 due to genetic drift and shift and repeated annual seasonal epidemics (Urbaniak and Markowska-Daniel 2014). The H5N1 strain of IAV was the first recorded strain that has crossed the barrier between animals and humans, resulting in zoonotic fatal infections (Mostafa et al. 2018). Emerging and re-emerging threats of influenza viruses remain a persistent public health challenge. Some studies suggested the potential for cross-species transmission and zoonotic transmission of ICV and IDV (Guo et al. 1983; White et al. 2016; Yuanji and Desselberger 1984). Therefore, clinical surveillance of ICV and IDV is beneficial to virus prevention and public health.

Because both IAV and IBV cause annual epidemics, strict surveillance programs are being implemented worldwide to monitor the slightest changes in genome caused by genetic drift or shift (Khanna et al. 2012; Khanna et al. 2008; Kumar et al. 2018; Taubenberger and Morens 2006; Treanor 2004). ICV and IDV only cause mild upper respiratory symptoms in host, and ICV occasionally causes severe lower respiratory symptoms in children. Thus, ICV and IDV are not considered as clinically important as IAV and thus have not been effectively monitored. Given the lack of effective monitoring data, the prevalence of ICV and IDV in China

remains unclear. ICV is distributed worldwide, and serological studies have shown that most humans can develop antibodies against the virus (Salez et al. 2014). One report showed that genetic similarity between porcine ICV and human ICV results in interspecies transmission (Guo et al. 1983; Yuanji and Desselberger 1984). Emerging IDV strains have also shown potential for interspecies transmission and zoonosis. Ferrets are model animals for human influenza virus infection, and studies have shown that IDV can replicate and be transmitted among ferrets (Sreenivasan et al. 2015). Analyses of the zoonotic potential of IDV in people who had occupational contact with cattle revealed that they had a high seroprevalence of IDV (94-97%). Thus, IDV may have the ability of interspecies transmission (White et al. 2016). Cross-species transmission of the virus poses a significant threat to human and livestock health. (Olival et al. 2017).

Analysis of the maximum-likelihood phylogenetic tree based on PB1 sequences of influenza A, B, C and D viruses revealed that IDV and ICV clustered most closely. Studies have also shown approximately 50% homology between IDV and ICV, and swine is the common host of ICV and IDV. Thus, it is necessary to identify the two pathogens in samples. Accurate and rapid detection method is an important prerequisite for effective monitoring of ICV and IDV. Nucleic acid detection with good specificity and repeatability offers great advantages in clinical detection; for example, the TaqMan-based real-time PCR method exhibits good specificity and sensitivity (Arya et al. 2005; Heid et al. 1996). In this study, a duplex real-time PCR method was developed to detect ICV and IDV.

The possibility of false-positive results will be increased given the improvement in sensitivity, so contamination should be avoided during sampling and experimental operations. Good operation is necessary to obtain credible results in the laboratory. To avoid interference between primers and probes designed for real-time PCR, the target fragments of ICV and IDV were designed to be similar in size and annealing temperature, and the reaction system was optimized. Finally, the limit of detection (LOD) of this assay were as low as 1×10^1 copies/μL for both pathogens, and no interference was noted between the two pairs of primers and probes. Faccini et al. developed and evaluated a singleplex real-time PCR detection method that can detected IDV in a short period time with an LOD of 20 copies/μL (Faccini et al. 2017). Kanti et al. developed a real-time PCR detection method for ICV and used this method to detect ICV infection in human respiratory tract samples from Alberta, Canada (Pabbaraju et al. 2013). Henritzi et al. developed a multiplex real-time PCR method for the detection of influenza A, B, C and D viruses. LOD of this

method reached 1×10^1 copies/μL (Henritzi et al. 2019), but the fluorescence intensity decreased obviously when plasmid standards with concentrations of 1×10^2 copies/μL and 1×10^1 copies/μL were detected. Thus, this detection method exhibited poor stability in low-concentration samples. Compared with previously reported methods, the duplex real-time PCR detection method developed in this study has better sensitivity and detection efficiency. Given that clinical samples with ICV and IDV coinfection were not detected in this study, a coinfection simulation test was performed. The maximum concentration difference between the two pathogen recombinant plasmids was selected as the template to simulate the possible interference between them in clinical environment. The detection method developed in this study still maintains high sensitivity and specificity when simultaneously assessing different concentrations of two pathogens in the same sample. There are some limitations in this study. Due to the difficulty of obtaining human clinical samples, we were unable to use this method to detect human clinical samples. In addition, no ICV-positive samples were detected.

In summary, ICV and IDV exhibit potential public health significance given the potential for cross-host transmission and zoonosis. At present, ICV and IDV are not effectively monitored in China. The high homology between them makes it necessary to establish a rapid and effective duplex detection method. Thus, a duplex real-time PCR assay for the detection of ICV and IDV was developed. Given the lower LODs, this new detection method with lower false-negative rates than currently available methods facilitates more effective ICV and IDV monitoring. The method can detect duplex pathogens simultaneously in a single reaction, representing a convenient coinfection detection method that is useful for effective clinical detection and scientific research.

Conclusions

In this study, a TaqMan-based duplex real-time PCR method was developed for the simultaneous detection of ICV and emerging IDV simultaneously. This method had good specificity and repeatability with an LOD as low as 1×10^1 copies/μL for both pathogens. At present, no efficient method is available to detect ICV and IDV simultaneously. This duplex real-time PCR detection method can be used for the rapid detection of ICV and IDV in a large number of samples in the context of clinical assessments and viral research, which also benefits animal and public health.

Materials and Methods
Construction of plasmid standards

RNA of ICV from positive samples was extracted as a template, and the designed primers were used for PCR amplification. Following the manufacturer's instructions,

cDNA of ICV was obtained by reverse transcription using the HiScript III RT SuperMix for qPCR (+gDNA wiper) Kit (Nanjing Vazyme Biotechnology Co., Ltd.), and then target fragments of ICV were amplified *via* high-fidelity PCR using Phanta Max Super-Fidelity DNA Polymerase (Nanjing Vazyme Biotechnology Co., Ltd.). After the selection of TA colonies and confirmation by DNA sequencing (Sangon Biotech (Shanghai) Co., Ltd.), PCR fragments were cloned into the pMD18-T vector. Sequencing confirmed that the recombinant plasmid pMD18-T-ICV contained an ICV insertion fragment. The recombinant plasmid pUC57-IDV containing IDV targeted fragment was synthesized by Nanjing Genscript Biotech Co., Ltd.

RNA extraction and reverse transcription

Samples positive for PCV2, PCV3, JEV, CSFV, IAV, TTsuV1, TTsuV2 and PRRSV were stored in our laboratory. Clinical samples collected from 2019 to 2020 were stored at -80 °C. Nasal swab and lung tissue samples were treated with 3 to 5 volumes of PBS. Then, samples were mixed with supernatant by vortexing and collected after centrifugation at 12,000×g at 4 °C for 15 min. Viral nucleic acids were extracted using the Viral DNA/RNA Kit (Jiangsu Cowin Biotech Co., Ltd.), and the HiScript III RT SuperMix for qPCR (+gDNA wiper) Kit was used to perform reverse transcription following the manufacturer's instructions.

Primers and probes

All ICV and IDV sequences available in GenBank were analyzed to improve the detection performance of the primers. Finally, we designed primers and probes for the conserved *NP* and *M* genes for ICV, and *PB1* and *P42* genes were chosen for IDV. Among them, ICV-detect-F/R and IDV-detect-F/R are two primer pairs used for real-time PCR detection method, and ICV-RT-F/R and IDV-RT-F/R are two primer pairs used for conventional PCR. The specific primers and probes used for the construction of plasmid standards were designed with Oligo 7 (V. 7.60) (Table 5) and synthesized by Sangon Biotech (Shanghai) Co., Ltd.

Reaction condition optimization for duplex real-time PCR

Amplification was performed on a Roche LightCycler® 96 Instrument (Roche Life Science) with the following program: 95 °C for 600 s; 45 cycles of 95 °C for 10 s, 55 °C for 10 s, and 72 °C for 20 s. Fluorescence signals were collected automatically at the end of each cycle. The duplex real-time PCR included the following components: 2× AceQ qPCR Probe Master Mix (AceQ qPCR Probe Master Mix kit, Nanjing Vazyme Biotechnology Co., Ltd.), primers, probes, and templates for ICV and IDV. To determine the optimal template concentration,

Table 5 Primer and probe sequences for ICV and IDV

Primer/Probe	Sequence 5'-3'	Gene	Product size	Position
ICV-Detect-F	AAGAGCCATGCAAGGAATTGA	*NP*	117 bp	710-826[a]
ICV-Detect-R	TCTGCGATGACATTTAGAGCTTC			
ICV-Probe	VIC-AAAGCGTTTCCTGGACCTTTAA-MGB			
ICV-RT-F	ATGGGRCGAATGGCDATGAAATGGT	*M*	257 bp	731-987[a]
ICV-RT-R	TCCCATCTGCCRAGYACAAAACC			
IDV-Detect-F	CCCTCCTACAGGAACAGAGTTTTC	*PB1*	124 bp	1880-2003[b]
IDV-Detect-R	GCGTTGATACAACTGCCTCG			
IDV-Probe	FAM-AGGAACCCCTTTACACAGTTTGAGAAGACAG-BHQ1			
IDV-RT-F	GTGGTGCACATATGCCTGAG	*P42*	487 bp	169-655[b]
IDV-RT-R	CCATTGCTCTTGAYCCATAYGC			

[a] GenBank accession NC_006311.1; [b] GenBank accession NC_036615.1

the duplex reaction system was optimized by using different primer (10 μM) and probe (10 μM) concentrations. In this stage, we used the primers and probes in the system at concentrations ranging from 400 nM to 1100 nM and 100 nM to 450 nM, respectively.

Sensitivity of the duplex real-time PCR assay
To explore LOD of the duplex real-time PCR method, PCRs for both viruses separately using standard plasmid templates with concentrations ranging from 1×10^7 copies/μL to 1×10^1 copies/μL were performed. At this stage, result is only considered the presumable LOD. To confirm LOD value, a duplex real-time PCR was performed using plasmid templates at the concentration of the presumable LOD, tenfold and 1/10 concentration of the presumable LOD with 15 replicates for each concentration. The lowest concentration that was consistent with the positive detection rate of ≥95% was regarded as the reliable LOD.

Specificity of the duplex real-time PCR assay
To exclude potential false-positive results caused by other viruses that may be present in the sample, duplex real-time PCR detection method was used to detect ICV and IDV standard plasmids and other respiratory virus-positive samples preserved in our laboratory, including PCV2, PCV3, JEV, CSFV, IAV, TTsuV1, TTsuV2 and PRRSV. RNase-free water was served as a negative control.

Repeatability of the duplex real-time PCR assay
Duplex real-time PCR using the standard plasmids of ICV and IDV at concentrations ranging from 1×10^7 copies/μL to the LOD was performed with three replicates for each reaction. The experiment was repeated thrice with a seven-day interval between replicates. The same concentrations of ICV and IDV standard plasmids were mixed to form the detection template. To evaluate the repeatability, CV of Cq values was calculated for different concentrations of virus in samples based on the three tests.

Standard sample simulated coinfection test
Coinfection with two pathogens may be encountered in clinical testing. To simulate actual coinfection events, two types of simulation experiments were performed. First, the same concentrations of plasmid standards of ICV and IDV were mixed as templates and detected using the duplex real-time PCR assay. The standard plasmid of each pathogen at concentrations ranging from 1×10^7 copies/μL to the LOD was tested. In another simulation experiment, different concentrations of standard samples were combined. The plasmid standard of one pathogen at a concentration of 1×10^7 copies/μL and the other at the LOD were mixed. Then, sample mixture was assessed using the duplex real-time assay.

Clinical sample detection
We tested 31 nasal swab and lung tissue samples collected from pigs using duplex real-time method. Clinical performance of this methods was evaluated by comparing the results with those of conventional PCR and DNA sequencing. DNA sequencing was performed by Sangon Biotech (Shanghai) Co., Ltd. to confirm positive samples detected using this detection method.

Authors' contributions
S.S. and P.Z.Z. were involved in the experimental design and provided guidance on the experimental operation. Z.L.T., L.M., L.J.X. and W.N.N. performed the experiments and data analysis. All authors contributed to writing the manuscript. All authors have read and approved the final version of the manuscript.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing financial interest.

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