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Development of a multiplex real-time RT-PCR assay for simultaneous detection and differentiation of influenza A, B, C, and D viruses

Hewei Zhang [a,d], Yin Wang [a], Elizabeth Porter [a], Nanyan Lu [a], Yanhua Li [b], Fangfeng Yuan [a], Molly Lohman [a], Lance Noll [a], Wanglong Zheng [a], Colin Stoy [a], Yuekun Lang [b], Victor C. Huber [c], Wenjun Ma [b], Lalitha Peddireddi [a,b], Ying Fang [b], Jishu Shi [e], Gary Anderson [a,b], Xuming Liu [a,b,*], Jianfa Bai [a,b,**]

[a] Kansas State Veterinary Diagnostic Laboratory, College of Veterinary Medicine, Kansas State University, Manhattan, KS, USA
[b] Department of Diagnostic Medicine and Pathobiology, College of Veterinary Medicine, Kansas State University, Manhattan, KS, USA
[c] Division of Basic Biomedical Sciences, Sanford School of Medicine, The University of South Dakota, Vermillion, SD, USA
[d] College of Food and Drugs, Luoyang Polytechnic, Luo Yang, Henan, China
[e] Department of Anatomy and Physiology, College of Veterinary Medicine, Kansas State University, Manhattan, KS, USA

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ABSTRACT

Influenza is a common and contagious respiratory disease caused by influenza A, B, C, and D viruses (IAV, IBV, ICV, and IDV). A multiplex real-time RT-PCR assay was developed for simultaneous detection of IAV, IBV, ICV, and IDV. The assay was designed to target unique sequences in the matrix gene of IBV and ICV, the RNA polymerase subunit PB1 of IDV, and combined with USDA and CDC IAV assays, both target the matrix gene. The host 18S rRNA gene was included as an internal control. *In silico* analyses indicated high strain coverages: 97.9% for IBV, 99.5% for ICV, and 100% for IDV. Transcribed RNA, viral isolates and clinical samples were used for validation. The assay specifically detected target viruses without cross-reactivity, nor detection of other common pathogens. The limit of detection was approximately 30 copies for each viral RNA template, which was equivalent to a threshold cycle value of ~37.

1. Introduction

Influenza is a highly contagious viral respiratory disease caused by influenza viruses, which are single-strand, negative sense RNA viruses. The viruses belong to the family *Orthomyxoviridae* and are classified into four influenza virus genera. Each genus contains only one species, designated as influenza A, B, C, and D virus (IAV, IBV, ICV, and IDV) (King et al. 2018; Vemula et al. 2016). Influenza genomes are highly variable due to frequently occurred reassortments and mutations (Brockwell-Staats et al. 2009; Lowen 2017; Lyons and Lauring 2018; Steel and Lowen 2014).

IAV is the most common and widely distributed pathogen that can infect humans, pigs, cattle, birds, and other animals (Nelson and Vincent 2015; Vemula et al. 2016). The natural host and reservoir of IBV was long assumed to be restricted exclusively to humans, but recent serological, molecular, and experimental infection studies demonstrated that pigs are also susceptible to IBV infection (Ran et al. 2015), which supports and extends the previous observations made in the 1960s (Takátsy et al. 1969; Takatsy et al. 1967). ICV was first identified in humans in 1947, and was originally thought to be exclusively a human pathogen until 1981, when it was also isolated from pigs in China (Yuanji and Desselberger 1984; Yuanji et al. 1983), and recently identified in cattle in the US (Zhang et al. 2018a; Zhang et al. 2018b). IDV was first isolated and characterized in swine in 2011, and later identified in cattle (Collin et al. 2015; Hause et al. 2014; Hause et al. 2013; Mitra et al. 2016). Most recently, IDV infections in swine and bovine herds have been widely reported in China, Italy, Mexico, France, and Japan (Chiapponi et al. 2016; Foni et al. 2017; Horimoto et al. 2016; Rosignoli et al. 2017; Zhai et al. 2017).

Human and animal IAV and IBV have been well studied and various well-characterized diagnostic assays are available (Banerjee et al. 2018; Bell et al. 2014; Bruning et al. 2017; Cho et al. 2013; Vemula et al. 2016). However, there is a pressing need to develop new diagnostic assays, particularly those that can detect and differentiate multiple pathogens for rapid and accurate detection and differentiation of the two understudied and under-diagnosed influenza viruses, ICV, and IDV.

Real-time PCR is considered as the most practical and sensitive approach for identification of influenza viruses and other pathogens in most diagnostic laboratories (Hoffmann et al. 2009; Kralik and Ricchi 2017; Vemula et al. 2016). Currently, the United States Department of

* Corresponding author. Tel.: +1-785-532-3407.
** Corresponding author. Tel.: +1-785-532-4332; fax: +1-785-532-3909.
E-mail addresses: xmliu@vet.ksu.edu (X. Liu), jbai@vet.ksu.edu (J. Bai).

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Agriculture (USDA)-validated real-time RT-PCR (RT-qPCR) assay for detection of IAV in swine samples (adapted from an avian influenza assay) can generally cover the Eurasian and North American swine IAV and the 2009 pandemic H1N1 lineages of swine-origin (Slomka et al. 2010; Spackman and Suarez 2008; Zhang and Harmon 2014). In addition, the Centers for Disease Control and Prevention (CDC) RT-qPCR protocol for the universal detection of type A influenza viruses has been recommended by the World Health Organization (WHO) (Selvaraju and Selvarangan 2010). Singleplex RT-qPCR assays were developed for the detection of IBV in humans (Ran et al. 2015; Selvaraju and Selvarangan 2010), and ICV in humans (Faux 2010; Howard et al. 2017; Pabbaraju et al. 2013; Salez et al. 2014), as well as IDV in swine and cattle (Faccini et al. 2017; Hause et al. 2013). Multiplex real-time RT-PCR (RT-mqPCR) assays were also developed for simultaneous detection of IBV and IAV (Hindiyeh et al. 2013; Hindiyeh et al. 2005; Smith et al. 2003; Templeton et al. 2004; Van Elden et al. 2001). However, no RT-mqPCR assay has been reported so far for simultaneous detection of any three or four influenza viruses. Due to frequent mutations in the viral genomes (Stellrecht 2018), and more sequences have been generated every year, the previously designed RT-qPCR assays may not be able to detect the majority of current field strains and variants especially for ICV and IDV viruses. *In silico* analyses indicated that many published primers and probes have low coverage rates against the currently available influenza virus sequences, and the coverage rates of the above mentioned RT-qPCR assays are lower than 90%, and most of them are ranging from 40% to 60%. Such coverage rates are not sufficient to meet the needs for influenza surveillance and diagnosis.

The main objective of this study was to develop and validate a high-coverage, highly sensitive and specific RT-mqPCR assay to simultaneously detect and differentiate IAV, IBV, ICV, and IDV strains. In this study, RT-mqPCR assays for specific detection of IBV, ICV, and IDV (plus an internal control) were developed by designing primers and probes based on all currently available sequences. Then a 4-plex assay was formed and optimized by combining the IBV, ICV, and IDV assays with the internal control. The newly developed 4-plex IBV, ICV, and IDV assay was further combined with the currently used IAV assays recommended by USDA as well as OIE (World Organization for Animal Health), CDC and WHO. The resulting 5-plex RT-mqPCR assay was optimized and validated for simultaneous detection and differentiation of all four influenza viruses.

2. Materials and methods

The American Association of Veterinary Laboratory Diagnosticians (AAVDL) and OIE standard guidelines on the basic procedures and quality control for the development, optimization, and validation of molecular assays were followed. The major protocols are described similarly in our previous publication (Shi et al. 2016).

2.1. Samples and virus isolates

A total of 720 swine clinical samples and 1525 bovine clinical samples (including nasal swab, oral fluid, lung, blood, feces and stomached organ tissue homogenate) were collected from Kansas State Veterinary Diagnostic Laboratory (KSVDL) during 2016 and 2017 and were subjected to testing with the newly developed 5-plex assay. Additional 38 known swine IAV positive samples were collected at an abattoir in Iowa during 2012–2014, and 1 IAV, 4 IBV, 1 ICV, and 6 IDV cell isolates were kindly provided by Drs. Wenjun Ma and Jishu Shi of Kansas State University College of Veterinary Medicine.

2.2. Design of real-time RT-PCR assays

As the matrix (M) segment of IBV and ICV and the RNA polymerase complex subunit, PB1 segment of IDV are the most conserved segment to each virus, they were selected as the molecular targets for the assay design. According to all complete and near-complete segment sequences that were available from the GenBank, 6593 IBV and 196 ICV M gene sequences, and 29 IDV PB1 sequences were used for primer and probe designs to achieve highest possible coverage rate. The host housekeeping gene, 18S rRNA gene that amplifies from swine, bovine, and human samples, was chosen to serve as an internal control to monitor the nucleic acid extraction efficiencies and potential PCR inhibitions (Bai et al. 2018). Real-time PCR primers were designed with annealing temperature (Tm) of approximately 60 °C and the probes with Tm of approximately 63–65 °C. The cloning and sequencing primer pairs were designed flanking the real-time RT-PCR targets for the construction of positive control plasmids and Sanger sequencing confirmation of the positive clinical samples. The IAV universal primers and probes were adopted from two RT-qPCR assays that are recommended by the USDA (OIE 2015, http://www.oie.int/standard-setting/terrestrial-manual/access-online/) and the CDC IAV assay (WHO 2017, http://www.who.int/influenza/resources/laboratories/en/). Both the USDA and CDC IAV assays are targeted on the matrix gene, and equal amount of primers and probes were used in the 5-plex RT-mqPCR assay. All primers and probes are listed in Table 1.

In this study, the singleplex assay is defined as test on single target. The duplex assay is defined as test on one of the four influenza viruses along with the internal control (IC), 18S rRNA gene. The 5-plex assay includes IAV, IBV, ICV, and IDV plus the IC in a single reaction.

2.3. Extraction and purification of nucleic acid

The influenza virus RNA was extracted from 140 μL of clinical samples by QIAamp Viral RNA Mini Kit (Qiagen, Valencia, CA), or 100 μL cell culture (supernatants for IAV and IDV, allantoic fluids for IBV and ICV) by Direct-zol RNA MiniPrep kit (Zymo Research, Irvine, CA) according to the manufacturer's recommendations. Plasmid DNA was purified using Qiagen QIAprep MiniPrep kit, and PCR products (for cloning or sequencing) were purified using Qiagen QIAquick PCR purification kit.

2.4. Construction of positive control plasmids and preparation if in vitro transcribed RNA

The IBV and ICV M gene and IDV PB1 gene fragments flanking the RT-qPCR targets were reverse transcribed into cDNA using SuperScript III first-strand synthesis system (Invitrogen/ThermoFisher, Carlsbad, CA), then PCR amplified using TaKaRa LA Taq PCR kit (TaKaRa, Mountain View, CA). Purified PCR products were cloned into pCR4 vector and transformed into *E. coli* cells using Invitrogen TOPO TA PCR Cloning kit. *In vitro* transcribed RNA was produced using MEGAscript T7 Transcription kit (LifeTechnologies/ThermoFisher, Carlsbad, CA). Both the constructed plasmid DNA and the *in vitro* transcribed RNA were used as the templates for initial analytical analysis of the real-time PCR assays.

2.5. Real-time PCR protocols

All real-time PCR reactions were carried out in a 20 μL reaction that consists of 0.4 μM of each forward and reverse primer, 0.2 μM of each probe, 3 μL of template, 2 μL of 10x Multiplex Enzyme Mix, and 10 μL of 2 × Multiplex RT-PCR Buffer from Path-ID Multiplex One-Step RT-PCR Kit (Applied Biosystems/ThermoFisher, Grand Island, NY). The thermocycling parameters include a reverse transcription step at 48 °C for 10 min and RT inactivation and denaturation at 95 °C for 10 min, followed by 45 cycles of denaturation at 95 °C for 15 s and annealing/extension at 60 °C for 45 s. The reactions were run with the Bio-Rad CFX96™ Touch™ Real-time PCR Detection System (Bio-Rad, Hercules, CA). The threshold cycle (Ct) values were generated and results were analyzed using Bio-Rad CFX Manager 3 software. Graphs were generated by GraphPad Prism 7 (GraphPad Software, La Jolla, CA).

Table 1
Primers and probes used for real-time RT-PCR, molecular cloning, and sequencing confirmation.

Assay	Target Gene	Primer probe	Sequence (5′-3′)	Tm (°C)	Amplicon size (bp)	*In silico* coverage
			Real-time PCR primers and probes			
IBV1	M	IBV1-Fa	GCAGAGCAGCGAGATCTTCAG	62.8	96	IBV1:89.7%
		IBV1-Fb	CAGAGCAGCGAGATCCTCA	60.4		(5911/6593)
		IBV1-R	CTTTYCCCATTCCATTCATTGT	60.1–62.1		
		IBV1-Pr	TEX615-CTGTGTTCATAGCTGAGACCATCTGCIBRQ	65.5		
IBV2	M	IBV2-F	TCCTGGAAATTATTCAATGCAAG	60.3	78	IBV2: 87.9%
		IBV2-R	CTGTGTGAATGTGATGCTTGTTT	60.1		(5794/6593)
		IBV2-Pr	TEX615-CGCAYAAAGCACAGAGYGTTCC-IBRQ	65.4–69.5		97.9% combined (6456/6593)
ICV1	M	ICV1-F	TCGGCAGATGGGAGAGATG	62.3	144	ICV1:95.4%
		ICV1-R	GAATTGGTGAGTTGTCGGTTTC	60.8		(187/196)
		ICV1-pr	MAX-CTCCCAGGTCAAGTCTCTCCCT-IBFQ	63.3		
ICV2	M	ICV2-F	TGGCCTTGGAGAAGAAGCA	62.0	100	ICV2:74.0%
		ICV2-R	CAAGTGGGGTCTCATTATATTACTTCC	61.4		(145/196)
		ICV2-Pra	MAX-TGATTGCATAATATGGCCAAACTTTCT-IBFQ	64.6		99.5% combined (195/196)
		ICV2-Prb	MAX-TGTGATTACATAATATGGCCAAACTTTCTG-IBFQ	64.9		
IDV	PB1	IDV-F	AATTCTGTGCCAATGAAGCTG	60.3	104	IDV:100%
		IDV-Ra	TGGCATATTTCTTTCACTTGTCC	60.4		(29/29)
		IDV-Rb	TGGCATATTTCTTTCGCTTGTC	61.4		
		IDV-Rc	GCATGTTTCTTTCACTCGTCC	59.7		
		IDV-Pr	Q705-CATAAGTTTGYCTTCCTTCAGTGAGCTT-BHQ3	63.4–66.7		
IAV1-USDA	M	M25-F	AGATGAGTCTTCTAACCGAGGTCG	62.3	100	USDA (OIE 2015)
		M124-Ra	TGCAAAAACATCTTCAAGTCTCTG	60.8		
		M124-Rb	TGCAAAGACACTTTCCAGTCTCTG	63.5		
		M64-Pr	FAM-TCAGGCCCCCTCAAAGCCGA-BHQ1	71.5		
IAV2-CDC	M	InfA-F	GACCRATCCTGTCACCTCTGAC	61.0–63.4	106	CDC (WHO 2017)
		InfA-R	AGGGCATTYTGGACAAAKCGTCTA	64.9–67.2		
		InfA-Pr	FAM-TGCAGTCCTCGCTCACTGGGCACG-BHQ1	76.5		
18S rRNA	18S rRNA	18S-F	GGAGTATGGTTGCAAAGCTGA	60.3	100	
		18S-R	GGTGAGGTTTCCCGTGTTG	61.4		
		18S-Pr	Cy5-AAGGAATTGACGGAAGGGCA-BHQ3	64.0		
	Cloning and sequencing primers					
IBV	M	IBV-*cF*	TGCTTTTTAAAACCCAAAGACCAG	62.4	505	
		IBV-cR	GCACTTCCATTACATCCTTTGCA	63.4		
ICV	M	ICV12-cF2	AAAGCCAGCACAGCAATGAA	61.9	590	
		ICV12-cR1	TCAAAAATACCATCATTGGAAAAAGG	63.2		
IDV	PB1	IDV1-*cF*	GTCAAGAACTTACTCCTACAGTAGAAC	55.6	360	
		IDV1-cR	GTTCCTTTGAATGTTCCCTGTGT	61.6		

2.6. Specificity analyses and diagnostic performance of the assays

Samples used for specificity analysis include: 1 IAV isolate and 55 IAV clinical samples; 4 IBV isolates; 1 ICV isolate and 38 bovine ICV samples; 6 IDV isolates and 71 IDV samples. Clinical samples positive to the influenza viruses were confirmed by Sanger sequencing or by other validated RT-PCR assays. Swine and bovine samples positive to other common pathogens and negative to influenza viruses including 91 porcine circovirus type 2 (PCV2), 178 porcine circovirus type 3 (PCV3), 40 porcine deltacoronavirus (PDCoV), 58 porcine epidemic diarrhea virus (PEDV), 18 porcine parainfluenza virus (PPIV), 76 porcine reproductive and respiratory syndrome virus (PRRSV), 5 group A rotavirus, 1 group B rotavirus, and 2 group C rotavirus samples were also used for specificity analysis.

2.7. Sensitivity analyses with singleplex, duplex, and 5-plex assays

The sensitivity was determined by 10-fold serial dilutions of constructed plasmid DNA, *in vitro* transcribed RNA, RNA extracted from viral isolates, and RNA extracted from clinical positive samples, respectively. Standard curves were generated with singleplex, duplex, and 5-plex real-time RT-PCR reactions. The limit of detection (LOD) was determined using 95% Probit analysis at the detectable concentrations. Furthermore, the Ct values of duplex and 5-plex assays on 55 IAV, 71 IDV, and 21 ICV clinical samples were compared (Only 21 of the 38 ICV samples were available for this comparison).

3. Results

3.1. In silico analysis and prediction of assay coverage

Because of high level of divergence in influenza virus sequences, some of the primers and probes were designed with degenerate bases, and two pairs of primers and probes targeting different regions were designed for IBV and ICV assays. The coverage rate (%) is calculated as the percentage of total number of perfectly matched sets of "forward primer + reverse primer + probe" sequences over the total number of currently available sequences from the GenBank (as of November 15, 2017). The *in silico* analysis indicated that the coverage rates are 97.9% (6456/6593) for IBV, 99.5% (195/196) for ICV, and 100% (29/29) for IDV strains (Table 1).

3.2. Analytical sensitivity of the real-time PCR assays

From real-time PCR assays on plasmid DNA and *in vitro* transcribed RNA samples, the PCR amplification efficiencies (E%) were ranged 92–105% with correlations coefficient (R^2) greater than 0.99. The limit of detection (LOD) on *in vitro* transcribed RNA was approximately 30 copies per reaction for both singleplex and 5-plex assays. Specifically, 27 copies for IBV, 24 copies for ICV, and 35 copies for IDV, which corresponds to Ct values of 36–38. These were no noticeable Ct differences observed between the singleplex and 5-plex assays with plasmid DNA and *in vitro* transcribed RNA templates.

Since the constructed plasmids and *in vitro* transcribed RNA only contain a small portion of the influenza virus genome, to make sure

the assay is not compromised when tested on full viral genome, the RNA samples extracted from cell cultures of the influenza viral isolates were tested with the singleplex and 5-plex real-time RT-PCR assays. The standard curves plotted with Cts *versus* Log dilution factors or template concentrations showed that all the PCR amplification efficiencies (E%) and the correlations coefficient (R^2) are within the satisfactory range (E% = 90–110%, and $R^2 > 0.990$) (Fig. 1 and Table 2). The standard curves of the internal control 18S rRNA showed no significant difference between the singleplex and 5-plex assays (Fig. 1A. Similar IC standard curves were generated but not shown in Fig. 1B, C, and D). In addition, very similar Cts were generated by the singleplex and 5-plex real-time RT-PCR assays on the serial dilutions of RNA from viral cell cultures. The average Ct difference between the singleplex and 5-plex assays on serial dilutions of virus isolates was within a single Ct difference (<0.6), which indicates that multiplexing does not significantly affect analytical sensitivities of the real-time RT-PCR assay (Fig. 1 and Table 2).

3.3. Assay sensitivity with diagnostic samples

To make sure the assay sensitivity is not compromise when tested on diagnostic samples, IAV, ICV and IDV positive clinical samples were used for further study (no positive IBV clinical samples available). The internal control was detected in all samples except the plasmids and *in vitro* transcribed RNA samples, indicating a good practice in nucleic acid extraction and there were no PCR inhibitions. To determine the LODs in terms of Ct cutoff values, the clinical samples were serially diluted by 10-fold at the first 5 dilutions, and followed by a serial of 2-fold dilutions for more accurate LOD determinations. The 5-plex real-time RT-PCR assay generated similar sensitivities to that generated by duplex assays. The standard curves plotted with Cts *versus* dilution factors showed that all PCR amplification efficiencies (E%) are ranged 90%–100% and the correlations coefficient (R^2) are greater than 0.99.

The results also indicated that the reliable Ct cutoff values for influenza virus diagnosis can be defined as: Ct ≤36 are positives, Ct = 36–39 are weak positives, and Ct >39 are negatives. In addition, the average Ct difference between the 5-plex and duplex assays on serial dilutions of clinical samples was within a single Ct difference (< 0.6), which indicates that results from the two assays are very close (Fig. 2 and Table 3).

3.4. Specificity of the duplex and 5-plex assays

The specificity of primers and probes was first evaluated by *in silico* analysis with NCBI primer design tool, which presented unique influenza virus target for each set of assay. To experimentally distinguish pathogens among influenza viruses and from other swine and bovine pathogens, cell cultures of the IAV, IBV, ICV, and IDV isolates, clinical samples positive to the influenza viruses (confirmed by Sanger sequencing or by other validated assays), and clinical sample negative to influenza viruses but positive to other common swine and bovine pathogens were assayed with duplex and 5-plex real-time RT-PCR. The results demonstrated that the IAV, IBV, ICV, and IDV assays detected the specific target viruses without cross detection of any non-target pathogens, including those that were positive to common swine and bovine pathogens listed in the Materials and Methods section.

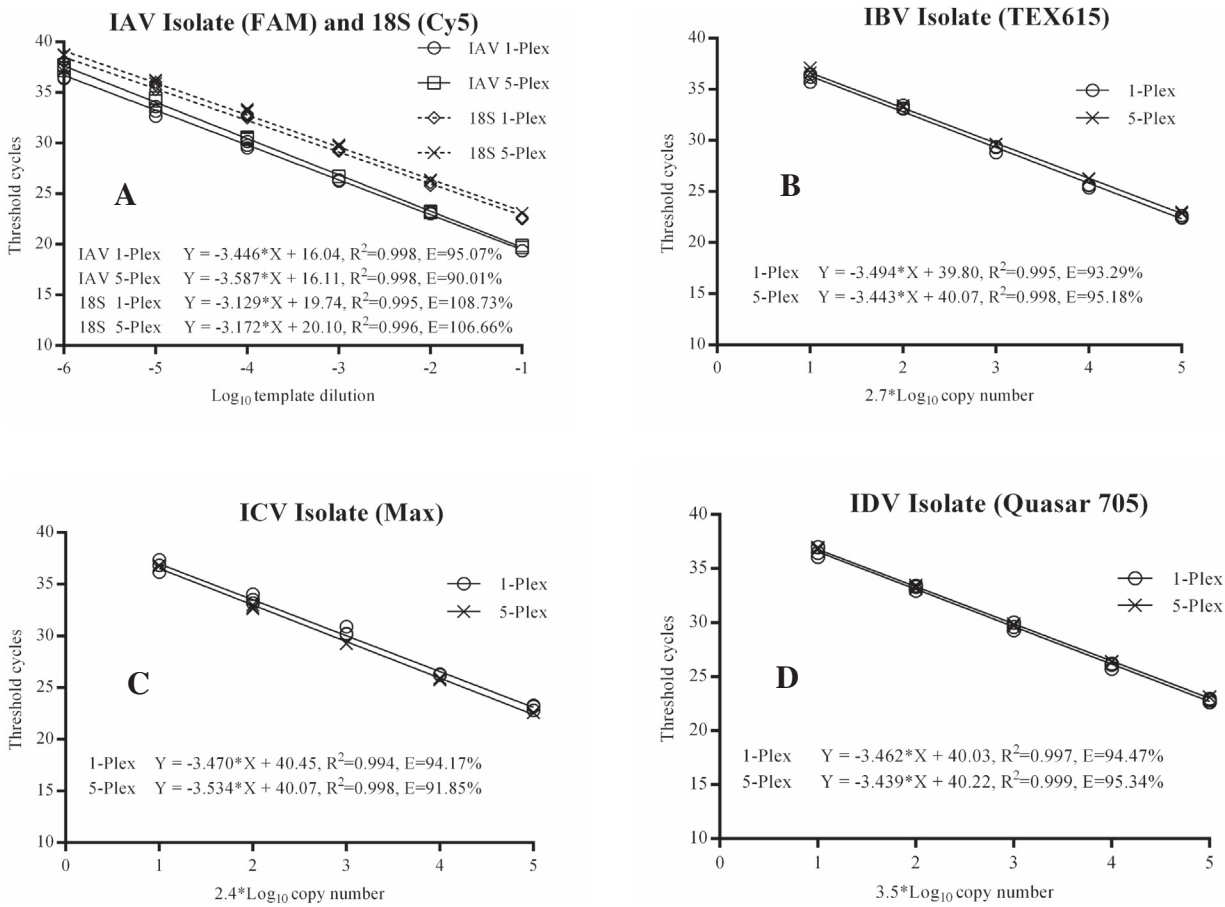

Fig. 1. Standard curves of RT-qPCR assays with serial dilutions of cultured virus isolates. Panel A: Standard curves of IAV and 18S rRNA by singleplex and 5-plex RT-qPCR assays; Panel B: IBV standard curves of singleplex and 5-plex RT-qPCR at starting viral isolate RNA concentration of 2.7×10^6 copies/reaction; Panel C: ICV standard curves of singleplex and 5-plex RT-qPCR at starting concentration of 2.4×10^6 copies/reaction; Panel D: IDV standard curves of singleplex and 5-plex RT-qPCR at starting concentration of 3.5×10^6 copies/reaction.

Table 2
Standard curve comparison of 5-plex and singleplex assays on viral isolates.

Assay	Target Gene	Primer / probe	Sequence (5'-3')	Tm (°C)	Amplicon size (bp)	*in silico* Coverage
Real-time PCR primers and probes						
IBV1	M	IBV1-Fa	GCAGAGCAGCGAGATCTTCAG	62.8	96	IBV1:89.7%
		IBV1-Fb	CAGAGCAGCGAGATCCTCA	60.4		(5911/6593)
		IBV1-R	CTTTYCCCATTCCATTCATTGT	60.1-62.1		
		IBV1-Pr	TEX615-CTGTGTTCATAGCTGAGACCATCTGC-IBRQ	65.5		
IBV2	M	IBV2-F	TCCTGGAAATTATTCAATGCAAG	60.3	78	IBV2: 87.9%
		IBV2-R	CTGTGTGAATGTGATGCTTGTTT	60.1		(5794/6593)
		IBV2-Pr	TEX615-CGCAYAAAGCACAGAGYGTTCC-IBRQ	65.4-69.5		97.9% combined (6456/6593)
ICV1	M	ICV1-F	TCGGCAGATGGGAGAGATG	62.3	144	ICV1:95.4%
		ICV1-R	GAATTGGTGAGTTGTCGGTTTC	60.8		(187/196)
		ICV1-pr	MAX-CTCCCAGGTCAAGTCTCTCCCT-IBFQ	63.3		
ICV2	M	ICV2-F	TGGCCTTGGAGAAGAAGCA	62.0	100	ICV2:74.0%
		ICV2-R	CAAGTGGGGTCTCATTATATTACTTCC	61.4		(145/196)
		ICV2-Pra	MAX-TGATTGCATAATATGGCCAAACTTTCT-IBFQ	64.6		99.5% combined (195/196)
		ICV2-Prb	MAX-TGTGATTACATAATATGGCCAAACTTTCTG-IBFQ	64.9		
IDV	PB1	IDV-F	AATTCTGTGCCAATGAAGCTG	60.3	104	IDV:100%
		IDV-Ra	TGGCATATTTCTTTCACTTGTCC	60.4		(29/29)
		IDV-Rb	TGGCATATTTCTTTCGCTTGTC	61.4		
		IDV-Rc	GCATGTTTCTTTCACTCGTCC	59.7		
		IDV-Pr	Q705-CATAAGTTTGYCTTCCTTCAGTGAGCTT-BHQ3	63.4-66.7		
IAV1-USDA	M	M25-F	AGATGAGTCTTCTAACCGAGGTCG	62.3	100	USDA (OIE 2015)
		M124-Ra	TGCAAAAACATCTTCAAGTCTCTG	60.8		
		M124-Rb	TGCAAAGACACTTTCCAGTCTCTG	63.5		
		M64-Pr	FAM-TCAGGCCCCCTCAAAGCCGA-BHQ1	71.5		
IAV2-CDC	M	InfA-F	GACCRATCCTGTCACCTCTGAC	61.0-63.4	106	CDC (WHO 2017)
		InfA-R	AGGGCATTYTGGACAAAKCGTCTA	64.9-67.2		
		InfA-Pr	FAM-TGCAGTCCTCGCTCACTGGGCACG-BHQ1	76.5		
18S rRNA	18S rRNA	18S-F	GGAGTATGGTTGCAAAGCTGA	60.3	100	
		18S-R	GGTGAGGTTTCCCGTGTTG	61.4		
		18S-Pr	Cy5-AAGGAATTGACGGAAGGGCA-BHQ3	64.0		
Cloning and sequencing primers						
IBV	M	IBV-cF	TGCTTTTTAAAACCCAAAGACCAG	62.4	505	
		IBV-cR	GCACTTCCATTACATCCTTTGCA	63.4		
ICV	M	ICV12-cF2	AAAGCCAGCACAGCAATGAA	61.9	590	
		ICV12-cR1	TCAAAAATACCATCATTGGAAAAAGG	63.2		
IDV	PB1	IDV1-cF	GTCAAGAACTTACTCCTACAGTAGAAC	55.6	360	
		IDV1-cR	GTTCCTTTGAATGTTCCCTGTGT	61.6		

Note: (1) The starting viral RNA concentration for IBV, ICV, and IDV RT qPCR assays were 2.7×10^6, 2.4×10^6, and 3.5×10^6 copies/reaction, respectively. (2) Ct difference is the average absolute Ct value difference between the singleplex and 5-plex assays.

3.5. Diagnostic performance of the duplex and 5-plex assays on clinical samples

A total of 55 IAV, 38 ICV, and 71 IDV positives were identified from clinical samples with the newly developed 5-plex assay. The 55 IAV positive samples include 37 positive IAV samples (swine nasal swabs) from the Iowa abattoir, 15 positives detected from 127 KSVDL archived swine nasal swabs (11.8%), 2 positives from 27 lung samples (7.4%), and 1 positive from 56 oral fluids (1.8%). From the 1525 archived bovine clinical samples with suspected respiratory diseases, 38 samples were assayed as positive to ICV, four were dual-positive to ICV and IDV, and 12 ICV positives (Ct <30) were confirmed by Sanger sequencing. Of these 38 positive ICV samples, 23 were identified from 952 nasal swabs (2.4%) and 15 positives from 498 lung samples (3.0%). Among the 71 IDV positive samples assayed in this study, 69 were positives and 2 were negatives as previously tested by the IDV assay currently used at KSVDL. Of these 71 positive IDV samples, 42 positives were identified from 952 nasal swabs (4.4%), 28 positives from 498 lung samples (5.6%), and 1 positive from 4 oral fluids. A total of 30 IDV positives (Ct < 30) were confirmed by Sanger sequencing of the PB1 gene fragment. The four samples co-positive for ICV and IDV were also confirmed by Sanger sequencing. For the ICV and IDV positive samples that were not sequenced, some did not have enough amount of extracted RNA, and most samples with Ct >30 were not amplified by the sequencing primers using regular PCR, likely due to the low concentration and degradation of RNA samples during the storage, as the fragment sizes for Sanger sequencing (590 nt for ICV and 360 nt for IDV) are much larger than the real-time RT-PCR targets (100 or 144 nt for ICV and 104 nt for IDV).

The newly developed duplex and 5-plex assays were compared by testing 55 IAV, 21 ICV, and 71 IDV positive clinical samples, and generated similar Ct values with ranges of: 19.02–34.74 by IAV duplex and 19.22–35.35 by IAV 5-plex; 15.66–36.52 by ICV duplex and 15.33–36.61 by ICV 5-plex; 12.23–36.67 by IDV duplex, and 12.12–36.34 by IDV 5-plex. The averages of absolute differences in Ct values between the duplex and 5-plex assays on these 55 IAV, 21 ICV, and 71 IDV positive samples were 0.30 (ranging 0–0.98), 0.49 (0.07–1.91), and 0.43 (0–1.60), respectively, which were within a single Ct difference.

4. Discussion

Although a number of real-time PCR assays have been published and currently used for diagnosis of influenza viruses, due to the difficulty of real-time PCR design and the challenges of multiplexing, none is highly multiplexed and are with high strain coverage. Most of those assays especially ICV and IDV assays are not capable of detecting the majority of current field strains and variants, and are not sufficient to meet the needs of influenza surveillance and diagnosis. In this study, we developed a high coverage and highly multiplexed (5-plex) real-time RT-PCR assay for simultaneous detection and differentiation of all four influenza viruses. The *in silico* analysis indicated that the binding sites of our designed primers and probes are highly conserved, therefore the newly developed real-time PCR assays can potentially maintain high coverage against additional field influenza strains.

The benefits of multiplexing real-time PCR are obviously economics and convenience, such as cost-saving with fewer reactions and less reagents, time-saving, and enhanced throughput. In a multiplex real-time PCR, multiple targets (usually between 2 and 4) are detected

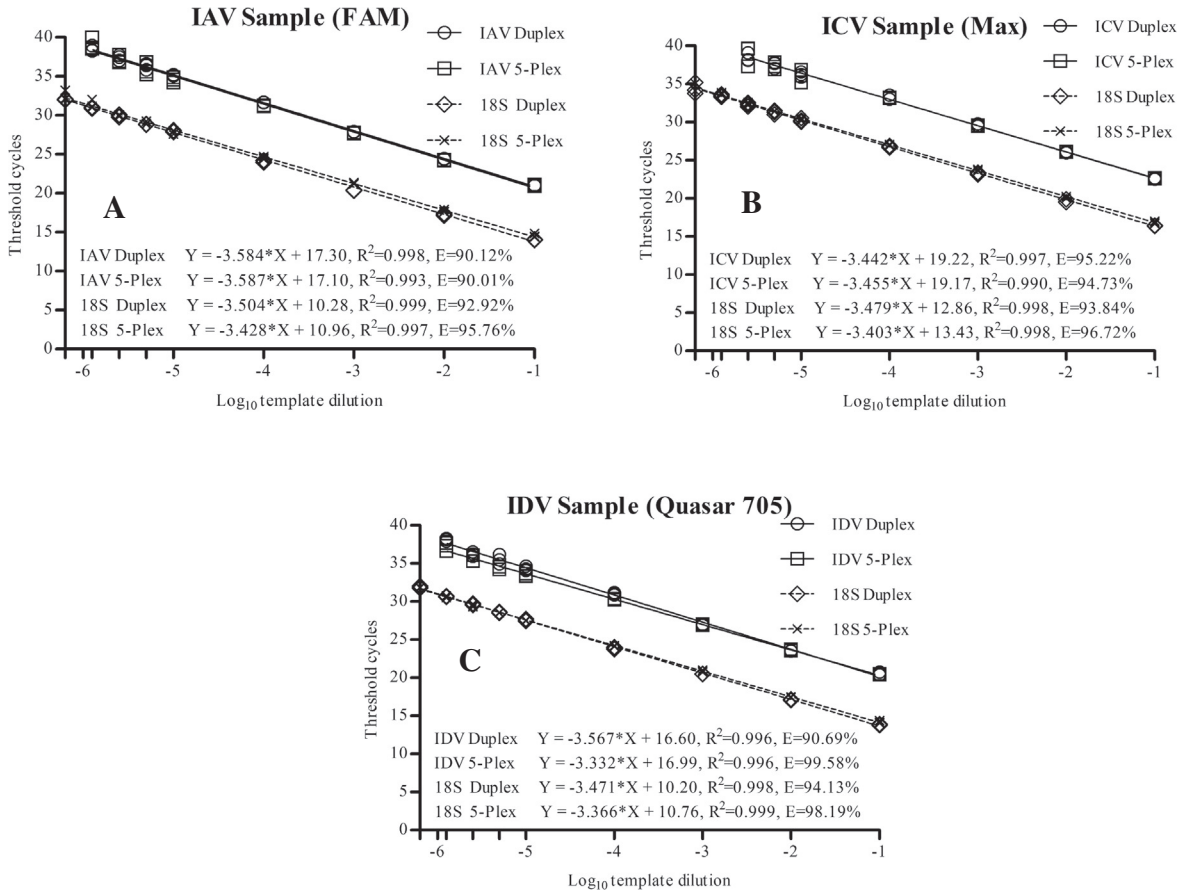

Fig. 2. Standard curves generated by the RT-qPCR assays with positive clinical samples of IAV, ICV and IDV. Panel A: IAV standard curves of duplexed condition (with 18S rRNA) and compared with 5-plex reactions (addition of IBV, ICV and IDV); Panel B: ICV standard curves of duplexed condition and compared with 5-plex reaction; Panel C: IDV standard curves of duplexed condition and compared with 5-plex reaction.

simultaneously in the same reaction. However, the challenge of a probe-based multiplex real-time PCR assay is potentially reduced sensitivity by multiplexing, which is affected by the competition of reagents among different assays and potential interactions among different oligomers (Bai et al. 2018). With improved design of the assays and optimized PCR conditions in this study, our validation data demonstrate that the newly developed 5-plex assay is highly sensitive and specific in detection and differentiation of influenza A, B, C, and D viruses, from both virus isolates and clinical samples.

Among the 71 IDV positive samples, 2 samples were tested negative by the IDV assay currently used at KSVDL, but were positive for IDV tested with the newly developed 5-plex assay (Ct 28.36 and 32.84). This suggested that the new assay has a higher strain coverage or is more sensitive.

A total of 38 ICV positives were identified from cattle (with suspected respiratory diseases) in Kansas, Colorado, Minnesota, Missouri, Montana, Nebraska, Oklahoma, and Texas in the United States. Sequencing results confirmed the ICV positive PCR data. Although ICV of pig-origin was reported in China and Japan in 1980s and 1990s (Kimura et al. 1997; Yamaoka et al. 1991; Yuanji and Desselberger 1984; Yuanji et al. 1983), influenza C virus has not been reported in bovine prior to our recent study (Zhang et al. 2018a).

Considering the interspecies transmission and infection of the emerging influenza D virus (first identified in Oklahoma) between pigs and cattle in recent years (Chiapponi et al. 2016; Collin et al. 2015; Foni et al. 2017; Hause et al. 2014; Hause et al. 2013; Mitra et al. 2016; Zhai et al. 2017), the newly identified ICV positive cases in

Table 3
Comparison of assay sensitivities between 5-plex and duplex reactions on clinical positive samples.

Target		IAV		IBV		ICV		IDV	
Assay		singleplex	5-plex	singleplex	5-plex	singleplex	5-plex	singleplex	5-plex
R^2		0.998	0.998	0.995	0.997	0.994	0.995	0.997	0.999
E%		95.07	90.01	93.29	95.18	94.17	90.70	94.47	95.34
Ct on serial dilutions	10^{-1}	19.37	19.80	22.52	22.92	23.10	22.61	22.81	23.14
	10^{-2}	23.11	23.18	25.53	26.25	26.28	25.86	26.02	26.38
	10^{-3}	26.36	26.74	29.18	29.64	30.44	29.27	29.64	29.82
	10^{-4}	29.83	30.49	33.24	33.32	33.58	32.81	33.23	33.31
	10^{-5}	33.15	34.35	36.13	36.60	36.80	36.80	36.51	36.87
	10^{-6}	36.77	37.45	N/A	N/A	N/A	N/A	N/A	N/A
Ct difference		0.57		0.43		0.57		0.26	

the United States give us a timely alert of the potential risk that the emerging ICV may cause infections in pig and cattle populations.

The influenza viruses are zoonotic pathogens that can be transmitted between animals and human. Although the recently discovered influenza D viruses have been only identified from pigs and cattle, there are potential risks for IDV to infect human. In addition, previously the influenza C viruses were reported mainly from human infections, now cattle with ICV infections have been identified and confirmed in a number of states in the USA. Our assay can be used to monitor and diagnose ICV and other influenza viruses in cattle with suspected respiratory diseases.

Since the primers and probes of the real-time PCR assays on IBV, ICV, and IDV in this study were designed based on all available sequences from the GenBank database, and the IAV RT-qPCR primers and probes were adopted from the well-established USDA IAV assay (OIE 2015) and the CDC IAV assay (WHO 2017), the newly developed multiplex real-time PCR assay of IAV, IBV, ICV, and IDV can be used for the detection and differentiation of different influenza viruses in swine and bovine, and potentially for human and other animal species as well, although we need to keep validating the assay with more diagnostic samples, especially IBV samples. In addition, we may need to adjust the primer and probe sequences of any influenza assay due to the continuous variation of the viruses over time. Validation and modification of a molecular diagnostic assay appear to be a continuous process.

In conclusion, a high-coverage (inclusivity up to 98%–100%), highly sensitive, and highly multiplexed (5-plex), one-step real-time RT-PCR assay was developed and validated in this study. It should facilitate the detection and differentiation of influenza A, B, C, and D viruses under high throughput settings. To the best of our knowledge, this may be one of a few 5-plex real-time PCR assays that have been developed and validated for diagnostic applications.

Declaration of competing interest

The authors declare that they have no conflicts of interest.

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