Pakistan Veterinary Journal

ISSN: 0253-8318 (PRINT), 2074-7764 (ONLINE)
DOI: 10.29261/pakvetj/2018.086

RESEARCH ARTICLE

Rapid Detection of Feline Calicivirus and Feline Herpesvirus by Duplex Nested RT-PCR

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ARTICLE HISTORY (17-413)

Received: December 14, 2017
Revised: April 19, 2018
Accepted: April 25, 2018
Published online: September 19, 2018

Key words:
Detection
Duplex nested RT-PCR
Feline calicivirus
Feline herpesvirus

ABSTRACT

The aim of this study was to develop a novel duplex nested RT-PCR (dnRT-PCR) method which can simultaneously detect and distinguish feline herpesvirus type 1 (FHV-1) and feline calicivirus (FCV) in cats with upper respiratory tract disease (URTD) and evaluate the performance of this assay in clinical screening. Four pairs of primers based on TK gene of FHV-1 and ORF2 gene of FCV were designed, respectively, and a dnRT-PCR assay was developed and evaluated. A total of 115 clinical swaps of cats collected from private veterinary clinics and animal shelters in Jilin province between November 2016 and May 2017 were used to evaluate this newly developed dnRT-PCR method compared with conventional assay. The results indicated that the detection limits of this method were $10^{1.0}$ $TCID_{50}$/ml, $10^{2.2}$ $TCID_{50}$/ml and $10^{2.0}$ $TCID_{50}$/ml for FHV-1, FCV and a mixture of the two viruses which were 100-fold and 1000-fold higher than single and duplex conventional RT-PCR/PCR, respectively, and was also highly specific. Clinical screening indicated the concordance rates for dnRT-PCR and single conventional RT-PCR/PCR were 99.13% (kappa=0.955) for FHV-1 and 98.26% (kappa=0.956) for FCV, respectively. These results suggest that the duplex nested RT-PCR is a rapid and sensitive method that is useful for clinical diagnosis and etiological investigation of feline caolicivirus and feline herpesvirus.

To Cite This Article: Yi S, Niu J, Dong H, Hu G, Guo Y, Wang K, Zhang S and Zhao Y, 2018. Rapid detection of feline calicivirus and feline herpesvirus by duplex nested RT-PCR. Pak Vet J, 38(4): 347-352. http://dx.doi.org/10.29261/pakvetj/2018.086

INTRODUCTION

Feline upper respiratory tract disease (URTD) is most commonly seen in cats that are grouped together in crowded, high-stress environments, such as animal shelters, boarding kennels and breeding centers (Cohn, 2011). URTD is caused by numerous pathogens, including feline calicivirus (FCV) (Berger *et al.*, 2015), feline herpesvirus type 1 (FHV-1) (Mcgregor *et al.*, 2016), *Bordetella bronchiseptica* (*Bb*) (Garbal *et al.*, 2016), *Chlamydia felis* (*Cf*) (Kang *et al.*, 2016), *Streptococcus canis*, *staphylococcus* (Nemoto *et al.*, 2017) and *Mycoplasma felis* (*Mf*) (Le Boedec, 2017). Among these pathogens, FCV and FHV-1 are highly contagious and are responsible for most cases of URTD (Holst *et al.*, 2010; Sykes, 2014). Clinical cases of FCV are generally more common, but FHV-1 appears to induce more severe clinical symptoms than FCV (DiGangi *et al.*, 2012; Fernandez *et al.*, 2017). Despite active vaccination programs, the high prevalence of these viruses (10-34% and 20-53% for FHV-1 and FCV, respecitively), which is due to their antigenic diversity and the inabilities of current vaccines, remains a major cause of veterinary care costs and euthanasia in animal hospitals (Najafi *et al.*, 2014; Jas *et al.*, 2015; Fernandez *et al.*, 2017).

It is difficult to distinguish the infections caused by FCV and FHV-1 due to similar clinical symptoms, such as sneezing, conjunctivitis and nasal discharge, as well as latent and multiplex infections in the subclinical carrier stage (Radford *et al.*, 2007; Rodriguez *et al.*, 2016). This makes pathogen identification necessary for clinical diagnosis and treatment. Because current detection methods such as viral isolation, antigen-detection ELISAs, immunofluorescence tests and serological testing are costly, time-consuming and nonspecific and/or insensitive, rapid, inexpensive and sensitive assays that

can both test and differentiate the two viruses in a single procedure are required for their differential detection and etiological investigation. Now, this problem has been solved with the development of molecular biology techniques.

Several studies have developed and evaluated PCR or reverse transcription PCR (RT-PCR) based assays, including conventional, nested, real time and multiplex RT-PCR/PCR, for rapid differentiation and detection of FCV and FHV-1(Sykes *et al.*, 2001; Marsilio *et al.*, 2005; Wang *et al.*, 2017b; Meli *et al.*, 2018). However, the comparative results of these assays in previous reports revealed that the sensitivity of multiplex RT-PCR/PCR is lower than that of conventional and nested RT-PCR/PCR and that nested PCR/RT-PCR is more time-consuming than conventional and multiplex RT-PCR/PCR.

Therefore, the main objective of this study was to develop a duplex nested RT-PCR (dnRT-PCR) method that combines both multiplex and nested RT-PCR/PCR to detect and differentiate these two common respiratory viruses.

MATERIALS AND METHODS

Viral and bacterial strains: Two viral strains were used as positive controls in this study: cell cultured adapted "CH-JL_2" strain ($10^{8.2}$ $TCID_{50}$/ml) of FCV and cell cultured adapted "B" strain ($10^{8.0}$ $TCID_{50}$/ml) of FHV-1, which were identified and stored in our laboratory. To determine the specificity of our methods, the following viruses and bacteria were used: Feline parvovirus (FPV), *Mycoplasma felis* (*Mf*), *Streptococcus canis* (*S. canis*), *Staphylococcus aureus* (*S. aureus*), *Bordetella bronchiseptica* (*Bb*) and *Chlamydia felis* (*Cf*). In addition, 115 oropharynx and/or conjunctival swabs collected from private veterinary clinics and animal shelters in Jilin province between November 2016 and May 2017 were used to evaluate this newly developed dnRT-PCR method.

Genome extraction: Genome of reference strains and clinical swabs were extracted using the AxyPrep™ Body Viral DNA/RNA Miniprep kit (CORNING Bio., China), and reverse transcribed to synthesize cDNA using the RevertAid first strand cDNA synthesis kit (Invitrogen, USA) according to the manufacturer's instructions.

Duplex nested RT-PCR: The oligonucleotide primers were designed based on the TK gene of FHV-1 and ORF2 gene of FCV were designed, respectively (Table 1). For the first reaction, the amplification was carried out in a total volume of 25 μl of reaction mixture containing 2.5 μl of 10×Ex Taq™ buffer (Mg^{2+} plus), 7.5 mM of dNTPs mixture, 10 pM of forward and reverse primer of each virus, 3 U Ex Taq™ DNA polymerase (Takara Bio., China) and 2.0 μl of synthetic cDNA mixture. Rnase free water was added to a total volume of 25 μl. The amplification was performed with a thermocycler (Bioer Co., China) using the following procedure: 1 cycle of predenaturation at 94°C for 5 min followed by 35 cycles of denaturation at 94°C for 45 sec, annealing at 63°C for 45 sec, extension at 72°C for 30 sec, and a final extension at 72°C for 10 min, and then stored at 4°C. The PCR product of the first reaction was used as a genetic template for the nested PCR. The reaction mixture was the same as the first reaction, but the amplification procedure's annealing temperature was set at 59°C. The amplified products from the 2 reactions were separated after electrophoresis on 1.5% agarose gels at 140 V for 20 min, and visualized using a gel documentation system (Wealtec, USA). φX174-Hinc Ⅱ digest and DL 1000 DNA marker (Takara Bio., China) were used as the molecular weight markers.

Specificity and sensitivity of the dnRT-PCR: The specificity of the dnRT-PCR assay was evaluated using FPV, *Mf*, *S. canis*, *S. aureus*, *Bb* and *Cf*. To determine the sensitivity of the dnRT-PCR, 10-fold serial dilutions of individual and a mixture of reference viral strains, ranging from $10^{8.0}$ $TCID_{50}$/ml to $10^{1.0}$ $TCID_{50}$/ml for FHV-1; $10^{8.2}$ $TCID_{50}$/ml to $10^{1.2}$ $TCID_{50}$/ml for FCV; and $10^{8.0}$ $TCID_{50}$/ml to $10^{1.0}$ $TCID_{50}$/ml for a mixture of the two viruses, were carried out in minimum essential medium (MEM) (HyClone Co., USA) before use. In addition, the sensitivity of the dnRT-PCR was compared with that of conventional RT-PCR/PCR and multiplex PCR developed previously by Sykes *et al.* (2001).

Evaluation of the dnRT-PCR on clinical samples: To evaluate the performance (sensitivity and specificity) of the duplex nested RT-PCR on clinical specimens, a total of 115 oropharynx and/or conjunctival swabs were tested by this newly developed assay. The sensitivity and specificity were estimated by comparing this assay with a single conventional RT-PCR/PCR that acted as the "gold standard" for the detection of FHV-1 and FCV in this study. The data were calculated and analyzed as described in previous papers (Nguyen *et al.*, 2013).

Table 1: Primers designed for the duplex nested RT-PCR/PCR in this study

Virus	Target gene	Primer	Sequence	Position	Product size	Tm	Accession no.[a]
FHV-1	Thymidine kinase	HerpAF[1st]	5'-AGATTTGCCGCACCATACCTTC-3'	566-587	513-bp	63.1	M26660
		HerpAR[1st]	5'-CCGGGCTTTGAAAACACTGAAT-3'	1078-1057		63.2	
		HerpBF[2nd]	5'-TTGCCGCACCATACCTTCTTTTA-3'	570-592	403-bp	63.9	
		HerpBR[2nd]	5'-TTAAGCTTCCCCCACCCATCA-3'	972-952		64.0	
FCV	Capsid protein	CaliAF[1st]	5'-CAACCTGCGCTAACGTGCTTA-3'	5318-5338	681-bp	61.7	U13992
		CaliAR[1st]	5'-TTCCCCCAAAAACYCCAGATC-3'	5998-5978		61.2	
		CaliBF[2nd]	5'-CACTCAYGAGGCCATYGAC-3'	5332-5340	336-bp	59.2	
		CaliBR[2nd]	5'-TTCCCTTGGGTTTCAGATGTGC-3'	5867-5848		63.7	

Note: [a] Accession no. indicates the GenBank accession number of reference sequence; [1st] HerpAF, HerpAR, CaliAF and CaliAF were the primers in the first round of duplex nested RT-PCR/PCR; [2nd] HerpBF, HerpBR, CaliBF and CaliBF were the primers in the second round of duplex nested RT-PCR/PCR.

RESULTS

Strategy and optimization of the duplex nested RT-PCR: As expected, two viruses were amplified when using our primers designed in this study. The 513-bp and 681-bp specific fragments were displayed for FHV-1 and FCV using outer primers in the first reaction, respectively. And the 403-bp and 336-bp specific PCR products were obtained for these two viruses using inner primers (Fig. 1). Thus, we develop scientific strategy of dnRT-PCR to detect clinical samples. Firstly, extracting genome from clinical oropharyax and/or conjunctival swabs collected from cats; and then screening and distinguishing FCV and FHV-1 by dnRT-PCR (Fig. 1).

Specificity of the dnRT-PCR: The specificity of the dnRT-PCR was evaluated for the target organism using conventional and nested primers. First, we tested the specificity using positive reference strains in the following reactions: reaction mixture containing one primer pair and two positive templates, two primer pairs and one positive template, and two primer pairs and two positive templates. A single amplification product was obtained for each viral target in the different reactions, and no non-specific amplification products were observed. To further evaluate the specificity of the method, all specific amplification products were sequenced and compared with the reference gene in GenBank, and their identities were confirmed as FHV-1 and FCV (data not shown). Second, the other pathogens associated with feline upper respiratory disease, including FPV, *Mf*, *S. canis*, *S. aureus*, *Bb* and *Cf*, were tested using the dnRT-PCR assay to evaluate its analytical specificity. The detection results showed that there was no amplification product for the negative control or the pathogens in the first reaction, and there was also no band for the second reaction (Fig. 2).

Comparison of the sensitivity of the dnRT-PCR with conventional RT-PCR/PCR: The results of the sensitivity of the dnRT-PCR compared with that of conventional RT-PCR/PCR are shown in Fig. 3. For the conventional RT-PCR/PCR, the detection limits of FHV-1 and FCV were $10^{3.0}$ $TCID_{50}$/ml and $10^{4.2}$ $TCID_{50}$/ml, respectively. For the conventional duplex RT-PCR/PCR, the detection limits of FHV-1, FCV and the mixture of the two viruses were $10^{5.0}$ $TCID_{50}$/ml, $10^{5.2}$ $TCID_{50}$/ml and $10^{5.0}$ $TCID_{50}$/ml, respectively. The dnRT-PCR detected the viral samples with a titer of $10^{1.0}$ $TCID_{50}$/ml, $10^{2.2}$ $TCID_{50}$/ml and $10^{2.0}$ $TCID_{50}$/ml for FHV-1, FCV and their combination, respectively. The results of the comprehensive comparison revealed that the analytical sensitivity of the dnRT-PCR was higher than that of the conventional single and multiplex RT-PCR/PCR, and the detection limits of the conventional assay were 100-fold and 1000-fold lower than that of dnRT-PCR, respectively. In addition, the sensitivity of dnRT-PCR using inner primers was 1000-fold greater than the sensitivity of the single PCR using outer primers.

Testing results of the dnRT-PCR on clinical specimens: Table 2 summarizes the test data for the 115 clinical specimens detected using dnRT-PCR. Of these samples, 11.3%, 27.8% and 6.1% were positive for FHV-1, FCV and co-infection, respectively. Analysis results of a two-sided chi-square test indicated that there were significant differences ($P<0.05$) in the prevalence of FHV-1 and FCV, while the differences in the prevalence from samples obtained specifically from veterinary clinics and animal shelters for the same virus were not significant ($P>0.05$).

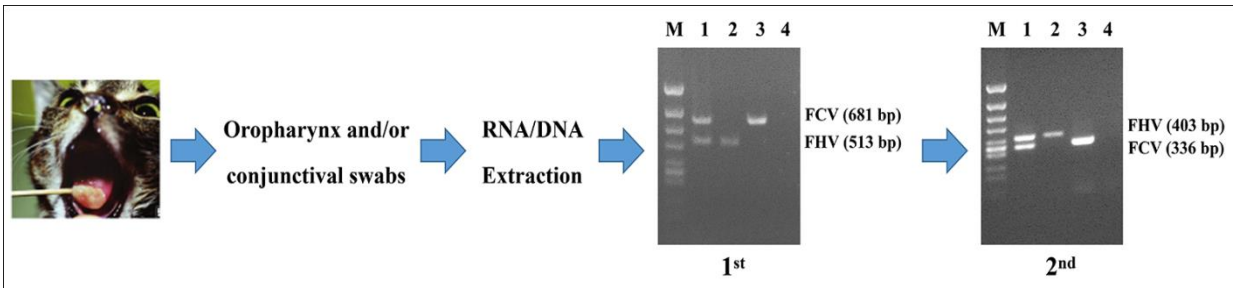

Fig. 1: Procedure and detection results of the duplex nested RT-PCR/PCR for FHV-1, FCV, and a mixture of the two viruses. M, molecular size marker (φX174-Hinc Ⅱ digest); lane 1, FHV-1 and FCV; lane 2, FHV-1 (513-bp and 403-bp for the first (1st) and second (2nd) round amplifications, respectively); lane 3, FCV (681-bp and 336-bp for 1st and 2nd amplifications, respectively); lane 4, negative control.

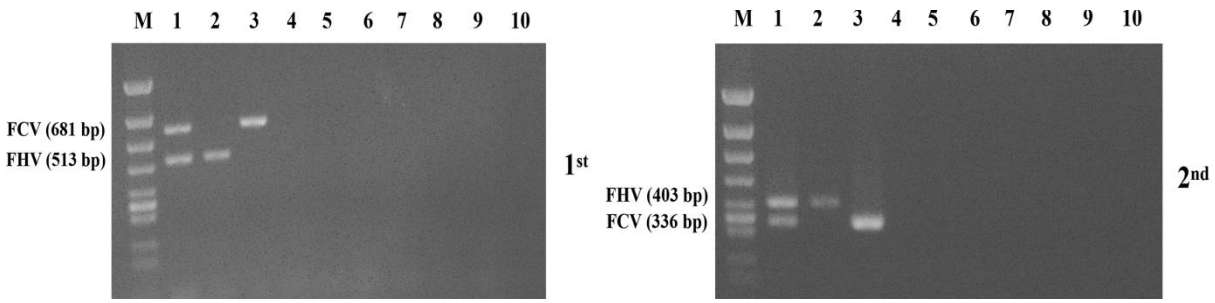

Fig. 2: Specificity of the duplex nested RT-PCR/PCR for other pathogens of the infected feline respiratory tract. M, molecular size marker (φX174-Hinc Ⅱ digest); lane 1, FHV-1 and FCV; lane 2, FHV-1; lane 3, FCV; lane 4, Feline parvovirus; lane 5, *Mycoplasma felis* (*Mf*); lane 6, *Streptococcus canis* (*S. canis*); lane 7, *Staphylococcus aureus* (S. aureus); lane 8, *Bordetella bronchiseptica* (Bb); lane 9, *Chlamydia felis* (Cf); lane 10, negative control.

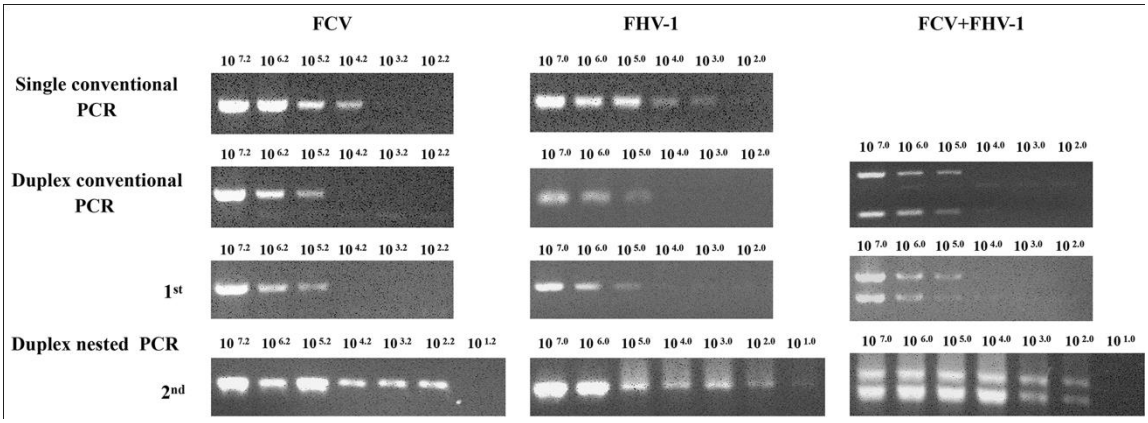

Fig. 3: Comparison of sensitivity among the newly developed duplex nested RT-PCR/PCR, single and duplex conventional RT-PCR/PCR described in previous papers for FHV-1, FCV, and a mixture of the two viruses. The numbers at the top indicate the tissue culture infectious doses of the virus dilutions ($TCID_{50}$/ml).

Table 2: Screening test data of 115 clinical specimens by the dnRT-PCR/PCR method

Sample source	No. of samples	No. of positive samples (positive %)			P[a]
		FHV-1	FCV	FHV-1+FCV	
Veterinary clinics	63	9 (14.3)	21 (33.3)	5 (7.9)	0.020
Animal shelter	52	4 (7.7)	11 (21.2)	2 (3.9)	0.046
total	115	13 (11.3)	32 (27.8)	7 (6.1)	0.003
p [b]		0.377	0.209	0.454	

Note: Positive rate = number of positive samples/total number of tested samples. Statistical analysis was performed by a two-sided chi-square test using PASW Statistics 17.0, and probability (p) value < 0.05 was considered as statistically significant. [a] The differences of prevalence among FHV-1 and FCV; [b] the differences of prevalence among veterinary clinics and animal shelters for the same virus.

Table 3: Performance comparison of the duplex nested RT-PCR/PCR with single conventional RT-PCR/PCR for the detection of clinical specimens

dnRT-PCR		Gold standard*		Performance evaluation of the dnRT-PCR/PCR VS "gold standard"					
		CP	CN	Sensitivity	Specificity	PPV	NPV	concordance rates	Kappa
FHV-1	P	12	1	100%	99.03%	92.31%	100%	99.13%	0.955
	N	0	102						
FCV	P	30	2	100%	97.65%	93.75%	100%	98.26%	0.956
	N	0	83						

Note: P, positive; N, negative; CP, conditional positive; CN, conditional negative. * Single conventional RT-PCR/PCR acted as the "gold standard". Sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and concordance rates were calculated and kappa values were obtained by the kappa statistic using the PASW Statistics 17.0.

Performance evaluation: The performance of dnRT-PCR was evaluated by comparing it with conventional assay. The sensitivity and specificity of the dnRT-PCR method were 100% and 99.03% for FHV-1, and 100% and 97.65% for FCV, respectively (Table 3). In addition, the concordance rates for dnRT-PCR and single conventional RT-PCR/PCR were 99.13% (kappa=0.955) for FHV-1 and 98.26% (kappa=0.956) for FCV (Table 3).

DISCUSSION

Feline upper respiratory tract disease caused by FHV-1 and FCV is a significant disease that increases mortality in cats and wild felines, as well as the cost of clinical diagnosis. This may be due to the high prevalence of these two viruses (ranging from 10 to 53%) and the unreliability of available control measures (Cohn, 2011; Fernandez *et al.*, 2017; Thomas *et al.*, 2017). To optimize prevention and treatment programs and to reduce the spread of the disease, large-scale epidemiological data are required. The development of molecular biological techniques provides the possibility of achieving this goal. Multiplex RT-PCR/PCR has been developed and applied as rapid, sensitive and time-saving method to simultaneously detect and differentiate various respiratory and enteric viruses in previous reports (Choudhary *et al.*, 2013; Radko *et al.*, 2017; Wang *et al.*, 2017a; Kowada *et al.*, 2018). However, it is confirmed that the sensitivity of multiplex RT-PCR/PCR is lower than conventional assays due to the competition among the primer pairs in the same reaction (Bellau-Pujol *et al.*, 2005). By contrast, the nested RT-PCR/PCR amplifies the reaction by obtaining amplification products based on the PCR products used as templates from the first round in the second reaction, making it more sensitive than conventional assays (Ben Salem *et al.*, 2010). Therefore, we developed a novel duplex nested RT-PCR method, which combined the higher sensitivity of nested PCR with the simultaneous detection of multiplex PCR for feline upper respiratory viruses. We then analyzed and compared the sensitivity and specificity between dnRT-PCR and conventional RT-PCR/PCR for the detection of clinical specimens.

As reported in previous studies, optimizing all compositions in the reaction and amplification programs are critical for improving the specificity and sensitivity of dnRT-PCR (Ben Salem *et al.*, 2010; Saito *et al.*, 2018). In current study, the step-by-step protocol described by Nguyen *et al.* (2013) was used to optimize the strategy. First, the annealing temperature was determined using gradient PCR ranging from 55 to 68°C for each reaction, and the optimal annealing temperature of the multiplex PCR was then selected by analyzing that of each primer pairs. Next, all component concentrations (dNTP, primers, and DNA polymerase) were optimized based on the

results of the last optimization step. The optimization results showed that the annealing temperature of these two reactions was similar, and the annealing temperatures of the first reaction was set at 4°C higher than the nested reaction. The primer concentrations in the multiplex reaction mixture were lower than that of the traditional PCR. Finally, the optimized strategy was used to assess the performance of this novel assay developed in this study.

For nested PCR, the unspecific amplification product frequency was very high due to the many recombinants that formed between different primer pairs in the second reaction. Bellau-Pujol *et al.* (2005) developed three multiplex hemi-nested RT-PCR assays to detect 12 respiratory RNA viruses, but the unspecific bands that appeared in the hemi-nested RT-PCR deterred researchers from its using it in clinical testing. This is resolved in the newly developed dnRT-PCR assays by ensuring that the PCR products are well separated in the agarose and that the bands are single for each target virus (Fig. 1). Furthermore, all the amplified DNA fragments for each virus in the different reactions were sequenced, and the homology between the above sequences and their reference genomes were analyzed. The analysis results showed that the homology was higher than 99.7% (data not shown), which indicates the newly designed primers were reliably amplified the expected target genes. There were no amplification products for Feline parvovirus, *Streptococcus canis*, *Mycoplasma felis*, *Staphylococcus aureus*, *Bordetella bronchiseptica* or *Chiamydia felis*, assuring the specificity of this assay (Fig. 2).

To assess analytical sensitivity, successive 10-fold dilutions of viral strain combinations (FHV-1, FCV, and mixtures of FHV-1 and FCV) were tested by dnRT-PCR, conventional and duplex RT-PCR/PCR. The sensitivity of the novel dnRT-PCR ranged from $10^{1.0}$ $TCID_{50}$/ml to $10^{2.2}$ $TCID_{50}$/ml for FHV-1, FCV and a mixture of the two viruses and was 100-fold and 1000-fold higher than that of the single and duplex conventional RT-PCR/PCR (Fig. 3). Thus, this newly developed dnRT-PCR method would be highly applicable to the screening of clinical samples with low viral titers. In previous studies, the sensitivity of single conventional RT-PCR/PCR for FCV and FHV-1 was compared with cell culture, and the results showed that RT-PCR/PCR was more sensitive than cell culture (Sykes *et al.*, 2001; Marsilio *et al.*, 2005). However, the comparison of single RT-PCR/PCR and multiplex RT-PCR/PCR for FCV and FHV-1 was lacking. In our study, this comparison was carried out using reference primers, and the results indicated that single RT-PCR/PCR was 10-fold and 100-fold more sensitive than duplex RT-PCR/PCR, which is consistent with other studies on pathogen detection. In brief, the sensitivity of dnRT-PCR was higher than that of both single and duplex RT-PCR/PCR.

The clinical specimens from veterinary clinics and animal shelters in Jilin province were screened using the dnRT-PCR method. The results showed that the prevalence of FHV-1 and FCV was 11.3 and 27.8%, respectively, and the prevalence of the two viruses was statistically significant (Table 2). This result was the same as that of previous epidemiological studies, which revealed that infections with FCV were more common (Henzel *et al.*, 2015; Ravicini *et al.*, 2016; Fernandez *et al.*, 2017). In addition, the screening results using dnRT-PCR and conventional RT-PCR/PCR are consistent, and the concordance rates were 99.13% for FHV-1 and 98.26% for FCV (Table 3), respectively, suggesting that the dnRT-PCR could be used as a highly specific and sensitive method to differentiate FHV-1 and FCV in clinical samples.

Conclusions: We have developed a novel duplex nested RT-PCR assay that can simultaneously detect FHV-1 and FCV in feline upper respiratory tract disease in this study. This assay is more sensitive and specific in detecting feline respiratory viruses than the conventional single and duplex PCR. In addition, this assay can reduce time and testing costs and will be useful for clinical diagnosis and etiological investigations in animal shelters and veterinary diagnostic laboratories, thereby optimizing the management programs and reducing the spread of disease.

Acknowledgments: This work was supported by National Key R&D Program of China [grant numbers 2017YFD0501700]; National Key R&D Program of China [grant number 2016YFD0501002]; and an agriculture industry project of China [grant number 201303042].

Authors contribution: SSY, JTN and GXH: conceived and designed the experiments. SSY, JTN and YBG: performed the experiments. KW and SZ: analyzed the data. SZ, HD and YLZ: contributed reagents/materials/ analysis tools. SSY, JTN and GXH: wrote the paper. SSY, HLW and HD: revised the paper.

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