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Development of a duplex real-time RT-PCR for the simultaneous detection and differentiation of Theiler's murine encephalomyelitis virus and rat theilovirus

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A B S T R A C T

Theiler's murine encephalomyelitis virus (TMEV) and rat theilovirus (RTV), the member of the genus *Cardiovirus*, are widespread in laboratory mice and rats, and are potential contaminants of biological materials. Cardioviruses infection may cause serious complications in biomedical research. To improve the efficiency of routine screening for Cardioviruses infection, a duplex real-time reverse transcriptase polymerase chain reaction (RT-PCR) assay was developed for simultaneous detection and differentiation of TMEV and RTV. The duplex assay was specific for reference strains of TMEV and RTV, and no cross-reaction was found with seven other rodent viruses. The limits of detection of both TMEV and RTV were 4×10^1 copies RNA/reaction. Reproducibility was estimated using standard dilutions, with coefficients of variation <3.1%. 439 clinical samples were evaluated by both duplex real-time RT-PCR and conventional RT-PCR. For 439 clinical samples, 95 samples were positive for TMEV and 72 samples were positive for RTV using duplex real-time RT-PCR approach, whereas only 77 samples were positive for TMEV and 66 samples were positive for RTV when conventional RT-PCR was applied. Mixed infections were found in 20 samples when analyzed by conventional RT-PCR whereas 30 samples were found to be mixed infection when duplex real-time RT-PCR was applied. This duplex assay provides a useful tool for routine health monitoring and screening of contaminated biological materials of these two viruses.

1. Introduction

Theiler's murine encephalomyelitis virus (TMEV) is a positive-sense, single-stranded RNA virus classified as a member of the genus *Cardiovirus* in the family *Picornaviridae*, along with encephalomyocarditis virus (EMCV) and Mengovirus (Ozden et al., 1986; Pevear et al., 1987). TMEV is a common cause of asymptomatic enteric infections in mice housed in non-barrier animal colonies (Brownstein et al., 1989). In addition, TMEV spreads to the central nervous system (CNS) to cause poliomyelitis (flaccid paralysis) and more rarely, encephalitis (Thompson et al., 1951). TMEV is divided into two subgroups based on neuro-virulence in mice after intra-cerebral inoculation. The first subgroup consists of highly neuro-virulent strains represented by GDVII and FA that cause acute and fatal encephalitis. The second subgroup comprises less virulent strains, such as BeAn and DA, that produce a persistent infection in the CNS of mice that results in mononuclear cell inflammation and demyelination (Lehrich et al., 1976; Lipton, 1975). This demyelinating disease has immune parameters and histopathology similar to those of chronic progressive multiple sclerosis (MS) and thus is extensively studied as an infectious model for MS.

TMEV infection is one of the most common viral infections detected in contemporary laboratory mouse colonies. Recent reports document that laboratory rats can also be naturally and experimentally infected with the TMEV virus, the newborn rats inoculated intracerebrally with the TMEV virus showed clinical signs similar to those in mice, characterized by flaccid paralysis of the hind limbs, ruffled hair coat, tremor and weight loss (Rodrigues et al., 2005).

Rat theilovirus (RTV), also referred to as Theiler-like virus of rats (Ohsawa et al., 2003), or rat encephalomyelitis virus, is a newly characterized rat *Cardiovirus* that has been shown to be related, but genetically distinct from, other viruses in the TMEV group. The first report of a TMEV-like pathogen infecting rats occurred in 1964

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when a small group of Sprague-Dawley (SD) rats were identified with central nervous system deficits and histopathologic lesions that resembled those of mice infected with TMEV (McConnell et al., 1964). The original isolate, known as MHG, was reported to cause paralysis in suckling rats and mice following intracranial inoculation. It was further recognized that subclinically infected rats developed serum antibodies that cross-reacted with TMEV antigens (Hemelt et al., 1974). The seroprevalence data indicate RTV is one of the most prevalent viral pathogens infecting research rat colonies (Drake et al., 2008; Dyson, 2010; Livingston and Riley, 2003; Ohsawa et al., 2003; Rodrigues et al., 2005).

Since cardioviruses infection poses a greater epizootic threat for colony-bred mice and rats used in biomedical research, rapid diagnosis is essential to prevent transmission of infection throughout a research animal facility. Viral diagnosis is based commonly on antigen or antibody detection. Serologic methods such as multiplexed fluorescence immuneassay (MFIA), enzyme-linked immunosorbent assay (ELISA) and indirect immunofluorescence assay (IFA) has typically been used to diagnose TMEV and RTV infections in rodents (Drake et al., 2011; Khan et al., 2005; Laborde et al., 2008; Lipton et al., 2001; Pritchett-Corning et al., 2009). However, serologic assays cannot detect TMEV and RTV infections directly in immunodeficient strains of rodents that do not generate a humoral immune response, and the time required for host seroconversion in immunocompetent rodents may prevent rapid definitive diagnosis by serologic testing during an epizootic. Serologic assays also do not give information if an agent is still present in an animal and if it is still infective. In addition, serologic assays to detect antibodies to RTV in rats have historically used mouse TMEV strains as antigens, exploiting the antigenic cross-reactivity of these viruses with RTV (Easterbrook et al., 2008; Pritchett-Corning et al., 2009). Therefore, the two groups of viruses may not be distinguishable. For the detection of contamination in biological materials, the mouse/rat antibody production (M/RAP) test is still applied occasionally, although polymerase chain reaction (PCR) tests have been developed (Bootz et al., 2003). This test requires the use of animals, is time consuming and expensive.

PCR has the advantages of high sensitivity and high specificity, which enables it to detect infections in experimental mice and biological materials and even environmental contamination. Different PCR methods, either conventional or real-time PCR based assay, have been developed and have proven a useful adjunct diagnostic method for detection of TMEV and RTV (Bootz et al., 2003; Drake et al., 2008; Trottier et al., 2002). More recently, multiplex real-time PCR has been reported to be as robust as simplex real-time PCR in detecting a broad range of different rodent pathogens but its application for the detection of cardioviruses has not yet been reported (Pang et al., 2014).

The aim of this study is to develop a rapid, sensitive and specific duplex real-time RT-PCR assay for the simultaneous detection of TMEV and RTV, and provide a useful tool for the routine health monitoring of these two viruses in laboratory rodents and for the screening of contaminated biological materials.

2. Materials and methods

2.1. Viruses

Theiler's mouse encephalomyelitis virus BeAn8386 strain (TMEV), mouse hepatitis virus A59 strain (MHV), reovirus type 3 Dearing strain (Reo-3), rat coronavirus 8190 strain (RCV), sendai virus Sendai/52 strain (Sendai), pneumonia virus of mice number 15 strain (PVM) were obtained from American Type Culture Collection (ATCC). rat theilovirus Y17 strain (RTV) and murine norovirus Guangzhou/K162/09/CHN strain (MNV) were obtained from intramural stocks, and RNA for lymphocytic choriomeningitis virus Armstrong strain (LCMV) were kindly provided by Dr. Zheng ming HE, Laboratory Animal Institute, National Institutes for Food and Drug Control. TMEV, RTV, Reo-3, Sendai, and PVM were propagated in BHK-21 cells (ATCC CCL-10), MHV and RCV was propagated in NCTC 1469 (ATCC CCL9.1), MNV was propagated in RAW264.7 cells (ATCC TIB-71). All viral stocks were stored at −70 °C until use.

2.2. Clinical samples

Clinical samples were collected from four different sources: (1) 296 stool and cecum samples were collected from 244 mice and 52 rats in Guangdong province of China during the period 2012–2014 for routine health monitoring services, these laboratory animals had been reared under barrier colonies; (2) 49 Sprague-Dawley (SD) rats and 52 KM mice were obtained from a non-barrier laboratory rodent colony, all animals were euthanized under CO_2 anesthesia, and the cecum specimens were collected; (3) 32 wild rats (*Rattus norvegicus*) were live-trapped around the non-barrier laboratory rodent colony mentioned above, and the ceca were collected from the rats according to the same sampling procedures; (4) 15 different cultured cell line samples were obtained from intramural stocks. In addition, twenty cecum content and spleen samples collected from eight specific pathogen free (SPF) mouse strains (BALB/c, C57BL/6, DBA, FVB, 129, ICR, KM and NIH) and two rat strains (SD and Wistar) were used to evaluate specificity of the duplex real-time RT-PCR assay, these animals were reared under barrier colonies and were confirmed as serology negative for TMEV and RTV by a commercial ELISA kit (XpressBio, Maryland, USA). Subsequently, a 10%(w/v) suspension of all stool, cecum and spleen specimens was prepared with phosphate-buffered saline (PBS; 0.01 M, pH 7.2), clarified by centrifugation at 4000*g* for 10 min at 4 °C, and stored at −70 °C until used. The experimental protocols were approved by the Institutional Animal Care and Use Committee at Guangdong Laboratory Animals Monitoring Institute.

2.3. RNA extraction

The RNAs of the reference viruses and clinical samples were extracted using QIAamp Viral RNA Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. RNA concentrations were measured using a UV–vis spectrophotometer NanoDrop 2000c (Thermo Scientific, USA) and adjusted to about 10 ng/μl in nuclease-free H_2O to normalize the different extractions. All RNA samples were stored in −70 °C until use.

2.4. Design of primers and probes

The whole genomic nucleotide sequences of TMEV and RTV were obtained from GenBank database. Sequence alignments were performed using the Clustal W method from the MEGA5.0 program (DNAStar Inc., Madison, WI). Specific primer and probe combinations were created using Primer express software (Applied Biosystems Inc., Foster City, CA, USA) for individual detection of TMEV and RTV. Specific primers and probe for TMEV were developed from the 2A protein region of the viral genome (GenBank accession no. X56019) and labeled with FAM as a fluorescent detector. Primer/Probe combinations for RTV were developed using sequence from the leader peptide (GenBank accession no. EU542581) region of the viral genome and labeled with JOE as the fluorescent dye. The primers and probes used in the present study are listed in Fig. 1 and Table 1.

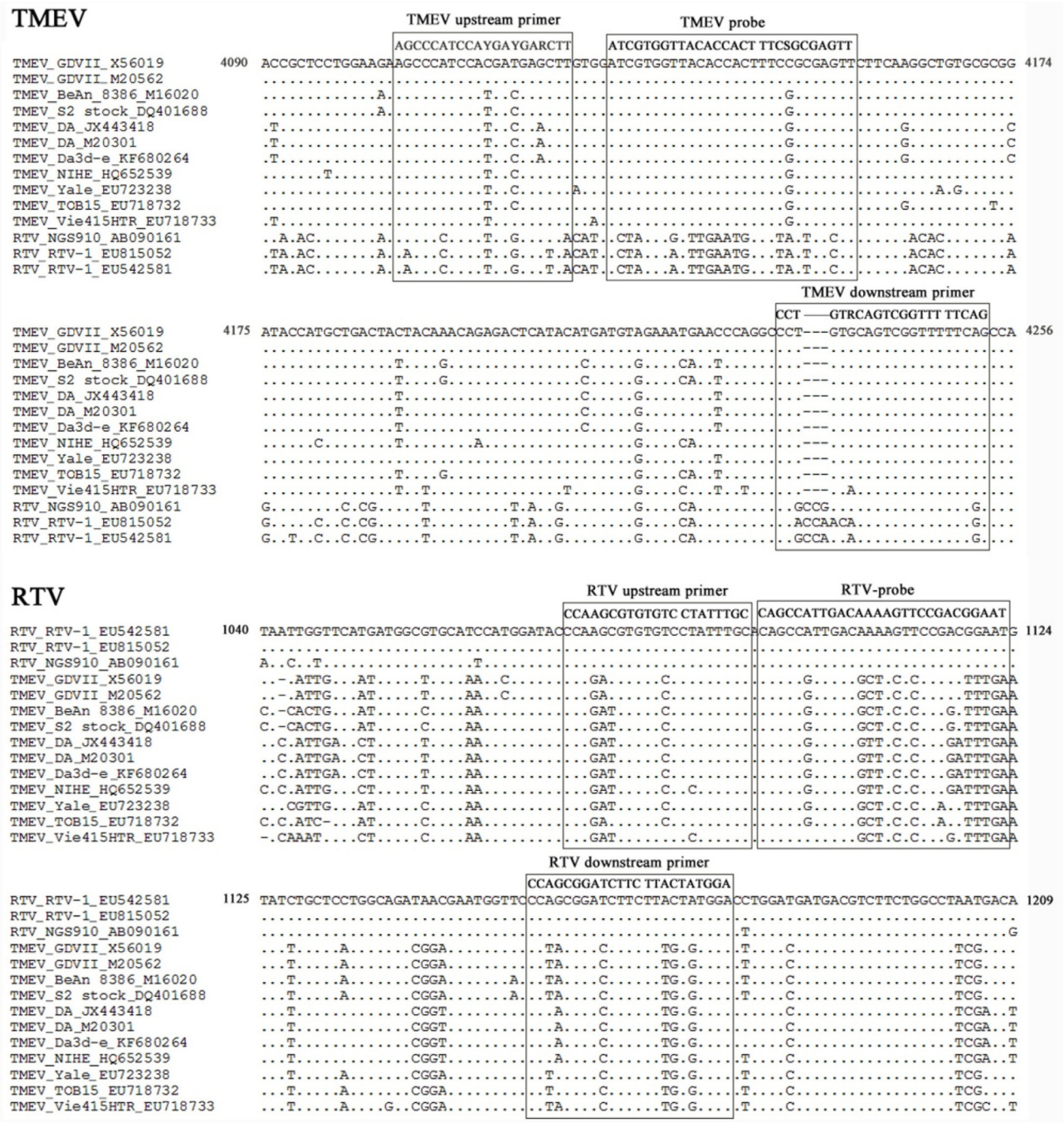

Fig. 1. Alignment of primers and probes for TMEV and RTV. Nucleotide sequences of the primers and probes are shown at the top of the frames while the corresponding nucleotide sequences of TMEV and RTV are shown at the bottom. Dots indicate that nucleotides are identical to those of the first line sequence. The upstream primers and probes were used as shown while the downstream primers were used as antisense oligonucleotides. The upstream and downstream primers and probe of TMEV had 3, 1, and 1 degenerate nucleotide, respectively.

2.5. One-step duplex real-time RT-PCR

The duplex real-time RT-PCR was performed in a one-step format, using the One-step PrimeScript™ RT-PCR Kit (TaKaRa Biotechnology Co., Dalian, China). A reaction mixture was prepared containing (per vial) 4 μl of sample RNA, 0.4 μM of each primer and 0.2 μM of each probe (Table 1), 12.5 μl of 2× One-Step RT-PCR Buffer, 2.5 U TaKaRa Ex Taq™ HS, 0.5 μl PrimeScript™ RT Enzyme Mix II, and the remaining volume was adjusted to 25 μl with RNase-free water. The passive reference dye Rox was included in the reaction mixture. The cycling parameters for RT-PCR included 5 min at 42 °C for reverse transcription, 10 s at 95 °C for Taq HS activation, followed by 45 cycles for amplification with 95 °C for 5 s and 60 °C for 34 s. Fluorescence signals were collected at the end of each amplification step. The reaction was carried out using ABI7500 equipment and software (Applied Biosystems Inc., Foster City, CA, USA). A simplex real-time RT-PCR assay was performed in the same manner as the duplex assay, except that only one set of primers/probe was included.

Table 1
Primer pairs and probes used in this study.

Method	Primer pairs/probe set	Sequence (5′–3′)	Location[d,e]	Amplicon size (bp)	Reference
Duplex real-time RT-PCR	TMEV-F1	AGCCCATCCAY[a]GAY[a]GAR[b]CTT	4105–4124[d]	149	This study
	TMEV-R1	CTGAAAAACCGACTGY[a]ACAGG	4253–4233[d]		
	TMEV-P	FAM-ATCGTGGTTACACCACTTTCS[c]GCGAGTT-BHQ1	4129–4156[d]		
	RTV-F1	CCAAGCGTGTGTCCTATTTGC	1074–1094[e]	104	This study
	RTV-R1	TCCATAGTAAGAAGATCCGCTGG	1177–1155[e]		
	RTV-P	JOE-CAGCCATTGACAAAAGTTCCGACGGAAT-BHQ1	1096–1123[e]		
conventional RT-PCR	TMEV-F2	CCTATTTGCACAGCCGT	1101–1117[d]	403	(Bootz et al., 2003)
	TMEV-R2	GGAGAGGTGCCATAGTAGC	1485–1503[d]		
	RTV-F2	GACCTCTTTCAACGCGACG	363–381[e]	363	This study
	RTV-R2	CGATGTCTGTTCTAAGTTTCC	705–725[e]		

[a,b,c]Degenerate nucleotides: Y = C or T; R = A or G and S = C or G.
Fluorophores: FAM (6-carboxyfluorescein) and JOE (6-carboxy-4′, 5′-dichloro-2′, 7′-dimethoxyfluorescein).
Quenchers: BHQ-1 (black hole dark quencher 1).
[d,e]Numbering according to the sequences of TMEV GDVII (X56019) and RTV-1(EU542581), respectively.

2.6. *Preparation of* in vitro-*transcribed RNA standards for viral RNA quantification*

For sensitivity determination, RNA transcripts of the selected region of TMEV and RTV were synthesized *in vitro*. The amplification of each target sequence was performed by conventional RT-PCR on template RNAs from TMEV (strain BeAn8386) and RTV (strain Y17) using the primers indicated in Table 1 (TMEV-F1/TMEV-R1 for TMEV, and RTV-F1/RTV-R1 for RTV). The DNAs obtained were gel-purified, quantified by spectrophotometry and stored at −20 °C until use. Each DNA was inserted into a pGEM-T vector (Promega, Madison, WI, USA) using a T-A cloning strategy according to the manufacturer's instructions, and transferred into competent *Escherichia coli* bacteria for propagation. Plasmid DNA was purified from insert-containing colonies, using TIANprep Mini Plasmid Kit (Tiangen, Beijing, China). Virus-derived DNA inserts were revealed by conventional PCR using specific primers (as above), the identity and integrity of the positive plasmids had been verified by sequencing. The homogeneity of the plasmid preparations was assessed by agarose gel electrophoresis. The DNA concentration of each plasmid preparation was determined by spectrophotometry using a NanoDrop 2000c (Thermo Scientific, USA). *In vitro* transcription was performed using the RiboMAX™ Large Scale RNA Production Systems (Promega, Madison, WI, USA) with T7 RNA polymerase over linearized plasmid DNA, following the manufacturer's instructions. Transcribed RNA was treated with RNase-free DNase and purified with the QIAamp Viral RNA Mini kit (Qiagen, Hilden, Germany). RNA transcripts were stored at −70 °C until use. Additionally, RNA concentration was determined by spectrophotometry. RNA copy number was determined by the following formula: Amount (RNA copies/μl) = (X(g/μl)RNA × 6.022×10^{23})/(nt transcript length × 340). After the transcription, purification and RNA quality evaluation, 10-fold series dilutions containing 1×10^8 to 1×10^0 RNA copies were prepared using healthy mice total RNA extracts (10 ng/μl).

2.7. *Specificity assay*

The specificity of the duplex real-time RT-PCR assay was determined by evaluation of RNA extracted from positive cultures of the following rodent viral pathogens: TMEV, RTV, MHV, Reo-3, RCV, Sendai, PVM, MNV and LCMV, and from twenty cecum content and spleen samples of ten SPF rodent strains.

2.8. *Sensitivity assay*

Ten-fold serial dilutions of the RNA standard of TMEV and RTV ranged from 1.0×10^8 to 1.0×10^0 copies/ng of total RNA were tested to determine the detection limits of the duplex real-time RT-PCR assay. The serially diluted RNA standard was also used to establish a standard curve for TMEV and RTV by plotting the threshold cycle (Ct) and the viral copy logarithm. Based upon the duplex real-time PCR standard curve, the correlation coefficient (R^2) was obtained. These assays were performed in parallel and in triplicate on the same dilutions.

2.9. *Reproducibility assay*

The standard RNA ranged from 1.0×10^7 to 1.0×10^1 copies/ng of total RNA of TMEV and RTV were used to evaluate the inter-assay and intra-assay reproducibility of duplex real-time RT-PCR. Ct values were measured in multiple replicates. The threshold cycle of each concentration was obtained and calculated.

2.10. *Conventional RT-PCR*

For comparative purposes, conventional RT-PCR were developed for detection TMEV and RTV in clinical samples. Two sets of primers used for conventional RT-PCR were designed and described in Table 1. The conventional RT-PCR was performed for the separate detection of TMEV and RTV as previously described (Bootz et al., 2003) with little modification. Briefly, The first strands of cDNA were synthesized with PrimeScript™ 1st Strand cDNA Synthesis Kit (TaKaRa Biotechnology Co., Dalian, China) according to the manufacturer's instructions, the PCR reaction mixture in 20 μl reaction volume comprised of 10 μl 2 × Premix Taq™ (TaKaRa Biotechnology Co., Dalian, China), 0.4 μl of forward and reverse primers (10 μM), 4 μl of cDNA, the remaining volume was adjusted with RNase-free water. The Reactions were carried out using a program that included initial denaturation at 95 °C for 5 min, followed by 35 cycles of denaturation at 95 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s, and a final extension at 72 °C for 10 min. PCR products were analyzed by electrophoresis in 2% agarose gels, stained with GelRed (Biotium, Hayward, CA, USA) and visualized by ultraviolet light.

3. Result

3.1. *Sensitivity and standard curve of the duplex real-time RT-PCR*

Serial dilutions of each RNA standards ranged from 1×10^8 to 1×10^0 copies/ng of total RNA were tested with the duplex assay, the fluorescent signals were detected with 1×10^1 to 1×10^8 copies/ng of total RNA; however, no specific signals were detected with 1×10^0 copies/ng of total RNA and the negative control for the duplex assay (Fig. 2). Therefore, the detection limit of the duplex real-time RT-PCR assay was approximately 4×10^1 copies of RNA per reaction for both TMEV and RTV. Standard curves were constructed from the Ct value; the duplex assay was able to detect

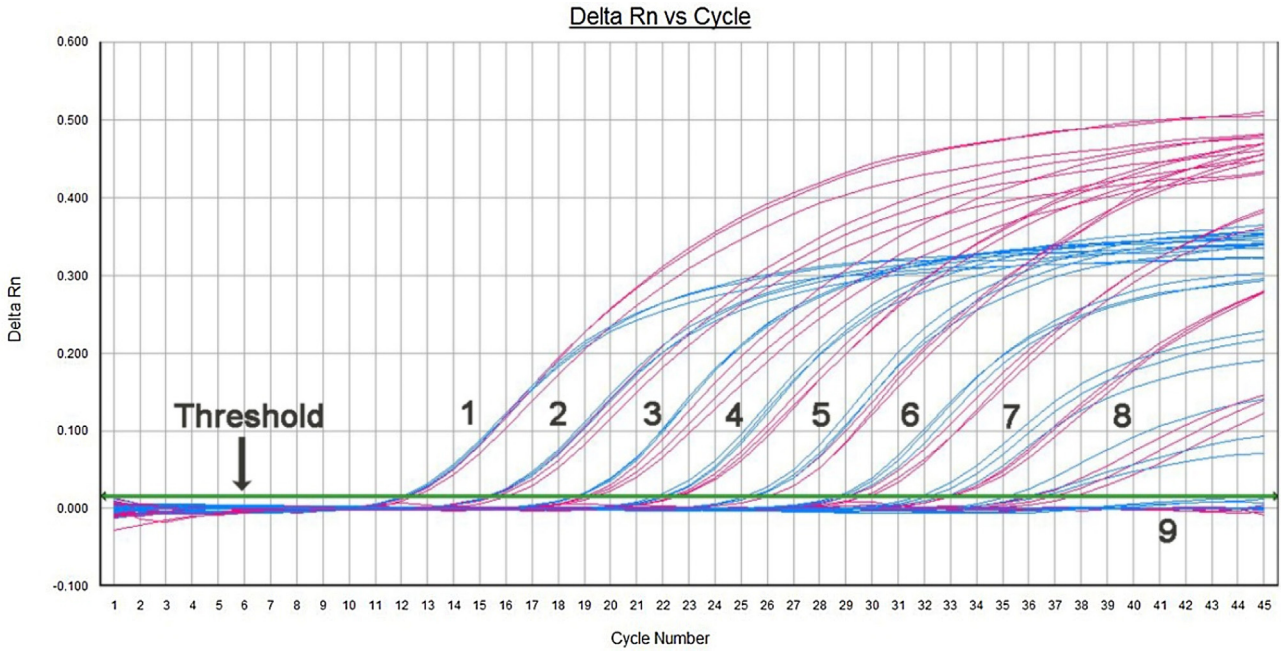

Fig. 2. Sensitivity of duplex real-time RT-PCR. Serial dilutions of *in vitro*-transcribed RNA standards containing sequences of TMEV and RTV were evaluated. Dilutions from left to right on the amplification plot (labeled 1–8, red amplification curves represent TMEV, and blue amplification curves represent RTV) correspond to 1×10^8, 1×10^7, 1×10^6, 1×10^5, 1×10^4, 1×10^3, 1×10^2, and 1×10^1 copies/ng of total RNA, respectively. The threshold cycles <40 are considered positive results. The 1×10^0 copies/ng of total RNA dilution and negative control amplification plot (labeled 9) did not reach the threshold value, and therefore is considered to be negative result. The detection sensitivity was 4×10^1 copies of RNA per reaction for both TMEV and RTV.

each RNA standard over a linear span of 1×10^1 to 1×10^8 copies/ng of total RNA (Fig. 3A, B). The dynamic range of the assay encompassed eight orders of magnitude, with a strong linear relationship between the Ct values and the log 10 of the input number of copies (TMEV, $R^2 = 0.9982$; RTV, $R^2 = 0.9989$). The slopes of each standard curve were −3.490 for TMEV and −3.366 for RTV. The standard curves generated from the duplex assay were similar to those generated from the single assay (Fig. 3C, D). No obvious interference or inhibition was observed in duplex real-time RT-PCR when compared with simplex assay

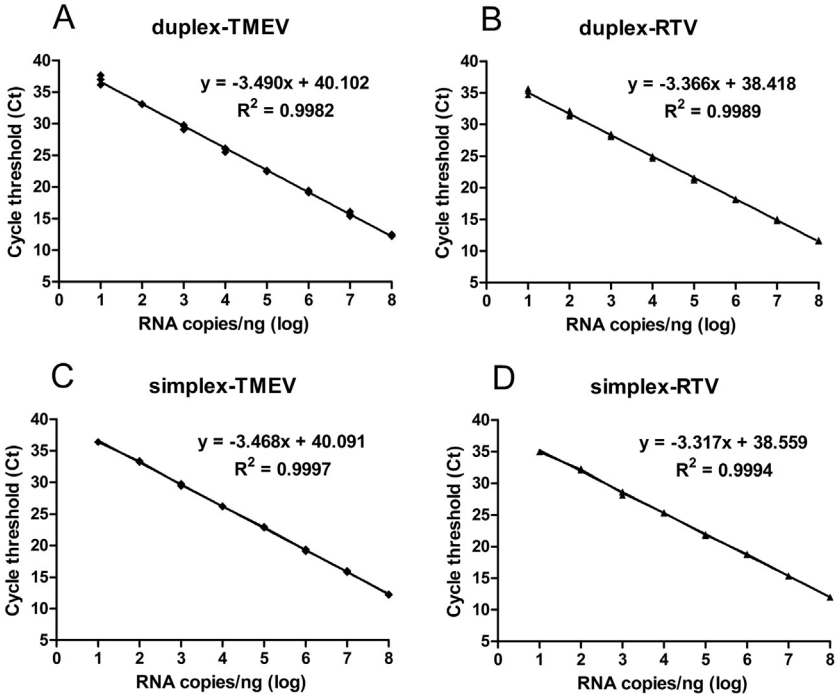

Fig. 3. Standard curves for the duplex and simplex real-time RT-PCR. Serial dilutions of each RNA standard ranging from 1×10^1 to 1×10^8 copies/ng of total RNA were used to generate the standard curves. The cycle of threshold values that corresponded to each PCR cycle number was plotted against the copy number logarithm of each RNA standard. (A) Duplex real-time RT-PCR of TMEV. (B) Duplex real-time RT-PCR of RTV. (C) Simplex real-time RT-PCR of TMEV. (D) Simplex real-time RT-PCR of RTV.

3.2. Specificity of the duplex real-time RT-PCR

The specificity of the duplex real-time RT-PCR assay was validated using genomes RNA of TMEV, RTV, seven other rodent viruses and samples of ten SPF rodent strains. In the FAM channel, all samples tested negative except for TMEV, while in the JOE channel, all samples tested negative except for RTV (Fig. 4). The results suggest that the designed primer pairs and probes were highly specific and selective for their target viruses and exhibited no cross-reactivity with other viruses.

To determine whether viral genomic RNA influences amplification efficiency, we tested the duplex assay in the presence of large amounts of another viral RNA. In the presence of 1×10^6 copies/ng of RTV genomic RNA, the TMEV standard curves were similar to those generated from the standard dilutions alone (Fig. 5A). Similarly, in the presence of 1×10^6 copies/ng of TMEV genomic RNA, the RTV standard curves were similar (Fig. 5B). These results indicate that the genomic RNA does not inhibit PCR amplification each other.

3.3. Reproducibility of the duplex real-time RT-PCR

To evaluate intra- and inter-assay variability, a tenfold serial dilution of the mixed RNA standards from 1×10^7 to 1×10^1 RNA copies/ng of total RNA was used in the duplex assay. Ct values were measured in multiple replicates, and the mean of each Ct value and standard deviation (SD) and coefficients of variation (CV) were calculated. Intra-assay variability was determined using five replicates per batch, and inter-assay variability was examined by running the same standards with three replicates on four consecutive days (Table 2). The coefficients of variation were less than 5% in the intra- and inter-assays, indicating that the assay was highly reproducible.

Table 2
Intra- and inter-assay variability.

Copy number of RNA standard	TMEV		RTV	
	Ct (mean ± SD)	CV (%)	Ct (mean ± SD)	CV (%)
Intra-assay(n = 5)				
4×10^7	15.80 ± 0.12	0.75	15.17 ± 0.25	1.66
4×10^6	19.27 ± 0.12	0.61	18.87 ± 0.19	0.99
4×10^5	22.87 ± 0.19	0.83	21.73 ± 0.18	0.83
4×10^4	26.23 ± 0.07	0.25	25.18 ± 0.21	0.83
4×10^3	29.64 ± 0.13	0.45	28.39 ± 0.28	0.98
4×10^2	33.13 ± 0.32	0.98	32.06 ± 0.26	0.80
4×10^1	36.97 ± 0.39	1.06	35.00 ± 0.47	1.35
Inter-assay (n = 12, three replicates × four times)				
4×10^7	15.80 ± 0.22	1.42	15.47 ± 0.47	3.06
4×10^6	19.30 ± 0.13	0.69	18.80 ± 0.45	2.39
4×10^5	22.68 ± 0.21	0.94	22.07 ± 0.51	2.31
4×10^4	26.13 ± 0.31	1.20	25.56 ± 0.53	2.08
4×10^3	29.59 ± 0.38	1.29	28.94 ± 0.63	2.19
4×10^2	33.19 ± 0.53	1.59	31.99 ± 0.49	1.54
4×10^1	37.29 ± 0.74	2.00	34.96 ± 0.61	1.74

3.4. Detection of clinical samples by duplex real-time RT-PCR and conventional RT-PCR

A total of 439 clinical samples obtained from four different sources were simultaneously tested using the duplex real-time RT-PCR method and conventional RT-PCR. As shown in Table 3, from duplex real-time RT-PCR analysis, 95 (21.6%) and 72 (16.4%) of the total 439 clinical specimens were positive for TMEV and RTV, respectively, and the viral loads of positive TMEV and RTV were 5.0×10^1 to 4.6×10^5 copies/ng of total RNA and 4.0×10^1 to 6.1×10^3 copies/ng of total RNA, respectively (Fig. 6). 77 (17.5%) and 66 (15.0%) samples were positive for TMEV and RTV by conventional RT-PCR, respectively. All positive samples of TMEV and RTV detected by conventional RT-PCR were positive by the duplex

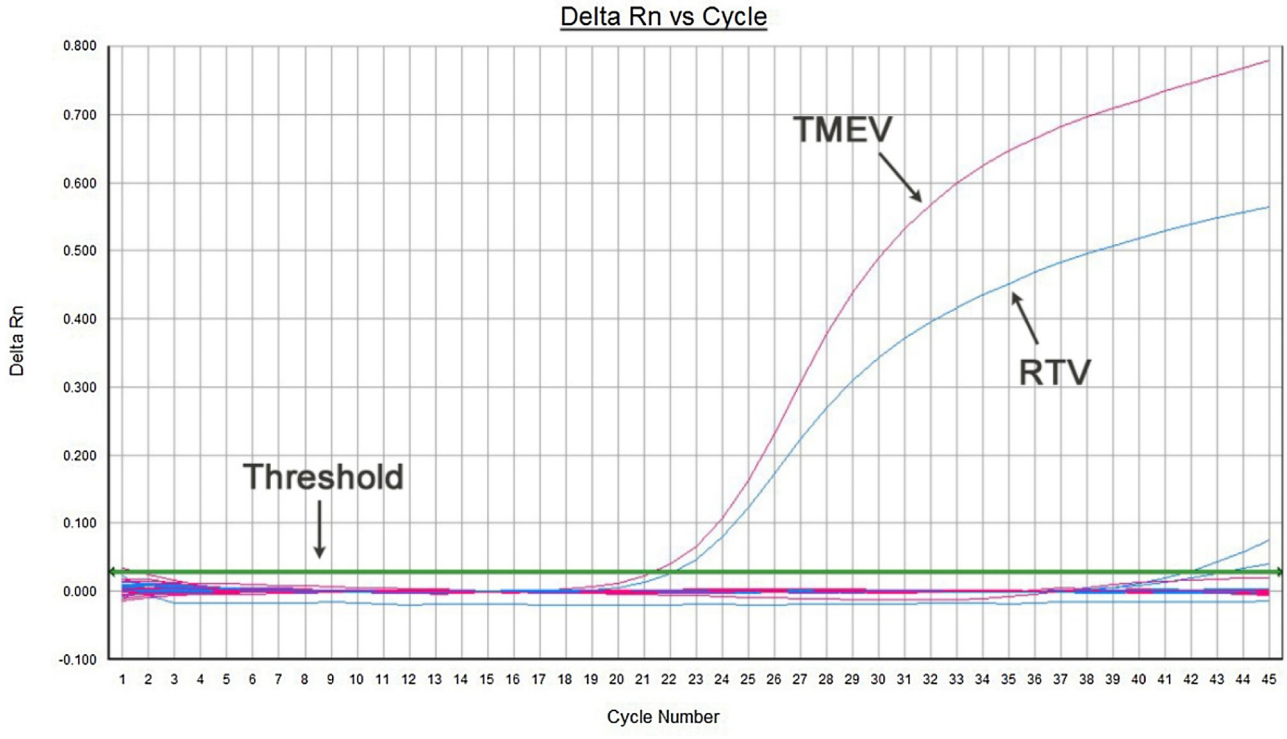

Fig. 4. Specificity of duplex real-time RT-PCR. The specific fluorescent signals were detected from RNA of TMEV and RTV. No cross-reactions were detected from MHV, Reo-3, RCV, Sendai, PVM, MNV, LCMV, and the cecum content and spleen samples collected from ten SPF mouse and rat strains.

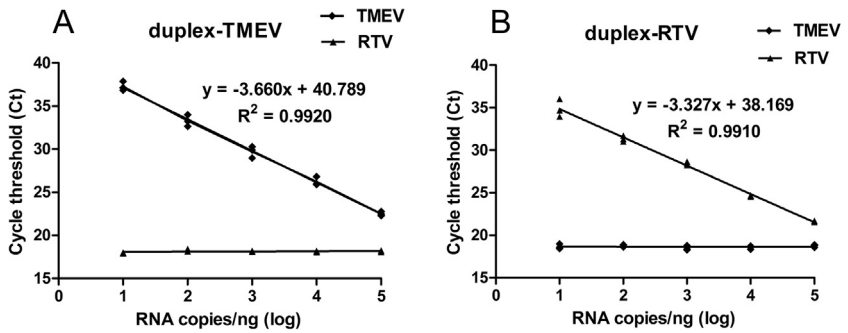

Fig. 5. Inhibition of PCR amplification by large amounts of viral genomic RNA during duplex real-time RT-PCR. (A) Tenfold dilution series of TMEV RNA standard mixed with 1×10^6 copies/ng of RTV genomic RNA were analyzed by duplex real-time RT-PCR. (B) Tenfold dilution series of RTV RNA standard mixed with 1×10^6 copies/ng of TMEV genomic RNA. (♦),TMEV RNA; (▲), RTV RNA.

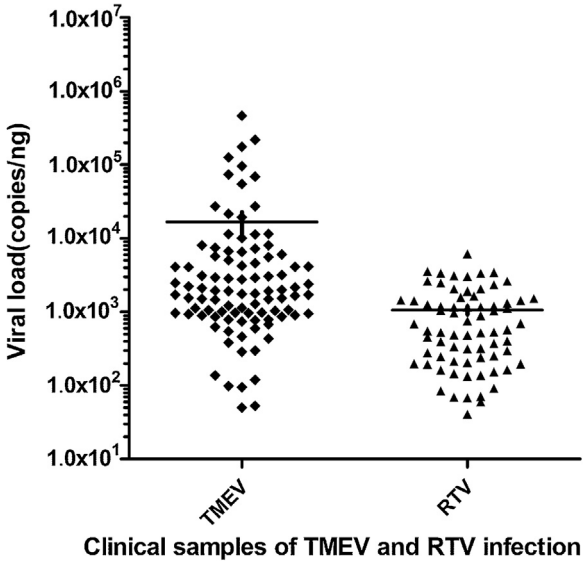

Fig. 6. TMEV and RTV viral loads were quantified by duplex real-time RT-PCR in 439 clinical samples. Ninety-five clinical samples were TMEV (♦) and seventy-two were RTV (▲).

real-time RT-PCR. Moreover, there are 18 more samples positive for TMEV and 6 more samples positive for RTV by duplex real-time RT-PCR when compared with the result from conventional RT-PCR. These results indicated that duplex real-time RT-PCR was more sensitive than conventional RT-PCR. In addition, 30 samples co-infected with TMEV and RTV were all from 128 rodent samples collected from non-barrier colony and wild colony. No co-infections of the two viruses were found in the barrier colony. 15 different cultured cell line samples were also negative for TMEV and RTV.

4. Discussions

Cardioviruses infection is ubiquitous and worldwide in its distribution in colony-bred and feral mice and rats. The greater prevalence of cardioviruses in rodent had made TMEV and RTV a more serious concern to biomedical researchers. Since cardioviruses are easily spread by the fecal-oral route, and viruses are characterized by resistance to inactivation, periodic reintroduction, and relatively long shedding periods. Elimination of these viruses from research facilities requires improved detection. The diagnosis of TMEV or RTV in clinical as well as symptomless infections depends upon positive serology, M/RAP, or RT-PCR amplification of viral RNA. This study describes the development of a new duplex real-time RT-PCR assay for the detection of TMEV and RTV using differently labeled Taqman probes for each virus. The primer/probe combinations were highly specific, and no cross-reaction was detected by using seven other viral pathogens of rodent. Meanwhile, the cecum content and spleen samples collected from ten SPF mouse and rat strains were also negative by the duplex real-time RT-PCR assay. This clearly demonstrates that the TMEV and RTV primer/probe combinations are specific to their specified virus.

Cell-adapted virus culture supernatant, recombinant plasmids and *in vitro*-transcribed RNA can be used for determination of the sensitivity of real-time PCR (Trottier et al., 2002). In this study, the *in vitro*-transcribed RNA containing the partial gene of the TMEV and RTV virus were used to determine the sensitivity of duplex real-time RT-PCR. By using different concentrations of primers, probes, and other reagents, reaction temperatures and times, the conditions of the duplex real-time RT-PCR were optimized (data not shown). The duplex assay was sensitive enough to detect and quantify a very low copy number without mutual interference; the limits of detection of both TMEV and RTV were 4×10^1 copies RNA/reaction. The sensitivity of this duplex assay is equivalent to that of the simplex assay. Nevertheless, duplex PCR takes the same

Table 3
Detection of TMEV and RTV from clinical samples.

Samples	Number of samples tested	Number of positive samples					
		TMEV		RTV		Co-infection	
		Duplex real-time RT-PCR	Conventional RT-PCR	Duplex real-time RT-PCR	Conventional RT-PCR	Duplex real-time RT-PCR	Conventional RT-PCR
Mice in barrier colony	244	27	25	0	0	0	0
Rats in barrier colony	52	0	0	3	3	0	0
Mice in non-barrier colony	52	36	29	4	4	4	4
Rats in non-barrier colony	49	25	19	38	35	19	12
Wild rats	27	7	4	27	24	7	4
Cultured cell lines	15	0	0	0	0	0	0
Total	439	95	77	72	66	30	20

amount of time and effort but produces more data. The system was also specific and sensitive even in the presence of large amounts of another viral RNA. The low mean CV of the duplex real-time RT-PCR indicated that this assay was able to generate reproducible results.

Furthermore, clinical samples collected from four different sources were simultaneously evaluated using the duplex real-time RT-PCR and conventional RT-PCR. The results showed that the duplex real-time RT-PCR assay was more sensitive and effective than conventional RT-PCR in the examination of 439 clinical samples, where 18 samples and 6 samples were negative by conventional RT-PCR, respectively, but they were positive by the duplex real-time RT-PCR. The TMEV and RTV positive rate of clinical samples obtained from barrier colony-raised mice or rats was 11.1% (27/244) and 5.8% (3/52), respectively, which is much higher than the rate of 0.26–1.43% reported in North America and Europe (Pritchett-Corning et al., 2009), indicating that cardioviruses had a high prevalence in China in the past 3 years. The positive rate of non-barrier colony and wild colony was higher than that of barrier colony. In additional, mixed infection of TMEV and RTV was detected in non-barrier raised laboratory rodent and wild rats. As wild mice and rats are the natural host of cardioviruses (Lipton et al., 2001; Ohsawa et al., 2003; Rodrigues et al., 2005), it is necessary to prevent introduction of non-barrier colony raised or feral mice and rats, or contaminated bedding from an outside source, into a clean (barrier conditions) laboratory rodent colony. Thus, strict adherence to standard biological containment measures and barrier conditions is advisable.

In conclusion, duplex real-time RT-PCR assay developed in this study can be used to specifically and simultaneously detect TMEV and RTV. It is a convenient, rapid, and sensitive diagnostic tool for routine health monitoring of viral infection in laboratory mice and rats, and for the screening of contaminated biological materials.

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