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Evaluation of a duplex reverse-transcription real-time PCR assay for the detection of encephalomyocarditis virus

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Abstract. We evaluated a fluorogenic probe–based assay for the detection of encephalomyocarditis virus (EMCV) by comparing a set of published primers and probe to a new set of primers and probe. The published reagents failed to amplify a range of Australian isolates and an Italian reference strain of EMCV. In contrast, an assay based on 2 new sets of primers and probes that were run in a duplex reverse-transcription real-time PCR (RT-rtPCR) worked well, with high amplification efficiency. The analytical sensitivity was ~100-fold higher than virus isolation in cell culture. The intra-assay variation was 0.21–4.90%. No cross-reactivity was observed with a range of other porcine viruses. One hundred and twenty-two clinical specimens were tested simultaneously by RT-rtPCR and virus isolation in cell culture; 72 specimens gave positive results by RT-rtPCR, and 63 of these were also positive by virus isolation. Of 245 archived cell culture isolates of EMCV that were tested in the RT-rtPCR, 242 samples were positive. The new duplex RT-rtPCR assay is a reliable tool for the detection of EMCV in clinical specimens and for use in epidemiologic investigations.

Key words: Encephalomyocarditis virus; reverse-transcription polymerase chain reaction.

Introduction

Encephalomyocarditis virus (EMCV; order Picornavirales, family *Picornaviridae*, genus *Cardiovirus*, species *Cardiovirus A*) is a small, non-enveloped, positive-sense single-stranded RNA virus.[16] EMCV was first isolated in 1940 and has a worldwide distribution.[4] Rodents are considered as reservoirs or natural hosts of EMCV,[27] and they transmit the virus to a wide range of mammalian species including pigs,[1,2,4,8,9,12] cattle,[6,7] elephants,[11,17] marsupials,[23] other rodent species,[9] non-human primates,[3,13,19,23,33] and, rarely, humans.[15,20,26,32]

EMCV infection in different animals results in clinical signs ranging from asymptomatic persistence in natural reservoirs (rodents) to sudden death in most other animal species.[1,3,4,13,23] Pigs are considered to be the most commonly and severely affected domestic animal.[2,9] EMCV infection is known to be a cause of sudden death with high mortality rates as a result of myocarditis and encephalitis in young pigs, and reproductive failure in sows.[2,4] This may lead to serious economic losses in pig farms. Significant losses are also experienced in a wide range of wildlife species, especially non-human primates, particularly in a captive environment,[3,11,19,23,25,33] but also in the wild.[11,17]

Methods used to detect EMCV infection include virus isolation,[10,18,22] serology,[36] immunohistochemistry,[30] conventional reverse-transcription PCR (RT-PCR),[14,18,21,22,28,29] reverse-transcription loop-mediated isothermal amplification (RT-LAMP),[34] and real-time RT-PCR (RT-rtPCR) based on SYBR Green detection.[31] A fluorogenic probe–based RT-rtPCR assay has also been described.[35] In contrast to traditional assays, RT-rtPCR is considered to be a highly sensitive, specific, and time-saving method, and is of higher sensitivity than RT-LAMP.[34]

We evaluated a duplex fluorogenic probe–based RT-rtPCR assay by comparing it with a published assay[35] and virus isolation in cell culture. The capacity of the duplex RT-rtPCR to detect EMCV in clinical samples was also evaluated.

Materials and methods

Sample sources

At the Biosecurity Sciences Laboratory (BSL; Department of Agriculture and Fisheries, Coopers Plains, Queensland,

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Table 1. Details of samples tested in both laboratories.

Source (period)/Sample type	Species	Positive			Negative
		RT-rtPCR		Virus isolation	RT-rtPCR
		No. of positives	Ct value		
Queensland (1983–2016)					
Virus cultures	Porcine, bovine, primate, camelid, marsupial	25	13.2–30.9	25	
Heart	Porcine, bovine, primate, camelid, marsupial	44	13.4–30.0	38	44
Brain	Porcine	1	21.5		4
Lymph node	Porcine	1	36		1
Spleen	Porcine	1	33.7		1
Total		72		63	50
NSW (1970–2016)					
Viral cultures	Porcine, bovine, primate, marsupial	201	23.4 (11.4–38.1)*	202	1
Tissue	Porcine, bovine, primate, marsupial	41	17.6 (10.2–37.5)†	43	2
Total		242		245	3

NSW = New South Wales, Australia; RT-rtPCR = reverse-transcription real-time PCR. Cycle threshold (Ct) values are presented as ranges or means, with ranges in parentheses if applicable.
* Includes 3 samples with Ct values >32.5.
† Includes one sample with Ct value >32.5.

Australia), 122 samples collected from a wide range of diseased mammalian species were tested concurrently by virus isolation and with the new RT-rtPCR. A large archival collection of tissue samples and cell culture fluids from which EMCV had been isolated was also available for testing. These samples were collected in 1970–2016 from a wide variety of mammalian species and were stored at −80°C at the Elizabeth Macarthur Agriculture Institute (EMAI; Menangle, New South Wales, Australia; Table 1). Also included was a European strain of EMCV (detected as a result of fatal myocarditis in Novara, Italy in 1986; Gualandi GL, Cordioli P, unpublished data[12]). Forty-two heart and one brain homogenate from various mammalian species were also tested concurrently at EMAI.

Primers and probes

The new RT-rtPCR assay was designed at BSL (AlleleID, Premier Biosoft, Palo Alto, CA). Twenty published EMCV sequences, including EMCV ATCC strain VR-129B from the National Center for Biotechnology Information (NCBI), were aligned, and the consensus sequence was used for the design of both RT-rtPCR assays (Table 2). The assay included sets of primers and TaqMan fluorogenic probes (Table 3), targeting sequences of the 5′–nontranslated region (5′-NTR) and 2B of the polyprotein region of the genome, and were used in a duplex assay. The sequence specificity of the primers and probes was confirmed by nucleotide–nucleotide search using the basic local alignment search tool (BLAST) of GenBank (http://www.ncbi.nlm.nih.gov/BLAST/). The assay used for comparison was as described previously[35] (Table 3).

Table 2. List of encephalomyocarditis virus (EMCV) sequences used for assay design.

	EMCV strain	GenBank accession
1	D variant	M37588.1
2	B variant	M22457.1
3	D variant	M22458.1
4	K3	EU780148.1
5	K11	EU780149.1
6	Ruckert	M81861.1
7	1086C	DQ835185
8	BEL-2887A-91	AF356822.1
9	GXLC	FJ897755
10	GX0602	FJ604853.1
11	HB1	DQ464063
12	BJC3	DQ464062
13	EMCV	X87335.1
14	EMCV	X74312.1
15	EMCV	NC_001479
16	pEC9	DQ288856
17	PEMCV 30	AY296731
18	PEMCV CBN	DQ51424
19	PEMCV NJ08	HM641897.1
20	ATCC VR-129B	KM269482.1

Nucleic acid extraction

Total nucleic acid was extracted from 50 μL of a 1/10 dilution of tissue culture fluid or supernatant from tissue homogenates with a magnetic bead–based extraction kit (MagMax-96, Thermo Fisher Scientific, Austin, TX) according to the manufacturer's instructions, using a magnetic particle handling

Table 3. Primers and probes used in the reverse-transcription real-time PCR (RT-rtPCR) assays for encephalomyocarditis virus (EMCV).

Assay name/Target	Primer/probe	Nucleotide sequence (5′–3′)	EMCV strain ATCC VR-129B Position	Source
Duplex RT-rtPCR				
5′-NTR	EMCV 5NTR-F	GTCTGTAGCGACCCTTTG	519-536	This study
	EMCV 5NTR-R	CCTTGTTGAATACGCTTGAG	694-675	
	EMCV 5NTR-P	FAM-AGCCATTTGACTCTTTCCACAACTAT-BHQ1	671-646	
2B	EMCV 2B-F	ATGGGAAAATGTAAAAGAAACA	4173-4194	
	EMCV 2B-R	GCATCACTGCTATTGTCA	4273–4256	
	EMCV 2B-P	FAM-AGCTGCACACATCTGCTCAA-BHQ1	4244-4225	
Yuan et al. TaqMan assay				
3D	EMCV-F	TCATTAGCCATTTCAACCCA	7156-7175	[35]
	EMCV-R	GAGATACAAACCCGCCCTAA	7290-7271	
	EMCV-P	FAM-TCCCATCAGGTTGTGCAGCGA-TAMRA	7214-7234	
CN PCR				
5′-NTR	CN 5NTR-F	CTAACGTTACTGGCCGAAGC	293-312	This study
	CN 5NTR-R	GGTACCTTCTGGGCATCCTT	718-700	
2B	CN 2B-F	CAGCTTTTACGGCTTTGCTC	4088-4107	
	CN 2B-R	GTCCCAAACCAATCAACCAC	4523–4504	

5′-NTR = 5′–nontranslated region; 2B, 3D = polyprotein regions; CN = copy number; F = forward primer; P = TaqMan probe; R = reverse primer. FAM (6-carboxyfluorescein) was used as the reporter and BHQ-1 (black hole quencher) as the quencher.

system (KingFisher 96, Thermo Fisher). Extracts were eluted in 50 μL of nuclease-free water, and stored at –20°C if amplification by RT-rtPCR was not carried out immediately.

RT-rtPCR

The duplex RT-rtPCR was evaluated in 2 laboratories with slightly different methods.

EMAI protocol. The new duplex RT-rtPCR was performed using a commercial master mix (AgPath-ID one-step RT-PCR kit, Thermo Fisher) with 5 μL of purified nucleic acid in a total reaction volume of 25 μL. The reaction mixture included 0.15 μL of each forward and reverse primer (100 μM), 0.05 μL of each probe (100 μM), and the components of the RT-rtPCR kit as recommended by the manufacturer. The RT-rtPCR was run (ABI 7500 Fast thermocycler, normal mode, Thermo Fisher) under the standard reaction conditions for the master mix for a total of 45 cycles. Background fluorescence was adjusted automatically, and the threshold was set manually at 0.05. Results were expressed as cycle threshold (Ct) values, being the cycle at which the amplification curve crossed the 0.05 threshold.

In order to confirm that none of the samples contained factors that would either reduce the efficiency of RNA extraction or contain inhibitors of the RT-rtPCR, an extraneous RNA construct was used (XIPC).[24] This was added to the sample lysis buffer, and the corresponding primers and probe were then added to the EMCV reaction mix to form a triplex RT-rtPCR. The impact of the XIPC on the efficiency of the RT-rtPCR was assessed by testing a titration series of the reference strain of EMCV used along with RNA extracted from some of the samples to compare performance of the EMCV duplex assay with and without the XIPC assay components.

BSL protocol. The duplex RT-rtPCR was performed (Rotor-Gene Q, Qiagen, Chadstone, Victoria, Australia; SuperScript Platinum III one-step quantitative RT-PCR system master mix, Thermo Fisher) as recommended by the manufacturer. The reaction mix contained 2 μL of each of the primers (40 μM) and 1 μL of each of the probes (5 μM). The probes were labeled with 2 different fluorophores, FAM (6-carboxyfluorescein) and VIC (Thermo Fisher). The cycling parameters were reverse transcription at 37°C for 15 min followed by initial denaturation at 95°C for 2 min and 45 cycles at 95°C for 5 s, 52°C for 5 s, and 72°C for 15 s, at which step the fluorescence was acquired. The background fluorescence was adjusted manually, and the threshold was set at 0.05. The results were expressed as described above.

PCR competency (successful extraction and absence of PCR inhibitors) of the RNA extracts was monitored by multiplexing the duplex RT-rtPCR with an internal control RT-PCR that targets mitochondrial DNA.[5] The internal control RT-PCR probe was labeled with a different fluorophore, Cy5 (cyanine 5).

Analytical sensitivity, intra-assay variation, and amplification efficiency of the RT-rtPCR

An Australian reference strain of EMCV (F728) with a titer of $10^{7.8}$ $TCID_{50}$/mL was diluted in serial 10-fold dilutions from 10^{-1} to 10^{-8} in phosphate-buffered gelatin saline (PBGS). These dilutions were extracted and tested in the RT-rtPCR assays in duplicate to determine detection limits and construct standard curves. Intra-assay variation was assessed by calculating the coefficient of variation (CV) from the duplicates on each test plate. The amplification efficiency of each RT-rtPCR was calculated from the slope of the standard curve constructed from the serial dilutions of template.

Determination of copy number and limit of detection

The copy number (CN) and limit of detection (LOD) were determined as described previously.[6] Briefly, for the determination of CN and LOD of each of the RT-rtPCR assays, CN PCR assays were designed. The 5′-NTR CN PCR was designed to amplify a 425-bp region of the EMCV 5′-NTR, and the 2B CN PCR was designed to amplify a 436-bp region of the 2B region of the EMCV genome. The primers were designed to flank the amplicons of their respective RT-rtPCR assays (Table 3).

The resulting amplicon was purified and quantified (NanoPhotometer, Implen, München, Germany), and serial 10-fold (10^{-1}–10^{-15}) dilutions were prepared and assayed. The CN was calculated using the formula described by the University of Rhode Island Genomic and Sequencing Center (http://cels.uri.edu/gsc/cndna.html).

Analytical specificity of the RT-rtPCR

As well as comparison with the virus isolation results, the specificity of the new duplex RT-rtPCR was confirmed by testing high-titer samples of other relevant animal viruses including Bungowannah virus, Menangle rubulavirus, porcine parvovirus (PPV; species *Ungulate protoparvovirus 1*), porcine circovirus 2 (PCV-2), porcine epidemic diarrhea virus (PEDV), classical swine fever virus (CSFV; species *Pestivirus C*), and influenza A virus.

Virus isolation

Virus isolation in cell culture was undertaken on 10–20% homogenates of 122 tissues using standard techniques.[2,23] Virus isolates were identified as EMCV by virus neutralization test using a polyclonal antiserum against EMCV.

Results

The performance of the published and the new duplex assay was initially assessed under the same reaction conditions by testing serial dilutions of the Australian F728 strain of EMCV. The published EMCV RT-rtPCR failed to show amplification of any virus dilution. When testing a collection of isolates with Ct values of 15–19 in the new assay, it also failed to detect 7 strains (including the Italian Novara strain) or had a difference of >10 cycles (6 isolates) compared to the new assay. Acceptable results were only obtained for 2 isolates. Consequently, evaluation of this assay was discontinued. In contrast, there was good amplification with the new duplex RT-rtPCR over an 8 $\log_{10}$ range without reaching the LOD, with the 10^{-8} dilution of the virus stock, giving a mean Ct value of 34.4. The intra-assay CV was 0.21–4.90%. The amplification efficiency was 97.24%. The determination of the LOD at BSL suggested that the cutoff for the RT-rtPCR was a Ct value of 36, which corresponded to 0.9 $TCID_{50}$. The CN using amplified DNA was ~1.2×10^2 and 1.5×10^2 target copies, which corresponded to Ct values of 37.5 and 35.5 for 5′-NTR RT-rtPCR and 2B RT-rtPCR, respectively.

The high specificity of this duplex RT-rtPCR assay was demonstrated by the lack of amplification of high-titer nucleic acid extracts from all other porcine viruses tested (Bungowannah virus, Menangle virus, PPV, PCV-2, PEDV, CSFV, and influenza A virus).

Further evaluation of the duplex RT-rtPCR was conducted in Queensland in parallel with virus isolation on 122 clinical samples; 72 of the 122 samples were positive by RT-rtPCR, and EMCVs were isolated from 63 of these samples. Fifty of the 122 samples were negative by both methods (Table 1). When comparing the Ct values obtained from various tissues, heart tissue gave the lowest Ct values (13.4–30.0); lymph node and spleen gave the highest Ct values. The Ct values of heart tissues were comparable to those of viral culture (13.2–31.0). The single brain sample tested also gave a low Ct value (13.2). On the other hand, lymph node and spleen gave the highest Ct values (36.0 and 33.7, respectively), which are close to the LOD of the RT-rtPCR. There was no evidence of inhibition of the internal control including for the 50 negative samples.

Finally, when the RT-rtPCR was used to test the 43 archived tissue homogenates from which EMCV had been isolated at EMAI, and 202 EMCV isolates (including the Italian Novara strain), 242 of the 245 samples were positive, with mean Ct values of 17. 6 (range: 10.2–37.5) for the isolates, and 23.4 (range: 11.4–38.1) for the tissue homogenates. Three of the 245 samples were negative. There was no evidence of inhibition of the XIPC that was included in the RT-rtPCR (data not shown). The evaluation of the efficiency of the duplex EMCV RT-rtPCR was not affected by the inclusion of an XIPC construct or the internal control RT-rtPCR.

Discussion

Initial evaluation of the published set of primers and probe[35] gave extremely poor results. Conversely, the paired set of new primers and probe showed good amplification with a

high efficiency (97.24%). One possible interpretation is that the published primers and probe that failed to amplify most of the Australian isolates of EMCV were designed and validated using samples collected in China, but the published assay also failed to detect the Italian Novara strain. However, there is not a single consensus sequence to detect all Australian strains of EMCV, hence the need to use a paired set of primers and probe, run in a duplex assay (triplex with XIPC and internal RT-rtPCR). Two variant strains of EMCV have been described in Singapore.[33] These 2 variants were shown to be divergent from other EMCV strains based on sequencing of the 3D region of the polyprotein, the region targeted by the published assay, and the VP1 capsid region.[32] Based on sequence comparisons, we did not expect the published assay to detect these 2 variants. In silico evaluation of the published sequences of these variants suggests that these would also be detected by one set of primers and probe in the new duplex assay. Unfortunately, there was no Chinese positive control sample of EMCV available in either of the 2 participating laboratories to evaluate the performance of the new duplex RT-rtPCR against a Chinese strain of EMCV. Conversely, there was also no published genomic sequence of an Australian EMCV available to the Chinese scientists during the development of their assay.[35] Our study demonstrates the critical need to evaluate published assays using local strains of an agent.

The reliability of the new duplex assay from nucleic acid extraction through the RT-rtPCR was confirmed by titration of a high-titer virus preparation. This evaluation demonstrated good assay performance over a range of 8 $\log_{10}$ with a LOD equivalent to 0.3 $TCID_{50}$/mL (mean Ct value of 34.4) with good repeatability (CV = 0.21–4.90%).

The RT-rtPCR results for clinical samples suggest that the best sample for the detection of EMCV RNA is heart tissue. The Ct values for heart RNA extracts gave consistently lower Ct values when compared to other tissues such as spleen and lymph node. Brain was the alternative sample of choice but may depend on whether there are characteristic EMCV lesions in the brain (i.e., nonsuppurative encephalitis).

Because pigs are the species most frequently infected with EMCV, the high specificity of this assay was confirmed by a lack of reactivity with a number of porcine viruses, including Bungowannah virus, Menangle virus, PPV, PCV-2, PEDV, CSFV, and influenza A virus.

The duplex RT-rtPCR also has very high analytical sensitivity, and while correctly identifying all 63 positive clinical specimens from which EMCV was isolated, it also detected EMCV RNA in another 9 specimens that gave negative results by virus isolation. During retrospective testing of the collection of archived samples, negative results were obtained for 3 of 245 stored virus isolates or tissue that had originally given positive results; another 4 samples gave Ct values >32.5. Given that all 7 samples or isolates had been held for >20 y, it is likely that samples deteriorated during storage.

The ability of the duplex RT-rtPCR to detect EMCV RNA in clinical samples from which virus has been isolated underlines the detection sensitivity of the assay. Furthermore, the specificity of the RT-rtPCR was also demonstrated by the negative results obtained from 50 clinical samples from which EMCV was not isolated.

We validated a fluorogenic probe–based duplex RT-rtPCR method for detection of EMCV on a large number of samples collected from a variety of mammalian species and over a lengthy period of time; the oldest samples had been stored for 46 y. Our results indicate that this fluorogenic probe–based duplex RT-rtPCR method is reliable and can be used for the detection of EMCV. Although one Italian virus was successfully detected, there is, however, a need to evaluate this assay in other geographical regions to confirm its capacity to detect a wider range of strains of EMCV.

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Declaration of conflicting interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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