Animal health

Novel RT-qPCR workflow for simultaneous identification of rotavirus species A, B, and C

Introduction

Rotaviruses are among the most prevalent etiological agents causing severe diarrhea in young pigs. Although rarely fatal, disease severity can increase with concurrent infection or environmental stressors, which can further delay weight gain and time-to-market. Adult pigs become resistant to clinical disease as their immune system matures. Of the 9 known rotavirus species, rotavirus A, B, and C (RVA, RVB, and RVC) are the most important species associated with diarrhea in piglets.

Rotaviruses (*Reoviridae*) are double-stranded RNA (dsRNA) viruses with linear genomes comprising 11 gene segments. RNA viruses depend on RNA-dependent RNA polymerase (RdRp) to replicate their genome during infection. The poor fidelity of RdRps results in numerous mutations among members of the same RNA virus species [1]. Molecular detection of rotavirus must account for this complexity. Here we describe a multiplex reverse transcription quantitative PCR (RT-qPCR) design that can differentiate RVA, RVB, RVC, and an internal positive control in a single reaction. Applied Biosystems™ VetMAX™ Rotavirus A/B/C Reagents showed excellent ability to selectively amplify rotavirus species A, B, and C and showed no indications of amplifying non-rotavirus targets *in silico* and *in vitro*.

When compared to a commonly used published workflow against a large panel of rotavirus-infected porcine samples, this new design demonstrated a lower average C_q and identified a larger number of rotavirus-positive samples. The VetMAX Rotavirus RT-qPCR workflow successfully identified rotaviruses in porcine feces, intestine, and environmental samples, both in individual samples and in pools of 5.

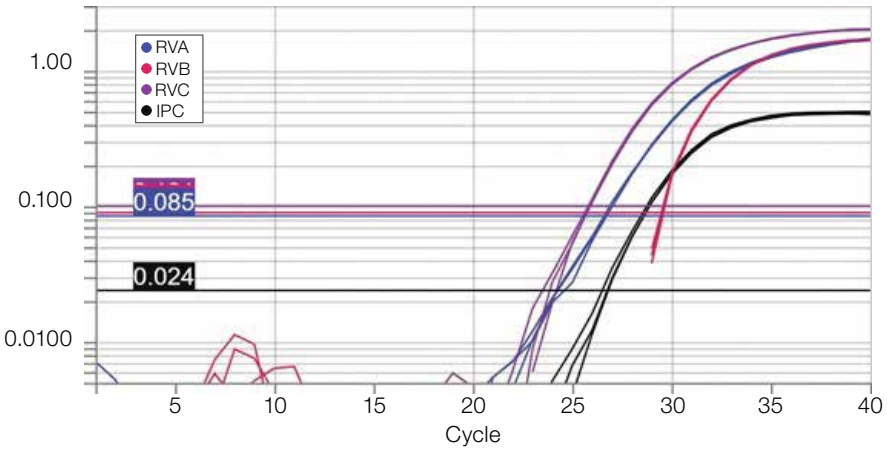

Figure 1. Amplification of RVA, RVB, RVC, and the internal positive control (IPC) in a single reaction mix.

Materials and methods

VetMAX Rotavirus A/B/C Reagents

VetMAX Rotavirus A/B/C Reagents includes the VetMAX Rotavirus Primer/Probe Mix, which uses Applied Biosystems™ TaqMan™ probes for real-time PCR detection. The primer/probe mix assigns different fluorescent dyes to each rotavirus target and the internal positive control to differentiate the four targets (Figure 1). The fluorescent labels are ABY™, FAM™, VIC™, and Cy®5 dyes for RVA, RVB, RVC, and the IPC, respectively.

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VetMAX Rotavirus primer/probe design

The VetMAX Rotavirus Primer/Probe Mix targets two separate RVA gene targets and two separate RVB gene targets to increase coverage of known rotavirus sequences. The primers and probes were selected based on a large panel of RVA, RVB, and RVC sequences obtained from NCBI, collaborators, and samples sequenced internally. Proprietary software was used to determine percent coverage of the rotavirus sequences by the primer/probe mix. *In silico*, the coverage was >96% for porcine rotaviruses A, B, and C (Table 1).

Table 1. Percent coverage of known rotavirus sequences by the VetMAX Rotavirus Primer/Probe Mix.

Sequences	RVA	RVB	RVC
Number analyzed	290	197	162
Number detected	288	194	156
Percent detected	99.3%	98.5%	96.3%

Samples

A total of 339 porcine samples infected with rotavirus were obtained from the Iowa State University Veterinary Diagnostic Laboratory (ISU-VDL), the University of Minnesota Veterinary Diagnostic Laboratory (UMN-VDL), and the Carthage Veterinary Service. Sample descriptions included feces (208), fecal swabs (51), rectal swabs (5), feedback (20), fluid (2), homogenate (13), oral fluid (3), processing fluid (1), tissue composite (2), intestinal tissue (25), environmental samples (5), and others (4).

Nucleic acid extraction

Total nucleic acid was extracted from porcine samples using the Applied Biosystems™ MagMAX™ CORE Nucleic Acid Purification Kit automated on the Thermo Scientific™ KingFisher™ Flex Purification System following the Complex Workflow as described in the MagMAX CORE kit user guide. Applied Biosystems™ Xeno™ RNA Control was added to the MagMAX CORE Lysis Solution as recommended in the user guide to gauge extraction efficiency and inhibitor presence. The extracted nucleic acid was used immediately in RT-qPCR testing and then stored long term at –20°C.

RT-qPCR

All extracted nucleic acid samples and controls were heat-denatured to allow for efficient RNA reverse transcription prior to PCR amplification. Samples and controls were heat-denatured at 95°C for 3 minutes, allowed to cool, and briefly centrifuged prior to adding to the RT-qPCR reaction mix. Three controls were included in each RT-qPCR run: (1) no-template control (NTC) consisting of nuclease-free water, (2) negative extraction control (NEC) prepared during nucleic acid extraction with 1X PBS, and (3) positive control consisting of the RVA/RVB/RVC/Xeno RNA Control. The RT-qPCR reactions were run on either the Applied Biosystems™ 7500 Fast Real-Time PCR System; the Applied Biosystems™ QuantStudio™ 5 Real-Time PCR System, 96-well, 0.1 mL; or the QuantStudio 5 Real-Time PCR System, 96-well, 0.2 mL. The real-time PCR instruments were run using the thermal profiles described in Tables 2 and 3.

Table 2. Fast run mode for the thermal cycling conditions.

Stage	Description	Step	Cycles	Temp.	Time
Reverse transcription	cDNA synthesis	1	1	50°C	5 minutes
Enzyme activation	*Taq* polymerase activation	2	1	95°C	10 minutes
PCR	Template denaturation	3a	40	95°C	3 seconds
	Template annealing and extension	3b		60°C	30 seconds

Table 3. Standard run mode for the thermal cycling conditions.

Stage	Description	Step	Cycles	Temp.	Time
Reverse transcription	cDNA synthesis	1	1	48°C	10 minutes
Enzyme activation	*Taq* polymerase activation	2	1	95°C	2 minutes
PCR	Template denaturation	3a	40	95°C	15 seconds
	Template annealing and extension	3b		60°C	45 seconds

Data analysis

Applied Biosystems™ QuantStudio™ Design and Analysis Software version 2.7.0 was used to analyze run files from both instruments. The run threshold for each dye channel was set based on the ΔRn of the rotavirus positive control using control-based threshold (CBT). 10% CBT was set by multiplying the ΔRn at cycle 40 for each dye in the control sample by 0.1. A manual baseline was set to 3–15. However, porcine samples with high concentrations of rotavirus having C_q values under 15 gave better results using auto baseline. When testing porcine field samples for the presence of rotavirus, we found that auto baseline worked best for RVA and RVC, but manual baseline worked best for RVB.

Data interpretation

Results from the controls (positive control, NTC, and NEC) were examined to determine if the PCR run was valid (see Table 4). Rotavirus samples with C_q values <37 were considered positive, between 37 and 39.9 were considered suspect positive, and ≥40 were considered negative.

Results

Analytical sensitivity

A total of 284 porcine samples identified as being infected with RVA, RVB, and/or RVC were obtained from state veterinary diagnostic labs. These samples were processed and tested with both theVetMAX Rotavirus Primer/Probe Mix and a published method [2] using the same master mix on the same QuantStudio 5 Real-Time PCR System. The VetMAX Rotavirus assay was run using fast PCR cycling conditions, and the published method was run under the PCR cycling conditions recommended by the authors. The results showed that the VetMAX Rotavirus Primer/Probe Mix identified 4 RVA targets, 7 RVB targets, and 1 RVC target that were not identified by the published method (Table 5). However, the VetMAX Rotavirus Primer/Probe Mix did miss 1 RVA target and 1 RVB target that were identified by the published method. The C_q of each target missed by one of the two primer/probe mixes is reported in Table 6. For samples identified by both methods, the average difference in C_q between the two primer/probe mixes was determined, and the VetMAX Rotavirus Primer/Probe Mix demonstrated an average C_q that was 1.9 less for RVA, 2.5 less for RVB, and 0.2 higher for RVC than with the published method.

Table 4. Valid runs gave results from the RT-qPCR controls within the listed parameters.

Stage	RVA C_q	RVB C_q	RVC C_q	Xeno control C_q
VetMAX Rotavirus A/B/C Controls	24–28	27–31	24–28	24–28
NTC (water)	Undetected	Undetected	Undetected	Undetected
NEC (PBS or water extraction)	Undetected	Undetected	Undetected	26–34

Table 5. Number of samples out of 284 samples not detected *in vitro* by the VetMAX Rotavirus Primer/Probe Mix and by a published rotavirus A/B/C method.

Method	RVA	RVB	RVC
VetMAX primer/probe mix	1	1	0
Published method	4	7	1

Table 6. C_q for samples missed by either the VetMAX Rotavirus Primer/Probe Mix or the published rotavirus A/B/C method.

	RVA		RVB		RVC	
Method showing missed call	VetMAX	Published	VetMAX	Published	VetMAX	Published
VetMAX primer/probe mix	Undetected	36.7	Undetected	32.2	–	–
Published method	8.7	Undetected	27.3	Undetected	13.5	Undetected
Published method	15.5	Undetected	33.9	Undetected	–	–
Published method	16.1	Undetected	35.1	Undetected	–	–
Published method	19.3	Undetected	36.2	Undetected	–	–
Published method	–	–	36.7	Undetected	–	–
Published method	–	–	36.8	Undetected	–	–
Published method	–	–	36.9	Undetected	–	–

Analytical specificity

The VetMAX Rotavirus Primer/Probe Mix demonstrated no detection of non-rotavirus sequences within the GeneBank™ database, indicating high analytical specificity for rotaviruses *in silico*. *In vitro* studies with the VetMAX Rotavirus Primer/Probe Mix showed no false-positive detection with a panel of porcine samples infected with non-rotavirus pathogens associated with porcine enteric diseases (Table 7).

Limit of detection (LOD)

The LOD of the VetMAX Rotavirus Primer/Probe Mix was determined using dilutions of the rotavirus RNA control templates. Each rotavirus RNA template was tested separately with 20 replicates at each concentration. The LOD was defined as the lowest concentration giving a minimum of 19 out of 20 replicates (i.e., 95%) testing positive. For this study, a C_q below 40 was considered positive. The analytical sensitivity for RVA, RVB, and RVC was 25 copies, 40 copies, and 10 copies, respectively, as shown in Table 8.

Table 7. Exclusion panel consisting of non-rotavirus enteric pathogens used to determine the analytical specificity of the primer/probe mix.

ID	Agent positive for	Sample type	Univ16S*	RVA	RVB	RVC	Xeno
1	Porcine sapovirus (C_q = 15.7)**	Fecal swab	13.2	Und.	Und.	Und.	29.2
2	Porcine sapovirus (C_q = 32.3)	Fecal swab	10.4	Und.	Und.	Und.	29.9
3	Porcine sapovirus (C_q = 13.4)	Fecal swab	5.2	Und.	Und.	Und.	30.9
4	Porcine sapovirus (C_q = 20.3)	Fecal swab	5.2	Und.	Und.	Und.	31.2
5	Porcine sapovirus (C_q = 28.6)	Fecal swab	4.5	Und.	Und.	Und.	31.0
6	*Lawsonia* (C_q = 16.2)	Feces	11.2	Und.	Und.	Und.	30.0
7	*Lawsonia* (C_q = 16.2)	Feces	5.0	Und.	Und.	Und.	30.5
8	*Lawsonia* (C_q = 16.4)	Feces	9.5	Und.	Und.	Und.	30.1
9	*Brachyspira hyodysenteriae*	Medium	6.8	Und.	Und.	Und.	29.7
10	*Salmonella choleraesuis*	Medium	<3.0	Und.	Und.	Und.	29.9
11	*Salmonella typhimurium*	Medium	<3.0	Und.	Und.	Und.	29.2
12	*Clostridium perfringens*	Medium	4.5	Und.	Und.	Und.	29.3
13	*Clostridium difficile*	Medium	4.4	Und.	Und.	Und.	29.5
14	PEDV positive	Environmental	4.9	Und.	Und.	Und.	30.1
15	PDCoV positive	Environmental	5.0	Und.	Und.	Und.	29.9
16	TGEV positive	Environmental	5.0	Und.	Und.	Und.	29.7

* Univ16S is a universal real-time PCR assay that detects bacterial DNA based on the 16S rRNA gene sequence, and it was included in this study to demonstrate the presence of microbial nucleic acid in the samples.

** The C_q values in parentheses were provided by the donating institution, where available.

Table 8. LOD for RVA, RVB, and RVC.

Species	Copies/reaction	Number positive	C_q range	LOD
RVA	10	12	37.6–39.7	25
	25	20	35.4–37.7	
	50	20	34.1–35.8	
	100	19	32.7–33.8	
RVB	5	0	Undetected	40
	20	18	38.0–39.7	
	40	20	36.3–38.4	
	80	20	36.1–37.0	
RVC	5	13	37.1–40.0	10
	10	20	35.7–38.1	
	20	20	35.2–36.8	
	40	20	34.2–35.3	

Sample pooling

The VetMAX Rotavirus Primer/Probe Mix was used on sample pools consisting of one rotavirus-positive porcine sample mixed with 4 negative porcine samples in equal volumes prior to nucleic acid extraction. Each rotavirus-positive sample was tested with and without pooling (Figure 2). The results show amplification of rotavirus-positive samples with and without pooling; however, the pooled sample give a higher C_q value due to dilution. A rotavirus-positive sample in a pool of 5 shows an average C_q increase of 3.0 compared to the same sample run individually.

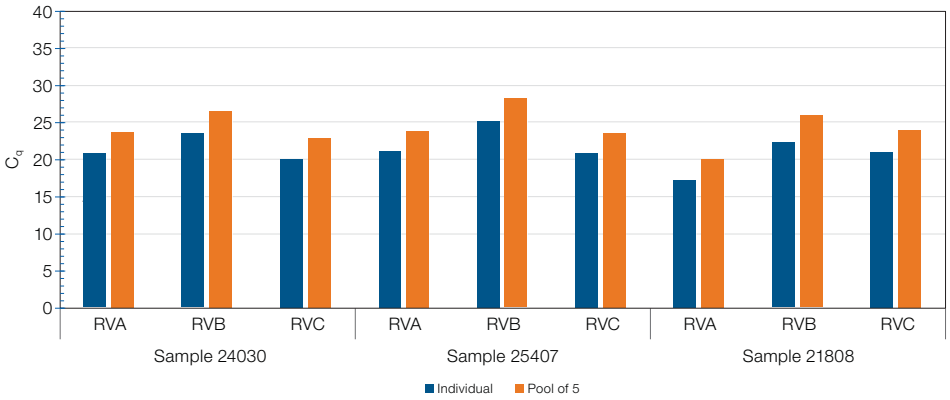

Figure 2. Amplification of RVA, RVB, RVC, and the IPC in a single reaction mix.

Discussion

Designing molecular workflows for rotaviruses is challenging due to high strain diversity and lack of conserved genomic targets. Furthermore, the GenBank database contains rotavirus sequences that are often poorly annotated, and the number of sequences differs greatly among gene segments. The VetMAX Rotavirus Primer/Probe Mix was developed from a database of rotavirus sequences obtained from collaborations and from whole-genome sequencing done within Thermo Fisher Scientific as part of this project, in addition to porcine rotavirus sequences from the GenBank database. Amplification of RVA and RVB was increased by combining two independent designs for these two species. Amplification of all three rotavirus species was further expanded by addition of degenerate primers and probes to compensate for the high degree of genetic variability associated with rotaviruses. The VetMAX Rotavirus Primer/Probe Mix incorporates a complex RT-qPCR design based on the number of primers and probes needed for optimal coverage, but the complexity of the primer/probe mix showed no negative impact on analytical specificity, level of detection, or ease of use in these studies.

Authors

Robert Tebbs, Aaron Balogh, Bob Qiu, Pius Broska, Kellsie Kice, Denise Meza, Gail Ulloa, Rick Conrad, and Yasmine Zein-Phillipson, Thermo Fisher Scientific.

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