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Development of a Real-time PCR test for porcine group A rotavirus diagnosis[1]

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ABSTRACT.- Marconi E.C.M., Bernardes N.T.C.G., Beserra L.A.R., Silva F.D.F. & Gregori F. 2015. **Development of a Real-time PCR test for porcine group A rotavirus diagnosis.** *Pesquisa Veterinária Brasileira 35(1):39-43*. Departamento de Medicina Veterinária Preventiva e Saúde Animal, Universidade de São Paulo, Av. Professor Dr. Orlando Marques de Paiva 87, São Paulo, SP 05508-270, Brazil. E-mail: fabiogregori@gmail.com

Group A Rotavirus (RVA) is one of the most common causes of diarrhea in humans and several animal species. A SYBR-Green Real-Time polymerase chain reaction (PCR) was developed to diagnose RVA from porcine fecal samples, targeting amplification of a 137-bp fragment of nonstructural protein 5 (NSP5) gene using mRNA of bovine NADH-desidrogenase-5 as exogenous internal control. Sixty-five samples were tested (25 tested positive for conventional PCR and genetic sequencing). The overall agreement (kappa) was 0.843, indicating 'very good' concordance between tests, presenting 100% of relative sensitivity (25+ Real Time PCR/25+ Conventional PCR) and 87.5% of relative sensitivity (35- Real Time PCR/40- Conventional PCR). The results also demonstrated high intra- and inter-assay reproducibility (coefficient of variation ≤1.42%); thus, this method proved to be a fast and sensitive approach for the diagnosis of RVA in pigs.

INDEX TERMS: Rotavirus, diagnosis, PCR, Real-Time PCR, NSP5, swine.

RESUMO.- [**Desenvolvimento de um teste de PCR em Tempo Real para o diagnóstico de rotavírus suínos do Grupo A.**] Rotavírus do grupo A (RVA) é uma das causas mais frequentes de diarreias em humanos e várias espécies animais. Um teste de PCR em Tempo Real com SYBR-Green foi desenvolvido visando o diagnóstico de RVA a partir de fezes suínas, através da amplificação de um fragmento de 137 pares de bases do gene da proteína não estrutural 5 (NSP5) viral e de mRNA de NADH-desidrogenase-5 bovina como controle interno exógeno. Foram testadas 65 amostras (25 delas positivas por PCR convencional e sequenciamento nucleotídico). A concordância entre os testes foi de 0,843, considerada "muito boa", apresentando 100% de sensibilidade relativa (25+ PCR Tempo Real/25+ PCR convencional) e 87,5% de sensibilidade relativa (35- PCR Tempo Real/40- PCR convencional). Os resultados também demonstraram elevada reprodutibilidade inter e intra-ensaio (coeficiente de variação ≤ 1,42%); portanto, este método demonstrou ser uma rápida e sensível alternativa para o diagnóstico de RVA em suínos.

TERMOS DE INDEXAÇÃO: Rotavírus, diagnóstico, PCR, PCR em Tempo Real, NSP5, suínos.

INTRODUCTION

Group A rotavirus (RVA) is one of the main causative agents of diarrhea in a variety of animal species, as reported in several countries (Estes & Kapikian 2007). RVA infection is common in swine farms, and it results in decreased growth performance, increased mortality and economic loss (Papp et al. 2013).

RVA belongs to Family *Reoviridae*, Subfamily *Sedoreovirinae*, Genus *Rotavirus*, and its genome consists of 11 double-stranded RNA (dsRNA) segments, coding for 6 structural (VP1, VP2, VP3, VP4, VP6, VP7) and 6 non-structural proteins (NSP1 through 6) (King et al. 2012).

Diagnosis of RVA infection plays a central role in establishing measures for control and prevention. The methods currently available, the polyacrylamide gel electrophoresis (PAGE) technique (Herring et al. 1982) allows the detection of all rotavirus strains regardless of the group to which

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they belong; however, it is time consuming and inefficient to process a large number of clinical samples. The enzyme--linked immunosorbent assay (ELISA) overcomes these difficulties; however, it is capable of detecting only a restricted number of viral groups and presents limitations of availability and quality of antibodies required (Jerez 1997, Zhu et al. 2013). Conventional polymerase chain reaction (PCR) is advantageous as a rapid procedure, described in routine practice by several authors (Dhama et al. 2009), while Real-time PCR provides higher sensitivity, throughput, and may reduce cross-contamination, since there is no need of post-PCR manipulations (Pestana et al. 2010, Mijatovic-Rustempasic et al. 2013).

RVA have undergone various changes during evolution, including point mutations, reassortment, rearrangement, and intragenic recombination (Ghosh & Kobayashi 2011), which has resulted in a large variability in genotypes (Matthijnssens et al. 2011) as well as intra-genotypic variations (Gregori et al. 2012, Amimo et al. 2013). Therefore, molecular diagnosis should take into consideration the fact that the primer hybridization sites are not always conserved, especially among viral strains that infect different host species, resulting in a greater likelihood of false-negative results, and requiring periodic review of these primers.

Despite their importance, diagnostic methods, such as PCR, which take genetic diversity into consideration, in particular, the diversity presented by porcine RVA, are scarce. This study describes the development of a SYBR-Green Real-time PCR assay for rapid and sensitive detection of porcine RVA in fecal material.

MATERIALS AND METHODS

RNA extraction and reverse transcription. Fecal suspensions (50% w/v) were prepared in ultrapure water previously treated with 0.1% diethyl pyrocarbonate (DEPC) and clarified by centrifugation at 12,000 g for 15min at 4°C. The supernatant from fecal suspensions and controls were subjected to RNA extraction with TRIzol reagent (Invitrogen Carlsbad, CA, USA) according to the manufacturer's instructions. At the end of procedure, RNA was solubilized in 15µL of ultrapure water pre-treated with 0.1% DEPC.

The reverse transcription (RT) reaction was performed according to the following protocol: The RNA sample (7µL) was denatured at 95°C for 5 min and immediately transferred into an ice bath for 10min. Then, denatured RNA was added to a RT-mix solution containing 1× First Strand Buffer, 1mM of each dNTP, 10mM of DTT, 50ng of random primers (Invitrogen, Carlsbad, CA, USA), 1µL of ultrapure water, and 200U of Superscript Reverse Transcriptase III (Invitrogen Carlsbad, CA, USA) in a final reaction volume of 20µL. This was incubated at 37°C for 60min and then at 70°C for 15min.

Primer design. A total of 34 RVA sequences encoding the non-structural protein NSP5, available in the GenBank database, were aligned using the ClustalW 2.1 software (Larkin et al. 2007). Primers were selected visually from conserved regions of the alignment, being designated NSP5FW1 (5' GGCTTTTAAAGCGCTACAG 3') and NSP5RV137 (5' TGTTCACTCCTACCAATAGATTT 3'), generating a fragment of 137-bp (base pairs). These sequences were submitted to BLAST/n to verify its analytical specificity, whereas the formation of dimers, hairpins, and melting temperature (T_m) were assessed with the OligoAnalyzer 3.1 software (www.idtdna.com).

Production of plasmids. To establish the Real-time PCR standard curve, a 137-bp PCR product of the gene encoding NSP5 of porcine RVA was used, with a previously known nucleotide sequence, cloned into pTZ57R/T vector (Fermentas, Waltham, MA, USA), according to the manufacturer's protocol. The resulting plasmid was transformed into *Escherichia coli* DH5α cells and then purified using the NucleoSpin Plasmid Prep kit (Macherey--Nagel, Düren, Germany). The cloned DNA fragments were linearized by digestion with the enzyme *Eco*RI (BioLabs Ipswich, MA, USA) following manufacturer's instructions, quantified by NanoDrop 2000 (Thermo Scientific, Waltham, MA, USA) spectrophotometer, and stored at -80°C until use.

Real-Time PCR. Assays were performed using Maxima SYBR Green qPCR/ROX Master Mix (Fermentas, Waltham, MA, USA) in a StepOne Real-time PCR System (Applied Biosystems, Waltham, MA, USA). The procedures were optimized for primer concentration and annealing temperature, using RVA 32/00 as reference strain (Rodriguez et al. 2004) and seven swine feces samples serially screened as positive by PAGE (Herring et al. 1982) and ELISA (Gregori et al. 2000).

SYBR Green Real-Time PCR reaction consisted of 12.5µL of Maxima SYBR Green/ROX qPCR Master Mix (2×), 0.3µM of each primer (NSP5FW1/NSP5RV137), 2µL of cDNA, and up to 25µL of ultrapure water. The conditions were as follows: 95°C for 10 min; 40 cycles at 95°C for 1min, 54°C for 1min, and 72°C for 1min, followed by a standard melting curve (95°C for 15 s, 60°C for 1 min, and 95°C for 15 s with a reading at every 0.3°C) to assess the specificity of PCR according to the T_m profile of the reference strain.

A total of 65 porcine fecal samples from piglets aged less than 40 days old were tested using the Real-time PCR assay, 25 of which were previously positive by conventional PCR using primers described by Salem et al. (2010) and confirmed by the respective genetic sequencing of the NSP5 gene. Ultrapure water without template strand was used as negative control (NTC).

The exogenous internal control consisted of MDBK cells maintained in 25-cm^2 flasks with MEM medium supplemented by 5% fetal bovine serum. Cells were removed by scraping the monolayer, centrifuged at 5,000 g for 10min, reducing their volume to 500µL.

Ten-µL MDBK cell precipitate were added to fecal suspension in the samples that were negative for rotavirus by Real-time PCR, and RNA extraction was performed as described previously. The samples were then tested by amplification of the messenger RNA (mRNA) of the gene encoding the mitochondrial bovine NADH-dehydrogenase-5 protein using a pair of primers (BOV-F and BOV--R), described by Caldwell, Raley & Levine (2007), which generates a product of 191-bp. The following amplification conditions were used: 95°C for 10 min; 40 cycles of 94°C for 1 min, 55°C for 2min, and 72°C for 1 min, followed by a standard melting curve of the product (95°C for 15s, 60°C for 1 min, and 95°C for 15s with a reading at every 0.3°C). Negative results from this test were interpreted as inconclusive detection of RVA, presumably due to presence of PCR inhibitors.

Agreement was assessed using the Kappa test adopting a confidence interval of 95% along with the calculation of relative diagnostic sensitivity and specificity, by comparing the results of conventional PCR and Real-Time PCR for RVA (Thrusfield 2007).

Definition of analytical sensitivity and specificity. In order to determine the analytical sensitivity of the Real-time PCR method, a standard linearized plasmid was used as a template and serially diluted 10-fold using ultrapure water previously treated with 0.1% DEPC, resulting in concentrations ranging from 1.04×10^1 to 1.04×10^5 DNA copies/µL in triplicate. The correlation coe-

fficient (r^2) and the reaction efficiency (E) were calculated using the formula $E = 10^{(-1 / slope)} - 1$ (Dorak 2006).

Six fecal samples classified as positive for rotavirus by Real-time PCR were selected along with the reference strain RVA 32/00 to check the analytical specificity for rotavirus group A. For this purpose, the products of reverse transcription of these samples were subjected to a standard PCR reaction, using the same primers used in Real-time PCR, which in brief included 2.5μL of cDNA added to the PCR-mix, consisting of 1× PCR Buffer (Invitrogen Carlsbad, CA, USA), 0.2mM of each dNTP, 0.5μM of each primer (NSP5FW1 and NSP5RV137), 2mM of $MgCl_2$, 1.25 U of Taq DNA Polymerase (Invitrogen Carlsbad, CA, USA), and water in a total of 25μL. The products were then amplified using the following conditions: 95°C for 2min; 35 cycles of 95°C for 1min, 54°C for 1min, 72°C for 1min; 72°C for 10 min, and storage at 10°C.

The 137-bp bands were purified directly from agarose gels using the Illustra GFX PCR DNA and Gel Band Purification Kit (GE Healthcare, Little Chalfont, Buckinghamshire, UK), added to bidirectional sequencing reactions using BigDye Reagent 3.1 (Applied Biosystems, Waltham, MA, USA), and analyzed in an ABI-3500 (Applied Biosystems, Waltham, MA, USA) genetic analyzer, in accordance with the manufacturer's protocol. The nucleotide sequences were submitted to BLAST/n to compare its identity with sequences from rotavirus group A.

Finally, the bovine coronavirus BCoV Kakegawa reference strain (Akashi et al. 1980) was subjected to Real-time PCR to identify possible cross amplification.

Assessment of reproducibility. The linearized standard plasmid was diluted in concentrations ranging from 1.04×10^1 to 1.04×10^5 DNA copies/μL and subjected to Real-Time PCR. Each dilution was repeated 3 times (intra-assay variation) and processed on 3 different days (inter-assay variation) to define the coefficients of variation (CV) and average values of C_t (cycle threshold).

RESULTS

Primer design

The T_m values of the designed primers were similar (51.8°C for NSP5FW1 and 51.4°C for NSP5RV137), presenting the most stable heterodimer with a ΔG value of -3.89 kcal × $mole^{-1}$. In silico primer analysis demonstrated that NSP5FW1 and NSP5RV137 had E values of 0.41 and 0.003 on BLAST/n, respectively, as well as, each one had 100% identity to at least 250 target sequences of rotavirus. The most stable homodimer had ΔG values of -13.09 kcal × $mole^{-1}$ and -3.42 kcal × $mole^{-1}$; and the hairpin had values of -1.72 kcal × $mole^{-1}$ and -3.42 kcal × $mole^{-1}$ for the primers NSP5FW1 and NSP5RV, respectively.

Detection in clinical samples and correlation with conventional PCR

Swine fecal samples were tested using Real-Time and conventional PCR tests, results of which are shown in Table 1. The positive samples showed a C_t between 19.53 and 39.43, with greater frequency (81.25%) between the range of 25.59 and 36.58.

The melting curve profile was similar in the positive and standard samples (average T_m =75.12±0.16°C). In the negative samples, fragments the coding gene of the mitochondrial bovine NADH-dehydrogenase-5 protein were subsequently detected, validating the extraction and amplification procedures, and their C_t values ranged from 22.07 and 27.11, with an average T_m of 78.76±0.28°C. With respect to the no template control (NTC), all replicates were negative as expected.

The relative diagnostic sensitivity between the tests was 100% (CI95% 0.863-1), and the relative diagnostic specificity was 87.5% (CI95% 0,732-0,958). The agreement can be considered 'very good', with a Kappa value of 0.843 (CI95% 0.713 - 0.974).

Analytical sensitivity and specificity

Adequate linear relationship was observed between concentrations ranging from 1.04×10^1 to 1.04×10^5 DNA copies/μL of the linearized plasmid with a correlation coefficient (r^2) of 0.998 and efficiency of 102.21%. Quantitative data showed that the detection limit of Real-Time PCR was 1.04×10^1 copies/μL.

The sequencing of seven PCR products, generated using primers NSP5FW1 and NSP5RV137 (six fecal samples and standard sample 32/00), showed high identity (98%–100%) with different strains of RVA, including KC117151 of porcine origin. This confirmed the analytical specificity of the fragment generated.

The bovine BCoV Kakegawa coronaviruse showed no sign of cross-amplification using Real-Time PCR.

Reproducibility

The reproducibility of the assay, using triplicates of each dilution of linearized standard plasmid for reactions of Real-Time PCR, are presented in Table 2. The coefficients of intra- and inter-assay variations were relatively small (< 1.42%), ranging between 0.27% to 0.87% and 0.53% to 1.42%, respectively.

DISCUSSION

The gene encoding NSP5, located in segment 11, is 667 bp in length (referring to the RV SA11 sample) and this protein interacts with other proteins and viral RNA (Contin et al.

Table 1. Results of Real-time (qPCR) and conventional PCR used for diagnosis of porcine RVA detection

		Conventional PCR		
		Positive	Negative	Total
qPCR	Positive	25	5	30
	Negative	0	35	35
	Total	25	40	65
Sensitivity		100% (CI95% 0.863-1)		
Specificity		87.5% (CI95% 0,732-0,958)		
Kappa value		0.843 (CI95% 0.713-0.974)		

Table 2. Reproducibility of Real-time PCR assay used for detection of a fragment of the NSP5 gene of porcine RVA

Concentration of the standard plasmid (DNA copies /μL)	n	Intra-assay			Inter-assay		
		Average Ct	SD	%CV	Average Ct	SD	%CV
1.04×105	3	18.28	0.16	0.87	18.39	0.09	0.53
1.04×104	3	21.83	0.06	0.27	21.42	0.30	1.42
1.04×103	3	25.25	0.18	0.71	25.52	0.19	0.76
1.04×102	3	28.35	0.14	0.49	28.40	0.24	0.85
1.04×101	3	31.37	0.19	0.60	31.49	0.14	0.46

2010, King et al. 2012). Its main function is to participate in the formation of viroplasms, which are cytoplasmic structures where genome replication and morphogenesis of new particles occurs (Criglar et al. 2014). Among porcine strains, previous data showed low rates of genetic variability NSP5/6, because their structural and functional constraints and lower exposure to immune selection (Barbosa et al. 2013), which is a desirable property when selecting targets for nucleic acid detection methods.

The primers designed for the coding segment of porcine group A rotavirus NSP5 protein showed consensus areas with the 34 porcine sequences retrieved from GenBank, while the BLAST/n query resulted in high nucleotide identity of the rotavirus strains. There was an effective amplification of target product during the assay, and despite the presence of homo-dimers and hairpins, these presented high ΔG values (Santalucia 2007), demonstrating the overall suitability of the primer design.

We opted for the reverse transcription step with random primers to allow for screening of other potential viral agents from cDNA, including coronaviruses and even of other rotavirus genes, giving greater versatility to the test. However, differences between the annealing temperatures of the primers prevented the detection of the target gene and the exogenous internal control in a single temperature cycle. Nevertheless, we included the mitochondrial mRNA encoding bovine NADH-dehydrogenase-5 (Caldwell et al. 2007) based on a favorable report by Asano et al. (2010), who used this control to validate a PCR assay for the VP1 gene of RV, in agreement with our finding of accurate detection in Real-Time PCR, where C_t values between 22.07 and 27.11 and low variability in T_m (78.76±0.28°C) was obtained.

Comparing results from conventional PCR with Real-Time PCR, 100% relative diagnostic sensitivity between tests and relative diagnostic specificity of 87.5% was obtained, as presented in Table 1. The agreement value (84.3%) showed a disparity in 5 samples between the results from conventional and Real-Time PCR techniques.

A possible explanation is an eventual 'false-positive' result generated by Real-time PCR. Meanwhile, these 5 samples presented a melting curve with the same profile shown by other samples and standard RVA (average T_m = 75.12±0.16°C), with C_t between 31.62 and 39.43. Additionally, genetic sequencing of the generated fragments confirmed that all had identity with rotavirus, implying that differences in detection thresholds of the tests were observed, thus increasing the diagnostic sensitivity of SYBR-Green Real-time PCR.

This was further corroborated by sequencing results from another field strain, positive in both tests, as well as the reference strain, which generated nucleotide sequences corresponding to RVA. Besides that, the NTC did not generate any signal as well as no cross-amplification of coronavirus was detected.

Salem et al. (2010) defined a detection limit of RVA detection primers after the second round PCR as being 10^1 $TCID_{50}$/mL, but in a multiplex system, also including primers for porcine coronavirus (PEDV and TGEV), and due to modifications adopted in the current study (different RNA extraction and PCR mastermixes, primer binding areas, and monoplex system) there are limitations when comparing these performance results.

Similarly, Pang et al. (2004) reported that Real-Time PCR targeting the RVA NSP3 gene, using TaqMan system, was 1,000-fold more sensitive than conventional RT-PCR.

The analytical sensitivity of Real-time PCR was determined by a standard curve of serial dilutions in base 10, which lower detection value was 1.04×10^1 copies/μL, with a correlation coefficient (r^2) of 0.998 and efficiency of 102.21%, which are values within the expected range for this type of test. Similarly, the values of inter- and intra-assay reproducibility exhibited low coefficients of variation, below 3% (Sariya et al. 2012). These results are similar to those reported by Ye et al. (2012), who develop a Real-Time PCR reaction for the NSP5 RVA gene and achieved an efficiency of 106% and r^2 = 0.997, with a detection limit between 10^8 and 10^1 copies/μL.

Considering the advantages of SYBR Green Real-Time PCR, which include a lower chance of cross contamination due to a closed-tube system, quicker sample processing, and higher diagnostic sensitivity as compared to conventional PCR, the system developed in this study is an alternative for the diagnosis of RVA infection prevalent in porcine farms. This method has the potential to assist in the control and prevention of the disease.

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