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Development of primers for sequencing the *NSP1*, *NSP3*, and *VP6* genes of the group A porcine rotavirus

Desenvolvimento de oligonucleotídeos iniciadores para sequenciamento dos genes codificadores das proteínas NSP1, NSP3 e VP6 de rotavírus suíno do grupo A

Fernanda Dornelas Florentino Silva[1*]; Paloma de Oliveira Tonietti[1]; Luis Ramiro Luna Espinoza[1]; Paulo Eduardo Brandão[2]; Leonardo José Richtzenhain[2]; Fabio Gregori[2]

Abstract

Rotavirus is the causative pathogen of diarrhea in humans and in several animal species. Eight pairs of primers were developed and used for Sanger sequencing of the coding region of the *NSP1*, *NSP3*, and *VP6* genes based on the conserved regions of the genome of the group A porcine rotavirus. Three samples previously screened as positive for group A rotaviruses were subjected to gene amplification and sequencing to characterize the pathogen. The information generated from this study is crucial for the understanding of the epidemiology of the disease.
Key words: Diarrhea, PCR, group A rotaviruses, sequencing, swine

Resumo

Rotavírus é responsável pela ocorrência de diarreia em humanos e em várias espécies animais. Oito pares de oligonucleotídeos iniciadores (*primers*) foram desenvolvidos e utilizados como estratégia para o sequenciamento do tipo Sanger da região codificadora dos genes NSP1, NSP3 e VP6 com base nas áreas conservadas do genoma dos rotavírus do grupo A de suínos. Um total de 3 amostras previamente triadas como positivas para rotavírus do grupo A, tiveram os respectivos genes amplificados e sequenciados por estes oligonucleotídeos iniciadores, possibilitando a caracterização das amostras circulantes, cujo conhecimento é essencial para a compreensão da epidemiologia da doença.
Palavras-chave: Diarreia, PCR, rotavírus grupo A, sequenciamento, suíno

[1] Pesquisadores, Pós-Graduação em Epidemiologia Experimental Aplicada às Zoonoses, Universidade de São Paulo, USP, SP, Brasil. E-mail: ferrota42@gmail.com; palomatonietti@gmail.com; luis_aries18@hotmail.com
[2] Profs. da Faculdade de Medicina Veterinária e Zootecnia, FMVZ/USP, São Paulo, SP, Brasil. E-mail: paulo7926@usp.br; leonardo@usp.br; fabiogregori@gmail.com
[*] Author for correspondence

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Rotavirus is the major causative agent of viral diarrhea in humans and animals and has an impact on public and animal health, especially in swine (MARTELLA et al., 2010). Its genome is composed of 11 double-stranded RNA segments surrounded by an icosahedral triple-layered capsid. Its genome encodes six structural viral proteins (VP1–4, VP6, VP7) and six nonstructural proteins (NSP1–NSP6) (ESTES; KAPIKIAN, 2007). The genotyping of the virus samples targets mainly the genes encoding the VP4 and VP7 proteins because these are responsible for producing neutralizing antibodies and are therefore important for vaccine development (DENNEHY, 2008). However, knowledge of the other genes enables a better understanding of the pathogenesis and epidemiology of the disease (MATTHIJNSSENS et al., 2008). These include the *NSP1* genes, whose coding region is located between nucleotides 32 and 1,492 (segment 5), *NSP3*, between nucleotides 26 and 967 (segment 7), and *VP6*, between nucleotides 24 and 1,217 (segment 6), with reference to sample PRG942 of the porcine rotavirus (Accession numbers: JF796718, JF796720, and JF796727).

The NSP1 and NSP3 proteins act by increasing the translation of viral mRNA, thus favoring the replication and production of virus progeny (CHUNG; MCCRAE, 2011). The VP6 protein, in turn, is the main structural component of the rotavirus and is highly immunogenic and antigenic (KAPIKIAN; HOSHINO; CHANOCK, 2001).

Considering the variability of rotaviruses that infect different animal species (GREGORI; BRANDÃO; JEREZ, 2012; PARRA et al., 2004; TANIGUCHI; URASAWA, 1995), eight pairs of primers were developed to amplify and sequence the complete coding regions of proteins NSP1, NSP3, and VP6 of the group A porcine rotavirus. Conserved areas of the genes were selected after alignment with 20 nucleotide sequences of group A rotavirus of porcine origin from Brazil and other countries that were available in GenBank by using the ClustalW 2.1 software (LARKIN et al., 2007). These regions were used to design the primers described in Table 1. With respect to *NSP1*, three different regions were targeted for amplification: nucleotides 18–817, 531–916, and 798–1,545, with comparison to the reference sample PRG942 (Accession number: JF796718). For *NSP3*, two regions were amplified, 25–654 and 374–1,032, with reference to sample PRG942 (Accession number: JF796720). Finally, for the amplification of *VP6*, we used a combination of the primers originally described by Shen, Burke and Desselberger (1994), which flank a 1,356-base pair (bp) region with two other primers internal to them (579–598 and 598–579), generating two fragments, one 598-bp long and the other, 778-bp long. Another pair of primers was designed targeting the segment 348–808 within the coding region, which results in an overlapping area, thus allowing the assembly of the final sequence (contig). The reference sample for *VP6* was PRG942 (accession number JF796727).

Each primer was submitted to BLAST/n to assess its similarity to sequences of the porcine rotavirus and possible non-specific amplifications. By employing the Oligo Analyzer program (© 2013 Integrated DNA Technologies, Inc.), no formation of dimers and hairpins was observed with $\Delta G < -10$ kcal/mol among the pairs of primers used in each of the reactions. To enhance PCR for the three different genes simultaneously, all primers were tested under a temperature gradient ranging from 48°C to 58°C, and the mean melting temperature (T*m*) for all primers was set at T*m* 50°C.

The fragments of the coding regions of *NSP1*, *NSP3*, and *VP6* were amplified by different PCRs, in which 2.5 µL of cDNA, previously extracted using TRIzol reagent (Invitrogen®) according to the manufacturer's protocol, was added to the PCR mix containing 1× PCR Buffer (Invitrogen®), 0.2 mM of each dNTP, 0.5 µM primers (sense and antisense according to Table 1), 1.5 mM $MgCl_2$, 1.5 U of Platinum Taq DNA Polymerase (Invitrogen®), and 25 µL water q.s.p. Porcine rotavirus sample 32/00p (RODRIGUEZ et al., 2004) was used as positive

control and DEPC-treated water was used as negative control. The amplification conditions were as follows: 95°C/3 min, 40 cycles of 95°C/1 min; 50°C/1.5 min; 72°C/1 min, followed by a final extension of 72°C/10 min. The PCR products were then purified using the EXOSAP-it (USB®) reagent, and Sanger-type bidirectional sequencing was performed using a Big Dye reagent 3.1 (Applied Biosystems®) and an ABI 3500 sequencer (Applied Biosystems®), according to the manufacturer's instructions. Because the primers defined in this study have overlapping regions, the final complete sequences encompassing the entire coding region of the three different genes were established using the Cap-Contig software available in the BioEdit software v. 5.1.3.0 (HALL, 1999). The nucleotide sequences were defined from all amplified fragments and submitted to Genbank as follows: *NSP1* (Accession numbers KC855054, KC855055, and KC855056), *NSP3* (Accession numbers KC855057, KC855058, and KC855059), and *VP6* (KC855060, KC855061, and KC855062); these fragments were highly homologous to the group A porcine rotavirus samples. Although the primers described in this study reflect various swine samples in different locations, a periodic review of new sequences submitted to Genbank should be performed because of the nature of this rapidly evolving rotavirus. This approach used a hybridization temperature common to all three genes studied, which renders the multigenic analysis approach practical. These primers may be potentially used for diagnostic purposes; however, determination of the detection threshold and the adoption of an endogenous internal control may be required. Considering the large number of genotypes described in the literature (MATTHIJNSSENS et al., 2011), gene sequencing techniques allow the characterization of samples, which is essential for the elucidation of the epidemiology of the disease.

Table 1. Primer sequences used for amplification and sequencing of the genes *NSP1*, *NSP3*, and *VP6* of the group A porcine rotavirus, according to the fragment generated and the position on the fragment.

Gene	Primer	Sequence (5′-3′)	Fragment (bp)	Position
NSP1	NSP1S (sense)	GTCTTGTGKRAGCCATGG	800[a]	18–35
	NSP1AS (antisense)	AAGTTTACATGCTYCATTCC		798–817
	NSP1FW531 (sense)	TYAATCAGACTCCATTTTCATT	386[b]	531–552
	NSP1RW893 (antisense)	TAGTTTATRCTGCGTAAACATTCT		893–916
	NSP1S798-817 (sense)	GGAATGRAGCATGTAAACTT	748[c]	798–817
	NSP1AScomp (antisense)	GGCGCTACTCTAGTGCAG		1528–1545
NSP3	NSP3S (sense)	GATGCTCAAGATGGAGTCTAC	630[a]	25–45
	NSP3AS641 (antisense)	TGAAGAGAGTACATATTTTCAAT		632–654
	NSP3S374-396 (sense)	ATGAGAGTACTTAATGCWTGTTT	658[b]	374–396
	NSP3AS (antisense)	AGCTTTTAACTATTGTGCTCA		1012–1032
VP6	VP6F* (sense)	GGCTTTTAAACGAAGTCTTC	598[a]	1–20
	VP6AS579-598 (antisense)	CCAGCTACYTGAATTTCTGA		579–598
	VP6EMS	ATAGCACCACAATCTGAAGC	461[b]	348–579
	VP6EMAS	TCAACCTCTACATTACTTGGTCTTA		784–808
	VP6S579-598 (sense)	TCAGAAATTCARGTAGCTGG	778[c]	579–598
	VP6R*	GGTCACATCCTCTCACTA		1339–1356

*Described by Shen, Burke and Desselberger (1994)
[a] Initial portion of the fragment
[b] Middle portion of the fragment
[c] Final portion of the fragment.
Source: Elaboration of the authors.

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