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Primer design for complete sequencing of the *Avastrovirus* ORF2 gene

Desenho de *primers* para o sequenciamento completo do gene ORF2 em *Avastrovirus*

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Abstract

Avastrovirus infection is associated with enteric disease, nephritis, and hepatitis in birds. In this study, we present a protocol for the complete sequencing of the ORF2 gene of avian nephritis virus (ANV), chicken astrovirus (CAstV), and turkey astrovirus type 1 (TAsTV-1) using a conventional Sanger technique. Previously and newly designed primer pairs targeting both the conserved flanking and internal regions of the ORF2 gene of these three viruses were used. The information derived from the astroviral sequences obtained in this study is fundamental for characterizing this virus and providing data regarding several aspects of disease epidemiology and prevention.

Key words: PCR. Sequencing. *Avastrovirus*. Avian. ORF2.

Resumo

As infecções por avastrovírus estão associados à doença entérica, nefrite e hepatite em aves. Aqui, nos apresentamos um protocolo planejado para o sequenciamento completo do gene ORF2 em *Avian Nephritis Virus* (ANV), *Chicken Astrovirus* (CAstV) e *Turkey Astrovirus tipo 1* (TAsTV-1), usando a técnica de sequenciamento convencional de Sanger. Foram usados *primers* previamente descritos e desenhados neste estudo, tendo como alvo as regiões conservadas flangeadoras e internas dentro do gene ORF2 nos três vírus. O conhecimento destas sequências é um elemento chave para caracterizar o vírus e prover de dados em diversos aspectos da epidemiologia e prevenção da doença.

Palavras-chave: PCR. Sequenciamento. Avastrovírus. Aves. ORF2.

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Avastroviruses have been associated with enteric disease in avian species (DE BENEDICTIS et al., 2013). In addition to diarrhea, infection has been linked to kidney lesions and nephritis in chickens, fatal hepatitis in ducks, and poultry enteritis mortality syndrome (PEMS) in turkeys, chickens, and guinea fowl (PANTIN-JACKWOOD et al., 2011). The virus has been isolated worldwide, infecting different avian species (KOCI; SCHULTZ-CHERRY, 2002). In Brazil, avastroviruses have been shown to infect chickens with clinical symptoms of enteric diseases (METTIFOGO et al., 2014). *Avastrovirus* are non-enveloped, positive-sense, single-stranded RNA viruses, classified as a genus within the *Astroviridae* family (MACLACHLAN; DUBOVI, 2011; GUIX et al., 2013).

According to the International Committee on Taxonomy of Viruses (ICTV, 2014), the genus *Avastrovirus* can be divided into three species: *Avastrovirus* 1 (turkey astrovirus type 1, TAsV-1), *Avastrovirus* 2 (avian nephritis virus, ANV), and *Avastrovirus* 3 (duck astrovirus, DAsV; and turkey astrovirus type 2, TAsV-2). However, Chicken astrovirus (CAstV), a common enteric virus of broiler flocks (TODD et al., 2009), has yet to be regarded as a species within the ICTV classification.

More recently, an additional sub-division into genotypes has been suggested because of differences in the virus capsid amino acid sequence (BOSCH et al., 2011, GUIX et al., 2013). Thus far, seven genotypes of *Avastrovirus* have been recognized using the mean amino acid genetic distance (p-dist) calculated based on the viral capsid sequence as the cut-off; the range between and within genotypes is 0.414–0.728 and 0.047–0.299, respectively (BOSCH et al., 2011, GUIX et al., 2013). Based on this, ANV from chicken isolates belong to genotype 2 and 5, CAstV to genotype 4, and TAsV-1 (one cases of TAsV-1 infection in chickens has been reported) to genotype 1 (GUIX et al., 2013).

The ORF2 gene encodes a precursor protein, comprising of 668–742 amino acids (2007–2229 nt)

and having a size of 73–81 kDa, known as the capsid protein (PANTIN-JACKWOOD et al., 2013). This protein is responsible for attachment and entry into host cells (BASS; UPADHAYAYULA, 1997), and it is a multi-domain protein containing a conserved N-terminal region (comprised of the basic S and P1 domains), a highly variable C-terminal region (spike or P2 domain), and acidic domains (KRISHNA, 2005), which are removed by host cell caspases (MENDEZ et al., 2004). Changes in receptor binding can play an important role in cross species transmission (DUBOIS et al., 2013).

Although ORF2 sequencing is a key element in the classification of *Avastrovirus* (BOSCH et al., 2011), PCR primers for complete gene amplification and sequence information are limited. In this study, we present a protocol for complete Sanger sequencing of the ORF2 gene of ANV, CAstV, and TAsV-1, combining new and previously designed primers.

Avastrovirus positive samples, ANV, CAstV, and TAsV-1 previously characterized by Chu et al. (2008) using ORF1b amplification and sequencing, were used for the complete amplification and sequencing of the ORF2 gene. Briefly, sixty pooled fecal samples (5–6 randomly selected fecal samples/pool; 1 pooled sample/flock) from apparent healthy broiler, layer hens, and breeders were collected from chicken flocks in Brazil and submitted for RNA extraction, using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol, and retro-transcribed with a reverse transcriptase enzyme (SuperScript III, Invitrogen). The ORF1b fragment amplification and sequencing primers and PCR conditions used for *Avastrovirus* species determination were as previously described by Chu et al. (2008).

For complete ORF2 gene amplification (2007–2229 nt), three different primer pairs were selected: ANV PreCAP / ANV PostCAP (TODD et al., 2011), CAstV PreCAP / CAstVPostCAP (SMYTH et al., 2012), and TAsV-1 PreCAP / TAsV-1 PostCAP; the

latter was designed to pair to the flanking nucleotide regions of turkey astrovirus USA Strain (GenBank accession number NC002470; Table 1). The respective PCR mixes were prepared as follows: 1× PCR Buffer (Invitrogen), 0.2 mM of each dNTP, 0.5 µM of each primer (Table 1), 1.5 mM MgCl₂, and 1 U of Platinum Taq DNA Polymerase (Invitrogen), in a 22.5 µL final volume. A total of 2.5 µL of each cDNA (ANV, CAsTV, and TAsTV-1) was added to each tube and submitted to 94°C/2 min followed by 40 cycles of 94°C/15 sec, 57°C/30 sec, 50°C/30 sec, or 60°C/30 sec (ANV, CAsTV, and TAsTV-1, respectively), and 72°C/2.5 min (increasing 5 sec from the cycle 25) and a final extension at 72°C/10 min. The amplicons were analyzed by agarose gel (2%) electrophoresis, stained with SYBR Safe DNA Gel Stain (Invitrogen, Paisley, UK) according to the manufacturer's recommendations, and visualized using UV light. The expected amplicon size is approximately 2,000-2,500 bp for ANV and CAsTV and 2,000 for TAsTV-1.

The complete ORF2 gene PCR products of ANV and CAsTV were gel excised and purified using the GeneJET Gel Extraction Kit (Thermo Scientific, CA, USA) and subsequently cloned using the InsTAclone PCR Cloning Kit (Thermo Scientific). Plasmids containing the respective inserts were sequenced using the Sanger technique in duplicates with the BigDye Terminator v.3.1 Cycle Sequencing Kit (Applied Biosystems, CA, USA), resolved using an ABI 3500 Genetic Analyzer (Applied Biosystems), and the obtained sequences were further edited with BioEdit v. 7.2.5 (HALL et al., 1995).

As the average read length obtained using the Sanger sequencing technique is approximately 800–1,000 nt (SAEED et al., 2009), the complete sequencing of the ANV and CAsTV ORF2 gene required such a design of the internal primers which could help to obtain a set of overlapping products using a primer walking strategy (STUDIER, 1989; MARTIN-GALLARDO et al., 1992) (Table 1, Figure 1). Two internal primers pairs (ANV InterCAPF/ANV InterCAPR and ANV InterCF/ANV InterCR)

were designed for ANV ORF2 sequencing because of the high heterogeneity observed in the obtained sequences (Table 1, Figure 1), while for CAsTV, only one pair was designed (CAsTV InterCAPF/CAsTV InterCAPR). For TAsTV-1, the ORF2 gene could be successfully sequenced with a flanking primer pair designed in this study (the total size of the TAsTV-1 ORF2 gene is approximately 2050 bp). These primers were submitted to BLAST/n analysis to assess their identity and specificity, and the dimer and hairpin formations were evaluated using the Oligo Analyzer 3.1 software tool (IDT, Integrated DNA Technologies, Inc., Coralville, IA, USA). Each of the primers was directly added to the sequencing reaction mix and the previously obtained ORF2 gene plasmid inserts were used as the template. Sequences were edited using BioEdit v. 7.2.5 (HALL et al., 1995) and sequenced fragments of the same gene were assembled using CAP (Contig Assembly Program) (HUANG, 1992).

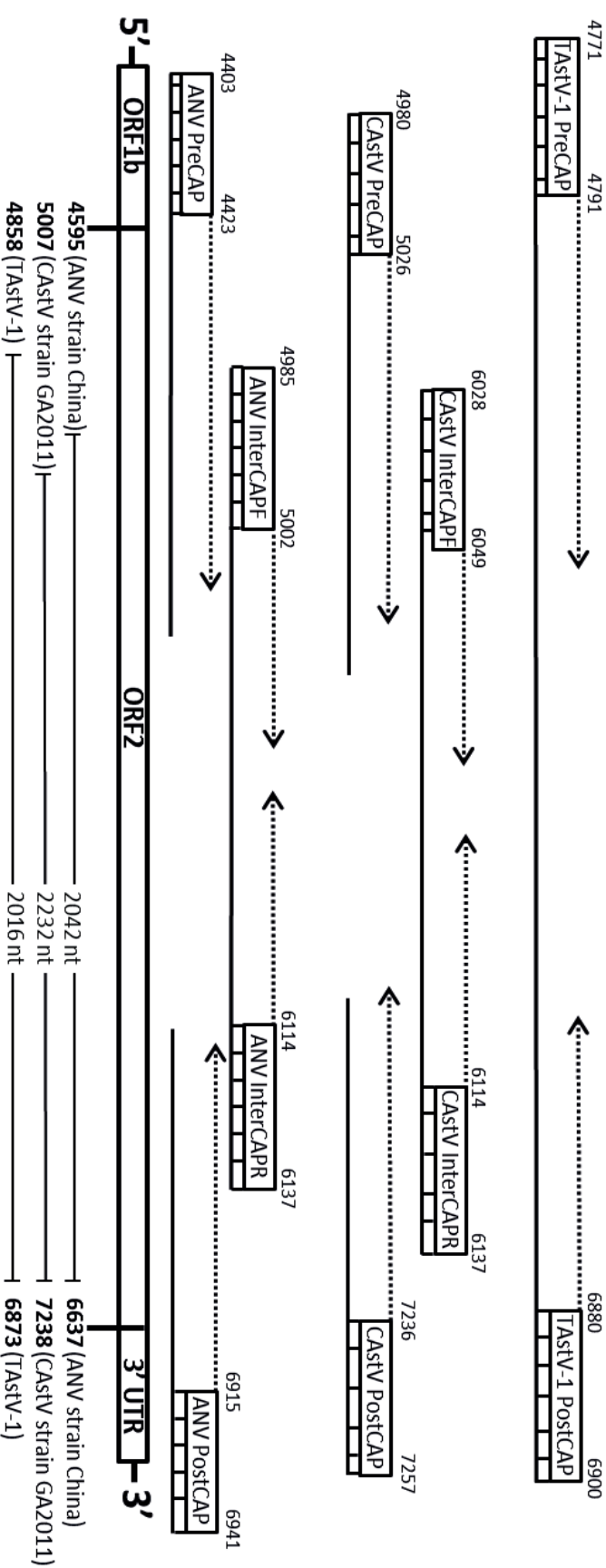
Amplification and sequencing of ORF1b from the 60 chicken fecal samples enabled ANV (21/60), TAsTV-1 (6/60), and CAsTV (2/60) species identification. Complete ORF2 gene amplification was achieved in 8/21 ANV-, 1/6 TAsTV-1-, and 1/2 CAsTV-positive samples. In addition, detection of ANV ORF2 polymorphic genes in the same sample was possible (via gene cloning) and up to four different ORF2 sequences were observed in the ANV-positive samples (except for two samples in which two different sequences were observed). ORF2 complete gene analysis (p-dist) showed that the ANV sequences belonged to genotype 5, previously described in chickens, and confirmed the CAsTV and TAsTV-1 fit with genotype 4 and 1, respectively. The p-dist percent within each genotype detected in this study was 0.027 ± 0.011 , 0.018 ± 0.02 , and 0.043 ± 0.01 for Genotype 5, 4, and 1, respectively (the cut off value within each genotype was 0.047-0.299). The ANV, CAsTV, and TAsTV-1 ORF2 sequences generated in this study were deposited with the GenBank database under accession numbers ANV (KU711050-KU711077), CAsTV (KU711078), and TAsTV-1 (KU711079).

Table 1. Primer sequences used for amplification and sequencing of the ORF2 gene in ANV, TAsV-1, and CasV (according to the fragment generated and the position of the primers).

Species	Primer	Sequence (5' - 3')	Annealing T _o	Size (nt)	Position ¹	Gene	Reference
ANV	ANV PreCAP	ACCTTGAATCCCTGTGGGCA	57°C	2538	4403-4423	ORF1b / ORF2 /	Todd et al., 2011
	ANV PostCAP	AAAAGTTAGCCCAATTCAAAATTAATTC			6915-6941	3' UTR	
	ANV InterCAPF	TCKCARTCRCCDGCCTGA	*	1152	4985-5002	ORF2	This study
	ANV InterCAPR	GTGTCNSTVRYRYTTGCATTTG			6114-6137		
	ANV InterCF	TCGCCWGCAGATKCGTATGG	*	1131	4990-5010	ORF2	This study
	ANV Inter CR	TGCAITTTGRAGAAARTAGTC			6101-6121		
CasV	CasV PreCAP	TAGAGGGATGGACCGAAATATAGCAGC	50°C	2277	4980-5006	ORF1b / ORF2 /	Smyth et al., 2009
	CasV PostCAP	TGCAGCTGTACCCTCGATCCTA			7236-7257	3' UTR	
	CasV InterCAPF	TWYTRMGAGGTGGCTGGTGGGT	*	1150	6028-6049	ORF2	This study
	CasV InterCAPR	TGTTTTTGCTGCTCAGGTCC			7159-7178		
TAsV-1	TAsV-1 PreCAP	CGCCATGCCCAAGGAAGCCGGC	60°C	2050	4771-4791	ORF1b/ORF2	This study
	TAsV-1 PostCAP	TCGATCCTACCCGGCGTGCC			6880-6900		

* Added directly to the sequencing reaction mix.
¹ Primer position is indicated in reference to the genomes of strains ANV (HM029238), CasV (JF832365), and TAsV-1 (NC002470).

Figure 1. Schematic representation of the complete ORF2 sequencing strategy. Arrows represents the direction of nucleotide addition for each new DNA strand created. Dashed lines represent the estimated fragment to be generated by the primer. Solid lines represents the DNA fragment generated.



In silico analysis of the designed primers using the BLAST online tool and BioEdit v. 7.2.5 software (HALL et al., 1995), demonstrated that the primers designed in this study enabled the amplification of all previously obtained Brazilian ANV, CAstV, and TAsTV-1 sequences, as well as others recovered from the GenBank database. However, considering the large number of genotypes and high genetic diversity of these viruses, continuous analysis of *Avastrovirus* in chickens and other bird species should be performed. As expected, the BLAST/n results matched their respective viral species, with identities ranging from 65–100%, and these species showed no similarity with other enteric viruses. These relatively low values were generated mostly for the 5' ends of the primer sequences. With regard to dimers and hairpins, although some of the primers were presented as theoretical hairpin or (self/cross) dimer formation, their respective ΔG (Free Gibbs Energy) was high, and therefore, their performance was not affected.

The previously described primers for the complete ORF2 gene (TODD et al., 2011; SMYTH et al., 2012) allowed for the amplification of 8/21 ANV- and 1/2 CAstV- positive samples. These primers, designed based on ANV and CAstV European and Asian sequences, demonstrated reduced specificity with South American samples, which can be explained by the high heterogeneity and diversity of Brazilian ANV and CAstV sequences. The complete TAsTV-1 ORF2 gene was successfully amplified, although the primers were designed based on the single TAsTV-1 sequence available in the GenBank database. The limited information available in the database regarding sequences and primers for ORF2 amplification restricts the analysis of this gene in *Avastrovirus*.

The application scope of the designed ANV primers enabled the amplification of sequences, including European, Brazilian, and Asian strains from the GenBank database, classified as genotypes

2 and 5, both previously described in chickens (IMADA et al., 2000; ZHAO et al., 2011). However, there is limited genetic information pertaining to CAstV and TAsTV-1 ORF2 in the database, and reports of these viruses in Brazil and South America are very limited; thus, we cannot speculate regarding the designed primers scope, although during standardization, we did successfully amplify and sequence one CAstV and TAsTV-1 strain.

The complete sequencing of ORF2 by primer walking is a well-known strategy used in ANV and CAstV strains (TODD et al., 2011; SMYTH et al., 2012). The 3' Rapid Amplification of cDNA Ends (RACE) technique has also been used for the complete sequencing of ORF2 in ANV strains (CHAMINGS et al., 2015). In fact, the unique complete TAsTV-1 ORF2 sequence available in GenBank was obtained by RACE methodology (JONASSEN et al., 2001). Primer walking has the advantage of enabling the direct sequencing of unknown DNA, such as the ORF2 insert in a plasmid vector (MARTIN-GALHARDO et al., 1992). Moreover, the RACE technique is relatively more expensive.

The approach presented in this study provides a method to be used for genotypic and diversity studies of *Avastrovirus* in chickens, which are essential for the elucidation of the epidemiology of this virus.

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