Mini Review

Avian coronavirus Main Replicase Enzymes at a Glance

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Abstract | Avian Coronavirus (AvCoV) belongs to the *Gammacoronavirus* genus from the *Coronaviridae* family and *Nidovirales* order. Coronaviruses have a single-stranded positive RNA genome, corresponding to the largest known RNA genome, with about 27kb for AvCoV. The first 5' two thirds of this genome, correspondent to a wide ORF1 (also called replicase gene), code for non-structural proteins (nsps) that are enrolled in viral transcription, replication and pathogenesis. The last 3' one-third of the genome codes the four structural and accessory proteins. Nsps act in a complex replication process, made possible by the special characteristics of AvCoV genome. This manuscript aims to present an overview of the main aspects of the current knowledge on the main AvCoV replicase genes.

Editor | Muhammad Munir, The Pirbright Institute, UK.
Received | October 05, 2016; **Accepted** | October 20, 2016; **Published** | December 15, 2016
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DOI | http://dx.doi.org/10.17582/journal.bjv/2016.3.6.161.165
Citation | Ayres, G.R.R., and P.E. Brandão. 2017. *Avian coronavirus* main replicase enzymes at a glance. *British Journal of Virology*, 3(6): 161-165.
Keywords: Avian coronavirus, Replicase, ORF1, Review

Avian coronavirus (AvCoV) belongs to the *Gammacoronavirus* genus from the *Coronavirinae* subfamily on the *Coronaviridae* family and *Nidovirales* order (ICTV, 2015). This is a virus, with a virion composed by four structural proteins: spike protein (S), envelope protein (E), membrane protein (M) and nucleocapsid protein (N). Spike proteins are composed by 2 subunits (S1 and S2) that occur as trimers on the virion surface. S1 subunit emerges from virus envelope, is responsible for the interaction with cell receptors and is the main target to neutralizing antibodies, therefore being used for AvCoV serotyping and its correspondent gene being the main target to virus genotyping. S2 subunit anchors S protein to the envelope and it is enrolled in the fusion process between viral envelope and cell membrane during infection. M protein anchors the viral envelope to the nucleocapsid, composed by phosphoprotein N that, in turn, binds to genomic RNA (as reviewed in Cavanagh, 2007).

Coronaviruses have single-stranded positive RNA genomes, corresponding to the biggest RNA genome known, with about 27kb in AvCoV. Interestingly, the first 5' two thirds of this genome, correspondent to ORF1, code for non-structural proteins (nsps) that are enrolled in viral transcription, replication and pathogenesis. The last 3' one-third of the genome codes the four structural proteins described above and accessory structural proteins, as well as some non-structural proteins that take part on the replication process (Masters, 2006).

Nidoviruses transcript mRNAs in a discontinuous hemi-nested pattern through a complex process mediated by viral RNA dependent (RdRp) RNA polymer-

ase that results in general in 6 mRNAs: 5 subgenomic mRNAs (2-7kb) and one mRNA corresponding to entire genome. This complex process is only possible because of some interesting aspects of viral genome which will be briefly described herein focused on the main proteins involved in this process.

The 3' genomic region is polyadenylated, similarly to cellular mRNAs. Moreover, a 5' genomic sequence (70 to 100 nucleotides) corresponds to a leader sequence that is followed by a transcription regulatory sequence (TRS), with 8 or 9 nucleotides. Besides, TRS sequences are also present between each genome ORF and in the negative version of genomic RNA the TRS sequence is complementary to the final region of leader sequence.

Therefore, during cell infection, the genomic RNA is released into the cytoplasm and acts as mRNA (once it is a positive RNA with a polyadenylated 3' region) leading to the translation of the replicase polyprotein, coded by ORF1. Through a self-cleavage process, this complex polyprotein releases subunits that will act in transcription process (such as the enzymes RdRp, RNA helicase, 3'-5'exonuclease). RdRp binds to 3'UTR genomic region and starts the negative sense transcription until the following TRS sequence that leads to the releasing of the new negative RNA strand. However, as described above, this TRS sequence is able to complementarily bind to the final region of the near 5' leader sequence, allowing continuing the transcription of the nascent negative strand. Therefore, a hemi-nested set of negative subgenomic RNAs is produced and further used as template to the transcription of positive subgenomic mRNAs (sg-mRNAs), by the same RdRp (Sawicki et al., 2007).

This complex process shows how refined is the Coronavirus transcription machinery and provokes attention to viral nsps.

ORF1 (also called ORF1ab and polyprotein replicase gene) has already been pointed as fundamental in *Avian coronavirus* pathogenesis. In a fundamental study, a virulent strain of AvCoV of the M41 genotype has a large part (8.4kb) from the final region of ORF1ab replaced by its correspondent residue taken from the non-virulent Beaudette genotype. The recombinant virus showed reduced virulence both *in vitro* and *in vivo*. As a result, the replicase gene was pointed as one of the pathogenicity factors in AvCoV (Armesto et al., 2009).

The ORF1 is also called ORF1ab because, due to a ribosomal frameshift, it translated as two sections, polyprotein 1a and 1ab that are self-cleaved during and after the translation process (Cavanagh, 2007; Graham et al., 2005). In AvCoV, it was predicted that ORF1a codes nsp2 to nsp11 and ORF1b nsp12 to nsp16 (Sawicki et al., 2007; Ziebuhr and Snijder, 2007). In other Coronavirus species nsp1 gene is included in 5'region of ORF1a (Ziebuhr and Snijder, 2007).

Although not all of the nsps had had their functions unveiled, some different active sites were recognized along replicase polyprotein: papain-like protease (Plpro), placed in nsp3; main protease (Mpro), in nsp5 and RNA polymerase RNA dependent (RdRp), in nsp11 and nsp12; helicase (HEL) in nsp13; exoribonuclease (ExoN), in nsp14; uridylate-specific endoribonuclease (NendoU), in nsp15; and methyltransferase (MT) in nsp16 (Ziebuhr et al., 2000).

Nsp2 is the first encoded protein in AvCoV genome and its importance in early stages of infection has been speculated (Yang et al., 2009). It has been shown that some of hydrophobic residues in nsp2 might anchor the replicase complex to the Golgi during the infection (Hagemeijer et al., 2010). In murine hepatitis virus MHV (a *Betacoronavirus*) it was shown that mutations in nsp2 gene lead to differences in virulence and pathogeny (Roth-Cross et al., 2009). Even though its presence was proved not to be necessary to coronavirus replication, it is possible that nsp2 may act in general RNA synthesis (Graham et al., 2005).

Nsp3 contains the active Plpro site that cleaves the N-terminal region (between nsps 2-3 and 3-4) of polyproteins 1a and 1ab (Ziehbur et al., 2000). Several Plpros have already been described in viruses with positive RNA genomes. However, in different coronaviruses Plpros and its proteolitic patterns show important differences (Thiel, 2007). By comparing genetic sequences, two Plpro active domains were initially pointed in AvCoV genomes: Pl2pro, that would act cleaving amino-terminal region of 1ab polyprotein and modulating cell responses to AvCoV infection; and Pl1pro with a unknown function (Lim et al., 2000; Mondal and Cardona, 2004). However, it was latter demonstrated that for *Gammacoronavirus* and for some *Betacoronavirus* there is only one active Plpro, orthologous to Pl2pro from other coronaviruses (Ziebuhr and Snijder, 2007). In AvCoV, homologous

sequences of Pl1pro were also found in nsp3, however these fragments do not included regions required to proteolitic action, being considered inactive (Thiel, 2007).

In SARS-CoV (a *Betacoronavirus*) it is known that Plpro acts with Mpro cleaving polyproteins 1a and 1ab to release its 16 mature nsps (Tan et al., 2005). Besides, it was demonstrated a deubiquitinating action of Plpro in coronavirus (Clementz et al., 2010) lead to the expeculation that this protease may inhibit the cell response to the infection. Additionally, some *in vitro* data obtained in MHV (also a *Betacoronavirus*) infections suggest that Plpro can inhibit interferon type 1 production (Zheng et al., 2008) and can modulate the expression of TNF, α-IL and IL-6 (Eriksson et al., 2008).

Besides Pl2pro active site and Pl1pro truncated sequence, nsp3 contains three conserved domains, thought Ac, ADRP and Y (Ziebuhr and Snijder, 2007). The ADP-ribose 1"- phosphatase (ADRP), also referred as X domain, seems not to be necessary to virus replication *in vitro*, being also suggested a possible role in virus-host interaction (Putics et al., 2005).

Regarding its importance in proteolytic processing of replicase polyprotein, the active site of nsp5 was named main protease (Mpro). However, this protein was earlier designated 3C-like cysteine proteinase (3CL), due to its homologous in picornavirus sequences. Mpro mediates itself its releasing from pp1a and acts in cleavage sites from nsp4 to nsp16. Besides, in MHV, it was demonstrated the action of Mpro in RNA synthesis. The smaller nsps 7 to 10, placed in the 3' region of pp1a, seems to join RdRp in RNA transcription and replication (Ziebuhr et al., 2000; Ziebuhr and Snijder, 2007).

The 3'region of nsp12 gene codes coronavirus RNA-dependent RNA-polymerase RdRp, that share similarities with group I polymerases in N' terminal region and with group III in C' terminal portion, thus being classified as an outgroup of superfamily I RdRps (Koonin, 1991). Recently, it was sown that this RdRp shows increased fidelity, a fundamental characteristic for viruses with such a large genome. However, RdRP fidelity is under epistatic action of ExoN domain of nsp14. Those findings suggest that CoV nucleotide sequences are built under the action a multiprotein replicase-fidelity complex (Sexton et al., 2016). This more refined machinery is fundamental for AvCoV to keep such a large genome.

AvCoV helicase domain is located in nsp13, being classified in helicase superfamily 1. Besides unwind double stranded RNAs, CoV helicase also acts in DNA substrates and seems to play different roles as nucleic acid stimulated NTPase and dNTPase; and on 5' capping os RNAs (as reviewed in Gorbalenya et al., 2006; Ziebuhr and Snijder, 2007).

Nsp15 encodes a highly conserved ribonuclease, called NendoU that consists in a genetic marker for *Nidovirales* order (Snijder et al., 2003; Ivanov et al., 2004). This is a U-specific endoribonuclease that does not cleave DNA but can cleave total cellular RNA and single stranded RNA (Cao et al., 2008), however, further studies are still necessary to unveil biological features in which NendoU may be enrolled. More information is also necessary to a better comprehension over nsp16 that contains a predicted methyltransferase domain (MT). It is possible that MT acts in RNA processing and maturation jointly with other nsps (Ivanov et al., 2004).

Coronaviruses shows high genetic diversity, due to point mutations and due to high recombination frequency, which may be due to the error rate RdRp and limited proof-reading ability and due to discontinuous transcription pattern (Jackwood et al., 2012).

Complete genome analysis in AvCoVs showed that nsp2, nsp3 and nsp16 genes, joinly with S gene, are amongst the main targets to recombination events (Thor et al., 2011).

Comparing different sequences of ORF1ab, from heterologous lineages of AvCoV, it was possible to verify that Plpro shows higher divergence than Mpro and RdRp (Mondal and Cardona, 2004). Additionally, nsp3 was associated with the attenuation process, and showed to be more variable than S gene sequences, in comparisons of AvCoV sequences from attenuated and virulent strains from a same serotype (Phillips et al., 2012). Besides, recombinations between field and vaccine virus lineages in this ORF have been reported (Liu et al., 2013). Those findings may, probably, be a result of divergences in selective pressures in different targets. Thus, it is possible that differences in these genes (and their products) may confer adaptive advantages to AvCoV lineages (Mondal and Cardona, 2004).

In AvCoVs isolated in USA, a high identity between ORF1ab sequences retrieved from field and vaccinal samples, regardless of soro-genotype, has been reported (Mondal and Cardona, 2004). Studies based on complete genome sequences revealed a possible recombination event between AvCoV strains from Asia and United States, probably due to the evolution of these linages from a common ancestor (Thor et al., 2011).

This rich genetic diversity, due to mutations and recombination, submitted to strong positive selection, was also demonstrated to impact in Coronavirus evolution, epidemiology and pathogenesis (Brandão et al., 2009; Jackwood et al., 2012).

Acknowledgments

The authors are grateful to FAPESP (grant numbers 2011/00874-5 and 2011/00603-1) and CNPq (grant number 301225/2013-3) for the financial support.

Conflict of Interest

There is no conflict of interest.

Authors' Contribution

Both authors contributed equally to this paper.

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