On the evolution of *Avian infectious bronchitis* virus in VERO cells

Sobre a evolução do vírus da bronquite infecciosa das galinhas em células VERO

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Abstract

Avian infectious bronchitis virus (IBV) (*Nidovirales*: *Coronaviridae*) is a chicken *Gammacoronavirus* with the highest evolution rate in the genus and, despite the recently reported proofreading activity of its polymerase, intra and inter-host diversity is a well documented phenomenon. This study aimed to assess the genetic variation of serial passages of a variant genotype IBV strain *in vitro*. Strain CRG-BETA, propagated in chicken embryos, was inoculated in VERO cells monolayers up to the 4th passage and each passage was monitored with an RT-PCR targeted to the S1 gene (nt 705 to 1094) and an RT-PCR to the protein 5a mRNA. All passages were positive to RT-PCRs to S1 and passages 1 to 3 to 5a mRNA; S1 sequences showed no polymorphism. The finding of IBV mRNA in the cell cultures demonstrates that the CRG-BETA IBV strain is replicating in the VERO cells and regarding S1 sequence analysis, the lack of nucleotide mutations shows that CRG-BETA might have reached a fixed status. As a conclusion, different genotypes of IBV present different evolutionary patterns not only *in vivo* as previously known, but also *in vitro*, as described herein.

Keywords: IBV. Spike. Evolution. *In vitro.*

Resumo

O virus da bronquite infecciosa das galinhas (IBV) (*Nidovirales*: *Coronaviridae*) é um *Gammacoronavirus* com a maior taxa evolutiva no gênero e, apesar de uma recentemente relatada atividade corretiva de sua polimerase, a diversidade intra e inter-hospedeiros é um fenômeno bem documentado. Este estudo objetivou avaliar a variação genética após passagens seriais de uma amostra de IBV variante. A amostra CRG-BETA, propagada em embriões de galinhas, foi inoculada em monocamadas de células VERO até a quarta passagem e cada passagem foi monitorada com uma RT-PCR para a região S1 do gene S (nt 705 a 1094) e uma RT-PCR para o mRNA da proteína 5a do vírus. Todas as passagens foram positivas para S1 e as passagens 1 a 3 para mRNA 5a; sequências de S1 não apresentaram polimorfismos. O encontro de mRNA de IBV nos cultivos celulares demonstra que a amostra CRG-BETA está replicando nas células VERO e, em relação à análise de S1, a ausência de mutações de nucleotídeos demonstra que a amostra CRG-BETA pode ter atingido um estado fixo. Como conclusão, diferentes genótipos de IBV apresentam diferentes padrões evolutivos não apenas *in vivo*, como previamente conhecido, mas também *in vitro*, como aqui relatado.

Palavras-chave: IBV. Espícula. Evolução. *In vitro*.

Introduction

Avian infectious bronchitis virus (IBV), currently named as *Avian coronavirus* (*Nidovirales*: *Coronaviridae*: *Coronavirinae*: *Gammacoronavirus*) is a worldwide distributed major pathogen of breeders, broilers and layers, causing disease of the reproductive, respiratory, urinary and enteric systems of the birds, with a high attack rate and a major economic impact to the poultry industry[1].

IBV occurs as a wide range of genotypes and serotypes due to the high prevalence of infection and

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the high mutation rate, while the heterotypic protection amongst different serotypes distantly related is low[2]. This high evolutionary rate leads thus to a continued emergence of nucleotide and amino acids mutations and eventually to the emergence of novel types of the virus[3].

It's known that a same initial population of IBV, *i.e.*, a same quasispecies under a neutral network constraint, might go different evolutionary ways depending on the type of cell it infects *in vivo*[4] and that even a low number of *in vitro* passages might result in lineages divergent form the initial one[5].

A typical Brazilian IBV lineage has been described[6,7] with at least three sub lineages[3] consistently divergent from the archetypical strains already described. Nonetheless, there's no data on the effect of serial passages of these viruses neither *in vivo* nor *in vitro*.

As the knowledge on the molecular behavior of IBV is paramount for the knowledge on vaccine strain stability, the aim of this investigation was to assess the divergence of a Brazilian strain of IBV in cell culture based on a sequence of the spike envelope glycoprotein (S) gene, a major marker for diversity in this virus species.

Material and Method

Virus strains

The strain CRG-BETA isolated in chicken embryos (4th passage) and typed as Brazilian genotype was used for the *in vitro* inoculations. The H120 vaccine strain (Massachusetts serotype) was used as a positive control in the RT-PCRs (DEPC-treated water was used as the negative control).

Passages in VERO cells

Forty-eight hour-old confluent VERO cells grown in 25cm2 flasks with MEM (minimal essential medium) with 10% phoetal bovine serum (PBS) were used for all inoculations. The growth medium was discarded and 1mL of CRG-BETA (4th passage in embryos) was inoculated in the monolayers; 1mL of PBS-free MEM was inoculated in another flask of cells (mock-infected).

After an one-hour adsorption at 37ºC, the inocula were not discarded and 6mL of MEM plus 2.5% PBS were added and the monolayers were kept at 37ºC for 4 days. After this period, cells were frozen and thaw and 1 mL of each previous passage of CRG-BETA or control (mock-infected cells) was used for the next passage up to the 4th passage. Cells were observed daily for the presence of cytopathic effect (CPE).

RNA extraction

All RNA extractions were carried out with TRIzol Reagent™ (Invitrogen, Carlsbad, CA, USA) according to manufacturer`s instructions.

Reverse-transcription polymerase chain reaction (RT-PCR) to the 5a protein mRNA

For the detection of viral transcription activity, each passage (CRG-BETA and control) was monitored for the presence of mRNA of the non-structural protein 5a of IBV.

Complementary DNA was synthesized with M-MLV Reverse Transcriptase™ (Invitrogen, Carlsbad, CA, USA) according to manufacturer's instructions using the anti-sense primer M41N-AS 5' CACACGTTAAGATGTGTTTTGGTCC 3', complementary to positions 164 to 188 of the 5a gene sequences AY392047.1, X03448.1, DQ834384.1 and AY851295.1.

PCR was carried out with Platinum Taq DNA Polymerase™ (Invitrogen, Carlsbad, CA, USA) according to manufacturer`s instructions and an annealing temperature of 48ºC with the antisense (M41N-AS) and sense (Niña-S 5' TCTATCACACTAGCCTTGCGCTAGA 3', nucleotides 25 to 49 of sequences AY392047.1, X03448.1, DQ834384.1 and AY851295.1, correspondent to the leader sequence). After agarose gel electrophoresis strained with ethidium bromide, reactions with the predicted 164bp amplicons were considered as positives.

RT-PCR for the S1 subunit of the spike glycoprotein gene

Each passage (CRG-BETA and control) was submitted to an RT-PCR targeted to the amplification of a 390bp fragment of the region coding for the S1 subunit of the spike glycoprotein (nt 705 to 1094) as described by Worthington, Currie and Jones[8] using M-MLV Reverse Transcriptase™ (Invitrogen, Carlsbad, CA, USA) according to manufacturer's instructions and Random Primers ™ (Invitrogen, Carlsbad, CA, USA) for the cDNA synthesis and Platinum Taq DNA Polymerase™ (Invitrogen, Carlsbad, CA, USA) according to manufacturer`s instructions for the first round and nested PCRs.

DNA sequencing

S1 amplicons were purified from agarose gels using Ilustra™ GFX PCR DNA and Gel Band Purification kit (GE Healthcare, USA), bi-directional DNA sequencing was carried out with BigDye 3.1™ (Applied Biosystems Cidade, País) and sequences resolved in an ABI-3130™ Automatic Sequences (Applied Biosystems™).

Chromatograms were analyzed with Phred online (http://asparagin.cenargen.embrapa.br/phph/) and only positions with scores higher than 20 were used. Sequences were assembled with Cap-Contig and aligned with CLUSTAL/W using Bioedit v. 5.0.9[9].

RT-PCR for NewCastle Disease Virus (NDV)

All passages, including the mock-infected controls, were monitored for NDV as described by Tiwari et al.[10].

Results

Passages in VERO cells

Syncytia were observed from the first to the third passage (Figure 1B), while no alterations were detected in the mock-infected monolayers (Figure 1A). By the 4th passage, no CPE was detected.

RT-PCRs to the 5a protein mRNA and S1 subunit

Passages 1 to 4 and 1 to 3 of CRG-BETA in VERO cells resulted positive for the RT-PCR to the S1 region and to the 5a mRNA, respectively, while no amplifications were detected in the mock-infected cells.

DNA sequencing

The partial sequences of the S1 were obtained for passages 2 to 4 and from the original CRG-BETA strain in embryos. No mutations were detected amongst the four sequences in the 316nt alignment obtained (Figure 2).

RT-PCR for (NDV)

All VERO cells passages of CRG-BETA were found positive for NDV. No NDV was detected in the mock-infected monolayers.

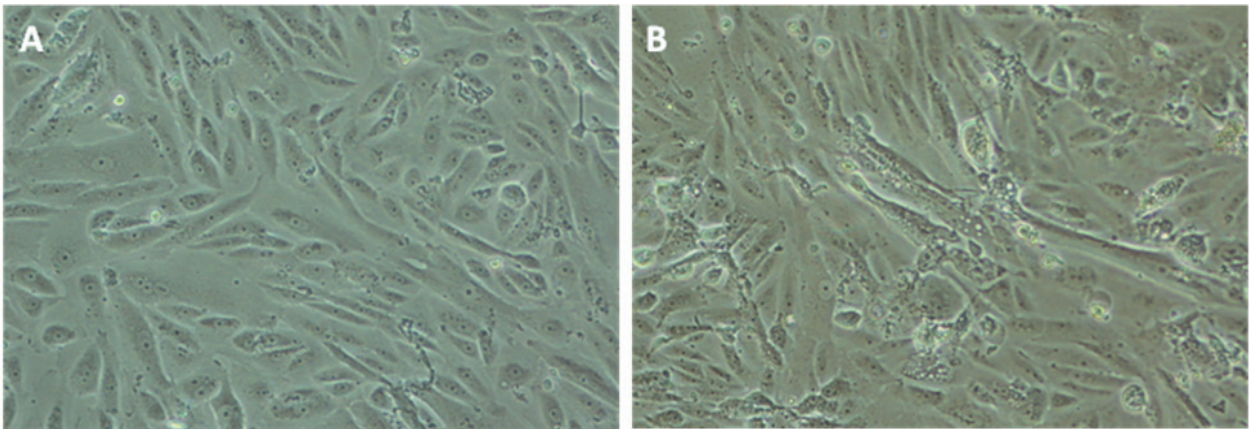

Figure 1 - VERO cells monolayers 100 hours post-infection with mock inoculum (A) or CRG-BETA IBV (B) at the 3rd passage; 100x magnification

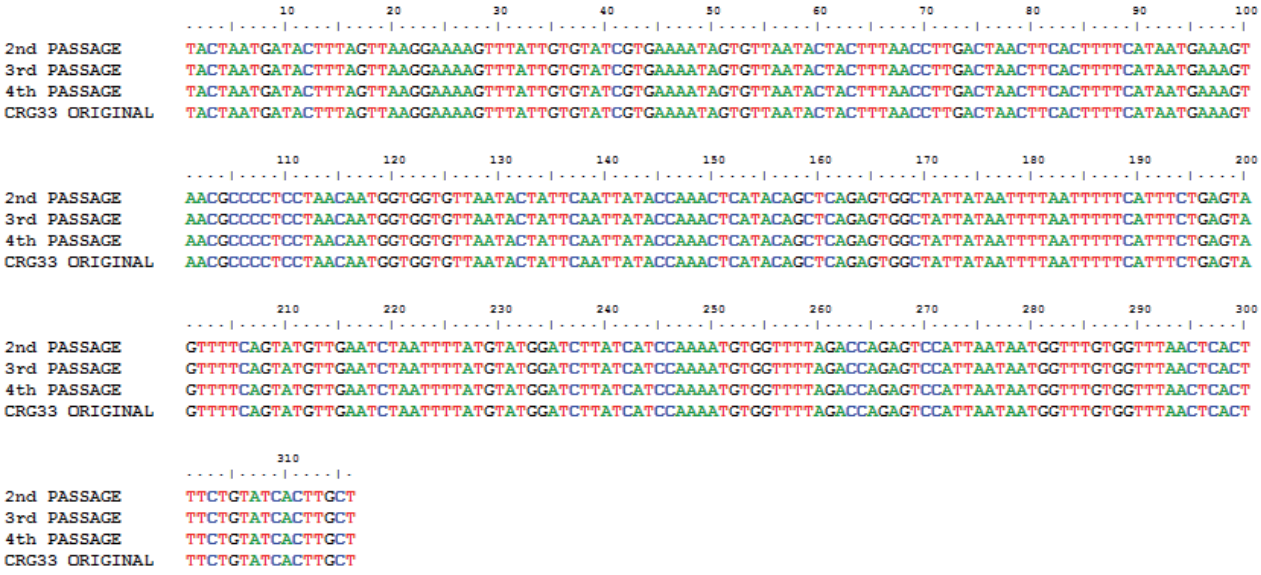

Figure 2 - Partial S1 subunit sequences of IBV strain CRG-BETA ate the second, third and fourth passages in VERO cells and the original strain in embryos

Discussion

In this study, a Brazilian genotype IBV strain was serially passaged in VERO cells and no mutations in a region of the S1 subunit nucleotide sequences were detected.

The detection of mRNA transcription, achieved in this study with an RT-PCR to the 5a gene mRNA, clearly demonstrates that the CRG-BETA strain is replicating in the cells and that the RNA detected is not genomic or inoculum-derived.

In association with the appearance of syncytia, a typical IBV cytopathic effect in cell culture[5], these results demonstrate that, at least up to the third passage, this strain was able to replicate and to spread from cell to cell with the methods employed herein.

Nonetheless, is noteworthy that passage 4 produced no CPE or mRNA was detected. One can hypothesize that CRG-BETA was present in a high titer in the original, embryo-derived, strain and was thus able to efficiently replicate in the first passages due to the high IBV population number present in the inoculum, but, arguably due to intense selection, the IBV population might have decrease to a low number at the 4th passage, entering in a bottleneck phase. A higher number of passages is necessary to verify this effect and the quantization of mRNA transcripts by qPCR would also shed some light on that.

The stability of the S1 region analyzed is also an interesting finding, as others[5] have described a less mutation-robust spike gene for IBV passages in VERO cells using the Beaudette strain as an inoculum.

The Beaudette strain belongs to the Massachusetts serotype of IBV, while CRG-BETA is a Brazilian genotype one, with a maximum reported nucleotide identity of 79.3% for this S1 region amongst these types[3]. As the spike protein plays a major role in receptor-binding[11], it can be speculated that this difference in S might have played a role in the different evolutionary pathways specifically endowing CRG-BETA with a more mutation-robust S when replicating in VERO cells. Anyhow, again this speculation must be taken with care, as further passages and large S sequences would be necessary to assess its consistency. Also, as non-structural proteins might play an important role in pathogenesis[2], differences in these proteins might

also have accounted for a different host-virus relationship in the cytoplasmic environment.

Finally, the presence of NDV must also be accommodated in the hypothesis for the evolutionary patterns described herein. It's known that co-infecting viruses, such as IBV and *Avian metapneumovirus* might interfere with each other's replication *in vivo* due to receptor competition[12] and thus NDV might have outreplicated IBV CRG-BETA in the monolayers and led it to extinction.

As a conclusion, an exclusive evolutionary pattern of a Brazilian genotype IBV strain has been detected which might serve as a model not only for the assessment of vaccine strain stability, but also for a deeper understanding of co-evolution co-infecting viruses *in vivo* and *in vitro*.

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