


# Evaluation of the genetic variability found in Brazilian commercial vaccines for infectious bronchitis virus

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**Abstract** Infectious bronchitis virus (IBV) is currently one of the most important pathogens in the poultry industry. The H120 and Ma5 are the only viral strains approved by the Brazilian government as the constituent of vaccines. Despite the systematic vaccination in Brazil, IBV has not yet been controlled and diseases associated with this virus have been reported in vaccinated chickens. Here, we investigated the genetic variability of H120 and Ma5 strains present in the IBV vaccines from different Brazilian manufacturers. We performed DNA sequencing analyses of the S1 spike glycoprotein gene to investigate its genetic variability and the presence of viral subpopulations among vaccines, between batches, and also in each vaccine after a single passage was performed in chicken embryonated eggs. Our results

revealed up to 13 amino acid substitutions among vaccines and some of them were localized in regions of the S1 glycoprotein that play a role in virus–host interaction. Secondary nucleotide peaks identified in the chromatogram for the S1 gene sequence revealed that all original vaccines (H120 and Ma5) were composed by different subpopulations of IBV. Moreover, new viral subpopulations were also found in vaccines after a single passage in chicken embryonated eggs. These findings indicate that H120 and Ma5 viral strains used in vaccines market in Brazil can still mutate very rapidly during replication, leading to amino acid substitutions in proteins involved in the stimulation of the immune response, such as the S1 glycoprotein. Therefore, our data suggest that the genetic variability of these viral strains should be taken into consideration to ensure an effective immune response against IBV.

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## Introduction

Infectious bronchitis virus (IBV) is a member of the *Coronaviridae* family, and it is currently one of the most important pathogens in the poultry industry due to the economic losses caused by the infectious bronchitis disease [1]. The most commonly used control measures against IBV infection in the world, including Brazil, are based on the combination of biosafety and immunoprophylactic programs. In Brazil, IBV vaccination is performed since the early 80's and the Massachusetts serotype is the only one approved by the Ministry of Agriculture and Supply [2]. Different laboratories produce IBV vaccines containing the H120 viral strain, which are attenuated after going through 120 passages in specific

pathogen-free (SPF) embryonated chicken eggs. The Ma5 strain was introduced in Brazil in the early 90's, and it was obtained from an essentially pure culture of the Massachusetts serotype. Despite the systematic vaccination in Brazil, IB has not yet been controlled efficiently and diseases associated with IBV have also been reported in vaccinated chickens [3–10].

Coronaviruses, such as IBV, are usually a mixture of genetic mutants in the same isolate. The main reason for that is the lack of a proofreading mechanism by the RNA polymerase during viral RNA replication, resulting in a heterogeneous progeny [11–13]. When high-performance genomic sequencing shows two or more nucleotide peaks on the chromatogram for the same position, this information can be used to identify any variance in the viral RNA genome within an isolate [14–16]. Furthermore, the ambiguous heights of these peaks can be used to identify predominant and minor viral subpopulations in the same isolate [17, 18]. The spike glycoprotein S1 binds the virus to the host cell and it contains important epitopes that induce the formation of neutralizing antibodies [5].

In this scenario, the present work investigated the composition and genetic stability of IBV vaccines marketed in Brazil, containing either H120 or Ma5 viral strains. Our goal was to find answers for the following questions: (i) Are the IBV strains in the vaccines genetically homogenous and generate viral subpopulations? (ii) Are the vaccines against IBV genetically stable? To address these questions, we performed DNA sequencing of S1 gene in IBV vaccines from different manufacturers. We investigated the presence of viral subpopulations in all vaccines and also between two consecutive batches of the same vaccine. Additionally, we investigated the presence of viral subpopulations in each vaccine after a single passage in chicken embryonated eggs.

## Materials and methods

### Vaccines

Two consecutive batches of live IBV vaccines produced by different laboratories were purchased for this study. We evaluated vaccines containing the H120 viral strain from 4 manufacturers, here called vaccines A, B, C, and D ( $n = 8$ ), and the cloned Ma5 viral strain vaccine ( $n = 2$ ). Vaccines for avian metapneumovirus, Newcastle disease virus, and Gumboro disease virus, which are not related to IBV, were used as a negative control in PCR reactions.

### Single passage of vaccines in embryonated chicken eggs

Specific pathogen-free (SPF) embryonated chicken eggs were purchased from VALO BioMedia, and used to perform

a single passage of H120 vaccines (A, B, C, and D) and Ma5 vaccine. For each vaccine, 100 microliters ( $\mu\text{L}$ ) of each vaccine suspension was inoculated in the chorioallantoic cavity of three eggs at 9–11 days of incubation, and after 40 h post-infection (hpi), eggs were refrigerated at 4 °C for 16 h [19]. Upon confirmation of embryos' death using an ovoscope, 3 mL of the chorioallantoic liquid (CAL) were collected. In the case of the Ma5 vaccine, necropsy of embryos was also performed, and fragments of trachea, lungs, kidneys, spleen, intestine, and bursa of Fabricius were collected for viral RNA extraction.

### RNA extraction and cDNA synthesis by RT-PCR

To obtain the S1 gene sequence of viral strains of commercial vaccines before and after viral reisolation, viral RNA extraction was performed using Trizol<sup>®</sup> Reagent (Invitrogen<sup>™</sup>), according to the manufacturer's instructions. Viral RNA was then reverse transcribed into cDNA by RT-PCR using the Veriti<sup>®</sup> Thermal Cycler (Applied Biosystems). Each reaction had a final volume of 20  $\mu\text{L}$ , and contained 700 ng of total RNA and SuperScript<sup>®</sup> III Reverse Transcriptase (Invitrogen<sup>™</sup>) enzyme, according to the manufacturer's recommendations. The RT-PCR product was used as a template in the conventional PCR assay.

### Conventional PCR

PCR reactions were performed in the Veriti<sup>®</sup> Thermal Cycler (Applied Biosystems) using the oligonucleotides S1OLIGO5' and S1OLIGO3' [20] (Online Resource 1). These oligonucleotides flank and amplify the entire IBV S1 gene, which comprises approximately 1720 base pairs (bp). Reactions were performed in 0.2 mL microtubes, and contained 25  $\mu\text{L}$  of Go Taq<sup>®</sup> Green Master Mix (Promega), 5  $\mu\text{L}$  of cDNA, 50  $\mu\text{M}$  of each oligonucleotide, and Nuclease-Free Water (Promega) for a final volume of 50  $\mu\text{L}$ . Amplification conditions were set as 94 °C/1 min, followed by 35 cycles (94 °C/30 s, 50 °C/45 s, and 72 °C/2 min), and a final extension step of 72 °C/10 min.

To obtain satisfactory amounts of the RNA from organs of embryos inoculated with Ma5 vaccine, PCR products were additionally amplified using the oligonucleotides CK2 and CK4 [19]. These oligonucleotides amplify a fragment of approximately 600 bp that flank the hypervariable regions I and II of the IBV S1 gene (Online Resource 1). Reactions contained 25  $\mu\text{L}$  of Go Taq<sup>®</sup> Green Master Mix (Promega), 5  $\mu\text{L}$  of cDNA, 50  $\mu\text{M}$  of each oligonucleotide, and Nuclease-Free Water (Promega) for a final volume of 50  $\mu\text{L}$ . Amplification conditions were set as 94 °C/1 min, followed by 40 cycles (94 °C/30 s, 50 °C/45 s, and 72 °C/1 min), and a final extension step of 72 °C/5 min. PCR products were stained with GelRed<sup>™</sup> (Biotium) upon electrophoresis in

1% agarose gel, and observed under a UV transilluminator. The amplification of the targeted IBV genomic segment was performed in triplicate for each sample in the same run, and PCR products were sequenced separately.

### DNA sequencing of the S1 gene

PCR products were sequenced by the Sanger method through Macrogen (Seoul, Rep. of Korea), and chromatograms obtained were analyzed using CLC Genomics Workbench version 7.5 software (Qiagen). During analysis, sequences were trimmed (quality score limit: 0.01; ambiguous nucleotide residues: 0), and S1 gene contigs were assembled using the Assemble Sequences tool. After the assembly of contigs, chromatograms were manually reviewed, and any genetic variations found, including secondary nucleotide peaks, were further investigated for the presence of viral subpopulations in the vaccines. Consensus sequences for the S1 gene of original vaccines were obtained using CLC software and were deposited in the GenBank with the following Access Numbers: vaccine A (KU736749), vaccine B (KU736748), vaccine C (KU736750), vaccine D (KU736751), and Ma5 vaccine (KU736747).

### Structural model

The IBV S1 protein still does not have its 3D structure completely elucidated, therefore, we used its amino acid sequence to build a structural model based on its homology with the trimer spike protein (S1 and S2 domains) from HCoV-NL63 coronavirus (pdb id: 5SZS). The IBV S1 DNA sequence of vaccine A was modeled using Modeller v 9.17 program [21]. A total of 10 models were generated and the one with the best quality structure was selected. Stereochemistry and energetic qualities of the model were assessed using the Ramachandran plot [22], Prosa [23], ERRAT [24], and WHAT\_CHECK [25]. The structures were visualized using the Pymol v0.99 [26]. Epitope regions for B and T lymphocytes [27–30] were aligned with S1 sequence of H120 vaccine A (Genbank: KU736749) using omega clustal software [31].

### Cloning and high-resolution melting analysis (HRM)

PCR was performed to amplify fragments of the region containing the secondary nucleotide peak at the position 125 that was detected in the chromatograms of the original Ma5 vaccine. The oligonucleotides C2U (114–133 nucleotides) and C3L (341–325 nucleotides) [32] were used to amplify approximately 230 bp (Online Resource 1), which comprise the amino-terminal region of the S1 protein. This region is responsible for the induction of antibodies against IBV, and it is used to define the different viral serotypes [27].

PCR reactions were performed in 0.2 mL microtubes in the Veriti<sup>®</sup> Thermal Cycler (Applied Biosystems), containing 25  $\mu$ L of Go Taq<sup>®</sup> Green Master Mix (Promega), 5  $\mu$ L of cDNA, 10  $\mu$ M of each oligonucleotide, and Nuclease-Free Water (Promega) for a final volume of 50  $\mu$ L. Amplification conditions were set as 94 °C/1 min, followed by 35 cycles (94 °C/30 s, 50 °C/40 s, and 72 °C/30 s). The PCR product from each reaction (~ 230 bp) was purified based on the 1.8% agarose gel electrophoresis results. Purification of DNA fragments was performed using Wizard<sup>®</sup> SV Gel (Promega), followed by cloning in pGEM<sup>®</sup>-T Easy Vector (Promega), according to the manufacturer's instructions. Vector constructs were transformed in bacteria, in which 38 transformants were selected and their plasmid DNA were purified using GeneJET Plasmid Miniprep Kit (MBI Fermentas) to be used in qPCR reactions, followed by DNA sequencing.

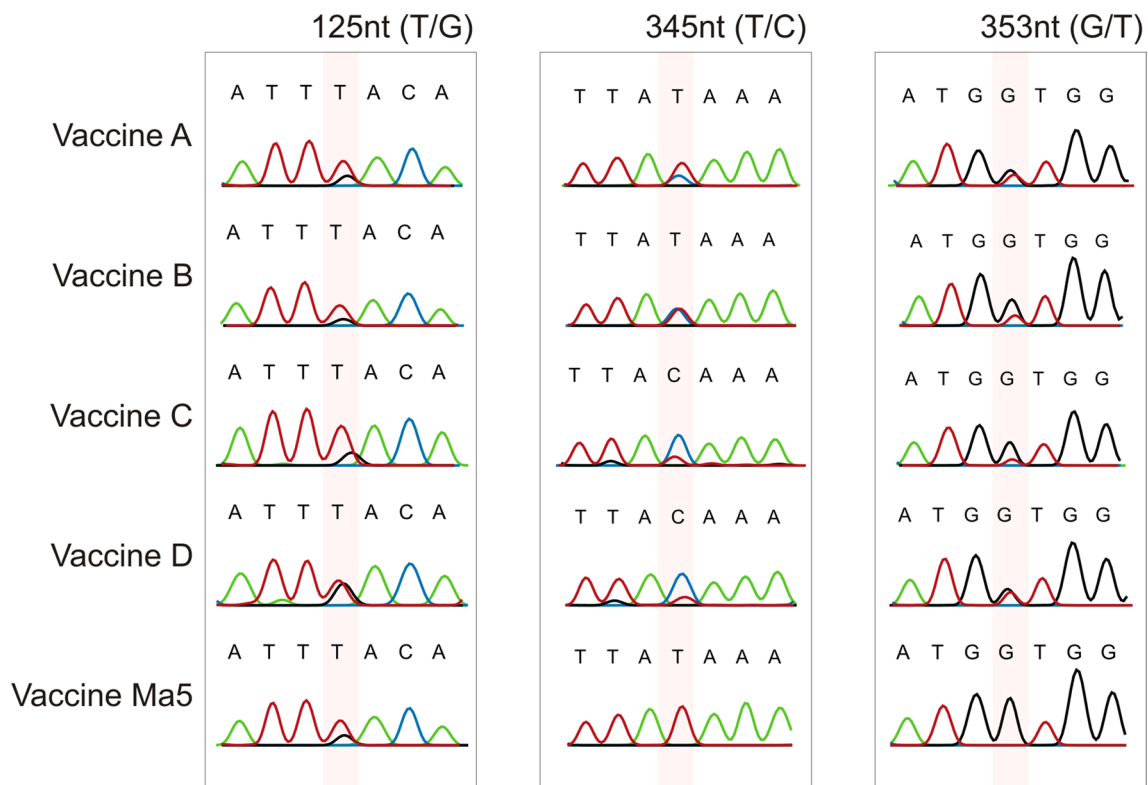
Real-time PCR reactions (qPCR) were performed using Eco<sup>™</sup> Real-Time PCR System (Illumina<sup>®</sup>). Each reaction contained 12.5  $\mu$ L of SYBR<sup>®</sup> Select Master Mix (Life Technologies), 100 ng of plasmid DNA, and 2  $\mu$ M of each oligonucleotide (C3L and C2U) for a total volume of 25  $\mu$ L. Amplification conditions comprised 40 cycles (94 °C/30 s, 45 °C/30 s, and 72 °C/1 min). Products of each qPCR reaction were immediately subjected to High-Resolution Melting analysis, with a temperature range of 65–95 °C, with increments of 0.1 °C. Results were analyzed using the EcoStudy v5.0.4890 software (illumina<sup>®</sup>). After the melting cycle, DNA products along with a 100 bp DNA marker (Invitrogen<sup>™</sup>), were stained with GelRed<sup>™</sup> (Biotium) and visualized in a 2% agarose gel under UV light.

## Results

### DNA sequencing analysis of the S1 gene in commercial vaccines containing either H120 or Ma5 viral strains

DNA sequencing of the IBV S1 gene was performed in commercial vaccines marketed in Brazil, containing either H120 or Ma5 strains. H120 vaccines are produced in Brazil by four different manufacturers, here called vaccines A, B, C, and D, while the Ma5 vaccine is produced by only one manufacturer. Gene sequencing analyses were performed in two consecutive batches of each vaccine, and DNA sequencing results obtained were deposited in the GenBank with the following access numbers: vaccine A (KU736749), vaccine B (KU736748), vaccine C (KU736750), vaccine D (KU736751), and Ma5 vaccine (KU736747).

Analysis of chromatograms revealed that vaccines A, B, C, and D exhibited secondary nucleotide peaks at the positions 125, 345, and 353 (Fig. 1) while vaccine Ma5 only exhibited a secondary peak at the position 125. In



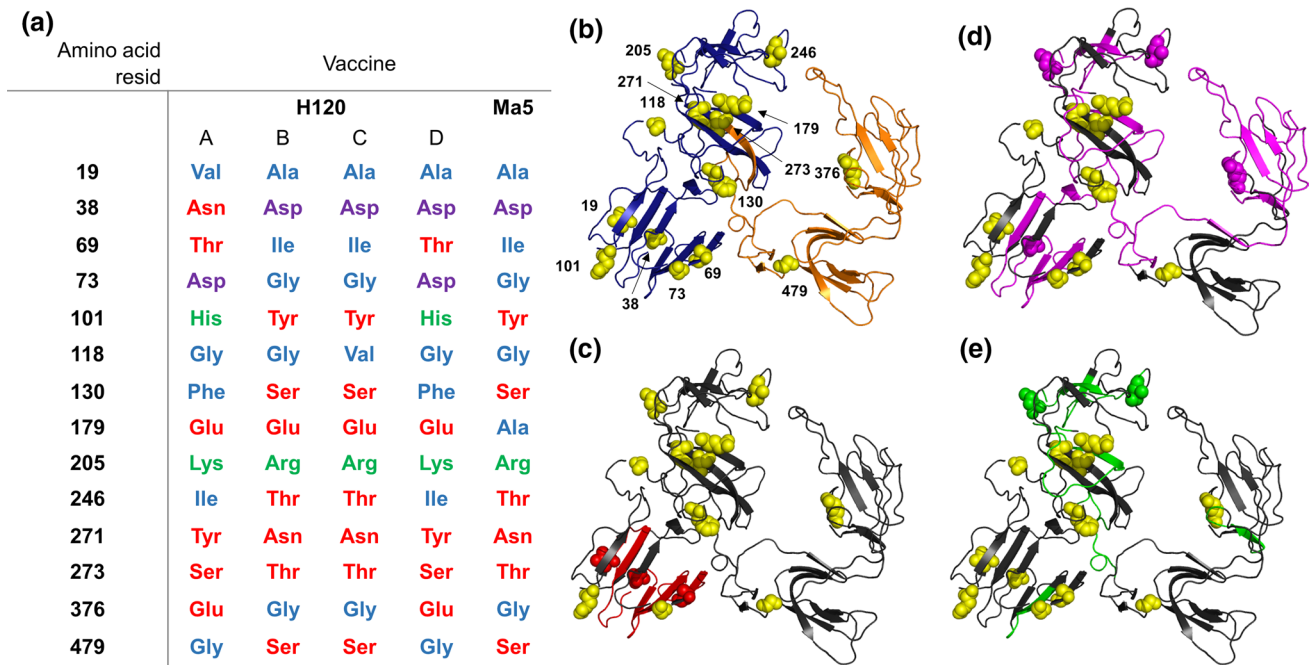
**Fig. 1** Chromatograms showing secondary nucleotide peaks in the S1 gene sequence in IBV commercial vaccines (A, B, C, D, and Ma5). Nucleotide peaks are highlighted showing predominant and minor viral subpopulations

this position, vaccines A, B, C, D, and Ma5 exhibited a main peak of thymine and a secondary peak of guanine, suggesting the presence of two viral subpopulations. At the position 345, vaccines A and B exhibited a main peak of thymine and a secondary peak of cytosine, whereas vaccines C and D exhibited the opposite. Ma5 vaccine did not present a secondary peak at the position 345. At the position 353, vaccines A, B, C, and D exhibited a main peak of guanine and a secondary peak of thymine while the Ma5 vaccine did not present a secondary peak either. Of note, we did not observe any changes in the nucleotide sequence of the S1 gene between two consecutive batches of any vaccine produced by the same manufacturer.

Alignment analyses of the amino acid sequence of the S1 spike protein revealed that vaccines A, B, C, D, and Ma5 had up to 13 amino acid (aa) substitutions (Fig. 2a). We found 12 and 13 aa substitutions on vaccine A compared to vaccines B and C, respectively, while vaccine B exhibited only 1 aa substitution compared to vaccine C. Also, vaccine A only had 2 aa substitutions compared to vaccine D. Furthermore, 10 and 11 amino acid substitutions were found on vaccine D compared to vaccines B and C, respectively. We also found 13, 1, 2, and 11 aa substitutions on vaccine Ma5 compared to vaccines A, B, C, and D, respectively.

To investigate the impact of the polymorphisms found on the S1 protein in each vaccine, their three-dimensional (3D) structures were predicted using an *in silico* method. The best model generated a protein structure with 88.6% of the amino acid residues in accessible regions while 97.5% of amino acid residues were in energetically allowed regions according to Ramachandran plot analyses, and they exhibited a high-quality stereochemistry based on calculations made using ProSa, ERRAT, and WHAT\_CHECK programs. The predicted model for the S1 spike protein in vaccine A showed a protein structure essentially formed of beta sheets and loops (Fig. 2b), similar to what has been observed for other coronaviruses genera [33].

Protein structure analyses revealed amino acid substitutions in regions of the S1 protein that play a role in the virus–host cell interaction. The S1 protein region that interacts with receptors of the respiratory tract in chickens exhibited 3 polymorphisms among vaccines in the residues 19–69 (Fig. 2c), and it has been shown that residues 38 and 69 are essential for this interaction [34]. At the position 38, vaccines B, C, D, and Ma5 showed the acidic polar Asp while vaccine A showed the neutral polar Asn. On the other hand, in the position 69, vaccines A and D showed the neutral polar Thr while vaccines C, D, and Ma5 showed the neutral non-polar Ile. We also found 4 and 2 variations among



**Fig. 2** Location of polymorphisms in the structural model of the IBV S1 glycoprotein. **a** Polymorphisms found in the amino acid sequences of the Ma5 vaccine strain and in the amino acid sequences of the H120 vaccine strains derived from four different Brazilian manufacturers (A, B, C, and D). Different colors equal to different polarities of the amino acids: Blue is for non-polar, red is for neutral polar, purple is for acidic polar, and green is for basic polar. **b** Structural model of IBV S1 protein based on the sequence found in vaccine A (Gen-

bank: KU736749); the N-terminal region is in blue and the C-terminal region is in orange [34]. Amino acid residues that showed polymorphisms are represented as spherical-shaped and in golden color. **c** The S1 protein region is essentially involved in binding of host cell receptors [34]; **d** epitopes region for B lymphocytes antigen presentation in magenta [27–29], **e** epitopes region for T lymphocytes antigen presentation in green [28, 30] (Color figure online)

vaccines in epitope regions for B lymphocytes (residues 24–61, 132–149, 181–196, 196–215, 232–247, 282–300, 291–398, and 409–415) (Fig. 2d) and T lymphocytes (residues 44–51, 181–196, 196–215, 232–247, 282–300, and 406–414) (Fig. 2e), respectively.

**S1 gene sequencing analyses of H120 vaccines after viral reisolation from embryonated chicken eggs**

We investigated the genetic stability of the commercial vaccines containing H120 strain by comparing the S1 gene sequence found in the original vaccines with the ones obtained after an additional passage in SPF embryonated chicken eggs. Chromatogram analyses of the S1 gene sequences of vaccines before and after viral reisolation showed different genetic variations. Vaccines B and D did not exhibit genetic variations in the S1 gene after viral reisolation. Figure 3 depicts the genetic variations observed in other vaccines; the S1 sequence from the original vaccine C exhibited a main peak of cytosine (shown in blue) at the position 345, which represented the predominant viral subpopulation, and a secondary peak of thymine (shown in red), indicating the presence of a smaller subpopulation. On the contrary, after viral reisolation, this smaller subpopulation

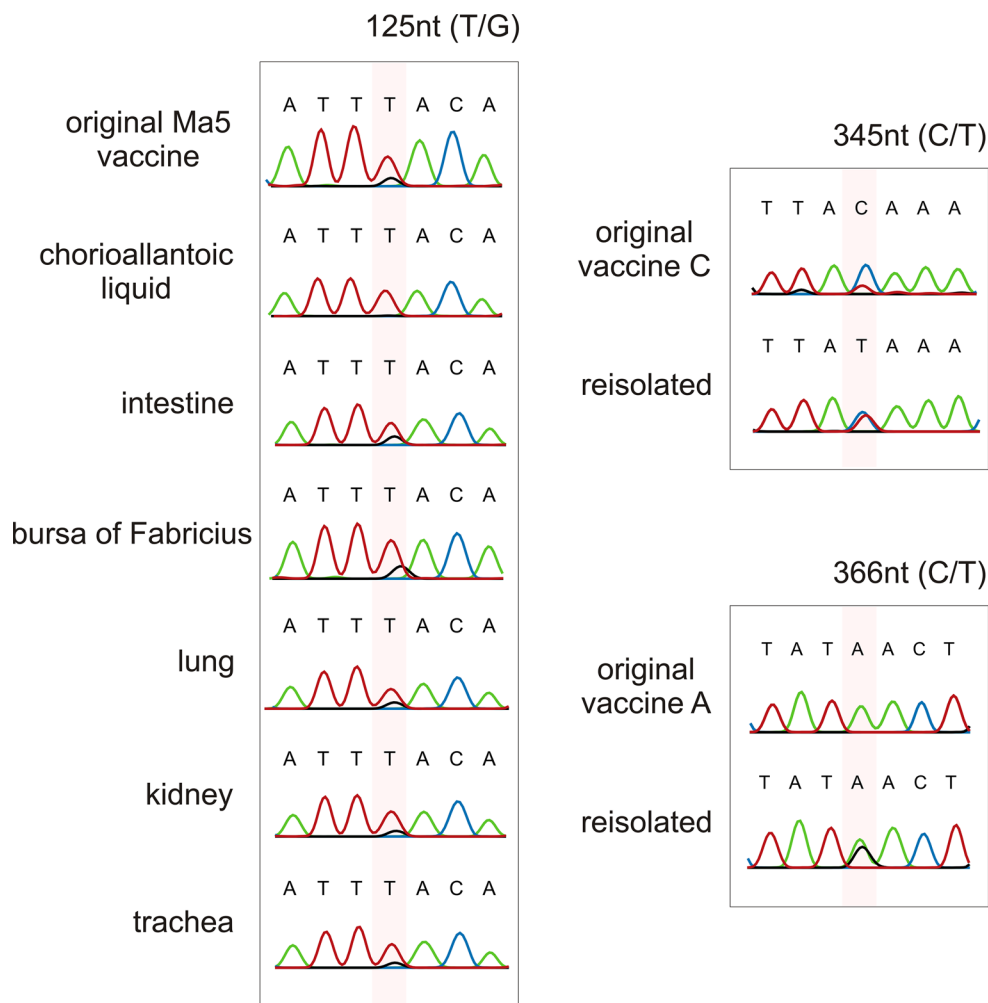
stood out, even as the predominant subpopulation in one of the sequencing reactions. On the other hand, the original vaccine A only exhibited one peak of adenine (shown in green) at the position 366, but after the reisolation, a secondary peak of guanine (shown in black) was also identified, indicating that a point mutation happened during that extra passage in embryonated chicken eggs before viral reisolation.

**S1 gene sequencing analysis of the Ma5 vaccine after viral reisolation from embryonated chicken eggs**

The genetic variation in the commercial Ma5 vaccine was examined by comparing the S1 gene sequence in the original vaccine with the one found in the viral vaccine reisolated from the chorioallantoic liquid (CAL) after a single passage in SPF embryonated chicken eggs. We also isolated the virus from different organs of chicken embryos to identify any genetic variation in the S1 gene compared to the original vaccine.

Chromatogram analyses of the S1 gene showed that the original Ma5 vaccine exhibited two peaks at the nucleotide position 125, indicating the presence of two viral subpopulations. After inoculation of SPF embryonated eggs, two viral

**Fig. 3** Chromatograms showing sites exhibiting secondary nucleotide peaks in the S1 gene sequence after reisolation of viral strains from vaccines A, C, and Ma5. The following nucleotide positions are highlighted: 125, 345, and 366. DNA sequences were analyzed for vaccines A, C, and Ma5, in which viral strains were reisolated from the chorioallantoic liquid. The S1 gene sequence was also analyzed for the Ma5 viral strains isolated from fragments of the intestine, bursa of Fabricius, lung, kidney, and trachea



subpopulations were also identified at the same nucleotide position in all organs collected from the chicken embryos, i.e., trachea, lungs, kidney, intestine, and bursa of Fabricius. On the contrary, the S1 gene sequence found in the virus reisolated from the chorioallantoic liquid only exhibited the predominant nucleotide peak at the same position (Fig. 3).

### Analysis of the Ma5 vaccine by HRM

To further investigate the presence of viral subpopulations in the Ma5 vaccine, we specifically amplified the region containing the nucleotide position 125. PCR products were then cloned in a plasmid and transformed in bacteria to be analyzed by HRM, followed by DNA sequencing. Due to the large number of transformant colonies obtained, we performed a real-time PCR assay to amplify each clone and amplicons were then subjected to High-Resolution Melting analyses (HRM). This method was used to help select clones exhibiting different melting temperatures for the same amplicon and to reduce the need to sequence all clones obtained.

To evaluate all 38 clones in triplicate, we performed three runs in different plates, and after careful analyses, we selected six clones that exhibited different melting profiles (clones 5, 6, 11, 16, 18, and 20). After this first screening, a new run was performed with the selected clones. Clone 5 was used as a control (blue horizontal line), in which clones 6, 11, 16, 18, and 20 as well as the original Ma5 vaccine were compared to. Among the six selected clones, five exhibited a different melting profile compared to the one exhibited by the original Ma5 vaccine and only the clone 20 exhibited a melting profile similar to the original Ma5 vaccine. The original Ma5 vaccine and clone 20 exhibited positive peaks while clone 11 exhibited a negative peak. On the other hand, clones 6, 16, and 18 exhibited a sinusoidal curve (Online Resource 2).

Selected clones were then subjected to DNA sequencing to identify viral subpopulations. Sequencing alignments indicated a common polymorphism in all clones in the nucleotide position T129C when compared to the original Ma5 vaccine. Additional polymorphisms were also found on clones 20 (A140G), 5 (T193C), and 11 (G284A), whereas

clones 6, 16, and 18 exhibited identical sequences (Table 1). However, we did not confirm the presence of viral subpopulations based on the nucleotide position 125.

## Discussion

The occurrence of Infectious bronchitis disease in poultry farms in Brazil and around the world, mostly in vaccinated poultry, has aroused concern in the scientific community, especially in regard the viral strains constituents of IBV vaccines.

Here, we found that vaccines against IBV containing the H120 viral strain, Massachusetts serotype, marketed in Brazil exhibited genetic variability in the S1 gene in least three sites, as the chromatogram sequencing analyses showed clear secondary nucleotide peaks. Based on the S1 sequence, previous studies have also identified the presence of viral subpopulations in commercial IBV vaccines using the Arkansas serotype (Ark) [18, 35, 36]. Another study confirmed the presence of two viral subpopulations in a commercial IBV vaccine using the VicS strain, which is currently available only in Australia [37].

Although the use of IBV vaccines is economically useful for the poultry industry, especially for broilers production, conventional and molecular epidemiological studies have confirmed the virus ability to rapidly evolve and evade successful vaccination programs. It has been demonstrated that the immunological pressure exerted by vaccines has contributed to the evolution of viral strains [38]. An evidence for this is the frequent identification of IBV strains in the poultry in Brazil, mostly in vaccinated poultry farms, in which viral strains are isolated from clinical cases of infectious bronchitis disease [6–10].

**Table 1** Polymorphisms found in the S1 gene sequences of six clones selected by High-Resolution Melting analyses (HRM) compared to the one from the original Ma5 vaccine

Samples	Nucleotide position			
	129	140	193	284
Ma5 vaccine	T	A	T	G
Clone 20	<b>C</b>	<b>G</b>	T	G
Clone 5	<b>C</b>	A	<b>C</b>	G
Clone 11	<b>C</b>	A	T	<b>A</b>
Clone 6	<b>C</b>	A	T	G
Clone 16	<b>C</b>	A	T	G
Clone 18	<b>C</b>	A	T	G

Sequencing alignments indicated a common polymorphism in all clones in the nucleotide position T129C. Additional polymorphisms were also found on clones 20, 5, and 11. The polymorphisms are highlighted in bold

In the present study, we built a 3D model for the IBV S1 protein using homology modeling. Based on the same approach, other researchers have also generated a 3D model for S1 protein using the I-TASSER (Iterative Threading Assembly Refinement) [39, 40] but in our 3D model, we further evaluated the structural domains containing the polymorphisms among the vaccines tested.

Another study using the IBV Arkansas viral strain showed that polymorphisms in the S1 protein affect its interaction with the host tissue, in addition to changing protein antigenicity [41]. The region between the amino acid residues 19 and 69 of the S1 protein is essential to bind to the host cell receptors, specifically, residues 38, 43, 63, and 69 [34]. Our analysis showed genetic variations corresponding to amino acids with different physico-chemical properties in the positions 38 and 69. In addition, the significant difference found in the S1 amino acid sequence among commercial vaccines containing the H120 viral strain, particularly in epitopes for B and T lymphocytes antigen presentation, may have contributed to the antigenic differences of vaccines.

In overpopulated poultry areas, the risk of viral escape between close poultry farms is likely, which makes vaccination a risky practice, especially when using live vaccines against IBV produced by different laboratories, which exhibit genetic variability, as we have shown here. The use of vaccines manufactured by different laboratories on the same poultry farms or the introduction of a new virus escaped from a vaccine between batches may increase the chances of genetic variability of IBV strains. There is evidence demonstrating the recombination between IBV strains from the Massachusetts serotype and environmental samples under natural conditions [42].

Some researchers have suggested that the genetic variability found in the IBV vaccines provides an advantage to the virus, allowing it to adapt to changes in the environment and to infect different host tissues [43] while others have suggested that it increases antigenic diversity, and thus, broadening the spectrum of protection, although this has not been demonstrated [35]. IBV vaccine strains from the same serotype that exhibit differences in the S1 gene sequence and are manufactured by different laboratories may present features that point to the original virus seed strain selected to produce the vaccine, as well as the number of passages performed in embryonated chicken eggs to attenuate the virus. However, it was not clear how IBV vaccines produced from the same virus seed by different manufacturers could exhibit important genetic variability and differences in the S1 gene sequence. Therefore, to investigate that, we performed a single additional passage of each H120 viral strain vaccine in embryonated chicken eggs, which is the same standard procedure used by manufacturers. Our results showed that after viral reisolation, two of the four commercial vaccines exhibited differences in the S1 gene sequence compared to

the original vaccines. For instance, vaccine C exhibited a minor viral subpopulation that was detected as a secondary nucleotide peak in the original vaccine but it turned out to be even as a predominant subpopulation after reisolation. On the other hand, a new viral subpopulation emerged in the vaccine A after reisolation since a secondary nucleotide peak was detected at the 366 position; however, the original vaccine A did not contain any viral subpopulations in that position.

The S1 gene sequence was also amplified directly from the commercial Ma5 vaccine, in which the results matched 100% with the reference strain (AY561713, GenBank). However, chromatogram analyses of the S1 gene sequence showed a secondary peak at the nucleotide position 125, indicating the presence of two viral subpopulations in the vaccine. A major peak of thymine and a minor peak of guanine were detected, in which the predominant viral subpopulation encoded for the amino acid asparagine and the minor viral subpopulation encoded for threonine.

To further investigate the presence of viral subpopulations in the Ma5 vaccine, the RNA region containing the nucleotide position 125 was amplified by PCR, and the amplicon was cloned in a plasmid, transformed, and then, transformant colonies were subjected to HRM analyses, followed by DNA sequencing. Out of 38 clones obtained, 6 were selected for DNA sequencing. This clone selection was based on the HRM results, in which selected clones exhibited different melting profiles for the same amplicon compared to the original Ma5 vaccine. The minor viral subpopulation initially identified in the chromatogram analyses of the S1 gene sequence due to the presence of a secondary nucleotide peak at the position 125 was not confirmed by HRM analyses, suggesting that perhaps its DNA melting profile might be very similar to the original Ma5 vaccine. Nevertheless, the HRM analyses identified other four viral subpopulations, indicating that the Ma5 vaccine is constituted by different IBV strains.

Additionally, the genetic variability observed in the S1 gene sequence found in the Ma5 vaccine was investigated after being inoculated once in SPF embryonated chicken eggs and different organs of the embryos were collected to reisolate the virus. The S1 gene sequence found in the original vaccine was consistently detected in all organs of the embryos, in which chromatogram analyses showed the same two nucleotide peaks (T/G) at the position 125 as seen in the original Ma5 vaccine. On the other hand, only the predominant viral subpopulation found in the original vaccine was detected in the virus reisolated from the chorioallantoic liquid, even though it is possible that the minor viral subpopulation was negatively selected in this case.

In the present study, genetic variations were found in the S1 gene sequence among the commercial IBV vaccines sold in Brazil. The amino acid substitutions caused

by this genetic variability suggest that the binding of the S1 glycoprotein to different cell receptors may be different, indicating the emergence of different IBV strains in the vaccines. In fact, it has been suggested that amino acid substitutions near the receptor binding domain of the S1 glycoprotein may change the affinity of coronaviruses to different cellular receptors [44].

Our results showed that H120 IBV vaccines marketed in Brazil exhibit genetic variability among different manufacturers, even though vaccines originate from the same virus seed. The results indicate the presence of viral subpopulations in the original vaccines, even after one single passage in SPF embryonated chicken eggs. Viral subpopulations were also found in the original cloned Ma5 vaccine. Nevertheless, when we tested consecutive batches of original vaccines from the same manufacturer, additional genetic variations in the S1 gene sequence between batches were not found. The genetic variabilities found in the H120 and Ma5 viral strains used in Brazilian commercial vaccines should be a reason of concern to manufacturers to ensure an effective immune response against IBV, especially because outbreaks are still common in vaccinated poultry farms in Brazil.

Thus, the current strategy used to control IBV does not seem to be suitable because the live attenuated vaccines can mutate rapidly during virus replication in host cells, leading to a generation of viral subpopulations that could recombine with environmental viral strains, contributing even more to IBV genetic diversity. An alternative strategy to overcome genetic variability in the final vaccine product would be the development of vaccines containing recombinant viral subunits or DNA vaccine vectors.

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**Compliance with ethical standards**

**Conflict of interest** The authors declare that they have no conflict of interest.

**Ethical approval** This project complied with the principles of the Commission for Ethics in Animal Experimentation of the Federal University of Viçosa (UFV) under Protocol No. 74/2013.

**Research involving human participants** This article does not contain any studies with human participants performed by any of the authors.



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