# Diversity of IBV Avian Coronavirus S1-region Under Different Vaccination Schemes in Poultry in Brazil.

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ABSTRACT. Infectious Bronchitis Virus (IBV) is the causative agent of a highly contagious disease that severely harms the Brazilian poultry industry. The virus is present in all parts of the country and occurs in various genotypes. This paper reports a study in which sequences of Brazilian IBV field strains are compared to the used vaccination schedule in order to find a possible relation. Samples from commercial chicken flocks were collected and screened for the presence of IBV by a nested RT-PCR targeted to the 3´UTR of all currently known members of the Gammacoronavirus genus. The viral RNA from the positive samples was submitted to a hemi-nested RT-PCR for partial amplification of the S1-gene and DNA sequencing. With phylogenetic analysis the sequences were compared to reference sequences from GenBank to determine the genotype and also compared to the used vaccination schedule. The results show that all the sequences belong to the Brazilian GI-11 genotype and consequently differ considerably from the used Massachusetts-serotype vaccine strains. Furthermore, the results suggest that there is IBV variety within one farm and that IBV field strains vary regionally. Considering IBV variety triggered by vaccination, it is due to limitations in this trial not possible to draw any conclusions. In order to make this matter clearer further research should be done on this subject.

RESUMO. Projeto de Pesquisa em Medicina Veterinária, Universidade de Utrecht - *Diversidade de região S1 do Coronavírus Aviário IBV sob diferentes programas de vacinação, em aves domésticas no Brasil* - O vírus da bronquite infecciosa (IBV) é o agente causador de uma doença altamente contagiosa que causa perdas econômicas à indústria de avicultura brasileira. O vírus está presente em todas as partes do país com registros de ocorrência de diversos genótipos. O objetivo desse estudo foi analisar a correlação de sequências de cepas de campo do IBV brasileiro comparado àquelas utilizadas no programa de vacinação das granjas estudadas. Amostras de matrizes comerciais de aves foram coletadas e analisadas quanto à presença de IBV, por meio de RT-PCR nested, com alvo molecular sendo a região 3'UTR do genêro Gammacoronavirus. O RNA viral das amostras positivas foi submetido a RT-PCR hemi-nested para amplificação parcial do gene S1 e sequenciamento do DNA. Com análise filogenética, as sequências foram comparadas às sequências de referência do GenBank - para determinação de genótipo - e também foram comparadas ao programa de vacinação empregado. Os resultados demonstram que todas as sequências pertencem ao genótipo GI-11 brasileiro e, consequentemente, diferem consideravelmente das cepas utilizadas nas vacinas, as quais utilizam o sorotipo Massachusetts. Além disso, os resultados sugerem que há variedade de IBV dentro de uma mesma granja e que as cepas de campo do IBV variam regionalmente no país. Considerando a comparação filogenetica entre a variedade de IBV e a vacinação empregada, os resultados não indicam que possa haver uma possível correlação. Almejando elucidar esse ponto, mais pesquisas devem ser feitas na área.

Key words: Avian infectious bronchitis virus, vaccination, phylogenetic analysis, IBV, Brazil

Abbreviations: IBV=Infectious bronchitis virus, RT-PCR = Reverse transcription – polymerase chain reaction, UTR=untranslated region

# Introduction

Infectious Bronchitis (IB) is a highly contagious viral disease that causes respiratory problems, decreases growth rate and drops in egg-production in domestic fowl (Gallus gallus). The main symptoms include cough, nasal discharge, watery eyes, reduced egg quality, decreased feed conversion efficiency, and increased carcass condemnation (Fraga *et al.*, 2013). Furthermore the virus can induce reduced fertility in breeders (Villarreal *et al.*, 2007). Especially in broiler IB causes a higher vulnerability for colibacillosis (Matthijs *et al.*, 2003; Matthijs *et al.*, 2005). The virus is able to replicate in all epithelial tissues and therefore harms the respiratory tract and several other organs (Cavanagh, 2007a). Currently this virus is one of the biggest sanitary problems in the Brazilian poultry industry and is responsible for considerable economic losses (Colvero *et al.*, 2015). The causative agent is Infectious Bronchitis Virus (IBV), which is an enveloped single-strand RNA virus with positive-sense genome from the Gammacoronavirus genus, Avian coronavirus species (Caron, 2010; www.ictvonline.org/virustaxonomy.asp). IBV has a high mutation rate due to a poor proofreading activity of the polymerase complex, which is typical for coronaviruses (Woo *et al.*, 2012; Abreu *et al.*, 2010; Cavanagh, 2005). This and other facts result in a huge amount of genotypes and therefore problems when the disease is being controlled by vaccination (Hashemzadeh *et al.*, 2015). Although cross-protection is described by Toro *et al.*, (2015) clinical cases show that the Massachusetts-strain (Mass) vaccination does not offer enough cross-protection against the Brazil-strain IBV lineages (Brandão, 2010; Colvero *et al.*, 2015; Villarreal *et al.*, 2007). According to the new classification the Brazil-type strains are further in this study referred to as lineage GI-11 (Valastro *et al.*, 2015). The effects of this poor cross protection are IB outbreaks, even in vaccinated chicken flocks (Brandão, 2010; Di Fabio *et al.*, 2000). As said before, the mutation speed of IBV is high, resulting in several Brazil-specific genotypes and (sub) lineages (Villarreal *et al.*, 2010). Since a Mass- IBV was first detected in Brazil in 1957 the number of variants increased strongly (Silva, 2010). A 2008 study showed that only 8.0% of the 75 studied Brazilian IBV samples appeared to be of the Mass serotype (Sandri *et al.*, 2008). This large variety of not-Mass serotype IBVs is also shown by Chacón *et al.*, (2007), who found that from his studied samples over 63.4% was positive for a not-Mass type serotype of IBV. In a later study the genetic similarity between the IBV lineages was taken into account and the results showed that the present GI-11 strain IBVs differ from 62.4 up to 70.7% from the Mass serotype (Chacón *et al.*, 2011). This lack of genetic similarity supports the occurrence of IB outbreaks in vaccinated flocks on account of insufficient cross-protection from the used vaccination. The capability of cross protection is an important matter on account of the Brazilian agricultural policy. Since in 1979 two more live Mass-type vaccinations against IBV were admitted, no further legal alterations were made ever since (Silva *et al.*, 2010). This means that the Brazilian poultry industry at this moment is only allowed to use live vaccinations against the Mass-type IBV while the most dominantly present strains are of the GI-11 type, resulting in outbreaks and economic losses.

For the comparison of different types of IBV the most chosen target is the S- gene (Valastro *et al.*, 2016; Caron, 2010). The S-gene is a region in the genome that codes for a protein (S) that is post-translationally cleaved into two subunits, S1 and S2. The carboxyl-terminal S2 is anchored in the viral membrane and the amino-terminal S1 forms a spike on the outside of the virion (Cavanagh *et al.*, 1986a). The S1 is the most important protein when it comes to the neutralization by antibodies (Cavanagh and Naqi, 1997; Winter *et al.*, 2008). Besides being neutralized by antibodies the S-protein is also responsible for being bound there by the host cell and entering the cell though the membrane (Cavanagh *et al.*, 1986b). The S1-gene is not only responsible for the actions mentioned above but it is one of the mutation hot-spots in the genome (Brandão, 2013; Thor *et al.*, 2011; Caron, 2010). Caron (2010) describes 20 to 25% difference in S1 amino acids, presumably even more. Caron (2010) states that cross-protection is poor when amino acid difference is higher than 5%. This means that immunity to one serotype does not automatically mean immunity to another. Cavanagh, *et al.*, (2007b) even described S-gene recombination rate of 50%.

The aim of this study is to compare partial S1-gene sequences of Brazilian field IBV strains in poultry under different vaccination Schedules. The main question is whether IBV variation is triggered by vaccination.

# Materials and Methods

## Sampling

For this study 20 samples were taken from the trachea, lung, kidney, oviduct and cecal tonsils of commercial chickens in the municipality of Santa Maria de Jetibá, Espírito Santo state, Brazil. The samples were taken from layer, rearing and layer farms. The farms were managed according to modern views on management but reported problems of continually emerging respiratory symptoms and high mortality. The dataset was enlarged with samples sent to the Laboratório de Biologia Molecular Aplicada e Sorologia, Departamento de Medicina Veterinária Preventiva e Saúde Animal, Universidade de São Paulo, Brazil, including sequences already available and further FTA cards that had to be retested during this work. These samples were sent by local veterinarians, farm managers and staff from a pharmaceutical company (MSD Animal Health) for IBV detection in commercial chicken flocks from both healthy and sick birds (disease status was not considered in this study). Layer, broiler and broiler-breeder flocks were present among the sampled flocks. From each sample the information considering the state of origin and flock-specific vaccination scheme against IB was collected. The sample-collectors pressed fresh organ samples on Whatman® FTA® cards (GE Healthcare Life Sciences). On one card, samples from a maximum of five chickens were collected. The samples were taken during a two year (2014-2016) period in the states of Paraná, São Paulo, Minas Gerais, Goiás, Espírito Santo and Pernambuco (figure 1).


Figure 1: Map with the Brazilian states Pernambuco: PE; Espírito Santo: ES; Minas Gerais: MG; São Paulo: SP; Paraná: PR; Goiás: GO

## IBV screening

RNA was extracted out of a quarter of each circle of the FTA the card with PureLink® RNA Mini Kit (Ambion™ Brazil), in accordance with the manufacturer's instructions. To make cDNA the reverse-transcription reaction was performed with 3.5 µl of denatured RNA-sample and 6.5 µl of RT mix. The RT mix contained 1X First-Strand Buffer, 0.01 M of DTT, 1 mM of each dNTP, 25 ng of Random primers (Life Technologies Brazil), 100 U of M-MLV Reverse Transcriptase™ (Life Technologies Brazil) and RNase free water to final volume of 10 µl. The reaction was carried out in accordance with the manufacturer's instructions. The obtained cDNA was subjected to a hemi-nested PCR targeted to the highly conserved 3´UTR of the virus genome (figure 2) with primers designed by Cavanagh *et al.,* (2002). Firstly, sense specific primer UTR41+$^{c}$ was used in combination with anti-sense specific primer UTR11-$^{c}$ to amplify a 266 base pair (bp) DNA fragment (See table 1 for primer sequences). For this PCR, 2.5 µl of cDNA sample was used with GoTaq™ Green Master Mix 1X (Promega Brazil), 400 nM of each forward and reverse primers and RNAse-free water to final volume of 25 µl. From this PCR product, 1 µl was amplified in a Hemi-nested PCR with sense specific primer UTR41+ and anti-sense

specific primer UTR31- with the same concentration of reagents and amplification conditions of the first round PCR. With this Hemi-nested PCR a DNA fragment of 179bp was obtained. Hemi-nested PCR products were screened for the presence of a specific fragment in comparison with GeneRuler 100 bp DNA Ladder (Thermo Scientific) with electrophoresis using 1.5% agarose gel stained with SYBR Safe DNA Gel Stain (Invitrogen, Carlsbad, CA, EUA) and 0.5x TBE buffer. The gel was checked for positive bands under UV-light. Samples that were positive for IBV were then tested using a hemi-nested PCR targeted to the S-gene. With every step positive and negative controls were included. As a positive control a H120 IBV vaccine-strain was used and DEPC-treated water functioned as negative control.

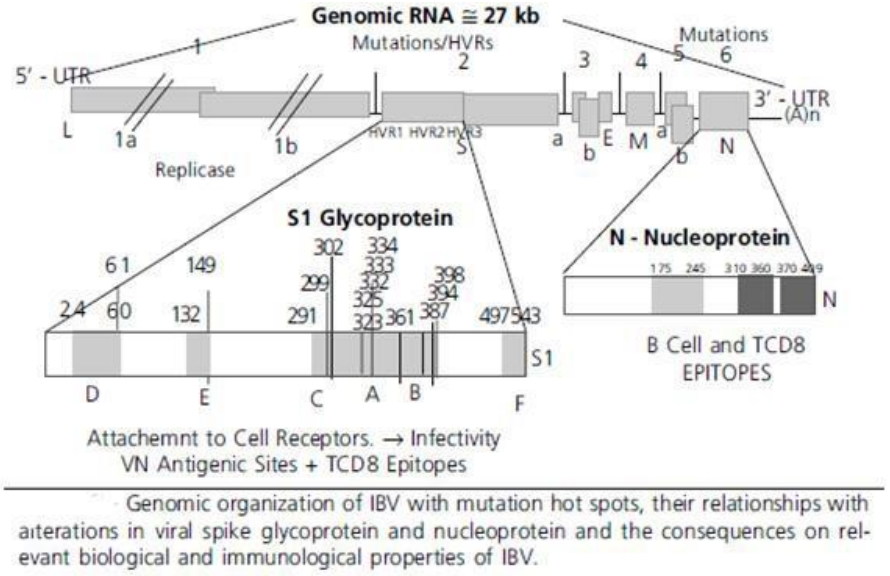


Figure 2 - Illustration from: (Montassier, 2010

S-gene targeted PCR and sequencing

Samples positive to the screening PCR were subjected to a partial amplification of the S1-coding region. To this end, the S-gene targeted PCR the forward primer IBVS was used in combination with IBVAS1 (see table 1) and for the Hemi-nested PCR the IBVAS1 primer was replaced by the primer IBVAS2 as described by Torres *et al.,* (2014) for a 445bp amplicon. Under UV light positive bands were cut out of the agarose gel and the DNA was purified with the Gel Band purification Kit™ (GE Healthcare), in accordance with the manufacturer's instructions. The purified DNA was submitted to bidirectional DNA sequencing with Big Dye™ Terminator v3.1 (Cycle Sequencing Kit, (Applied Biosystems™)) using IBVS and IBVAS2 and ABI-3500 *Genetic Analyzer* (Applied Biosystems™). The dataset was completed with 27 other sequences obtained from the laboratory´s routine IBV detection.

Table 1: Primers

| Name | Sequence | Position in genome |
|---|---|---|
| UTR41+[c] | ATGTCTATCGCCAGGGAAATGTC | 27342 to 27364[a] |
| UTR31-[c] | GGGGCGTCCAAGTGCTGTACCC | 27501 to 27520[a] |
| UTR11-[c] | GCTCTAACTCTATACTAGCCTA | 27586 to 27607[a] |
| IBVS | ACTCTTTTGTGTGCACTAT | 544 to 568[b] |
| IBVAS1 | CTTTGGTCTCATTAGAAGTAAAAAC | 28 to 46[b] |
| IBVAS2 | TAGYYACASDAACTYTTAAATTATA | 448 to 472[b] |

(UTR: Cavanagh *et al.,* 2002; IBV: Torres *et al.,* 2014). [a]The nucleotide positions correspond to those in the sequence of the IBV Beaudette genome, GenBank accession number: M95169. [b]The nucleotide positions correspond to those in the sequence of H120, GenBank accession number: M21970.

Sequence and phylogenetic analyses

The obtained sense and anti-sense sequences were submitted to quality evaluation using Phil's Read Editor (Phred) online application (http://asparagin.cenargen.embrapa.br/phph/). The sense and anti-sense sequences were assembled together with the Cap-Contig application implemented in Bioedit 7.0.9.0 software. The sequences were aligned with retrieved reference sequences from GenBank (National Center of Biotechnology Information). For the alignment with the reference sequences all the sequences were shortened to 112bp, in order to achieve a dataset with same-length sequences. With MEGA7 software a nucleotide phylogenetic tree was created (Neighbor-joining, Maximum Composite Likelihood model, 1,000 bootstrap replications). This tree was used to determine the serotype of the found IBV lineages. After this, the same type of tree (Neighbor-joining, Maximum Composite Likelihood model, 1,000 bootstrap replications) was constructed with only the GI-11 type sequences for an easier visualization of the sequences and the vaccine schedule dependent pattern of segregation. For this tree the sequences were shortened to 283bp in order to achieve same-length sequences only. This tree was used to determine whether there are any correlations between IBV evolution and vaccination schedules.

To compare the S1-gene variety of IBV with the vaccination program the used vaccine schedules should be divided into groups, certainly because not only the vaccine type is taken into account but also the number of vaccinations. In order to clarify this, the groups are to be seen in table 2.

Table 2, Vaccination schedules divided in groups:

| Vaccination schedule | Reference number |
|---|---|
| Live Mass-strain (Ma5 /H120) (MSD Animal Health) at Day 1 | V1 |
| Live Mass-strain (Ma5 /H120) (MSD Animal Health) + B-48 (CEVAC®) at Day 1 | V2 |
| IBmulti, killed D274 and H120) (MSD Animal Health) at Day 1 | V3 |
| Several (>1) live Mass-strain vaccinations | V4 |
| Several (>1) live Mass-strain (Ma5/H120) (MSD Animal Health) vaccinations in early life combined with several (>1) inactivated IBmulti (MSD Animal Health) vaccinations afterwards | V5 |
| No information available | V(x) |

# Results

From the 20 collected samples 8 proved to be positive for IBV. After processing these samples 2 DNA sequences were obtained.

The dataset was completed with 27 other sequences that were either already available at the laboratory or obtained after retesting of stored FTA cards.

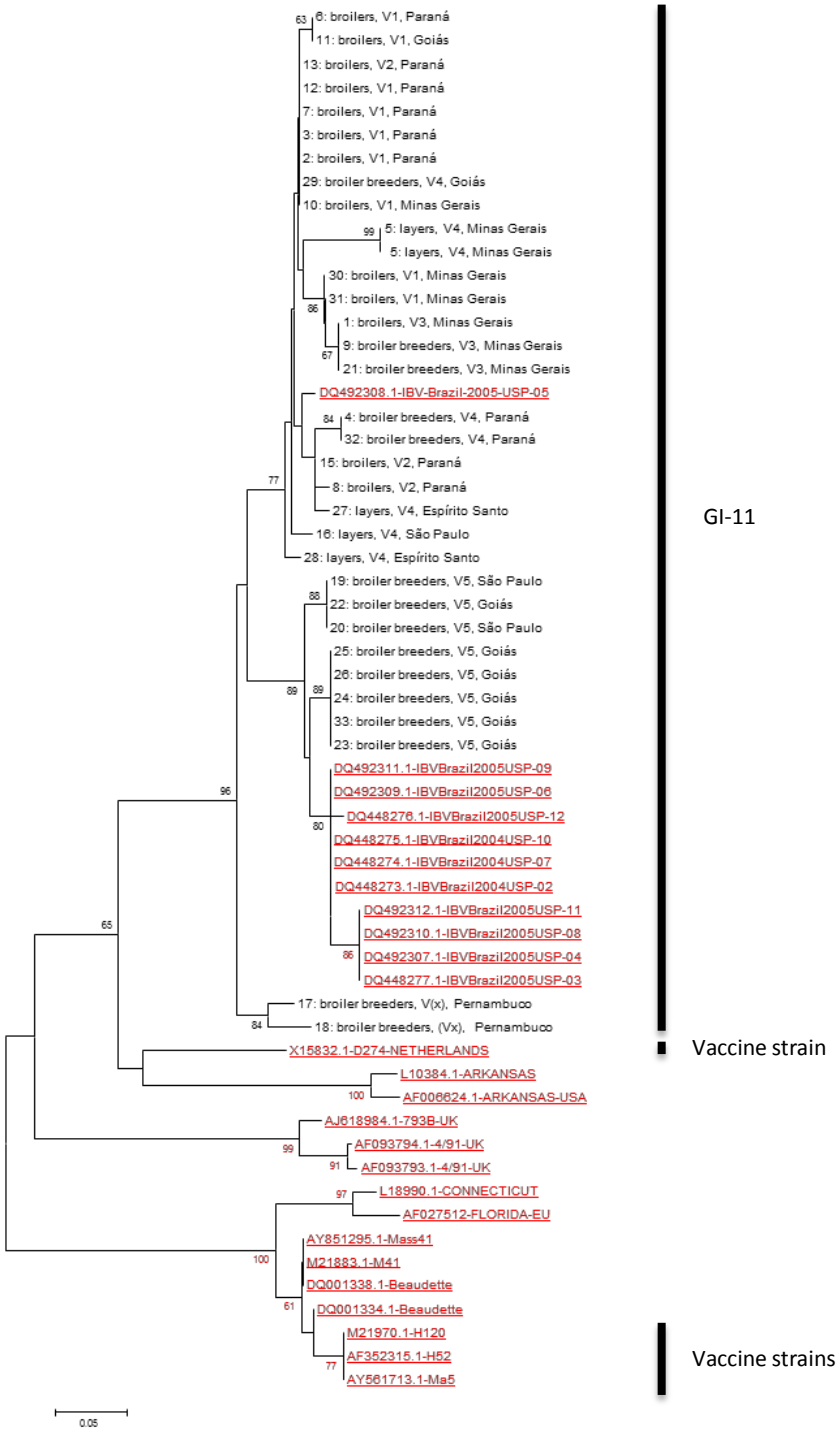


Figure 3: Phylogenetic distance tree with the neighbor-joining algorithm and MCL model for 112bps of the S1-gene showing serotypes/genotypes (with the access number from GenBank; red and underlined) and samples included in this study. The numbers above each node represent the bootstrap values for 1000 replicates (only values greater than 50% are shown). The bar represents the number of nucleotides per site. The samples included in this study are designated as: sample number, bird-type, vaccination schedule and state of origin.

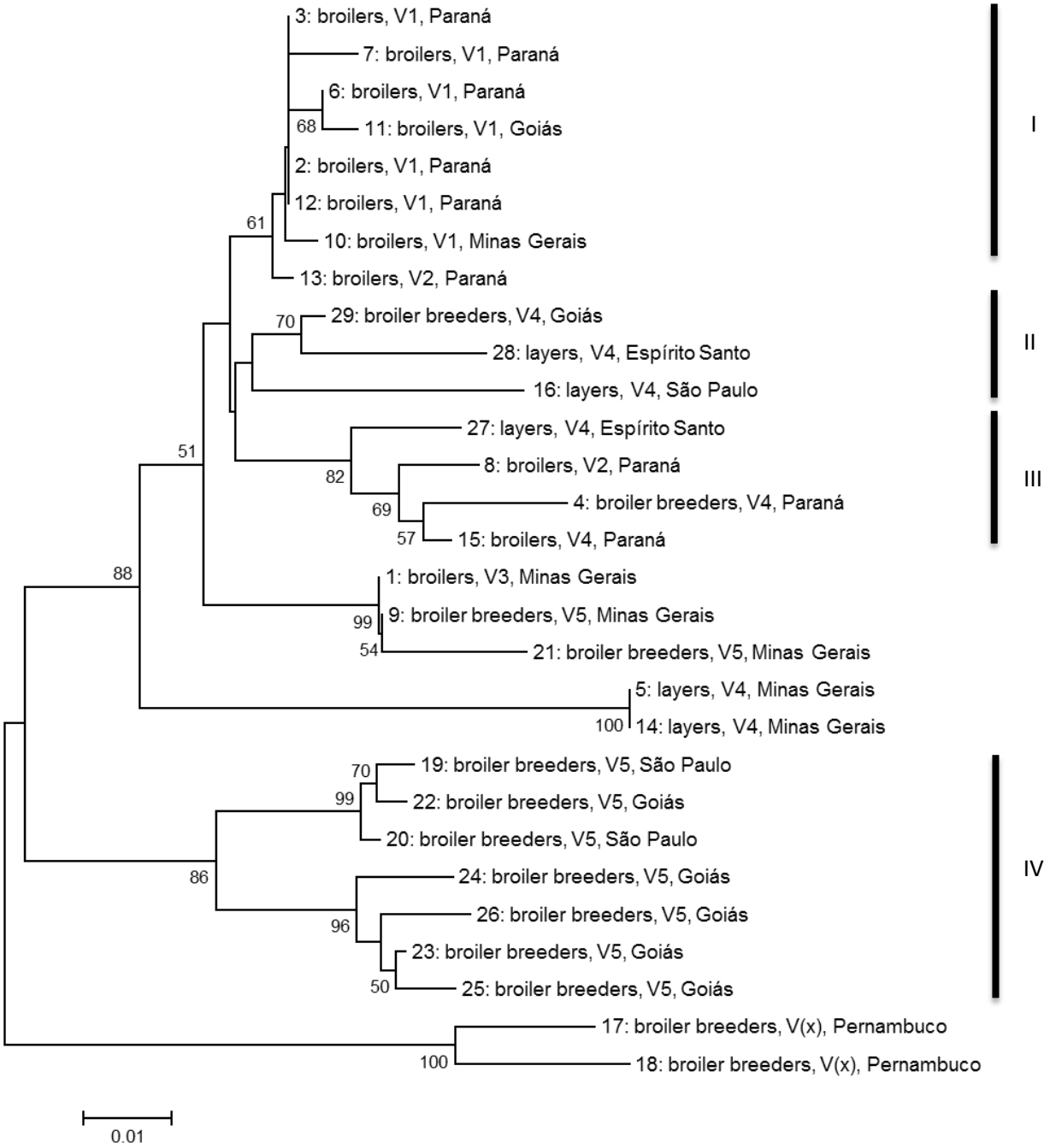


Figure 4: Phylogenetic distance tree with the neighbor-joining algorithm and MCL model for 283bps of the S1-gene showing samples included in this study. The numbers above each node represent the bootstrap values for 1000 replicates (only values >50% are shown). The bar represents the number of nucleotides per site. The samples are designated as: sample number, bird-type, vaccination schedule and state of origin.

According to the two phylogenetic trees (figure 3 and 4) there can be stated that all the found sequences belong to the Brazilian GI-11 genotype; consequently the sequences differ considerably from the used vaccination strains. Figure 3 shows a separation based on state of origin as well as a separation based on the used vaccination schedule. Figure 4 shows that there are different types of IBV present on the same farm and provides a clearer visualization of clusters based on the used vaccination schedule and the state of origin.

# Discussion

In this study, chicken-samples were collected in the field and screened for the presence of IBV and the S1 coding region was partially sequenced for the positive samples. The results were used to make a phylogenetic tree in which the genetic distance between the samples was compared with the geographical location and the used vaccination schedule.

When viewing the results, the first notable characteristic is that all the studied IBV strains are of the GI-11 type. In figure 3 is to see that all the studied virus strains are together in one cluster with the reference GI-11 strains from GenBank (strains retrieved from GenBank are underlined). When one takes into account that these samples have been taken in Brazil and that the vast majority of the Brazilian field strains belong to the GI-11 lineage this is an expected finding (Sandri *et al.,* 2008; Chacón *et al.,* 2007; Villarreal *et al.,* 2007).

Secondly, the results show that the found sequences differ notably from the used (Massachusetts) vaccine strains Ma5, H120 and D274. The mean amino acid identity of GI-11 strains and Massachusetts strains is described by Villarreal *et al.,* (2007) and was only 68.93%. This fact may support the inadequate protection of Brazilian chicken flocks with IB outbreaks and production loss as a consequence (Brandão, 2010; Colvero *et al.*, 2015; Villarreal *et al.,* 2007; Di Fabio *et al.,* 2000). The main purpose of this study is to investigate whether the evolution of IBV is triggered by vaccination. As stated in the introduction, all the used live vaccinations are of the Mass-strain IBV, since no other type of live vaccination is permitted in Brazil (Silva *et al.,* 2010). The used vaccination types are Ma5 (MSD Animal Health), H120 (MSD Animal Health), B-48 (CEVAC®), and IBmulti (MSD Animal Health). The Ma5- vaccine is an attenuated live Mass 5 virus strain which is often used by spray or drinking water application (Bande *et al.,* 2015). It induces local as well systemic immunity against a wide range of IBV´s (MSD Animal Health). The H120-vaccine is the most common among the live IBV vaccines. It is a mild vaccine which is usually used for first time vaccinations. The H120-vaccine is known for spreading quickly within the flock (Matthijs *et al.,* 2008). It does, however, not offer long lasting immunity (MSD Animal Health). CEVAC®´s live B-48 vaccine contains the live strain of Mass B-48 strain. The only inactivated vaccine that was used is IBmulti (MSD Animal Health). This vaccine contains inactivated strains of Mass41 and Netherlands D274. According to MSD Animal Health this vaccine should be used for re-immunization only. The first vaccination should be performed with a live vaccine. In this study not only the type of vaccination was taken into account but also the number of vaccinations. Normally broilers get vaccinated on day 1 with a live mass-type vaccine. Layers and broiler breeders receive several vaccinations during the rearing and productive period (Bande *et al.,* 2015). The birds in this study usually got vaccinations during early life with live vaccines and booster vaccinations with inactivated vaccine.

As broilers and longer living birds are vaccinated according to different schedules, usually V1/2 for broiler and V4/5 for layers and breeders, the bird type is also a variable to be taken into account in the analysis of vaccine schedules. In cluster I from figure 4 only broilers are assembled. The middle part shows a mixture of broilers, layers and breeders and the lowest part shows layers and breeders only. When we see cluster I in figure 4 we can state that all the lineages originate from broilers that received the same vaccination schedule (V1). In the middle part of the tree some broiler-strains are present as well. The case is that these birds received a for broilers uncommon vaccination schedule (V3/V4) (Marangon and Busani, 2006). This can mean that the difference in S1 sequence might depend on the vaccination schedule, otherwise these broiler strains should be assembled in cluster I. Mainly because the strains are from the same state (Paraná) as the ones in cluster I (see figure 1 for the Brazilian states).

Regarding the layers and broiler breeders IBV sequences, the first thing to notice is that there are no bird-type specific clusters. When the vaccination schedules are being taken into account, there can be seen that there are two clusters in which V4 dominates (clusters

II and III). The fact that these samples have been collected in different states may be evidence for the distribution in clusters according to the same vaccination schedule.

The same sort of clustering can be observed for V5. The clusters with broiler breeders from the states of Minas Gerais, São Paulo and Goiás were all vaccinated according to V5. Their gathering into one cluster (cluster IV) may point out the importance of vaccination in S1 variety. Nonetheless, the clusters from, mainly, the broiler breeders are also based on the state they come from and several strains that originate from the same farm, such as sequences 22, 23, 24, 25 and 26. These sequences all come from the same breeder-farm in the state of Goiás. Because of these confounding variables, an influence of the vaccination schedule for long-living birds cannot be extracted from the data.

About the decision to divide the vaccination programs into groups some comments have to be made. The choice was made to make the first division on the number of vaccinations and the schedules were divided into two groups. The first group (V1, V2 and V3) received only one vaccination on day 1. The second group (V4 and V5) consisted of the birds that received multiple vaccinations. This is basically a division between the schemes for broiler and layers/breeders. To classify further the type of vaccine was taken into account. The vaccination types were divided in Ma5/H120, B-48 and IBmulti. The choice to combine Ma5 and H120 into one is made because both strains are of the same Mass-serotype (Li *et al.,* 2012). These choices combined led to the division in groups as seen in table 2. Regarding the speculation that there is a relation between S1 variety and vaccination schedule there are several points that need to be considered. At first this study includes only 29 samples, a quite low number, and a very short sequence of the spike gene (283bp). To get a broader view on the distribution of IBV in Brazil there should have been more samples included from flocks with different vaccination schedules. Hopefully a study with a higher number of samples based on statistical sampling design and full S sequencing will show a clearer picture of the possible correlations between vaccinations and S1- variety. Together with the insufficient number of samples, some samples have been included in the study without having information on vaccination history.

Furthermore, understanding the IBV clustering pattern based on vaccination schedule is difficult when most of the vaccination schedules are highly similar. The broiler breeders included in this study received in general V5. A number of them are from the same farm and are clearly clustered together for this reason. Broiler breeders that received vaccination schedule V4 are not included in this cluster and unfortunately, the number of samples is not big enough to make any bold speculations.

In the results it is stated that a possible explanation for the division in clusters is the state from which the sample is originative. Abreu *et al.,* (2010) proved that there are several regional variants of IBV present in Brazil. Especially in figure 3 is to be seen that the samples taken in the same state clearly cluster together. This could possible also explain the position of the two virus strains from the state of Pernambuco. Most unfortunately no information on vaccination history was available from these two samples. The samples from Pernambuco have a special position in both the phylogenetic trees. This could be due to the location of Pernambuco state in the very Northeast of Brazil (see figure 1). Considering the vast distances between the sampling locations and the study of Abreu *et al.,* (2010), the special position of the Pernambuco samples in the tree might be based on regional differences between the various IBV field strains. An interesting finding is that IBV variety also occurs on a much shorter distance, even on the same farm. The sequences 22, 23, 24, 25 and 26 are from samples that are collected on the same farm. As seen in cluster IV of figure 4 there is variety even within one farm.

There are a few things that should be remarked on the materials and method of this study. During the processing of the Whatman® FTA® cards (GE Healthcare Life Sciences) there were lots of samples that proved to be positive for IBV but from which it was not possible to obtain a usable sequence. This can be due to the insufficient viral load on the card or any other problem with the cards. In this study the use of the easy usable Whatman® FTA® cards(GE

Healthcare Life Sciences) is chosen because of the long distances between the sampling locations and the high temperatures in the Brazilian countryside (Biswal *et al.*, 2016). The biggest limitation factor in this study is the insufficient amount of samples. More samples are needed to draw firm conclusions.

As far is known to the author this study is the first attempt to find any relation between IBV diversity and vaccination. As a consequence, no previously reported information is available on this subject. If such a relation or its absence is found, this could make a difference to the poultry industry as better predictions of IBV evolution and adequacy of vaccination schedules could be designed in order to control not only IB occurrence but also IBV evolution. Although the outcomes of this trial show no clear relation between IBV variety and vaccination, vaccination and the enhanced variety of IBVs should be investigated more thoroughly in order to keep up with the ongoing emergence of new IBV strains.

As a consequence there can be stated that there probably is no relation between diversity within the GI-11 type IBV and vaccination. The most likely explanation for the found IBV variety is that the variation between the strains is based on the region of occurrence.

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