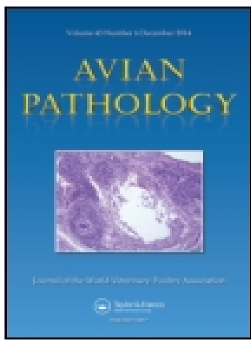

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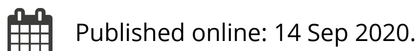

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ORIGINAL ARTICLE

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Genotypic characterization and molecular evolution of avian reovirus in poultry flocks from Brazil

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ABSTRACT
Avian reovirus (ARV) is one of the main causes of infectious arthritis/tenosynovitis and malabsorption syndrome (MAS) in poultry. ARVs have been disseminated in Brazilian poultry flocks in the last years. This study aimed to genotype ARVs and to evaluate the molecular evolution of the more frequent ARV lineages detected in Brazilian poultry-producing farms. A total of 100 poultry flocks with clinical signs of tenosynovitis/MAS, from all Brazilian poultry-producing regions were positive for ARV by PCR. Seventeen bird tissues were submitted to cell culture and ARV RNA detection/genotyping by two PCRs. The phylogenetic classification was based on σC gene alignment using a dataset with other Brazilian and worldwide ARVs sequences. ARVs were specifically detected by both PCRs from the 17 cell cultures, and σC gene partial fragments were sequenced. All these sequences were aligned with a total of 451 ARV σC gene data available in GenBank. Phylogenetic analysis demonstrated five well-defined clusters that were classified into lineages I, II, III, IV, and V. Three lineages could be further divided into sub-lineages: I (I vaccine, Ia, Ib), II (IIa, IIb, IIc) and IV (IVa and IVb). Brazilian ARVs were from four lineages/sub-lineages: Ib (48.2%), IIb (22.2%), III (3.7%) and V (25.9%). The Bayesian analysis demonstrated that the most frequent sub-lineage Ib emerged in the world around 1968 and it was introduced into Brazil in 2010, with increasing spread soon after. In conclusion, four different ARV lineages are circulating in Brazilian poultry flocks, all associated with clinical diseases.

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ARV; tenosynovitis; arthritis; malabsorption syndrome; phylogeny; viral evolution

RESEARCH HIGHLIGHTS

- One-hundred ARV-positive flocks were detected in all main poultry-producing regions from Brazil.
- A large dataset of 468 S1 sequences was constructed and divided ARVs into five lineages.
- Four lineages/sub-lineages (Ib, IIb, III and V) were detected in commercial poultry flocks from Brazil.
- Brazilian lineages shared a low identity with the commercial vaccine lineage (I vaccine).
- Sub-lineage Ib emerged around 1968 and was introduced into Brazil in 2010.

Introduction

Avian reovirus (ARV) belongs to the genus *Orthoreovirus*, family *Reoviridae*, and can infect different species of birds worldwide. Chickens can present stunting syndrome, respiratory/enteric diseases, immunodepression, malabsorption syndrome (MAS), and even secondary infections by other microorganisms (Jones, 2013). The most common disease caused by ARV is arthritis/tenosynovitis, with birds having difficulty to move in the flocks and presenting poor feed conversion and low weight gain. Flock mortality increases due to starvation and dehydration in ARV-infected flocks. These clinical manifestations are pronounced in birds from 4–7 weeks old. Therefore, ARV is a serious health problem for broiler producers (Jones, 2013; Nham *et al.*, 2017). In addition, chicken carcasses must be discarded at slaughter due to the unsightly appearance of affected hock joints, resulting in additional economic losses (Souza *et al.* 2018).

ARV has a non-enveloped, icosahedral virion with a double capsid structure, about 80 nm in diameter (van der Heide, 2000). The ARV genome is a double-stranded RNA (dsRNA) with 10 segments classified into three size classes: large (L1, L2 e L3), medium (M1, M2 e M3), and small (S1, S2, S3 e S4). M1 (coding for µA protein) is the most conserved (95% similarity) among all segments, so it has been the target region for the detection by RT–PCR (Tang *et al.* 2016). S1 (coding for σC minor capsid protein) and M2 (coding for µB outer-capsid protein)

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are the most variable segments in the whole genome (Su *et al.* 2006). S1 is also involved in the cell attachment and production of the neutralizing antibodies (Kant *et al.* 2002; Day, 2009; Jones, 2013). The diversity in the σC gene has been used to classify ARVs into five lineages since the first taxonomy proposal (Kant *et al.* 2002; Liu *et al.*, 2004). More recent studies based on S1 re-classified this virus into six lineages: I to VI (Lu *et al.*, 2015; Ayalew *et al.*, 2017).

ARV horizontal transmission occurs by the shedding of virus particles through the digestive tract, since most birds are likely to become infected via the faecal-oral route, but transmission by the respiratory tract may also occur. In addition, reoviruses may enter via the broken skin of the chicks from the litter, and the infection can spread to hock joints. Vertical transmission to the progeny was also reported at a low rate (Jones, 2013). Infections by low pathogenic strains are usually asymptomatic, but contamination with virulent strains in immunodepressed birds results in tenosynovitis and/or MAS (Jones, 2013). Free-living birds were demonstrated to carry virulent ARVs genetically related to chicken ARVs, implicating free-living birds as reservoirs for the transmission among the poultry farms (Lawson *et al.*, 2015).

Dissemination of pathogenic ARVs in poultry flocks can be controlled by immunization with live and inactivated vaccines. The main goal is to eliminate vertical transmission and to provide maternal antibodies to the progeny, preventing infection of the chicks (Sellers, 2016). Four main strains (S1133, 1733, 2408, and 2177) have been used as live vaccines to immunize poultry flocks. However, these strains have not provided adequate protection against field challenges (Chénier *et al.*, 2014; Tang & Lu, 2015; Tang *et al.*, 2016; Chen *et al.*, 2019). All these commercial vaccine strains are from lineage I, while lineages from II to VI have been frequently reported in the field in the last years. Previous reports have already demonstrated that immunization with one lineage does not cross-protect against other lineages (Tang & Lu, 2015). In addition, most ARVs detected in clinical cases of tenosynovitis/MAS are from lineages II to VI, so they are genetically and antigenically distinct from current reovirus vaccine strains (Sellers, 2013; Sellers, 2016). ARVs from lineage I were also detected in vaccinated chickens presenting tenosynovitis; therefore, immunization with the same lineage could not provide adequate protection to the flocks (Troxler *et al.*, 2013).

An increased rate of ARV-related diseases, caused by pathogenic strains of different lineages, has been demonstrated in North and South America in the last decade (Sellers, 2016; Ayalew *et al.*, 2017). In Brazil, previous reports demonstrated that arthritis was responsible for partial/total condemnation of several carcasses in slaughterhouses (Souza *et al.*, 2018; Reck *et al.*, 2019). Therefore, this study aimed to investigate the ARV lineages/genotypes in commercial poultry flocks from Brazil in recent years. To provide adequate genotype identification, molecular phylogeny, and classification of these viruses into lineages and sub-lineages was reviewed based on ARV σC gene sequences from different poultry-producing regions in the world. Additional temporal analyses were performed to determine the time of the most recent common ancestor (tMRCA) and to study the recent evolutionary history of the sub-lineages detected in the Brazilian field samples.

Materials and methods

Clinical samples

Tissue samples (tendons, intestines, heart, liver, joints) were obtained from necropsied birds presenting clinical signs of ARV infection in producing broiler flocks, or from chicken carcasses with tenosynovitis lesions detected in slaughterhouses from 2015 to 2017. One-hundred samples were positive in ARV-specific PCR in a diagnostic laboratory (Simbios Biotecnologia, Cachoeirinha, Brazil) in these two years, demonstrating the wide occurrence of ARV in Brazil. The broiler farms and the slaughterhouses were located in four important poultry-producing regions: South – S (73%), Southeast – SE (20%), Midwest – MW (5%) and Northeast – NE (2%) (Figure 1).

Some tissue samples were selected by convenience to represent the main clinical diseases, collecting at least one tissue from each flock. Clinical samples from chicken carcasses presented visual alterations in the tibiotarsal joint, detected in the slaughterhouse (Supplementary Figure 1). Histopathological findings from these samples were characterized by synovial cell hyperplasia, predominantly mononuclear inflammatory infiltrate, associated with cellular debris, erythrocytes and fibrin in the joint space, as previously reported (Souza *et al.*, 2018). Some tissues from necropsied birds presenting MAS clinical signs were also collected. These samples were stored at −20°C and later submitted to ARV isolation in cell culture as previously described (van der Heide *et al.*, 1981).

Virus was recovered from a total of 17 samples after the viral culture, including 12 samples from chicken carcasses and five from necropsied birds presenting MAS (three from flocks with poor feed conversion rate and presence of soft faeces, and two presenting bone fragility, necrosis of the head of the femur with purulent exudates). Sixteen samples were from farms/slaughterhouses located in the South, and one sample was from the Southeast Brazilian region (Figure 1, Table 1).

RNA extraction, RT–PCR, PCR and sequencing

RNA was extracted from cell cultures using silica adsorption method with NewGene Prep and Preamp

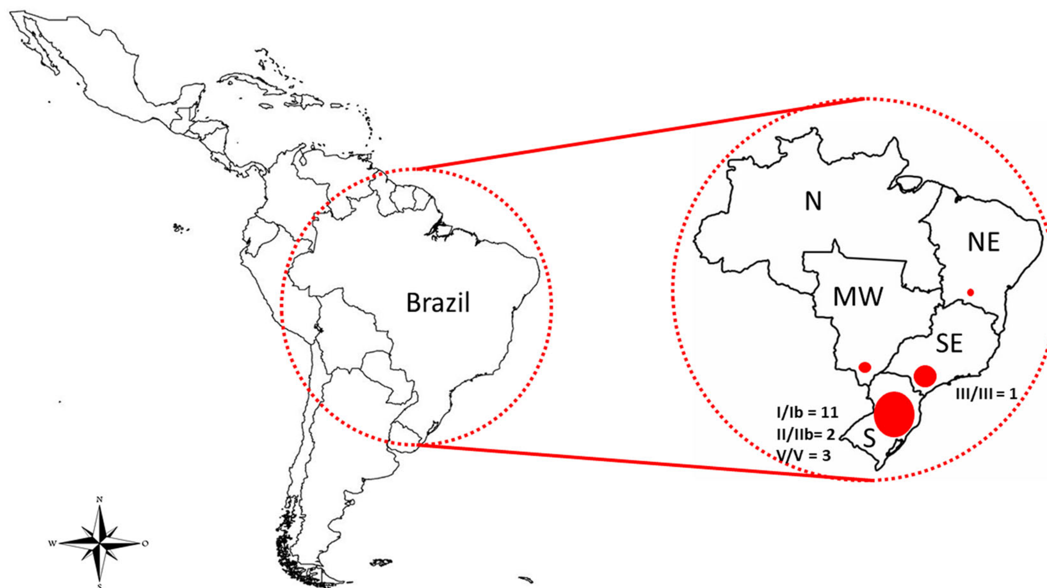

Figure 1. Geographical distribution of ARVs in the four main poultry-producing Brazilian regions. The diameter of each solid circle is proportional to the number of samples analysed in each region.

Table 1. General data of the 17 ARV-positive Brazilian poultry tissue samples included in the study.

Id Sample	Lineage	Year of isolation	State	Clinical signs	Accession number
BR/2290	II	2016	Parana	Arthritis	MK297893
BR/2991	I	2016	Parana	Arthritis	MK297894
BR/2992	I	2016	Parana	Arthritis	MK297895
BR/3118	I	2016	Parana	Arthritis	MK297896
BR/3121	I	2016	Parana	Arthritis	MK297897
BR/3290	I	2016	Parana	Arthritis	MK297898
BR/3292	V	2016	Parana	Arthritis	MK297899
BR/3522	II	2016	Parana	Arthritis	MK297901
BR/3525	I	2016	Parana	Arthritis	MK297902
BR/5237	V	2016	Parana	Arthritis	MK297907
BR/5246	V	2017	Parana	Arthritis	MK297908
BR/5881	III	2017	São Paulo	Arthritis	MK297909
BR/4154	I	2016	Parana	MAS	MK297903
BR/4526	I	2016	Santa Catarina	MAS	MK297904
BR/4527	I	2016	Parana	MAS	MK297905
BR/4535	I	2016	Santa Catarina	MAS	MK297906
BR/3295	I	NI	Parana	MAS	MK297900

Note: NI = not informed.

kits according to the manufacturer's instructions (Simbios Biotecnologia). A specific reverse transcription – real-time quantitative PCR (rqPCR) for M1 segment was used to detect ARV in cell culture (Tang & Lu, 2016). All rqPCR assays were carried out in the StepOne thermocycler (Applied Biosystems, Foster City, CA) with the following conditions: 37°C for 30 min (reverse transcription), 95°C for 3 min, 40 cycles of 95°C for 15 s, 60°C for 60 s.

A σC gene region, encompassing the main antigenic fragments of the protein, was selected for reverse transcription – nested PCR (rnPCR) amplification as previously described (Lu *et al.*, 2015; Ayalew *et al.*, 2017; Palomino-Tapia *et al.*, 2018; Souza *et al.*, 2018). Briefly, reverse transcription and the first round PCR were performed with primers ReoS-F1 5 "- TCR CAG CGA AGA GAR GTC G-3" and ReoS-R1 5 "- GTC GAT GCC SGT ACG CAC G – 3" (targeting a region of 938 bp) and using the following conditions: 37°C for 30 min (reverse transcription), 95°C for 3 min, 30 cycles of 95°C for 20 s, 50°C for 40 s and 72°C for 60 s (Veriti – Applied Biosystems). Second-round PCR was carried out with primers ReoS-F2 5 "- TGA TAC TST CMT TGA CTT CGA ACG-3" and ReoS-R2 5 "- CAT CTA CAT ATT RAC TAT CAG TKC G -3" (targeting a region of 652 bp) and the following amplification conditions: 95°C for 3 min, 35 cycles of 95°C for 20 s, 50°C for 40 s and 72°C for 60 s. The rnPCR amplification products were then evaluated in polyacrylamide gel electrophoresis. All amplified DNA samples were purified using the NewGene Preamp kits (Simbios Biotecnologia) and quantified by agarose gel electrophoresis 2%. Amplified products were sequenced using sense and antisense primers of the second-round PCR (ReoS-F2 and ReoS-R2) and BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems Inc., Norwalk, CT, USA). The sequencing rounds were performed in the thermocycler Veriti 96 (Applied Biosystems Inc.) with the same conditions of the second-round rnPCR above. All samples were purified by ethanol/ EDTA/sodium acetate protocol and the precipitated DNA products were diluted in formamide Hi-Di, denatured (95°C for 2 min) and injected in the automated DNA sequencing ABI-Prism 3500 Genetic Analyzer (Applied Biosystems Inc.) by the company ACTGene (Porto Alegre, RS, Brazil). The chromatogram sequences from both strands were inspected and assembled into contigs by using Geneious 11.1.5 program (https://www.geneious.com).

Sequence dataset compilation and maximum likelihood phylogenetic analysis

All worldwide available ARV σC gene sequences under the taxon ID 38170 (*Avian orthoreovirus*) were downloaded from GenBank (https://www.ncbi.nlm.nih.gov/). ARV sequences without information of sampling location were excluded from further analysis. This sequence dataset plus the Brazilian ARV σC gene sequences generated in the present study were aligned with Mafft (Katoh & Standley, 2016) and visually inspected in AliView (Larsson, 2014), where sequences which were too short were excluded. The algorithms RDP (Martin & Rybicki, 2000), GENECONV (Padidam *et al*., 1999), BOOTSCAN (Posada & Crandall 2001), MAXCHI (Maynard-Smith, 1992), CHIMAERA (Posada & Crandall, 2001), SISCAN (Gibbs *et al*., 2000) and SEQ (Boni *et al*., 2007) were used with default settings as implemented in RDP4 software (Martin *et al*., 2015) to look for the presence of chimeric genomes. Recombination was considered credible in sequences only if they were detected by more than three methods having significant *P*-values coupled with strong phylogenetic support for recombination.

A maximum likelihood phylogenetic tree was constructed with IQ-TREE software and branch support was calculated by using ultrafast bootstrap approximation (UFBoot) in 1000 replicates (Nguyen *et al*.. 2014). Prior to tree reconstruction, ModelFinder application (Kalyaanamoorthy *et al*., 2017) was used to select the best-fitted model from the analysed dataset. Finally, trees were visualized and edited in FigTree v1.4.3 (http://tree.bio.ed.ac.uk/software/figtree/).

ARV lineages were determined according to the topology of the phylogenetic tree and the position of previously classified reference strains (Liu *et al*., 2003; Lu *et al*., 2015; Palomino-Tapia *et al*., 2018). Additional criteria to classify ARV sequences into lineages and sub-lineages were: (i) clustering with support higher than 90 UFBoot; (ii) a median pairwise genetic identity greater than 80% in sub-lineage candidates; (iii) statistically smaller pairwise genetic distance for intra sub-lineage sequences than inter sub-lineage sequences; (iv) clustering that included multiple sequences from at least two different outbreaks. Pairwise genetic distances were calculated by using adegenet package (Jombart, 2008), available in R platform (http://www.R-project.org/). The Kruskal–Wallis test was then used to verify overall significant differences between the pairwise genetic distances of the sub-lineages. Tukey's *post-hoc* test was used to verify the specific differences between the sub-lineages. All statistical tests were two-sided and *P*-values < 0.05 were considered statistically significant.

Phylodynamic analyses

The temporal signal of relevant phylogenetic clusters, as identified in the ML tree, was investigated with TempEst software (Rambaut *et al*., 2016). Time-scaled phylogenetic tree estimation was performed using BEAST/BEAGLE v 1.10.4 software (Bouckaert *et al*., 2014). The SRD06 codon position substitution model, which was shown to be statistically superior in tests with different sets of organisms (Shapiro *et al*., 2006), and the non-parametric Bayesian Skygrid coalescent model, which estimates the viral effective population size (N_e) trajectories throughout evolutionary history (Gill *et al*., 2013), were applied. The best molecular clock model was tested by marginal likelihood estimation (Baele *et al*., 2013).

Monte Carlo Markov Chains were run for 50 million generations to ensure stationary and adequate effective sample size (ESS) for the main parameters. Tracer v.1.6 software (Rambaut *et al*., 2018) was used to diagnose Monte Carlo Markov Chains, adjust initial burn-in, and to perform the Skygrid demographic reconstruction. Uncertainty in parameter estimates was evaluated in the 95% highest posterior density (HPD 95%) interval. TreeAnnotator v1.8.2 (https://beast.community/treeannotator) was used to summarize the maximum clade credibility tree from the posterior distribution of trees and the maximum clade credibility tree was visualized and edited in FigTree v.1.4.4 (available at http://tree.bio.ed.ac.uk/software/figtree/).

Results

Detection of ARV by PCRs

All 17 samples were amplified by rqPCR and rnPCR. The amplified fragments of approximately 652 bp (nucleotide positions 44–696 in the segment S1 of the reference sequence AF004857) of the rnPCR from all samples were further sequenced by Sanger methodology. ARV sequences generated here were deposited under the GenBank accession numbers MK297893 - MK297909 (Table 1).

Molecular diversity and classification

To assess the robustness of the current ARV lineages classification, and to identify the circulating Brazilian genotypes, 634 σC gene sequences from 16 countries (including 10 previously sequenced Brazilian samples), were downloaded from GenBank. Identical nucleotide sequences and data without the place of origin were removed, to leave 451 sequences remaining. The 17 Brazilian ARVs sequences from this study were added, resulting in a final number of 468 sequences. Then, all sequences were trimmed from nucleotides 44–696 in the coding region of the σC gene. Chimeric forms were not detected using the RDP4 software.

ARV lineages were identified by the occurrence of strongly supported nodes (UFBoot = 90) in the phylogenetic tree. The designation of sub-lineage was

assigned to monophyletic groups of at least three viruses sampled from at least two different outbreaks. Strains not clustering into any sub-lineage according to these criteria ($n = 25$) were defined as unique variants. ARV lineages were distributed on the phylogenetic tree into five well-supported and genetically divergent groups (Figure 2). In addition, lineages I and II presented three sub-lineages (Vaccine, Ia and Ib; IIa, IIb, and IIc, respectively), while lineage IV had two (IVa and IVb). The other two lineages (III and V) did not present any subdivision.

Lineage I had 121 ARV sequences from 12 different countries that were sampled from 1970 to 2017. Sequences in lineage I presented a median nucleotide identity of 77.7% and were further divided into the sub-lineages Vaccine, Ia and Ib. Sub-lineage Vaccine had 53 highly similar sequences (98.7% of identity) and included the four commercial vaccine strain sequences (S1133, 1733, 2408, and 2177). The sub-lineage Ia included 36 sequences from the United States of America, Hungary, Canada, and Israel, sharing a median identity of 91.3%. Sub-lineage Ib grouped the remaining 30 sequences, including 13 isolates from Brazil (11 sequenced from this study), with a median identity of 94.9%. Besides Brazil, sub-lineage Ib included sequences from France, Hungary, the United States of America, Israel, Germany, Taiwan, and South Korea, isolated from 1992 to 2016. All sub-lineages showed a significant difference in genetic pairwise distances when comparing intra and inter sub-lineages distances (Figure 3).

Lineage II comprised 117 sequences from ARV samples isolated in seven different countries from 1992 to 2016. Nucleotide identity among sequences from lineage II was smaller (69.2%) than other ARV lineages. Based on genetic distances, lineage II was also sub-divided into three sub-lineages (IIa – 68 sequences with 80.8% of identity, IIb – 17 sequences with 87.1%; and IIc – 32 sequences with 81.6%). Four Brazilian sequences previously described and two from this study clustered into sub-lineage IIb with

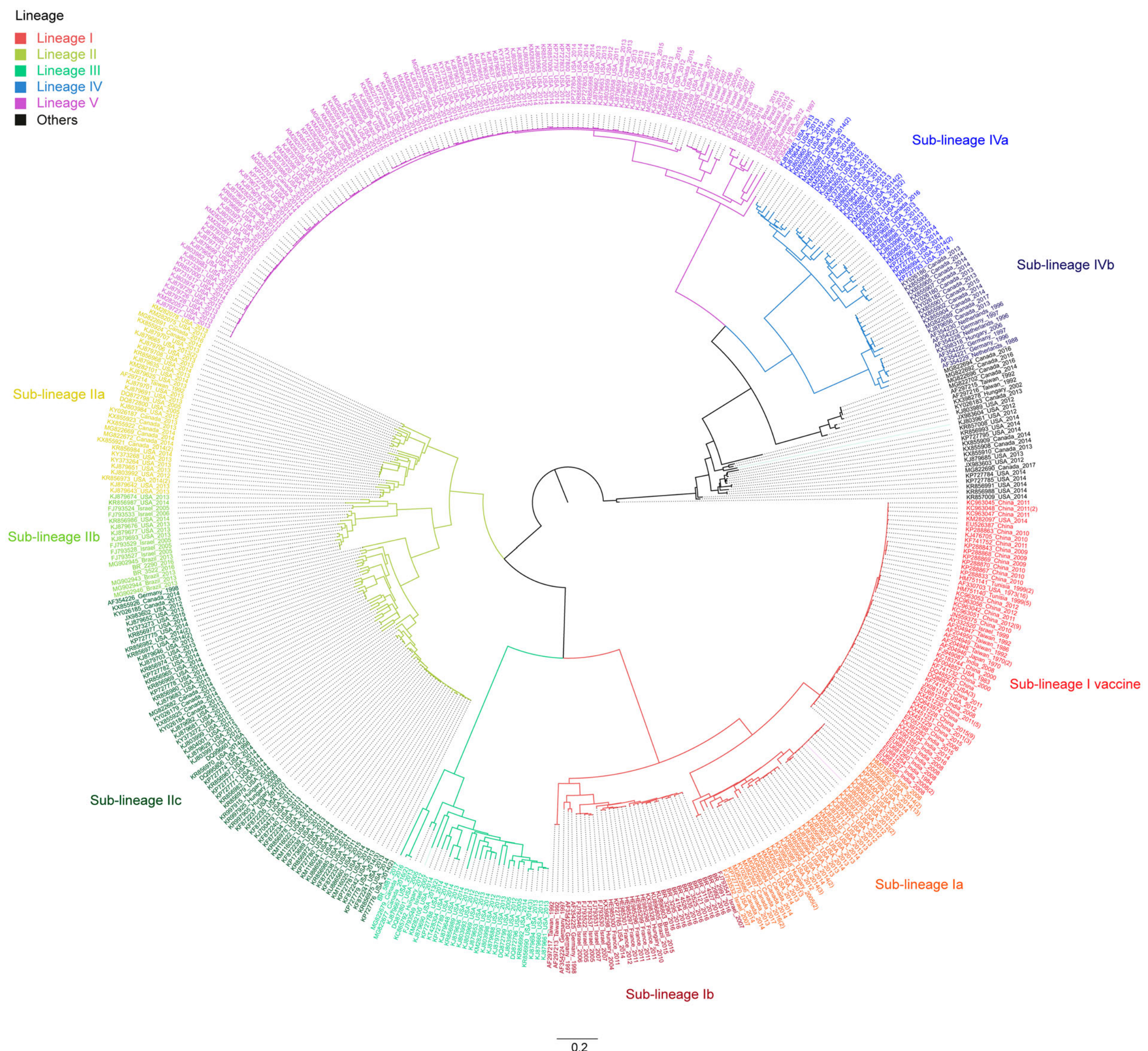

Figure 2. Maximum Likelihood phylogenetic tree of 468 ARV strains based on the σC sequence variability. Branches represent lineage classification and sub-lineages are identified by the name.

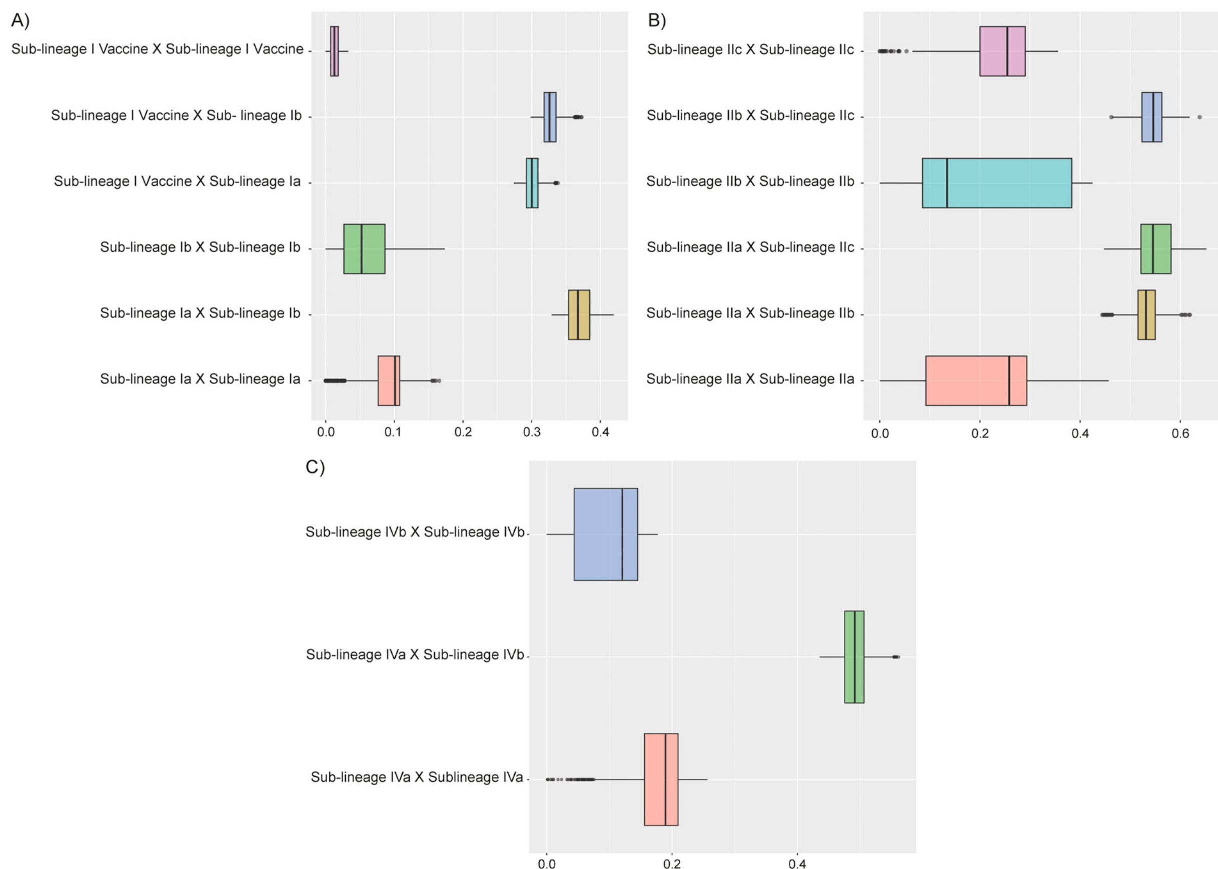

Figure 3. Pairwise genetic distances between sequences within the same sub-lineage (intra) and sequences from different sub-lineages (inter). (A) Lineage I. (B) Lineage II. (C) Lineage IV.

five isolates from Israel and the United States of America that were sampled between 2005 and 2016.

Lineage III was composed of 30 sequences collected from five different countries from 2005 to 2017. They had a mean identity of 80.9%. Although the tree topology shows two clades, this lineage was not divided into sub-lineages, because one of the clades was composed of two Canadian sequences isolated in the same year and only one Brazilian sequence. Moreover, pairwise genetic distance analysis did not support two sub-lineages. Lineage IV showed a median of 83.9%, including 50 sequences collected in five different countries from 2005 to 2016. It could be divided into two sub-lineages: IVa with 32 sequences (83.9%) and IVb with 18 sequences (89.2%). Lineage V presented 125 sequences isolated between 1971 and 2017 from six countries. All sequences presented a high identity (98.3%), and pairwise genetic distances tested between different clades did not identify any sub-lineage. Seven Brazilian isolates belonged to this lineage, six of them formed an independent clade at the base of the lineage separated from other countries, and the remaining sequence grouped with isolates from the United States of America.

Twenty-five sequences did not cluster with any one of the five well-established lineages. Seven of them positioned basally to the lineage IV in small paraphyletic clusters (UFBoot = 43–94), including four previously characterized as lineage IV in a previous study (Palomino-Tapia *et al.*, 2018). The 18 remaining sequences were dispersed basally in the phylogeny, including nine previously classified as lineage VI (KR856993, KR857008, KP727795, KP727784, KP727785, KR856991, KR856988, KR857009, and MG822690) (Lu *et al.*, 2015), four previously analysed as lineage V (KY026183, KX855908-10) (Lu *et al.*, 2015; Ayalew *et al.*, 2017) and five without any previous classification.

In summary, Brazilian ARVs from this study were classified into four lineages: I ($n = 11$; all Ib), II ($n = 2$; all IIb), III ($n = 1$) and V ($n = 3$) (Figure 1). With the inclusion of previously sequenced Brazilian ARVs from GenBank, there are 13 samples (48.2%) from lineage I (all Ib), six (22.2%) from lineage II (all IIb), one (3.7%) from lineage III and seven (25.9%) from lineage V, demonstrating the occurrence of at least four different lineages/sub-lineages in poultry-producing flocks from Brazil.

Phylodynamic analyses

In an attempt to understand the evolutionary history of ARV lineages in Brazil, the main phylogenetic clades were submitted to TempEst. This analysis showed that only sub-lineage Ib presented a good temporal signal ($R^2 = 0.40$), with a positive correlation between genetic divergence and sampling dates. The time to

the Most Recent Common Ancestor (tMRCA) of sub-lineage Ib was estimated to be 1968 (1953–1982, 95% HPD) (Figure 4) and the estimated mean rate of evolution of the analysed fragment of σC gene was 2.3×10^{-3} substitutions/site/year (1.47×10^{-3}–3.09×10^{-3}, 95% HPD). According to our analysis, the introduction of this sub-lineage into Brazil occurred in approximately 2010 (2007–2012, 95% HPD) and one Hungarian sequence appears basally to the Brazilian clade, suggesting a likely geographic origin. The Skygrid plot reconstructed an effective population size (N_e) trajectory with small variations across time since the onset of this sub-lineage, but with a possible decrease from the middle 1990s until 2010 (Figure 5).

Discussion

ARV has been increasingly associated with viral arthritis in Brazilian and other American broiler chicken-producing countries in the last years. Field ARV strains (from different lineages/genotypes) have been detected in chickens with tenosynovitis and MAS in commercial poultry farms worldwide, even in vaccinated flocks (Troxler *et al.*, 2013; Lu *et al.*, 2015; Sellers, 2016; Ayalew *et al.*, 2017; Souza *et al.*, 2018; Chen *et al.*, 2019).

In an attempt to classify the field Brazilian ARVs, our first approach was to generate a large and complete dataset of ARV σC gene sequences. This dataset was constructed with a region of the σC gene with high availability of sequences in GenBank, and which was previously used for the differentiation and classification of ARVs (Kant *et al.*, 2002; Liu *et al.*, 2003). The complete analysis resulted in a more complete phylogenetic tree. In addition, a new classification (into lineages and sub-lineages) was proposed to improve ARV classification, previously identified with different nomenclatures to define clades/phylogenetic clusters, such as lineages, genotypes, clusters and sub-clusters (Liu *et al.*, 2003; Lu *et al.*, 2015; Palomino-Tapia *et al.*, 2018; Egaña-Labrin *et al.*, 2019; Zhang *et al.*, 2019).

In this large dataset, the 468 ARV sequences could be clearly divided into five genetic groups (defined as lineages I, II, III, IV, and V) presenting high phylogenetic support (100%). A previous report classified ARVs into six main lineages/genotypes (Liu *et al.*, 2003), but lineage VI was composed of only one isolate (L07069) that was later re-classified as lineage V (Lu *et al.*, 2015). More recent studies proposed lineage VI with strains from the United States of America and Canada. All these ARVs were characterized from chicken field samples collected after 2014 (Lu *et al.*, 2015; Palomino-Tapia *et al.*, 2018, Egaña-Labrin *et al.*, 2019). In the revision of this classification procedure here, all these sequences did not cluster together in a monophyletic clade with high branch support. Therefore lineage VI was still not considered in the ARV classification in this study.

The present study also proposes the classification of some lineages into sub-lineages. This new subdivision was defined based on tree topology (occurrence of clades with high branch support), genetic similarity, as well as the cluster composition (the diversity of sampling locations). Three lineages could be clearly divided into sub-lineages: I (I vaccine, Ia, Ib), II (IIa, IIb, IIc), and IV (IVa and IVb). The other two lineages did not present any subdivision. Other studies also demonstrate virus classification in lineages and sub-lineages according to criteria similar to those used here (Li *et al.*, 2017; Rkachev *et al.*, 2017).

Brazilian ARVs were also classified into lineages and sub-lineages according to the criteria proposed here.

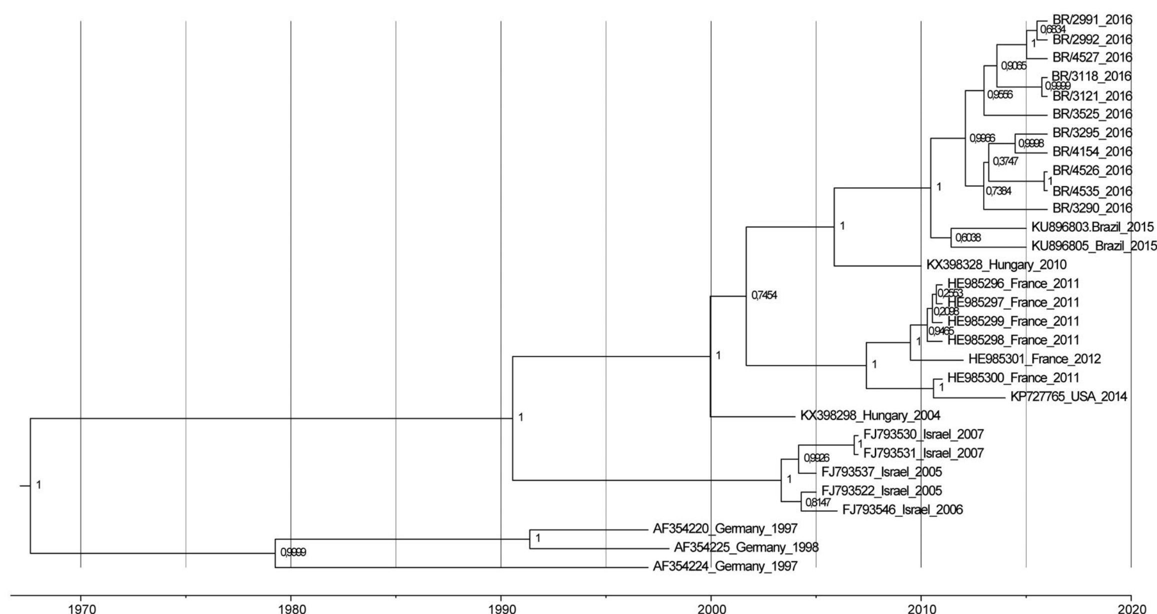

Figure 4. Bayesian maximum clade credibility phylogenetic tree of sub-lineage Ib.

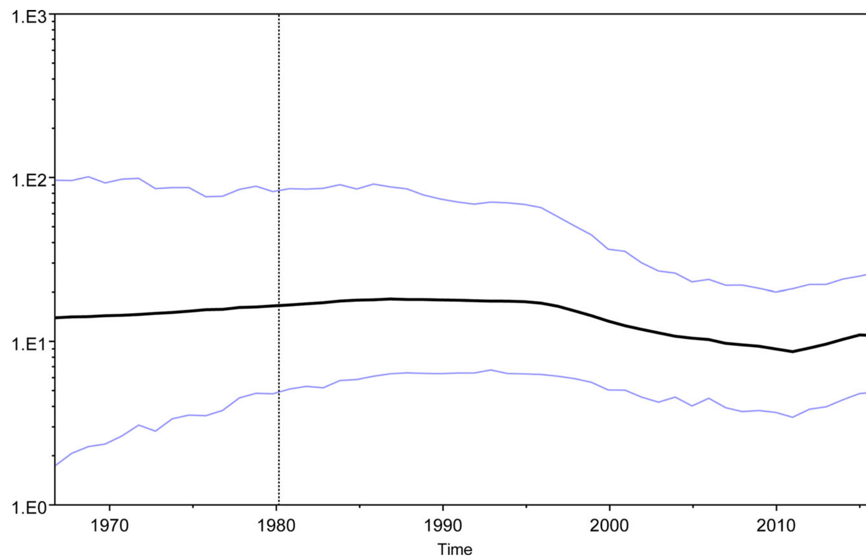

Figure 5. Bayesian skyline plot of partial σC gene of Sub-lineage Ib. *Y*-axis represents the effective number of infections and *X*-axis shows the timeline. The fine lines correspond to the intervals of highest probability density (95% HPD).

Data of the 17 ARVs sequenced here demonstrated the occurrence of four lineages in Brazil. Lineage I was the most frequent, with 11 isolates (64.7%), mainly from two poultry-producing states (Parana and Santa Catarina). ARVs from lineages II and V were also frequent (11.8% and 17.6%, respectively). These lineages are also circulating in the South region of Brazil as previously described (Souza *et al.*, 2018). Further, one unique sample (5.9%) of the lineage III was detected for the first time in Brazil. Taken together with other previous results, lineage I predominated in Brazil with 48.2%, followed by lineages V (25.9%), II (22.2%), and III (3.7%) (Sellers, 2016, Souza *et al.*, 2018).

The potential recombination within the σC gene was also evaluated here. Homo- and heterologous reassortment and recombination are potential events in *Reoviridae* (McDonald *et al.*, 2016). In addition, these genetic modifications shape the evolution of chicken orthoreoviruses according to previous reports (Farkas *et al.*, 2016, Ayalew *et al.*, 2017). Intrasegmental recombination was already reported in different ARV genes (Farkas *et al.*, 2016). However, no possible recombination was observed in any evaluated S1 sequences in this study.

The possible association among specific diseases (tenosynovitis and MAS) and lineages were evaluated, but ARV clinical manifestations could not be related to any of the four detected lineages. Birds with MAS were mostly infected with ARVs from lineage I (the most frequent), whereas chickens with tenosynovitis could be harbouring ARVs from the four different lineages. Notably, ARVs from five different lineages (especially including lineage I) were already detected in birds with tenosynovitis and MAS, even in flocks immunized with commercial vaccines (Chen *et al.*, 2019). A more recent study showed the presence of ARV in cases of MAS in broiler chickens in Brazil; however, they could not prove that ARV was the causal agent of this disease because healthy birds also presented the virus in the intestinal tract (Lima *et al.*, 2019).

In addition, the present study reported a detailed evolutionary analysis of the sub-lineage Ib (the most frequent lineage in Brazil). The nucleotide substitution rate estimated in our analyses for this sub-lineage was similar to a previous evaluation of lineage I (3.1×10^{-3} nucleotide substitutions/site/year) (Liu *et al.*, 2003). Further, our data demonstrated that the introduction of this sub-lineage into Brazil occurred in approximately 2010, which is the same time that the diagnosis of reovirus increased drastically in several countries (Sellers, 2016). Dynamic study of the Ib population size presented here also reinforces the increasing spread of ARVs from this lineage after 2010. Unfortunately, no other sub-lineage presented a good temporal signal to estimate time-scaled trees and investigate in detail their evolutionary history. Therefore, more studies are necessary to elucidate the real evolution of ARVs and their spread around the world.

Finally, the high rate of variation among circulating ARV lineages makes it difficult to develop a vaccine to cross-protect against all strains, including strains from lineage I (Sellers, 2013; Troxler *et al.*, 2013; Zhang *et al.*, 2019). Alternatively, autogenous vaccines have been proposed to assist commercial poultry companies to control ARV disease (Sellers, 2016). A safe recommendation for a competent autogenous vaccine programme should include ARV isolates with at least 95% amino acid identity in σC protein to circulating field strains (Palomino-Tapia *et al.*, 2018). There is also some evidence about the importance of the M2 gene and the protein encoded by it (μB) in viral antigenicity and pathogenicity (Su *et al.*, 2006; Egaña-Labrin *et al.*,

2019). New genome-wide studies are necessary to understand the participation of these two different genes (S1, M2), as well as all the other genes and their proteins, to define ARV serogroups and pathotypes. Finally, the continuous emergence of new lineages, sub-lineages, and even unique variants requires an efficient epidemiological surveillance programme, assisted with molecular biology tools, to monitor ARV strains in the poultry flocks.

Conclusions

The present study demonstrated the occurrence and spread of four ARV lineages in Brazilian commercial poultry flocks with tenosynovitis or MAS. These Brazilian lineages shared a low identity with the vaccine lineage.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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