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Molecular and phylogenetic analyses of *Salmonella* Gallinarum trace the origin and diversification of recent outbreaks of fowl typhoid in poultry farms

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ABSTRACT

Fowl typhoid (FT) and pullorum disease (PD) are two important poultry infections caused by *Salmonella enterica* subsp. *enterica* serotype Gallinarum (*S.* Gallinarum). *S.* Gallinarum strains are adapted to birds and classified into biovars Gallinarum (bvGA) and Pullorum (bvPU) as they are the causative agent of FT and PD, respectively. In Brazil, FT/PD outbreaks have been reported along the last 50 years, but there was a recent increase of FT field reports with the suspicion it could be due to virulence reversion of the attenuated live vaccine SG9R. In this study, we applied molecular biology assays and phylogenetic methods to detect and investigate *S.* Gallinarum isolates from commercial poultry flocks in order to understand the evolutionary history and origin of the recent FT outbreaks in Brazil. *S.* Gallinarum isolates were obtained from thirteen different poultry flocks with clinical signs of FT/PD from 2013 to 2015. These isolates were serotyped, tested with three specific PCR (for the detection of bvGA, bvPU and live vaccine strain SG9R) and submitted to sequencing of a variable genome region (ISR analysis). The complete genome of one bvGA strain (BR_RS12) was also compared to other *S.* Gallinarum complete genomes (including other two Brazilian ones: bvGA 287/91 and bvPU FCVA198). PCR detected all thirteen isolates as *S.* Gallinarum (eight bvGA and five bvPU), none positive for SG9R strain. ISR analysis revealed that all eight bvGA isolates showed exactly the same nucleotide sequences with 100% similarity to reference strains, while two patterns were observed for bvPU. Genome phylogeny demonstrated distinct clades for bvGA and bvPU, with the bvGA clade showing a clear subdivision including three genomes: SG9R vaccine, the respective SG9 parent strain and one SG9R revertant field isolate (MB4523). The evolutionary rate of the total *S.* Gallinarum genome was calculated at 6.15×10^{-7} substitutions/site/year, with 2.8 observed substitutions per year per genome (1 SNP per 4292 bases). Phylodynamics analysis estimated that at least two introductions of *S.* Gallinarum bvGA happened in Brazil, the first in 1885 and the second in 1950. The Brazilian bvGA genomes 287/91 and BR_RS12 analyzed here were related to the early and the late introductions, respectively. In conclusion, these results indicate the occurrence of *S.* Gallinarum strains associated with FT outbreaks that have been circulating for more than 50 years in Brazil and are not originated from virulence reversion of the SG9R vaccine.

1. Introduction

The genus *Salmonella* has more than 2500 serologically distinguishable variants (serotypes). *Salmonella enterica* subsp. *enterica* of the serotype Gallinarum (*S.* Gallinarum) is the causative agent of two important poultry diseases: fowl typhoid (FT) and pullorum disease (PD). Birds with these diseases present a severe infection with systemic clinical signs and can result in high rates of mortality and/or morbidity in poultry-producing flocks worldwide (Gast, 2008).

FT is an acute septicemic or chronic disease that occurs most often

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Table 1
Overview and some molecular characteristics of the *S.* Gallinarum isolates used in this study according to biovar, geographic origin, year of isolation and type of bird.

Strain	Biovar	State of isolation	Year of isolation	Type of bird	ISR	*invA*	*speC*	*glgC*	*glgC 9R*
BR_RS4245	bvGA	Rio Grande do Sul	2013	Breeder	Gall	+	+	+	−
BR_RS11[a]	bvGA	Rio Grande do Sul	2014	Breeder	Gall	+	+	+	−
BR_RS12[a]	bvGA	Rio Grande do Sul	2014	Breeder	Gall	+	+	+	−
BR_RS13[a]	bvGA	Rio Grande do Sul	2014	Breeder	Gall	+	+	+	−
BR_RS91	bvGA	Rio Grande do Sul	2014	Layer	Gall	+	+	+	−
BR_RS146	bvGA	Rio Grande do Sul	2015	Layer	Gall	+	+	+	−
BR_RS183	bvGA	Rio Grande do Sul	2016	Layer	Gall	+	+	+	−
BR_SC61	bvGA	Santa Catarina	2014	Broiler	Gall	+	+	+	−
BR_RS10	bvPU	Rio Grande do Sul	2014	Layer	Pull 1	+	+	−	−
BR_RS74	bvPU	Rio Grande do Sul	2014	Layer	Pull 1	+	+	−	−
BR_RS145	bvPU	Rio Grande do Sul	2015	Layer	Pull 1	+	+	−	−
BR_SC71[b]	bvPU	Santa Catarina	2014	Broiler	Pull 2	+	+	−	−
BR_SC72[b]	bvPU	Santa Catarina	2014	Broiler	Pull 2	+	+	−	−

[a] and [b]: samples from same integration and hatchery.

in adult birds through horizontal transmission, although chicken of all ages can be infected. FT was recognized in 1888 and the bacterial agent was firstly named *Bacillus gallinarum*, after *B. sanguinarum* and finally *Salmonella gallinarum*. PD is also characterized by a septicemic infection especially in young birds as a result of a transovarial/vertical transmission. It was first described in young chicks with fatal septicemia and defined as bacillary white diarrhea (BWD). The etiological agent was discovered in 1899 and named *Salmonella pullorum* (Bullis, 1977). Only more recently these two bacteria were taxonomically defined to be of the same species and serotype: *S.* Gallinarum. It is characterized to belong to D serogroup, to have the same O antigens (1, 9, 12) and to be non-motile (because they do not express the flagellum proteins). However, FT and PD *Salmonella* isolates have still been differentiated as biovars Gallinarum (bvGA) and Pullorum (bvPU), respectively. In addition, laboratorial analyses demonstrated that bvGA and bvPU are biochemically differentiated in the fermentation of dulcitol (metabolized only by bvGA strains) and decarboxylation of ornithine (metabolyzed only by bvPU strains), although some exception were already described (Barrow and Freitas Neto, 2011).

FT and PD were widely spread and became endemic in the poultry-producing countries around the world since the early 20th century. Due to the economic losses in commercial flocks, national programs were implemented to control both diseases in the 1950s and 1960s worldwide. In the USA, for example, the control of these avian *Salmonella* typhoid infections was included in the National Poultry Improvement Plan (NPIP). The first strategy was to eradicate the bacterial agents with the elimination of any infected poultry flock in the farms (Gast, 2008). Meanwhile, one vaccine was produced in the United Kingdom with the attenuation of the virulent smooth strain 9 (S9) to a rough (R) form in laboratory. Eradication and/or immunization with this live vaccine (named SG9R) have been used to prevent FT and PD in the several endemic regions of the world (Lee et al., 2005; Wigley, 2016).

In Brazil, FT and PD were detected in Minas Gerais state in 1919 and 1928, respectively. Both diseases were identified in other Brazilian state (São Paulo) in the end of the second decade of the 20th Century. Outbreaks of these diseases were more frequently reported in all country after the introduction of the intensive poultry production systems in the 1950s. Later, FT and PD were recognized as an important problem in poultry farms with the increased isolation of *S.* Gallinarum in the 1980s (Berchieri Júnior and Oliveira, 2010). Economic losses to the national poultry industry, affecting the exportation of Brazilian poultry-derived products, resulted in the implementation of the control of *S.* Gallinarum in the National Poultry Health Program (PNSA – *Plano Nacional de Sanidade Avícola*; Brasil, 2009). Despite PNSA, there was a new and high increase of cases between 2005 and 2016 and more than one hundred outbreaks of avian typhoid salmonelosis in layers and broilers flocks (most of them related to FT) were reported in different poultry-producing states of the country (OIE, 2016). Two main possibilities have been raised to explain the high incidence of FT in recent years: (i) virulence reversion of the live vaccine SG9R, as previously described (Van Immerseel et al., 2013); and (ii) failure in the farm biosecurity programs (Secundo de Souza et al., 2015; Celis-Estupiñan et al., 2017). Furthermore, vertical transmission of *S.* Gallinarum strains in the hatchery, increasing the presence in breeder flocks, could not be discarded as a possible reason to spread this bacterial pathogen in layers and broilers (Gast, 2008; Kwon et al., 2014).

Salmonella is a well-studied bacteria and a key example of the occurrence of genomic signatures that distinguish lifestyles and host adaptation (Thomson et al., 2008; Langridge et al., 2015). Whole-genome sequencing (WGS) of several *Salmonella* strains has increasingly evidenced the genetic differences between serotypes. The genome-wide comparison between the similar serotypes Gallinarum and Enteritidis having different lifestyles demonstrated that around 125 genes are responsible for the physiological differences, including phage-related genes, fimbriaes and type III secreted proteins. In the specific comparison between bvGA and bvPU strains, some minor differences were observed: fourteen genes were present in bvGA and not in bvPU, while three genes were present in bvPU and not in bvGA (Feng et al., 2013).

Meanwhile, single nucleotide polymorphisms (SNPs) analyses in the whole genomes have been used to differentiate *Salmonella* lineages and to trace strains of the same serotype in humans and poultry (Feasey et al., 2016; Van Immerseel et al., 2013). It was even possible to study population dynamics and evolutionary history of other *Salmonella* serotypes, providing subtyping resolution and phylogenetic precision (Deng et al., 2014; Leekitcharoenphon et al., 2016). In this study, we applied molecular biology assays (including whole-genome sequencing) and phylogenetic methods to investigate *S.* Gallinarum strains isolated in outbreaks from Brazil and other regions of the world. The main objective was to understand the evolutionary history of this important *Salmonella* serotype and to elucidate recent FT outbreaks in Brazil.

2. Materials and methods

2.1. Bacterial samples

Thirteen *S.* Gallinarum strains were isolated from poultry flocks with clinical signs of FT and/or PD in South Brazil from 2013 to 2016 (Table 1). These strains were obtained from flocks (layers, broilers and breeders) in ten different integration and hatchery production systems. Bacterial isolates were obtained by enrichment with buffered peptone water at 36 °C for 24 h, selective enrichment in Rappaport-Vassiliadis and Tetrathionate broths at 43 °C for 24 h and plating on MacConkey agar at 36 °C for 24 h. Typical colonies were serotyped according to the White-Kauffman-Le Minor (WKL) scheme (Issenhuth-Jeanjean et al., 2014) and tested for the biochemical profile (ornithine and lysine decarboxylation, hydrogen sulfide production, urea decomposition, and

the use of sucrose, lactose, maltose and citrate) to differentiate bvGA from bvPU (Gast, 2008).

2.2. Molecular biology assays

Genomic DNA of all isolates was extracted and submitted to molecular characterization by the following experimental approaches: i) *invA* real-time PCR for *Salmonella* generic detection (Hoorfar et al., 2000); ii) *glgC* conventional PCR for the detection of *S.* Gallinarum bvGA and bvPU (Kang et al., 2011); iii) *speC* conventional PCR for the detection of *S.* Gallinarum bvGA only (Kang et al., 2011); iv) *glgC* SG9R conventional PCR for detection of the vaccine strain (Kang et al., 2012); and v) operon *rrnH* intergenic sequence ribotyping (ISR) analysis to observe intra-serotype variation (Pulido-Landínez et al., 2013). The *S.* Gallinarum bvGA strain BR_RS12 has been previously sequenced, assembled and annotated by our group and a detailed explanation of the applied methodology is explained elsewhere (De Carli et al., 2016).

2.3. Phylogenetic analyses

A phylogenetic comparative analysis was performed with all *S.* Gallinarum bvGA and bvPU genomes available in GenBank, including BR_RS12 (Table 2). As an outgroup for the analysis, a *S.* Enteritidis (P125109) genome was used. The bvGA genomes SG9, SG9R, MB4523, ST572 and VTCCBAA614 and bvPU FCVA198 were assembled using Geneious 6.0.3 Read Mapper algorithm available in the software Geneious 10.0.5 (Kearse et al., 2012). A minimum read's quality score of 30 was applied and the reference genomes used in the assembly were *S.* Gallinarum bvGA 287/91 and *S.* Gallinarum bvPU CDC1983-67. The *S.* Gallinarum bvPU RKS5078, ATCC9120 and S06004 genomes were obtained already assembled in the GenBank. The assembled contigs were then submitted to CSI Phylogeny 1.2 web application (Kaas et al., 2014), where they were aligned against *S.* Enteritidis (P125109) genome and SNPs positions were extracted with default parameters. The best-fitting nucleotide substitution model for the SNPs' alignment was tested in the software jModelTest (Posada, 2008) and maximum likelihood (ML) phylogenetic tree was constructed in RAxML (Stamatakis, 2014).

2.4. Bayesian phylogenetic analysis

The time of origin and population dynamics of bvGA genomes were evaluated by applying Bayesian phylogenetic analyses. Prior to that, the temporal signal of the ML SNPs' tree was assessed using Tempest software (Rambaut et al., 2016) where the year of isolation of each strain was used to establish a temporal framework for the phylogeny. Sequences outliers in a regression of root-to-tip divergence versus sampling time were removed. Time-scaled phylogenetic tree reconstruction was performed using BEAST/BEAGLE software (Drummond et al., 2012) as available in the Cipres Science Gateway (https://www.phylo.org). Marginal likelihood estimation (MLE) (Baele et al., 2013) was applied to compare alternative site and clock models in a Bayesian framework. Several combinations of site and molecular clock models were evaluated to find the best-fitted models. Trees were reconstructed using GTR substitution model and the relaxed gamma molecular clock, which outperformed alternative models. To allow for effective population size multiple changes across time, the non-parametric Bayesian Skyline coalescent model was applied in the analyses.

3. Results

3.1. Biochemical characterization and molecular detection of bvGA and bvPU

According to the biochemical and serological profile, all thirteen isolates were confirmed as serotype Gallinarum. In addition, eight isolates were identified as bvGA and the remaining five as bvPU. All the DNA samples of these isolates presented positive results for the generic amplification of *Salmonella* by the real-time PCR based on the *invA* gene. All eight bvGA isolates were also positive for the *glgC* and *speC* conventional PCR, while the five bvPU isolates were positive only for the *glgC* PCR amplification, as expected for these biovars (Kang et al., 2011). The specific *glgC* gene PCR from the vaccine strain SG9R did not present positive result for all thirteen field isolates (Table 1).

Furthermore, ISR analysis revealed that all eight bvGA strains (BR_RS11, BR_RS12, BR_RS13, BR_SC61, BR_RS4245, BR_RS91, BR_RS183 and BR_RS146) presented the same fragment size (729 bp) and nucleotide sequences, also with 100% of similarity to other reference strains of this biovar (including SG9 strain). On oppose, the five bvPU Brazilian isolates presented another fragment size (655 bp) with two divergent nucleotide sequences named Pull 1 and Pull 2 (Table 2). BR_RS74, BR_RS10 and BR_RS145 (Pull 1 group) presented nucleotide sequences with 100% of identity with the reference strains of this biovar (ATCC9120, FCVA198, RKS5078 and CDC1983-67), while BR_SC71 and BR_SC72 (Pull 2 group) presented a different sequence with 97% of identity to reference bvPU strains (data not shown).

3.2. Whole-genome comparison and SNP analysis

In order to understand the molecular phylogeny of avian typhoid *Salmonella*, complete genome data were obtained from thirteen *S.* Gallinarum strains isolated in different geographic regions of the world (Table 2). Three of these strains were isolated in Brazil: one bvPU (FCAV198) isolated in 2008 and sequenced in 2014 (Batista et al., 2014) and two bvGA, the first isolated from a commercial poultry flock in 1991 and sequenced in 2008 (Thomson et al., 2008) and the second

Table 2
Overview of the *S.* Gallinarum and *S.* Enteritidis complete genomes used in this study according to the strain characteristics.

Strain	Biovar	Country	Collect	Source	Author
SG9	bvGA	United Kingdom	1955	Chicken Feces	Richardson et al. (2011)
SG9R	bvGA	United Kingdom	2004	Vaccine	Van Immerseel et al. (2013)
ST572	bvGA	United Kingdom	2009	NA[a]	Yoshida et al. (2016)
MB4523	bvGA	Belgium	2009	Chicken Spleen	Van Immerseel et al. (2013)
BR_RS12	bvGA	Brazil	2014	Chicken Viscera	De Carli et al. (2016)
287/91	bvGA	Brazil	1991	Brown Egg	Thomson et al. (2008)
VTCCBAA614	bvGA	India	2012	Chicken Liver	Vaid et al. (2015)
CDC1983-67	bvPU	NA[a]	NA[a]	NA[a]	Feng et al. (2013)
ATCC 9120	bvPU	NA[a]	2012	Human	Yao et al. (2016)
S06004	bvPU	China	2006	NA[a]	Li et al. (2015)
RKS5078	bvPU	NA[a]	NA[a]	NA[a]	Feng et al. (2013)
FCVA198	bvPU	Brazil	2008	Ovary	Batista et al. (2014)
P125109	Enteritidis	United Kingdom	1991	Human	Thomson et al. (2008)

[a] Not Available.

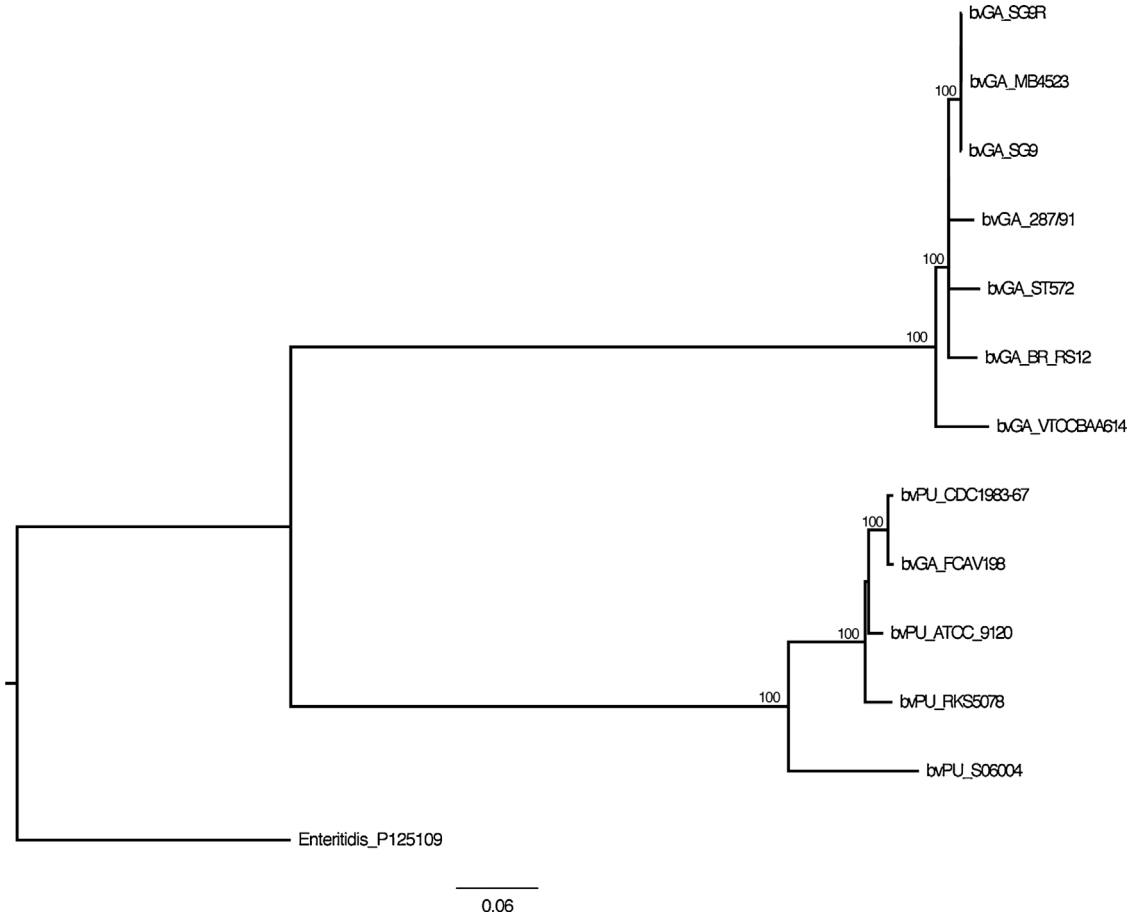

Fig. 1. Maximum-likelihood phylogeny of *Salmonella* Gallinarum bvGA and bvPU based on the twelve strains with complete genomes rooted to *S.* Enteritidis.

recently sequenced and announced by our group (De Carli et al., 2016).

All bvGA and bvPU genomes available in public databanks until 2016 were analyzed here. After assembling them using proper reference genomes for bvGA and bvPU biovars, SNPs were extracted using *Salmonella enterica* serotype Enteritidis strain P125109 as a reference sequence. This procedure originated an alignment containing 9195 variable sites used to construct the ML phylogenetic tree (rooted with the reference *S.* Enteritidis P125109 strain) and to demonstrate the evolutionary relationship among the *S.* Gallinarum strains. As expected, it was observed that bvGA and bvPU isolates formed two distinct strong supported clades in the phylogeny (Fig. 1). *Salmonella* Enteritidis outgroup presented a mean of 4842 SNPs in relation to all isolates from Gallinarum serotype, while bvPU and bvGA clusters showed a mean distance of 2971 SNPs between them. Internal distance in the bvGA clade was of only 339 SNPs. It is noteworthy that the bvGA vaccine SG9R, the respective parent SG9 and the reverted virulent phenotype strain (MB4523) formed a highly supported sub-clade in the tree (Fig. 1). SG9, SG9R and the MB4523 virulence revertant strain presented less than 12 SNPs of difference among them. Brazilian strains BR_RS12 and 287/91 presented 364 SNPs of difference between them and they differed in 282 and 260 SNPs in comparison with SG9R vaccine strain, respectively. The Indian strain (bvGA_VTCCBAA614) showed the greater distance (522 SNPs) to the SG9R strain and was grouped externally in the bvGA clade.

The bvPU cluster showed an average distance of 724 SNPs among isolates from this biovar. It is important to highlight that Brazilian field strain FCAV198 clustered together and with high support to one reference pathogenic strain from CDC (CDC 1983-67), isolated from poultry ovary in the 1970s (Fig. 1). The distance between these two strains was only 74 SNPs. As happened in the bvGA clade, an Asian strain (bvPU_S06004 isolated in China) was grouped more externally in the bvPU clade, with a median distance of 1405 SNPs to the other bvPU genomes.

3.3. Bayesian phylogenetic analysis

In order to reconstruct demographic history and time of introduction in Brazil of *S.* Gallinarum bvGA strains related to FT outbreaks, Bayesian phylogenetic analyses were performed with five complete bvGA genomes: SG9, 287/91, ST572, VTCCBAA614 and BR_RS12. The vaccine SG9R and the revertant MB4523 sequences were excluded because they were clear outliers in the TempEst regression analysis. A total of 1084 SNPs were observed among these five genomes. BEAST analysis estimated a mean evolutionary rate of 2.64×10^{-4} substitutions/site/year (1.82×10^{-4}–3.96×10^{-4}, 95% HPD) for the SNP's alignment, giving a calculated evolutionary rate of 6.15×10^{-7} (4.24×10^{-7}–9.23×10^{-7}, 95% HPD) for the whole genome. A mean of 2.9 (2.0–4.3, 95% HPD) substitutions were observed per year per genome. Our analyses estimated the time of most recent common ancestor (tMRCA) of the whole bvGA clade to 1855 (1806 to 1912, 95% HPD) (Fig. 2). The Brazilian strain 287/91 was shown to be a descendant of these early lineages and should be introduced in Brazil in the mid to late XIX century. In the late 1940's (mean 1949, 1944 to 1955, 95% HPD) there was a new bvGA introduction in Brazil, of a lineage descending from the vaccine parent strain SG9. The BR_RS12 strain described by our group seems to be a descendant of this second introduction. Skyline plot analysis shows an increasing in the bvGA effective population size (N_e) around late 1940's and early 1950's (Fig. 2).

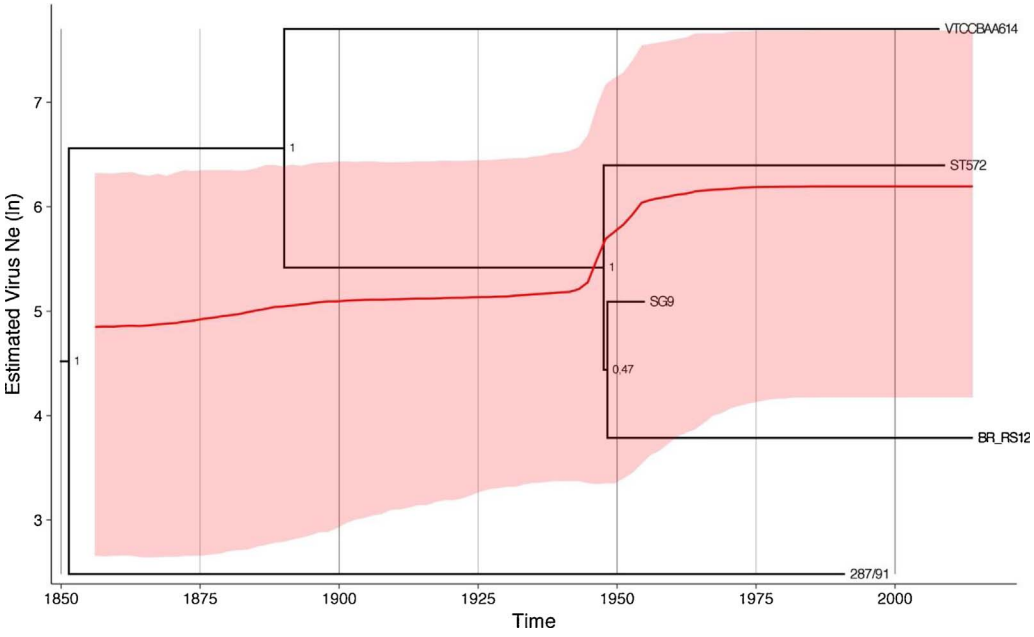

Fig. 2. Phylogenetic analysis through bayesian method to determinated the source of *Salmonella* Gallinarum in Brazil and Skyline chart to determine the population distribution.

4. Discussion

Brazil is the second largest producer of chicken meat and the sixth egg producer in the World. There are intensive avian farms in almost all regions of the country. South Brazil is the country's main region of large-scale poultry production with each farm hosting tens of thousands birds susceptible to several diseases, among them FT and PD. According to OIE information, there were 138 cases of avian typhoid salmonellosis (87% FT and 13% PD) formally reported in Brazil in the last 10 years. In several years in this period (2009, 2010, 2011, 2013, 2014 and 2015), FT and PD were not reported to OIE (although there were some outbreaks reported by veterinarians in poultry farms) and the status was considered of “present diseases but without quantifiable data” (OIE, 2016). A significant percentage of the reported cases (32%) occurred in avian farms located in the three states from South Brazil (Paraná, Santa Catarina and Rio Grande do Sul). In the present study, thirteen *S.* Gallinarum isolates (including eight bvGA and five bvPU strains) from intensive poultry-producing farms located in Rio Grande do Sul and Santa Catarina states were submitted to molecular genetic analyses.

All thirteen *S.* Gallinarum field isolates (bvPU and bvGA) were detected by *glgC* PCR, while only the eight *S.* Gallinarum bvGA isolates were detected by *speC* as previously described (Kang et al., 2011, 2012). In addition, none isolate presented positive result with the PCR procedure for SG9R detection, a strong indicative that bvGA field isolates were not originated from SG9R vaccine as described by Van Immerseel et al. (2013). We further proceeded with ISR analysis to confirm the *Salmonella* serotype and biovars, since bvGA and bvPU have different fragment sizes and nucleotide sequences in this genome region (Pulido-Landínez et al., 2013). All bvGA and three bvPU isolates presented complete identity to bvGA and bvPU reference strains, respectively. However, two bvPU isolates presented 3% of divergence in comparison to bvPU reference sequences. This diversity was not really unexpected, since *Samonella enterica* strains has at least seven multiple operons of rRNA (*rrn*) that can be rearranged due to the occurrence of homologous recombination among operons (Matthews et al., 2010). Interestingly, this organization depends on the interaction between serotype and host. Specific host bacteria, such as typhoid *Salmonella*, colonize several organs, grow slowly and rely on the absence of competition in systemic infections. Therefore, they are under less selective pressure to maintain the order of their genes, consequently presenting greater genetic diversity in this region (Helm et al., 2003; Matthews et al., 2010).

S. Gallinarum was previously demonstrated to be a host adapted serotype with specific genetic components associated to invasiveness phenotype (Langridge et al., 2015). However there is not any previous study analyzing the spread and dissemination of the both biovars (Gallinarum and Pullorum) using a whole-genome analysis approach. The phylogenetic analyses of the complete genomes presented in this study showed two clearly distinct clades: one with the five bvPU and other with the seven bvGA strains. These results demonstrated that these two avian typhoid biovars probably evolved separately from a common ancestor with under similar pressure selection, reinforcing previous studies (Langridge et al., 2015; Feng et al., 2013). In addition, it was possible to observe that no clear structure was presented in the tree for the five bvPU genomes. This was expected since all isolates were obtained in independent geographic regions and collected in different years. A similar situation was observed for four of the seven bvGA genomes, including the two Brazilian ones (287/91 and BR_RS12). The three other bvGA strains (SG9, SG9R and MB4523) were much more related among them due to the origin of these strains. SG9R is the attenuated form of SG9, while MB4523 was obtained in a poultry Belgium flock with FT outbreak due to reversion of the SG9R vaccine strain to a virulent phenotype (Van Immerseel et al., 2013). In a preliminary analysis of the topology of the phylogenetic tree, both Brazilian strains seem to be different strains. Moreover they clearly clustered outside the SG9 bvGA sub-clade, so they did not originated from a recent event of SG9R vaccine reversion. Another studies also found genetic differences among the field strains circulating in Brazil and the vaccine strain (Secundo de Souza et al., 2015; Celis-Estupiñan et al., 2017).

The reconstruction of the history and time of introduction of *S.* Gallinarum bvGA Brazilian strains with the Bayesian phylogenetic approach revealed distinct origins of the two FT outbreaks strains (one of the 1990s and other from this decade). Our results suggest that these bvGA strains are probably circulating in the field for a long time, since the 1850s for 287/91 and since the 1950s for BR_RS12. These results are in agreement with previous reports that demonstrated more than one pattern of *S.* Gallinarum bvGA spreading in Brazil by ERIC-PCR and PFGE (Secundo de Souza et al., 2015; Celis-Estupiñan et al., 2017). Noteworthy, the results presented here are based only in two Brazilian strains, what might not be representative of all genetic diversity of bvGA field strains in the country. Moreover, 287/91 and BR_RS12 strains were isolated more than 20 years apart, and these two distinct

lineages might not be currently co-circulating in Brazil. Therefore, more discriminatory techniques, as multi locus sequence typing (MLST) and preferentially WGS, should be used to further characterize the recent Brazilian strains, drawing more definitive conclusions as previously performed to other serotypes (Achtman et al., 2012; Feasey et al., 2016).

Furthermore, Bayesian Skyline plot (Fig. 2) reconstructed a steep increase in bvGA effective population size around1950, exactly when the old commercial poultry farms (1920s and 1930s) were replaced for a new and more intensive system of creation and monitoring (Belusso and Hespanhol, 2010). In spite of including all bvGA full genomes available in public databanks, only five sequences were submitted to Bayesian phylogenetic analyses in the current study and more detailed and accurate estimations might be reported as new genomes become available.

In conclusion, our comparative analysis of *S.* Gallinarum genomes improves the understanding about Brazilian FT outbreak behaviors. SG9R strain vaccination is very unlikely to be the origin of the current FT cases in Brazil and is very likely that more than one field strain is currently circulating in the country. The current FT outbreaks are probably related to farm biosecurity programs failures to control the spread of local strains. In the last three decades Brazil has witnessed a marked growth in poultry flocks for chicken and egg production. It is probable that industry and government veterinary services were not able to respond adequately to this enormous increase in production, and biosecurity measures may not have been so stringent as a result of this increase (Gonçalves and de Moraes, 2017). The results presented here reinforce the importance of epidemiological surveillance to provide support to animal health policies.

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