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Genotypic diversity of *Salmonella* ser. Gallinarum strains isolated from 2012 to 2016 in Brazil

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Abstract: *Salmonella* Gallinarum biovar Gallinarum (SG) is a host-specific avian pathogen causing fowl typhoid (FT), a notifiable disease that causes septicemia in poultry and significant economic losses. In the state of Santa Catarina, Brazil, several cases of FT were reported from 2012 to 2016. However, strains of these outbreaks have not yet been characterized, and the genotypic characterization of isolates is essential for epidemiological surveillance and outbreak investigations. The objective of this work was to study the genetic diversity of SG isolated from the FT outbreaks, aiming to identify similarity or dissimilarity among strains isolated from the reported foci. For this study, 56 SG strains were submitted to pulsed-field gel electrophoresis (PFGE). Through the PFGE analysis, a prevalent genotypic profile (57.1%) of SG was identified, suggesting the occurrence of an endemic clone, whose dissemination is possibly linked to the transport of infected birds across the regions. Fifteen other genotypic profiles were also obtained, evidencing the genetic variability of circulating strains and multiple contamination sources.

Key words: *Salmonella* Gallinarum, pulsotypes, Brazil

Salmonella Gallinarum biovar Gallinarum (SG) is a gram-negative, rod-shaped, nonspore-forming bacterium and a highly pathogenic agent causing fowl typhoid (FT), a severe systemic disease of chickens and other galliform birds that has a significant economic impact in the poultry industry [1]. Therefore, SG is potentially an economical and sanitary threat to poultry-producing regions, mainly in developing countries.[1]

Brazil is currently the largest exporter of poultry meat, and the state of Santa Catarina accounts for almost a quarter of this amount [2]. SG has reemerged in the last decade in Brazilians flocks, causing great concern of the poultry sector, since it involves the slaughtering of birds affected by the disease [3]. In the state of Santa Catarina, from 2012 to 2016, 96 outbreaks were reported, mostly involving commercial poultry farms in the western region of the state.

Studies to monitor and trace the pathogen may be useful in programs for the prevention and control of *Salmonella* spp., but they require phenotypic and genotypic differentiation of circulating serotypes on farms [4]. The genotypic characterization of the isolates can provide useful information to evaluate the genetic connections among strains and epidemiological investigations, and it is crucial for monitoring their circulation in different geographic regions [5]. Currently, pulsed-field gel electrophoresis (PFGE) is considered a gold-standard method for genotyping of *Salmonella* and the only suitable molecular method for all serotypes [6–8].

In this regard, this study aimed to perform PFGE analysis to evaluate genetic relationships of 56 SG strains, isolated during five years from 18 regions, two bird species, and five types of production.

A total of 56 SG strains were obtained from the Brazilian Official Laboratory (LANAGRO), Campinas, Brazil. Samples were collected from FT outbreaks that occurred in 18 municipalities of the state of Santa Catarina, two avian species, and five types of production from 2012 to 2016. Serotypes and biovars of *Salmonella* isolates were determined by the LANAGRO following conventional methods [9] and cryopreserved at –70 °C.

The PFGE technique was performed according to the Centers for Disease Control and Prevention's PulseNet

[1] World Organization for Animal Health (2019). World Animal Health Information Database (WAHIS Interface) – Version 1 [Online]. Website http://www.oie.int/wahis2/public/wahid.php/ [accessed 17 Jun 2019].

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protocol [10]. Briefly, cryopreserved SG strains were inoculated in 5% sheep blood agar (Oxoid, UK) and incubated at 37 °C for 18 to 24 h. The obtained colonies were transferred to trypticase soy agar (BD, USA) and incubated as previously described. The growths were collected in suspension buffer (1 M Tris-HCl [Invitrogen, USA], 0.5 M EDTA [Invitrogen, USA]) and the cell suspensions were adjusted to 5.0 McFarland turbidity standard. Agarose plugs were produced with 400 µL of 2.7% low melting agarose (Bio-Rad, USA), 400 µL of cell suspension, and 20 µL (20 mg/mL) of proteinase K (Invitrogen, USA). The plugs were incubated in 5 mL of lysis buffer (1 M Tris-HCl, 0.5 M EDTA, 10% lauryl sarcosine [Merck, Germany], and 25 µL of proteinase K [20 mg/mL]), at 54 °C under stirring for 2 h. Soon after, plugs were washed twice with ultrapure water and four times with elution buffer (1 M Tris-HCl [Invitrogen, USA], 0.5 M EDTA [Invitrogen, USA]) at 50 °C for 10 min and stored in TE solution (1 M Tris-HCl [Invitrogen, USA], 0.5 M EDTA [Invitrogen, USA], pH 8.0) at 4 °C. Chromosomal DNA was digested using 50 U/gel plug of restriction endonuclease *Xba*I (New England BioLabs, USA). The plug slices were placed in a 1.2% agarose gel (Bio-Rad, USA) and subjected to pulsed-field electrophoresis using the CHEF-DR III system (Bio-Rad, USA). The parameters used were as follows: 22 h of running time; 14 °C; low MW of 30 kb; high MW of 700 kb, 6 V/cm of voltage gradient; an included angle of 120° with 2.16 s of pulse time and 63.8 s of final pulse time. Electrophoresis was performed in 0.5 TEB solution (Tris Base [Invitrogen, USA], boric acid [Invitrogen, USA], and 0.5 M EDTA [Invitrogen, USA]). Gels were stained with 0.5 µg/mL ethidium bromide (Invitrogen, USA) and photographed under ultraviolet light.

The gel images were processed and analyzed by BioNumerics software (version 3.0; Applied Maths). DNA banding patterns were compared with *Salmonella enterica* serotype Braenderup H9812, digested by the enzyme *Xba*I (New England BioLabs, USA), and the similarity analysis was performed using the Dice coefficient with 1.7% tolerance [11]. The dendrogram was generated by the unweighted pair group method, and the isolates were considered to have the same pulsotype when the numbers and locations of the bands were indistinguishable.

Sixteen pulsotypes were obtained, showing 9 to 12 bands and 95.7% to 63.1% similarity among themselves (Figure). The most prevalent pulsotype (P1) corresponds to 57.1% of the samples analyzed (32/56), followed by P3 with 10.7% (6/56) and P2 with 5.4% (3/56). Still, pulsotypes with double or single samples were obtained. No pulsotype grouped the strains by region, type of production, or year of the isolation, except P3.

FT is a disease that affects avian species and causes considerable economic losses in the world poultry industry [12]. Although it is considered a disease under control in several developed countries [13], FT continues to be a severe problem in developing countries, particularly in Africa, Asia, and Latin America.[2]

During outbreaks of this disease, the molecular characterization of SG isolates can contribute to the surveillance service, pointing out the origin of the pathogen and supporting more effective measures to control the disease. The gold-standard method applied is PFGE, a discriminatory technique used in several public health networks (e.g., PulseNet, FoodNet, and VetNet) that allows the differentiation of isolated strains through genotyping [14] and interlaboratory standardization, consequently supporting comparisons between different studies [15].

In this study, the PFGE patterns analysis resulted in 16 pulsotypes. The largest group of pulsotypes obtained was P1, grouping SG strains regardless of their geographical region, type of production, or year of isolation and suggesting the existence of an endemic clone circulating in the state. Similar results were found in a Korean PFGE-based study, where no clear relationship among the geographic region or the breed was observed among SG strains grouped in the same cluster [16]. In a Brazilian study, six DNA banding patterns were found using the ERIC-PCR technique and some of the SG isolates recovered from different regions and years were clustered with 100% similarity, indicating the spread of the genotypes among these regions [17].

The transport of asymptomatic birds across these regions was likely responsible for the introduction of SG into healthy flocks, leading to the dissemination of the genotype detected in the P1 group. This assumption is supported by the fact that SG has limited survival outside the host since, under these conditions, the number of metabolic and biosynthetic routes is restricted [18–20]. Therefore, the transportation of animals plays a crucial role in spreading SG over long distances. Furthermore, horizontal transmission is the main route for the pathogen's propagation, which requires a source of infection, such as commercialized birds [21].

Strains of SG clustered in P1 were recovered over five years, indicating that there were favorable conditions for the propagation and perpetuation of the genotype. Failures in biosafety programs and the adoption of inadequate hygiene measures are among the factors that may have contributed to the persistence of this pathogen. Because the epidemiology of *Salmonella* in poultry farming is quite complex and bacteria can be introduced through different sources (such as food, water, insects, rodents, and rubber

[2] World Organization for Animal Health (2019). World Animal Health Information Database (WAHIS Interface) – Version 1 [Online]. Website http://www.oie.int/wahis2/public/wahid.php/ [accessed 17 Jun 2019].

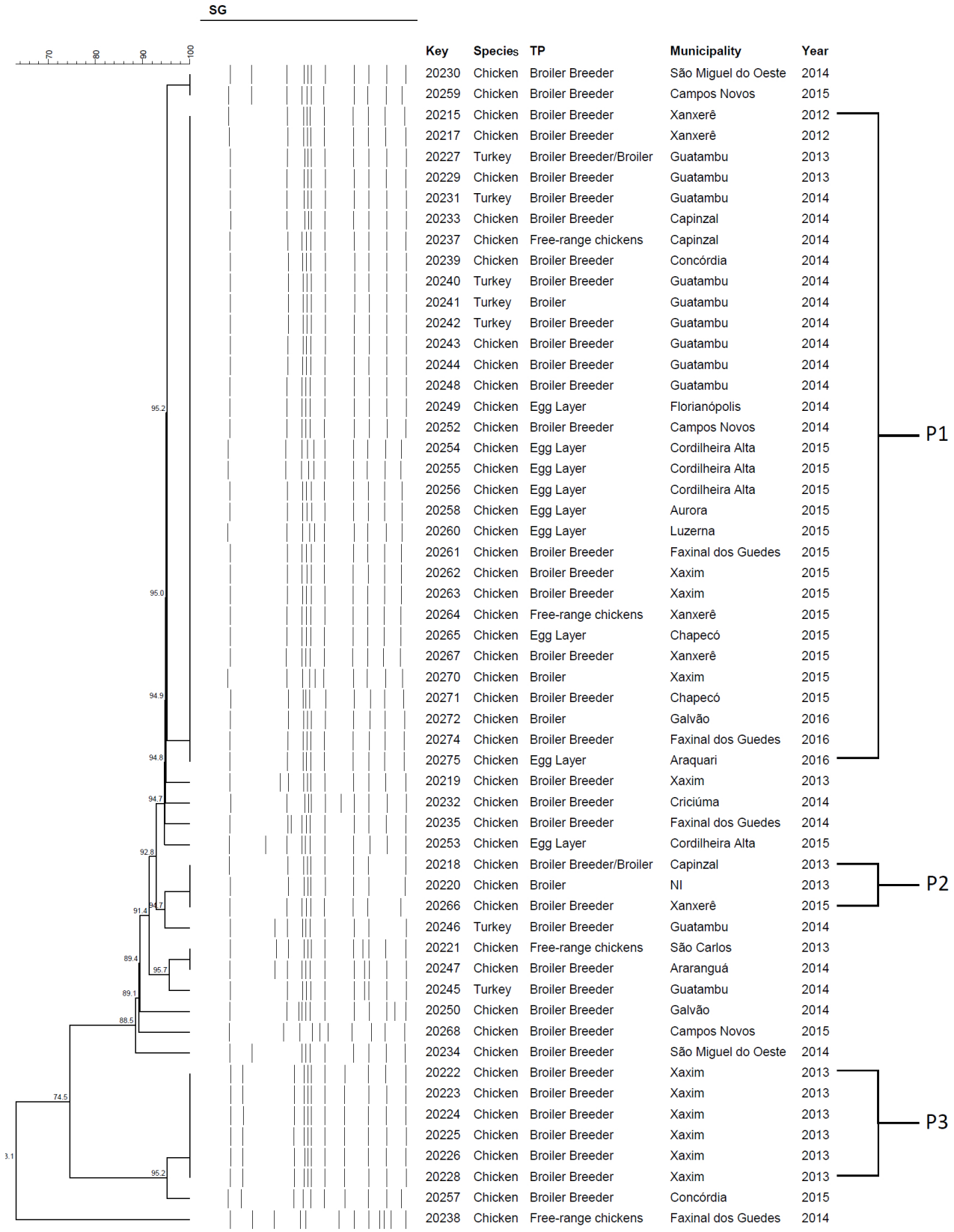

Figure. Dendrogram displaying PFGE profiles using restriction enzyme *Xba*I for *Salmonella* Gallinarum biovar Gallinarum isolates. *Xba*I pulsotypes are given with ID number (Key), species, type of production (TP), municipality, and year of the reported FT outbreaks.

boots), biosecurity programs must mitigate the risk of pathogens entering [22].

The P3 pulsotype strains were grouped from a single region, which suggests that that genotype has circulated specifically in that geographical area. For the other pulsotypes, no direct relationship of the strains with the year, type of production, or region was observed. The 16 pulsotypes obtained show the genetic diversity of the circulating strains across the state and demonstrate the multiple contamination sources of the flocks.

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