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ORIGINAL ARTICLE

ERIC-PCR genotyping of field isolates of *Salmonella enterica* subsp. *enterica* serovar Gallinarum biovars Gallinarum and Pullorum

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Salmonella Gallinarum (SG) and *Salmonella* Pullorum (SP) have been classified as biovars belonging to *Salmonella enterica* subsp. *enterica* serovar Gallinarum. Genetic diversity among isolates of the same biovar can be detected by DNA fingerprinting techniques which are useful in epidemiological investigations. In this study, we applied the PCR amplification of Enterobacterial Repetitive Intergenic Consensus sequences (ERIC-PCR) to analyse 45 strains of SG and SP, most of which were isolated from diseased poultry of different Brazilian regions over a period of 27 years until 2014. The ERIC-genotypes obtained were used to describe the epidemiological relationship amongst the strains. Our findings showed that there were six ERIC-patterns for SG strains at 80% similarity. In addition, some of the SG isolates recovered from different regions and years clustered with 100% similarity, suggesting that transfer of genotypes between these regions has taken place. The commercial rough vaccine strain 9R showed a unique profile. Meanwhile, more genetic diversity was observed among SP strains where ten ERIC-patterns were also formed at 80% similarity.

Introduction

Salmonella Gallinarum (SG) is the aetiological agent of fowl typhoid (FT), a septicaemic disease observed mainly in adult birds, characterized by variable morbidity and high mortality. *Salmonella* Pullorum (SP) triggers an illness, pullorum disease (PD), a severe systemic disease that generally results in high mortality only in young birds (Barrow & Freitas Neto, 2011; OIE, 2012). Some of the birds that recover from PD, or immunocompetent adult birds which generally do not present clinical disease, may develop a carrier state and bacteria may be thus transmitted vertically at sexual maturity (Berchieri Junior *et al*., 2001; Shivaprasad & Barrow, 2008). In Brazil, both FT and PD have decreased in incidence in commercial flocks since 1994 when the National Programme of Poultry Health (Programa Nacional de Sanidade Avícola – PNSA) was adopted and standard procedures towards poultry biosecurity established (Brazil, 2009). However, in the last few years, FT has again emerged in breeder and commercial flocks reared in several regions of the country (OIE, 2015) although official data do not make this evident.

During a disease outbreak, a detailed epidemiological investigation would directly influence measures that should be adopted for the application of a successful control plan. DNA fingerprinting techniques are powerful tools by providing genetic information about the isolates related to an ongoing outbreak and may thus be used for this purpose (Liebana *et al*., 2001).

The Enterobacterial Repetitive Intergenic Consensus (ERIC) technique has been used in order to identify genetic diversity in enterobacteria (Versalovic *et al*., 1991; Sachdeva & Virdi, 2004; Yu *et al*., 2011; Fendri *et al*., 2013; Dorneles *et al*., 2014). This method has allowed separation of clonal strains by the detection of specific chromosomal segments (ERIC sequences) suggesting this could be applied in epidemiological investigations (Dorneles *et al*., 2014). It is noteworthy that the current gold standard method to discriminate bacteria involved in epidemiological surveys is Pulsed-Field Gel Electrophoresis (PFGE) (Ribot *et al*., 2006). However, PFGE is both time consuming and requires staff skilled in the method to be reproducible. The ERIC method, generally considered to be both reliable and sensitive, is a much simpler procedure than PFGE (Kosek *et al*, 2012; Fendri *et al*., 2013; Dorneles *et al*., 2014).

To the best of our knowledge, there are no studies that have applied ERIC-PCR analysis to SG and SP isolates. Therefore, the present study aimed at assessing the applicability of ERIC-PCR in epidemiological studies involving SG and SP field strains.

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Materials and Methods

Strains. Forty-four field strains, 22 of SG and 22 of SP, and one commercial SG rough vaccine strain, 9R, were used in this study (Table 1). All field strains were isolated from chickens presenting clinical signs of FT or PD. They were identified by serotyping and biochemical tests at The National Agriculture and Livestock Laboratory (LANAGRO) or The Oswaldo Cruz Foundation (FIOCRUZ), which are official laboratories associated with the Brazilian Ministry of Agriculture, Livestock and Supply (MAPA). SG strain 9R was obtained from a commercial live vaccine. All strains were stored at −80°C at the Laboratory of Avian Diseases in Faculdade de Ciências Agrárias e Veterinárias, until use.

DNA extraction. Strains were inoculated in 10 ml of sterile Lysogeny broth (Becton Dickinson and Company, San Jose, CA, USA) which was incubated at 37°C overnight (18 h) with constant shaking (150×*g*). Bacteria were pelleted from 1 ml of this culture and chromosomal DNA extracted using the method developed by Marmur (1961). DNA quantity (ng/µl) and quality (ratios of absorbance) were measured in a spectrophotometer (DeNovix, Wilmington, DE, USA). Values of ratios in the range of 1.8–2.0 at 260/280 nm and 1.8–2.2 at 260/230 nm of absorbance indicate that DNA samples have very low concentration or absence of contaminants such as salts, proteins and solvents. Only DNA samples which showed quality parameters of approximately two were used in this study.

ERIC-PCR reaction. ERIC-PCR was performed according to Fendri *et al.* (2013) with the modifications described below. Each PCR reaction was made up of 1x DreamTaq™ Green Buffer with $MgCl_2$ (Thermo Fisher Scientific Inc., Asheville, NC, USA – EP0711), 1.0 pmol of primers ERIC1R (5′-ATG TAA GCT CCT GGG GAT TCA C-3′) and ERIC2 (5′-AAG TAA GTG ACT GGG GTG AGC G-3′) (Sigma-Aldrich, Saint Louis, MO, USA), 5 mM of $MgCl_2$ (Invitrogen Corp., Carlsbad, CA, USA), 240 µM of each deoxynucleotide triphosphate (dNTP) (Invitrogen Corp.), 5 µl of DNA template (approximately 60 ng), 2 U of Taq DNA polymerase (Thermo Fisher Scientific Inc.) and ultrapure water (Sigma-Aldrich) to a final volume of 25 µl. The PCR conditions were 5 cycles of 3 min at 94°C, 1 min at 49°C and 2 min at 72°C and then followed by 35 cycles of 1 min at 94°C, 1 min at 56°C and 2 min at 72°C, with the last extension at 72°C for 5 min. A 5 µl aliquot of each amplified reaction was mixed with 1 µl of 1:500 diluted Gel-Red™ Nucleic Acid Stain, 10.000 in water (Biotium Inc., Hayward, CA, USA) and then loaded on a 1.5% agarose gel. Electrophoresis was performed at 4 V/cm for 1 h and then the agarose gel was exposed to UV light using the Gel

Table 1. Salmonella *Gallinarum and* Salmonella *Pullorum strains used in this study.*

Strain	Original name	Biovar	Source	State of isolation	Year of isolation
1	SP CEPÁRIO M 191 – CR 31/06-1	Pullorum	LANAGRO	No information	2005
2	SP CEPÁRIO 578 – CR 350/06	Pullorum	LANAGRO	No information	2006
5	SG ATCC 9184	Gallinarum	Fiocruz/INCQS 00378	No information	No information
7	SP CEPÁRIO M 328 – CR 366/08	Pullorum	LANAGRO	No information	2008
8	SP ATCC 9120	Pullorum	Fiocruz/INCQS 00261	No information	No information
10	SG 345/11	Gallinarum	LANAGRO	No information	2011
11	SP CEPÁRIO 783 – CR 135/07	Pullorum	LANAGRO	No information	2007
12	SP CEPÁRIO 459 – CR 32/06	Pullorum	LANAGRO	No information	2005
13	SG 33 – IOC 1780/96 SP	Gallinarum	Fiocruz	São Paulo	1996
14	SG 34 – IOC 4623/98 MG	Gallinarum	Fiocruz	Minas Gerais	1998
15	SG 35 – IOC 4295/02 MG	Gallinarum	Fiocruz	Minas Gerais	2002
17	SG 31 – IOC 3558/99 RS	Gallinarum	Fiocruz	Rio Grande do Sul	1999
18	SP 8 – IOC 1771/96 SP	Pullorum	Fiocruz	São Paulo	1996
19	SP 7 – IOC 9771/04 RS	Pullorum	Fiocruz	Rio Grande do Sul	2004
20	SP 4 – IOC 1467/97 SP	Pullorum	Fiocruz	São Paulo	1997
21	SP 1 – IOC 332/01 SC	Pullorum	Fiocruz	Santa Catarina	2001
23	SP CEPÁRIO 104 – AV 363/07	Pullorum	LANAGRO	No information	2007
26	SG 36 – IOC 2003/01 MT	Gallinarum	Fiocruz	Mato Grosso	2001
31	SG 291/90	Gallinarum	FCAV/Unesp	São Paulo	1990
35	SP 449/87 Nal	Pullorum	FCAV/Unesp	São Paulo	1987
36	SG257/87-a	Gallinarum	FCAV/Unesp	São Paulo	1987
40	SP 6 – IOC 1783/96 SP	Pullorum	Fiocruz	São Paulo	1996
44	IBB1811 SP	Gallinarum	BASTOS	São Paulo	2011
48	31086/2012	Gallinarum	Mercolab	South region	2012
49	22124/2012	Gallinarum	Mercolab	South region	2012
50	24530/2010	Gallinarum	Mercolab	South region	2010
53	7514/2012	Pullorum	Mercolab	South region	2012
54	19228/2012	Pullorum	Mercolab	South region	2012
55	19035/2012	Pullorum	Mercolab	South region	2012
56	19551/2009	Gallinarum	Mercolab	South region	2009
58	28880/2012	Pullorum	Mercolab	South region	2012
59	15154/2012	Pullorum	Mercolab	South region	2012
60	27496/2012	Pullorum	Mercolab	South region	2012
64	20811/2012	Pullorum	Mercolab	South region	2012
65	22301/2012	Pullorum	Mercolab	South region	2012
111	SG287/91	Gallinarum	FCAV/Unesp	São Paulo	1991
194	1035/12	Gallinarum	FCAV/Unesp	São Paulo	2012
A	1044/13	Gallinarum	FCAV/Unesp	São Paulo	2013
B	1047/14	Gallinarum	FCAV/Unesp	São Paulo	2014
C	1050/14	Gallinarum	FCAV/Unesp	São Paulo	2014
D	1039/12	Gallinarum	FCAV/Unesp	São Paulo	2012
E	1049/14	Pullorum	FCAV/Unesp	São Paulo	2014
F	1005/12	Gallinarum	FCAV/Unesp	São Paulo	2012
G	1006/12	Gallinarum	FCAV/Unesp	São Paulo	2012
9R	–	–	–		–

Doc Ez (Bio-Rad, Hercules, CA, USA) system for imaging. A 1 Kb Plus DNA Ladder (Invitrogen Corp.) was included on the gel as a marker for each set of five samples.

Data analysis. The similarity matrix was obtained from the dissimilarity matrix generated after the application of Dice coefficient with 1% of tolerance and 0.5% of optimization to data. The UPGMA (Unweighted Pair Group with Arithmetic Mean) method was applied to this matrix in order to construct a dendrogram by means of the software Bionumerics version 7.1 (Applied Maths, Sin Martens Latem, Belgium). Data from SG or SP strains were analysed separately and two dendrograms were thus built. The 9R vaccine strain was analysed in parallel with the other SG strains.

Results

ERIC-PCR band profiles from each SG and SP strain tested (ERIC-genotype) was used to generate two dendrograms by cluster analysis (Figures 1 and 2). The DNA profile from isolates was analysed by biovar. Vertical lines were positioned in both plots, cutting them off at 80% similarity. Six distinct ERIC-patterns for SG (G1–G6) and 10 for SP (P1–P10) strains were thus identified.

A common ERIC-pattern, designated G1, encompassed 12 out of the 22 SG strains tested (Figure 1). These strains were isolated during a broad range of years and from the South and Southeast regions of Brazil (Table 1). Strains 13, 15 and 17 (group G1) showed identical ERIC-genotypes. They were isolated in different states (São Paulo, Minas Gerais and Rio Grande do Sul) between 1996 and 2002. Hundred percent similarity was also observed for strains B and C (group G1) and 5, 10, 111 and 194 (group G5). *Salmonella* Gallinarum strain 287/91 (111), isolated in 1991 and characterized by *in vivo* experimental work (Freitas Neto *et al.*, 2013), was identical to two other strains isolated in 2011 and 2012, in addition to the ATCC 9184 (5). Groups G2, G3, G4, and the 9R rough vaccine strain were represented by one strain each, suggesting a distant genetic relationship between them and the others. Groups G5 and G6 comprised strains geographically more distant, isolated from poultry reared in São Paulo and southern states, respectively.

SP strains presented a wider variety of genotypes than SG strains (Figure 2). Bacteria forming group P1 were isolated from a more recent outbreak occurring between 2012 and 2014. By contrast the three strains (19, 20 and 21) in group P4 were isolated from poultry reared in Rio Grande do Sul, São Paulo and Santa Catarina, respectively, between 1997 and 2004, and were identical by ERIC analysis.

Discussion

FT and PD are diseases that affect avian species and cause considerable economic losses to the poultry industry worldwide (Barrow & Freitas Neto, 2011). Both illnesses are considered under control in many developed countries (OIE, 2012). Nevertheless, they remain a serious problem in developing countries in Africa, Eastern Asia, and Central and South America (OIE, 2015). During an ongoing outbreak full characterization of the isolates involved would be an advantage to the surveillance service indicating the source of the pathogen thus helping the adoption of more efficient measures to control further spread of the disease.

ERIC-PCR has been shown as a powerful tool for demonstrating variability among isolates (Sachdeva & Virdi, 2004; Fendri *et al.*, 2013). It has been suggested that ERIC sequences would in all likelihood have some functional role in the bacterial cell (Asinmov *et al.*, 2005; De Gregorio *et al.*, 2005) although this has not yet been demonstrated.

The dendrogram generated from the SG ERIC-genotypes presented a large ERIC-pattern, here designated as G1, which clustered most of SG strains, regardless of their

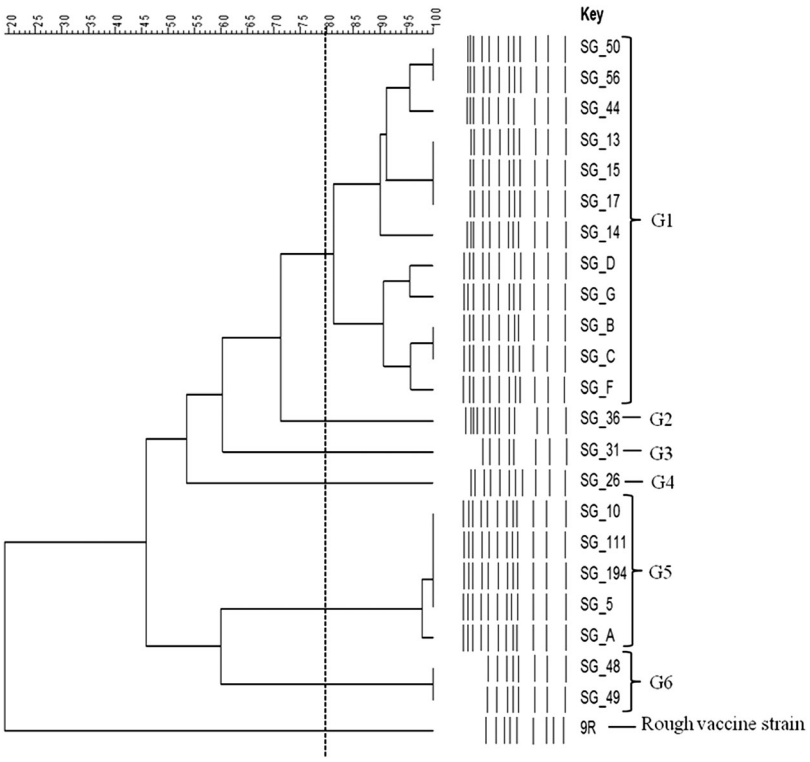

Figure 1. *Dendrogram clustering the 22 field strains of* Salmonella *Gallinarum (SG) and a rough vaccine strain (9R) through UPGMA method and Dice similarity coefficient.*

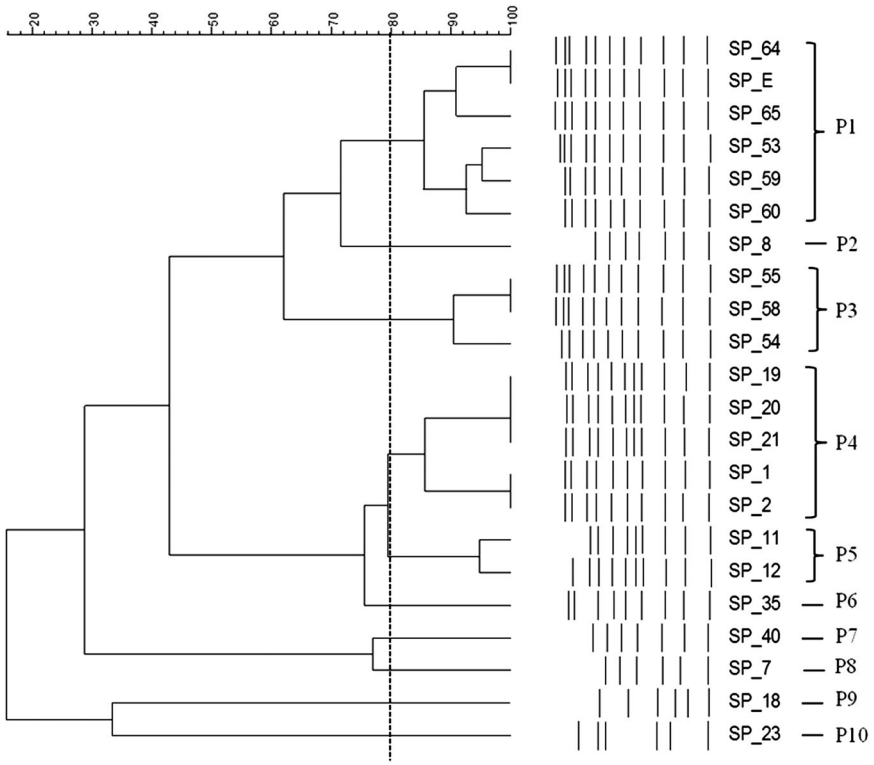

Figure 2. *Dendrogram grouping the 22 field strains of* Salmonella *Pullorum (SP) by using UPGMA method and Dice similarity coefficient.*

geographic region or year of isolation. Since most SG strains tested here were isolated in the South and Southeast regions of Brazil, this datum suggests the existence of an endemic clone predominant in these regions. A similar hypothesis was previously proposed by Kwon *et al*. (2010), who applied the PFGE-technique to SG strains isolated in Korea, and could not observe any relationship between year and geographic region of isolation for most SG strains studied.

Reinforcing the independence of the year of isolation, we noted that strains 10 (isolated in 2011) and 194 (isolated in 2012) presented ERIC-genotypes identical to the highly pathogenic strain SG 287/91 (111), isolated in 1991 from infected poultry (ERIC-pattern G5). These findings suggest that the resurgence of FT in Brazilian flocks may not be related to the evolution of more virulent SG strains, but rather more likely to the result of failure in biosecurity programmes and neglect of implementation of hygiene measures.

Interestingly, SG strains 13, 15 and 17 also showed 100% similarity, although they have been isolated in different Brazilian states between 1999 and 2002. Such an observation suggests that the bacteria would have been continually present in these states during this period. It is believed that SG and SP have a low viability outside the host because they are impaired in a number of metabolic and biosynthetic pathways (McMeechan *et al.*, 2005; Thomson *et al*., 2008; Feng *et al*., 2013). Thus, SG propagation over long distances is probably unlikely to happen naturally. Moreover, horizontal transmission is thought to be the main route of SG propagation (Berchieri Junior *et al*., 2001). Commercial exchange of poultry would likely have taken place between these regions, and asymptomatic birds, harbouring this pathogen, could have been introduced into healthy poultry flocks.

The acquisition of a more virulent phenotype by the rough vaccine strain 9R (reversion of virulence) was previously suggested by Van Immerseel *et al*. (2013) in order to explain the genetic relatedness found, by PFGE, between the vaccine and some field strains. In this study, similar evidence was not supported by our data. SG 9R presented a very different ERIC-genotype and, due to this low similarity with the other SG strains, it clustered separately from the others.

PD has been controlled in Brazil since the implementation of the National Programme of Poultry Health, in 1994 (Brazil, 2009), although some cases occur sporadically (OIE, 2015). SP strains showed more variable ERIC-genotypes in comparison with SG strains (10 ERIC-patterns). Nevertheless, contrary to what was observed for SG strains, there was no predominant SP ERIC-pattern. Pseudogene formation in DNA repair coding sequences has been described in SP strains, suggesting that this biovar would be more susceptible to mutations (Feng *et al*., 2013). This could explain the genetic diversity observed among the SP strains across the different regions in this study. For instance, in São Paulo state (Southeast region) SP strains from five distinct ERIC-patterns were isolated. A similar picture has also been observed in the South region where 3 ERIC-patterns were identified.

In this study the ERIC-PCR method was able to discriminate strains within individual biovars and could be adopted in investigations of FT and/or PD outbreaks.

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