Pesq. Vet. Bras. 33(12):1416-1422, dezembro 2013

Detection of virulence-associated genes in *Salmonella* Enteritidis isolates from chicken in South of Brazil[1]

Karen A. Borges[2]*, Thales Q. Furian[2], Anderlise Borsoi[3], Hamilton L.S. Moraes[2], Carlos T.P. Salle[2] e Vladimir P. Nascimento[2]

ABSTRACT.- Borges K.A., Furian T.Q., Borsoi A., Moraes H.L.S., Salle C.T.P. & Nascimento V.P. 2013. **Detection of virulence-associated genes in *Salmonella* Enteritidis isolates from chicken in Southern Brazil.** *Pesquisa Veterinária Brasileira 33(12):1416-1422*. Centro de Diagnóstico e Pesquisa em Patologia Aviária, Faculdade de Veterinária, Universidade Federal do Rio Grande do Sul, Avenida Bento Gonçalves 8824, Porto Alegre, RS 91540-000, Brazil. E-mail: karen.borges@ufrgs.br

Salmonella spp. are considered the main agents of foodborne disease and *Salmonella* Enteritidis is one of the most frequently isolated serovars worldwide. The virulence of *Salmonella* spp. and their interaction with the host are complex processes involving virulence factors to overcome host defenses. The purpose of this study was to detect virulence genes in *S.* Enteritidis isolates from poultry in the South of Brazil. PCR-based assays were developed in order to detect nine genes (*lpfA*, *agfA, sefA, invA, hilA, avrA, sopE, sivH* and *spvC*) associated with the virulence in eighty-four isolates of *S.* Enteritidis isolated from poultry. The *invA*, *hilA, sivH, sefA* and *avrA* genes were present in 100% of the isolates; *lpfA* and *sopE* were present in 99%; *agfA* was present in 96%; and the *spvC* gene was present in 92%. It was possible to characterize the isolates with four different genetic profiles (P1, P2, P3 and P4), as it follows: P1, positive for all genes; P2, negative only for *spvC*; P3, negative for *agfA*; and P4, negative for *lpfA*, *spvC* and *sopE*. The most prevalent profile was P1, which was present in 88% of the isolates. Although all isolates belong to the same serovar, it was possible to observe variations in the presence of these virulence-associated genes between different isolates. The characterization of the mechanisms of virulence circulating in the population of *Salmonella* Enteritidis is important for a better understanding of its biology and pathogenicity. The frequency of these genes and the establishment of genetic profiles can be used to determine patterns of virulence. These patterns, associated with *in vivo* studies, may help develop tools to predict the ability of virulence of different strains.

INDEX TERMS: *Salmonella* Enteritidis, PCR, virulence profile, poultry.

RESUMO.- [Detecção de genes associados à virulência em cepas de *Salmonella* Enteritidis isoladas de frangos na região sul do Brasil.] *Salmonella* spp. estão entre os principais agentes causadores de doenças transmitidas por alimentos, e o sorovar *Salmonella* Enteritidis é o mais frequentemente isolado no mundo. A virulência de *Salmonella* spp. e a sua interação com o hospedeiro são processos complexos que envolvem fatores de virulência para sobreviver às defesas do hospedeiro. O objetivo deste estudo foi detectar genes de virulência em cepas de *S.* Enteritidis isoladas a partir de fontes avícolas no sul do Brasil. Ensaios de PCR foram desenvolvidos para a detecção de nove genes (*lpfA*, *agfA, sefA, invA, hilA, avrA, sopE, sivH* e *spvC*) associados à virulência em oitenta e quatro amostras de *S.* Enteritidis. Os genes *invA*, *hilA, sivH, sefA* e *avrA* estavam presentes em 100% dos isolados; *lpfA* e *sopE* estavam presentes em 99%; *agfA* em 96%; e o gene *spvC* estava presente em 92%. Foi possível caracterizar os isolados em quatro perfis genéticos distintos (P1, P2, P3 e P4), sendo P1 positivo para todos os genes; P2 negativo apenas para *spvC*; P3 negativo para *agfA* e P4 negativo para *lpfA*, *spvC* e *sopE*. O perfil de maior frequência foi P1, presente em 88% dos isolados. Apesar de

[1] Received on June 27, 2013.
Accepted for publication on October 26, 2013.

[2] Centro de Diagnóstico e Pesquisa em Patologia Aviária (CDPA), Faculdade de Veterinária, Universidade Federal do Rio Grande do Sul (UFRGS), Av. Bento Gonçalves 8824, Porto Alegre, RS 91540-000, Brazil. *Corresponding author: karen.borges@ufrgs.br

[3] Tuiuti Universidade do Paraná, Rua Sydnei Antônio Rangel 238, Curitiba, PR 82010-330, Brazil.

todos os isolados pertencerem ao mesmo sorovar, foi possível observar variações na presença de genes associados à virulência entre os mesmos. A caracterização dos mecanismos de virulência circulantes na população de *Salmonella* Enteritidis é importante para um maior entendimento da sua biologia e patogenicidade. A frequência destes genes e o estabelecimento de perfis genéticos podem ser utilizados para determinar os padrões de virulência dos isolados. Estes padrões, associados a estudos *in vivo*, podem auxiliar na elaboração de ferramentas que permitam predizer a capacidade de virulência das diferentes cepas.

TERMOS DE INDEXAÇÃO: *Salmonella* Enteritidis, PCR, perfil de virulência, frangos.

INTRODUCTION

Salmonella spp. are considered the major cause of foodborne disease in humans, and *Salmonella* Enteritidis is the most frequently isolated serovar in Europe, South America and Asia (Vieira et al. 2009). Phylogenetic analyses show the influence of different factors in the existence and persistence of *Salmonella* spp in animals, such as cross-contamination among animals, environment and feed (Mello et al. 2011). The virulence of *Salmonella* spp. is associated with a combination of chromosomal and plasmid factors (Oliveira et al. 2003), and many studies have identified genes that encode these factors. Some virulence factors are associated with the cellular structure of the bacteria, such as fimbriae (Edwards & Puente 1998). The long polar fimbria (*lpf operon*) contributes to the affinity of the bacteria for Peyer's patches and adhesion to intestinal M cells (Bäumler & Heffron 1995, Bäumler et al. 1996). One of the main functions of aggregative fimbria (*agf operon*) is to promote the initial interaction of the bacteria with the intestine of the host and stimulate bacterial self-aggregation, resulting in higher rates of survival (Collinson et al. 1992, 1993). The *Salmonella*-encoded fimbria (*sef operon*) promotes a better interaction between the bacteria and the macrophages (Collinson et al. 1996).

Salmonella spp. pathogenicity islands (SPI) are of critical importance for *Salmonella* spp. virulence, once they encode a molecular apparatus called the type III secretion system (TTSS), which is able to inject bacterial effector proteins through bacterial and host membranes to interact with host cells (Marcus et al. 2000). The *hilA* gene encodes the central regulator HilA, which is necessary for the expression of the TTSS components. HilA is also required to invade epithelial cells and induce apoptosis of macrophages (Bajaj et al. 1996). The protein InvA is essential for epithelial invasion (Galán & Curtis III 1989) and AvrA is an effector protein of the TTSS complex that contributes to the virulence of *Salmonella* spp. by limiting the host's inflammatory responses through the inducement of cell apoptosis, especially of macrophages, and by the innibition of IL-8 and TNF-α (Collier-Hyames et al. 2002, Ben-Barak et al. 2006). *Salmonella* spp.'s outer proteins (Sops) contribute to the invasion by these bacteria through the generation of membrane deformations (Hardt et al. 1998) and the rearrangement of the cytoskeleton of the host cells (Galán & Zhou 2000). The *sivH* gene encodes an outer membrane protein associated with intestinal colonization (Kingsley et al. 2003). Other important *Salmonella* spp. virulence factors are found on virulence plasmids. All of the virulence plasmids share a highly conserved region designated *spv*RABCD (*Salmonella* plasmid virulence). The spv region promotes rapid growth and survival of *Salmonella* spp. within the host cells and it is important for systemic infection (Libby et al. 1997). The purpose of this study was to evaluate the virulence potential of *S*. Enteritidis isolates from poultry by detecting the presence of nine virulence-associated genes by polymerase chain reactions (PCRs), as well as to determine the distribution patterns of these genes.

MATERIALS AND METHODS

Bacterial isolates. This stduy was developed at the Diagnostic Center and Research in Avian Pathology (CDPA) of Federal University of Rio Grande do Sul (UFRGS). Eighty-four isolates of *Salmonella* Enteritidis were selected from CDPA collection. These bacteria were isolated between 1996 and 2010 from different avian sources in Rio Grande do Sul state in the south of Brazil. The sources included broiler systems and slaughterhouses; in addition we also used one sample of hatchery. Additional data of isolates (year and source of isolation) are shown in Table 1. A complete antigenic characterization and serovar identification were performed by the Enteric Pathogens Laboratory in the Oswaldo Cruz Institute Foundation (Fiocruz, Rio de Janeiro, Brazil). The bacterial isolates were kept frozen at -70°C in brain heart infusion broth and glycerol.

DNA extraction. The bacteria were retrieved from frozen culture stocks and cultured overnight at 37°C in brain heart infusion broth (Oxoid; Cambridge, United Kingdom). An aliquot of 1 mL of each bacterial culture was separated for DNA extraction by heat treatment as described by Borsoi et al. (2009).

Polymerase chain reaction (PCR). The PCRs were conducted in individual reactions using primers for the following genes: *invA, hilA, avrA, agfA, lpfA, sefA, sopE, spvC* and *sivH*. The sets of primer pairs, sizes of the PCR products and references used in the PCR assay are described in Table 2. All PCR mixtures (25μL) were performed with 2.5μL of 10X PCR buffer (Centro de Biotecnologia UFRGS; Porto Alegre, Rio Grande do Sul, Brazil), 1 U of Taq DNA polymerase (Centro de Biotecnologia UFRGS; Porto Alegre, Rio Grande do Sul, Brazil) and 2 μL of template DNA. The reagent concentrations, amplification conditions and number of cycles are described in Table 3. The cycling program was perfomed in the Esco Swift MaxPro thermal cycler (Esco, Singapore). The amplified products were separated by electrophoresis in a 1.2% agarose gel and stained with ethidium bromide. Fragments were transilluminated with UV light. *Escherichia coli* ATCC 25922 and *Salmonella* Enteritidis ATCC 13076 were used as negative and positive controls, respectively, for all PCR reactions, except for that of the *agfA* gene, for which *Salmonella* Typhimurium ATCC 14028 was used as a positive control. In all PCRs, a mixture of all constituents of the PCR mixed without the addition of extracted DNA was used as PCR control.

RESULTS

The *Salmonella* Enteritidis strains had different frequencies of the target genes, regardless the year and the source of isolation (Table 1). The *invA*, *hilA, sivH, sefA* and *avrA* genes were present in 100% (84/84) of the isolates. *lpfA* and *sopE* were present in 99% (83/84). Gene *agfA* was pre-

Table 1. Virulence genes and genetic profile of *Salmonella* Enteritidis isolates from chicken in South of Brazil

Strain	Year of isolation	Source	Present genes	Genetic Profile
1	1996	Broiler carcass	*invA, hilA, agfA, sefA, lpfA, sopE, avrA, sivH, spvC*	P1
2	1996	Broiler carcass	*invA, hilA, agfA, sefA, lpfA, sopE, avrA, sivH, spvC*	P1
3	1996	Broiler carcass	*invA, hilA, agfA, sefA, lpfA, sopE, avrA, sivH, spvC*	P1
4	1996	Broiler carcass	*invA, hilA, agfA, sefA, lpfA, sopE, avrA, sivH, spvC*	P1
5	1996	Broiler carcass	*invA, hilA, agfA, sefA, lpfA, sopE, avrA, sivH, spvC*	P1
6	1996	Broiler carcass	*invA, hilA, agfA, sefA, lpfA, sopE, avrA, sivH, spvC*	P1
7	1996	Broiler carcass	*invA, hilA, agfA, sefA, lpfA, sopE, avrA, sivH, spvC*	P1
8	1996	Broiler carcass	*invA, hilA, agfA, sefA, lpfA, sopE, avrA, sivH, spvC*	P1
9	1996	Broiler carcass	*invA, hilA, sefA, lpfA, sopE, avrA, sivH, spvC*	P3
10	1996	Broiler carcass	*invA, hilA, sefA, lpfA, sopE, avrA, sivH, spvC*	P3
11	1996	Broiler carcass	*invA, hilA, agfA, sefA, lpfA, sopE, avrA, sivH*	P2
12	1996	Broiler carcass	*invA, hilA, agfA, sefA, lpfA, sopE, avrA, sivH, spvC*	P1
13	1996	Broiler carcass	*invA, hilA, agfA, sefA, lpfA, sopE, avrA, sivH*	P2
14	1996	Broiler carcass	*invA, hilA, agfA, sefA, lpfA, sopE, avrA, sivH, spvC*	P1
15	1996	Broiler carcass	*invA, hilA, agfA, sefA, lpfA, sopE, avrA, sivH, spvC*	P1
16	1996	Broiler carcass	*invA, hilA, agfA, sefA, lpfA, sopE, avrA, sivH, spvC*	P1
17	1999	Broiler carcass	*invA, hilA, agfA, sefA, lpfA, sopE, avrA, sivH, spvC*	P1
18	1999	Drag swab	*invA, hilA, agfA, sefA, lpfA, sopE, avrA, sivH, spvC*	P1
19	1999	Drag swab	*invA, hilA, agfA, sefA, lpfA, sopE, avrA, sivH, spvC*	P1
20	1999	Drag swab	*invA, hilA, agfA, sefA, lpfA, sopE, avrA, sivH, spvC*	P1
21	1999	Drag swab	*invA, hilA, agfA, sefA, lpfA, sopE, avrA, sivH, spvC*	P1
22	1999	Drag swab	*invA, hilA, agfA, sefA, lpfA, sopE, avrA, sivH*	P2
23	1999	Drag swab	*invA, hilA, agfA, sefA, lpfA, sopE, avrA, sivH, spvC*	P1
24	1999	Drag swab	*invA, hilA, agfA, sefA, lpfA, sopE, avrA, sivH, spvC*	P1
25	1999	Liver	*invA, hilA, agfA, sefA, lpfA, sopE, avrA, sivH, spvC*	P1
26	1999	Liver	*invA, hilA, agfA, sefA, lpfA, sopE, avrA, sivH, spvC*	P1
27	1999	Liver	*invA, hilA, agfA, sefA, lpfA, sopE, avrA, sivH, spvC*	P1
28	1999	Liver	*invA, hilA, agfA, sefA, lpfA, sopE, avrA, sivH, spvC*	P1
29	1999	Liver	*invA, hilA, agfA, sefA, lpfA, sopE, avrA, sivH, spvC*	P1
30	1999	Liver	*invA, hilA, agfA, sefA, lpfA, sopE, avrA, sivH, spvC*	P1
31	1999	Liver	*invA, hilA, agfA, sefA, lpfA, sopE, avrA, sivH, spvC*	P1
32	1999	Liver	*invA, hilA, agfA, sefA, lpfA, sopE, avrA, sivH, spvC*	P1
33	1999	Liver	*invA, hilA, agfA, sefA, lpfA, sopE, avrA, sivH, spvC*	P1
34	1999	Liver	*invA, hilA, agfA, sefA, lpfA, sopE, avrA, sivH, spvC*	P1
35	1999	Liver	*invA, hilA, agfA, sefA, lpfA, sopE, avrA, sivH, spvC*	P1
36	1999	Liver	*invA, hilA, agfA, sefA, lpfA, sopE, avrA, sivH, spvC*	P1
37	1999	Liver	*invA, hilA, agfA, sefA, lpfA, sopE, avrA, sivH, spvC*	P1
38	1999	Liver	*invA, hilA, agfA, sefA, avrA, sivH*	P4
39	1999	Drag swab	*invA, hilA, agfA, sefA, lpfA, sopE, avrA, sivH, spvC*	P1
40	1999	Liver	*invA, hilA, agfA, sefA, lpfA, sopE, avrA, sivH, spvC*	P1
41	1999	Liver	*invA, hilA, agfA, sefA, lpfA, sopE, avrA, sivH, spvC*	P1
42	1999	Liver	*invA, hilA, agfA, sefA, lpfA, sopE, avrA, sivH*	P2
43	2000	Drag swab	*invA, hilA, agfA, sefA, lpfA, sopE, avrA, sivH, spvC*	P1
44	2000	Drag swab	*invA, hilA, agfA, sefA, lpfA, sopE, avrA, sivH, spvC*	P1
45	2000	Drag swab	*invA, hilA, sefA, lpfA, sopE, avrA, sivH, spvC*	P3
46	2000	Drag swab	*invA, hilA, agfA, sefA, lpfA, sopE, avrA, sivH, spvC*	P1
47	2000	Drag swab	*invA, hilA, agfA, sefA, lpfA, sopE, avrA, sivH, spvC*	P1
48	2000	Drag swab	*invA, hilA, agfA, sefA, lpfA, sopE, avrA, sivH, spvC*	P1
49	2000	Drag swab	*invA, hilA, agfA, sefA, lpfA, sopE, avrA, sivH, spvC*	P1
50	2000	Drag swab	*invA, hilA, agfA, sefA, lpfA, sopE, avrA, sivH, spvC*	P1
51	2000	Feed	*invA, hilA, agfA, sefA, lpfA, sopE, avrA, sivH, spvC*	P1
52	2000	Drag swab	*invA, hilA, agfA, sefA, lpfA, sopE, avrA, sivH*	P2
53	2000	Drag swab	*invA, hilA, agfA, sefA, lpfA, sopE, avrA, sivH, spvC*	P1
54	2000	Drag swab	*invA, hilA, agfA, sefA, lpfA, sopE, avrA, sivH, spvC*	P1
55	2000	Liver	*invA, hilA, agfA, sefA, lpfA, sopE, avrA, sivH, spvC*	P1
56	2000	Liver	*invA, hilA, agfA, sefA, lpfA, sopE, avrA, sivH, spvC*	P1
57	2000	Liver	*invA, hilA, agfA, sefA, lpfA, sopE, avrA, sivH, spvC*	P1
58	2000	Liver	*invA, hilA, agfA, sefA, lpfA, sopE, avrA, sivH, spvC*	P1
59	2000	Liver	*invA, hilA, agfA, sefA, lpfA, sopE, avrA, sivH, spvC*	P1
60	2000	Liver	*invA, hilA, agfA, sefA, lpfA, sopE, avrA, sivH, spvC*	P1
61	2000	Liver	*invA, hilA, agfA, sefA, lpfA, sopE, avrA, sivH, spvC*	P1
62	2000	Liver	*invA, hilA, agfA, sefA, lpfA, sopE, avrA, sivH, spvC*	P1
63	2000	Liver	*invA, hilA, agfA, sefA, lpfA, sopE, avrA, sivH, spvC*	P1
64	2000	Liver	*invA, hilA, agfA, sefA, lpfA, sopE, avrA, sivH, spvC*	P1
65	2000	Liver	*invA, hilA, agfA, sefA, lpfA, sopE, avrA, sivH*	P2
66	2000	Liver	*invA, hilA, agfA, sefA, lpfA, sopE, avrA, sivH, spvC*	P1

Table 1 (cont.). Virulence genes and genetic profile of *Salmonella* Enteritidis isolates from chicken in South of Brazil

Strain	Year of isolation	Source	Present genes	Genetic Profile
67	2000	Drag swab	*invA, hilA, agfA, sefA, lpfA, sopE, avrA, sivH, spvC*	P1
68	2001	Drag swab	*invA, hilA, agfA, sefA, lpfA, sopE, avrA, sivH, spvC*	P1
69	2001	Drag swab	*invA, hilA, agfA, sefA, lpfA, sopE, avrA, sivH, spvC*	P1
70	2001	Drag swab	*invA, hilA, agfA, sefA, lpfA, sopE, avrA, sivH, spvC*	P1
71	2001	Liver	*invA, hilA, agfA, sefA, lpfA, sopE, avrA, sivH, spvC*	P1
72	2001	Liver	*invA, hilA, agfA, sefA, lpfA, sopE, avrA, sivH, spvC*	P1
73	2001	Drag swab	*invA, hilA, agfA, sefA, lpfA, sopE, avrA, sivH, spvC*	P1
74	2001	Drag swab	*invA, hilA, agfA, sefA, lpfA, sopE, avrA, sivH, spvC*	P1
75	2001	Drag swab	*invA, hilA, agfA, sefA, lpfA, sopE, avrA, sivH, spvC*	P1
76	2001	Drag swab	*invA, hilA, agfA, sefA, lpfA, sopE, avrA, sivH, spvC*	P1
77	2001	Liver	*invA, hilA, agfA, sefA, lpfA, sopE, avrA, sivH, spvC*	P1
78	2001	Drag swab	*invA, hilA, agfA, sefA, lpfA, sopE, avrA, sivH, spvC*	P1
79	2001	Liver	*invA, hilA, agfA, sefA, lpfA, sopE, avrA, sivH, spvC*	P1
80	2001	Liver	*invA, hilA, agfA, sefA, lpfA, sopE, avrA, sivH, spvC*	P1
81	2001	Liver	*invA, hilA, agfA, sefA, lpfA, sopE, avrA, sivH, spvC*	P1
82	2001	Drag swab	*invA, hilA, agfA, sefA, lpfA, sopE, avrA, sivH, spvC*	P1
83	2001	Pipped eggs	*invA, hilA, agfA, sefA, lpfA, sopE, avrA, sivH, spvC*	P1
84	2010	Cecal content	*invA, hilA, agfA, sefA, lpfA, sopE, avrA, sivH, spvC*	P1

Table 2. Virulence factors genes identified in *Salmonella* Enteritidis from avian origin in South Brazil

Gene	Virulence factor	Primer sequence (5'-3')	Base pair	Reference
lpfA	Fimbria	CTTTCGCTGCTGAATCTGGT		
		CAGTGTTAACAGAAACCAGT	250	Bäumler &
agfA	Fimbria	TCCACAATGGGGCGGCGGCG		Heffron 1995
		CCTGACGCACCATTACGCTG	350	Cesco et al. 2008
sefA	Fimbria	GATACTGCTGAACGTAGAAGG		
		GCGTAAATCAGCATCTGCAGTAGC	488	Oliveira et al. 2002
invA	Invasion	GTGAAATTATCGCCACGTTCGGGCAA		
		TCATCGCACCGTCAAAGGAACC	284	Oliveira et al. 2002
hilA	Invasion	CTGCCGCAGTGTTAAGGATA		
		CTGTCGCCTTAATCGCATGT	497	Guo et al. 2000
avrA	Effector protein	GTTATGGACGGAACGACATCGG		
		ATTCTGCTTCCCGCCGCC	385	Prager et al. 2003
sopE	Effector protein	ACACACTTTCACCGAGGAAGCG		
		GGATGCCTTCTGATGTTGACTGG	398	Prager et al. 2003
sivH	Invasion	CAGAATGCGAATCCTTCGCAC		
		GTATGCGAACAAGCGTAACAC	763	Kingsley et al. 2003
spvC	Plasmid - virulence	CGGAAATACCATCTACAAATA		
		CCCAAACCCATACTTACTCTG	669	Swamy et al. 1996

sent in 96% (3/84) and the *spvC* gene was present in 92% (7/84). All isolates showed at least five virulence-associated genes. The results of the PCRs are summarized in Table 4. Based on the combination of present and absent genes, the *S.* Enteritidis were divided in four different gene profiles. In order to facilitate the analysis, these profiles were named P1, P2, P3 and P4. Regarding the profiles, among the 84 isolates analyzed, 88% (74/84) were categorized as P1 (positive for all genes), 7% (6/84) as P2 (*spvC* absent), 4% (3/84) as P3 (*agfA* absent) and 1% (1/84) as P4 (*lpfA*, *sopE* and *spvC* absent).

DISCUSSION

All South Brazilian *Salmonella* Enteritidis isolates were positive for *invA* and *hilA;* similar observations have been reported by other studies around the world (Amini et al. 2010, Campioni et al. 2012, Craciunas et al. 2012). It was expected that these genes would be detected in all of the isolates due to their importance for cell invasion. PCR is a useful tool for the rapid detection of *Salmonella* spp., and *inv*A and *hilA* genes can be considered target genes for the detection of this genus.

Although results for the *sopE* and *avrA* genes were similar to the 100% frequency found in previous studies on *Salmonella* Enteritidis (Hopkins & Threlfall 2004), other works have found lower frequencies (Rahman et al. 2004, Streckel et al. 2004, Zou et al. 2011, Liu et al. 2012). This frequency variation could be caused by the recombinations that frequently occur in the location of these genes (Hopkins & Threlfall 2004). These findings are important, since changes in the repertoire of proteins, such as SopE and AvrA, can lead to changes in the ability of this serovar to adapt to new hosts and, consequently, the emergence of novel virulent strains (Prager et al. 2000). In our study, a high percentage (99%) of isolates had the *avrA* and *sopE* genes. However, only 17.1% and 9.7% of *Salmonella* Hadar isolates had the *avrA* and *sopE* genes, respectively (Cesco 2010). When comparing this data, it is clear that there is

Table 3. PCR assay conditions used in this study to detect the virulence-associated genes in *Salmonella* Enteritidis isolates

Gene	Reagent quantities and concentrations	Thermal amplification conditions *	Number of cycles
lpfA	0.2 µL dNTPs (2.5 mM), 1 µL each primer (20 pmol), 2 µL $MgCl_2$ (4 mM)	94°C, 1 sec 55°C, 1 sec 74°C, 21 sec	35
agfA	2 µL dNTPs (2.5 mM), 1 µL each primer (20 pmol), 1.25 µL $MgCl_2$ (2.5 mM)	94°C, 1 sec 58°C, 1 sec 74°C, 21 sec	35
sefA	2 µL dNTPs (2.5mM), 1 µL each primer (20 pmol), 1.25 µL $MgCl_2$ (2.5mM)	94°C, 1 sec 55°C, 1 sec 74°C, 21 sec	35
invA	2 µL dNTPs (2.5 mM), 1 µL each primer (20 pmol), 1.25 µL $MgCl_2$ (2.5 mM)	94°C, 1 sec 58°C, 1 sec 74°C, 21 sec	35
hilA	2 µL dNTPs (2.5 mM), 1 µL each primer (20 pmol), 0.75 µL $MgCl_2$ (1.5 mM)	94°C, 120 sec 62°C, 60 sec 72°C, 60 sec	30
avrA	2 µL dNTPs (2.5 mM), 1 µL each primer (20 pmol), 1 µL $MgCl_2$ (2 mM)	94°C, 60 sec 64°C, 60 sec 72°C, 60 sec	30
sopE	2 µL dNTPs (2.5 mM), 1 µL each primer (20 pmol), 1 µL $MgCl_2$ (2 mM)	94°C, 60 sec 55°C, 60 sec 72°C, 60 sec	30
sivH	2 µL dNTPs (2.5 mM), 1 µL each primer (20 pmol), 1 µL $MgCl_2$ (2 mM)	94°C, 30 sec 56°C, 45 sec 72°C, 45 sec	30
spvC	3 µL dNTPs (2.5 mM), 3.5 µL each primer (20 pmol), 1 µL $MgCl_2$ (2 mM)	93°C, 60 sec 42°C, 60 sec 72°C, 120 sec	30

* sec = seconds.

Table 4. Frequency of detection of virulence-associated genes in isolates of *Salmonella* Enteritidis from avian in South of Brazil

Gene	Positive strains	
	Total (n=84)	Total (%)
lpfA	83	99
agfA	81	96
sefA	84	100
invA	84	100
hilA	84	100
avrA	84	100
sopE	83	99
sivH	84	100
spvC	77	92

a difference in the pattern of proteins among different serovars. Some authors consider this high frequency of *avrA* gene is present only in serovars that are considered to be the most important etiological agents of salmonellosis (Ben-Barak et al. 2006). All of these isolates of *S*. Enteritidis were *sivH* gene positive. Although there are few studies on the frequency of this gene in populations of *Salmonella* spp., our results are similar to previous works (Kingsley et al. 2003). Many of these effectors proteins were shown to play an important role in *Salmonella* virulence. However, their absence in some isolates, such as *sopE*, suggests that they are not essential for invasive manifestation in the human host (Suez et al. 2013).

It was verified that all isolates presented had at least two of the fimbrial genes analyzed in this study, highlighting the importance of fimbriae in the infection process. It is possible that there are additive effects of the adhesins Lpf and Agf in the colonization of the intestine and systemic virulence (Wagner & Hensel 2011). The high frequency of *lpfA* and *agfA* were similar to other data obtained by previous works that studied different serovars (Doran et al. 1993, Borsoi et al. 2009, Cesco 2010). Besides being important in the adhesion during the infection process, the *agfA* gene is also associated with biofilm formation (Barnhart & Chapman 2006, Yoo et al. 2013). Our results show this gene is present in isolates from carcasses, which can pose greater risk of contamination on the slaughterhouses. The negative isolates may have lost the gene during their evolution. Studies concerning the frequency of these genes are important in tracking the adaptation of different serovars of *Salmonella* spp. to an increasing number of hosts because the acquisition and loss of fimbrial genes are involved in this process (Bäumler et al. 1997). The high frequency of *sefA* is consistent with previous findings (Amini et al. 2010, Craciunas et al. 2012), and it can be considered a target gene to identify the serovar *S.* Enteritidis by PCR (Amini et al. 2010).

Our results for the virulence plasmid gene *spvC* were similar to those found by other authors (Oliveira et al. 2003, Castilla et al. 2006, Amini et al. 2010). There are also other studies that have found lower frequencies for this gene in strains of avian origin (Okamoto et al. 2009, Derakhshandeh et al. 2013, Moussa et al. 2013). It is possible that the presence of this gene is related to the host from which the sample was isolated (Amini et al. 2010). Amini et al. (2010) compared the frequencies of *spvC* in strains isolated from

humans (100%) and cattle (90%), which are not similar to those found in *S.* Enteritidis strains isolated from poultry. Comparing different serovars, it was observed that 92% of *S.* Enteritidis strains had the *spvC* gene, whereas only 28% of *S.* Typhimurium strains (Moussa et al. 2013) and 0% of *S.* Hadar strains (Cesco 2010) were positive for the gene. The different frequencies found for this gene showed that the virulence of *Salmonella* Enteritids alternates among the plasmid and chromosomal factors, according to the genetic profile of each isolate.

Despite the antigenic similarities among the *S.* Enteritidis isolates used in this study, the gene pattern was not the same for all bacteria. Although all the serovars of *Salmonella* spp. can be considered as potentially pathogenic, there are some differences in their virulence (Karasova et al. 2009). The highest frequency of P1 profile demonstrate that these genes are widely distributed in the population of *Salmonella* spp. The presence of more than one genetic profile may suggest acquisitions or deletions of genes in different clones, which could promote different levels of strain adaptation to the host (Bäumler et al. 1997, Prager et al. 2000, Moussa et al. 2013).

It is known that *Salmonella* spp. isolates lost and acquire new virulence factors over time in order to adapt to new hosts or to the environment (Bäumler et al. 1997, Suez et al. 2013). Currently, the foremost challenges are determining how *Salmonella* acquires virulence factors and what the most important genetic traits conferring virulence to *Salmonella* spp. The knowledge of these characteristics allows a better approach in the study of *Salmonella* virulence and hence the development of strategies to reduce this virulence. Studies involving the exact involvement of each gene in the pathogenesis of this bacterium would be possible to establish criteria for predicting the virulence of this microorganism.

CONCLUSION

The understanding of the virulence of *Salmonella* spp. requires several steps. Nevertheless, the results of this study support, through the provision of protocols and gene profiles, the premise that there is a genetic differentiation in isolates from the same serovar, which provides a basis for criteria to determine possible variations for *in vivo* virulence of different strains, as well as for further studies in phylogenetic analysis.

REFERENCES

Amini K., Salehi T.Z., Nikbakht G., Ranjbar R., Amini J. & Ashrafganjooei S.B. 2010. Molecular detection of *invA* and *spv* virulence genes in *Salmonella* Enteritidis isolated from human and animals in Iran. Afr. J. Microbiol. Res. 4(21):2202-2210.

Bajaj V., Lucas R.L., Hwang C. & Lee C.A. 1996. Co-ordinate regulation of Salmonella typhimurium invasion genes by environmental and regulatory factors is mediated by control of hilA expression. Mol. Microbiol. 22(4):703-714.

Barnhart M.M. & Chapman M. 2006. Curli biogenesis and function. Annu. Rev. Microbiol. 60:131-147.

Bäumler A.J. & Heffron F. 1995. Identification and sequence analysis of lpfABCDE, a putative fimbrial operon of *Salmonella typhimurium*. J. Bacteriol. 177(8):2087-2097.

Bäumler A.J., Tsolis R.M. & Heffron F. 1996. Contribution of fimbrial operons to attachment to and invasion of epithelial cell lines by *Salmonella* Typhimurium. Infect. Immun. 64(5):1862-1865.

Bäumler A.J., Gilde A.J., Tsolis R.M., Van der Velden W.M., Ahmer B.M.M. & Heffron F. 1997. Contribution of horizontal gene transfer and deletion events to development of distinctive patterns of fimbrial operon during evolution of *Salmonella* serovars. J. Bacteriol. 179(2):317-322.

Ben-Barak Z., Streckel W., Yarona S., Cohenc S., Prager R. & Tschape H. 2006. The expression of the virulence-associated effector protein gene avrA is dependent on a Salmonella enterica-specific regulatory function. Int. J. Med. Microbiol. 296:25-38.

Borsoi A., Santin E., Santos L.R., Salle C.T.P., Moraes H.L.S. & Nascimento V.P. 2009. Inoculation of newly hatched broiler chicks with two brazilian isolates of *Salmonella* Heidelberg strains with different virulence gene profile, antimicrobial resistance and pulsed field gel electrophoresis pattern to intestinal changes evaluation. Poult. Sci. 88:750-758.

Campioni F., Bergamini A.M.M. & Falcão J.P. 2012. Genetic diversity, virulence genes and antimicrobial resistance of *Salmonella* Enteritidis isolated from food and humans over a 24-year period in Brazil. Food Microbiol. 32:254-264.

Castilla K.S., Ferreira C.S.A., Moreno A.M., Nunes I.A. & Ferreira A.J.F. 2006. Distribution of virulence genes *sefC*, *pefA* e *spvC* in *Salmonella* Enteritidis phage type 4 strains isolated in Brazil. Braz. J. Microbiol. 37:135-139.

Cesco M.A.O. 2010. Pesquisa de Fatores Associados à Virulência de *Salmonella* Hadar através da Reação em Cadeia da Polimerase (PCR). Dissertação de Mestrado em Ciências Veterinárias, Faculdade de Veterinária, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS. 84p.

Cesco M.A.O., Zimermann F.C., Giotto D.B., Guayba J., Borsoi A., Rocha S.L.S., Camilotti E., DalMolin J., Moraes H.L.S. & Nascimento V.P. 2008. Pesquisa de genes de virulência em *Salmonella* Hadar em amostras provenientes de material avícola. Anais 35º Congresso Brasileiro de Medicina Veterinária, Gramado/RS, R0701-0.

Collier-Hyames L.S., Zeng H., Sun J., Tomlinson A.D., Bao Z.Q., Chen H., Madara J.L., Orth K., Chen H. & Neish A.S. 2002. Cutting edge: *Salmonella* AvrA effector inhibits the key proinflammatory, anti-apoptotic NF-kB pathway. J. Immunol. 169:2846-2850.

Collinson S.K., Emody L., Trust T.J. & Kay W.W. 1992. Thin aggregative fimbriae from Diarrheagenic *Escherichia coli*. J. Bact. 174(13):4490-4495.

Collinson K., Doig P.C., Doran J.L., Clouthier S., Trust T.J. & Kay W.W. 1993. Thin aggregative fimbriae mediate binding of *Salmonella* Enteritidis to fibronectin. J. Bacteriol. 175(1):12-18.

Collinson K., Liu S.L., Clouthier S.C., Banser P.A., Doran J.L., Sanderson K.E. & Kay W.W. 1996. The location of four fimbrin-enconding genes, agfA, fimA, sefA and sefD, on the Salmonella Enteritidis and/or *S*. Typhimurium XbaII-BlnI genomic restriction maps. Gene 169:75-80.

Craciunas C., Keul A.L., Flonta M. & Cristea M. 2012. DNA-based diagnostic tests for *Salmonella* strains targeting *hilA, agfA. spvC* and *sefC* genes. J. Env. Man. 95:512-218.

Derakhshandeh A., Firouzi R. & Khoshbakht R. 2013. Association of three plasmid-encoded spv genes among different *Salmonella* serotypes isolated from different origins. Indian J. Microbiol. 53(1):106-110.

Doran J.L., Collinson S.K., Burian J., Sarlos G., Todd E.C.D., Munro C.K., Kay C.M., Banser P.A., Peterkin P.I. & Kay W.W. 1993. DNA-based diagnostic tests for *Salmonella* species targeting agfA, the structural gene for thin, aggregative fimbriae. J. Clin. Microbiol. 31(9):2263-2273.

Edwards R.A. & Puente J.L. 1998. Fimbrial expression in enteric bacteria: a critical step in intestinal pathogenesis. Trends Microbiol. 6(7):282-287.

Galán J.E. & Curtis III R. 1989. Cloning and molecular characterization of genes whose products allow *Salmonella* typhimurium to penetrate tissue culture cells. Proc. Natl Acad. Sci. USA 86:6383-6387.

Galán J.E. & Zhou D. 2000. Striking a balance: modulation of the actin cytoskeleton by *Salmonella*. PNAS 97(16):8754-8761.

Guo X., Chen J., Beuchat L.R. & Brackett R.E. 2000. PCR detection of *Salmonella enterica* serovar Montevideo in and on raw tomatoes using primers derived from hilA. Appl. Environm. Microbiol. 66(12):5248-5252.

Hardt W.D., Urlab H. & Galán J.E. 1998. A substrate of the centisome 63 type III protein secretion system of *Salmonella* typhimurium is encoded by a cryptic bacteriophage. Proc. Natl Acad. Sci. USA 95:2574-2579.

Hopkins K.L. & Threlfall E.J. 2004. Frequency and polymorphism of *sopE* in isolates of *Salmonella enterica* belonging to the ten most prevalent serovars in England and Wales. J. Med. Microbiol. 53:539-543.

Karasova D., Havlickova H., Sisak F. & Rychlik I. 2009. Deletion of *sodCI* and *spvBC* in *Salmonella enterica* serovar Enteritidis reduced its virulence to the natural virulence of serovars Agona, Hadar and Infantis for mice but not for chickens early after infection. Vet. Microbiol. 139:304-309.

Kingsley R.A., Humphries A.D., Weening E.H., Zoete M.R., Winter S., Papaconstantinopoulou A., Dougan G. & Bäumler A.J. 2003. Molecular and phenotypic analysis of the CS54 island of *Salmonella enterica* serovar Typhimurium: identification of intestinal colonization and persistence determinants. Infect. Immun. 71(2):629-640.

Libby S.J., Adams G., Ficht T.A., Allen C., Whitford H.A., Buchmeier N.A., Bossie S. & Guiney D.G. 1997. The spv genes on the *Salmonella* Dublin virulence plasmid are required for severe enteritis and systemic infection in the natural host. Infect. Immun. 65(5):1786-1792.

Liu Y., Yang Y., Liao X., Li L., Lei C., Li L., Sun J. & Liu B. 2012. Antimicrobial resistance, resistance genes and virulence genes in *Salmonella* isolates from chicken. J. Anim. Vet. Adv. 11(23):4423-4427.

Marcus S.L., Brumell J.H., Pfeifer C.G. & Finlay B.B. 2000. *Salmonella* pathogenicity islands: big virulence in small packages. Microbes Infect. 2:145-156.

Mello R.T., Guimarães A.R., Mendonça E.P., Coelho L.R., Monteiro G.P., Fonseca B.B. & Rossi D.A. 2011. Identificação sorológica e relação filogenética de *Salmonella* spp. de origem suína. Pesq. Vet. Bras. 31(12):1039-1044.

Moussa I.M., Aleslamboly Y.S., Al-Arfaj A.A., Hessain A.M., Gouda A.S. & Kamal R.M. 2013. Molecular characterization of *Salmonella* virulence genes isolated from different sources relevant to human health. J. Food Agrc. Environ. 11(2):197-201.

Okamoto A.S., Andreatti Filho R.L., Rocha T.S., Menconi A. & Marietto-Gonçalves G.A. 2009. Relation between the *spvC* and *invA* genes and resistance of *Salmonella* Enteritidis isolated from avian material. Int. J. Poult. Sci. 8(6):279-582.

Oliveira S.D., Santos L.R., Schucha D.M.T., Silva A.B., Salle C.T.P. & Canal C.W. 2002. Detection and identification of salmonellas from poultry-related samples by PCR. Vet. Microbiol. 87:25-35.

Oliveira S.D., Rodenbusch C.R., Michael G.B., Cardoso M.I.R., Canal C.W. & Brandelli A. 2003. Detection of virulence genes in *Salmonella* Enteritidis isolates from different sources. Braz. J. Microbiol. 34(1):123-124.

Prager R., Mirold S., Tietze E., Strutz U., Knüppel B., Rabsch W., Hardt W.D. & Tschäpe H. 2000. Prevalence and polymorphism of genes encoding translocated effector proteins among clinical isolates of *Salmonella enterica*. Int. J. Med. Microbiol. 290(7):605-617.

Prager R., Rabsch W., Streckel W., Voigt W., Tietze E. & Tschäpe H. 2003. Molecular properties of *Salmonella enterica* serovar Paratyphi B distinguish between its systemic and its enteric pathovars. J. Clin. Microbiol. 41(9):4270-7278.

Rahman H., Streckel W., Prager R. & Tschäpe H. 2004. Presence of *sopE* gene and its phenotypic expression among different serovars of *Salmonella* isolated from man and animals. Indian J. Med. Res. 120:35-38.

Streckel W., Wolff A.C., Prager R., Tietze E. & Tschäpe H. 2004. Expression profiles of effector proteins SopB, SopD1, SopE1 and AvrA differ with systemic, enteric and epidemic strains of *Salmonella enterica*. Mol. Nutr. Food Res. 48:496-503.

Suez J., Porwollik S., Dagan A., Marzel A., Schorr Y.I., Desai P.T., Agmon V., McClelland M., Rahav G. & Gal-Mor O. 2013. Virulence gene profiling and pathogenicity characterization of non-typhoidal *Salmonella* accounted for invasive disease in humans. PLoS ONE 8(3):e58449.

Swamy S.C., Barnhart H.M., Lee M.D. & Dreesen D.W. 1996. Virulence determinants *invA* and *spvC* in Salmonellae isolated from poultry products, wastewater and human sources. Appl. Environ. Microbiol. 62(10):3768-3771.

Vieira A., Jensen A.R., Pires S.M., Karlsmose S., Wegener H.C. & Wong D.L.F. 2009. WHO global foodborne infections network country databank: a resource to link human and non-human sources of *Salmonella*. Proc. 12th Symposium of the International Society for Veterinary Epidemiology and Economics, Durban, South Africa, p.1.

Wagner C. & Hensel M. 2011. Adhesive mechanisms of *Salmonella enterica*, p.17-34. In: Linke D. & Goldman A. (Eds), Bacterial Adhesion - Chemistry, Biology and Physics. Springer, New York.

Yoo A.Y., Yu J.E., Yoo H., Lee T.H., Lee W.H., Oha J.I. & Kang H.Y. 2013. Role of sigma factor E in regulation of *Salmonella* Agf expression. Biochem. Biophys. Res. Commun. 430:131-136.

Zou W., Al-Khaldi S.F., Branham W.S., Han T., Fuscoe J.C., Han J., Foley S.L., Xu J., Fang H., Cerniglia C.E. & Nayak R. 2011. Microarray analysis of virulence gene profiles in *Salmonella* serovars from food/food animal environment. J. Infect. Dev. Ctries 5(2):94-105.