A comparative survey between non-systemic *Salmonella* spp. (paratyphoid group) and systemic *Salmonella* Pullorum and *S.* Gallinarum with a focus on virulence genes[1]

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ABSTRACT.- Astolfi-Ferreira C.S., Pequini M.R.S., Nuñez L.F.N., Santander Parra S.H., Chacon R., Torre D.I.D., Pedroso A.C. & Ferreira A.J.P. 2017. **A comparative survey between non-systemic *Salmonella* spp. (paratyphoid group) and systemic *Salmonella* Pullorum and *S.* Gallinarum with a focus on virulence genes.** *Pesquisa Veterinária Brasileira 37(10):1064-1068*. Departamento de Patologia, Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, Av. Prof. Dr. Orlando Marques de Paiva 87, São Paulo, SP 05508-270, Brazil. E-mail: ajpferr@usp.br

A comparative survey between non-systemic (paratyphoid *Salmonellae*) and systemic (*S*. Pullorum and *S*. Gallinarum) *Salmonella* strains was performed to produce a virulence gene profile for differentiation among the groups. The following virulence genes were evaluated: *inv*A, *spv*C, *sef*C, *pef*A, *fim*Y, *sop*B, *sop*E1, *stn and avr*A. There are substantial differences among paratyphoid *Salmonellae*, *S*. Pullorum, and *S*. Gallinarum regarding the genes *sef*C, *spv*C, *sop*E1 and *avr*A. A higher frequency of *sef*C, *spv*C, *sop*E1 and *avr*A genes were detected in *S*. Gallinarum and *S*. Pullorum when compared with strains from the paratyphoid group of *Salmonella*. These results may be useful for differentiating among different groups and serotypes.

INDEX TERMS: *Salmonella* spp., paratyphoid group, *Salmonella* Pullorum, *Salmonella* Gallinarum, virulence genes. pullorum disease, fowl typhoid, chicken.

RESUMO.- [**Uma investigação comparativa entre *Salmonella* spp. não-sistêmicas (grupo paratifoide) e sistêmicas *Salmonella* Pullorum e *S*. Gallinarum com enfoque nos genes de virulência.**] Uma investigação comparativa entre amostras de *Salmonella* não-sistêmicas (grupo paratifoide) e sistêmicas (*S*. Pullorum and *S*. Gallinarum) foi desenvolvida para produzir um perfil de genes de virulência para diferenciação entre os grupos. Os seguintes genes de virulência foram avaliados *inv*A, *spv*C, *sef*C, *pef*A, *fim*Y, *sop*B, *sop*E1, *stn* e *avr*A. Detectou-se uma diferença substancial entre *Salmonella* do grupo paratifoide, *S*. Pullorum e *S*. Gallinarum considerando os genes *sef*C, *spv*C, *sop*E1 e *avr*A. Os genes *sef*C, *spv*C, *sop*E1 e *avr*A foram detectados, em maior número, em *S*. Gallinarum e *S*. Pullorum quando comparados com as amostras de *Salmonella* do grupo paratifoide. Estes resultados podem ser úteis para a diferenciação entre os diferentes grupos e sorotipos de *Salmonella*.

TERMOS DE INDEXAÇÃO: *Salmonella* spp., grupo paratifoide, *Salmonella* Pullorum, *Salmonella* Gallinarum, genes de virulência, pulorose, tifo aviário, galinha.

INTRODUCTION

The paratyphoid Salmonellae is responsible for food poisoning in humans when transmitted by food derived from infected chicken meat. The paratyphoid group of *Salmonella*, generally, is not considered pathogenic for chickens when orally ingested. *Salmonella* Pullorum and *S*. Gallinarum, regardless of the route of infection, cause mortality in young and adult birds, respectively (Pomeroy & Nagaraja 1991).

Virulence factors determine the ability of a given bac-

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terial strain to cause disease. The invasion of and adhesion to the intestinal epithelium are essential events in salmonellosis pathogeny. *Salmonella's* adherence to intestinal epithelial cells has been associated with the presence of type 1 fimbria (Isaacson & Kinsel 1992), coded by the *fim operon*. This operon harbors 10 genes including the *fim Y* gene, which is a regulatory gene used to detect the *Salmonella* genus (Yeh et al. 2002). The SEF14 fimbriae operon was described as a 3.9 kilobase (kb) region in *Salmonella* Enteritidis (Thorns et al. 1990). The *sef*ABC gene(s) is located in this region. The *sef*C gene encodes an external membrane protein that leads the *sef*A subunit and the *sef*D adhesin to the cellular surface (Edwards et al. 2000). The *sef*C gene encodes a protein homologous to fimbrial outer membrane proteins, and it has been suggested that *sefC* is a component of this external membrane operon. The *pef* fimbriae are encoded by the *pef* (plasmid-encoded fimbriae) *operon* and are associated with adhesion to small intestine epithelial cells in mice (Bäumler et al. 1996).

Several factors contribute to host invasion, including the type III secretion system, which is a structure present in the bacterial membrane that is capable of transferring effector proteins into the host cell. *Inv*A is one of the proteins that comprises the secreted protein export complex (Kimbrough & Miller 2002). The *Sop*E1 and *Sop*B proteins that are associated with other secreted proteins have the capacity to alter the cytoskeleton and thus provoke intestinal epithelial cell membrane ruffling and induce bacterial internalization. The *sop*E1 gene is horizontally transmitted by prophages and is present in a few *Salmonella* spp. serotypes (Zhang et al. 2002). AvrA is another protein secreted by the type III secretion system that inhibits NF-κB transcription factor activation and increases human epithelial cell apoptosis *in vitro* (Collier-Hyams et al. 2002).

Some *Salmonella* serotypes cause gastroenteritis, and although the enterica subspecies produce the gene for enterotoxin (*stn*), only some strains produce this phenotype when cultured using conventional methods (Prager et al. 1995). High molecular weight plasmids (Helmuth et al. 1981) contain a highly conserved region called the *spv* region. The spv proteins are necessary to establish systemic infection (Roudier et al. 1992, Libby et al. 1997, Matsui et al. 2001).

The aim of this study was to compare the presence of virulence genes *fim*Y, *inv*A, *sef*C, *pef*A, *sop*E1, *sop*B, *stn, avr*A and *spv*C among the *Salmonella* paratyphoid group (non--systemic serovars), *S*. Pullorum and *S*. Gallinarum (systemic infection biovars) via Polymerase Chain Reaction (PCR), between systemic and non-systemic *Salmonella* strains, respectively.

MATERIALS AND METHODS

***Salmonella* spp. strains and bacterial cultivation.** *Salmonella* Typhimurium ATCC 14028 was used as a positive control strain for the detection of the *inv*A, *spv*C, *pef*A, *sop*B, *fim*Y and *avr*A genes. *S. enteritis* SA193 or *S.* Gallinarum SA 158 were used as positive control strains for the detection of *sef*C or *sop*E1 genes, respectively. The *S.* Pullorum, *S.* Gallinarum, *S.* Typhimurium, *S.* Senftenberg, *S.* Agona, *S.* Braenderup, *S.* Bredeney and *S.* Cerro strains belong to the Laboratory of Avian Diseases - FVMZ-USP, São Paulo, SP, Brazil. The strains were grown in LB Agar at 37ºC for 24 hours. Table 1 lists the studied *Salmonella* strains' numbers, origins and isolation years.

Genomic Bacterial DNA Extraction and Polymerase Chain Reaction. Genomic DNA extraction was carried out according to Boom et al. (1990). The primers specific to the *inv*A, *spv*C, *sef*C, *pef*A, *fim*Y, *sop*B, *sop*E1 and *avr*A genes, references and amplicon sizes are presented in Table 2. All PCR reactions were carried out with Taq DNA polymerase (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

For *inv*A or *spv*C gene amplification, the PCR machine was programmed for 30 cycles, as follows: one minute at 93°C for denaturation, one minute at 42°C for annealing and 2 minutes at 72°C for extension. For the *pef*A or *sef*C genes, the following program of 30 cycles was used: one minute at 95°C for denaturation, one minute at 50°C for annealing and 6 minutes at 72°C for extension. Finally, for *fim*Y, a*vr*A, *sop*B, *stn* or *sop*E genes, 35 cycles of one minute at 94°C for denaturation, one minute at 55°C for annealing and a minute and a half at 72°C for extension were used. In each amplification, a positive control (*Salmonella* spp. reference strains) and a negative control (ultra-pure water) were added.

Detection of PCR products. The PCR products (10 µl) were visualized after separation by electrophoresis in an agarose gel (1.5%) stained with ethidium bromide (10 µg/ml) and photogra-

Table 1. The origins and years of isolation for *Salmonella* strains used in this study for the detection of virulence genes

Serotype	Group classification*	Number of isolates	Origin	Year of isolation
S. Agona	B	1	Ceca – Monitoring	1985
S. Typhimurium	B	2	Chicken sick	1985
S. Typhimurium	B	10	Ceca – Monitoring	1985
S. Cerro	K	11	Feed – Monitoring	1989
S. Gallinarum	D1	28	Chicken sick	1990
S. Pullorum	D1	18	Chicken sick	1990
S. Senftenberg	E4	13	Ceca - Monitoring	1999
S. Bredeney	B	1	Ceca - Monitoring	2001
S. Braenderup	C1	1	Ceca - Monitoring	2001

* According to Kauffmann-White-LeMinor serotyping scheme.

Table 2. Primer sequences used for *Salmonella* spp. virulence gene detection

nes	Primer sequences 5´- 3´		Amplicon size - bp
*inv*A	F: TTGTTACGGCTATTTTGACCA	R: CTGACTGCTACCTTGCTGATG	521
*spv*C	F: CGGAAATACCATCTACAAATA	R: CCCAAACCCATACTTACTCTG	669
*pef*A	F: GGGAATTCTTGCTTCCATTATTGCACTGGG	R:TCTGTCGACGGGGGATTATTTGTAAGCCACT	526
*sef*C	F: GCGAAAACCAATGCGACTGTAG	R: CCCACCAGAAACATTCATCCC	1104
*fim*Y	F: GAGTTACTGAACCAACAGCT	R: GCCGGTAAACTACACGATGA	526
*avr*A	F: GTTATGGACGGAACGACATCGG	R: ATTCTGCTTCCCGCCGCC	389
*sop*B	F: CAACCGTTCTGGGTAAACAAGAC	R: AAGATTGAGCTCCTCTGGCGAT	1349
*sop*E1	F: ACACACTTTCACCGAGGAAGCG	R: GGATGCCTTCTGATGTTGACTGG	399
stn	F:TTGTCTCGCTATCACTGGCAACC	R:ATTCGTAACCCGCTCTCGTCC	618

Table 3. A comparison of the number of detected genes among *Salmonella* serotypes isolated from paratyphoid infection, pullorum disease and fowl typhoid origins

Salmonella strains	Associated diseases	Genes detected								
n= 85		*inv*A	*fim*Y	*stn*	*sop*B	*sef*C	*spv*C	*sop*E1	*pef*A	*avr*A
S. Agona (n=1)	Paratyphoid infection	1	1	1	1	0	0	0	0	1
S. Typhimurium (n=12)	Paratyphoid infection	12	12	12	12	1	1	2	1	12
S. Senftenberg (n=13)	Paratyphoid infection	13	13	13	13	1	1	1	4	13
S. Cerro (n=11)	Paratyphoid infection	11	11	11	11	0	0	0	3	11
S. Braenderup (n=1)	Paratyphoid infection	1	1	1	1	0	0	0	0	1
S. Bredeney (n=1)	Paratyphoid infection	1	1	1	1	0	0	0	1	1
S. Pullorum (n=18)	Pullorum Disease	18	18	18	14	12	13	5	2	18
S. Gallinarum (n=28)	Fowl Typhoid	28	28	28	23	21	16	20	2	28
Total of positives (%)		85 (100)	85 (100)	85 (100)	76 (89,4)	35 (41,1)	31 (36,4)	28 (32,9)	13 (15,2)	85 (100)

phed under ultraviolet light. A 100 bp DNA ladder (Invitrogen™) was used as a molecular size marker.

RESULTS

In this study, the *inv*A, *fim*Y and *stn* genes were detected in all of the analyzed strains. All *Salmonella* Typhimurium, *S*. Senftenberg, *S*. Agona, *S*. Cerro, *S*. Braenderup and *S*. Bredeney serotypes were positive (100%) for the *sop*B gene, while 14 (77.8%) and 23 (82.1%) of the *S*. Pullorum and *S*. Gallinarum strains were positive for this same gene, respectively.

The *sop*E1 gene was detected in three (7.7%) of the *Salmonella* strains in the paratyphoid group, 5 (27.8%) of the *S.* Pullorum strains and 20 (71.4%) of the *S.* Gallinarum strains.

The *pef*A gene was observed in nine (23.1%) paratyphoid group strains, two (11.1%) *S.* Pullorum strains and in two (7.1%) *S.* Gallinarum strains.

Regarding the *avr*A gene, all of the *S.* Pullorum and *S.* Gallinarum serotype strains and 38 (97.4%) strains of the paratyphoid group were positive (Table 3).

DISCUSSION

For the genes studied, differences were detected in the genetic profile of *Salmonella* strains in the paratyphoid group that is responsible for fowl typhoid.

The *inv*A gene is considered a target for the molecular detection of the *Salmonella* genus (Rahn et al. 1992, Stone et al. 1994, Swamy et al. 1996). All of the analyzed strains produced the 521 bp *inv*A-derived PCR amplicon, confirming the results of Swamy et al. (1996). However, these results did not agree with Dodson et al. (1999), who found four negative *Salmonella* Pullorum strains. The presence of the *fim*Y gene in the studied strains, including Cerro, Agona and Braenderup serotypes, was also verified; these results were not found in the study performed by Yeh et al. (2002). Serotypes not yet studied for the *stn* gene (Prager et al. 1995, Dinjus et al. 1997, Rahman 1999) were also included in this study. This gene was found in 100% of the strains, which is in agreement with the report by Prager et al. (1995), furthermore, in the present study, the *stn* gene was also detected in the *S*. Senftenberg, *S*. Agona, *S*. Cerro, *S*. Braenderup, *S*. Bredeney and *S*. Pullorum serotypes.

The *spv* (*Salmonella plasmid virulence*) operon was found in a few serotypes of *Salmonella enterica* subspecies I, mainly those frequently associated with disease (Bäumler et al. 1998). The *spv* genes are essential for a given *Salmonella* spp. to be able to cause systemic infection in laboratory animals (Libby et al. 1997, Matsui et al. 2001). In this study, *S.* Pullorum and *S.* Gallinarum strains were isolated from internal organs and were *spvC*-gene positive in 13 (72.2%) and 16 (57.1%) of the strains, respectively. Thus, the *spvC* gene might have contributed to the systemic infections caused by these strains. However, other virulence factors may be important because strains negative for this gene were capable of causing pullorum disease or fowl typhoid. Only two (5.1%) paratyphoid *Salmonella* strains isolated from cecum possessed this gene; this result is in agreement with Swamy et al. (1996), who detected a low frequency of this gene (3.8%) in strains from this source.

The *pef*A gene was detected in 15.3% of the strains of *S*. Typhimurium, *S*. Senftenberg, *S*. Cerro, *S*. Bredeney, *S*. Pullorum and *S*. Gallinarum serotypes in the present study. However, Bäumler et al. (1997) detected this gene in *S*. Typhimurium, but did not detect it in *S.* Gallinarum or *S.* Pullorum; this gene was also not found among *S.* Pullorum strains analyzed by Dodson et al. (1999). Bäumler et al. (1996) suggested that acquisition of the fimbrial operons might have been one of the mechanisms that enabled the spread of *Salmonella* spp. to a large range of domestic animal host species. It was verified that 82.2% of *S*. Gallinarum and *S*. Pullorum strains possessed the *sef*C gene. This result is in agreement with other data in the literature that indicates the SEF14 fimbriae operon is distributed in group D *Salmonella* spp. (Thorns et al. 1992, Turcotte & Woodward 1993). However, other *Salmonella* groups also contain this gene, but at a low frequency (5.13%, in one strain of *S*. Typhimurium or *S*. Senftenberg).

Only one (1.2%) *S*. Typhimurium strain was *avr*A-gene negative. Although, *avr*A gene action is still poorly understood, Du & Galan (2009) and Jones et al. (2008) showed specifically inhibits the *Salmonella*-induced activation of the JNK pathway through its interaction with other secretion systems. The function of the protein coded for by this gene has recently been described as inhibiting pro--inflammatory activity by blocking the NF-κB transcription factor and increasing *in vitro* epithelial cell apoptosis (Collier-Hyams et al. 2002). These authors suggested that the protein could be a host defense mechanism for rapidly eliminating infected cells. Our results confirmed the results of Prager et al. (2000) in *S*. Typhimurium, *S.* Gallinarum and *S.* Pullorum serotypes. However, this gene has also been

found in *S*. Senftenberg, *S*. Cerro, *S*. Braenderup and *S*. Bredeney serotypes, showing its vast distribution within *Salmonella enterica*. Monack et al. (2001) proposed an *in vivo Salmonella*-induced macrophage death model in which *Salmonella* induces an early or late macrophage death under physiological conditions. That is, in the intestinal stage, the inflammation induced after macrophages apoptosis would help to disperse *Salmonella* in the gastrointestinal tract. During this phase, the *SPI 1*-encoded type III protein secretion system and the binding *of Sip*B to caspase-1 would be involved. Once the systemic infection has been established, the cells killed by apoptosis would be phagocyted or ingested by newly migrated cells, which would allow a new intracellular dissemination cycle. In this phase, the proteins encoded by *SPI-2* and *spv* genes would also be involved. AvrA is an effector protein secreted by the type III secretion system (Hardt & Galán 1997) and, therefore, it plays a role in the enteric phase of the infection. It is capable of inducing *in vitro* epithelial cell apoptosis (Collier-Hyams et al. 2002). It is possible that AvrA protein causes macrophage apoptosis in the lamina propria of the mucosa, which would make it another effector protein capable of activating caspase-1.

We found *sop*B genes in 89.4% of the strains, which is in accordance with the results of other authors (Prager et al. 2000, Mirold et al. 2001).

*Sop*E1 genes were detected in 20 and 5 strains of *S*. Gallinarum and *S*. Pullorum serotypes (54.3%), respectively, while only 7.7% of *S.* Typhimurium and *S.* Senftenberg strains possessed this gene within other serotypes. These results agree with those reported by Prager et al. (2000), which indicated that this gene is found in a higher frequency in *S.* Gallinarum and *S.* Pullorum strains when compared to *S.* Typhimurium strains. Taking into account that the *sop*E1 gene is transmitted by prophages and was associated with *S*. Typhimurium enteropathogenicity in bovines (Zhang et al. 2002), a greater pathogenic potential for *sop*E1-positive *S.* Gallinarum and *S.* Pullorum strains may be expected, especially considering that the first two strains were isolated from sick fowls in this study.

Taking into account the presence of the *spv*C, *sop*E1 and *pef*A genes in *S.* Gallinarum and *S.* Pullorum strains, the different genetic profiles that were observed suggest the presence of different clones causing the disease in fowls. Comparisons of *S.* Pullorum and *S*. Gallinarum strains with the paratyphoid group of *Salmonella* revealed substantial differences concerning the presence of the *sop*E1, *spv*C and *sef*C genes. More comprehensive studies must be conducted to determine which virulence genes might be important to *Salmonella* virulence in fowls.

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