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REVISTA DO INSTITUTO DE MEDICINA TROPICAL DE SÃO PAULO

JOURNAL OF THE SÃO PAULO INSTITUTE OF TROPICAL MEDICINE

ORIGINAL ARTICLE

http://doi.org/10.1590/S1678-9946201961036

Detection of virulence genes in *Salmonella* Heidelberg isolated from chicken carcasses

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ABSTRACT

During the last years, Brazilian government control programs have detected an increase of *Salmonella* Heidelberg in poultry slaughterhouses a condition that poses a threat to human health However, the reasons remain unclear. Differences in genetic virulence profiles may be a possible justification. In addition, effective control of *Salmonella* is related to an efficient epidemiological surveillance system through genotyping techniques. In this context, the aim of this study was the detection of 24 virulence-associated genes in 126 *S.* Heidelberg isolates. We classified the isolates into 56 different genetic profiles. None of the isolates presented all the virulence genes. The prevalence of these genes was high in all tested samples as the lowest number of genes detected in one isolate was 10/24. The *lpfA* and *csgA* (fimbriae), *invA* and *sivH* (TTSS), and *msgA* and *tolC* (intracellular survival) genes were present in 100% of the isolates analyzed. Genes encoding effector proteins were detected in the majority of SH isolates. No single isolate had the *sefA* gene. The *pefA* gene was found in only four isolates. We have also performed a screening of genes associated with iron metabolism: 88.9% of isolates had the *iroN* geneand 79.4% the *sitC* gene. Although all the isolates belong to the same serotype, several genotypic profiles were observed. These findings suggest that there is a diversity of *S.* Heidelberg isolates in poultry products. The fact that a single predominant profile was not found in this study indicates the presence of variable sources of contamination caused by SH. The detection of genetic profiles of *Salmonella* strains can be used to determine the virulence patterns of SH isolates.

KEYWORDS: Salmonellosis. *Salmonella* Heidelberg. Poultry. PCR. Virulence genes.

INTRODUCTION

Salmonella spp. is one of the main pathogens causing foodborne bacterial infections in humans and poultry products are the main sources of bacterial contamination[1]. The presence of this microbiological hazard affects negatively the poultry industry. Brazilian products of avian origin are exported to five continents. These poultry products should meet certain sanitary requirements of control and certification in order for this trade to occur properly. The control of *Salmonella* spp. in slaughterhouses is strict. In addition to having its relevance as a cause of foodborne disease, this bacterial pathogen has a major economic impact causing great losses to the domestic market and to export as well[2].

It is worth mentioning that the performance of the Brazilian government in programs aimed to control *S. enteritidis* and *S. typhimurium* in food processing led to the reduction of their prevalences. In contrast, during the last couple of

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Received: 4 April 2019

Accepted: 27 June 2019

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years there has been an increase in the prevalence of other serotypes, as *Salmonella* Heidelberg (SH). SH is one of the most widely distributed serotypes worldwide and is among the ten *Salmonella* serotypes most frequently associated with human diseases[3,4]. In a retrospective study spanning three decades (between 1962 and 1991) carried out in Brazil, *S.* Heidelberg was identified in birds and poultry products[5]. According to the Rapid Alert System for Food and Feed Safety Alerts (RASFF), *S.* Heidelberg was among the serotypes most often isolated in the years 2013, 2014, and 2016[6].

The successful control of this microorganism is also related to efficient national epidemiological surveillance systems using genotyping and phenotyping techniques. This methodology helps to study characteristics of the various serotypes of *Salmonella* as the ability and the way the pathogen adapts to the host and to the environment, depending on the virulence of each strain. Among the *Salmonella* that cause foodborne infections in humans, *S.* Heidelberg has been shown to be more invasive compared to other serotypes that cause gastroenteritis. Systemic disease occurs in approximately 13% of the cases of *S.* Heidelberg infections[7,8].

S. Heidelberg's increasing importance is due to the frequent development of resistance to antimicrobial drugs, limiting treatment options in cases of salmonellosis[9] and leading to a number of studies on this subject, carried out by many researchers around the world. The main issues are closely related to the pathogenicity of the microorganisms as they depend on a number of factors, including virulence factors of each strain, the serotype involved, the amount of inoculum, virulence factors expressed by the microorganism and the immunological status of the host. Therefore, *Salmonella* spp. may cause a broad spectrum of clinical diseases that vary in severity ranging from a mild gastrointestinal infection to a severe systemic illnesses[10].

Virulence factors are encoded by a number of genes located on the bacterium own chromosome, the so-called housekeeping genes, which give specific and basic characteristics to bacteria from the same family. These genes can be found in the so-called pathogenicity islands, or in mobile genetic elements such as transposons, plasmids and bacteriophages[11-13]. These genes confer advantages for bacteria such as adaptation to the host cell, resistance to antimicrobials and the ability to overcome host defense mechanisms.

The increase in the occurrence of SH in food products and in SH-related infections is of concern to Brazilian authorities, since SH control and elimination from poultry farms is a major problem (data not shown). Changes in the acquisition of genes by SH strains have been reported in other animal species and have been considered as important factors that may have contributed to the increase of virulence[14]. In this context, the present study aimed to screen the 24 genes associated with virulence of SH isolates by polymerase chain reaction (PCR).

MATERIALS AND METHODS

Bacterial isolates

The present study was carried out at the Laboratory of Bacteriology and Mycology, Veterinary Hospital of the Universidade de Passo Fundo (UPF) and Centro de Diagnostico e Pesquisa em Patologia Aviaria (CDPA) of the Universidade Federal do Rio Grande do Sul (UFRGS). A total of 126 *Salmonella* Heidelberg isolates from chilled and frozen chicken carcasses collected in 2015 in a poultry slaughterhouse and serotyped at the Osvaldo Cruz Foundation, Rio de Janeiro State, Brazil, were used in this study. These samples were kindly provided by Eduardo Cesar Tondo from the Institute of Food Science and Technology (ICTA) at UFRGS, Porto Alegre, Rio Grande do Sul State, Brazil. Samples were stored at -20 °C in a Brain Heart Infusion (BHI) broth with 20% glycerol. All stored isolates were reactivated and re-identified biochemically and serologically.

Polymerase chain reaction (PCR)

The molecular characterization of the 126 isolates of *S.* Heidelberg consisted of screening 24 genes involved in the virulence and pathogenicity of *Salmonella* spp. The following genes were screened in this study: *invA, hilA, lpfA, lpfC, sefA, csgA* [*agfA*], *spvB, pefA, sopE, avrA, sivH, orgA, prgH, spaN, tolC, sipB, sitC, pagC, msgA, spiA, iroN, sopB, cdtB* and *sifA*. DNA extraction was carried out through a heat treatment[15]. Only samples with at least 10 ng of DNA were subjected to amplification. The method used was adapted from the technique published by Borges *et al.*[15]. Amplifications were performed in a MaxPro Swift thermocycler (Esco, Singapore) and afterwards electrophoresis was performed in 1.5% agarose gels stained with ethidium bromide. The analysis of the amplified products was performed by the visualization of the corresponding bands under ultraviolet light using a transilluminator (MacroVue, Pharmacia LKB Biotechnologies, Uppsala, Sweden) coupled to digital photodocumentation of these bands/transilluminated gels (Alpha Innotche, San Leandro, USA). The sequence of primers, as well as the size of amplicons and the reference of protocols for detection of each gene are presented in

Table 1. Cycling conditions and reaction mixtures (25 µL of total volume) were previously described[16]. Standard strains of *Mannheimia haemolytica* (ATCC 29694) and *Salmonella* enteritidis (ATCC 13076) were used as negative and positive control, respectively. PCR assays were repeated three times.

RESULTS

The absolute and relative frequencies of the 24 investigated virulence-associated genes grouped according to their respective functions are shown in Table 2. The 126

Table 1 - *Salmonella* spp. virulence genes (n=24), sequence of *primers* to amplify *Salmonella* Heidelberg virulence genes, the amplicons' molecular weight (in base pairs) and the corresponding references.

Genes	*Primers* sequences (5'-3')	Base pair (bp)	References
lpfC	GCCCCGCCTGAAGCCTGTGTTGC AGGTCGCCGCTGTTTGAGGTTGGATA	641	26
spvB	CTATCAGCCCCGCACGGAGAGCAGTTTTTA GGAGGAGGCGGTGGCGGTGGCATCATA	717	26
pefA	GCGCCGCTCAGCCGAACCAG GCAGCAGAAGCCCAGGAAACAGTG	157	26
orgA	CAGCGCTGGGGATTACCGTTTTG TTTTTGGCAATGCATCAGGGAACA	255	26
prgH	GCCCGAGCAGCCTGAGAAGTTAGAAA TGAAATGAGCGCCCCTTGAGCCAGTC	756	26
spaN	AAAAGCCGTGGAATCCGTTAGTGAAGT CAGCGCTGGGGATTACCGTTTTG	504	26
tolC	TACCCAGGCGCAAAAAGAGGCTATC CCGCGTTATCCAGGTTGTTGC	161	26
sipB	GGACGCCGCCCGGGAAAAACTCTC ACACTCCCGTCGCCGCCTTCACAA	875	26
sitC	CAGTATATGCTCAACGCGATGTGGGTCTCC CGGGGCGAAAATAAAGGCTGTGATGAAC	768	26
pagC	CGCCTTTTCCGTGGGGTATGC GAAGCCGTTTATTTTTGTAGAGGAGATGTT	454	26
msgA	GCCAGGCGCACGCGAAATCATCC GCGACCAGCCACATATCAGCCTCTTCAAAC	189	26
spiA	CCAGGGGTCGTTAGTGTATTGCGTGAGATG CGCGTAACAAAGAACCCGTAGTGATGGATT	550	26
iroN	CCGCGTTATCCAGGTTGTTGC ACTGGCACGGCTCGCTGTCGCTCTAT	1205	26
sopB	CGGACCGGCCAGCAACAAAACAAGAAGAAG TAGTGATGCCCGTTATGCGTGAGTGTATT	220	26
cdtB	ACAACTGTCGCATCTCGCCCCGTCATT CAATTTGCGTGGGTTCTGTAGGTGCGAGT	268	26
sifA	TTTGCCGAACGCGCCCCCACACG GTTGCCTTTTCTTGCGCTTTCCACCCATCT	449	26
lpfA	CTTTCGCTGCTGAATCTGGT CAGTGTTAACAGAAACCAGT	250	29
csgA (agfA)	TCCACAATGGGGCGGCGGCG CCTGACGCACCATTACGCTG	350	30
sefA	GATACTGCTGAACGTAGAAGG GCGTAAATCAGCATCTGCAGTAGC	488	31
invA	GTGAAATTATCGCCACGTTCGGGCAA TCATCGCACCGTCAAAGGAACC	284	31
hilA	CTGCCGCAGTGTTAAGGATA CTGTCGCCTTAATCGCATGT	497	32
avrA	GTTATGGACGGAACGACATCGG ATTCTGCTTCCCGCCGCC	385	33
sopE	ACACACTTTCACCGAGGAAGCG GGATGCCTTCTGATGTTGACTGG	398	33
sivH	CAGAATGCGAATCCTTCGCAC GTATGCGAACAAGCGTAACAC	763	34

isolates of *S.* Heidelberg showed 56 different genotypic profiles based on the detection of genes associated with virulence and pathogenicity. It is worth noting that none of the isolates evaluated had all the 24 screened genes. However, the prevalence of these genes was high since the lowest number of genes detected in one isolate was 10/24. The *lpfA* and *csgA* (fimbriae), *invA* and *sivH* (type III secretion system) and *msgA* and *tolC* (intracellular survival) genes were present in 100% of the analyzed isolates (Table 2).

DISCUSSION

Virulence factors are encoded by a number of genes and may be located on the bacterium's own chromosome. These molecules may be present in the so-called pathogenicity islands, or in mobile genetic elements[12,13]. It is widely reported that when these genes are present and especially when gene expression is induced, they confer a number of advantages to bacteria.

Fimbrial virulence genes play an important role in bacterial pathogenicity since they promote bacterial binding to intestinal epithelial cells (enterocytes)[17]. In the present study, the fimbrial genes *sefA*, *lpfA*, *lpfC*, *csgA*, and *pefA* were analyzed; 100% of the isolates had the *lpfA* and *csgA* genes (Table 2). The *lpfA* gene encodes the major subunit of long polar fimbriae which promotes the adhesion of bacteria to the epithelial cells lining the Peyer's patches of the intestine conferring cross-immunity between serotypes[18-20].

Fine aggregative fimbriae are essential to the synthesis of extracellular polymeric substance (EPS) which is involved in biofilm formation and environmental persistence. The *csgA* (formerly *agfA)* gene is one of the genes encoding the presence of these fimbriae which also have properties related to pathogenesis and auto-aggregation (increased survival), are pro-inflammatory and increase invasion of eukaryotic cells[19]. The *csgA* gene is also associated with bacterial adhesion to various inert surfaces, including those used in food production. For this reason, it is also considered an important gene for the production of biofilms and the maintenance of the bacteria in the environment[20,21]. Other researchers have reported a high detection frequency of the *csgA* gene in different serotypes of *Salmonella* spp.[22-24]. Our findings corroborate theirs. Doran *et al.*[25] analyzed 604 strains of *Salmonella* belonging to different serotypes. These authors noted that 603 samples presented the *csgA* gene, suggesting that this gene is well conserved among serotypes.

No isolate had the *sefA* gene. The *pefA* gene was found in four isolates only (Table 2). The *pefA* gene may be absent

Table 2 - Absolute and relative frequencies of 24 virulence genes screened by PCR in *Salmonella* Heidelberg isolates.

Gene Function		Gene	Absolute frequencies (n=126)	Relative frequencies (%)
Fimbriae		*sefA*	0	0
		lpfA	126	100
		lpfC	107	84.9
		agfA	126	100
		pefA	4	3.17
TTSS	Structure	*sipB*	91	72.2
		invA	126	100
		orgA	84	66.7
		prgH	65	51.6
		spaN	103	81.7
	Effector protein	*avrA*	124	98.4
		sopE	112	88.9
		sopB	123	97.6
		sivH	126	100
		sifA	108	85.7
	Regulatory protein	*hilA*	84	66,6
Survival inside cells (macrophages)		*pagC*	120	95.2
		spiA	120	95.2
		msgA	126	100
		tolC	126	100
Plasmid		*spvB*	1	0.79
Iron metabolism		*iroN*	112	88.9
		sitC	100	79.4
Toxins		*cdtB*	11	8.73

in some salmonellae due to the original localization of the gene, which is plasmidial. Plasmids are present in only a few serotypes of *Salmonella* spp.[26]. It is worth mentioning that the *sefA* gene is considered one of the target genes for detecting and serotyping *S. enteritidis*[22]. The acquisition and loss of specific fimbrial *operons* may be one of the mechanisms by which different *Salmonella* serotypes have been able to adapt to an increasing number of hosts[27]. Studies have shown that fimbriae have tropism by different cell types in distinct hosts. For this reason, it is possible that the absence of certain fimbriae could alter the virulence of the strain[19].

All SH (100%) presented the *invA* gene which is related to host recognition and internalization of the bacterium during invasion of epithelial cells. This gene is associated with the structure of TTSS (Type Three Secretion System) and is considered the main target gene for the detection of strains belonging to this genus by PCR[22,28]. In this study, 66.6% of the SH isolates presented the *hilA* gene. This gene encodes the hyperinvasive protein *hilA* which is the main regulator of TTSS components and also encodes SPI-1[29]. This gene is related to the cell recognition and invasion process. Both the *hilA* gene and *invA* gene are considered target genes for the detection of the genus *Salmonella*[28].

The *orgA, sipB, prgH*, and *spaN* genes are also related to the structure of these systems[26]. In the SH isolates, the frequencies of the *spaN* gene (81.7%) and *sipB* (72.2%) were high, followed by other genes with lower frequencies. The *spaN* gene is one of the 12 genes that form a cluster associated with host invasion properties[30]. Borges *et al.*[24], reported the presence of *orgA, sipB, prgH*, and *spaN* genes in different *Salmonella* serotypes. These authors noted that the *spaN* gene is associated with strains isolated from poultry sources and is a potential epidemiological marker of *Salmonella* of avian origin.

Genes encoding the effector proteins secreted by TTSS, such as: *sif*A, *avr*A, *sop*E, *sop*B e and *siv*H were detected in the majority of tested SH strains (Table 2). It is important to search for these genes because the detection of possible changes in the repertoire of the resulting proteins can mean changes in the ability of *Salmonella* serotypes to adapt to new hosts, allowing them to become emergent and may be related to the appearance of salmonellosis outbreaks[31].

In order to assess survival rate and inside cells, especially the ability to survive within macrophages, the following genes were investigated in our study: *msgA, spiA, pagC,* and *tolC*. We detected the msgA and tolC genes in 100% of the tested isolates (126 SH samples). The *pagC* and *spiA* genes were detected in 95.2% of the analyzed specimens (Table 2). It is worth mentioning that the *spiA* gene is also related to the ability of the isolates to produce biofilms. Our findings corroborate those published by Borges *et al.*[24] who detected the presence of the *msgA* gene in 100% of the tested samples whereas the *spiA* and *pagC* genes showed a frequency of 93.8% in these samples. Rizzo *et al.*[23] detected the genes *msgA, spiA, tolC* and *pagC* in all of the *Salmonella* Gallinarum strains.

Only one SH isolate had the *spvB* gene (0.79%). This gene is of plasmid origin, is associated with the *Salmonella* virulence plasmid and is responsible for the maintenance and bacterial survival within the cell[31]. The low frequency of this gene was expected among SH, since the presence of plasmids in *Salmonella* is closely related to the involved serotype. Its detection is more common in serotype Enteritidis[32,33]. According to Rizzo *et al.*[23] the *spvB* gene was expressed in 80% (12/15) of the serotype Gallinarum samples tested showing that this gene may also be common to other serotypes. DNA extraction by heat treatment could have interfered with the detection of this gene. However, the use of DNA samples extracted through commercial kits presented similar results (unpublished data).

In the present study, we investigated genes associated with iron metabolism, including the *iroN* gene and the *sitC* gene. Of the analyzed SH isolates, 88.9% presented the *iroN* gene whereas 79.4% had the *sitC* gene (Table 2). Bacteria rely on a number of mechanisms and molecules to acquire iron for survival. The *sitC* gene encodes membrane proteins in bacterial cell which are associated with iron uptake. In contrast, *iroN* gene encodes proteins that function as receptors of siderophores[26,34]. After cell invasion, *Salmonella* spp. finds an environment with restricted amount of iron, an essential element for their survival and growth within the host cell[26,34]. Consequently, bacteria have developed a variety of iron procurement systems, which are generally redundant and are not simultaneously expressed. For this reason, the absence of these genes is unlikely to be a problem for the survival and multiplication of *Salmonella* within the host cell. The *iroN* gene is positively associated with the serotype Heidelberg[24]. According to the study carried out by Borges *et al.*[24], in which different *Salmonella* serotypes were analyzed, the absence of the i*roN* gene and the presence of the *spaN* gene are associated with bacterial strains from poultry sources.

The *cdtB* gene is one of the genes encoding toxins that induce apoptosis of infected cells. Of the tested isolates, only 11 (8.7%) had this gene. The finding of a low frequency *cdtB* gene is in agreement with previous studies in which different serotypes of *Salmonella* spp. were analyzed[23,24]. According to the published literature, this gene is possibly restricted to some serotypes of *Salmonella* spp[26].

Some results were unexpected, such as the low frequency of *hilA, orgA* and *prgH* genes, presenting

differences in relation to the molecular profiles found in SH strains isolated in Brazil between 1996 and 2006[24]. Gene frequency variation and genetic profile may be influencing the survival and multiplication capacity of the strains over the years, subsequently leading to an increase in the frequency of this serotype isolated from poultry sources in Southern Brazil. Aravena *et al.*[27] analyzed SH strains isolated in Chile between 2006 and 2011 and did not observe these variations. However, Antony *et al.*[14] detected the operon *safABCD* in strains isolated in USA between 2009 and 2017. This operon has been previously described as absent in this serotype[14]. These results support the theory that modification in genetic profile of circulating SH strains are a possible reason for the increased detection of this particular serotype. Further studies are needed to confirm this hypothesis. A preliminary analysis performed by our laboratory (data not shown) demonstrated that strains isolated in Brazil in 2016 and 2017 presented a greater genetic variability when compared to those isolated until 2006[28]. In more recent isolates, two new ribotypes were identified. The emergence of two new ribotypes may indicate a trend in changing circulating clones, which could be one of the reasons for the increased frequency of this serotype isolation[28].

The knowledge on *Salmonella* serotypes and detection of genetic profiles of these bacterial pathogens can be used to determine different virulence patterns of isolates. These data allow a better understanding of the characteristics of each serotype and the establishment of criteria to predict the virulence of these microorganisms. The information generated in these studies would help and improve *Salmonella* control strategies in the food industry, as well as the control measures for the prevention of salmonellosis in humans.

In the present study, we noted that among the isolates of *S.* Heidelberg tested there are genotypic profile differences. This finding suggests the presence of a great diversity of *S.* Heidelberg isolates in poultry products. The fact that a single predominant profile was not found in our survey indicates that there are a variety of S. Heidelberg contamination sources in the food industry.

CONFLICT OF INTERESTS

All authors state that there is no conflict of interests regarding the publication of this manuscript.

ACKNOWLEDGMENTS

The authors of this manuscript gratefully acknowledges Eduardo Cesar Tondo from the Institute of Food Science and Technology (ICTA) at UFRGS, Porto Alegre, Rio Grande do Sul State, Brazil who kindly provided the *Salmonella* Heidelberg samples for this study.

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