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Relation Between the *SpvC* and *InvA* Virulence Genes and Resistance of *Salmonella enterica* Serotype Enteritidis Isolated from Avian Material

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Abstract: Pathogenic bacteria possess genes responsible for virulence, that when expressed, determine not only bacterial invasion and persistence and destruction of host cells but also survival capacity under inappropriate culture conditions. This study utilized 100 samples of *Salmonella enterica* subspecies *enterica* Serotype Enteritidis (SE) isolated from avian material, detected the virulence genes *InvA* and *SpvC* by medium of Polymerase Chain Reaction (PCR) and compared them for possible expression of virulence factors for survival in hostile conditions of temperature, pH and reduced concentration of nutrients necessary for SE multiplication. Of all the samples analyzed, two presented the genes *SpvC* and *InvA*, simultaneously, with one probable expression of them being verified in growth with pH 10.0 or temperature of 25°C. But in relation to nutrient concentration, neither sample obtained growth when seeded in medium containing 0.5% nutrient.

Key words: Gene *InvA*, gene *SpvC*, resistance, *Salmonella* enteritidis, virulence

INTRODUCTION

The pathogenicity of *Salmonella* depends on a series of factors associated with the bacterium, the bird itself and conditions in which the bird is raised. Association and penetration of the bacterium into digestive mucosa is a prerequisite for systemic infection (Barrow, 1995; Rychlik *et al*., 1998).

For *Salmonella* to be virulent, the expression of numerous genes is necessary, which encode some factors with the ability to be located in transmissible genetic elements such as plasmids, bacteriophages and transposons and may be part of specific regions in the chromosome of the bacterium (Hacker *et al*., 1997). Pathogenic bacteria regulate the expression of genes necessary for virulence and this action is frequently associated with specific environmental conditions. Various studies have demonstrated that *Salmonella* within macrophages express different proteins when grown in culture media (Abshire and Neidhardt, 1993; Buchmeier and Heffron, 1990; Burns-Keliher *et al*., 1998).

The virulence of *Salmonella* is linked to a combination of chromosomal and plasmid factors. The *Inv*A invasion gene enables the invasion da *Salmonella* in epithelial cell cultures (Galan and Curtiss III, 1989), while the *operon* (*SpvRABCD*), which contains five genes and is present in plasmids commonly associated with many serotypes, determines the ability to augment the severity of enteritis and persistence at extra-intestinal sites (Libby *et al*., 2000).

The invasion gene (*Inv)* and the presence of virulence plasmids (*Spv*) are essential for the virulence expression of *S.* Typhimurium in mice. However, the relation between the presence of the *InvA* and *SpvC* genes and the reduction of invadability of *Salmonella* isolated from diverse sources associated with broiler chickens has not yet been determined (Swamy *et al*., 1996). Serotypes of *Salmonella* that do not possess the *InvA* gene are not capable of expressing *InvABC* genes, making them unable to invade mammalian cells (Galán *et al*., 1992).

Plasmids involved in virulence are also related to survival and growth of the bacterium in host cells, although they apparently are not involved in the initial interaction between *Salmonella* and intestinal mucosa. Nevertheless, the plasmids enable the bacterium to persist in cells of the reticuloendothelial system such as the spleen and liver (Gulig and Curtiss III, 1987; Swamy *et al*., 1996).

Salmonella virulence plasmids were not necessary in mice to dislocate the bacterium via the intestinal mucosa, demonstrating that *Salmonella* samples, even without plasmids, were capable of colonizing and persisting in the spleen and liver, although in the systemic phase of the infection there had been inadequate bacterial growth (Guiloteau *et al*., 1996).

Pathogenic bacteria (with *Spv operon*) express virulence factors that help them survive in hostile conditions such as elevated temperature, acidic pH and privation of nutrients (Valone *et al*., 1993).

This work aimed to detect the virulence genes *SpvC* and *InvA* in samples of *Salmonella* Enteritidis isolated from avian material by means of PCR, correlating the presence of these genes with the possible expression of virulence factors for survival in hostile conditions of temperature, pH and nutrient privation.

MATERIALS AND METHODS

The 100 samples of *Salmonella enterica* subspecies *enterica* Serotype Enteritidis (SE) utilized were isolated from viscera (liver, cecum, yolk sac or ovary), swabs of the cloaca or carcasses of chickens and stocked in nutrient agar until the moment of use.

The SE samples were reactivated in tubes containing 3 mL of Brain-heart Infusion (BHI) and incubated in aerobiosis at 37°C for 24 h. Next, each sample was seeded on a Petri dish containing Brilliant Green Agar (BGA) and incubated as described previously. After this period, five colonies from each sample were seeded in tubes containing 3 mL of nutrient broth. Subsequently, each of the SE samples was submitted to variation in pH, temperature or concentration of nutrients (Fig. 1).

To verify bacterial growth at different pH levels, the samples were seeded in tubes containing complete nutrient broth with the pH corrected to 2.0, 4.0, 6.0, 8.0 and 10.0 while utilizing hydrochloric acid (1 N) and sodium hydroxide (0.1 N) for acidic and alkaline pH correction, respectively.

The growth of SE samples in different concentrations of nutrients was found to be seeded in nutrient broth with pH 7.0±0.2 and prepared at concentrations of 0.5%, 1.0, 2.5, 5.0 and 10.0% based on the recommendation of the manufacturer (Oxoid®). The nutrient broth is composed of 1 g/L of meat extract, 2 g/L of yeast extract, 5 g/L of peptone and 5 g/L of sodium chloride.

Complete nutrient broth with pH 7.0±0.2 at temperatures of 10, 25, 40, 55 and 70°C was utilized to verify the growth of each SE sample.

All the cultures were accomplished in triplicate and incubated for 24 h at 37°C, except those submitted to different temperatures. After the incubation period the cultures were evaluated in relation to growth of SE samples, verified by turbidity of the culture medium and subsequent seeding in BGA. Three tubes containing complete nutrient broth, pH 7.0±0.2, seeded with SE sample and incubated at 37°C for 24 h and three tubes containing the same medium and not seeded, constituted, respectively, the positive and negative controls. For detection of virulence genes, bacterial lysate was obtained containing the DNA from each SE sample, cultivating the bacterium for 24 h at 37°C in BGA. Colonies were transferred to an assay tube containing sterile bidistilled water until achieving a turbidity equivalent to three on the McFarland scale and centrifuged at 15,000 rpm for 5 min. Next, the supernatant was discarded and 1 mL of sterile bidistilled water was added. This suspension was submitted to 100°C for 10 min and after refrigeration (4°C) centrifuged at 15,000 rpm, with the supernatant being stored at -20°C until the moment of use (Álvarez *et al*., 2003).

The oligonucleotides (primers) utilized for the virulence genes *InvA* and *SpvC* (Swamy *et al*., 1996) are specified in Table 1.

After obtaining the lysate from SE samples, the presence of genes was investigated by Polymerase Chain Reaction (PCR).

For the virulence genes (*InvA* and *SpvC*) amplification was performed in a sterile microtube, adding 2 µL of DNA from each SE sample, 15.55 µL of ultrapure water, 2,5 µL of PCR buffer 10 X (100 mM Tris-HCl pH 8.3, 500 mM KCl, 15 mM $MgCl_2$ and 0.01% gelatin), 0.75 µL of $MgCl_2$ (50 mM), 0.5 µL of each dNTP (deoxynucleotide triphosphates) to 2.5 mM, 1 µL of each primer (20 ρmoles) and 0.2 µL of DNA Taq polymerase (5 U/µL), for a total volume of 25 µL. Amplification was initiated with denaturation at 94°C for 5 min, followed by 35 cycles at 94°C for 30 sec, annealing at 60°C for 30 sec and extension at 72°C for 30 sec, then a final extension for 4 min at 72°C.

To each 7 µL of PCR product from each sample was added 3 µL of loading buffer (6X). After homogenization 10 µL was placed in each well of 1.0% agarose gel. The PCR products together with the marker of molecular weight (100 bp DNA *ladder*) were separated by electrophoresis in a 100 V current for 1 h and 30 min. The gel was stained with ethidium bromide solution (10 mg/mL) for 10 min and analyzed under ultraviolet light.

RESULTS AND DISCUSSION

PCR revealed the presence of specific bands referencing molecular weights of the genes *InvA*-521 bp and *SpvC*-669 bp (Fig. 2).

The investigation of the virulence genes *InvA* and *SpvC* from SE samples showed that only 3% and 26%, respectively, did not present the gene. Furthermore, all the samples that were negative for the *SpvC* gene, coincidentally, were also negative for the *InvA* gene, which grew only within the pattern ideal for *Salmonella* spp. culture (37°C/24 h) according to Franco and Landgraf (1996) and Silva (1999).

None of the samples tested grew in culture with pH 2.0, demonstrating the inability of SE to grow in highly acidic

Table 1: Oligonucleotides (primers) referent to virulence genes (*InvA* and *SpvC*) of *Salmonella* Enteritidis

Genes	*Oligonucleotides (5' → 3')*	Base pair (bp)
InvA	TTGTTACGGCTATTTTGACCA	521
	CTGACTGCTACCTTGCTGATG	
SpvC	CGGAAATACCATCTACAAATA	669
	CCCAAACCCATACTTACTCTG	

Table 2: Number de samples positive for the virulence genes at the different pH tested in relation to the total number of *Salmonella* Enteritidis samples

	pH				
Gene	2.0	4.0	6.0	8.0	10.0
InvA	0/97	2/97	97/97	97/97	2/97
SpvC	0/74	2/74	74/74	74/74	2/74
SpvC e *InvA*	0/74	2/74	74/74	74/74	2/74

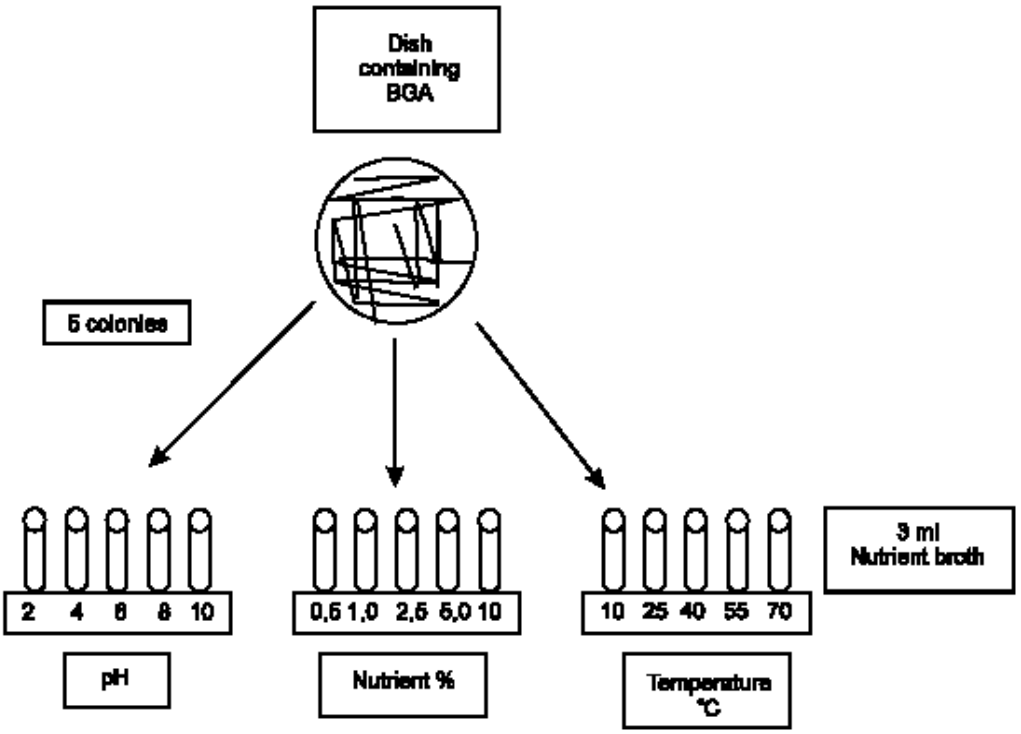

Fig. 1: Experimental delineation displaying culturing of *Salmonella* Enteritidis samples in medium with variation in pH, nutrient concentration and temperature

media. In cultures with pH 4.0 there was growth in only two SE samples, as occurred when the pH was 10.0, although they were not the same. In both cases the genes *SpvC* and *InvA* were present in four samples, demonstrating a possible expression of these virulence genes in bacterium. But in cultures with pH 6.0 and 8.0, all the samples grew, independent of whether they possessed the genes, in agreement with Silva (1999) who described the pH ideal for growth as being between 6.5-7.5 (Table 2).

In relation to the quantity of nutrients, only 11 SE samples were able to grow when culture medium had only 0.5% of the quantity recommended by the manufacturer, with all being positive for the presence of studied virulence genes. At the remaining nutrient concentrations all the samples grew independently of the presence of the researched genes (Table 3).

Considering the different temperatures of culturing, there was growth only at 25ºC (four samples) and at 40ºC (all samples), with or without the researched genes. The four samples that grew at 25ºC all presented the *InvA* and *SpvC* genes. At the other temperatures (10, 55 and 70ºC) there was no growth in any of the SE samples. Franco and Landgraf (1996) reported that the ideal temperature for *Salmonella* spp. growth is from 35-37ºC, with 5ºC and 47ºC being the minimum and maximum temperatures for growth, respectively (Table 4).

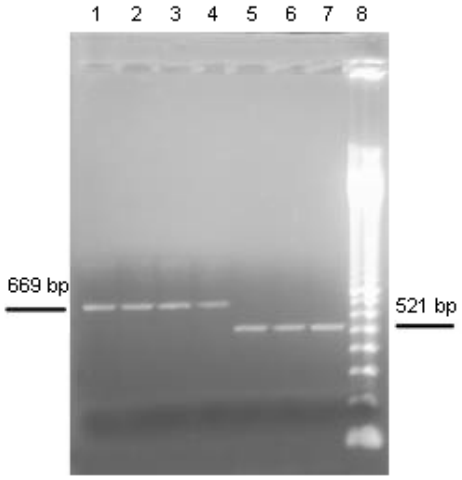

Fig. 2: Columns 1, 2, 3 and 4 represent *SpvC* virulence gene (669 base pairs) and columns 5, 6 and 7 the *InvA* gene (521 base pairs). Molecular weight marker 100 bp DNA *Ladder* (column 8)

The two SE samples that had grown at pH 10.0 and presented the genes *InvA* and *SpvC*, also were able to multiply at the temperature of 25ºC, demonstrating a possible capacity to survive in hostile conditions when the virulence genes are present and expressed. However, these two SE samples were not capable of growing at the nutrient concentration of 0.5%.

Table 3: Number of samples positive for virulence genes at different concentrations of nutrients relative to the total number of *Salmonella* Enteritidis samples

	Concentrations of nutrients (%)				
Gene	0.5	1	2.5	5	10
InvA	11/97	97/97	97/97	97/97	97/97
SpvC	11/74	74/74	74/74	74/74	74/74
SpvC e *InvA*	11/74	74/74	74/74	74/74	74/74

Table 4: Number of samples positive for virulence genes at different culture temperatures in relation to the total number of *Salmonella* Enteritidis samples

	Temperatures (°C)				
Gene	10	25	40	55	70
InvA	0/97	4/97	97/97	0/97	0/97
SpvC	0/74	4/74	74/74	0/74	0/74
SpvC e *InvA*	0/74	4/74	74/74	0/74	0/74

Not all bacteria that possess virulence genes are capable of expressing them. Nevertheless, when expressed they can be fundamental to multiplication and survival, thus amplifying the danger in relation to both animal and public health.

Conclusion: *Salmonella* Enteritidis can present genes related to invasion and virulence (*InvA* and *SpvC*), but are not always capable of expressing them and may affect growth in unsuitable conditions of pH, temperature and nutrient availability.

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