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DISTRIBUTION OF VIRULENCE GENES *SEF*C, *PEF*A AND *SPV*C IN *SALMONELLA* ENTERITIDIS PHAGE TYPE 4 STRAINS ISOLATED IN BRAZIL

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ABSTRACT

The distribution of virulence genes, *sef*C, *pef*A and *spv*C, was investigated in 110 *Salmonella* Enteritidis phage type 4 strains by polymerase chain reaction. Their influence in the caecal colonization and invasion of liver and spleen of one-day-old chickens was studied. Eight isolates were negative for the *spv*C gene, three for the *pef*A gene and one, for the *sef*C gene. These results allowed grouping the strains into four genotypes. Presence of these genes did not influence bacteria invasion in the liver and spleen of the chickens ten days after infection, although the presence of more than one fimbrial gene can be related to caecal colonization.

Key words: *Salmonella,* virulence genes, colonization, invasion

INTRODUCTION

Salmonella Enteritidis (SE) is one of the serotypes of the genus *Salmonella,* which causes diseases in many animal species and in human beings (23). In humans, the disease can develop from gastroenteritis to septicemia, causing severe damage and even death (13,23).

In commercial poultry breeding, the clinical form of the disease is more common in young birds. Adult chickens are one of the most important reservoirs of this serotype; they are carriers and the main cause of bacteria introduction in human food (12,13,18). Phage type 4 (PT4) SE strains are the most frequently described in outbreaks of human and poultry salmonellosis (19,27,32).

The adherence of bacteria to the cell surface is essential to the pathogenesis of the disease. Adherence to the cell surface is a key factor for bacteria invasion and survival inside the host cells. Fimbriae are one of the most important surface structures to guarantee bacterial fixation to the cell (10). SEF14 (9) and PEF fimbriae (4) play a role in the colonization of Peyer's patches and in the adhesion and invasion of intestine epithelial cells (5,20,29,31). Different authors described that SEF14 fimbriae contributed to the adherence of the pathogen to chicken ovarian granulosa cells, and egg-yolk specific antibodies for these fimbriae reduced the invasion and colonization in the first stages of infection (22,28).

Although serotype Enteritidis and other *Salmonella* serotypes contain virulence plasmids of different sizes and genetic composition, all contain a preserved region of approximately 8 Kb, called operon *spv* (14,17,24). This operon is important for the survival and multiplication of the bacteria inside the cells of the reticuloendothelial system, as the liver and the spleen (17,25).

This study aimed to detect the presence of *sef*C, *pef*A and *spv*C genes in isolates of SE PT4, using PCR, and to evaluate their role in the colonization of the caecum and invasion of liver and spleen in one-day-old chicks infected by oral route.

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MATERIALS AND METHODS

Bacterial strains

The SE PT4 isolates from humans (n=27), foods (n=6), pigs (n=8), bedding samples from poultry farms (n=8), chickens (n=41) and poultry meats (n=20) were obtained from the culture collection of the Ornithopathology Laboratory of the University of São Paulo (19). The 110 strains have been isolated between 1995 and 1997. Strains were stored at -80ºC, and subjected to a maximum of two passages. Isolates were grown in McConkey agar for 24 hours at 37ºC, and one colony of each strain was cultured in 3 mL of LB broth for 24 hours at 37ºC. A 200 µL sample of this cell suspension was used for the extraction of the bacterial DNA as described by Boom *et al*. (6).

PCR

The PCR amplification mixture (50 µL) consisted of 1X PCR buffer, 1.5 mM $MgCl_2$, 200 µM each dATP, dCTP, dGTP, dTTP, 50 pmol of each primer, and 1.0 U of Taq DNA polymerase (Invitrogen, NY) and sterile, ultrapure water. All DNA samples were diluted to a concentration of 5 ng. Commercially synthesized primers were used. Table 1 lists the primers used and the respective annealing temperatures. Multiplex was used for *pef*A and *sef*C genes. Gene *spv*C was amplified separately. *Salmonellla* Typhimurium ATCC 14028 was used as positive control for the three genes searched in this study, and *Escherichia coli* K12 served as negative control.

Detection of the amplified product

Amplified products were separated by electrophoresis in 1.5% agarose gel stained with ethidium bromide. The gels were photographed by means of the Image Master System (Amershan-Pharmacia Biothec). The 100 bp DNA ladder (Invitrogen, NY) was used as molecular size marker.

Experimental infection in Specific Pathogen Free (SPF) chicks

A SE strain from each of the four genetic profiles found by PCR analysis was orally inoculated in a group of 15 SPF White Legorhn chicks (one-day-old), supplied by Biovet Ltda. Approximately 1 x 10^1 colony-forming units (CFU) in 0.1 mL of medium was administered to each chick. The control group (n=20) received 0.1 mL of sterile oral solution of NaCl 0.85%.

Birds were sacrificed by cervical dislocation ten days after infection. The liver and the spleen of each chick were collected and placed together in sterile plastic bags. The caecum was collected separately. Organs were macerated and diluted in 0.1% peptone water and a 0.1 mL aliquot was streaked in XLT4 agar. Plater were incubated for 24 to 96 hours at 37ºC. Colonies suggestive of *Salmonella* were confirmed with polyvalent antisera (Promicro, São Paulo).

RESULTS

The *spv*C gene was absent in 7.2% (8/110) of the isolates; gene *pef*A was absent in 2.7% (3/110) and only 0.9% (1/110) of the isolates were negative for the *sef*C gene. Genes *pef*A and *sef*C were simultaneously present in 96.3% (106/110) of the isolates. Based on these results, the isolates were classified in four genetic profiles.

The first profile (P1), negative for the *spv*C and *pef*A genes and positive for the *sef*C gene, was observed in 2.7% (3/110) of the isolates. The second profile (P2), negative only for the *spv*C gene, was found in 4.5% (5/110) of the isolates. The third profile (P3), positive for the three genes considered in this study, was found in 91.8% (101/110), and the fourth profile (P4), negative for the *sef*C gene and positive for genes *spv*C and *pef*A, was found in 0.9% (1/110) of the isolates only (Table 2).

Table 2. Distribution of 110 *S*. Enteritidis PT4 strains according to the genetic profile.

Profile	Number of SEPT4 Strains (%)	Genes		
		*spv*C	*sef*C	*pef*A
P1	3 (2.7)	-	+	-
P2	5 (4.5)	-	+	+
P3	101 (91.8)	+	+	+
P4	1 (0.9)	+	-	+

Table 1. Primers used in PCR for the detection of virulence genes in *S.* Enteritidis PT4

Gene	Sequence of Nucleotides	Amplicon	Annealing Temperature	References
*spv*C	F: CGGAAATACCATCTACAAATA R: CCCAAACCCATACTTACTCTG	669	42ºC	Sway *et al.* (27)
*sef*C	F: GCGAAAACCAATGCGACTGTAG R: CCCACCAGAAACATTCATCCC	1103	50ºC	Bäumier *et al.* (4)
*pef*A	F: AGGGAATTCTTCTTGCTTCCATTCCATTATTGCACTGGG R: TCTGTCGACGGGGGATTATTTGTAAGCCACT	520	50ºC	Bäumier *et al.* (5)

Bacteria isolation from the caecum was possible in 13.3% and 6.6% of the birds infected with the P2 and P3 profiles, respectively. Bacteria isolation from the liver/spleen was possible in all groups. Percentage of chicks presenting SE in these organs were 40% for P1 and P4 profiles; 60% and 46.6%, for P2 and P3 profiles, respectively. These results are shown in Table 3.

Table 3. Positivity of isolation of *S.* Enteritidis PT4 from liver/spleen and caecum of chicks inoculated with strains with profiles P1, P2, P3 and P4.

		Number of positive chicks (%)		Control group	
Profile	Number of orally inoculated chicks	Liver/ Spleen	Caecum	Number of chicks	Number of positive chicks
P1	15	6 (40)	0	5	0
P2	15	9 (60)	2 (13.3)	5	0
P3	15	7 (46.6)	1 (6.6)	5	0
P4	15	6 (40)	0	5	0

DISCUSSION

Despite the different sources of isolation, 91.8% SE PT4 strains were positive for the investigated genes, suggesting the existence of similarities among them.

Fimbriae play an important role in the pathogenicity of bacteria, because they promote their attachment to intestinal epithelial cells (10,31). Caecal colonization is important for egg contamination (1). PEF fimbria is encoded by the *pef* operon located in a plasmid (11). Among the isolates analyzed in this study, gene *pef*A was absent in 2.7% (3/110). These results are similar to those presented by Woodward *et al*. (34), who found 2% (1/49) of SE negative for this gene. Bäumler *et al*. (4) studied the phylogenetic distribution of fimbrial genes in *Salmonella* spp and verified that, in the serotype Enteritidis, the *pef* operon presented two distantly related lineages: one that did not hybridize with the *pef*A gene, and another that hybridized and represented 93% of isolates in the global distribution of this serotype. SEF14 fimbria is encoded by the *sef* operon, which contains *sef*C gene. This gene encodes an outer membrane protein that contain the *sef*A subunit and the s*ef*D adhesin. In the present study, the *sef*C gene was detected in more than 99% (109/110) of the isolates. Other authors described the expression of SEF14 fimbriae in 100% of *S.* Enteritidis studied, which explains the high frequency of the *sef*C gene, since the absence of *sef*C affects the expression of adhesin (8).

In this study, the observed level of caecum colonization during experimental infection was low (1 x 10^1 UFC). 13.3% of the poultry inoculated with the P2 profile, and 6.6% of those inoculated with the P3 profile. This result is different from that reported by Asheg *et al.* (2), who studied poultry infected with low (2 x 10^2) and high dose of SE (2 x 10^8). These authors observed that in the first week of infection, continuous colonization of the caecum occurred. Caecal colonization was observed even in the group infected with the lowest dose, resulting in 80% of the poultry positive after ten days of infection. The difference in the results might be related to the dose used in the present study.

Since isolation in the caecum was only possible in the chicks infected with P2 and P3 profiles, positives for *sef*C and *pef*A genes, it is also important to consider the virulence potential of the strain used. In a trial where chickens were orally inoculated with SE, Thiagarajan *et al*. (29) described higher caecal colonization with bacteria that had SEF21 and SEF14 fimbriae, compared with bacteria that had one or none of the fimbriae. Experimental infection of mice with bacteria presenting mutation in fimbrial operons showed that the absence of at least two fimbrial structures may significantly decrease adherence to murine intestinal tissue and further reduce virulence (31). Aslanzadeh and Paulissen (3) demonstrated that synergic action occurs among fimbriae. In this study, only two fimbrial operons were studied. It is possible that the absence of caecal colonization in the groups inoculated with P1 and P4 profiles may have occurred due to the absence of other fimbrial operons not included in this study, as the *agf*B (31).

In the present study, 7.2% (8/110) of the isolates were negative for the presence of gene *spv*C (P1 and P2 profiles). In a study carried out with 245 *Salmonella* isolates, Swamy *et al.* (26) reported that 84.9% (208/245) were negative for the *spv*C gene. The majority of the positive isolates (81%) belonged to the Enteritidis serotype and were obtained from egg contents or from the egg production environment. Based on the results presented here, the presence of *spv*C gene probably did not influence caecum colonization or the invasion of the liver and the spleen.

Operon *spv* is conserved among different virulence plasmids of several *Salmonella* serotypes that produce systemic diseases (14). Other authors (15,16) did not observe any difference in chicks orally inoculated with SE PT4 strains with or without plasmids. This shows that the plasmid was not essential for bacterial location in the liver, spleen and even ovaries of laying-hens. In a trial with Dublin serotype in catlle, Wallis *et al*. (33) described that both wild-type and plasmid-cured strains were detected with similar frequencies at intestinal and systemic sites three days after challenge. Six days after challenge, the wild-type strain appeared to predominate in systemic sites. The authors concluded that virulence plasmids are not involved in the enteric phase of infection or the dissemination of bacteria, but probably mediate their persistence at systemic sites.

Results presented in this study suggest that the two fimbrial operons in the same isolate play a role in caecal colonization of poultry. However, due to the high number of genes involved in *Salmonella* virulence, complementary studies must be carried out to determine the importance of each one of them in the intestinal colonization and ability to invade systemic sites.

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RESUMO

Distribuição de genes de virulência *sef*C, *pef*A e *spv*C em cepas de *Salmonella* Enteritidis fago tipo 4 isoladas no Brasil

A distribuição dos genes de virulência *sef*C, *pef*A e *spv*C foi investigada em 110 amostras de *Salmonella* Enteritidis pertencentes ao fagotipo 4 através da reação em cadeia da polimerase. A influência destes genes na colonização do ceco e invasão do fígado e baço em pintinhos de um dia de idade foi avaliada. Oito amostras foram negativas para o gene *spv*C, três para o gene *pef*A e uma amostra para o gene *sef*C. Estes resultados permitiram a classificação das amostras em quatro genótipos. A presença destes genes não influenciou a invasão da bactéria no fígado e baço das aves dez dias após a infecção, entretanto, a presença de mais de um gene fimbrial pode ter relação com a colonização cecal.

Palavras-chave: *Salmonella*, genes de virulência, colonização, invasão

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