DISTRIBUTION OF VIRULENCE GENES SEFC, PEF A AND SPVC IN SALMONELLA ENTERITIDIS PHAGE TYPE 4 STRAINS ISOLATED IN BRAZIL

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ABSTRACT

The distribution of virulence genes, sefC, pefA and spvC, 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 spvC gene, three for the pefA gene and one, for the sefC 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 sefC, pefA and spvC 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 MgCl₂, 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 pefA and sefC genes. Gene spvC was amplified separately. Salmonella 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 Biotech). 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 10⁴ 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 spvC gene was absent in 7.2% (8/110) of the isolates; gene pefA was absent in 2.7% (3/110) and only 0.9% (1/110) of the isolates were negative for the sefC gene. Genes pefA and sefC 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 spvC and pefA genes and positive for the sefC gene, was observed in 2.7% (3/110) of the isolates. The second profile (P2), negative only for the spvC 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 45.5% (51/110) 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 sefC gene and positive for genes spvC and pefA, was found in 0.9% (1/110) of the isolates only (Table 2).
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.

<table>
<thead>
<tr>
<th>Profile</th>
<th>Number of orally inoculated chicks</th>
<th>Number of positive chicks (%)</th>
<th>Control group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Liver/ Spleen Caecum</td>
<td>Number of chicks</td>
<td>Number of positive chicks</td>
</tr>
<tr>
<td>P1</td>
<td>15</td>
<td>6 (40)</td>
<td>0</td>
</tr>
<tr>
<td>P2</td>
<td>15</td>
<td>9 (60)</td>
<td>2 (13.3)</td>
</tr>
<tr>
<td>P3</td>
<td>15</td>
<td>7 (46.6)</td>
<td>1 (6.6)</td>
</tr>
<tr>
<td>P4</td>
<td>15</td>
<td>6 (40)</td>
<td>0</td>
</tr>
</tbody>
</table>

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 pefA 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 pefA 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 sefC gene. This gene encodes an outer membrane protein that contain the sefA subunit and the sefD adhesin. In the present study, the sefC 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 sefC gene, since the absence of sefC affects the expression of adhesin (8).

In this study, the observed level of caecal 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 10^6). 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 sefC and pefA 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 agB (31).

In the present study, 7.2% (8/110) of the isolates were negative for the presence of gene spvC (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 spvC 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 spvC 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 cattle, 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.
Distribuição de genes de virulência sefC, pefA e spvC em cepas de Salmonella Enteritidis fago tipo 4 isoladas no Brasil


A presença destes genes não influenciou a invasão da bactéria permitiram a classificação das amostras em quatro genótipos. 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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Virulence genes in S. Enteritidis PT4


