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Establishment of a pathogenicity index in *Salmonella* Enteritidis and *Salmonella* Typhimurium strains inoculated in one-day-old broiler chicks

[*Estabelecimento de um índice de patogenicidade em cepas de* Salmonella *Enteritidis e* Salmonella Typhimurium *inoculadas em pintos de um dia de idade*]

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ABSTRACT

Salmonella Enteritidis and Salmonella Typhimurium are responsible for causing huge economic loses in aviculture, as they lead young broiler chicks to develop clinical disease and thus increase mortality. Salmonella's pathogenicity is considered complex and multifactorial, demanding more studies that could elucidate the interaction between host and pathogen. The present study aims to evaluate the virulence of 130S. Enteritidis isolates and 70S. Typhimurium inoculated in one-day-old chicks through the establishment of a pathogenicity index. For each strain, 10 commercial chicks from the Cobb lineage were used. Then, 200µL of a solution containing $2x10^8$ CFU of S. Enteritidis or S. Typhimurium were inoculated in the birds by intraperitoneal via. Mortality and presence of lesions such as aerosaculitis (A), perihepatitis (Ph), pericarditis (Pc), peritonitis (Pt), onfalitis (O) and cellulitis (C) were registered daily for seven days. From the second to the seventh day there was a proportional decrease in the punctuation of the time of death (TD) for each day that the bird had survived. The pathogenicity index was calculated using the following formula: PI = (TD x 5) + A + Ph + Pc + Pt + O + C. The obtainment of the PI of each bacterial sample was achieved by calculating the rate of the ten inoculated birds. Based on the obtained results, it was possible to attribute the pathogenicity value for each strain, which enabled us to classify them in groups of low (27/200), intermediate (95/200) and high (78/200) pathogenicity. The utilization of standards like time of death and presence of septicemic lesions made it possible to determine the pathogenicity rate for each strain. Besides that, the proposed model has presented dramatic differences between the high, intermediate and low pathogenicity groups, which makes this mechanism useful for further classification of strains isolated in poultry farms.

Keywords: birds, Salmonelosis, virulence

RESUMO

Salmonella Enteritidis *e* Salmonella Typhimurium *são responsáveis por imensos prejuízos econômicos ao setor avícola, podendo levar ao desenvolvimento de doença clínica e ao aumento da mortalidade em aves jovens. A patogenicidade de* Salmonella *é considerada complexa e multifatorial, necessitando de estudos que possam esclarecer a interação entre patógeno e hospedeiro. O presente trabalho teve por objetivo avaliar a virulência de 130 isolados de* S. Enteritidis *e 70 de* S.Typhimurium*, inoculadas em pintos de um dia de idade, por meio do estabelecimento de um índice de patogenicidade. Para cada cepa, foram utilizados 10 pintos comerciais da linhagem Cobb. As aves foram inoculadas com 200µL de uma solução contendo $2x10^8$ UFC de* S. Enteritidis *ou* S. Typhimurium*, por via intraperitoneal. A mortalidade e a presença de lesões como aerossaculite (A), peri-hepatite (Ph), pericardite (Pc), peritonite (Pt), onfalite (O) e celulite (C) foram registradas diariamente durante sete dias. Do segundo ao sétimo dia, houve uma diminuição proporcional da pontuação no tempo de morte (TM) a cada dia em que o animal sobrevivia.*

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O cálculo do índice de patogenicidade de cada pintinho inoculado (IP) obedeceu à seguinte fórmula: IP = (TMx5) + A + Ph + Pc + Pt + O + C. Para obtenção do IP de cada amostra, foi realizada a média do IP obtido com as 10 aves inoculadas. Com base nos resultados observados, foi possível atribuir um valor de patogenicidade a cada uma das cepas, permitindo classificá-las em grupos de baixa (27/200), intermediária (95/200) e alta patogenicidade (78/200). A utilização de critérios, como tempo de morte e presença de lesões septicêmicas, permitiu a determinação de um índice de patogenicidade para cada cepa. Além disso, o modelo proposto apresentou diferença significativa entre os grupos de alta, intermediária e baixa patogenicidade, permitindo, assim, a sua aplicação para classificação futura das cepas isoladas em granjas avícolas.

Palavras-chave: aves, salmonelose, virulência

INTRODUCTION

Bacteria from the S*almonella* genus, especially S*almonella* (*S*.) Enteritidis and *S*. Typhimurium are considered one of the main etiological agents causing foodborne diseases in many countries (CDC, 2014; PHAC, 2014). Frequently, poultry products act as reservoirs for these bacteria, being responsible for the human infection through meat and egg contamination (Von Huckert *et al*., 2009).

Most birds infected by *S*. Enteritidis and *S*. Typhimurium don't show any clinical signs, remaining asymptomatic for long periods (Back, 2004). However, the occurrence of the clinical disease associated with mortality has been observed in birds that are submitted to stress conditions and young broiler chicks that have an immature immune system (Akthar *et al*., 2013). Studies based on experimental inoculation of these bacteria in the first week of life demonstrate that both *S*. Enteritidis and *S*. Typhimurium cause infections that can lead to the development of acute septicemia, characterized by the presence of lesions such as peritonitis, perihepatitis, onfalitis, pericarditis, tiflitis, aerossaculitis and pneumonia (Desmidt *et al*., 1997; Dhillon, *et al*., 2001; Roy *et al*., 2001). Similarly, high mortality taxes are mentioned after experimental inoculation of those serovars in one-day-old chicks (Smith and Tucker, 1980).

Information about morbidity and mortality related to *Salmonella* could be important to quickly establish the virulence of an isolate in poultry farms as well as to evaluate the possible consequences of the infection (Millemann *et al*., 2005). Therein, this study aims to evaluate the virulence of 130 strains of *S*. Enteritidis and 70 strains of *S*. Typhimurium through its inoculation in one-day-old chicks. A score was assigned for each strain according to the number of animals that died during the observation period, the presence of septicemic lesions and the course of time between inoculation and death.

MATERIAL AND METHODS

The experiment was conducted with previous approval of the Ethical Committee in Animal Usage of Desiderio's Finamor Veterinary Research Institute (DFVRI), situated in Eldorado do Sul, Rio Grande do Sul State, identified by protocol number 23/2012.

One-day-old chicks from the Cobb lineage from commercial flocks of breeding hens at 58 weeks of age were used for the experiment. The birds were kept in 32°C heated environment and housed in isolated boxes with 30cm of length x 55cm of height x 35cm of breadth. Drinkable water and antibiotic free commercial food were provided *ad libitum*.

One hundred thirty strains of *S*. Enteritidis and 70 strains of *S*. Typhimurium from avian sources stored in Brain Heart Infusion (BHI, Oxoid® CM225B) broth and 25% glycerol kept frozen at -70°C were used in the experiment. The chosen strains belong to the bacteria acquis of the Center of Diagnosis in Avian Pathology and Research (CDAPR) that is part of the Federal University of Rio Grande do Sul State and to the Health Avian Laboratory from the DFVRI. To confirm the pureness of the strains, an aliquot from each sample was grown in BHI broth overnight and plated in Xilosis-Lisine-Desoxicolato (XLD, CN 278850 Difco®) agar. After a 24h incubation period in 37°C, compatible colonies were submitted to biochemical reactions (Triple-Sugar-Iron Agar – TSI, CM0277; Sulphide-Indol-Motility Agar – SIM, CM0435; Lysine-Iron-Agar – LIA, CM0381; and urea broth,

CM0071, Oxoid®). An aliquot from the samples was also selected for the detection of the gene *inv*A through the Polymerase Chain Reaction (PCR), according to Salehi *et al.* (2005).

The strains were defrosted and cultivated overnight in BHI broth at 37°C. Posteriorly the samples were spread on XLD agar and incubated for 24h at 37°C. Three colonies from each sample were selected and removed to 10mL of BHI broth and incubated for 24 hours at 37°C. The bacterial cell concentration in each culture was estimated through optical density lecture using a spectrophotometer (SP22, Bioespectro®, Brazil) with 600nm wave-length.

Each strain was inoculated in a group of ten one-day-old chicks associated with a mock-inoculated group also formed by ten birds. The chicks were individually inoculated with 200µL of a solution containing approximately 2x 10^8 colony-forming units (CFU) of *S.* Enteritidis or *S.* Typhimurium by intraperitoneal route. The mock-inoculated group was inoculated by the same route, with 200µL of 0.85% sterile saline solution. The assay was carried out in eight sets of 250 chicks and in each set 25 strains were evaluated. After reception and accommodation in each set, ten birds were randomly chosen in order to detect the antibodies titles to *S.* Enteritidis through the ELISA test (IDDEXX SE – Laboratories, Inc. Westbrook, USA) and to detect the presence of *Salmonella* spp. from conventional bacterial isolation (Borsoi *et al.*, 2009). After having 2mL of blood collected through cardiac puncture, the chicks were euthanized through cervical disarticulation. Liver and spleen samples were analyzed through bacterial isolation in order to discard previous contamination by *Salmonella* spp.

The animals were observed every 12h for seven consecutive days and their mortality was registered. The day of death and macroscopic lesions were combined to generate a Pathogenicity Index (PI) based on the methodology developed by Souza (2006) with modifications. The birds that were found dead were necropsied and the presence/absence of the following lesions was observed: aerossaculitis (A); peritonitis (Pt); onfalitis (O); perihepatitis (Ph); pericarditis (Pc) and cellulitis (C). The values "0.833" and "0" were attributed to the presence or absence of lesions. The animals that survived until the seventh day were killed by cervical dislocation, necropsied and evaluated as explained before. The time of death (TD) corresponds to the day that the chick died, which is "1" when it happened in the first day post-inoculation (dpi) reducing 0.14 for each day that the animal survived (Table 1). According to Souza (2006), this value is called Surviving Bonus Factor (SBF).

Table 1. Time of death values assigned according to the day of death after the birds' inoculation challenge with *Salmonella* Enteritidis or *Salmonella* Typhimurium

Day of death	1	2	3	4	5	6	7	S*
TD	1	0.86	0.72	0.58	0.44	0.30	0.16	0

S* – surviving chicks on the seventh day post-inoculation.

Liver and spleen from birds that died in the first and in the seventh day post inoculation were collected and inoculated in BHI broth at 37°C for 24 hours. After that, 100µL from this solution were transferred to 10mL of Rappaport-Vassiliadis (CM866 Oxoid®) broth and incubated for another 24h at 42°C. Then, each sample was plated in XLD agar and Brilliant Green Agar (CM263B Oxoid®), supplemented with 4% novobiocin, and once again incubated for another 24h at 37°C. Two characteristic colonies were submitted to biochemical tests and to agglutination reaction with somatic polyvalent serum to *Salmonella* spp. (Borsoi *et al*., 2009).

The software GraphPad Prism® for Windows 6.3 version was used to analyze data adopting 5% significance level ($P<0.05$). The results have been submitted to non-parametric statistic tests (Mann-Whitney Test, Kruskal-Wallis, Chi-Square Test and Wilcoxon Signed Rank) because the data did not follow a normal distribution.

RESULTS AND DISCUSSION

Antibodies for *S.* Enteritidis have not been detected in the serum samples collected before the beginning of the bird challenge. Similarly, lesions have not been found on these chicks during their necropsy, and all the spleen and liver

samples were negative for the *Salmonella* spp. isolation.

The medium values of the pathogenicity index of *S.* Enteritidis and *S.* Typhimurium have not differed significantly among themselves (Table 2). However, when the strains were clustered according to the classificatory index (1 to 10) in low categories (1-4), intermediate (5-7) and high pathogenicity (8-10), it was possible to detect statistic differences between those groups (Table 3). The only exception occurred between the intermediate and low pathogenicity *S.* Typhimurium strains, which have not shown any statistic difference. It is possible that this result is a consequence of the small number of strains that have been classified in the last group.

Table 2. Average of Pathogenicity Index (PI) from *S.* Enteritidis and *S.* Typhimurium strains inoculated in one-day-old chicks

Serotype	Number of strains	Number of birds in each group	PI*
S. Enteritidis	130	1300	6.55 ± 1.97a
S. Typhimurium	70	700	7.08 ± 1.56a

*The same letters in a column mean there was nosignificant difference (P=0,082).

Table 3. *S.* Enteritidis and *S.* Typhimurium strains inoculated in one-day-old chicks classified in groups of low (L), intermediate (I) and high (H) pathogenicity

	S. Enteritidis		*S.* Typhimurium	
Group	Number of strains	PI*	Number of strain	PI*
L	24	3.46 ± 0.66c	3	4 ± 0b,c
I	59	6.01 ± 0.80b	36	6.19 ± 0.82b
H	47	8.72 ± 0.77a	31	8.55 ± 0.68a

*Different letters in the same column mean significant difference (P<0.0001).

The grouping of strains in different categories according to the PI demonstrates that there was a variation in their virulence after the intraperitoneal route inoculation. Perhaps these variations are related to the capability that each strain has to survive and replicate in the host tissues, particularly inside the phagocytic lineage cells (Barrow *et al.*, 1987; Thompson *et al.*, 2011). According to Barrow *et al.* (1994), after intravenous inoculation the primary location of the microorganisms has been observed in organs from the reticuloendothelial system. In these organs, the bacterial multiplication is controversial. However, the macrophages have been traditionally pointed as the main cellular target in the host (Berchieri *et al.*, 2001). This possibility has been suggested by Barrow *et al.* (1987), who determined the virulence of *S.* Typhimurium in young birds isolating the bacteria mainly from reticuloendothelial organs just after the infection.

After being captured by phagocytic cells, *Salmonella* inhibits the processing and presentation of antigens. In addition, *Salmonella* produces enzymes that inactivate reactive oxygen and nitrogen species (Ochoa and Rodríguez, 2005). Those mechanisms are induced by multifactorial virulence systems encoded by genes that are present at pathogenicity islands (Osman *et al.,* 2010; Thompson *et al.*, 2011). Therefore, the phenotypic changes in *S.* Enteritidis and *S.* Typhimurium samples tested in this study might be the result of diversity in its genotypes.

Gast and Beard (1989) suggest that the systemic disease development requires more than the simple passage of the microorganism through the intestinal barrier, since a high percentage of *S.* Typhimurium re-isolation was obtained from the spleen and the liver of the inoculated birds, even from the groups with low or no mortality. Possibly, some virulence factors act after the invasion interacting with the organism defenses to determine the infection course and the taxes of elimination of the pathogen from internal organs (Gast and Beard, 1989; Porter and Curtis III, 1997).

Figure 1 presents the lesion frequency that has been observed after the inoculation of *S.* Enteritidis and *S.* Typhimurium in one-day-old chicks. Between the evaluated lesions,

aerossaculitis, peritonitis and onfalitis were the most common occurrences in both studied serotypes. These findings are similar to the ones described by many researches after experimental infection with *S.* Enteritidis (Desmidt *et al*., 1997; Dhillon *et al*., 1999; Alisantosa *et al*., 2000; Dhillon *et al*., 2001; Akthar *et al*., 2011; Akthar *et al*., 2013) and *S.* Typhimurium (Roy *et al*., 2001) in Specific Pathogens Free (SPF) birds between one and twenty-eight days.

On the other hand, the lesion with lowest frequency between the *S.* Typhimurium strains was pericarditis. Similarly Dhillon *et al.* (2001) describes that pericarditis was not common in birds that were found dead after the inoculation with different fagotypes of *S.* Enteritidis. Nevertheless, this finding differs from the ones observed by Roy and contributors (2001) who describe the presence of fibrinous exudate in the pericardium as one of the main macroscopic lesions observed in SPF chicks after oral inoculation of 10^7 CFU of *S.* Typhimurium.

Regarding *S.* Enteritidis strains, cellulitis was the less common lesion. This result agrees with what was expected, since bacteria from the *Salmonella* spp. genus have not been described in literature as an etiologic agent of this pathology under natural conditions (Fallavena, 2009). According to Norton *et al.* (1999), it is indispensable that the skin has injuries for the occurrence of avian cellulitis. Therefore, the occurrence of these lesions in the present study might be related to the inoculation route that was used, which permitted the pathogen to invade and proliferate in the subcutaneous tissue with the production of fibrinous-purulent plaques that were observed.

When the lesions were analyzed individually, no significant differences were found in the median values obtained for both *S.* Enteritidis and *S.* Typhimurium strains (P>0.05). Based on this, it is suggested that both serotypes have the potential to cause septicemic lesions such as the ones caused by the avian specific serotypes. This hypothesis is suggested by other authors who have found the same macroscopic alterations after experimental inoculation with *S.* Enteritidis, *S.* Typhimurium, and *S.* Pullorum (Alisantosa *et al*., 2000; Dhillon *et al*., 2001; Roy *et al*., 2001).

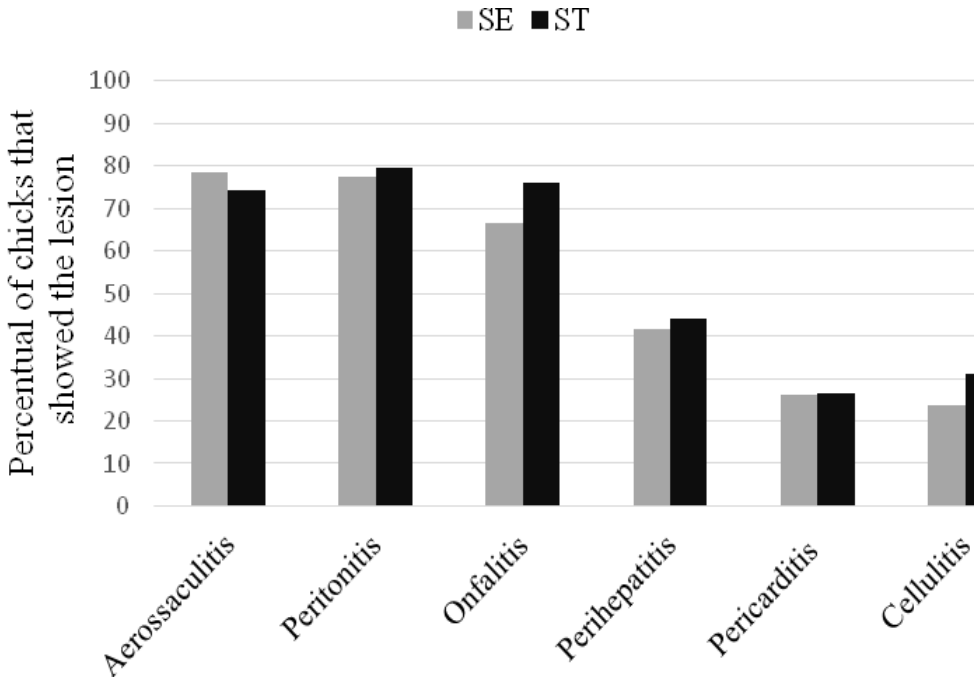

Figure 1. Lesion frequency presented by *S.* Enteritidis and *S.* Typhimurium strains after inoculation in one-day-old chicks.

Although the medium mortality percentage was bigger in the *S.* Typhimurium isolates (74.9%) than in the *S.* Enteritidis ones (69.5%), there was no significant statistical difference between them (P>0.05). Huge variation in mortality taxes induced by *S.* Enteritidis and *S.* Typhimurium have been reported after experimental inoculation in one-day-old chicks. Roy *et al.* (2001) demonstrate a mortality percentage of 33.33% for *S.* Typhimurium, 12.5% for *S.* Enteritidis phage type (PT) 4 and 8.5% for PT 8. Osman and contributors (2010) have also observed highest mortality caused by the same serovar, but with higher percentage values: 88% for *S.* Typhimurium and 72% for the *S.* Enteritidis isolates. On the other hand, Milleman *et al.* (2005) reported mortality between 0 and 36% for *S.* Typhimurium strains and between 6 and 17% for *S.* Enteritidis, demonstrating that mortality is not associated only with the serovar. Based on this information and on the results that have been obtained in this study, it is suggested that virulence may not be determined only by *Salmonella's* spp. serotype or phage type, but specially by individual characteristics of each strain (Smith and Tucker, 1980). Moreover, the severity or absence of clinical signs are related to many factors such as dose, inoculation route, age, lineage and immune state of the host (Gast and Beard, 1989; Gorham *et al.*, 1991; Desmidt *et al.*, 1997; Akthar *et al.*, 2011; Akthar *et al.*, 2013).

In the present work, *Salmonella* spp. was recovered from all samples collected from the birds that died in the first 24h post-inoculation, wherein a significant reduction was found in the re-isolation rates in bacteria collected from chicks that died in the seventh day post-inoculation (Table 4). Similarly, Gorham *et al.* (1991) have reported the recovery of *S.* Enteritidis from all the tissues collected (lungs, liver, spleen, reproductive organs and brain) from birds that died during the experiment. However, the percentage of re-isolation in animals that were euthanized in the seventh day post-inoculation varied between 60% and 80%. The reason why there is a lower *Salmonella* persistence in the surviving animals is not clear. After evaluating the role of the immune humoral system in the *S.* Enteritidis fagotype 4 infection, Desmidt *et al.* (1998) have observed a lower quantity of the pathogen present in the bursectomized birds during the first weeks after inoculation, despite the lack of antibodies. According to these authors, this conclusion indicates that other important mechanisms are involved in *Salmonella* suppression, as the immunity mediated by cells. Beal *et al.* (2006) obtained similar results after challenging bursectomized birds with *S.* Typhimurium, from what they concluded that cells B and antibodies are not required for the elimination of *Salmonella* in primary infections. According to Bumstead and Barrow (1993) the main responsible for bird's resistance against salmonellosis is the phagocytic system's ability to contain the disease in the initial phase.

Table 4. Re-isolation percentage of *S.* Enteritidis and *S.* Typhimurium observed in the first and in the seventh day post-inoculation in broiler chicks

	Recovery of *Salmonella* from liver and spleen	
DPI	*S.* Enteritidis**	*S.* Typhimurium
1	100(518/518)a	100(313/313)a
7*	94.9(376/396)b	90.3(158/175)b

* Surviving chicks on the seventh day post-inoculation.
** Values followed by different letters mean a significant difference at5% level of significance.

CONCLUSIONS

The utilization of standards such as time of death and presence of septicemic lesions after the inoculation of different isolates from *S.* Enteritidis and *S.* Typhimurium in one-day-old chicks allowed the determination of a pathogenicity score for each strain. Besides that, the proposed model has presented significant differences between the high, intermediate and low pathogenicity groups, allowing the use of this mechanism for further classification of strains isolated in poultry farms.

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