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Dissemination of Salmonella Enteritidis by Experimentally-Infected Pigeons

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ABSTRACT

Two groups of domestic pigeons (*Columba livia*) were experimentally infected orally with doses of 9.5 x10^7 and 9.5 x10^9 CFU/mL (group A and B, respectively) of a *Salmonella* Enteritidis (SE) strain isolated from chickens. None of the used doses caused mortality of the inoculated birds; however, the pathogen was successfully recovered from the liver and spleen of group B birds on day 7 post-inoculation (dpi). Pathogen shedding, as evaluated through cloacal swabs, occurred in both groups until the 14th day of observation ($p < 0.05$). Among all fecal samples collected from group B (n=4), three different birds shed the pathogen in their feces, out of which two were positive on 3 dpi and one on 7 dpi. The same number of fecal samples was evaluated in group A and only one bird shed the pathogen, on 7 and 14 dpi. The concentration of the microorganism in the feces was lower in group A than any sample from Group B. *Salmonella* Enteritidis isolated from chickens, when inoculated in pigeons, may be recovered from feces, cloacal swabs and organs, and these birds may contaminate poultry causing economic losses as well as posing a risk to the public health.

INTRODUCTION

The domestic pigeon (*Columba livia*) belongs to the order of Columbiformes and Columbidae family (Harrison, 1994). This bird rapidly reproduces in environments that offer sufficient shelter and large quantities of food available, but also lack of predators (Nunes, 2001). The domestic pigeon has a varied diet, which favors its multiplication, resulting in environmental disturbance where they inhabit due to the accumulation of feathers, feces and nest remains , leading to blockages of gutters or storm drain pipes (Nunes, 2001).

Concerning the public health, pigeons play an important role in the transmission of diseases that affect humans and domestic animals, such as toxoplasmosis (Karatepe *et al.* 2011), Newcastle disease (Alexander, 1985), aspergillosis (Pal, 1991) and salmonellosis (Sousa *et al.* 2010). According to Tauxe (1991), birds are considered the main source of salmonellosis dissemination in humans. Several authors have researched and isolated *Salmonella* spp. in the feces, cloacal swabs and organs of pigeons (Casanovas *et al.* 1995; Adesiyun *et al.* 1998; Toro *et al.* 1999; Passamonti *et al.* 2000; González-Acuña *et al.* 2007 and Sousa *et al*. 2010). *Salmonella* Enteritidis has been considered the serovar responsible for the largest number of outbreaks in humans and birds due to its wide distribution in nature, while the intestinal tract of animals are the main natural reservoir of this pathogen. Birds have a particularly important role in the spread of bacteria and may act as asymptomatic carriers, shedding the pathogen continuously in their feces (Franco, 2002).

The mechanism by which the infection of *Salmonella* spp. develops in Columbidae birds is still poorly understood, especially regarding the *Salmonella* Enteritidis. Therefore this study aimed at evaluating the route and duration of shedding in the environment of *Salmonella enterica* serovar Enteritidis by its inoculation in domestic pigeons (*Columba livia*). The study was approved by the Ethics Committee for Animal Use in the registry number 10244384-0/22 (CEUA-UECE).

MATERIAL AND METHODS

Experimental Groups

In this experiment, 56 domestic pigeons (*Columba livia*) were obtained from the Laboratory of Ornithological Studies (LABEO). A fourth generation of pigeons in captivity, between six and twelve months of age was selected to the study. Pigeons were housed in pairs per cage and separated into two groups, which orally received two different inoculum concentrations of *Salmonella* Enteritidis: Group A (lower dose) and Group B (higher dose). The following variables were analyzed: SE concentration in the feces and in organs, frequency of positive swabs, organ and fecal samples. The inoculation and evaluation of the concentration of *Salmonella* Enteritidis in organs and feces was performed according to the methodology adopted by Oliveira *et al.* (2005).

Inoculum Preparation

A broth containing a *Salmonella* Enteritidis strain resistant to nalidixic acid (SENal^r) was incubated overnight at a temperature of 37^{o}C, Birds in group A received 1 mL of the inoculum obtained from the culture SENal^r containing approximately 9.5x10^7 colony forming units/mL (CFU/ mL), whereas for group B, the culture was diluted by a factor of 10^{-3}, containing approximately 9.5 x 10^9 CFU/mL.

Microbiological Evaluations

On the 3, 7, 14, 21, 28, and 35 day post-inoculation (dpi), fecal samples were collected from each cage, and four birds from each group were euthanized for spleen and liver collection for analysis of CFU and frequency of positive SENal^r.

In the first step, the organs were individually homogenized and diluted in decimal series (1:10) in Phosphate Buffered Saline solution (PBS). The dilutions were plated on brilliant green agar (Oxoid CM265), containing nalidixic acid (25 µg/mL) and novobiocin (2 µg/mL) (BGNNov). The plates were incubated overnight at 37°C and the CFU/mL were transformed into $\log_{10}$ for analysis. In the second step, a part from the remaining macerated organ samples was removed and incubated in selenite broth (CM395 Oxoid.) with novobiocin (40 mg/L) overnight at 37°C, and then were plated on BGNNov and incubated at 37°C for 24 hours to enhance the growth of any potential SE cells in the organs, when CFU counting was not possible. The methodology used for the fecal samples was the same as the mentioned in the first stage of organ samples. Also, two cloacal swab samples were collected per week, and the methodology applied in the second stage of organ samples was used. Before starting the experiment, all birds were inspected according to Zancan *et al.*(2000) to confirm that they were free of *Salmonella* spp.

Statistical Analysis

The results of positive swab and fecal frequencies were compared using the $\chi 2$ test at a significance level of $p < 0.05$.

RESULTS AND DISCUSSION

During the experiment, there was no mortality. The concentration of bacteria used in both inocula may have been insufficient to cause bird death (Rocha e Silva *et al.*, 2013). However, the absence of mortality may be expected as literature often demonstrates that this serotype may infect the bird, but do not cause any visible clinical signs (Hogue *et al.*, 1997). The infected birds may act as carriers of the disease, infecting other birds (Van Immerseel *et al.*, 2004). Therefore, the age of the studied pigeons, which were six months or older, may be the most plausible explanation of the absence of mortality.

Studies performed with poultry show that infections by *Salmonella* Enteritidis of birds with more than one month of age rarely result in mortality (Suzuki, 1994). Ishola (2009) infected two groups of adult hens (33 wk) with different doses (1.3x10^4 and 1.3x10^8 CFU) of a SE strain previously isolated from chickens and did not report any mortality. The age-dependent immunity may favor the development of the immune system and the production of antibodies (Desmidt *et al.* 1998; Beal et al. 2004). In chickens, the susceptibility to the intestinal colonization by *Salmonella* spp. is higher during the first days of life, and reduces as the local intestinal microbiota develops (Nurmi & Rantala, 1973; Pivnick *et al.* 1982). Adult birds are relatively resistant

to the systemic multiplication of SE, but may suffer colonization of the intestinal tract in the absence of clinical manifestations and remain as carriers, shedding the microorganism intermittently (Gast, 2003). Differently to what is observed in adult birds, SE is capable of causing high mortality in young birds. Gorham *et al.* (1991) inoculated in newly-hatched chicks ($1x10^7$ CFU) a SE strain isolated from a poultry-rearing environment and verified that during seven days the mortality rate reached 21% (11/53).

When the concentration of SENal^r was evaluated in the collected organs, no bacterial growth was detected. However, SENal^r was successfully recovered from the organs submitted to enrichment in Selenite-cystine broth followed by plating on BGN, which shows that the liver and spleen were invaded by the bacterium, albeit in a small number (Table 1).

Table 1 – Viable number (log_{10}) of *Salmonella* Enteritidis per gram of samples after inoculation

	3 days		7 days		14 days		21 days		28 days		35 days	
Pigeon Nº	L	S	L	S	L	S	L	S	L	S	L	S
1	-	-	-	-	-	-	-	-	-	-	-	-
2	-	-	-	-	-	-	-	-	-	-	-	-
3	-	-	-	-	-	-	-	-	-	-	-	-
4	-	-	-	-	-	-	-	-	-	-	-	-
5	-	-	+	-	-	-	-	-	-	-	-	-
6	-	-	-	-	-	-	-	-	-	-	-	-
7	-	-	+	+	-	-	-	-	-	-	-	-
8	-	-	+	+	-	-	-	-	-	-	-	-

L: liver, S: Spleen;

Group A: Birds 1-4: inoculated with 1mL of diluted broth overnight (9.5 x 107 CFU/mL);

Group B: birds 5-8: inoculated with 1mL of diluted broth overnight (9.5 x 109 CFU/mL);

+/-: Positive/negative after incubation in selenite broth.

Relative to the time required for the pathogen to successfully colonize the spleen and liver of pigeons, our results show that the recovery was only possible on 7 dpi. Gast *et al.* (2011) reported that both the frequency of microbial invasion and the recovery of the number of pathogen cells in the liver can vary significantly depending on the exposure dose administered. Oliveira *et al.* (2005) showed that in hens orally inoculated with a lower dose of *Salmonella* (8.5×10^5 CFU/mL), the pathogen was recovered in the liver and the spleen only on 7 dpi, while in birds inoculated with the highest concentration (8.5×10^8 CFU/mL), it was recovered already did on 3 dpi. This indicates that both concentrations used in the present experiment may not have been sufficient to promote invasion of the liver and the spleen by the pathogen already on 3 dpi.

Although scientific literature demonstrates that infectious bacterial load and bird age are important factors and may justify the absence of mortality or of the dissemination of the pathogen in the organism, other factors, such as genes associated to virulence of some *Salmonella* Enteritidis strains, anatomic differences between pigeons and other bird species, and immune status must be considered. The ability of *Salmonella* serotypes to cause systemic or localized infections or even to establish a carrier state is associated with host immunity and virulence-associated genes present in the bacteria (Cunningham-Rundles, 2004). Considering the anatomical aspects, differently from poultry, pigeons present rudimentary ceca. This may affect the presence of *Salmonella* in the organism because the predominant colonization site of salmonella is the ceca (Desmidt *et al.*, 1997). Therefore, Gerlach (1994) asserts that birds that have rudimentary ceca or no ceca, such as psittacines, seem to be more susceptible to *Salmonella* sp. infection when compared with birds that have fully functional ceca. That may be explained by the presence of Gram-negative autochthonous anaerobic microorganisms present in the cecal flora, such as *Bacteroides* sp. and *Spherophorus* sp., which may function as natural antagonists of *Salmonella* sp. (Gerlach, 1994; Ritchie, 1994).

The results of the analysis of the frequency of cloacal swabs collected from both groups of pigeons showed that pathogen shedding occurred up to 14 dpi in both groups, and that the frequency of bacteria shedding in group B (9.5×10^9 CFU/mL) was significantly higher ($p < 0.05$) when compared with group A (9.5×10^7 CFU/mL) only on 3 and 7 dpi (Table 2).

Table 2 – Absolute (n) and relative (%) frequencies of swabs positive for *Salmonella* Enteritidis during the post-inoculation period

	Dose/CFU/mL 9.5×10^7		Dose/CFU/mL 9.5×10^9	
	N	(%)	N	(%)
3 dpi	2/24 [a]	8.3	8/24 [b]	33.3
5 dpi	2/20[a]	10.0	8/20 [b]	40.0
7 dpi	5/20	25	10/20	50.0
14 dpi	2/16	12.5	1/16	6.3
21 dpi	0/8	0	0/8	0
28 dpi	0/8	0	0/8	0
35 dpi	0/4	0	0/4	0

The absence of major findings with swabs in this study appears to be due to false negatives. Literature results with paratyphoid infections suggest this possibility, such as in Gast (2003), who worked with chickens and reported that, in sub-clinical infections,

infected chickens continued harboring the bacterium in their bodies and shedding it in feces for a variable period of time. The use of a strain that is not adapted to pigeons also seems to have influenced the low frequency of *Salmonella* in samples obtained from cloacal swabs. The use of strains adapted to the species under study can result in higher rates of fecal excretion. Ishola (2009) inoculated adapted strains in laying hens (10^{-8} CFU) and found that the highest frequency of positive results for *Salmonella* in feces within five weeks of experimental evaluation was 100% and the lowest was 40%. This demands greater efficacy of *Salmonella* control programs, not only by adopting the method of cloacal swab incubation as a determinant, but also by increasing the amount and the frequency of sample collection in order to know the real status of the birds (Muir *et al.,* 1998).

Relative to the frequency of SENal^r fecal shedding, the group of pigeons inoculated with the lower concentration started shedding only on 7 dpi ($1.5x10^4$), and continued until 14 dpi ($2x10^5$). Among the pigeons inoculated with the higher concentration of SENal^r, birds from two cages started shedding on 3 dpi ($1.9 x10^7$ and $2x10^9$) and from one cage on 7 dpi ($1x10^7$), but no cells were counted on the other days of observation (Table 3).

Table 3 – Recovery of *Salmonella* Enteritidis in feces of pigeons post-inoculation (CFU/g)

	3 dpi	7 dpi	14 dpi	21 dpi	28 dpi	35 dpi
Cages	CFU	CFU	CFU	CFU	CFU	CFU
2	0	0	0	0	0	0
5	0	0	0	0	0	0
7	0	0	0	0	0	0
10	0	$1.5x10^4$	$2x10^5$	0	0	0
16	0	0	0	0	0	0
18	0	$1x10^7$	0	0	0	0
19	$1.9x10^5$	0	0	0	0	0
23	$2x10^9$	0	0	0	0	0

Group A: cages 2, 5, 7 and 10: pigeons inoculated with 1mL of a diluted broth (9.5 x 107 CFU/mL); Group B: cages 16, 18, 19 and 23: pigeons inoculated with 1mL of diluted broth (9.5 x 109 CFU/mL)

When a microorganism is detected in the feces, the colonization of a part or all of the digestive tract may be implied. Barrow *et al.* (1988) said that the colonization of bacteria in the digestive tract can be observed through the excretion in feces and the detection of the pathogen is carried out by incubation of feces or cloacal swabs.

Since the strain used in this experiment is not adapted to the inoculated species, the doses administered in this study do not seem to be sufficient to generate a greater systemic dissemination, as no bird presented any significant quantity or frequency of the bacterium, considering all the pigeons assessed. Gast (2003) stated that inoculated chickens and turkeys can shed paratyphoid *Salmonella* spp. during the first two weeks after experimental oral infection and that the bacterium can be isolated from the intestinal tract; however, there may be a high percentage of non-contaminated feces.

The short period of fecal pathogen shedding presented by the pigeons maybe explained by the absence or temporary colonization of the intestinal tract by the the pathogen (Barrow *et al.* 1988). Another hypothesis for the decline in the frequency of SENal^r fecal shedding is that the pigeons may have been able to reduce the level of systemic infection probably by humoral immune response (Hassan *et al.* 1991; Muir *et al.* 1998). However, even with low quantities of contaminated feces, González-Acunã *et al.* (2007) argued that both dried and fresh pigeon feces pose a high risk of *Salmonella* transmission to humans, particularly children, the elderly, or immune-compromised individuals, as well as domestic animals.

The doses administrated to pigeons, although relatively low considering the results found in the organs, cloacal swabs and feces until 35 dpi, show that these birds can excrete *Salmonella* Enteritidis strains that not adapted to their species. Because pigeons have access to rural and urban environments, they may enter commercial poultry houses, defecate on the litter and cause major economic losses and public health issues. Experiments with chickens on the litter showed that reinfection may play an important role in the persistence of the infection (Gast, 2000; Beal *et al.* 2004).

In conclusion, this study shows that *Salmonella* Enteritidis isolated from chickens, when inoculated in pigeons, may be recovered from feces, cloacal swabs and organs. This demonstrates that pigeons may play a role in the dissemination of this pathogen, because they may shed it when defecating on the litter in commercial poultry houses or in feed production facilities, causing economic losses as well as posing a risk to public health.

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