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Experimental Infection by *Salmonella enterica* Subsp *Enterica* serovar Kottbus in Day-Old Broiler Chickens

■ **Author(s)**

Ribeiro SAM[1,3]
Berchieri Jr A[1]
Orsi MA[2]
Mendonça AO[2]
Ferrati AR[3]

[1] UNESP (Universidade Estadual Paulista), Faculdade de Ciências Agrárias e Veterinárias – Departamento de Patologia Animal, Campus de Jaboticabal.
[2] LANAGRO/SP - MAPA (Ministério da Agricultura, Pecuária e Abastecimento)
[3] LANAGRO/SP - FACTA (Fundação APINCO de Ciência e Tecnologia Avícolas)

■ **Mail Address**

Simone Alves Mendes Ribeiro
R. Raul Ferrari, s/n
Caixa Postal 5538
13.091-907. Campinas, SP, Brazil
Phone: +55 +19 3252 0155 extension 193

E-mail: simone_ribeiro2002@yahoo.com.br

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ABSTRACT

The strain used in this work was a *Salmonella enterica* subsp *enterica* serovar Kottbus (6,8:e,h:1,5) isolated from imported day-old ducklings in Laboratório Nacional Agropecuário (LANAGRO/SP) of the Ministry of Agriculture of Brazil (MAPA). In view of the lack of information available about this *Salmonella* isolate and also because it was detected in day-old imported birds, this study was carried out to investigate the dissemination of *S.* Kottbus among newly hatched chicks. The birds were placed in three groups: one group of 20 birds received 0.1 mL of *S.* Kottbus culture containing 1.2 x 10^8 CFU/mL, the second group of 20 birds was inoculated with 1.2 x 10^5 CFU/mL and the third group of 10 birds was untreated (control group). Results were similar for both infected groups. The bacterium was recovered from cloacal swabs collected from the first day following the experimental infection until the end of the trial (42 days post-inoculation). At 15 and 42 days post-inoculation (dpi), half of the birds of each group were killed for bacteriological examination of cecal contents, liver and spleen. At 15 dpi, viable cell counts of *S.* Kottbus were obtained in all kinds of samples. At 42 dpi, *Salmonella* was present in the liver and spleen of few birds, but in large amounts in the cecal contents of almost all birds.

INTRODUCTION

Large scale production of poultry meat and egg has demanded the control of Pullorum disease and Fowl typhoid in the modern poultry industry. However, commercial poultry are susceptible to infections caused by other *Salmonella* serotypes that may cause clinical disease (Berchieri Jr. *et al.*, 2000). Nevertheless, infections caused by such *Salmonella* serotypes are usually asymptomatic; the pathogens are transmitted vertically and are excreted with the feces. In addition, these serotypes may be associated with human foodborne salmonellosis (OFFICE INTERNATIONAL DES EPIZOOTIES, 2000; Forsythe *et al.*, 1967).

Once infectious pathogens are introduced in a poultry flock, they might spread rapidly. *Salmonella* Enteritidis has been focused by surveillance programs in animal farms since it was responsible for several outbreaks of human foodborne salmonellosis related to food prepared with poultry and eggs (Gast *et al.*, 1998; Gast & Beard, 1992; Lirio *et al.*, 1998; Okamura *et al.*, 2001; Peresi *et al.*, 1998). It is supposed that *Salmonella* Enteritidis reached poultry breeder farms through rodents and was vertically transmitted to the progeny. Eventually, it reached commercial flocks of broilers and laying hens and was spread worldwide. However, there are many other *Salmonella* serotypes that cause currently enteric human salmonellosis, such as *Salmonella* Infantis, *S.* Senftenberg, *S.* Anatum, *S.* Agona, *S.* Heidelberg and the always-important *Salmonella* Typhimurium (Clark & Thatcher, 1973; Okamura

et al., 2001; Parimal *et al.,* 2001; Taunay *et al.*, 1996; Tavechio *et al.*, 1996; Tavechio *et al.*, 2002). Based on the episode caused by *Salmonella* Enteritidis, the Brazilian Government has established that imported birds must be free of *Salmonella* serotypes Gallinarum, Pullorum, Enteritidis and Typhimurium (BRASIL, 1999). However, the surveillance plan does not consider other serotypes of *Salmonella*, which allowed them to be introduced in the country and flocks containing day-old infected birds were contaminated during rearing (Gama *et al.*, 2003).

Recently, *Salmonella* Kottbus was isolated in Brazil from imported day-old ducklings (Galletti *et al.*, 1999). This serotype had been previously isolated from other imported flocks (Ribeiro *et al.*, 2004) and no prevention measures were taken. *Salmonella* Kottbus has been implicated in several cases of salmonellosis. Over a period of 11 years, 5.7% of 245 hospitalized horses were infected by *S.* Kottbus, and 21.4% of these died due to the bacterium. Sick animals excreted fluid feces with large amounts of bacteria and contaminated the environment, other animals and human beings (Carter *et al.*, 1986).

It has been reported that 4.29% of carcasses and organs of healthy pigs were contaminated by *Salmonella*, and *S.* Kottbus was one of the isolated serotypes. In regard to environmental contamination, *Salmonella* was isolated from adult muscoid flies collected from commercial poultry farms, including *Salmonella* Kottbus (Mian *et al.,* 2002). According to Hoszowski & Wasyl, 2002, the majority of *Salmonella* strains isolated in veterinary laboratories in Poland in 2001 were recovered from poultry; and *S.* Kottbus was isolated from ducks. *Salmonella* Kottbus was isolated from lambs in a group of 200 animals, from which 16 died over a period of 3 days (VETERINARY LABORATORIES AGENCY, 2003).

According to the Information System of Public Health Laboratory in USA (1968-1998), the mean number of cases of human salmonellosis was 43 per year and in 2001 *Salmonella* Kottbus infection was observed in 23 people who had eaten alfafa sprouts (CENTER OF DISEASE CONTROL AND PREVENTION, 2002). In Lybia, a multi-drug resistant strain of *S.* Kottbus was among 21 *Salmonella* strains isolated from 16 children with diarrhea (El_Ghodban *et al.,* 2002). According to Nelius *et al.* (1969), most *Salmonella* infections remain as enteric diseases, however, *S.* Kottbus may cause septicemia that is often related to immunodeficiency. Human salmonellosis is one of the most important foodborne diseases, and foods containing poultry meat and eggs are usually involved in outbreaks (Jakabi *et al.,* 1999).

Since *Salmonella* serotypes may be introduced by vertical transmission through imported birds and some of these serotypes are not compulsorily investigated according to the surveillance plan adopted by the poultry breeding industry, this work was carried out to study the pathogenicity of a *Salmonella enterica* subsp *enterica* serovar Kottbus strain in commercial broiler chicks reared from one to 42 days of age.

MATERIAL AND METHODS

Salmonella strain

It was used a *Salmonella enterica* subsp *enterica* serovar Kottbus (6,8:e,h:1,5) strain that had been isolated from imported day-old ducklings and was resistant to nalidixic acid and novobiocin (Galletti *et al.*, 1999). The culture of *S.* Kottbus was prepared in 5 mL of nutrient broth (Oxoid, CM225), and incubated at 37°C/24h in a shaking water bath (100 rpm).

Experimental infections

Fifty newly hatched chicks from a broiler breeder flock were hatched at Laboratório Nacional Agropecuário (LANAGRO/SP) to be used in the experiment. Ten of these were sacrificed by cervical dislocation for bacteriological examination of the liver, spleen, egg yolk and cecal contents. The remaining birds were separated in three groups: one group of 20 birds received 0.1 mL of S. Kottbus culture containing 1.2×10^8 CFU/mL, the second group of 20 birds was inoculated with 1.2×10^5 CFU/mL and the third group of 10 birds was untreated (control group). Infected birds were housed in isolation units in a Biosafety Level 2 research facility and given water and antibiotic-free diet ad libitum. All birds were evaluated daily for clinical signs of disease and mortality. Dead chickens were removed from the isolation units, necropsied and examined for the presence of *Salmonella*.

Fifteen and 42 days post-infection, half of the birds were sacrificed by cervical dislocation and necropsied. Liver, spleen and cecal contents were collected to determine the number of *Salmonella* using Brilliant Green Agar plates containing 25 µg/mL nalidixic acid and 40 µg/mL novobiocin (Merck 7232) (VBNalNov), as described by Smith *et al.* (1980) (Table 1).

Cloacal swabs were taken at 24hs, 3 days, 7 days and then weekly after the infection, until the birds were 42 days old. Swabs were streaked directly onto VBNalNov plates and then kept in a tube containing 2

Table 1 - Recovery of Salmonella Kottbus in liver, spleen and cecal contents of chickens after infection. (log_{10}/g).

Bird #	*Salmonella* Kottbus inoculum											
	1.2 x 10^8 (CFU/mL)						1.2 x 10^5 (CFU/mL)					
	15 dpi			42 dpi			15 dpi			42 dpi		
	L	S	C	L	S	C	L	S	C	L	S	C
01	3.78	0	7.00	0	0	6.90	0	0	8.30	0	0	0
02	4.30	3.78	7.70	0	0	7.00	0	<1	7.30	0	0	6.30
03	3.30	3.30	<1	0	0	6.30	5.30	0	6.30	<1	0	4.00
04	<1	<1	7.70	0	0	6.60	<1	0	7.60	0	<1	4.30
05	<1	<1	7.90	0	0	6.00	<1	0	7.30	<1	0	5.48
06	3.30	<1	6.60	0	0	7.30	0	0	8.30	<1	<1	4.60
07	3.60	3.30	7.70	0	0	6.30	<1	<1	8.00	0	0	6.30
08	4.60	4.90	7.60	0	2.48	6.30	<1	0	8.60	0	<1	7.30
09	3.78	3.60	7.60	0	0	<1	<1	<1	7.95	ND	ND	ND
10	4.90	4.30	7.78	ND	ND	ND	ND	ND	ND	ND	ND	ND

L= liver; S= spleen; C=cecal contents; ND= not done (mortality in the first week).

mL Selenite Broth (Merck 107717). Both plates and tubes were incubated at 37ºC/24h. In case there was no growth, the swab was plated again onto VBNalNov and incubated at 37ºC/24h (Barrow *et al.*, 1988).

RESULTS

No *Salmonella* was recovered from the 10 newly-hatched birds that were evaluated.

Although four birds died in the first week after oral inoculation with *S.* Kottbus, there were no clinical signs of disease throughout the experiment.

Table 1 presents the viable counts of *Salmonella* in birds sacrificed 15 and 42 days post inoculation (dpi). Higher numbers of S. Kottbus were recovered in the groups inoculated with culture of *S.* Kottbus containing 1.2 x 10^8 CFU/mL. In both groups, isolation from liver and spleen decreased with time, but remained high in cecal contents.

Isolation of *Salmonella* Kottbus from cloacal swabs using either direct plating (D) or plating after enrichment (T) is shown in Table 2. Organisms were detected in the feces from 24 h post-inoculation until 42 dpi.

DISCUSSION

Salmonellosis is still nowadays the main foodborne disease in humans. Modern practices in the poultry industry are even now very favorable to the maintenance and dissemination of *Salmonella* serotypes. The pathogenicity of many serotypes in chickens, including *S.* Kottbus, remains unknown. The host-parasite relationship between *Salmonella* and chicken can be assessed following oral inoculation of day-old birds (Smith & Trucker, 1980; Gast & Beard, 1992; Gast *et al.*, 1998). In the present study, day-old broiler chicks were orally infected with *Salmonella* Kottbus to assess fecal shedding until 42 days of age and to examine the liver, spleen and cecal contents at 15 and 42 days of age.

Table 2 - *Salmonella* Kottbus isolated from the cloaca after infection.

Time (pi)	*Salmonella* Kottbus					
	1.2 x 10^8 CFU/mL			1,2 x 10^5 CFU/mL		
	D	E	T	D	E	T
24 h	03	04	07/20	02	03	05/20
03d	06	03	09/20	05	04	09/18*
07d	16	01	17/19*	06	02	08/17*
14d	10	07	17/19	13	03	16/17
21d	05	04	09/09**	06	02	08/08**
28d	09	00	09/09	04	02	06/08
35d	08	01	09/09	06	01	07/08
42d	04	01	05/09	03	04	07/08

D = *S.* Kottbus isolated by direct plating on VBNalNov agar; E = *S.* Kottbus isolated after enrichment in selenite broth; T = D + E / total number of examined birds;* = total number reduced due to mortality; ** = total number reduced due to necropsy. pi = post-inoculation; d = days post-inoculation; h = hours post-inoculation

The results of oral inoculation in day-old commercial broiler chicks demonstrated that *S.* Kottbus colonized the liver, spleen and ceca at diverse intensity. It was recovered from these organs and persisted in the intestinal tract resulting in cecal colonization. Pathogenesis studies associated with virulent strains suggested that organisms multiply in the liver and spleen after invasion and then disseminate to other organs, producing a systemic infection (Barrow *et al.*, 1987). Gast & Holt (1998) recovered *S.* Enteritidis from the liver and spleen at seven dpi and in the ceca 24 weeks post-inoculation. Similar situations have been reported in chicks experimentally inoculated with other *Salmonella* serotypes (Smith & Turker, 1980; Barrow *et al.*, 1987; Gast & Beard, 1992; Parimal *et al.*, 2001).

In the present study, birds showed no signs of disease caused by *S.* Kottbus, but there was

colonization of the cecum and shedding of the pathogen in the feces. *S.* Kottbus was detected in the feces since 24 h post-infection until 42 dpi, similarly to other serotypes of public health concern, including *Salmonella* Typhimurium and *Salmonella* Enteritidis (Barrow *et al.*, 1988; Gast & Holt, 1998; Smith & Tucker, 1980). In the alimentary tract, the main site of colonization is the cecum in both young and old chickens, but persistence is greater in younger birds. This has been previously reported (Smith & Turcker, 1980) and may be due to the fact that the gut flora in newly hatched chicks is simpler than in older birds (Barrow *et al.*, 1988). Since this serotype persists for a long time in birds, they may be contaminated until slaughter age. The stress to which infected birds are subjected during transportation to the abattoir enhances *Salmonella* shedding in the feces (Smith *et al.*, 1980). Consequently, bacteria numbers in the slaughterhouse are also increased, which makes it harder to obtain *Salmonella*-free carcasses (Boes *et al.*, 2001).

According to Jakabi *et al.* (1999), food prepared using poultry meat, eggs and egg products have been the most common source of *Salmonella* infections to humans. Synnot *et al.* (1998) reported human foodborne salmonellosis due to *Salmonella* Agona present in turkey meat. The authors also said that the disease was controlled after the etiologic agent had been investigated and the government had established a plan, including a monitoring program and general measures of hygiene and disinfection. This report should be considered as an alert to the programs adopted in the breeding poultry industry, i.e., control directed to only a few serotypes of *Salmonella* may not protect commercial poultry farms from the presence of *Salmonella* in their flocksIn Brazil, the National Plan of Surveillance of Avian Diseases (PNSA, Brasil, 1991) is currently focused on serotypes Gallinarum, Pullorum, Enteritidis and Typhimurium. Based on the present experiment with *Salmonella* Kottbus, any unexpected serotype present in imported birds might be intensively shed in the feces in the first days of life. It should be remembered that any food of animal origin should be free of *Salmonella*. In conclusion, not only *Salmonella* Kottbus but other serotypes might be introduced in Brazilian poultry farms through imported genetic material.

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