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EXPERIMENTAL INFECTION OF LAYING HENS WITH *SALMONELLA ENTERICA* SEROVAR GALLINARUM

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ABSTRACT

Experimental infections were set up in commercial laying birds, comprising a white relatively resistant line and a red susceptible line infecting with *Salmonella enterica* serovar Gallinarum. The major findings were that in susceptible birds clinical disease occurred in a dose-dependent manner. Faecal excretion occurred in susceptible birds almost up to death but also occurred in the more resistant line and in birds, which were convalescing. Removal of birds, which had died from the disease, from the environment, reduced the resultant mortality/ morbidity and may be regarded as a useful measure for control.

Key words: laying hens, *Salmonella* Gallinarum, contact infection

INTRODUCTION

The disease induced by infection with *Salmonella enterica* serovar Gallinarum in chickens is usually observed in adult birds, although the etiologic agent is very pathogenic for chickens of all ages (1,8). There is considerable information available on the biology of the microorganism and some aspects of its relationship with the host, although this is relatively elementary in nature (8,10,12). The disease remains an important economic problem and its control has been attempted using vaccination with a live attenuated strain of *Salmonella* Gallinarum or by chemotherapy. Both of these are not, however, completely satisfactory since the live vaccine retains some virulence (6,13) and many drugs are prohibited for use in animal production. Besides, drug therapy is expensive and sometimes increases mortality because of toxicity resulting from misuse (3). Another possibility for the future is the replacement of susceptible varieties of birds by others that are more resistant to clinical disease, as the light white varieties usually are (4). Light white varieties of chickens may not exhibit typical symptoms of fowl typhoid but can show sub-clinical disease with the bacterium persisting in the bird longer than it does in susceptible lines (1).

The present study was carried out to define some aspects of the progress of the infection of *Salmonella* Gallinarum in commercial laying hens. In the white variety which is often resistant to the clinical disease it was assessed the persistence of the bacterium despite of the absence of signals of fowl typhoid and in the brown birds it was assessed the transmission by feces, by eggs, and by contact between infected and non-infected birds.

MATERIALS AND METHODS

The method for enumeration of *Salmonella* Gallinarum was that adopted by Berchieri Jr. *et al.* (1). Briefly, an overnight broth culture of *Salmonella* Gallinarum strain 9, resistant to nalidixic acid (SGNal^r) was prepared and incubated in a shaking (100 strokes/min) incubator at 37ºC.

In experiment one, 48 newly-hatched chickens from a light white variety of Hy-Line were separated into two groups. In one group (group B) the birds were inoculated orally with 0.1 mL of the neat culture containing approximately 8.5 x 10^8 colony forming units/mL (cfu/mL) and in the other group (group A) the culture was diluted by a factor of 10^{-3}, thereby containing approximately 8.5 x 10^5 cfu/mL. The 5 days old birds were inoculated. After 3, 7, 14, 21, 28 and 35 days post-inoculation

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(dpi), birds were killed for bacteriological examination of the liver, spleen, ovary, blood and caecal contents. The samples were diluted in PBS (1:10) and homogenised and further diluted in a decimal series. 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 42ºC and the cfu/mL were transformed to $\log_{10}$ for analysis. In the absence of bacterial growth the sample was cultured in selenite broth (Oxoid .CM395) containing novobiocin (40 mg/L) overnight at 42ºC and then plated on BGNNov, which was incubated at 42ºC overnight. In addition, two groups of day-old commercial brown egg laying variety of Hy-Line were infected in the same way to observe morbidity and mortality.

A second experiment was carried out with 48 adult (18 weeks old) laying hens from a brown egg-layer flock (Hy line) placed in two groups. The birds in group B were inoculated orally with 1 mL of a neat culture containing approximately 8.5×10^8 cfu/mL and in the other (group A) 1 mL of the culture diluted to 10^{-3}, thereby containing approximately 8.5×10^5 cfu/mL. Birds from both groups were killed after 3, 7, 14, 21, 28 and 35 dpi for bacteriological examination as described above. At this time, eggs laid were also examined. They were collected in a sterile glass jar, broken by agitation and incubated overnight at 42ºC. From the jar, a cotton swab was used to collect a sample of egg, which was streaked out on BGNNov and incubated overnight at 42ºC. This experiment was repeated later with four groups of commercial brown egg laying hens, using four dilutions of the inoculum (neat: culture containing approximately 8.5×10^8 cfu/mL; 10^{-1}: 8.5×10^7 cfu/mL; 10^{-2}: 8.5×10^6 cfu/mL and 10^{-3}: 8.5×10^5 cfu/mL) for bacteriological examination of the faecal excretion and eggs laid.

A third experiment was done with 190 adult (18 weeks old) commercial brown egg laying hens (Hy line). The birds were separated in group A and group B. They were inoculated orally with 1 mL of an overnight broth culture containing approximately 8.5×10^8 cfu/mL of *Salmonella* Gallinarum. They were placed together with uninfected birds. In group A dead birds were taken out as soon as they were noticed and in group B dead birds were left remaining in the cage for 48 hours. Cloacal swabbing were taken twice a week and all eggs laid were done through out the experiment. Data from the two groups were compared using the χ^2 test of significance (14)

Two additional experiments were done to assess faecal excretion and egg contamination. In one of them birds from a new variety of brown egg Brazilian line were infected with neat or diluted $SGNal^r$ cultures. In the second additional experiment, the 40 adult (18 weeeks old) brown varieties of chickens (Hy line) were tested again, but using four doses of inoculum containing undiluted (8.5×10^8 cfu/mL), 10^{-1} (8.5×10^7 cfu/mL), 10^{-2} (8.5×10^6 cfu/mL) and 10^{-3} (8.5×10^5 cfu/mL) diluted cultures.

RESULTS

In all experiments cloacal swab and serological examination of the birds carried out at the beginning of each experiment did not show any evidence of *Salmonella.*

During experiment one, mortality was not observed among the variety of young light birds and *Salmonella* Gallinarum was not recovered from caecal or cloacal samples. Rates of organ infection were higher among birds that received the undiluted culture. However, in both groups *Salmonella* Gallinarum (SG) was found in liver and/or spleen 35 days post-infection (Table 1).

Table 1. *Salmonella* Gallinarum detected in samples of tissue of the light variety of laying hens.

	Number ($\log_{10}$) of viable cells per gram of the sample after any interval of time after infection															
	3 days		7days			14 days				21 days			28 days		35 days	
Bird nº	L	S	L	S	Ov	L	S	Ov	B	L	S	B	L	S	L	S
1	-	-	-	-	-	2.78	3.40	-	-	-	-	-	-	-	-	-
2	-	-	-	-	-	+	2.60	2.90	-	+	+	1.70	-	-	1.48	+
3	-	-	-	-	-	-	-	-	-	+	3.00	-	-	-	-	-
4	-	-	-	-	-	+	+	-	-	-	-	-	+	2.48	-	-
5	2.36	2.40	2.00	2.48	2.70	+	+	+	-	+	-	-	-	-	-	+
6	+	2.08	2.60	4.04	+	-	-	-	-	+	+	-	-	-	-	+
7	2.48	-	2.48	3.52	2.95	-	6.45	-	-	-	+	-	+	2.00	-	+
8	2.00	+	2.26	3.30	-	2.30	3.00	3.30	+	+	2.00	-	-	-	-	-

L: liver, S: spleen, Ov: ovary, B: blood. Negative results from the blood, ovary and caecal contents examination were excluded from the table;
Group B: birds number 1 to 4: inoculated with 0.1 mL of an overnight broth culture diluted at 10^{-3} (8.5×10^5 cfu/mL);
Group A: birds number 5 to 8: inoculated with 0.1 mL of a net (8.5×10^8 cfu/mL) overnight broth culture;
+/-: positive/negative after incubation in selenite broth.

Mortality was high in young red birds that received the neat culture of SG (16 died of 17 infected), whereas only one red bird died (1/17) in the group challenged with the broth culture diluted to 1:1000. At the end of the experiment, bacteriological examination failed to isolated SG from the liver or spleen samples of any of the surviving red birds.

Table 2 shows the results of bacteriological examination of adults birds of the commercial variety of brown egg line. Inspection was carried out up to 35 days post infection (dpi). However, 21 dpi only two birds remained in the flock inoculated with the higher dose and in the flock inoculated with the smaller dose three birds were examined at 28 and 35 dpi. As before, negative results related to samples collected 14, 21, 28, and 35 dpi are not shown in table two. The mortality among adult red birds was comparable with that seen with young red birds. SG was isolated in birds that received the lower dose in the first two weeks after infection but not thereafter. Those birds from which SG was countable in 14 and 21 dpi were clinically close to death. Mortality was high in the other group. No infected eggs were found during the experiment.

The third experiment (Table 3) was done to assess infection by contact between experimentally infected and uninfected birds. When dead birds were removed from the cage as soon as they were noticed the disease did not spread (7/110=6%) as it did when dead birds were allowed to remain in the cage (40/83=48%) indicating the importance of infection from infected dead animals, possibly through cannibalism. This is statistically highly significant (χ^2= 44.95, P< 0.01). In this experiment, cloacal swabs were taken twice a week and all 186 eggs were examined. No *Salmonella*-contaminated eggs were found. *Salmonella* was recovered by cloacal swab just before death in 16 birds. However, some birds presented a different behaviour, especially those that did not die. These birds excreted SGNal^r in the faeces for several days (Table 4).

Two additional experiments were done to assess faecal excretion and egg contamination. In one of them birds from a new variety of brown egg Brazilian line were infected with neat or diluted SGNal^r cultures. These birds were very resistant to infection with minimum mortality, and the birds excreted SGNal^r similarly to the white variety (data not shown). In the second

Table 3. *Salmonella* Gallinarum Infection by contact.

	Group* A		Group B	
Experiment	D/I**	D/C***	D/I	D/C
01	4/6	1/6		
02	7/10	0/30		
03	5/6	0/6	4/5	4/5
04	5/5	0/10	4/5	7/10
05	5/6	0/22	11/12	17/40
06	8/22	6/36	7/20	12/36
Total	34/55	7/110	26/42	40/83

*Group A: dead birds were taken out as soon as noticed; Group B: dead birds remained in cage for 48 hours;
**Number of dead birds/number of infected birds;
*** Number of dead birds/number of contact birds.

Table 2. *Salmonella* Gallinarum isolated from organs and caecal contents of a commercial brown egg adult laying hens

	Viable number (log_{10}) of *Salmonella* organisms per gram of the sample after any interval of time after infection															
	3 days					7 days					14 days				21 days	
Bird nº	L	S	Ov	B	C	L	S	Ov	B	C	L	S	Ov	C	S	Ov
1	-	-	-	-	-	4.62	4.08	+	2.08	-	-	-	-	-	-	-
2	-	-	-	-	-	-	-	-	-	-	3.20	4.08	+	4.45	-	-
3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2.78	+
4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
5	4.04	-	-	2.00	-	4.56	3.36	+	-	-	-	-	+	-	-	-
6	4.20	4.08	2.00	-	-	8.08	8.08	+	4.65	3.60	2.60	4.08	-	-	-	-
7	-	-	-	-	-	2.00	3.26	+	-	-	-	-	-	-	nd	nd
8	2.60	3.95	+	-	5.00	4.30	3.17	+	-	+	3.51	2.08	+	-	nd	nd

L: liver, S: spleen, Ov: ovary, B: blood, C: caecal contents;
Group B: birds number 1 to 4: inoculated with 1 mL of an overnight broth culture diluted at 10^{-3} (8.5 x 10^5 cfu/mL);
Group A: birds number 5 to 8: inoculated with 1 mL of a net (8.5 x 10^8 cfu/mL) overnight broth culture;
+/-: positive/negative after incubation in selenite broth;
nd: not done because bird died before

Table 4. *Salmonella* Gallinarum 9Nal^r isolated by cloacal swab from adult red laying hens.

	Days post-infection											
Bird nº	7**	8	9	10	12	13	20	21	22	23	25	26
65 – I*	+(9)											
53 – I		+(10)										
31 – I		+(10)										
21 – I			+(12)									
25 – I				+	+(13)							
44 – C				+								
87 – I				+(10)								
61 – I						+(14)						
94 – C							+(21)					
54 – C							+(21)					
78 – I								+(21)				
60 – C								+	+	+		
72 – C										+(24)		
60 – C												
72 – C												
82 – C										+		
98 – C											+	+

	27	28	29	30	31	32	33	34	36	44	45
60 – C				+	+		+		+		
82– C		+		+		+					
98 – C	+		+(30)								
42 – C				+							
85 – C					+(32)						
74 – C					+(32)						
95 – C						+	+(34)				
99 – C							+	+(35)			
36 – C										+	+

The swabs were daily after infection.
*I: Infected bird, C: Birds that acquired the infection by contact;
** +: positive cloacal swab. Number in parenthesis indicates the day that birds died;
NB: 46 – 66 dpi, cloacal swabs from all birds were negative.

additional experiment, the adult brown variety of chickens were tested again, but using four doses of inoculum containing undiluted 10^{-1}, 10^{-2} and 10^{-3} diluted cultures. As observed previously, *Salmonella* was isolated from the faeces almost to the point of death, while some of those that survived did excrete the bacteria for some days (Table 5). *Salmonella* Gallinarum was not recovered from any of 460 eggs laid.

DISCUSSION AND CONCLUSION

Previous work has shown that adult birds of light white varieties, which are considered from field data to be more resistant to fowl typhoid, survived the experimental infection. Nevertheless, some birds had transitory bacteraemia (1). We have also found that age is not a limiting factor for disease occurrence such was shown before (11,12). In the two groups of day-old red birds included in this experiment, mortality was also high among those inoculated with the neat culture and sub-clinical disease was seen among those that received the low dose as happened with the adult red birds.

In the experiment with adult red birds, the green/green yellowish diarrhoea started 2-4 days after infection, but the birds continued eating, drinking and laying. Strong changes occurred closer to death. Birds stopped eating, drinking and laying. At this point, *Salmonella* Gallinarum spread out rapidly through the population of adult birds, being present in the carcass and in the cloacae. These results suggest that the propensity towards disease-free carriage and the production of contaminated egg are less marked in susceptible birds, what agrees with previous studies (1,2), although similarities occur with the epidemiology of pullorum disease in different lines (10). In those birds that received the low dose no mortality occurred. Thus, when birds are susceptible, the occurrence of disease may be dose dependent as found previously (1) and birds that survived, whatever the dose is, did not necessarily become carriers as occurred with birds infected by *Salmonella* Pullorum (1,7).

Faecal excretion was eventually seen among varieties of birds considered resistant to *Salmonella* Gallinarum (2). In the present study, in the group infected with the low dose only one bird died (Table 2). *Salmonella* Gallinarum was not recovered from cloacae and eggs (1). However, in the experiment to assess contact infection (Tables 3 and 4), although frequent faecal excretion of *Salmonella* Gallinarum was not observed as occurred in *Salmonella* Enteritidis infection (5), some birds that did not die did excrete the bacterium for several days. Unlike previous results (1), these findings demonstrate that, among susceptible birds, survivors may contain and excrete *Salmonella*. Thus, faecal excretion is not related to the variety of the birds.

Table 5. *Salmonella* Gallinarum 9Nal^r isolated by cloacal swab from adult red laying hens.

	Days post-infection														
Bird nº	24h	48h	3	5	7	9	11	13	15	17	19	21	23	25	27
1						+	(11)								
2					+	(8)									
3					+	(8)									
4					+		(10)								
5						(9)									
6									(15)						
7						(8)									
8										+	(19)				
9						+	(11)								
10							+	+							
11							(11)								
12													+		
13											+	+			(28)
14						(8)									
15					(7)										
16							(10)								
17					+	(9)									
18										(16)					
19						+	(10)								
20					+	(9)									
21											+			+	
22								+			+				
23											+	(20)			
24							(10)								
25												+	+	+	+
26												+			+(28)
27															
28															
29						(8)									
30												+			
31							+								
32															
33							(11)								
34															
35															
36															
37							+	(12)							
38															
39															
40								(13)							

* Birds 1-10: receiving neat culture (8.5 x 10^8 cfu/mL), Birds 11-20: receiving culture diluted to 10^{-1} (8.5 x 10^7 cfu/mL), Birds 21-30: at 10^{-2} (8.5 x 10^6 cfu/mL), and birds 31-40: at 10^{-3} (8.5 x 10^5 cfu/mL). Number in parenthesis indicates the day that birds died.

The third experiment showed that by removing dead birds quickly, fowl typhoid might be prevented. In addition, the data from this work strongly suggest that the basic principles of hygiene and cleaning are very important in the prevention of fowl typhoid, possibly more so than the use of any kind of therapy (9).

In conclusion, the work have shown that 1.*Salmonella* Gallinarum was isolated from internal organs of birds with no disease up to 35 days as well as in feces; 2.The disease is dose dependant to the susceptible variety; 3.Eggs laid might not be contaminated by SG and the dissemination of the disease is strongly related with the presence of dead bird in the cage.

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RESUMO

Infecção experimental com *Salmonella enterica* serovar Gallinarum em poedeiras comerciais

Infecções experimentais por *Salmonella enterica* serovar Gallinarum foram realizadas em aves de postura comercial, incluindo uma linhagem branca resistente e uma linhagem vermelha susceptível ao desenvolvimento da enfermidade clínica. As aves de linhagem susceptível apresentaram doença clínica dependente da dose administrada. Excreção fecal foi observada em aves da linhagem susceptível próximo ao momento da morte e, eventualmente, em aves da linhagem resistente e aves convalescentes. A remoção das aves mortas do meio ambiente reduziu a taxa de mortalidade/morbidade, procedimento este que pode ser utilizado como medida de controle.

Palavras-chave: ave de postura comercial; *Salmonella* Gallinarum; infecção por contato

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