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Growth of *Salmonella* in Chickens' Yolk Sacs and Its Relationship to Pathogenicity[1]

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Newly hatched chicks were inoculated in the yolk sacs with standardized suspensions of *Salmonella anatum*, *S. heidelberg*, or *S. infantis*. At intervals between 3 and 48 hr postinoculation, chicks from each group were sacrificed, the average number of viable cells per yolk sac was determined, and liver tissue from each chick was examined for *Salmonella*. Growth patterns of the three species were almost identical when each chick was inoculated with about 3.5 million cells, but *S. heidelberg* was recovered more frequently from the liver, and caused a much higher percentage of mortality than did either *S. anatum* or *S. infantis*. When 100-fold dilutions of the suspension of *S. heidelberg* were used, mortality and recovery rates of the bacterium from the liver were directly related to the number of cells injected. The logarithmic growth phase was lengthened as the number of cells in the inocula was decreased; consequently, there was little difference in the average number of *S. heidelberg* cells per yolk sac at 36 or 48 hr postinoculation regardless of number of cells injected. Results of these trials indicated that factors other than rate of multiplication in the yolk sac are responsible for observed differences between *Salmonella* species in degree of pathogenicity for baby chicks.

Salmonella infections have become one of the most important groups of bacterial diseases affecting poultry, and, according to Williams (9), domestic poultry constitutes the largest single reservoir of salmonella organisms existing in nature.

Salmonella species were isolated from the shells of eggs laid by 11 of 40 healthy chicken breeder flocks surveyed by Stephens (7) in 1965, indicating the prevalence of these bacteria in the intestinal tracts of hens. Quist (5) stated that infection in newly hatched chicks may occur if hatching eggs are naturally infected or are contaminated in the incubator. Mundt and Tugwell (4) observed a high incidence of egg shell contamination, but an absence of salmonella in the meats of eggs laid by groups of hens experimentally infected with six species of *Salmonella*. Bierer (1) found that invasion of the navel appeared to be the principal route of infection of baby chicks when egg shells were sprayed with *S. typhimurium* on the 20th day of incubation. These observations suggest that the yolk sac of the newly hatched chick is the most important site of multiplication of the bacteria when incubators are contaminated with salmonellae.

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Vestal and Stephens (8) reported wide variations in the degree of pathogenicity for baby chicks of paratyphoids injected into the chicks' yolk sacs.

The principal objectives of this study were to determine whether the growth patterns of the individual *Salmonella* species were associated with their relative degrees of pathogenicity, and the effect of number of cells injected upon the growth patterns of *Salmonella* in the chick's yolk sac.

MATERIALS AND METHODS

Four trials were conducted to study the growth patterns of two mildly pathogenic (*S. anatum* and *S. infantis*) and one highly pathogenic (*S. heidelberg*) species of *Salmonella* in the yolk sacs of chicks. The trials were initiated with a total of 294 chicks. Three additional trials involving a total of 269 chicks were conducted to determine the effect of number of cells in the inoculum on the growth rate of *S. heidelberg* in the chicken's yolk sac.

The salmonellae used in this study were isolated from the shells of eggs laid by healthy chicken breeder flocks. The cultures were maintained in the lyophilized state until 4 days before injection into the chicks. Their relative degrees of pathogeneicity have been reported by Vestal and Stephens (8). The inocula were prepared by washing 48-hr cultures from Tryptose Agar slants, diluting the suspensions in sterile 0.1%

Proteose Peptone No. 3 solution to readings of 55% light transmittance at 525 mμ in a Baush & Lomb model 340 spectrophotometer, and further diluting the suspension 1:100 in the sterile Proteose Peptone solution.

A 1-ml amount of inoculum was injected by use of a sterile 2-ml syringe fitted with a 1-inch (2.54-cm), 22-gauge hypodermic needle, through the navel, into the yolk sac of each chick. The area around the navel was first cleaned with disinfectant. In the first four trials, the average number of cells of *S. anatum*, *S. heidelberg*, or *S. infantis* given each chick was about 3.5 million.

In the last three trials, each chick in one group was inoculated with about 2.87 million cells of *S. heidelberg*; a second group received about 28,700 each; a third group received about 287 cells each; and a fourth received an average of 2.87 cells per chick. All chicks were inoculated within 24 hr after hatching.

Plate counts were made to determine the number of viable cells in each inoculum.

At the intervals indicated in Table 1, two chicks inoculated with each species, or given each dilution of the *S. heidelberg* suspension in the last three trials, were sacrificed, and the yolk sacs were aseptically removed. The two yolk sacs representing each group were pooled and the volume was measured in a graduated cylinder. The yolk sacs were then diluted 1:10 in sterile Proteose Peptone solution and homogenized in a blender (John Oster Co., Milwaukee, Wis.). Further 10-fold dilutions were then made to reduce the concentration of cells to a countable number. A 0.1-ml amount of suspension per plate was placed on S S Agar (Difco), in triplicate, and streaked over the surfaces with sterile, bent glass rods. The agar plates were incubated at 37 C for 24 hr. Plates having 30 to 300 colonies were selected, the colonies were counted, and the average number of cells per yolk sac was calculated.

A small piece of liver tissue was aseptically removed from each chick sacrificed and pressed several times on the surface of an S S Agar plate to determine the time required for each species to reach this organ.

Mortality records, including approximate times of death, were kept in all trials. Most of the chicks that succumbed to the infections were examined by the same procedures outlined above.

The chicks were kept in chick boxes, without access to feed or water, at a temperature of 31 C for the 48-hr duration of each trial.

Data pertaining to number of cells per yolk sac at each time interval were analyzed by analysis of variance according to Snedecor (6). Significant treatment differences were determined by Duncan's multiple range test (3).

RESULTS AND DISCUSSION

The growth patterns of *S. anatum*, *S. infantis*, and *S. heidelberg* in the chicks' yolk sacs were almost identical (Fig. 1). No significant differences ($P < 0.05$) between species in number of viable cells per yolk sac were found at any of the nine time intervals.

The number of salmonella cells per yolk sac increased significantly ($P < 0.05$) with each time interval between 3 and 30 hr postinoculation (Table 1). No significant change in number of cells was observed between 0 and 3 hr postinoculation, or between 30 and 48 hr postinoculation.

The growth patterns of the *Salmonella* sp. in chickens' yolk sacs resemble a part of the classic bacterial growth curve outlined by Buchanan (2), when approximately 3.5 million cells are injected into each yolk sac. The lag phase ends at about 3 hr postinoculation, the logarithmic growth phase ends 9 to 12 hr postinoculation, the phase of negative growth acceleration occurs between the 12th and 30th or 36th hr postinoculation, and the stationary phase extends to, at least, the 48th hr postinoculation (Fig. 1).

When fewer than 3.5 million cells of *S. heidel-*

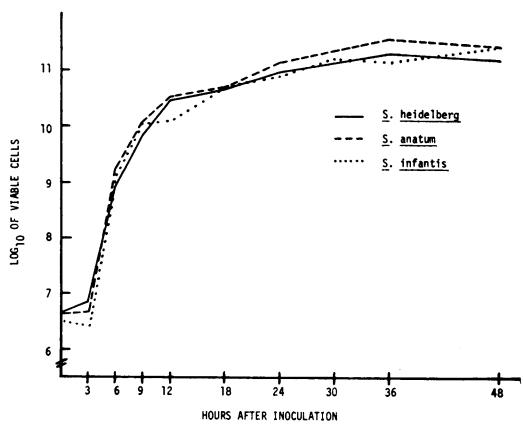

FIG. 1. *Growth patterns of Salmonella in the chick's yolk sac. Averages of four trials.*

TABLE 1. *Average growth rate of three Salmonella species in the chicken's yolk sac*[a]

Time after inoculation (hr)	Avg log no. of cells/yolk sac[b]
Inoculum (0)	6.5571 (1)
3	6.5513 (1)
6	9.0161 (2)
9	9.9366 (3)
12	10.3053 (4)
18	10.6353 (5)
24	10.9207 (6)
30	11.2235 (7)
36	11.2460 (7)
48	11.3201 (7)

[a] Each mean represents 24 chicks, 8 inoculated with *S. anatum*, 8 with *S. infantis*, and 8 with *S. heidelberg*.

[b] Means followed by different numbers in parentheses are significantly different at $P < 0.05$.

berg were injected into each yolk sac, the lag phase was either eliminated or drastically shortened (Fig. 2). The duration of the logarithmic growth phase was inversely related to the number of *S. heidelberg* cells in the inoculum; consequently, there was little difference in the number of viable cells per yolk sac at 36 and 48 hr postinoculation, regardless of the number in the inoculum (Fig. 2, Table 2). In chicks inoculated with an average of 2.87 cells, fewer than 100 cells per ml of yolk sac were present at 3 hr after inoculation.

S. heidelberg caused the highest percentage of mortality in each of the four trials in which three species were used (Table 3). Mortality caused by *S. anatum* and *S. infantis* was negligible.

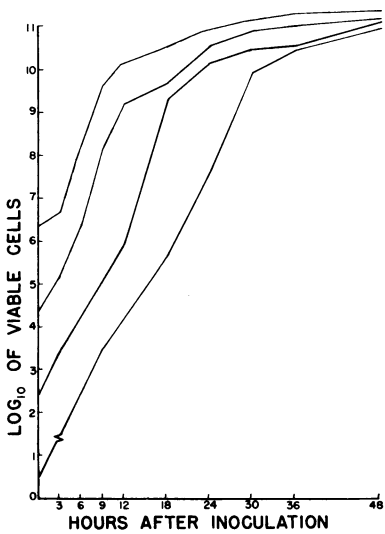

FIG. 2. *Effect of number of cells injected upon growth patterns of Salmonella heidelberg in the chick's yolk sac.*

The percentage of mortality caused by *S. heidelberg* was directly related to the number of cells injected (Table 4). When each chick was given only about three cells, no mortality occurred during the 48-hr duration of the trials; of 63 chicks given about 287 cells each, only 1 died; 6 of 68 given 28,700 cells died; and 24 of 81 given 2,870,000 cells died (Table 4). Most of the mortality occurred between 18 and 24 hr postinoculation (Tables 3 and 4).

The effects of species on rate and time of recovery of salmonellae from the chicks' livers are illustrated in Table 5. *S. heidelberg* was regularly recovered from the chicks' livers at 6 hr postinoculation and later. The rates of recovery of *S. anatum* and *S. infantis* from the chicks' livers did not equal that of *S. heidelberg* until 36 or 48 hr after inoculation of the yolk sacs.

Rate and time of recovery of *S. heidelberg* from the chicks' livers were directly related to the number of cells injected into the yolk sacs (Table 6).

Salmonellae were isolated from the livers of all chicks that died during this study. The average number of salmonella cells in the yolk sacs of chicks that died of the infections was not significantly different from the number in the yolk sacs of those that were sacrificed at about the same time.

Results of these trials indicate that differences in pathogenicity between *Salmonella* species are not caused by different rates of growth of the bacteria in the chick's yolk sac.

A comparison of mortality data from the first four trials (Table 3) with that from the last three (Table 4) indicates that a few thousand *S. heidelberg* cells are as detrimental, when injected into the yolk sacs of baby chicks, as are 3.5 million cells of *S. anatum* or *S. infantis*. These results are compatible with those reported by Vestal and Stephens (8).

TABLE 2. *Effect of number of cells injected on growth pattern of Salmonella heidelberg in the chicken's yolk sac*[a]

Time after inoculation (hr)	Avg $\log_{10}$ of no. of viable cells/yolk sac			
	Group I	Group II	Group III	Group IV
0 (inoculum)	6.3987 (1)	4.3987 (1)	2.3987 (1)	0.3987 (1)
3	6.6405 (1)	5.1873 (2)	3.4071 (2)	
6	8.3272 (2)	6.3990 (3)	4.2666 (3)	2.4211 (2)
9	9.6950 (3)	8.1953 (4)	5.0793 (4)	3.4183 (2)
12	10.2178 (3, 4)	9.2267 (5)	5.9839 ,5)	4.2244 (2, 3)
18	10.5899 (4, 5)	9.7060 (5)	9.3390 (6)	5.6416 (3)
24	11.0208 (4, 5, 6)	10.6114 (6)	10.1182 (7)	7.6333 (4)
30	11.2196 (5, 6)	10.9569 (6)	10.5286 (7, 8)	10.0168 (5)
36	11.3760 (5, 6)	11.0901 (6)	10.6073 (7, 8)	10.5094 (5)
48	11.4470 (6)	11.2630 (6)	11.1776 (8)	11.0244 (5)

[a] Means in each column followed by different numbers in parentheses are significantly different at $P < 0.05$.

TABLE 3. *Mortality following inoculation with Salmonella species*[a]

Time after inoculation	*S. anatum*	*S. infantis*	*S. heidelberg*
18	0	1	8
24	0	0	16
30	0	0	5
36	0	0	2
48	2	1	1
Total	2	2	32
Per cent	2.17	2.17	29.09

[a] A total of 92 chicks were inoculated with *S. anatum*, 92 with *S. infantis*, and 110 with *S. heidelberg*.

TABLE 4. *Mortality following injection of Salmonella heidelberg into chicks' yolk sacs*

Time after inoculation (hr)	Avg no. of cells injected per yolk sac			
	2.87×10^6	2.87×10^4	2.87×10^2	2.87
18	7	0	0	0
24	8	0	0	0
30	3	1	0	0
36	0	3	0	0
48	6	2	1	0
Total[a]	24/81	6/68	1/63	0/57
Per cent	29.63	8.82	1.59	0.00

[a] Denominator indicates total number of chicks inoculated.

TABLE 5. *Recoveries of Salmonella spp. from chicks' livers after inoculation of yolk sacs*[a]

Time after inoculation (hr)	*S. anatum*	*S. infantis*	*S. heidelberg*
3	2/8	6/8	3/8
6	3/8	2/8	7/8
9	3/8	3/8	8/8
12	1/6	5/8	8/8
18	4/8	4/8	8/8
24	5/8	4/8	8/8
30	4/8	5/8	8/8
36	6/8	7/8	8/8
48	7/8	7/8	7/7
Total	35/70	43/72	65/71
Per cent	50.0	52.79	92.96

[a] Denominator represents total number of chicks examined in four trials; numerator represents number of chicks from which *Salmonella* was recovered.

TABLE 6. *Effect of number of Salmonella heidelberg cells injected into yolk sacs on recovery rate from livers*[a]

Time after inoculation	Avg no. of cells injected per yolk sac			
	2.87×10^6	2.87×10^4	2.87×10^2	2.87
3	5/6	0/6	0/5	0/5
6	6/6	1/6	0/6	0/5
9	6/6	2/6	0/6	0/6
12	6/6	4/6	2/6	0/6
18	6/6	5/6	4/6	0/6
24	6/6	5/6	2/6	0/6
30	6/6	5/6	4/6	3/6
36	6/6	6/6	3/6	1/6
48	5/5	6/6	5/6	4/6
Total	52/53	34/54	20/53	8/52
Per cent	98.11	62.96	37.74	15.38

[a] Numerators indicate number of recoveries of *S. heidelberg;* denominators indicate number of chicks examined.

The consistency with which the salmonellae invaded the livers during the first 30 hr of the infection was found to be associated with mortality. *S. anatum* and *S. infantis* did not invade the liver as consistently as did *S. heidelberg*, and, subsequently, they caused less mortality. Decreasing the number of cells of *S. heidelberg* injected into each yolk sac caused an increase in time required for invasion of the liver, and, subsequently, less mortality.

The results of this study suggest that differences between species of *Salmonella* in degree of pathogenicity for chickens are due either to differences in quantity or quality of endotoxin released, or to differences in adaptability of the bacteria to the internal organs, other than the yolk sac, as an environment.

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