Epidemiol. Infect. (1999), **123**, 121–132. Printed in the United Kingdom

Salmonella infection in a commercial line of ducks; Experimental studies on virulence, intestinal colonization and immune protection

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(*Accepted 21 April 1999*)

SUMMARY

Experimental infections of different salmonella serotypes were established in a commercial line of ducks to provide baseline information on which control measures might be based. The ducks were very resistant to systemic infection with *Salmonella typhimurium*, *S. enteritidis* and *S. gallinarum* within 36 h of hatching. This was associated with an inherent inability of the strains to multiply in the reticulo-endothelial system. The resistance was not associated with poor invasiveness or serum sensitivity. Individual strains of *S. typhimurium*, *S. enteritidis*, *S. heidelberg* and *S. orion* colonized the gut well and were excreted in the faeces for at least 6 weeks by ducks when they were infected orally within 2 days of hatching. The main sites of colonization were the caeca and, to a lesser extent, the crop. Viable counts of each inoculated strain in the caeca remained in excess of 10^6 c.f.u. 3 weeks after infection although the organisms had been cleared from the spleen by this time. Much less excretion occurred when the birds were infected at 3 weeks of age. When infected ducks, which had cleared themselves of infection, were challenged orally with the homologous strain expressing a different genetic marker, very low levels of excretion of the challenge strain were detected when compared with a control group. After infection low titres of circulating lipopolysaccharide-specific IgG antibodies were detected by an ELISA. Intestinal colonization of newly hatched ducks with an *aroA* strain of *S. enteritidis* resulted in extensive colonization which exerted an exclusion effect on the parent strain inoculated 24 h later.

INTRODUCTION

Salmonella infections in poultry are probably the most important source of salmonella-associated food-poisoning in man. There is no reliable information on the relative prevalence of salmonella organisms in different species of domestic poultry and it is assumed that the contribution of different species to human infection bears some relationship to the quantity of meat from each species that is consumed. The number of commercial ducks slaughtered in 1995 within the UK was approximately 13 million (an increase from 8 million in 10 years), compared with 514 million broiler chickens, a ratio of approximately 1:40 [1]. On this basis it might be estimated that consumption of duck meat is responsible for only up to 1000 recorded cases per annum of food-poisoning of different degrees of severity. However, the consumption of duck meat is much greater in many other countries, particularly those in south east Asia [2], where the incidence of human infection originating from this source, although unquantified, is likely to be much greater.

The rational basis for salmonellosis control in the duck industry relies heavily on research into salmonellosis in chickens, which may not always be appropriate. Very little recent information is available on the characteristics and pathogenesis of salmonella infections in commercial ducks [3–5]. Many different serotypes of salmonella have been isolated from

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ducks, most of these being of public health significance but some, including *S. gallinarum*, *S. pullorum*, *S. typhimurium*, *S. enteritidis* and *S. anatum*, can cause considerable losses in birds of less than a few weeks of age [6, 7]. Death has been reported to result from a combination of systemic salmonellosis and diarrhoea, the disease being referred to, in the past, as 'keel' disease, so-called because animals remained apparently healthy until they keeled over and died [8–12].

Considerably more early work was carried out as a result of the realization that duck eggs were a source of salmonella infection for man. In experiments with birds that were infected naturally in the field, the ovary was frequently infected resulting in the production of infected eggs at a high frequency [9, 11, 13]. In laying birds the ovary was frequently the only site of infection. However, the incidence of human infection arising from consumption of duck meat is likely to be much greater, as it is with human infections arising from chickens [13].

It was our intention to provide information on the course of salmonella infection in a line of young commercial ducks reared in the United Kingdom. Information on the severity of systemic infection and its parameters and on patterns of faecal excretion could act as models suitable for the assessment of infection control regimens and different intervention strategies in birds reared for meat. Systemic salmonellosis in chickens may be characterized by studying morbidity and mortality following oral inoculation with different serotypes [14, 15]. The latter authors were able to explain virulence, or absence of, it by measuring invasiveness *in vivo* and the ability of the strain to survive and multiply in the reticuloendothelial system of the host. Faecal excretion has also been studied experimentally by assessing the amount and duration of excretion following direct inoculation or by the introduction of seeder birds [16, 17]. These systems were therefore applied to a study of experimental salmonellosis in the duck.

MATERIALS AND METHODS

Bacteria

S. typhimurium F98 (phage type 14), *S. enteritidis* P125589 (phage type 4) and *S. gallinarum* 9 were all isolated from diseased chickens. *S. typhimurium* strain Bangor (phage type 44) was isolated from a cow. All of the above strains are pathogenic for newly-hatched chickens and have been described previously [15]. *S. heidelberg* 17705 was isolated from a case of human food-poisoning. *S. orion* (3, 10:y:1, 5) was isolated from the UK duck industry. *S. enteritidis* P125109 *aroA*, prepared at this institute, has been described [18]. *E. coli* K12 (prototrophic) is a serum sensitive laboratory strain [19]. All broth cultures were made in 10 ml volumes of LB broth (Difco, West Molesey, Surrey, UK) incubated for 24 h in a shaking incubator (150 rev./min) at 37 °C. These cultures contained approximately 3×10^9 c.f.u./ml. For oral inoculation they were generally used as spontaneous mutants, resistant to nalidixic acid (Nal^r). For some experiments spectinomycin resistant (Spc^r) mutants were also used. In both cases mutants were selected by heavy inoculation of MacConkey plates containing 50 μg/ml sodium nalidixate or spectinomycin. These mutations have been found not to affect virulence or intestinal colonization ability [14].

Experimental animals

Very young ducks (6–36 h old but referred to as day-old), were a kind gift of Cherry Valley Farms (North Kelsey Moor, Lincolnshire, UK). There is greater variability in the hatch time in ducks than in chickens and some ducks were therefore older by several hours than others. They were reared on a commercial grower mix, free of antibiotics on wire floors in isolation rooms maintained at 20 °C. At 4 weeks of age they were transferred to an inert, non-medicated deep-litter (Litterite, Unitrition International Ltd, Selby, Yorkshire, UK) at 20 °C.

Pathogenicity assays

Bacterial pathogenicity was assessed by oral and parenteral inoculation of ducks that were either 1-day or 3 weeks old. Birds were inoculated orally with 0·3 ml of undiluted broth cultures containing approximately 10^9 c.f.u. LD_{50} estimations were made using intramuscular inoculation of 0·1 ml volumes of 10-fold dilutions of broth cultures. Mortality was recorded and LD_{50} values were calculated [20]. The livers of dead birds and birds surviving 3 weeks after inoculation were plated on MacConkey agar (Oxoid, CM7 Oxoid, Basingstoke, UK).

Intestinal invasiveness of strains was assessed by counting the number of viable bacteria in the spleen and intestinal samples at different times after oral inoculation. The contents of the ileum and caeca were gently squeezed out. These segments of the gut were

then opened longitudinally, washed gently in running water followed by cursory drying and the mucosal layers scraped off with a scalpel blade.

Survival and multiplication in the non-intestinal organs was assessed by counting bacteria in the liver, spleen and heart blood at times after intravenous (leg vein) inoculation of 10^6 c.f.u. in 0·1 ml. Viable counts were made using a derivation of the method of Miles and colleagues [21].

Intestinal colonization

Groups of 20–30 ducks which were either 1 day or 3 weeks old were caged. Separate groups were inoculated orally with Nal^r mutants of different salmonella strains. At weekly intervals fine cloacal swabs (throat swabs, Bibby-Sterilin, Stone, Staffordshire, UK) were used to assess faecal excretion. These were eluted in 2 ml of sodium selenite broth (Oxoid, CM395) and plated in a standard manner [22] on plates of Brilliant Green agar (Oxoid, CM263) containing sodium nalidixate (20 μg/ml) and novobiocin (1 μg/ml). The swabs were incubated overnight at 37 °C in the selenite broth and replated. At 3, 7, 14 and 21 days post-infection, groups of three ducks from each group were killed for post-mortem examination. In addition to the intestinal samples described above, samples of the contents of the crop-like enlarged oesophagus were taken. In one experiment twenty 1-day-old ducks were infected by placing them in contact (within the same group) with three ducks of the same age which had been infected with 0·3 ml of an undiluted culture of bacteria. The spread of infection from the infected to the non-infected birds was monitored as above.

Serum resistance

Blood was taken from the leg vein of six 1-week-old ducks and allowed to clot at 37 °C for 1 h. The sample was centrifuged at 500 g for 15 min at 4 °C and the serum was removed and used immediately. Overnight LB cultures of different organisms were centrifuged at 1800 g for 30 min and the cell pellets were resuspended in 10 ml of phosphate-buffered saline (PBS) and further diluted 1:100 in PBS, this was diluted 1:100 in serum samples. A 100 μl sample was withdrawn immediately after mixing. The serum/bacteria mixtures were warmed and held at 37 °C in a water bath and further samples were taken at 1, 2 and 3 h. Bacteria in the samples were counted on LB agar.

Immunity and colonization inhibition

In one experiment a group of day-old ducks was infected orally with a Nal^r mutant of *S. enteritidis* 125589. When they had ceased to excrete this strain they, and a separately housed group of ducks of the same age which were demonstrated bacteriologically and serologically to be free of salmonella infection, were infected orally with the same dose of a Spc^r mutant of the same strain. Faecal shedding was then monitored on Brilliant Green agar containing spectinomycin at 50 μg/ml.

In a second experiment, two groups of nine 1-day-old ducks were inoculated orally with 10^8 c.f.u. in 0·3 ml of a Spc^r mutant of either *S. enteritidis* 125109 or of 125109 *aroA* immediately prior to being given access to feed. A fourth group was left uninoculated. One day later three birds from each group were killed and the counts of the salmonella strain in the caecum were estimated as described above. At this time the remaining birds were inoculated with 10^6 c.f.u. in 0·3 ml of a Nal^r mutant of strain P125589. Two and 4 days later three ducks were killed and the counts of both organisms in the caeca were estimated on Brilliant Green agar containing either sodium nalidixate or spectinomycin.

Circulating IgG response to infection

Aliquots of 20 μl of blood were taken from a leg vein and diluted in 980 μl PBS containing 20 μl/ml Tween 20 (PBST). These were frozen at −20 °C until required. The titre of specific IgG antibodies was estimated for each sample by a standard indirect ELISA in Dynatech (Dynex Technologies Inc., Chantilly, Virginia, USA) microtitre plates using *S. enteritidis* lipopolysaccharide (LPS) as antigen [23] at a concentration of 60 μg per well and an alkaline phosphatase-linked rabbit anti duck IgG conjugate (Nordic) at a dilution of 1:1000.

Statistical analysis

Comparisons of rates of isolation from faeces and from tissues from different groups were made using χ^2 analysis.

RESULTS

Groups of between 18 and 21 1-day-old ducks, aged from 12 to 36 h old, were inoculated orally with approximately 3×10^8 c.f.u. in 0·3 ml of different

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Table 1. *Numbers of salmonella organisms in the spleen and alimentary tract following oral inoculation*

Days after inoculation	Tissue sampled	Log_{10} viable count/g in tissues after oral inoculation*			
		S. gallinarum 9	*S. enteritidis* 125589	*S. typhimurium* F98	*S. heidelberg* 17705
1	Spleen	< 2·5†	3·3	3·0	< 2
	Ileum contents	2·0	4·9	4·3	3·9
	Ileum wall	4·0	5·7	—‡	4·5
	Caecal contents	4·0	7·3	6·9	7·9
	Caecal wall	4·1	7·1	—	7·2
2	Spleen	3·2	3·4	3·2	3·5
	Ileum contents	3·5	4·4	< 2	5·0
	Ileum wall	4·8	5·9	—	6·2
	Caecal contents	3·3	6·6	5·2	7·3
	Caecal wall	6·8	7·5	—	7·6
3	Spleen	2·3	3·9	3·4	3·6
	Ileum contents	4·0	5·1	3·7	3·6
	Ileum wall	5·5	6·6	—	4·5
	Caecal contents	5·7	6·0	5·5	7·0
	Caecal wall	5·9	6·7	—	5·6

* All strains inoculated as Nal^r mutants, 5×10^8 c.f.u. in 0·2 ml.
† Values are median counts for three ducklings.
‡ Not done.

Table 2. *Survival of salmonella strains in duck tissues after intravenous inoculation with 10^6 c.f.u.*

Time after inoculation	Log_{10} median viable bacterial count/g of the following strains in the following tissues taken from three ducks								
	S. gallinarum 9			*S. enteritidis* 125589			*S. heidelberg* 17705		
	Liver	Spleen	Blood	Liver	Spleen	Blood	Liver	Spleen	Blood
30 min	5·6	5·7	2·5	5·6	5·8	2·6	5·7	5·5	3·2
1 d	5·5	5·9	2·3	5·8	5·2	2·3	6·1	5·3	2·9
2 d	5·8	6·2	2·0	4·4	4·7	2·6	5·1	5·1	2·9
5 d	4·1	5·6	< 2	4·2	4·7	2·0	3·7	4·6	< 2
12 d	2·0	2·0	< 2	2·3	3·3	< 2	2·3	3·7	< 2

antibiotic-sensitive salmonella strains. These were *S. gallinarum* 9, *S. typhimurium* strains F98 and Bangor, *S. enteritidis* 125589 and *S. heidelberg* 17705. During the ensuing 3 week period there was no mortality and no signs of morbidity. At post-mortem examination the livers and spleens appeared healthy and no growth of salmonella was obtained by culturing liver swabs on MacConkey agar. Two further groups were inoculated with 10× the inoculum size of the *S. gallinarum* strain and *S. typhimurium* F98. The results were the same.

The pathogenicity of four of these strains was tested by intramuscular inoculation and LD_{50} estimations made. The log_{10} values obtained for *S. gallinarum* 9, *S. enteritidis* 125589, *S. typhimurium* F98 and *S. heidelberg* 17705 were > 8·1, 5·20, 7·58 and 7·17 respectively.

The lack of pathogenicity of the strains indicated an inability to multiply in the tissues or tissue fluids but did not exclude poor invasiveness. These parameters were therefore tested. The ability of Nal^r mutants of different salmonella strains to invade as far as the spleen 1–3 days after oral inoculation of day-old ducks is shown in Table 1. *S. gallinarum* was isolated from the lumen of the intestinal sites in lower numbers more frequently than the other three strains and the intestinal wall counts of this strain were more frequently lower than the others (the wall counts of the *S. typhimurium* F98 were not estimated). However,

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Table 3. *Survival in fresh duck serum of different salmonella strains*

Bacterial strain	Log_{10} viable count of inoculated strain after the following number of hours incubation at 37 °C			
	0	1	2	3
S. gallinarum 9	5·4	5·5	5·7	5·9
S. enteritidis P125589	5·5	5·4	5·9	6·8
S. typhimurium F98	5·2	5·3	5·8	6·6
S. heidelberg 17705	5·7	5·5	5·9	6·9
S. orion	5·5	3·5	3·6	3·6
E. coli K12 prototroph	5·2	2·3	< 2	< 2

Table 4. *Faecal excretion of salmonella strains following oral inoculation*

Weeks after inoculation*	Number of birds per group	Number of ducks (expressed as a percentage) excreting the following *Salmonella* strains in the amounts indicated											
		S. typhimurium F98			*S. enteritidis* 125589			*S. heidelberg* 17705			*S. orion*		
		50†	D	T	50	D	T	50	D	T	50	D	T
1/7	29–30	93	100	100	100	100	100	100	100	100	100	100	100
1	20–23	52	78	100	70	100	100	15	30	100	—	—	—
2	17–20	30	90	100	76	94	100	75	100	100	50	70	100
3	9–12	8	50	88	11	33	88	88	100	100	24	75	100
4	8	0	38	75	0	38	100	50	100	100	12	12	100
5	5	0	0	60	0	20	40	60	80	100	0	20	80
6	5	0	0	60	0	0	100	0	25	75	0	0	100

* All ducklings inoculated orally with approximately 5×10^8 c.f.u. of Nal^r mutants in 0·2 ml.
† 50, 50 colonies per plate; D, 1 colony per plate; T, salmonella isolated by direct plating or by enrichment.

by 48 h after inoculation all four strains were present in the spleen at (median) counts of greater than 10^3 per g.

The survival of the *S. gallinarum*, *S. enteritidis* and *S. heidelberg* strains in the tissues after intravenous inoculation of day-old ducks with approximately 10^6 c.f.u. is shown in Table 2. Similar numbers of all three strains were found for 2 days after inoculation. A very slight reduction in numbers at 5 days was followed by a greater fall at 12 days although organisms were still detected in the liver and spleen.

The survival in fresh duck serum of the serum-sensitive *E. coli* K12 and five salmonella strains is shown in Table 3. The numbers of the *E. coli* strain had fallen considerably after 1 h incubation and the organism was not recovered subsequently. The numbers of *S. orion* fell 100-fold after 1 h but then stabilized. There was no reduction in the counts of any of the other strains that showed signs of multiplication after 2 h incubation.

The pattern of faecal excretion in groups of day-old ducks inoculated orally with Nal^r mutants of *S. typhimurium* F98, *S. enteritidis*, *S. heidelberg* and a strain of *S. orion*, a serotype isolated recently from ducks in the United Kingdom, is shown in Table 4. The groups consisted initially of 29–30 ducks but this number was reduced regularly by culling because of the fast growth rate of the birds and with post-mortem examination of the ducks killed. For uniformity the values are expressed as percentages throughout the experiment. There was no great difference in the changes in the rates of excretion of the strains during the first weeks of the experiment. However, the number of birds excreting each of the strains heavily declined as did (with some variation) the total number of birds excreting the *S. enteritidis* and *S. typhimurium* strains. It must be remembered that the number of birds at the end of the experiment was quite small. The majority of ducks were still excreting 7 weeks after inoculation (*c.* five birds per group at the end of the

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Table 5. *Intestinal colonization and isolation from the spleen of ducklings by salmonella strains following oral inoculation*

Days after inoculation*	Contents sampled at the following sites	Log_{10} median viable bacterial count/g of gut contents of the following strains			
		S. typhimurium F98	*S. enteritidis* 125589	*S. heidelberg* 17705	*S. orion*
3	Crop	< 2	< 2	4·5	< 2
	Ileum	3·2	4·3	3·6	3·9
	Caeca	6·8	6·8	7·4	5·6
	Spleen	3·9	4·1	3·9	2·0
7	Crop	3·0	4·4	< 2	3·1
	Ileum	4·2	4·2	2·9	3·3
	Caeca	6·7	6·3	6·5	4·9
	Spleen	3·2	3·5	3·3	< 2
14	Crop	< 2	2·7	3·3	3·1
	Ileum	< 2	2·6	2·3	4·6
	Caeca	5·9	5·7	7·7	5·3
	Spleen	< 2	2·0	< 2	< 2
21	Crop	< 2	2·0	2·0	< 2
	Ileum	2·5	< 2	3·1	< 2
	Caeca	6·2	5·2	6·2	5·9
	Spleen	< 2	< 2	< 2	< 2

* All ducklings inoculated orally with approximately 5×10^8 c.f.u. of Nal^r mutants in 0·2 ml.

experiment totalling 16/19 birds excreting the inoculated strains).

The viable numbers of these strains in sections of the alimentary tract at times after inoculation (the birds culled from the first half of this experiment) are shown in Table 5. The organisms were isolated most frequently and in highest numbers from the caeca and least frequently from the crop. Counts in excess of 10^5–10^6 were still present in the caeca 3 weeks after inoculation when the experiment ended. All strains had been eliminated from the spleen by 21 days post-inoculation.

The experiment on intestinal colonization by *S. enteritidis* provided information on the rate of excretion of this serotype in birds of different ages and birds infected by contact and on the evidence for a protective immunity against intestinal infection. Four groups of 20 1-day-old ducks were housed separately and infected with *S. enteritidis* 125589 Nal^r in different ways. In one group each bird was inoculated orally as in the previous section. When these birds had virtually ceased to shed the Nal^r strain in the faeces they were inoculated with a Spc^r mutant of the same strain. A second group was inoculated when it was 3 weeks old and a third was inoculated as ducklings by placing with them three ducklings of the same age which had been inoculated orally. In this case spread of infection to the initially uninfected birds was monitored. The fourth group was inoculated with the Spc^r mutant simultaneously with inoculation of the first group with this strain. All ducks inoculated when they were several weeks old were checked for freedom from salmonella infection by cloacal swab culture and an antibody response in a specific ELISA. The results of monitoring the faecal excretion of the inoculated strains in the ducks is shown in Table 6.

The number of ducklings excreting the Nal^r mutant in the first group fell at a faster rate than in the previous experiment. They had virtually ceased to shed the inoculated strain by 4 weeks post-inoculation (p.i.). The pattern of excretion in the ducklings infected by contact was very similar, there being slightly more ducks excreting at 2 weeks than 1 week p.i. More birds were excreting the inoculated strain when they were killed at 8 weeks p.i. than there were in the first group. Ducks infected when 3 weeks old, excreted the inoculated organism for only 1 week, isolations only being made for the first week p.i. When the first and fourth groups were infected with the Spc^r mutant this strain was excreted by a small number of ducks and for a short period of time. Isolations were made from the first group (previously infected with the Nal^r mutant) and from the fourth group (previously uninfected) on one and three occasions respectively.

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Table 6. *Faecal excretion of* S. enteritidis *125589 following oral inoculation of the Nal^r mutant in birds under different conditions and reinoculation with the Spc^r mutant of the same strain*

Mutant cultured	Weeks after initial inoculation	Number of birds per group	Number of ducks (expressed as a percentage) excreting the Nal^r or Spc^r mutant of *S. enteritidis* 125589 when inoculated orally*											
			Direct (ducklings)			Contact (ducklings)			Direct (3-week-old ducks)			Direct (8-week-old ducks)		
			50†	D	T	50	D	T	50	D	T	50	D	T
Nal^r	1	20	9	50	80	10	30	65	—	—	—	—	—	—
	2	19–20	11	63	84	10	30	80	—	—	—	—	—	—
	3	19–20	5	26	47	0	15	55	—	—	—	—	—	—
	4	16–17	6	18	21	0	10	25	0	22	50	—	—	—
	5	16–17	0	0	0	0	0	10	0	0	0	—	—	—
	6	15–17	0	0	6	0	0	20	0	0	0	—	—	—
	7	12–16	0	0	0	0	5	20	0	0	0	—	—	—
Spc^r	8	13	0	0	8	—	—	—	—	—	—	0	0	0
	9	13	0	0	0	—	—	—	—	—	—	0	0	8
	10	10–13	0	0	0	—	—	—	—	—	—	0	0	20
	10 (Caeca)	6–7	0	0	0	—	—	—	—	—	—	0	0	0

* All ducklings inoculated orally with approximately 8×10 c.f.u. of Nal^r mutants (day 0 or week 3) or Spc^r mutant (week 8) in 0·3 ml. The exception was group 2 (orally-contact) in which 3 of 23 ducklings were inoculated. The data for this group are taken from the excretion results of the 20 ducklings in contact with the 3 inoculated ducklings.
† 50, 50 colonies per plate; D, 1 colony per plate; T, salmonella isolated by direct plating or by enrichment.

Table 7. *Numbers of organisms of* S. enteritidis *125589 in the spleen and alimentary tract following oral inoculation of the Nal^r mutant in birds under different conditions and reinoculation with the Spc^r mutant*

Mutant counted	Weeks after initial inoculation	Tissue sampled	Log_{10} median viable count of *S. enteritidis* 125589 in the tissues of three ducks when inoculated orally*			
			Direct (ducklings)	Contact (ducklings)	Direct (3-week-old ducks)	Direct (8-week-old ducks)
Nal^r	$3\frac{1}{2}$	Crop	3·0	2·6	< 2	—
		Ileum contents	3·7	3·2	2·6	—
		Ileum wall	3·6	2·0	4·0	—
		Caecal contents	4·6	4·2	4·0	—
		Caecal wall	3·7	3·7	4·6	—
		Spleen	< 2	< 2	< 2	—
	7	Crop	< 2	< 2	< 2	—
		Ileum contents	< 2	2·7	< 2	—
		Ileum wall	< 2	2·0	< 2	—
		Caecal contents	2·5	2·7	< 2	—
		Caecal wall	< 2	< 2	< 2	—
		Spleen	< 2	< 2	< 2	—
	8	Crop	< 2	—	—	< 2
		Ileum contents	< 2	—	—	< 2
		Ileum wall	< 2	—	—	< 2
		Caecal contents	< 2(−)†	—	—	< 2(−)†
		Caecal wall	< 2(−)†	—	—	< 2(3·5, +)‡
		Spleen	< 2	—	—	< 2
Spc^r	9	Crop	< 2	—	—	< 2
		Ileum contents	< 2	—	—	< 2
		Ileum wall	< 2	—	—	< 2
		Caecal contents	< 2(−)†	—	—	< 2(−)†
		Caecal wall	< 2(−)†	—	—	< 2(+, +)§
		Spleen	< 2	—	—	< 2

* All ducklings and ducks inoculated orally with approximately 5×10^8 c.f.u. of Nal^r mutants (day 0 or week 3) or Spc^r mutant (week 8) in 0·3 ml. The exception were the contact group where the birds sampled were infected by placing them in contact with birds inoculated directly (see Table 5).

† All three samples also yielded no growth of *S. enteritidis* after enrichment in sodium selenite broth.

‡ The highest count was (log_{10}) 3·5 and one sample yielded growth of *S. enteritidis* after enrichment.

§ Two of the three samples yielded growth of *S. enteritidis* after enrichment.

The viable counts of the inoculated *S. enteritidis* mutants in the spleen and intestines of additional birds housed with the above groups on litter floors and which were removed at intervals for post-mortem examination are shown in Table 7. The salmonella counts in the ducks infected individually as ducklings and by contact were again similar, although no organisms were found in the spleen 2 weeks after inoculation. Despite the lower excretion rate only slightly lower salmonella numbers were isolated from the tissues of ducks infected at 3 weeks. No isolations of the Spc^r mutant were made from the group previously infected with the Nal^r mutant, including after enrichment culture of the caecal contents and wall, whereas positive isolations by enrichment culture were made from caecal wall samples from four ducks in the group infected first at 8 weeks of age. This difference was significant with $\chi^2 = 3{\cdot}08$ and $P = 0{\cdot}03$. Countable numbers of the Spc^r mutant were found in the caecal wall of one duck from this group but not from the group previously infected at 1 day of age. This was also significant ($\chi^2 = 3{\cdot}6$, $P = 0{\cdot}04$).

Table 8 shows that the circulating IgG antibody titres in the ducks on receipt (before infection) were high (between 1:256 and 1:1024, log_2 8–10). These titres fell to a value of log_2 5·6 (approx. 1:50) over several weeks in the ducks which were not infected until they were 8 weeks of age and increased in these birds after infection to log_2 12·1 (approx. 1:5000). However, although similar changes were seen in the

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Table 8. *Log_2 mean specific IgG antibody titre in serum from ducks*

Weeks after initial inoculation	Log_2 mean specific IgG titre (of seven ducks) in LPS based ELISA when ducks were inoculated*			
	Direct (ducklings)	Contact† (ducklings)	Direct (3-week-old ducks)	Direct (8-week-old ducks)
1	9·8	9·0	—	8·9
2	12·5	9·0	—	6·1
3	7·9	7·0	—	5·6
4	8·2	6·6	9·1	6·9
5	9·5	9·4	10·4	6·6
6	9·8	8·3	10·1	7·8
7	10·8	8·3	10·5	8·8
Birds challenged				
8	10·4	—	—	9·8
9	9·5	—	—	12·1
10	—	—	—	—

* See Table 6 for explanation of inoculations.
† Mean titre of sera from seven birds from contact group, i.e. the three ducks inoculated as the feeder group were not bled.

Table 9. *Exclusion effect on* S. enteritidis *P125109 Nal^r produced by pre-colonization with* S. enteritidis *P125109 Spc^r or* S. enteritidis aroA *Spc^r*

Bird group	Pre-colonizing strain*	Challenge strain†	Mean viable count of strain	Mean log_{10} viable count (of three birds) of pre-colonizing and challenge strains at the following days after inoculation of pre-colonizing strain		
				1	2	4
1	P125109 Spc^r	P125109 Nal^r	Pre-colonizing	7·03	7·75	8·14
			Challenge	—	< 2·8	< 2·8
2	*aroA*	P125109 Nal^r	Pre-colonizing	2·80	4·07	4·44
			Challenge	—	4·01	6·29
3	None	P125109 Nal^r	Challenge	—	5·87	6·87

* Ducks inoculated with 10^8 c.f.u. in 0·3 ml.
† Ducks inoculated with 10^6 c.f.u. in 0·3 ml.

other groups they were not nearly so marked as in this group. In fact, in the ducklings and older ducks infected directly by the oral route it was difficult to determine by ELISA whether or not they had been infected.

The effect of pre-colonization of the intestine of ducklings with a wild-type of *aroA* derivative of a *S. enteritidis* strain on the establishment in the gut of the parent strain inoculated orally 24 h later is shown in Table 9. The wild-type strain (P125109 Spc^r) colonized the gut well throughout the short experiment in contrast to the *aroA* mutant which colonized in concentrations 3–4 logs lower. In comparison with the control group which was challenged and showed good establishment in the caeca of the parent wild-type strain (P125109 Nal^r), pre-colonization with P125109 Spc^r completely prevented recovery of the challenge strain whereas this strain was found in increasing numbers in the ducklings infected with the *aroA* strain.

DISCUSSION

We have examined the pathogenicity of different salmonella strains for a line of commercial ducks bred extensively in the United Kingdom and elsewhere and have attempted to explain these findings in terms of the pathogenesis of salmonella as it is understood and modelled in chickens. The intestinal colonization parameters and some of the associated serological

https://doi.org/10.1017/S0950268899002605 Published online by Cambridge University Press

responses have also been studied. In addition the possibility of immune protection has been demonstrated.

In contrast to past findings indicating that certain serotypes, particularly *S. typhimurium*, are the cause of morbidity and mortality in ducklings, especially during the first 2 weeks of life [6, 7] our findings indicated a high level of resistance to systemic disease and diarrhoea, if this latter exists as a separate clinical entity. No lesions were present at post-mortem examination and the organism was not recovered from the liver indicating either poor invasiveness and/or, poor survival of the strain in the reticuloendothelial system. Similar findings were obtained with a 10× dose of *S. typhimurium* F98 and *S. gallinarum* 9, strains which, in susceptible newly-hatched chickens are able to produce levels of mortality reaching 100% [14, 15, 24]. The LD_{50} values also were much higher than might be expected even for resistant lines of newly hatched chickens [25, 26]. By this route of inoculation *S. enteritidis* was more virulent than the other strains tested whereas *S. gallinarum* was totally avirulent. This degree of avirulence in the avian typhoid organism was surprising but has been observed before [27, 28]. Circumstantial evidence suggests that there is little systemic salmonellosis in the field in very young ducks but that some occurs after cold stress, with the appearance of characteristic lesions (R. Henry, personal communication).

All four organisms tested were invasive, i.e. they were isolated in quantifiable numbers from the spleen by 2 days post-oral inoculation. The numbers of *S. gallinarum* in the intestine were lower than the other serotypes. This was expected as this strain also colonizes the gut of chickens poorly, probably because of its auxotrophic nutritional characteristic. Despite this, one day after inoculation, *S. gallinarum* was isolated from intestinal wall samples in densities greater than those found in the lumen, indicating active invasion as has been described for highly invasive salmonella organisms [15]. This was also the case with the other serotypes examined by 2 days post oral inoculation. That this relationship between mucosal and contents counts indicates invasion rather than adhesion as part of colonization is supported by the generally low counts of the strains in the ileum and by the fact that it has been found previously with invasive rather than colonizing salmonella strains [29].

Following intravenous inoculation none of the three strains (*S. gallinarum*, *S. enteritidis* or *S. heidelberg*) was eliminated rapidly from the liver or spleen. The time course of this was similar to that seen with elimination of *S. typhimurium* strains after intravenous inoculation of 3-week-old chickens. However, unlike the situation in chickens where bacteria are removed within minutes from the blood by the reticuloendothelial system, salmonella organisms could be isolated from the blood of the ducks in quantifiable numbers. This suggests that the cells of the reticuloendothelial system of the duck are less phagocytic than in the chicken, but supportive information is not available [30]. All the strains tested were complement resistant as indicated by their survival in normal duck serum. As with chickens this does not explain their persistence in the tissues which, with the greater counts in the spleen than in the blood, suggests intracellular survival [31]. The pathogenesis results indicate that as with innate resistance observable with a host and a salmonella serotype which is not adapted to it [15], and also with genetic lines of chickens resistant to systemic infection [25], the main site at which this resistance is expressed is the reticuloendothelial system.

Given the previous literature indicating that systemic disease in ducks with certain salmonella serotypes can occur it might indicate that the line of ducks studied here are fortuitously innately resistant to systemic salmonellosis. This would result in smaller losses in ducklings that might otherwise occur and might also indicate a higher resistance to ovarian infection in older birds.

There seemed to be no major differences amongst the four prototrophic salmonella strains tested for their ability to colonize the alimentary tract resulting in faecal excretion. All strains colonized well showing that salmonella infection in ducks represents a potential public health problem. Despite the fact that relatively few ducks were allowed to survive until the end of the experiment (6 weeks), infection was still present in a number of these (16/19). When the experiment was repeated on a second occasion using the *S. enteritidis* strain levels of excretion were reduced. Such variation between different batches of birds also occurs naturally with chickens and may result from differences in the gut flora acquired by different groups of birds. Similar levels of excretion were seen when the ducklings were infected by contact with orally inoculated ducklings but much greater reductions were recorded when 3-week-old ducks were infected, no isolations being made after the first week. This elimination is too rapid for an immuno-

logical explanation and it is most likely that acquisition of an inhibitory gut flora occurs at the birds mature. Theoretically, this could be exploited by adapting to ducks the competitive exclusion principle that is used extensively in chickens. In this, cultures of the gut flora found in salmonella-free adult hens are administered orally to newly-hatched chickens which, within hours, acquire the resistance possessed by the adult. This is currently being assessed in ducks but it does not seem to be as effective as in chickens (C. Impey, personal communication).

As occurred with salmonella colonization of chickens [29, 32–34] the caeca were the main sites of colonization and the crop-like oesophageal pouch less so. Organisms were still present in considerable numbers in the caecal contents after 3 weeks when none or very few were found in the crop. Very similar caecal counts were found at the beginning and the end of the experiment.

The ducks responded serologically to intestinal infection but with very low titres. There was evidence of a decline in the antibody titre with time in the very young birds suggesting transfer of maternal antibody by absorption of egg yolk. However, the titres did not increase greatly after infection. This was not due simply to non-responsiveness to LPS because the same occurred with flagella antigen (data not shown). High circulating antibody titres to both these antigens are induced following *S. enteritidis* in chickens [23]. Variable responses to infection measured by serology have been recorded before and the antibody responsiveness of ducks to infection is thought to be very poor in comparison with other species [30].

Ducks, infected at 8 weeks of age, were very resistant to oral challenge, resulting in very few isolates of the inoculated *S. enteritidis*. However, it was clear that some degree of immune protection had occurred in the ducks which had been infected as ducklings and had been allowed to clear themselves of infection before challenge at this time. This was apparent from the caecal swabs and to a greater extent from the quantitative bacteriology. This indicates that vaccination of ducks with a live attenuated salmonella may be an option for increasing the resistance of ducks to oral infection and this should be studied further. Extensive colonization of the gut of ducklings also produced the exclusion effect against subsequent colonization by a second strain as has been found in newly-hatched chickens [35, 36]. This suggests that, as with chickens, some live attenuated salmonella vaccines (not *aroA*) might be administered orally to ducklings immediately after hatching which should increase their resistance to sources of infection, such as hatchery-derived infections, the environment, etc. This is now being contemplated and recommended for chickens and it may help increase the resistance of ducks against intestinal salmonellosis. However, the possible entry of these vaccines into the human food chain must also be considered and vaccine strains should be no more virulent than the commensal *E. coli* which are undoubtedly consumed in much higher numbers and are normally present on poultry carcasses.

ACKNOWLEDGEMENTS

The authors would like to thank Mr A. Gray for technical assistance and Mr R. Henry, Cherry Valley Ducks, for assistance in various ways and scrutinizing the manuscript.

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