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Analysis of *Salmonella enterica* Serotype-Host Specificity in Calves: Avirulence of *S. enterica* Serotype Gallinarum Correlates with Bacterial Dissemination from Mesenteric Lymph Nodes and Persistence In Vivo

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Host and bacterial factors that determine whether *Salmonella* serotypes remain restricted to the gastrointestinal tract or penetrate beyond the mucosa and cause systemic disease remain largely undefined. Here, factors influencing *Salmonella* host specificity in calves were assessed by characterizing the pathogenesis of different serotypes. *Salmonella enterica* serotype Dublin was highly virulent intravenously, whereas *S. enterica* serotype Choleraesuis was moderately virulent. Both serotypes were virulent in calves infected orally. In contrast, *S. enterica* serotypes Gallinarum and Abortusovis were avirulent by either route. Serotypes Dublin, Gallinarum, and Abortusovis colonized the intestinal tract 24 h after oral inoculation, yet only serotype Dublin was consistently recovered from systemic tissues. Serotypes Dublin and Gallinarum invaded bovine intestines in greater numbers and induced greater enteropathogenic responses than serotypes Choleraesuis and Abortusovis. However, only serotype Dublin was able to persist within the intestinal mucosa, and use of a novel cannulation model demonstrated that serotype Dublin was able to pass through the mesenteric lymph nodes in greater numbers than serotype Gallinarum. Together, these results suggest that initial interactions with the intestinal mucosa do not correlate with host specificity, although persistence within tissues and translocation via efferent lymphatics appear to be crucial for the induction of bovine salmonellosis.

Within the species *Salmonella enterica* there are >2,000 different serotypes, including bacteria of great veterinary and medical significance, that can result in symptoms ranging from mild enteritis to severe systemic disease. Virulent *Salmonella* serotypes can differ greatly in their degree of host specificity (7, 27, 43). At present the genetic and phenotypic characteristics that limit the pathogenicity of certain serotypes in particular hosts are not fully understood. In cattle, *S. enterica* serotype Dublin is considered to be the predominant host-restricted serotype (41, 42, 46). Other serotypes, for example, *S. enterica* serotype Typhimurium, may also cause disease in cattle, but in contrast to serotype Dublin, serotype Typhimurium is frequently associated with intestinal infections in a wide range of phylogenetically unrelated species (29). Serotype Dublin, however, may also cause disease in a limited range of other mammals, including sheep and humans (10, 15, 41, 42). Similarly, *S. enterica* serotype Choleraesuis, which is regarded as being host restricted to pigs, may also cause disease in a limited number of other species (1, 10). On the other hand host-specific *S. enterica* serotypes such as Typhi, Gallinarum, and Abortusovis are typically associated with systemic disease and often abortion in phylogenetically closely related species only, namely, humans (22), fowl (39), and sheep and goats (24), respectively.

Several factors that affect the virulence of a given serotype for a particular host have been proposed. These include intes-

tinal invasion and destruction of the mucosal epithelium (35), avoidance of the induction of an intestinal inflammatory response (20, 32, 44), and survival within macrophages (47). In mice, serotype Typhimurium is more efficient at invading and destroying the intestinal epithelium than serotype Typhi (35), and serotype Typhimurium can persist in higher numbers than serotype Typhi in primary murine macrophages in vitro (47). These parameters correlate with the relative virulence of serotypes Typhimurium and Typhi in mice. However until the precise mechanisms of pathogenicity in different serotype-host combinations are fully understood, the importance of these two observations cannot be inferred. Furthermore, similar experiments quantifying the magnitude of intestinal invasion of different serotypes in cattle, pigs, and sheep (9, 44) and survival within porcine macrophages (53) clearly demonstrate that these parameters do not necessarily correlate with host specificity in other combinations of serotype and host.

The transmission of a given serotype to its specific host is also important in determining the incidence of salmonellosis (27). This has, however, received relatively little study. It is possible that the mechanism of transmission in diverse combinations of serotype and host may be different and may not directly correlate with virulence. For example, serotype Abortusovis infection of pregnant ewes typically has a low mortality rate, and animals may appear almost asymptomatic until the ewe aborts a heavily infected fetus, allowing the spread of the organism to other animals (24). Serotype Abortusovis is relatively poorly invasive and induces little damage in ovine intestines (44). Thus, this serotype could be described as having low

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virulence for adult sheep but high specificity attributable to its successful mode of transmission.

In order to elucidate the molecular basis of serotype-host specificity, one must consider as a whole the virulence, the multiple steps involved in pathogenesis, and the modes of transmission of different serotypes in a given host. In this study, the degrees of virulence of different *Salmonella* serotypes for cattle were compared. *Salmonella*-host interactions were characterized at various stages of pathogenesis, including initial colonization of tissues, intestinal invasion, induction of enteropathogenic responses, translocation to systemic sites, and survival within intestinal and systemic tissues. The results obtained in this study provide insight into the mechanisms involved in the ability of some serotypes, but not others, to be pathogenic in a given host.

MATERIALS AND METHODS

Bacterial strains. The serotypes used in this study were as follows: Typhimurium, strains ST4/74 (26) and ST12/75 (4); Dublin, strains SD3246 (19) and SD2229 (4); Choleraesuis, strains SCSA50 and SCS14/74 (9, 53); Gallinarum, strains SG9 (54) and SGJ91 (12); and Abortusovis, strains SAO44 (13) and SAO15/5 (28). The virulence and magnitude of intestinal invasion of these strains have recently been characterized in sheep (44), pigs (53), and chickens (personal communication). The strains were stored as mid-log-phase cultures in Luria-Bertani (LB) broth containing 10% glycerol at -70°C and were streaked onto LB agar plates and incubated overnight at 37°C for use.

Calf infection studies. All animal experiments were conducted according to the requirements of the Animal Scientific Procedures Act (1986). Friesian bull calves, aged approximately 28 days, were housed in a high containment unit and fed twice daily on a diet of reconstituted dried milk and unmedicated weaner nuts, with water freely available. All experimental animals were prescreened for the presence of *Salmonella* as described previously (49). None of the calves excreted *Salmonella* either at 1 week or immediately before infection. *Salmonella* cultures were prepared by inoculating Bacto Tryptose broth (25) with several bacterial colonies and incubating them statically at 37°C for 18 h. For oral inoculation, $8.9 \log_{10}$ CFU were diluted in 20 ml of antacid solution [sterile double-distilled water containing 5% (wt/vol) $\text{Mg}(\text{SiO}_3)_3$, 5% (wt/vol) NaHCO_3 , and 5% (wt/vol) MgCO_3]. The inoculum was administered to each calf with the aid of a syringe immediately before the morning feeding. For intravenous (i.v.) inoculation, cultures were diluted in 1 ml of isotonic saline to give a final concentration of $6.0 \log_{10}$ CFU and injected directly into the jugular vein.

Following inoculation by either route, the animals were monitored for clinical signs of disease, and rectal temperatures were recorded twice daily. A cumulative daily scoring scheme was used to record the severity of diarrhea as described previously (48). Calves were killed with an overdose of barbiturate if predefined clinical end points (48) were met during the course of infection. The surviving animals were killed either 24 h or 7 days after inoculation. At postmortem examination, the numbers of bacteria present in the lung, spleen, liver, ileum, cecum, and colon and their associated lymph nodes, together with the intestinal contents, were determined by the viable-count method (48). Typically, bacteria were plated on modified brilliant green agar (CM 329; Oxoid, Basingstoke, Hampshire, United Kingdom) with the addition of 120 mg of sulfadiazine/liter, while serotype Abortusovis SAO44 was plated on Rambach agar (Merck Ltd., Lutterworth, Leicestershire, United Kingdom). The mucosa and contents of the rumen, reticulum, omasum, and abomasum were sampled, in addition to the tissues listed above, in calves killed after 24 h. The limit of accurate quantification of the viable-count method is $2.0 \log_{10}$ CFU/g of tissue. Any samples containing fewer bacteria were enriched in Rappaport and selenite brilliant green broth (49). Samples that were positive on enrichment were assumed to contain between 1 and 100 CFU/g and were given a value of $2.0 \log_{10}$ CFU/g, while those that were negative were given a value of 0.

Bovine ligated-ileal-loop model for quantification of intestinal invasion, enteropathogenesis, and bacterial persistence. Modifications to the bovine ligated-ileal-loop model used by Watson et al. (51), Wallis et al. (48), and Bispham et al. (8) were employed to quantify intestinal invasion, enteropathogenesis (secretory and inflammatory responses), and bacterial persistence, respectively. *Salmonella* cultures were incubated overnight at 25°C with agitation, diluted approximately 1 in 3 in fresh LB broth, and incubated for a further 2 h at 37°C with agitation. The bacterial concentration was adjusted, by the addition of LB broth, to 6.7

\log_{10} CFU/ml for the invasion and persistence assays and $8.3 \log_{10}$ CFU/ml for the enteropathogenesis experiments. The calves were anesthetized, and intestinal loops either 9 (invasion and persistence) or 6 cm (enteropathogenesis) long, spaced 1 cm apart, were constructed in the distal or midileum, respectively, using surgical silk. In calves, the distal ileum contains a long strip of continuous Peyer's patch (34), and so a direct evaluation of the relative invasiveness of different serotypes for Peyer's patch mucosa and absorptive epithelium could be made. A total of 5 ml of *Salmonella* suspension was injected into each test loop, and the same volume of sterile LB broth was used as a negative control. For quantifying bacterial invasion and persistence, the loops were exteriorized after 1 h, and 5 ml of solution GC/Tcm₁₀ (3) containing 300 μg of gentamicin/ml was injected into each loop prior to reinternalization of the loop and repair of the wound. The invasion loops were left in situ for another hour, while those used for determination of bacterial persistence were left in situ for another 10 h. Different calves were used in experiments assessing either bacterial invasion or persistence. After the appropriate incubation period, the calves were killed with an overdose of barbiturate. The numbers of bacteria present in circular biopsy specimens of 6-mm radius were determined by the viable-count method as described previously (51).

For quantifying enteropathogenesis, 50 ml of blood was withdrawn immediately after loop inoculation and mixed with 9 ml of acid-citrate (trisodium citrate, 85 mM; citric acid, 46 mM; pH 4.9). Polymorphonuclear leukocytes (PMN) were isolated using the method of Carlson and Kaneko (11) and labeled with ^{111}In -oxinate (Mallinckrodt, Petten, The Netherlands) as described previously (48). At 12 h after incubation, the calves were killed with an overdose of barbiturate and the loops were removed. The secretory responses were defined as the ratio of fluid within the loop lumen to the length of the loop. The PMN influx was defined as the ratio of γ emission from radiolabeled PMN within the test loop mucosa and contents to that from the negative control loops.

Cannulation of efferent lymph vessels from mesenteric lymph nodes draining infected ileal loops. Inocula were prepared at a concentration of $\sim 8.9 \log_{10}$ CFU/ml, and calves were prepared for surgery as described above. A single ligated ileal loop of 80 cm flanked by two loosely tied spacers was constructed in the distal ileum. An efferent lymphatic vessel from a mesenteric lymph node draining the ligated section of the intestine was cannulated. The area of mesentery containing the cannulated vessel was sutured onto a piece of corrugated plastic; the ligated ileal loop, together with the attached cannula, was replaced within the abdominal cavity; and the wound was repaired. Preinfection lymph was collected continuously for 2 h at room temperature in tubes containing heparin at 50 U/ml. The distal ileum was exteriorized, and the ligated loop was inoculated with 50 ml of the test strain via the loosely tied spacers prior to loop replacement and wound repair. Care was taken not to inject straight into the loop in order to reduce the accidental administration of bacteria directly into a blood vessel. Lymph was collected continuously for another 10 h as described above, and the number of bacteria present in lymph collected over 2-h periods was determined by the viable-count method.

Statistical analyses. All viable-count data were normalized by logarithmic transformation. Unless otherwise stated, data were statistically analyzed using a two-way analysis of variance and the general linear model (software supplied by Minitab Inc., State College, Pa.) for the effect of the serotype and, when appropriate, the strain on the particular assay used. Subsequently, pairwise comparisons between strains and serotypes were performed. In the event that any differences were found, the Student *t* test was applied. The variance used in the *t* test was obtained from the adjusted mean square calculated in the analysis of variance. Standard errors of the mean in the histograms are based on an estimate of the variance calculated from the observations for each group and were not calculated using the pooled variance from the analysis of variance. The effects of serotype on rectal temperature were tested by means of an F test with the data taken as repeated measurements (Procedure Mixed [Statistical Analysis Systems; SAS Institute, Cary, N.C.]). Analyses of data from the cannulation and persistence assays were performed by means of paired *t* tests (Minitab Inc.).

RESULTS

Virulence of serotypes Dublin, Gallinarum, and Abortusovis, but not serotype Choleraesuis, in orally inoculated calves correlates with their predicted specificities for cattle. The virulence of serotypes Dublin, Choleraesuis, Gallinarum, and Abortusovis in cattle was assessed by daily monitoring of rectal temperatures and the production and severity of diarrhea for up to 7 days after oral inoculation. Bacterial recovery from

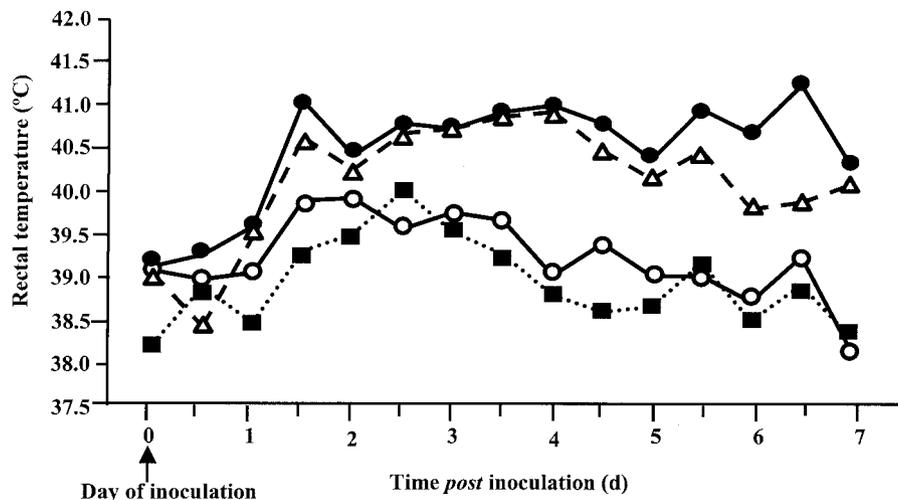


FIG. 1. Least-squares mean daily pyrexemic responses following oral inoculation of calves with serotype Dublin SD3246 (●), Choleraesuis SCSA50 (△), Gallinarum SG9 (○), or Abortusovis SAO44 (■). Each datum point is derived from the mean of six SD3246- or SCSA50-infected calves per group and three SG9- or SAO44-infected calves per group. The SEM for SD3246 and SCSA50 is 0.317, while the SEM for SG9 and SAO44 is 0.351.

selected systemic and enteric sites was determined following postmortem examination. All four *Salmonella* serotypes induced an increase in rectal temperature 1 to 2 days after inoculation. This response was severe and prolonged in six calves per group inoculated with either serotype Dublin or Choleraesuis but mild and transient in three calves per group inoculated with either serotype Gallinarum or Abortusovis (Fig. 1). From 2 days after inoculation, the rectal temperatures induced by serotypes Dublin and Choleraesuis were greater than those induced by serotype Gallinarum or Abortusovis ($P < 0.07$ to < 0.001). Only calves inoculated with serotype Dublin or Choleraesuis became dull and anorexic and developed diarrhea. Three out of six calves infected with each of these serotypes reached predefined clinical endpoints before 7 days after inoculation (Table 1) and were therefore humanely killed as indicated in Materials and Methods. At postmortem examination, only animals infected with either serotype Dublin or Choleraesuis had intestinal changes typical of acute salmonellosis, including necrotic, reddened ileal and colonic mucosa and enlarged, necrotic mesenteric lymph nodes. In general, serotypes Dublin and Choleraesuis were recovered from systemic and intestinal tissues in comparable numbers. Serotypes Gallinarum and Abortusovis were recovered from the intestinal mucosa and lymph nodes by enrichment culture only and were not recovered at all from intestinal contents or systemic

TABLE 1. Clinical findings for calves inoculated by the oral route with *S. enterica* serotype Dublin, Choleraesuis, Gallinarum, or Abortusovis

Strain	No. surviving/ no. inoculated	Mean time of euthanasia (days) \pm SEM	Mean diarrhea score \pm SEM
SD3246	3/6	5.8 \pm 0.6	12.7 \pm 4.2
SCSA50	3/6	5.3 \pm 0.8	11.8 \pm 2.3
SG9	3/3	7.0 \pm 0.0	0.7 \pm 0.7
SAO44	3/3	7.0 \pm 0.0	0.3 \pm 0.3

tissues (Table 2). These data demonstrate that serotypes Dublin and Choleraesuis are more virulent than serotypes Gallinarum and Abortusovis following oral inoculation of calves. With the exception of serotype Choleraesuis, these data correlate with the degree of specificity of these serotypes in cattle based on epidemiological data (41, 42). Thus, it can be concluded

TABLE 2. Recovery of *S. enterica* serotypes Dublin, Choleraesuis, Gallinarum, and Abortusovis from selected systemic and enteric sites up to 7 days after oral inoculation of calves^a

Tissue or material	Bacterial recovery (log ₁₀ CFU/ml \pm SEM)			
	SD3246	SCSA50	SG9	SAO44
Blood	0	0	0	0
Liver	3.02 \pm 0.21	2.31 \pm 0.55	0	0
HLN	2.62 \pm 0.23	2.89 \pm 1.11	0	0
Lung	3.48 \pm 0.24	3.15 \pm 0.34	0	0
MDLN	2.42 \pm 0.19	2.01 \pm 0.48	0	0
Spleen	2.94 \pm 0.22	E (3/6)	0	0
Intestinal contents				
Ileum	2.18 \pm 1.13	4.94 \pm 0.85	0	0
Cecum	4.35 \pm 0.99	5.23 \pm 0.64	0	0
Colon	3.63 \pm 1.38	5.37 \pm 0.69	0	0
Intestinal mucosa				
Ileum	4.47 \pm 0.69	5.80 \pm 0.72	E (1/3)	E (1/3)
Cecum	5.05 \pm 0.32	5.54 \pm 0.62	E (2/3)	E (2/3)
Colon	4.54 \pm 0.38	4.79 \pm 1.12	E (2/3)	0
Mesenteric lymph nodes				
Ileum	4.30 \pm 0.41	4.75 \pm 1.07	E (1/3)	E (1/3)
Cecum	4.67 \pm 0.21	5.40 \pm 0.63	E (3/3)	E (2/3)
Colon	4.12 \pm 0.31	4.85 \pm 1.05	2.43 \pm 0.43	E (1/3)

^a Triplicate samples were taken from each tissue or material, and means were calculated to give a value per animal. Each figure represents the mean of six calves each for serotypes Dublin (SD3246) and Choleraesuis (SCSA50) and three calves each for serotypes Gallinarum (SG9) and Abortusovis (SAO44) with the SEM. The limit of accurate quantification was 2.0 log₁₀ CFU/g, and samples that contained salmonellae in numbers too low to quantify are indicated by E (positive on enrichment culture) with the number of positive tissues/number of calves used in parentheses. HLN, hepatic lymph node; MDLN, mediastinal lymph node.

TABLE 3. Recovery of *S. enterica* serotypes Dublin, Gallinarum, and Abortusovis from selected enteric and systemic sites 24 h after oral inoculation of calves^a

Tissue or material	Bacterial recovery (log ₁₀ CFU/ml ± SEM)		
	SD3246	SG9	SAO44
Liver	E (5/5)	E (2/5)	E (0/3)
HLN	E (5/5)	E (0/5)	E (0/3)
Lung	E (4/5)	E (2/5)	E (0/3)
MDLN	E (2/5)	E (0/5)	E (0/3)
Spleen	E (5/5)	E (2/5)	E (0/3)
Intestinal contents			
Ileum	4.53 ± 0.61	2.88 ± 0.25	E (2/3)
Cecum	4.79 ± 0.42	3.01 ± 0.28	E (2/3)
Colon	5.14 ± 0.45	3.14 ± 0.27	E (2/3)
Intestinal mucosa			
Ileum	5.00 ± 0.43	3.87 ± 0.28	3.02 ± 0.36
Cecum	5.21 ± 0.21	3.89 ± 0.15	3.51 ± 0.76
Colon	3.89 ± 0.26	2.51 ± 0.21	2.57 ± 1.29
Mesenteric lymph nodes			
Ileum	4.67 ± 0.27	4.2 ± 0.03	3.28 ± 0.42
Cecum	4.78 ± 0.2	4.13 ± 0.1	3.25 ± 0.64
Colon	4.5 ± 0.3	2.87 ± 0.39	3.26 ± 0.66

^a Triplicate samples were taken from each tissue or material, and means were calculated to give a value per animal. Each figure represents the mean of five calves each for serotypes Dublin (SD3246) and Gallinarum (SG9) and three calves for serotype Abortusovis (SAO44) with the SEM. The limit of accurate quantification was 2.0 log₁₀ CFU/g, and samples that contained salmonellae in numbers too low to quantify are indicated by E (positive on enrichment culture) with the number of positive tissues/number of calves used parentheses. HLN, hepatic lymph node; MDLN, mediastinal lymph node.

that infection of cattle with different serotypes is a useful model to explore possible factors contributing to the low virulence of particular serotypes in a given host.

Serotype Dublin colonizes both intestinal and systemic sites in greater numbers than either serotype Gallinarum or Abortusovis during the initial stages of infection. To determine whether *Salmonella* serotypes, which differ in virulence for cattle, initially colonized the intestinal mucosa in similar numbers, two groups of five calves and one group of three calves were inoculated orally with serotype Dublin, Gallinarum, or Abortusovis, respectively. Bacteria were subsequently recovered from systemic and intestinal tissues 24 h after oral inoculation. Bacterial recovery from the stomach (omasum, abomasum, rumen, and reticulum) contents or mucosa was low, with serotypes Dublin and Gallinarum being occasionally isolated after enrichment culture only. However, serotype Dublin was the only serotype reproducibly recovered by enrichment culture from the majority of systemic tissues. Serotype Dublin was recovered in greater numbers than serotype Abortusovis from the ileum, cecum, and colon (P , <0.1 to <0.01) and in greater numbers than serotype Gallinarum from these sites (P , <0.1 to <0.01), with the exception of the lymph nodes associated with the ileum and cecum. In these tissues, despite the greater recovery of serotype Dublin, bacterial numbers in serotype Gallinarum-infected calves were statistically similar (P > 0.1) (Table 3). Serotype Gallinarum was recovered in numbers similar to those of serotype Abortusovis from all sites tested (P > 0.1). These data show that all three serotypes are

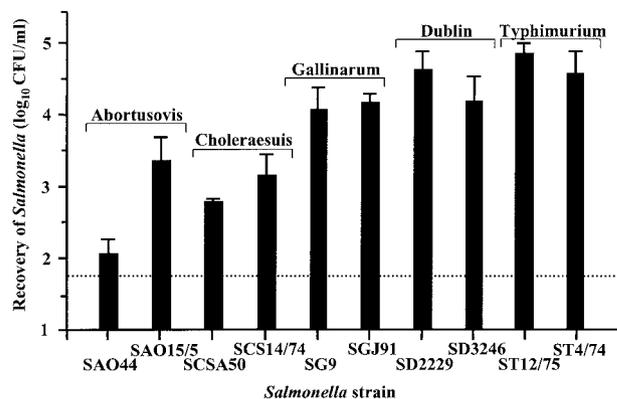


FIG. 2. Magnitudes of intestinal invasion of different *Salmonella* serotypes 2 h after infection of bovine ligated ileal loops. The results shown were derived from ligated loops constructed in the distal ileum containing absorptive epithelia. Triplicate samples were taken from two or three loops in each animal, and means were calculated to give a value per calf. Each bar represents the mean of two, three, or four calves plus the SEM. The dotted line represents the limit of accurate quantification.

able to colonize the intestinal mucosa and nodes. Therefore, the low rate of recovery of serotypes Gallinarum and Abortusovis from intestinal and systemic sites 7 days after oral inoculation cannot be accounted for by the killing of bacteria during passage through the stomachs and intestines. However, the reduced colonization of serotypes Gallinarum and Abortusovis correlated with the avirulence of these serotypes following oral inoculation. Possible reasons for this observation were investigated further.

Intestinal invasion and induction of enteropathogenic responses does not correlate with virulence of *Salmonella* serotypes following oral inoculation. *Salmonella* colonization of the intestines, as measured 24 h after oral inoculation, is affected by both initial intestinal invasion and subsequent bacterial persistence and growth. The contribution of intestinal invasion to both colonization and virulence was investigated using the bovine ligated-ileal-loop model. Between 6 and 12 individual loops per *Salmonella* strain were infected for 1 h, gentamicin was injected, and the loops were left for a further 1-h incubation prior to determination of the number of bacteria present within the mucosa by the viable-count method. The ligated-ileal-loop model allows several strains to be tested within one animal, and thus, it was possible to include two strains for each serotype. Serotype Typhimurium was also included in these experiments to allow a comparison to previous work performed in cattle (9, 51), pigs (9), sheep (44), and chickens (personal communication). For simplicity of interpretation, significance levels were determined from the mean values for two strains within a given serotype. The relative levels of invasiveness of the different serotypes for Peyer's patch mucosa and absorptive epithelia were also compared. As there were no significant strain differences in bacterial recovery from mucosa either with or without Peyer's patches or from mucosa in the distal compared to the midileum, results are presented based on data derived from the absorptive epithelia in the distal ileum only (Fig. 2). On average, serotype Typhimurium was more invasive than serotype Dublin (P < 0.01). These two

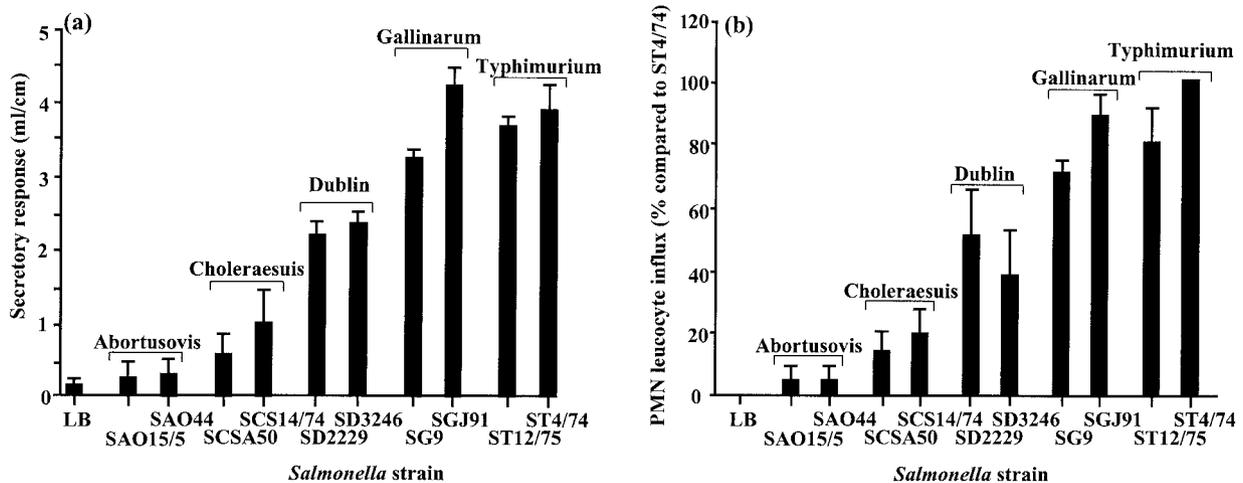


FIG. 3. Secretory and inflammatory responses induced by different *Salmonella* serotypes 12 h after infection of bovine ligated ileal loops. Each bar represents the mean response, per animal, derived from 2 or 3 loops per strain in three calves (total, 8 to 10 loops) plus the SEM. The secretory response (a) represents the mean volume/length ratio, and the PMN influx (b) is presented as a percentage compared to serotype Typhimurium ST4/74.

serotypes were more invasive than serotype Gallinarum ($P < 0.05$ to < 0.01), while serotype Choleraesuis was less invasive than serotype Gallinarum ($P < 0.01$) but more invasive than serotype Abortusovis ($P < 0.05$). The structural integrity of the intestinal mucosa was maintained during the infection, as assessed by microscopy (unpublished data), indicating that bacterial recovery would be unlikely to be affected by shedding of infected host cells or uptake of gentamicin, both of which may result from mucosal damage. These data show that serotypes Typhimurium, Dublin, and Gallinarum are more invasive in the bovine ileum than serotype Choleraesuis or Abortusovis, despite serotype Choleraesuis being more virulent than serotype Gallinarum following oral inoculation. In addition, the lower number of serotype Gallinarum cells than serotype Dublin cells able to colonize the bovine gastrointestinal tract after 24 h does not correlate with the intestinal invasion of this serotype 2 h after infection of ligated ileal loops.

The relationship between the enteropathogenicity and virulence of the different serotypes was investigated, again using the bovine ligated-ileal-loop model. Between 8 and 10 individual loops per strain were infected with *Salmonella*, and both the secretory response and PMN influx into the lumen and mucosa were quantified after 12 h. In order to obtain an average PMN influx for all calves, the results per animal were expressed as the mean percentage minus 1 (the value for the LB control loops) compared to the mean value for serotype Typhimurium ST4/74 (the strain that induced the greatest response) (Fig. 3). For simplicity of interpretation, significance levels were determined from the mean values for two strains within a given serotype. Serotypes Typhimurium and Gallinarum induced similar secretory responses ($P > 0.1$), while the PMN influx induced by serotype Typhimurium was greater than that induced by serotype Gallinarum ($P < 0.05$). These two serotypes induced greater secretory and inflammatory responses than serotype Dublin ($P < 0.001$), which in turn induced greater responses than serotype Choleraesuis ($P < 0.001$). Serotype Abortusovis induced little or no secretory

response or influx of inflammatory cells. The induction of enteropathogenic responses by the different serotypes clearly does not correlate with their relative virulence in cattle; however, there was a direct correlation between the magnitudes of the secretory and inflammatory responses. Furthermore, there was no direct correlation between the magnitude of enteropathogenic responses and intestinal invasion, as despite being similarly invasive, serotype Gallinarum was significantly more enteropathogenic than serotype Dublin, indicating that serotype-specific factors independent of intestinal invasion influence enteropathogenicity. Therefore, further studies were carried out using these two serotypes.

Bacterial translocation of serotypes Dublin and Gallinarum from mesenteric lymph nodes correlates with their virulence following oral inoculation. Both serotypes Dublin and Gallinarum initially colonize and invade bovine ileum and lymph nodes in relatively high numbers. However, only serotype Dublin results in severe systemic disease in calves, which is reflected by the greater frequency with which serotype Dublin colonizes systemic sites, compared with serotype Gallinarum, following oral inoculation.

The translocation of serotypes Dublin and Gallinarum from ileal lymph nodes was quantified in four calves per serotype, using a novel cannulation model. An efferent lymph vessel from a node directly draining an area of infected intestinal mucosa was cannulated, and the number of bacteria within the lymph was determined by the viable-count method over a 10-h period (Fig. 4). The initial recovery rates of serotypes Dublin and Gallinarum were similar ($P > 0.1$), with a peak of approximately $3.0 \log_{10}$ CFU/ml at 2 h falling to approximately $1.5 \log_{10}$ CFU/ml at 4 and 6 h. However, from 8 and 10 h post-loop infection, serotype Dublin was recovered in significantly greater numbers than serotype Gallinarum ($P < 0.05$). These results demonstrate the potential of serotype Dublin to disseminate in higher numbers than serotype Gallinarum and correlate with systemic colonization and virulence following the oral inoculation of calves.

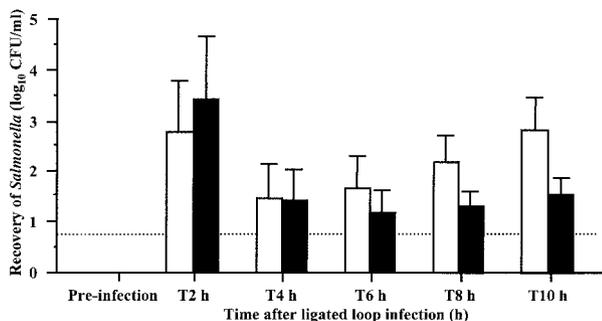


FIG. 4. Recovery of serotypes Dublin SD3246 (open bars) and Gallinarum SG9 (solid bars) from efferent lymph up to 10 h after infection of bovine ligated ileal loops. Each bar represents the mean of individual lymph samples from four calves per serotype plus the SEM. The dotted line represents the limit of accurate quantification.

Persistence of serotypes Dublin and Gallinarum within bovine ileal mucosa correlates with their virulence. The relative persistence of serotypes Dublin and Gallinarum in bovine tissue was assessed in ligated ileal loops. The loops were infected with either serotype for 1 h, as for the invasion assay, and then incubated with gentamicin for another 10 h, at which point the number of bacteria within the mucosa was determined by the viable-count method. Recovery of serotype Dublin was significantly higher than recovery of serotype Gallinarum ($P < 0.01$). The results shown (Fig. 5) are from a representative calf, with similar results obtained from two other animals (unpublished data). Recovery of serotype Dublin had increased by approximately 0.5 log₁₀ CFU/ml compared to the recovery in loops incubated for only 1 h with gentamicin, whereas the recovery of serotype Gallinarum fell by approximately 1.0 log₁₀ CFU/ml (Fig. 2). These results demonstrate that serotype Dublin is able to persist within bovine intestinal mucosa in greater numbers than serotype Gallinarum.

Recovery of serotypes Dublin, Gallinarum, and Abortusovis, but not Choleraesuis, from host tissues after i.v. inoculation correlates with virulence after oral inoculation. The persistence of bacteria within systemic tissues of three calves per *Salmonella* serotype was assessed 7 days after i.v. inoculation. Infection by this route allows direct bacterial access to systemic

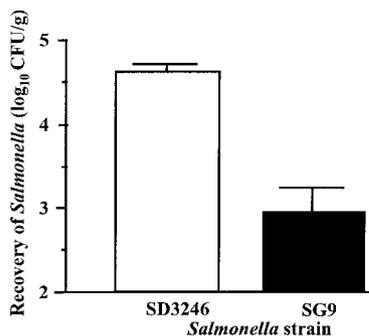


FIG. 5. Recovery of serotypes Dublin SD3246 and Gallinarum SG9 11 h after infection of bovine ligated ileal loops. Each bar represents the mean of three loops per serotype in a single animal plus the SEM based on the mean counts per serotype.

tissues, and any influence of the initial interaction with the intestines will have been bypassed. As none of the calves reached any of the predefined clinical endpoints, all of the animals were humanely killed 7 days after inoculation. All four *Salmonella* serotypes induced an increase in rectal temperature 1 to 2 days after inoculation. From 2 days onward, this increase was greater in calves infected with serotype Dublin than in calves infected with serotype Gallinarum or Abortusovis ($P, < 0.05$ to < 0.001). Infection with serotype Choleraesuis resulted in a response similar to that to serotype Dublin for up to 3 days after inoculation, followed by a drop and then a further rise in temperature toward the end of the experiment (Fig. 6). Calves inoculated with serotype Dublin or Choleraesuis became dull, lethargic, and anorexic after approximately 36 h. These symptoms, accompanied by diarrhea, persisted with different magnitudes of severity in calves inoculated with serotype Dublin but not in those inoculated with serotype Choleraesuis. Serotypes Choleraesuis, Gallinarum, and Abortusovis did not induce any diarrhea. At postmortem examination, serotype Dublin was recovered in greater numbers than serotype Choleraesuis from the intestinal contents, intestinal mucosa, and systemic tissues ($P, < 0.1$ to < 0.05) and in similar numbers from the intestinal lymph nodes and the lymph nodes associated with the systemic tissues ($P > 0.1$). Serotype Gallinarum was detected, by enrichment culture only, from a limited number of systemic and enteric tissues, while serotype Abortusovis was not recovered from any site sampled (Table 4). Bacterial recovery of serotypes Dublin, Gallinarum, and Abortusovis following i.v. inoculation correlates with virulence and bacterial recovery after oral inoculation. Despite being able to invade and translocate from the intestinal mucosa, serotype Gallinarum is unable to persist within systemic tissues, and this correlates with its low virulence in calves. In contrast, despite being less invasive, less enteropathogenic, and only moderately virulent when administered by the i.v. route, serotype Choleraesuis is as virulent as serotype Dublin following oral inoculation. These results suggest that the pathogenesis of serotype Choleraesuis is quite distinct from that of serotype Dublin.

DISCUSSION

The early host-pathogen interactions that influence the outcome of infection with different *Salmonella* serotypes remain largely undefined. In this study, factors that determine whether *Salmonella* serotypes remain restricted to the gastrointestinal tract or penetrate beyond the mucosa and cause systemic disease in calves were assessed. Bacterial persistence, in both enteric and systemic sites, appeared to be crucial in determining the virulence of serotype Gallinarum in calves, and this finding correlated with bacterial dissemination from the intestines to systemic tissues.

Serotypes Dublin and Choleraesuis were highly virulent in orally inoculated calves, while serotypes Gallinarum and Abortusovis had low virulence. These results confirm the expected specificities of serotypes Dublin, Gallinarum, and Abortusovis, but not serotype Choleraesuis (41, 42), in this host. In addition, they extend observations made in previous studies showing that the same strains of serotype Choleraesuis, Gallinarum, or Abortusovis used in this study are virulent in pigs (53), chickens (personal communication), and lambs (44), respectively.

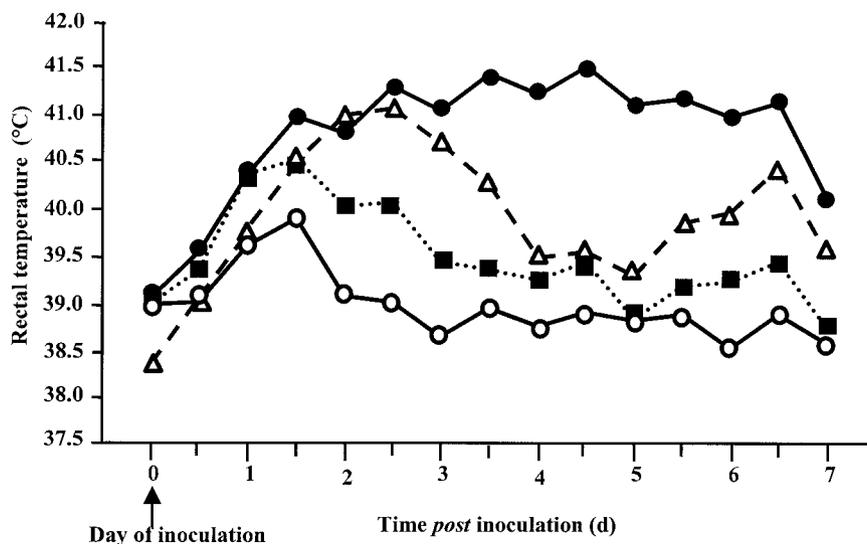


FIG. 6. Least-squares mean daily pyrexia responses following i.v. inoculation of calves with serotype Dublin SD3246 (●), *Choleraesuis* SCSA50 (△), *Gallinarum* SG9 (○), or *Abortusovis* SAO44 (■). Each datum point is derived from the mean of three calves per serotype. The SEM for all serotypes is 0.302.

Serotype *Choleraesuis* has previously been reported to be highly virulent in orally inoculated calves (40), so although our result is surprising, it is not without precedent. Infection of calves with either serotype *Gallinarum* or *Abortusovis* provides

TABLE 4. Recovery of *S. enterica* serotypes Dublin, *Choleraesuis*, *Gallinarum*, and *Abortusovis* from selected systemic and enteric sites 7 days after i.v. inoculation of calves^a

Tissue or material	Bacterial recovery (log ₁₀ CFU/ml ± SEM)			
	SD3246	SCSA50	SG9	SAO44
Blood	E (1/3)	0	0	0
Liver	3.90 ± 0.48	E (1/3)	0	0
HLN	3.37 ± 0.09	3.63 ± 0.07	E (1/3)	0
Lung	3.93 ± 0.51	2.57 ± 0.23	0	0
MDLN	3.05 ± 0.47	3.76 ± 0.21	E (1/3)	0
Spleen	3.84 ± 0.46	E (2/3)	0	0
Intestinal contents				
Ileum	4.24 ± 2.13	2.24 ± 0.24	E (1/3)	0
Cecum	5.21 ± 1.62	2.42 ± 0.42	0	0
Colon	5.16 ± 1.58	2.48 ± 0.48	0	0
Intestinal mucosa				
Ileum	4.45 ± 1.21	E (2/3)	0	0
Cecum	4.37 ± 1.19	2.50 ± 0.27	0	0
Colon	4.42 ± 1.24	E (2/3)	0	0
Mesenteric lymph nodes				
Ileum	4.13 ± 0.43	3.49 ± 0.25	E (1/3)	0
Cecum	3.85 ± 0.53	3.63 ± 0.24	0	0
Colon	3.67 ± 0.84	3.02 ± 0.51	0	0

^a Triplicate samples were taken from each tissue or material, and means were calculated to give a value per animal. Each figure represents the mean of three calves with the SEM. The limit of accurate quantification was 2.0 log₁₀ CFU/g, and samples that contained salmonellae in numbers too low to quantify are indicated by E (positive on enrichment culture) with the number of positive tissues/number of calves used in parentheses. HLN, hepatic lymph node; MDLN, mediastinal lymph node.

an excellent model to explore possible factors that may contribute to their low virulence in different animal hosts.

It is interesting to consider why serotype *Choleraesuis* is not associated with natural infection of calves despite being virulent and inducing severe diarrhea following experimental oral inoculation. One possible explanation is that serotype *Choleraesuis* is unable to circulate within or gain access to the bovine population in sufficient numbers to cause disease. Both fecal shedding from carrier animals and uptake into the nasal cavity as a result of the behavior and method of feeding of pigs are important in maintaining serotype *Choleraesuis* in pig herds (6, 17). Nasal inoculation results in more severe disease than oral inoculation, and so the infective dose for pigs by the nasal route may be lower. It could therefore be speculated that serotype *Choleraesuis* might be maintained in numbers high enough to infect pigs but too low to infect calves. An alternative explanation is that induction of infectious diarrhea in calves is insufficient to maintain serotype *Choleraesuis* on a long-term basis within the bovine population. Serotype Dublin is also able to establish a carrier state in adult animals, and this may act as a continual source of infection for calves. If serotype *Choleraesuis* is unable to establish its own carriage in cattle, this may limit its maintenance within the bovine population.

It has been proposed that serotype *Gallinarum* is avirulent in mice due to its inability to invade the murine intestinal tract (35). Here, we have shown that serotypes Dublin, *Gallinarum*, and *Abortusovis* are able to colonize and invade bovine intestines. This is evident from the recovery of bacteria from the mucosa and draining lymph nodes 24 h after inoculation and is suggested by an increase in rectal temperature 36 h after oral inoculation. Previously, a ligated-ileal-loop assay has been used to determine the ability of serotype Typhimurium to induce inflammatory and secretory responses in calves (49, 51, 52). The results obtained in this study confirm the reported highly invasive and highly enteropathogenic phenotypes of this sero-

type in calves. Intestinal invasion and the induction of inflammatory and secretory responses are key phases in the pathogenesis of serotypes Dublin and Typhimurium, as demonstrated by characterization of the phenotypes of isogenic mutants in bovine ligated ileal loops and orally inoculated calves. Strains that were noninvasive or nonenteropathogenic in ileal loops were attenuated following oral inoculation of calves (49, 51). Conversely, mutations that did not reduce enteropathogenic responses in ligated loops did not affect the severity of enteritis in orally infected calves (48). To our surprise, serotype Gallinarum proved to be as enteropathogenic as serotype Typhimurium and almost as invasive, yet more enteropathogenic, than serotype Dublin. Therefore, it is evident that serotype Gallinarum has the virulence determinants required for penetrating mammalian intestinal mucosa. However, for serotype Gallinarum, intestinal invasion and enteropathogenesis in ligated ileal loops do not correlate with virulence or serotype-host specificity. Clearly, the virulence factors necessary for invasion and enteropathogenesis in loops are not sufficient to induce disease in calves following oral infection. It is probable that in orally inoculated calves serotype Gallinarum is unable to grow or persist within the intestines to reach sufficient numbers to induce overt enteric disease. Interestingly, there have been some reports of serotype Gallinarum associated with outbreaks of enteritis in mammalian species, including humans (10, 18, 41). Most of these reports were published prior to the successful elimination of serotype Gallinarum from United Kingdom poultry flocks in the 1960s, and thus, it is possible that sufficiently high numbers of bacteria to cause disease entered the food chain. In complete contrast to serotype Gallinarum, serotype Choleraesuis is relatively poorly invasive and induces either low or no enteropathogenic responses in both calves and pigs, yet it is virulent following experimental oral and i.v. infection of both of these hosts (references 40 and 53, this study, and unpublished data). This is apparently analogous to the phenotypes of serotype Abortusovis in sheep (44), serotype Gallinarum in chickens (20), and serotype Typhi in humans (14, 33), where systemic pathogenesis in the natural target host species appears to correlate with the absence of an initial intestinal inflammatory response following invasion.

It would seem that factors other than those influencing intestinal invasion and elicitation of intestinal inflammatory and secretory responses contribute to *Salmonella* serotype-host specificity in calves. We have therefore developed a novel experimental model to study dissemination of *Salmonella* from intestinal to systemic sites. Both serotypes Dublin and Gallinarum were invasive and enteropathogenic yet exhibited contrasting virulence following oral or i.v. inoculation. Cannulation of efferent lymph vessels from mesenteric lymph nodes draining an infected ligated ileal loop demonstrated that serotypes Dublin and Gallinarum were initially able to translocate from the intestinal lumen to systemic sites in similar numbers. However, while the total number of disseminating bacteria decreased from 6 h after loop infection, serotype Dublin translocated in greater numbers than serotype Gallinarum. This result correlates positively with the ability of serotype Dublin, but not serotype Gallinarum, to induce disease in cattle. Thus, it appears that the mesenteric lymph nodes play an essential

role in the containment of serotype Gallinarum translocation from the intestinal mucosa to systemic tissues.

In this study, we have investigated the nature of host-pathogen interactions during the early phases of infection that might influence the development of systemic salmonellosis. Survival within cells of the reticuloendothelial system is believed to be a key stage in *Salmonella* pathogenesis (5, 16, 21, 30). Furthermore, there is some evidence to suggest that the ability to survive within such cells contributes to the host-specific phenotype in vitro (2, 23, 31, 37, 47) and in vivo (5). The situation in vivo is clearly much more complicated, as serotype Typhimurium has been shown to kill murine hepatic macrophages (36). Other studies using avian (20), murine (35), or porcine (53) macrophages infected with strains of defined virulence demonstrate no such correlation between bacterial persistence within macrophages and host specificity. Furthermore, comparing the relative persistence of serotypes Dublin and Gallinarum in bovine macrophages is difficult for two reasons. First, alveolar macrophages from this host are susceptible to *Salmonella*-induced cell lysis (50). Second, the difference between motile and nonmotile serotypes, for example, serotypes Gallinarum and Pullorum, has a profound impact on their ability to interact with macrophages in vitro, thereby making results from such studies difficult to interpret (20, 55).

The persistence of serotype Dublin was significantly greater than that of serotype Gallinarum in the intestinal mucosa both following oral inoculation and 11 h after the infection of ligated ileal loops. Furthermore, only serotype Dublin, and not serotype Gallinarum, persisted at systemic sites following i.v. inoculation. These differences will likely have a major effect on the ability of the bacteria to spread from mucosa to lymph nodes, from lymph nodes to systemic sites, and from systemic sites back to the intestine (reseeding). Factors affecting bacterial persistence include the ability to acquire nutrients, the avoidance of the host's bactericidal mechanisms, and the ability to proliferate within the host. The balance among such aspects of pathogenesis therefore appears to be crucial in determining *Salmonella* serotype-host specificity in calves. The importance of bacterial persistence for the virulence of serotype Dublin in cattle has been demonstrated by the analysis of isogenic *Salmonella* pathogenicity island 2 mutants (8). Disruption of type three secretion system 2 blocked the persistence of serotype Dublin in bovine intestines and almost completely attenuated serotype Dublin infection in cattle. Type three secretion system 2-secreted effector proteins have been shown to influence *Salmonella* net growth by both promoting bacterial replication (38) and blocking the killing of bacteria by macrophages (45). Clearly, identification of the factors contributing to the increased persistence of serotype Dublin compared to serotype Gallinarum will provide greater insight into the molecular basis of *Salmonella* host specificity. However, bacterial persistence in niches other than macrophages may also be of fundamental importance in determining the outcome of infection. Future work should concentrate on characterizing how, and with which cells, *Salmonella* interacts in vivo, which will allow the design of more biologically relevant in vitro assays. Only then can dissection of the molecular mechanisms involved in persistence of different serotypes in vivo begin.

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