NOTES

Development of an In Vivo Model for Study of Intestinal Invasion by *Salmonella enterica* in Chickens

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An in vivo loop test model for the investigation of the invasiveness of *Salmonella enterica* in chickens was developed. Ten jejunal loops were made in 10- to 12-week-old Lohman Brown chickens under isoflurane anaesthesia. *Salmonella* at 5.0×10^7 CFU was inoculated into each loop and left for 2 h, followed by a 1-h incubation with gentamicin in order to kill noninvasive bacteria. After euthanasia, *Salmonella* invasiveness was measured as tissue-associated counts relative to a reference strain. The ability of *Salmonella* invasion was 1 log₁₀ CFU higher per 42-mm² mucosal tissue in the anterior than in the posterior part of jejunum. A statistically significant ($P < 0.001$) sixfold difference in invasiveness was observed between a wild-type *S. enterica* serotype Typhimurium strain and the corresponding *invH* mutant. The model was shown to be able to show small differences in invasive capability and allows for comparison of strains tested in different animals, provided that the same reference strain is present in all animals.

Invasion of the gut epithelium is essential in the pathogenesis of *Salmonella* infections (4), and differences in virulence among serotypes might be related to differences in invasiveness. In order to study this, both in vivo and in vitro invasion models have been used (10, 13). As pointed out by Stone et al. (11), in vitro models may not incorporate all possible invasion pathways. In the case of the chicken, the lack of suitable epithelial cell lines is a further limitation in the use of in vitro models. In order to elucidate further *Salmonella* invasion in chickens, the present study was undertaken to develop and evaluate a chicken intestinal loop model.

Lohman Brown female chickens, 10 to 12 weeks of age, were used because they are immunologically mature and have an established gut flora. The birds were given feed and drinking water ad libitum and acclimatized for 5 to 6 days before the study commenced. All birds were tested for excretion of salmonellae by examining pools of 10 fecal swabs by standard culture procedures using Rappaport-Vassiliadis (Oxoid CM866) as the selective enrichment medium. In order to decrease intestinal content, the birds were starved 16 to 20 h prior to experiments.

Chickens were anesthetized by the administration of Isoflurane (Pharmacia) through a mask with continuous gas flow at a level of 1.5 to 3% depending on the depth of the anesthesia and an oxygen flow of 1.5 to 2.0 liters/min. The bird was covered with a sterile surgical blanket and defeathered at the abdominal surface. After disinfection of the incision area with 99% alcohol, the abdomen was opened by a transverse incision approximately 2 cm caudal to the sternum, and the jejunum was carefully exposed from the cavity by hand. Nine to ten ligated loops were constructed using epidermal polyester green

surgery suture (Kruuse, Martofte, Denmark). The loops were ca. 1.5 to 2.0 cm long and separated by smaller "spacer" loops of 0.5 to 1 cm in order to allow excision of the loops and to minimize the risk of leakage and contamination from adjacent dosed loops. Sufficient blood supply was ensured to both regular and spacer loops. The anatomic position of the loops is schematically shown in Fig. 1.

Loops were dosed with bacteria through an injection performed into the middle third of the loop opposite to the mesenteric line using a 0.4-mm (27 gauge) needle. After dosing, the loops were reintroduced into the abdomen, and the abdominal wall was clamped. Two hours later, 0.5 ml of phosphate-buffered saline (PBS) containing gentamicin (300 µg/ml) was added to each loop with an estimated final concentration of 150 to 200 µg/ml in the loop depending on the degree of absorption of the dose volume. The jejunum was relocated into the abdomen, and the abdominal wall was clamped again. After a further 1 h the chicken, still under anesthesia, was euthanized by decapitation. Loops were immediately removed under sterile conditions and placed on ice. In the laboratory they were cut clean at each ligature, opened along the mesenteric line, put into 5 to 10 ml of ice-cold mucosal medium (1), and gently rinsed in order to remove excessive gentamicin and non-tissue-associated salmonellae. Three circular biopsies of 6-mm diameter, a total of approximately 84-mm² mucosa, were subsequently taken from the gut tissues of each loop. The biopsies were placed in a 5-ml tube and homogenized with 2 ml of ice-cold PBS with 1% Triton X-100 using a Diax 900 dispersing machine (Heidolph Electro GmbH, Kelheim, Germany) for ca. 30 s to ensure homogeneity. After a lysis period of 10 min, 10-fold serial dilutions of the homogenate were made in physiological saline (0.9%), and subsequently 100 µl from each dilution was spread on xylose-lysine-desoxycholate agar (XLD-Agar; Oxoid CM469). The plates were incubated aerobically overnight at 37°C. The detection limit was approximately 10 bacteria per ml of biopsy homogenate, which is equivalent to

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FIG. 1. Schematic representation of the chicken intestinal tract. The jejunal section of the gut is shaded black, with black transverse bars to indicate the region for loop construction. Key: 1, proventriculus; 2, gizzard; 3, duodenum and pancreas; 4, jejunum; 5, diverticulum ventricularum; 6, ileum; 7, ceca; 8, rectum; 9, cloaca.

20 bacteria per 84-mm² mucosa. The counts per milliliter of homogenate, corresponding to counts per 42-mm² mucosa, were log₁₀ transformed, and this value was used to express invasion throughout the study. The first and the last loops were normally inoculated with 0.5 ml of PBS as control loops. The remaining loops were inoculated with test strains, which consisted of *Salmonella* serotype Typhimurium 4/74 and its corresponding *invH* mutant, *Salmonella* serotype Typhimurium 4/74 *invH201::TnphoA* (13), in addition to *Salmonella* serotype Choleraesuis A57 and *Salmonella* serotype Dublin 2229 (field isolates). All strains harbored serotype-specific virulence plasmids. The MIC values for gentamicin were 0.125 µg/ml for all strains.

The loop model is an indirect invasion assay, which demonstrates *Salmonella* counts from representative tissue sections rather than an exact measure of total internalized bacteria. *Salmonella* invades the loop mucosa during the 2-h incubation period, and the intracellular location leads to protection against the subsequent treatment with gentamicin. The efficacy of gentamicin for killing the noninvading bacteria is thus crucial to the outcome. To investigate this, cultures were made by adding 12 µl of an overnight broth culture to 10 ml of Luria-Bertani (LB) broth (CM-Lab) overlaid with 3 ml of paraffin oil and then incubating this mixture at 37°C for 6.5 h. This resulted in bacteria in late log phase and with low oxygen tension, which is favorable for invasion (6, 8). After incubation, the culture was harvested by centrifugation at 4,000 rpm for 15 min and suspended in 5 ml of PBS. The optical density at 620 nm was measured, and the dose culture adjusted to 0.1 optical density units corresponding approximately to 1.0×10^8 CFU/ml. For each loop a dose of 0.5 ml containing 0.5×10^8 CFU (log₁₀ 7.83) was desired. The bacterial concentration was verified by plate counts on XLD in duplicate. The mean dose (log₁₀ CFU) for all inoculations in this study was 7.81 ± 0.16 . Loops ($n = 5$) dosed with the wild-type strain *Salmonella* serotype Typhimurium 4/74 and treated with gentamicin showed 1.23-log₁₀ (17-fold)-lower mucosal counts than loops not treated with gentamicin. The corresponding difference for the *invH* mutant was 2.62 log₁₀ (417-fold) ($n = 5$) (Fig. 2), reflecting that this strain has a much lower invasion ability (see below) and hence is less protected against the effect of the antibiotic chase. The effect of gentamicin was more pronounced in this study

FIG. 2. Mean *Salmonella* counts (log₁₀ CFU/42-mm² mucosal tissue) in loops dosed with the wild-type *Salmonella* serotype Typhimurium 4/74 and the isogenic *invH* mutant with (G+) or without (G-) gentamicin treatment. The standard error of mean is also indicated.

than was seen in the cattle intestinal loop assay (13); however, the difference in effect between the wild type and its *invH* mutant was comparable for the two studies.

Preliminary investigations indicated that the ability of *Salmonella* to invade was not uniform throughout the jejunum (data not shown). Therefore, counts from 35 loops dosed with the *invH* mutant in eight loop positions using the two outermost loops (loop 1 and 10) as PBS controls, were analyzed. The inoculum was prepared as described above except that 12 µl of a 10^{-3} dilution of the primary culture was added to 10 ml of LB broth with a paraffin cover, followed by incubation for 8.5 h. The counts of the *invH* mutant were seen to decline from the anterior part to the posterior part of the jejunum as seen from Table 1. A statistical analysis of log₁₀ transformed bacterial counts was conducted as standard F tests in linear normal models, including effects of strain (*s*), loop position (*l*), and animal (*h*). The analysis, which also included loop counts for loops dosed with *Salmonella* serotype Typhimurium (wild type) ($n = 10$), *Salmonella* serotype Dublin ($n = 4$), and *Salmonella* serotype Choleraesuis ($n = 4$), supported an exponential decrease or a linear decrease after log₁₀ transformation of the bacterial counts (log linear decrease) in invasiveness in the craniocaudal direction. The decline in invasion was not *invH* specific, since the three other strains showed a similar trend. The description of the effect as a log linear decrease, depended on the exclusion of loop 10 (the most caudal loop) due to relative high counts of the *Salmonella* serotype Typhimurium 4/74 wild type at this position. Consequently, we decided to use loops 1 and 10 as blank PBS controls in the model. The analysis showed an average decline in *Salmonella* uptake over eight jejunal loops (loops 2 to 9) of 1.06 log units (10-fold) (95% confidence interval, 0.56 to 1.55) as a mere effect of the loop

TABLE 1. Mean counts (log₁₀ CFU) of *Salmonella* serotype Typhimurium 4/74 *invH201::TnphoA* from loops 2 to 9

Loop	Mean count (log ₁₀ CFU)	SEM	<i>n</i>
1	— ^a		
2	5.16	0.24	5
3	4.47	0.20	4
4	4.58	0.18	4
5	4.06	0.28	5
6	3.90	0.35	5
7	3.44	0.24	5
8	3.56	0.17	5
9	4.12	0.11	2
10	—		

^a —, Blank control.

position. In order to be able to compare the invasion of strains which are not dosed at the same loop positions a calculated correction for the effect of loop position was chosen. The calculated correction fits the actual data, and as such the validity of the model does not depend on the exclusion of loop 10. This exclusion is only for the convenience of a more perceivable description of the effect.

The jejunum was chosen as the intestinal site for the loop construction for practical surgical reasons. It has been shown that invasion can take place in all parts of the jejunum in day-old chicks (12), but it was not known whether *Salmonella* is able to invade the jejunum in immunocompetent animals with a fully developed gut flora. To directly demonstrate this, loops for histological examination were prepared separately. Sections of entire cross-sections of the intestine were fixed in 10% neutral buffered formalin and subsequently processed according to standard procedures. Slices of 3 μ m in thickness were mounted on Super Frost⁺/Plus slides (Menzel-Gläser). One slice of each sample was stained with Giemsa, hematoxylin, and eosin or stained by a peroxidase-antiperoxidase (PAP) immunohistochemical method according to standard procedures employing a polyclonal rabbit antiserum against serotype Typhimurium. Tissues from loops with both strains of serotype Typhimurium exhibited normal appearance of the mucosa with long slender villi. Minor early degenerative epithelial changes with vacuolization and loss of lateral cell adherence were only observed at the apex of villi. Immunohistochemical preparations demonstrated invasion of epithelial cells (enterocytes) of both wild-type serotype Typhimurium 4/74 and the corresponding *invH* mutant. Multiple intraepithelial salmonellae were demonstrated apically in several villi (Fig. 3). Some of the intraepithelial bacteria appeared to be within vacuoles, while others were free in the cytoplasm. Furthermore, the strains were seen to infiltrate the adjacent lamina propria of some villi. No infiltration with inflammatory cells were observed in the mucosa after the 2 h of incubation as demonstrated in Fig. 3.

In mammals, salmonellae have been reported to invade through specialized M cells in the Peyer's patches (5), but invasion outside the lymphoid tissue can also take place (12, 13) and may be more common than previously anticipated. Peyer's patch structures are present in chickens (2, 7). They undergo development from two Peyer's patch structures in day-old chicks, increasing to up to five structures diffusely scattered in the intestine of 12-week-old chickens with a subsequent decline to a single Peyer's patch structure in the ileocecal junction at the age of 56 weeks (2). In the present study the histopathological findings demonstrated internalization in enterocytes of both the wild-type strain and the *invH* mutant (Fig. 3), supporting the finding of Turnbull and Richmond (12) that invasion can take place through normal enterocytes. The Peyer's patch-like structures in the jejunum are not visible by the naked eye (2), and it was therefore not possible to evaluate whether Peyer's patches in a loop could influence the invasion rate due to the presence of phagocytic M-cell-like cells. Whether the surprising observation of the highest invasion of *Salmonella* in the anterior part of jejunum could be caused by the presence of more lymphoid tissue in the anterior part of the intestine compared to the posterior part is at present unknown.

The invasion of the wild-type serotype Typhimurium and its isogenic *invH* mutant was compared in order to evaluate the ability of the model to demonstrate differences between strains. The mean uptakes ($\log_{10}$ CFU) were 4.98 ± 0.16 from 10 loops dosed with serotype Typhimurium 4/74 and 4.1 ± 0.13 from 35 loops dosed with the *invH* mutant, giving a difference of 0.84 ± 0.29 equal to a sevenfold difference in invasiveness. The data were analyzed according to a model, including the

FIG. 3. Chicken intestinal villus with an intraepithelial apical (A) or basal (B) location of invading *Salmonella* serotype Typhimurium 4/74 (wild type). *Salmonella*-specific immunohistochemical PAP staining was used.

random effect of test strain, loop position, and animal as follows: $\log_{10}(\text{count}_{slh}) = \mu + \alpha_s + \beta_l + c_h + e_{slh}$, where μ represents a general level, α_s represents effect of the strains, β_l represents the effect of the loop position, c_h is the random effect of animal (chicken), and e_{slh} is the residual effect, which covers effects that cannot be explained by the factors included in the model (9). From the statistical model, estimated differences in invasiveness between the two strains ($\alpha_i - \alpha_j$) were derived using best linear unbiased predictors. This gives a confidence interval for the estimated difference in addition to a correction for the effect of the loop position. The results from this analysis can be interpreted as prediction intervals for mean differences between two strains, whether they are tested in the same or different animals provided that the same reference strain is present in all animals. When this statistical analysis was performed, a sixfold difference in invasiveness between the wild type and the *invH* mutant was observed that corresponded to a difference in counts of 0.78 $\log_{10}$ CFU with a 95% confidence interval between 0.37 and 1.19 ($P < 0.001$). The counts of loop 10 were included in the analysis in order not to underestimate the variance. The *invH* strain has previously been shown to have a decreased level of invasion compared to the

wild-type strain in an intestinal loop assay in calves and in cell cultures (11, 13), and the result gives direct evidence for a role of *invH*, which is part of the type III secretion system encoded by *Salmonella* pathogenicity island I (3) in the invasion process of *Salmonella* in chickens.

The small variations in dose did not significantly influence the uptake of any of the strains, which may be explained by a threshold effect, i.e., that the loop dose exceeded a biological level of what can possibly be internalized in the loop epithelium during the 2 h of incubation. Also, the small variation in age from 10 to 12 weeks did not have an apparent effect on the bacterial counts.

In conclusion, the differences in invasion demonstrated between the wild type and the corresponding *invH* gene highlight the potential of this model to show even small differences in the invasive potential of *Salmonella* serotypes. Another perspective of the model is the ability to show the mean difference in invasion between two strains even if they are not tested in the same animal. The model may further be used to study whether or not the same cellular mechanisms in mammals are important during *Salmonella* invasion in the avian host.

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REFERENCES

1. Amin, I. I., G. R. Douce, M. P. Osborne, and J. Stephen. 1994. Quantitative studies of invasion of rabbit ileal mucosa by *Salmonella* Typhimurium strains which differ in virulence in a model of gastroenteritis. *Infect. Immun.* **62**: 569–578.
2. Befus, A. D., N. Johnston, G. A. Leslie, and J. Bienenstock. 1980. Gut-associated lymphoid tissue in the chicken. I. Morphology, ontogeny, and some functional characteristics of Peyer's patches. *J. Immunol.* **125**:2626–2632.
3. Daefler, S., and M. Russel. 1998. The *Salmonella typhimurium invH* protein is an outer membrane lipoprotein required for the proper localization of *invG*. *Mol. Microbiol.* **28**:1367–1380.
4. Gulig, P. A. 1996. Pathogenesis of systemic disease, p. 2774–2787. In F. C. Neidhardt et al. (ed.), *Escherichia coli* and *Salmonella*. American Society for Microbiology, Washington, D.C.
5. Jones, B. D., N. Ghori, and S. Falkow. 1994. *Salmonella typhimurium* initiates murine infection by penetration and destroying the specialized epithelial M-cells of the Peyer's patches. *J. Exp. Med.* **180**:15–23.
6. Jones, B. D., and S. Falkow. 1994. Identification and characterization of a *Salmonella typhimurium* oxygen-related gene required for bacterial internalization. *Infect. Immun.* **62**:3745–3752.
7. Kato, A., Y. Hashimoto, Y. Kon, and M. Sugimura. 1992. Are there M-cells in the caecal tonsils of chickens? *J. Vet. Med. Sci.* **54**:999–1006.
8. Lee, C. A., and S. Falkow. 1990. The ability of *Salmonella* to enter mammalian cells is affected by bacterial growth state. *Proc. Natl. Acad. Sci. USA* **87**:4304–4308.
9. Little, R. C., G. A. Milliken, W. W. Stroup, and R. D. Wolfinger. 1996. SAS systems for mixed models. SAS Institute, Cary, N.C.
10. Popiel, I., and P. C. B. Turnbull. 1985. Passage of *Salmonella enteritidis* and *Salmonella thomson* through chick ileocecal mucosa. *Infect. Immun.* **47**:786–792.
11. Stone, B. J., C. M. Garcia, J. L. Badger, T. Hassett, R. I. F. Smith, and V. Miller. 1992. Identification of novel loci affecting entry of *Salmonella enteritidis* into eukaryotic cells. *J. Bacteriol.* **174**:3945–3952.
12. Turnbull, P. B. C., and J. E. Richmond. 1978. A model of *Salmonella* enteritis: the behaviour of *Salmonella enteritidis* in chicks intestine studies by light and electron microscopy. *Br. J. Exp. Pat.* **59**:64–75.
13. Watson, P. R., S. M. Paulin, A. P. Bland, P. W. Jones, and T. S. Wallis. 1995. Characterisation of intestinal invasion by *Salmonella typhimurium* and *Salmonella dublin* and effect of a mutation in the *invH* gene. *Infect. Immun.* **63**:2743–2754.