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Immunity to Experimental Fowl Typhoid in Chickens Induced by a Virulence Plasmid-Cured Derivative of *Salmonella gallinarum*

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Chickens were immunized by two intramuscular inoculations at 1 and 14 days of age with virulence plasmid-cured derivatives of *Salmonella gallinarum* and were challenged 14 days later by oral inoculation of ca. 50 50% lethal doses (LD_{50}) of fully virulent *S. gallinarum* 9. Mortality in the nonimmunized and immunized groups were 36 and 3%, respectively. This difference was highly significant ($P < 0.01$). A significant reduction in mortality was also produced following oral challenge with 5,000 LD_{50} doses. The LD_{50} values by intramuscular inoculation of the challenge organism into nonimmunized and immunized chickens were $\log_{10}$ (0.13 ± 1.57) and (9.74 ± 2.72), respectively. Immunization was effective whether chickens were immunized at 1 and 14 days of age or at 21 and 35 days of age. Serum agglutinins were present in immunized chickens. Immunization with plasmid-cured *Salmonella pullorum* gave less protection, and immunization with *Escherichia coli* K-12 possessing the virulence plasmid of *S. gallinarum* gave none. The plasmid-cured *S. gallinarum* was made both rough by virulent bacteriophage activity and nalidixic acid resistant (Nal^r) to produce a strain designated $9VP^-\phi^r$ Nal^r. It was compared with a Nal^r mutant of the rough 9R vaccine strain designated 9 Nal^r for virulence and immunogenicity. $9VP^-\phi^r$ Nal^r was slightly less protective and less virulent than was the 9R vaccine strain.

Fowl typhoid is still a disease of worldwide significance. It has largely been eradicated from those countries which have had an intensive poultry industry for many years and is now of particular economic importance in those countries which are beginning to intensify their industry, e.g., countries in Latin America, South America, the Middle East, the Indian subcontinent, and parts of Africa (18, 20).

Recent work has shown that the very high virulence for chickens of *Salmonella gallinarum*, the causative agent of fowl typhoid, is related to the possession of a high-molecular-weight (85-kilobase) plasmid (5). Barrow et al. (3, 5) found that elimination (curing) of this plasmid from a strain greatly reduced its virulence. In 3-week-old chickens, the plasmid-cured derivative was virtually avirulent, while in newly hatched chickens, there was some residual virulence. Reintroduction of the plasmid fully restored virulence. From pathogenesis studies, the plasmid was shown to be involved both in invasiveness in vivo and in the ability of the strain to survive and multiply in the cells of the liver and spleen. A similar plasmid was later shown to contribute to the virulence of *Salmonella pullorum* (2).

Because of the economic importance of fowl typhoid many studies have been carried out on the feasibility of vaccination to control it. Early studies showed that killed vaccines were of little practical use (12, 15, 22, 31, 32). Smith (22) developed two attenuated vaccine strains, one of which (a rough strain, 9R) was protective and did not induce the production of significant amounts of serum agglutinins. This latter characteristic is of particular importance where the disease is to be eradicated by using the whole-blood agglutination test (8, 19). Later work has shown the value of the 9R strain (9, 14, 21, 27). A number of authors have indicated that the strain still possesses some virulence for some breeds of chicken (9, 10, 21), although there is no evidence for reversion to full virulence in the field.

The worldwide prevalence of fowl typhoid, combined with increasing numbers of reports of furazolidone resistance in isolates of *S. gallinarum* resulting from extensive prophylaxis with this antibiotic (11, 26, 29, 30), indicates the need for continuing work on the development of an improved vaccine. Because the plasmid-cured derivative of *S. gallinarum* persisted in the reticuloendothelial system of the chicken for some time, it was considered that the strain might be used for vaccination purposes.

This article reports on the ability of the 85-kilobase (kb) plasmid-cured derivative of a strain of *S. gallinarum* to protect chickens against fowl typhoid. In addition, a mutant of this strain was produced and tested which was rough (to prevent the production of serum agglutinins) and resistant to nalidixic acid (to facilitate the maintenance of purity). The protective ability and virulence of this mutant were compared with these characteristics in a Nal^r mutant of the 9R strain.

MATERIALS AND METHODS

Chickens. Salmonella-free Rhode Island Red chickens aged 1 day or 3 weeks were used. They were kept under good hygienic conditions on wire-mesh floors in identically constructed pens in an animal house maintained at 21°C. The younger chickens were provided with extra heating from an infrared lamp suspended over each pen. They were fed ad libitum on a diet of the following composition: wheat meal, 40%; maize meal, 40%; British whitefish meal, 20%; mineral and vitamin supplement, 0.25%.

Bacterial strains. The strain used in most of the experiments was an 85-kb plasmid-cured derivative of *S. gallinarum* 9. The parent strain, which was also the challenge strain, has been maintained at the AFRC Institute for Animal Health for many years and is highly virulent for chickens. The method of "tagging" and curing the plasmid has been described previously (5).

Additional strains used included the 85-kb plasmid-cured derivative of *S. pullorum* 3 (2) and a prototrophic *Escherichia coli* K-12 strain (*proto*) into which the 85-kb plasmid

TABLE 1. Mortality following oral inoculation with *S. gallinarum* 9 after immunization with virulence plasmid-cured derivatives of *S. gallinarum* and *S. pullorum*

Immunization agent[a]	No. of chickens	*S. gallinarum* 9 oral challenge dose	No. of chickens dead at day (postchallenge): 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21	% Mortality over 3 weeks	χ^2	*P*	No. chickens/total with: Lesions in liver and spleen	Infected livers
S. gallinarum plasmid cured	23	10^{8b}	0 0 1 1 3 3 4 5 10 10 10 10 10 11 11 11 11	48	4.53	0.03	10/12	3/12
Nonimmunized	23	10^{8b}	0 2 10 14 16 18 18 18 18 18 18 18 18 18 18 18 18	78			0/5	0/5
S. gallinarum plasmid cured	30	10^{6c}	0 0 0 0 0 1 1 1 1 1 1 1 1 1 1 1 1	3	9.92	<0.01	8/29	2/29
Nonimmunized	28	10^{6c}	0 0 3 4 5 9 9 10 10 11 11 11 11 11 12 12 12	36			2/16	1/16
S. pullorum plasmid cured	30	10^{6c}	0 0 0 1 1 1 3 4 4 5 5 5 6 6 6 6 6	20	2.84	0.1	3/24	2/24
Nonimmunized	30	10^{6c}	0 0 3 4 5 9 9 10 10 11 11 11 11 11 12 12 12	40			1/18	0/18

[a] All chickens immunized intramuscularly with 10^6 organisms at day 1 (1 day of age) and 10^8 organisms at day 14, followed by challenge at day 28.
[b] 10^8 = 5,000 × LD_{50} by the oral route.
[c] 10^6 = 50 × LD_{50} by the oral route.

was mobilized from *S. gallinarum* 9 by the F plasmid. Briefly, this was carried out as follows. A three-factor mating was used in which the donor was *S. gallinarum* 9 containing the 85-kb plasmid which had been tagged with transposon Tn*3* (5). The mobilizing strain was *E. coli* K-12 mutant 711 (*lac his pro trp*) containing the F plasmid, and the final recipient was a Nal^r mutant of *E. coli* K-12 *proto*. The mating mixture was plated on MacConkey agar containing nalidixic acid (25 μg ml^{-1}) and ampicillin (100 μg ml^{-1}). Lactose-fermenting colonies were checked for the presence of the 85-kb plasmid and for the presence of the F plasmid by plasmid analysis (14).

In one experiment, the 9R vaccine strain developed by Smith from strain 9 (22) was compared with the plasmid-cured *S. gallinarum* strain. When analyzed for the possession of plasmids (14), the 9R strain was found to possess an 85-kb plasmid (unpublished data).

All cultures used were stored either on Dorset's egg slopes or in a lyophilized condition. All broth cultures were made in 10-ml volumes of nutrient broth (Oxoid CM 67) and were incubated at 37°C for 24 h in a shaking water bath (100 strokes min^{-1}). These contained between 5 × 10^8 and 1 × 10^9 CFU ml^{-1}.

Production of rough mutant. Spontaneous rough mutants were selected from broth cultures by their resistance to a bacteriophage φBL isolated from human sewage (4).

Production of nalidixic acid-resistant (Nal^r) mutants. Production of Nal^r mutants was done by the method of Smith and Gyles (24). Smith and Tucker (25) showed that Nal^r mutants of *Salmonella* strains were no less virulent for chickens than were the antibiotic-sensitive parent strains. However, Silva et al. (21) found that a Nal^r mutant of the 9R vaccine strain was less virulent than was the antibiotic-sensitive form.

Experimental plan. In all experiments, the challenge organism was *S. gallinarum* 9. In the first two experiments, the protective effect of vaccinating chickens intramuscularly with either the plasmid-cured derivatives of *S. gallinarum* 9 or *S. pullorum* 3 or an *E. coli* K-12 strain possessing the 85-kb *S. gallinarum* virulence plasmid, pSG090, was assessed under different conditions. The rough, Nal^r derivative of the plasmid-cured *S. gallinarum* strain, designated $9VP^-\phi^r$ Nal^r, and the Nal^r mutant of the 9R vaccine were compared for their protective abilities when inoculated via different routes. The virulence of these two mutants was compared by inoculating chickens via a variety of routes, followed by estimating viable counts of the strains in the spleens and recording lesions in this organ and in the liver. For all experiments, appropriate control groups were included. Fifty percent lethal dose (LD_{50}) values were calculated by using the MLP statistical package (Rothamstead Experimental Station, Harpenden, United Kingdom), which follows conventional methods of probit analysis (7). Viable bacterial counts were estimated by the method of Miles, Misra, and Irwin (16), plating the bacteria on MacConkey agar (Oxoid CM7) containing 25 μg of nalidixic acid per ml.

Detection of serum agglutinins. Chickens were bled from the wing vein. Serum was obtained and tested for the presence of agglutinins by slide agglutination of bacterial colonies from a nutrient agar (Oxoid CM 67) plate inoculated with *S. gallinarum* 9 and incubated overnight at 37°C.

RESULTS

Immunization with plasmid-cured strain (oral challenge). The effect of intramuscular immunization with either the plasmid-cured *S. gallinarum* or *S. pullorum* strains on mortality following oral challenge with *S. gallinarum* 9 is shown in Table 1. Considerable protection was afforded by the plasmid-cured *S. gallinarum*. Following oral challenge with 10^8 organisms, the mortality recorded was reduced from 78% (control chickens) to 48% (immunized chickens). The rate at which the immunized chickens died was significantly lower ($P < 0.01$) than that of the control birds when examined by the Wilcoxon-Mann-Whitney two-sample rank test (28). The difference in mortality rates was of marginal statistical significance. When the chickens were challenged with 10^6 organisms, the mortality rate was lower in both groups, 36 and 3% in the control and immunized chickens, respectively. This difference was statistically significant. Although immunization with the plasmid-cured *S. pullorum* strain reduced the mortality level produced by challenge from 40 to 20%, this reduction was not statistically significant.

In all of the groups, a small proportion of the surviving chickens had small areas of necrosis in the livers and spleens and, in some cases, the challenge strain was reisolated from their livers.

Immunization with plasmid-cured strains and plasmid-containing *E. coli* K-12 (intramuscular challenge). The LD_{50} values of control and immunized chickens challenged intra-

TABLE 2. Intramuscular challenge with *S. gallinarum* 9 following immunization with virulence plasmid-cured derivatives of *S. gallinarum* and *S. pullorum* and with *E. coli* K-12 containing the *S. gallinarum* virulence plasmid

Immunizing strain	Immunization schedule[a]	LD_{50} value (log_{10}) ± SE in:		χ^2	*P*
		Immunized chickens	Nonimmunized chickens		
S. gallinarum plasmid cured	10^6 CFU 1 day of age, 10^8 CFU 14 days of age, challenge 28 days of age	9.74 ± 2.72 (7.52 ± 1.55)[b]	0.13 ± 1.57 (0.80 ± 1.91)[b]	24.12 12.29	<0.01 <0.01
S. gallinarum plasmid cured[c]	10^7 CFU 21 days of age, 10^7 CFU 35 days of age, challenge 49 days of age	9.89 ± 2.58	0.80 ± 1.01	20.34	<0.01
S. pullorum plasmid cured	10^5 CFU 1 day of age, 10^8 CFU 14 days of age, challenge 28 days of age	3.77 ± 0.73	0.07 ± 1.10	7.34	<0.01
E. coli containing *S. gallinarum* plasmid	10^8 CFU 1 day of age, 10^8 CFU 14 days of age, challenge 28 days of age	1.23 ± 0.64	0.71 ± 0.66	0.35	0.6

[a] All immunizations were by the intramuscular route; five chickens were inoculated per dilution of culture.
[b] First value represents replicate one; value in parentheses represents replicate two.
[c] Serum agglutinins present in 8 of 10 chickens at time of challenge.

muscularly are shown in Table 2. Large increases in the LD_{50} values were observed in the chickens which had been immunized with the plasmid-cured *S. gallinarum* strain when compared with the values observed with the nonimmunized controls. In the chickens which were immunized at 1 day old, the log_{10} of the increase was greater than 9 on one occasion and greater than 6 on another, and in chickens immunized first at 3 weeks of age, it was also greater than 9. These differences were statistically significant. Immunization of newly hatched chicks with the plasmid-cured *S. pullorum* strain produced a log_{10} increase in the LD_{50} of the challenge strain of greater than 3; this difference was also highly significant, even though the degree of protection was considerably less than that afforded by the plasmid-cured *S. gallinarum* strain. Immunization with the *E. coli* K-12 strain possessing the *S. gallinarum* virulence plasmid had no protective effect, the log_{10} increase in LD_{50} in the immunized chickens compared with that in the control birds being less than 1.

Of 10 chickens immunized with *S. gallinarum* at 21 days old, 8 possessed agglutinins of sufficient titer to be detected by slide agglutination.

Immunization with vaccine strains 9R Nal^r or 9VP$^-\phi^r$ Nal^r (oral challenge). The effects of single oral immunization with either the derived strains 9VP$^-\phi^r$ Nal^r or 9R Nal^r and of parenteral immunization by different routes with 9VP$^-\phi^r$ Nal^r on mortality following oral challenge are shown in Table 3. When compared with the mortality in the nonimmunized control group (65%), oral immunization with 9R Nal^r produced considerably better protection (8% mortality) than did immunization with 9VP$^-\phi^r$ Nal^r (42%). The degree of protection produced by the former strain only was statistically significant, and the differences in degrees of protection produced by the two strains were also statistically significant.

Immunization with 9VP$^-\phi^r$ Nal^r by either intramuscular or subcutaneous routes produced better protection (24 and 27% mortality, respectively) than did immunization with this strain by oral administration. Although administration of 9R Nal^r by the oral route induced better protection, the difference between this and the protection induced by intramuscular immunization of 9VP$^-\phi^r$ Nal^r was not statistically significant.

Immunization with vaccine strains 9R Nal^r or 9VP$^-\phi^r$ Nal^r (intramuscular challenge). The protective effects of a single intramuscular immunization with 9R Nal^r or 9VP$^-\phi^r$ Nal^r were compared (Table 4). In both cases, considerable protection was produced when compared with the protection produced with an nonimmunized group of chickens. The log_{10} increase in the LD_{50} values produced by 9R Nal^r and strain 9VP$^-\phi^r$ Nal^r were greater than 11 and greater than 9, respectively. Both increases were statistically highly significant. The difference between the two LD_{50} values in the immunized chickens was of marginal statistical significance.

None of the 20 serum samples examined showed any trace of agglutination with *S. gallinarum* cells by slide agglutination.

When compared with those of the control group, the standard errors of the LD_{50} values for immunized chickens were high. This was caused by the unusual distribution of

TABLE 3. Mortality following oral inoculation with *S. gallinarum* 9 after immunization with vaccine strains 9VP$^-\phi^r$ Nal^r or 9R Nal^r given by different routes

Group no.	No. of chickens	Chickens immunized[a] with:	Route of immunization[a]	No. of chickens dead at day (post-challenge):												% Mortality over 3 weeks	Groups compared (χ^2 value)	*P* value from χ^2 comparison
				7	8	9	10	11	12	13	14	15	16	17	18			
1	26	9VP$^-\phi^r$ Nal^r	Oral	0	0	4	6	6	10	11	11	11	11	11	11	42	1 and 5 (2.8)	0.1
																	2 and 1 (8.3)	<0.01
2	26	9R Nal^r	Oral	0	0	0	0	1	2	2	2	2	2	2	2	8	2 and 3 (2.7)	0.1
																	2 and 5 (18.6)	<0.01
3	25	9VP$^-\phi^r$ Nal^r	Intramuscular	0	0	1	4	4	4	5	5	6	6	6	6	24	3 and 5 (8.8)	<0.01
4	26	9VP$^-\phi^r$ Nal^r	Subcutaneous	0	1	2	2	3	5	5	7	7	7	7	7	27	4 and 5 (7.7)	<0.01
5	26	Nothing	—[b]	0	3	6	9	11	13	15	15	15	15	17	17	65	—	—

[a] All chickens immunized orally with 3×10^8 organisms in 0.3 ml or intramuscularly with 1×10^8 organisms in 0.1 ml at 3 weeks of age followed by oral challenge with 3×10^8 organisms 3 weeks later.
[b] —, Not relevant.

TABLE 4. Intramuscular challenge with *S. gallinarum* 9 after intramuscular immunization with nalidixic-resistant mutants of either the 9R vaccine strain or a rough mutant of the virulence plasmid-cured derivative of *S. gallinarum*

Immunizing strain	Immunization schedule	No. of birds with serum agglutinins present at time of challenge/total	LD_{50} value ± SE (log_{10})	χ^{2a}	*P*
9R Nal^r	10^7 organisms day 21, challenge day 42	0/10	11.85 ± 4.70	19.56	<0.01
SG9VP$^-$ ϕ^r Nal^r	10^7 organisms day 21, challenge day 42	0/10	9.8 ± 4.06	8.48	<0.01
Nonimmunized	challenge day 42	ND[b]	0.80 ± 1.24		

[a] χ^2 values calculated by comparison with nonimmunized control. χ^2 value for comparison between the two immunized groups was 5.77; $P = 0.02$.
[b] ND, Not done.

deaths in the groups of immunized chickens. For the LD_{50} estimations, chickens were divided into groups of five and inoculated with serial dilutions of the challenge strain. The numbers of chickens which died in the groups inoculated with (log_{10}) 8, 7, 6, 5, 4, 3, 2, 1, and 0 organisms were 0, 0, 2, 0, 0, 0, 0, 0, and 0, respectively, in the case of 9R Nal^r and 2, 1, 0, 2, 2, 1, 0, and 1, respectively, in the case of 9VP$^-$$\phi^r$ Nal^r. These results reflected the outbred nature of the experimental chickens.

Isolation of challenge strain from spleens after immunization with vaccine strains. The viable numbers of *S. gallinarum* 9 Nal^r in the spleens of chickens which had been vaccinated twice intramuscularly with either 9VP$^-$$\phi^r$ Nal^r or 9R Nal^r and subsequently challenged with the parent strain are shown in Table 5. Selected bacterial colonies were all smooth when tested for agglutination with 0.2% acriflavine and were therefore the parent strain. Within a few days of challenge, large numbers of the challenge organisms were isolated from the spleens of the nonimmunized control group. Deaths were observed in this group, depleting the number of birds available for sampling. The numbers of organisms of the challenge strain in the spleens of chickens immunized with 9VP$^-$$\phi^r$ Nal^r were initially low but rose to levels comparable with those in the control group, despite the protection produced by 9VP$^-$$\phi^r$ Nal^r. No detectable challenge organisms (viable counts of log_{10} <2) were found in the spleens of chickens immunized with 9R Nal^r.

Virulence of vaccine strains. The virulence of 9R Nal^r and 9VP$^-$$\phi^r$ Nal^r for newly hatched chickens was calculated by LD_{50} estimations obtained by intramuscular inoculation. The log_{10} LD_{50} value ± standard error for 9R Nal^r was 6.85 ± 0.71 and for 9VP$^-$$\phi^r$ Nal^r was 8.19 ± 0.86.

The persistence of 9R Nal^r and 9VP$^-$$\phi^r$ Nal^r in the spleens of chickens inoculated with either of these strains by a number of routes is shown in Table 6. Following intramuscular inoculation, the viable counts of the two strains were initially comparable. However, whereas 9VP$^-$$\phi^r$ Nal^r was not detectable at 2 weeks postinoculation 9R Nal^r persisted for a week longer. Following oral inoculation, no organisms of 9VP$^-$$\phi^r$ Nal^r were detected at all in the spleen whereas 9R Nal^r persisted for at least 2 weeks. 9VP$^-$$\phi^r$ Nal^r was not detected in the spleens of chickens inoculated subcutaneously; no comparison was made with 9R Nal^r by this route.

TABLE 5. Isolation of *S. gallinarum* 9 from the spleens of chickens challenged with this organism after immunization with 9VP$^-$$\phi^r$ Nal^r or 9R Nal^r[a]

Days postchallenge	Log_{10} median viable *Salmonella* count (range) with:[b]		
	9VP$^-$$\phi^r$ Nal^r	9R Nal^r	Nonimmunized
4	<2 (<2–4.0)	<2 (<2)	4.0 (<2–4.6)
7	4.9 (<2–6.1)	<2 (<2)	5.5 (3.4–5.8)
14	4.4 (<2–5.0)	<2 (<2)	4.4[c]
21	1.7 (<2–3.1)	<2 (<2)	2.0[c]

[a] Chickens immunized by the intramuscular route 5 and 3 weeks before challenge with 10^7 organisms in 0.1 ml. Chickens challenged orally with 3 × 10^8 organisms in 0.3 ml.
[b] Counts and ranges are from spleens of five chickens at various times after inoculation with *S. gallinarum* 9 in chickens immunized with the given strains.
[c] Log_{10} mean count of two chickens only.

DISCUSSION

These experiments show that chickens can be protected against oral or intramuscular challenge with fowl typhoid by intramuscular or subcutaneous immunization with the 85-kb virulence plasmid-cured derivative of *S. gallinarum*. Not surprisingly, protection was less effective against large challenge doses. Protection was not complete, as was apparent from the death of some immunized chickens, from the presence of lesions in the liver and spleen and the isolation of the challenge organism from the liver. Incomplete protection was also induced by the 9R vaccine strain. This may have been a reflection of the fact that young chickens were used in the experiments, since Gordon, Garside, and Tucker (9) found that protection was better in older birds.

Protection against challenge with *S. gallinarum* was not as good when chickens were immunized with a plasmid-cured derivative of *S. pullorum*. This strain persists in the tissues for as long as does the plasmid-cured *S. gallinarum* strain (3). Thus, the degree of exposure of the host to the antigens is the same. It is possible that different antigens are possessed by these two biotypes. Smith (22) showed that fully virulent *S. pullorum* would protect chickens against challenge with *S. gallinarum*, but it is likely that the strain used by Smith possessed its virulence plasmid and this may be necessary to induce full protection.

Mice can be protected against *Salmonella enteritidis* and *Salmonella dublin* infection by being immunized with strains of these serotypes cured of their virulence-associated plasmids (6, 17). It is therefore not surprising that good protection in chickens was produced by a strain of *S. gallinarum* lacking a virulence plasmid. Plasmid-encoded antigens are clearly not essential for the production of good protective immunity. The 9R vaccine strain possesses a virulence plasmid, though it is not clear whether it is functional. The fact that the plasmid-cured strain was less immunogenic than was the 9R strain suggests that the virulence plasmid does contribute to complete immunogenicity. This was true for both parenteral and oral vaccination. The plasmid-cured strain was not expected to perform well by oral administration since, unlike *S. dublin* and *S. typhimurium*, its virulence plasmid contributes to in vitro invasiveness. However, this

TABLE 6. Persistence of 9VP$^-\phi^r$ Nal^r and 9R Nal^r vaccine strains when inoculated into chickens by various routes[a]

Days postinoculation	Log_{10} median viable *Salmonella* count (range) after inoculation[b]				
	Intramuscularly with:		9VP$^-\phi^r$ Nal^r (subcutaneous)	Orally with:	
	9VP$^-\phi^r$ Nal^r	9R Nal^r		9VP$^-\phi^r$ Nal^r	9R Nal^r
4	2.7 (<2–3.8)	3.2 (2.3–3.5)[c]	<2 (<2)	<2 (<2)	2.5 (<2–3.7)[c]
7	2.3 (<2–3.4)	2.5 (<2–4.0)[c]	<2 (<2)	<2 (<2)	3.0 (<2–3.7)[c]
14	<2 (<2)	2.5 (<2–3.9)[c]	<2 (<2)	<2 (<2)	2.5 (2.3–3.3)
21	<2 (<2)	<2 (<2)	<2 (<2)	<2 (<2)	<2 (<2)

[a] Chickens were inoculated at 21 days of age with 10^8 organisms in 0.1 ml (intramuscular and subcutaneous) or 3×10^8 organisms in 0.3 ml (oral).
[b] Counts and ranges are from spleens of five chickens at various times after inoculation via the given routes with the given strains.
[c] Liver lesions present.

is of little practical significance at the moment since, in the field, most chickens are vaccinated parenterally.

It is apparent that possession of the plasmid in the absence of as yet uncharacterized chromosomal genes does not give protection, since the *E. coli* K-12 strain possessing the 85-kb *S. gallinarum* plasmid was not protective. The presence of the F plasmid is unlikely to have affected the expression of the virulence plasmid, since its presence in fully virulent *S. gallinarum* and *S. pullorum* strains does not affect virulence (3). However, *E. coli* K-12 persists for a very short time in the liver and spleen of chickens and this may contribute to the poor immunogenicity. It is clear that for optimal immunogenicity and protection, expression of both plasmid and chromosomal genes are required.

Although the plasmid-cured strain induced protection in the chickens, it did not prevent multiplication of the challenge strain in the spleen except in the first few days after challenge. This suggests that the main mechanism of protection may be different from that induced by the 9R vaccine. Studies to characterize this mechanism more fully would require the use of inbred chickens.

The heterogeneous response to challenge in some groups of the Rhode Island Red chickens was an interesting and important practical observation. Since there is variation between breeds in susceptibility to fowl typhoid (23; N. Bumstead and P. A. Barrow, unpublished results) and Rhode Island Reds are outbred, this response is not surprising. It suggests that in some breeds of chicken bred primarily for performance, heterogeneity of response to vaccination might be expected in the field (9, 21).

It is important that live vaccines against fowl typhoid do not express somatic antigens, so that antibodies against these do not interfere with the whole-blood agglutination. Regardless, the somatic antigens are obviously not essential for protection. It would also be useful to have a vaccine expressing a genetic marker which increased the ease of purification and isolation. It was thus of practical significance that the rough Nal^r mutant of the plasmid-cured derivative, designated 9VP$^-\phi^r$ Nal^r, was also protective and was of slightly reduced virulence for chickens when compared with 9R Nal^r. How these two strains would compare under field conditions is unclear, but further work is necessary on the efficacy of the SG9VP$^-\phi^r$ Nal^r strain in breeding chickens housed under field conditions. The degree of protection induced by 9VP$^-\phi^r$ Nal^r was less than that induced by 9R under experimental conditions, but the former strain has the advantage of being less virulent. 9VP$^-\phi^r$ Nal^r might therefore be used on its own where the incidence of fowl typhoid is low, or it might be used to vaccinate more susceptible heavy breeds (23) prior to immunization with 9R, where the virulence of the latter strain might be a problem.

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