Safety Studies and Risk Analysis of an Attenuated *Salmonella gallinarum* Live Vaccine for Layer Chickens (1)

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Introduction

The control of salmonellae in commercial poultry raised under farm-intensive conditions involves a multi-factorial approach which includes vaccination. Vaccination has been shown to be effective not only in reducing morbidity and mortality, but more importantly, in reducing or preventing horizontal and/or vertical transmission of the bacteria. Though inactivated vaccines are available for use in commercial chickens, live vaccines have been suggested (3) to be a better approach for control of salmonellae because of the live vaccine's ability to induce a stronger cell-mediated response, which is an important immune mechanism against intracellular bacteria like the salmonellae.

The live rough strain of *Salmonella gallinarum*, designated 9R, was initially described by Smith (11) and subsequent laboratory and field studies showed the strain to be an effective vaccine candidate against *S. gallinarum, S. pullorum, and S. enteritidis* challenges following vaccination of poultry (1,2,4-10,12). Though the 9R strain was reported as an effective vaccine, several investigators indicated that the strain possessed some residual virulence for certain breeds of chickens and persisted in tissues such as the liver, spleen and ovaries, which in turn led to vertical transmission (4,5,10).

We, therefore, initiated studies to investigate 1) the safety of the 9R strain of *S. gallinarum* in various commercial layer breeds of chickens and non-host species, 2) the ability of the strain to revert to a virulent form following *in vivo* passages, 3) whether the 9R stain transmits horizontally using sentinel chickens, and 4) the ability of the strain to transmit vertically through the egg. All vaccine used in the studies was derived from well-characterized Master Seed stock.

Safety of Sg 9R Strain

- **Gordon and Luke, 1959**
  - "Pathological changes in the ovary in some (vaccinated) birds"; No clinical signs; No mortality.
  - Flocks previously/concurrently infected w/ field strains.
  - "No tendency to revert (to virulence)"
- **Gordon et al., 1959**
  - Mortality in one-day-old chicks (6/25 oral; 1/20 SQ)
- **Silva et al., 1981**
  - Hepatic and splenic lesions w/o mortality in meat-type and brown-egg-producing strains of(one-day-old) chicks"
- **No safety problems were mentioned in other publications** (Barrow et al., 1987; Barrow, 1990; Barrow et al., 1990; Bouzoubaa et al., 1989; Griffin and Barrow, 1993; Gupta and Mallick; 1977; Harbourne, 1957; Harbourne et al., 1963; Jaiswal and Mittal, 1984; Nassar et al., 1994; Smith, 1956; Smith, 1969 ...)
  - **Smith, 1956"**
  - Non-lethal to 1-day-old chicks and was never found to revert to the smooth virulent form *in vitro* or *in vivo*"
  - **Gordon and Luke, 1959"**
  - No tendency to revert (to virulence)"

Materials and Methods

I. Safety

Animals
Host Species

A total of 414 egg-producing chickens between the ages of one day and 10 weeks were used. The breeds included both specific pathogen free (leghorn) and commercial layers (Hyline-brown, Isa-brown and Dekalb-brown).

Other Species

Ten 4-week-old white turkeys and eight 3-5-week-old calves were tested.

Vaccine and Vaccination

An attenuated S. gallinarum strain 9R live vaccine (Sg9R), derived from a well-characterized Master Seed, was used in various studies. The animals were vaccinated at different ages by various routes of administration (Tables 1 and 2). The dose given was up to 100 times the recommended field dose.

Observations
Host Species

All birds were observed daily for the presence of clinical signs including depression, poor feathering, diarrhea and death. At 14 days post last vaccination, a total of 230 birds from five of the studies were subjected to postmortem examination. Any gross abnormalities in organs were recorded.

Other Species

From the calves, fecal samples were taken once a week. A 10 g fecal sample was placed in 90 ml Peptone water and incubated, followed by selective enrichment in Selenite broth and inoculation onto Rambach agar. Plates were checked for Salmonella colonies after overnight incubation. Rectal temperatures were measured.

The turkeys were monitored daily, after vaccination, for any clinical signs. Eight to eighteen days post inoculation, the birds were sacrificed for postmortem examination.

II. Reversion to virulence

Animals

A total of 89 one-day-old SPF chickens, obtained from 5 different hatches, were used in this study.

Vaccine and Vaccination

For the initial passage, ten one-day-old birds received a 0.1 ml oral dose of a live Salmonella gallinarum strain 9R vaccine at $10^{9.8}$ CFU (100X the field dose). Four days after inoculation, the liver and spleen were removed from each bird and homogenized in a minimal amount of saline. The tissue suspension was used as the inoculum for the next passage. A total of 5 sequential passages were performed at 4 day intervals in one-day-old SPF leghorn chickens. Each inoculum was cultured to attempt reisolation of the vaccine organism (Table 3).

Observations

All birds were observed daily for the presence of clinical signs until they were necropsied. Organs and/or tissues (heart, lung, liver, spleen, kidney, gastrointestinal tract) were examined. Any gross abnormalities were recorded and scored.

III. Horizontal Transmission

Animals

Egg-producing chickens, including both SPF (Leghorn) and commercial (Lohman-brown and Hyline-brown), were tested for horizontal transmission of the vaccine organism.

Vaccine and Vaccination

Sg 9R live vaccine was used in various studies. The animals were vaccinated at different ages by various routes of administration (Table 4). In each study 5-20 birds were left unvaccinated to serve as sentinels.

Tissue Colonization and Seroconversion

After 14 days to 22 weeks contact time between the vaccinates and the sentinels, a necropsy was performed to determine tissue colonization of the vaccine organism. The liver, spleen and cecum went through a Salmonella enrichment procedure. Serological testing, by rapid slide agglutination, was carried out in two of the studies to determine seroconversion.

IV. Vertical Transmission

Animals
137 SPF leghorns

Vaccine and Vaccination

Sg 9R live vaccine was used at 10^7 CFU/dose. At 6 weeks of age, 101 of the birds received a 0.2 ml subcutaneous vaccination and the remaining 35 birds received the vaccine by wing web stab. Four weeks later all of the birds received an oral booster vaccination in the drinking water.

Culturing of Eggs

For three months the eggs from the Sg9R vaccinated hens were collected. A total of 335 eggs were collected. The eggs and shells were enriched in tetrathionate (TT) broth. Egg and shell washing cultures were streaked onto Brilliant Green agar for Salmonella detection.

A second enrichment procedure followed.

Risk Analysis

To determine the potential risk of our Salmonella gallinarum 9R strain vaccine to animals, public health and the environment, a risk analysis was performed according to USDA guidelines.

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Safety Studies and Risk Analysis of an Attenuated *Salmonella gallinarum* Live Vaccine for Layer Chickens (2)

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Results:

Table 1. Safety of Salmonella gallinarum 9R Strain in Host Species

Study	Breed	Commercial/ SPF[a]	Age at 1st vac	No. of Birds	Vaccination				Clinical Signs	Gross Lesions
					First		Booster			
					Route	Level (log_{10})	Route	Level (log_{10})		
1	Leghorn	SPF	1 day	10	spray	7.9	DW[b]	8.6	None	None
	Leghorn	SPF	1 day	10	oral	8.0	DW	8.6	None	None
	Leghorn	SPF	2 wks	10	DW	8.5	DW	8.5	None	None
	Leghorn	SPF	6 wks	10	DW	9.6	N/A[c]	N/A	None	None
2	Isa-brown	Commercial	1 day	15	spray	8.6	DW	8.4	2/15 Dead[d]	None
	Hyline-brown	Commercial	1 day	15	spray	8.6	DW	8.4	None	None
	Leghorn	SPF	1 day	15	spray	8.6	DW	8.4	None	None
3	Isa-brown	Commercial	1 day	20	spray	8.6	DW	8.1	2/20 Dead[d]	None
	Isa-brown	Commercial	2 wks	10	WW[e]	8.8	DW	8.4	None	None
	Isa-brown	Commercial	2 wks	10	SQ[f]	8.9	DW	8.4	None	None
	Isa-brown	Commercial	4 wks	10	DW	8.6	DW	8.4	None	None
	Isa-brown	Commercial	4 wks	15	SQ	8.6	DW	8.4	None	None
	Isa-brown	Commercial	4 wks	15	WW	8.5	DW	8.4	None	None
	Isa-brown	Commercial	6 wks	10	SQ	8.8	DW	8.4	None	None
	Isa-brown	Commercial	6 wks	10	WW	8.8	DW	8.4	None	None
4	Hyline-brown	Commercial	4 wks	15	WW+	8.5	WW+	8.6	None	None
					SQ+	8.7	SQ+	8.6		
					DW[g]	8.7	DW	8.4		
	Hyline-brown	Commercial	6 wks	15	WW+	8.8	WW+	8.8	None	None
					SQ+	8.8	SQ+	8.8		
					DW	8.5	DW	8.5		
5	Dekalb-brown	Commercial	4 wks	15	WW+	8.7	WW+	8.8	None	None
					SQ+	8.8	SQ+	8.9		
					DW	8.4	DW	8.5		
6	Leghorn	SPF	6 wks	10	WW	7.3	WW	7.4	None	N/D[h]
	Leghorn	SPF	6 wks	10	WW	7.3	DW	7.0	None	N/D
	Leghorn	SPF	6 wks	10	SQ	7.2	DW	7.0	None	N/D
	Leghorn	SPF	6 wks	10	SQ	7.2	WW	7.4	None	N/D
7	Leghorn	SPF	6 wks	34	SQ	7.0	DW	7.1	None	N/D
	Leghorn	SPF	6 wks	35	WW	6.9	DW	7.1	None	N/D
8	Leghorn	SPF	6 wks	75	SQ	7.0	DW	6.6	None	N/D

[a] SPF = specific pathogen free
[b] DW = drinking water
[c] N/A = not applicable
[d] The vaccine is intended to be used in chickens at least 6 weeks of age.
[e] WW = wing web
[f] SQ = subcutaneous
[g] A combination of SQ, WW and DW routes.
[h] N/D = not determined

Table 2. Safety of *Salmonella gallinarum* 9R Strain in Turkeys and Calves

Animal	No.	1st Vaccination			2nd Vaccination			3rd Vaccination			Clinical Signs	Gross Lesions	Reisolation From Feces
		Age	Route	Level (log_{10})	Age	Route	Level (log_{10})	Age	Route	Level (log_{10})			
Turkey	10	4 wks	Im[a]+ Sq[b]+ oral[c]	7.8 7.8 8.1	-	-	-	-	-	-	none	none	N/D[d]
Calf	4	3 wks	oral	9.9	5 wks	oral	10.1	7 wks	SQ	9.0	temp.[e]	N/D	none
Calf	4	3 wks	SQ	8.9	7 wks	SQ	9.0	-	-	-	temp.	N/D	none

[a] IM = intramuscular
[b] SQ = subcutaneous
[c] A combination of IM, SQ and oral routes.
[d] N/D = not determined
[e] temp. = A transient temperature increase (0.9C) was measured in all calves beginning approximately 5 hours post last vaccination. All temperatures returned to normal

Summary of Animal Safety Data

- The Sg 9R vaccine has been extensively tested in our laboratory:
  - At different ages: from day-old to 10 weeks of age
  - 1X - 100X levels (10^7 CFU/dose - 10^9 CFU/dose)
  - By different routes (SQ, WW, Oral, DW, Spray, IM)
  - In different breeds of chickens, and turkeys and calves
  - Results:
    - our S. gallinarum 9R strain does not revert to virulence following sequential *in vivo* passages in chickens.
    - is safe for chickens when administered at two weeks of age or older.
- The vaccine organism has been extensively studied by independent researchers
  - for many years under both laboratory and field conditions
  - in small and large scale (field trial) throughout the world

Table 3. Reversion to Virulence (Backpassage) Study

Passage level	Dose	PI*, day	Recovery rate
1	$>10^{9.6}$	4	20/20
		14**	6/10
2	homogenate	4	3/10
3	homogenate	4	0/9
4	homogenate	4	0/10
5	homogenate	4	0/10
		14**	0/10
N/A	Saline	5	0/5
		14**	0/5

* Days post inoculation
**These birds were observed for clinical signs for 14 days post inoculation using passage level 1,passage level 5 or saline (control).
- Chickens were inoculated orally at day of age.
- No clinical signs, gross lesions or loss of weight gain were observed.

Possibility of Egg Transmission

Literature
- **From eggs:**
  9/190 (4.7%; 10 birds) *(Smith, 1956)***
  13/688 (1.9%; 14 birds) *(Gordon et al., 1959)****
  0/160 *(0% 16 birds) (Nassar et al., 1994)**
  0/495 (0%; 42 birds) *(Silva et al., 1981)*
- **From egg shell:**
  1/160 *(Nassar et al., 1994)**
- **From ovaries:**
  1/42 *(Silva et al., 1981)**
  ? *(Bouzoubaa et al., 1989)**

Our Laboratory
- **From eggs:**
  0/335 *(0%; 137 birds)*

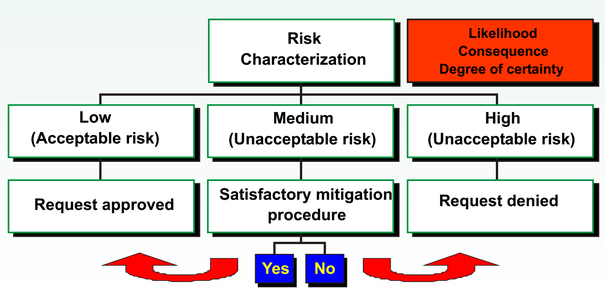
* In these studies, isolationswere done post challenge.
** In this dtudy, birds were vaccinated during lay
*** In this study, birds were vaccinated just before the onset of lay

Table 4. Horizontal Transmission (Spread)Studies*

Study	Dose/route	No. of Vac.	No. of Sentinel	Contact time	Sentinel Culture	Sentinel Seroconversion
A	10^8/Oral-SQ	5	5	14 days	0/5	ND
B	10^7/SQ	>10,000	20	22 wks	0/20	ND
C	10^7/SQ	>10,000	10	21 wks	0/10	ND
D	10^8/SQ,WW,DW	30	10	8 wks	0/10	0/10
E	10^7/SQ,DW	67	10	19 wks	0/10	0/10

* Studies used both commercial brown-egg layers and SPF Leghorns.
Summary:
- No *Salmonella* was isolated from any of the sentinel birds.
- No seroconversion in sentinel birds.
- Exaggerated conditions: close contact, high vaccinates/sentinel ratio, and highdose in two of the studies.

USDA Procedure for Risk Analysis
Gay and Orr, APHIS, 1994



Risk Ratings for Live S. gallinarum 9R Vaccine

	Likelihood		Consequence		Expec.	Risk
Safety	R	DC	R	DC	risk	rating
Animal	L(1)	C(1)	L(1)	MC(.75)	0.7500	Low
Human	L(1)	MC(.75)	L(1)	MC(.75)	0.5625	Low
Environ.	L(1)	C(1)	L(1)	MC(.75)	0.7500	Low

- R = rating (L = low, 1.0; M = medium, 0.5; H = high, 0.1)
- DC = degree of certainty (C = certain, 1.0 MC = moderately certain, 0.75; U = uncertain, 0.5)
- Expected risk = Risk rating = (Likelihood rating X DC) X (Consequence rating X DC)
- Low risk when risk rating > 0.375

Conclusions

The 9R strain was proved safe in all chicken breeds tested except for one-day-old Isa-brown commercial layers where some mortality occurred (4/35). In the reversion to virulence study, the 9R strain was isolated only from the chickens receiving the first two sequential passages when performed in day-old chickens. Under both laboratory and field conditions, no horizontal transmission was evident as monitored by reisolation attempts and serology. In several of these studies, conditions were exaggerated with overcrowding, high vaccinate/sentinel ratios, and high titer vaccine doses. Of 335 eggs collected from 137 birds in the vertical transmission study, there were no isolations of the vaccine strain.

The above studies further establish the safety and negligible transmissibility of the 9R strain and reinforce the already established attributes of the strain: 1) well-characterized and well-documented as an effective live vaccine strain, 2) S. gallinarum is avian species-specific and not a human pathogen, and 3) has been in existence for more than 40 years (strain stability) and has been used in commercial poultry in several countries for over 10 years with no reported problems (eggs and meat from vaccinated birds were consumed).

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