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<Original Article>

Establishment of a live vaccine strain against fowl typhoid and paratyphoid

Sun-Hee Cho[1], Young-Jin Ahn[1], Tae-Eun Kim[1], Sun-Joong Kim[1], Won Huh[2], Young-Sik Moon[2], Byung-Hyung Lee[2], Jae-Hong Kim[3,5], Hyuk Joon Kwon[4,5,]*

[1]*BioPOA Co., Yongin 17093, Korea*
[2]*Daesung Microbiological Lab. Co., Euiwang 16103, Korea*
Laboratories of [3]Avian Diseases and [4]Poultry Production Medicine, and [5]Reserch Institute for Veterinary Science,
College of Veterinary Medicine, Seoul National University, Seoul 08826, Korea

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Abstract : To develop a live vaccine strain against fowl typhoid and paratyphoid caused by *Salmonella* serovar Gallinarum biovar Gallinarum (*Salmonella* Gallinarum) and *Salmonella* serovar Enteritidis (*Salmonella* Enteritidis), respectively, several nalidixic acid resistant mutants were selected from lipopolysaccharide (LPS) rough strains of *Salmonella* Gallinarum that escaped from fatal infection of a LPS-binding lytic bacteriophage. A non-virulent and immunogenic vaccine strain of *Salmonella* Gallinarum, SR2-N6, was established through *in vivo* pathogenicity and protection efficacy tests. SR2-N6 was highly protective against *Salmonella* Gallinarum and *Salmonella* Enteritidis and safer than *Salmonella* Gallinarum vaccine strain SG 9R in the condition of protein-energy malnutrition. Thus, SR2-N6 may be a safe and efficacious vaccine strain to prevent both fowl typhoid and paratyphoid.

Keywords : attenuation, fowl typhoid, live vaccine, paratyphoid, rough strain

Introduction

Fowl typhoid is an acute septicemic disease of adult chickens that is characterized by anemia, leukocytosis, and hemorrhage [28]. It is a disastrous disease in the poultry industry because it is extremely difficult to eradicate, which can cause enormous economic loss. The causative agent, *Salmonella* Gallinarum biovar Gallinarum (*Salmonella* Gallinarum), is non-motile and host-adapted [1, 28].

Salmonella Gallinarum was identified in South Korea during the 1992 fowl typhoid outbreaks and has since been isolated nationwide [12, 23]. The lipopolysaccharide (LPS) rough vaccine strain of *Salmonella* Gallinarum, SG 9R, has been used in fowl typhoid and paratyphoid prevention [2, 7, 15]. However, vertical transmission via eggs and chicken virulence were suspected [29]. SG 9R is a rough strain that lacks O-side chain repeats of LPS due to a single nonsense mutation in *rfaJ* [13, 17]. The *Salmonella* plasmid virulence (*spv*) genes, *spvB* and *spvC*, (among *spvR, A, B, C,* and *D*) on the large virulence plasmids of pathogenic *Salmonella* serotypes can replace the virulence of the entire plasmid [9, 19]. Since SG 9R possesses intact *spvB* and *spvC* and is frequently isolated in the field cases of fowl typhoid, its high pathogenicity to chicks in the condition of protein-energy malnutrition (PEM) has raised questions concerning the safety of SG 9R in the field [13].

Salmonella serovar Enteritidis (*Salmonella* Enteritidis) causes paratyphoid in poultry, which is important as the major causative agent of food-poisoning related to poultry products in the world [27]. In Korea, efforts to eradicate *Salmonella* Enteritidis have involved an extensive monitoring system to detect anti-D-group O-antigen including the application of antibody in the field [22]. Recently another attenuated fowl typhoid vaccine strain was developed by an allelic exchange method using a suicide vector [18]. The vaccine strain has intact LPS genes and may induce anti-D-group O-antigen antibody, which may cause confusion to the *Salmonella* Enteritidis monitoring system. Thus, a live vaccine strain that is safe and avoids confusion to the monitoring system is needed. Test and slaughter is the basic strategy to prevent *Salmonella* Enteritidis. However, fowl typhoid vaccine protecting against *Salmonella* Enteritidis may be more valuable in countries where fowl typhoid is endemic. SG 9R was reported to be protective against *Salmonella* Enteritidis [7, 24].

In this study we established a rough, safe and efficacious vaccine strain, SR2-N6, and evaluated its vaccine efficacy against fowl typhoid and paratyphoid, and safety by animal experiments.

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*Corresponding author
Tel: +82-2-880-1226, Fax: +82-2-885-6614
E-mail: kwonhj01@snu.ac.kr

Materials and Methods

Bacteria and bacteriophage

The rough vaccine strain SG 9R (Intervet, The Netherlands) and virulent field strains (SG002, SG120, and SG0197) of *Salmonella* Gallinarum and *Salmonella* Enteritidis (SE38) were cultured with Mac Conkey agar plate (Difco, USA) and LB broth (Difco) at 37°C. To select a LPS mutant of SG002, the *Salmonella* Gallinarum-specific bacteriophage, ϕSG-JL2, which possesses a receptor comprised of O-side chain repeats of LPS was prepared as previously described [14].

Rescue of a rough, nalidixic acid resistant, avirulent, and immunogenic *Salmonella* Gallinarum strain, SR2-N6

A virulent field strain of *Salmonella* Gallinarum, SG002 [5×10^7colony forming units (CFU)/mL] was co-cultured with ϕSG-JL2 at a multiplicity of infection (moi) of 10 in LB broth (Difco) for 48 h to select rough mutants of SG002. The cultured bacteria were spread on MacConkey agar plates containing 100 μg/mL of nalidixic acid and cultured for 48 h at 37°C. The nalidixic acid-resistant (Nal^r) rough colonies were picked and cultured in LB broth at 37°C overnight. Each rough and Nal^r mutant was diluted to 1×10^7 CFU/mL and subcutaneously inoculated into 10 1-day-old (do) commercial male brown layer chicks. Each chick was observed for 7 days. Rough and Nal^r mutants that did not cause mortality and development of lesions on the liver and spleen, and which showed higher spleen to body ratio were selected. The selected mutants were subcutaneously inoculated (approximately 1×10^7 CFU/mL) into 20 1-day-old commercial male brown layer chicks. After 7 days each chick was challenged with 1×10^6 CFU/mL of SG120 orally.

When all the animal experiments were conducted, the Institutional Animal Care and Use Committee of Seoul National University was not established yet. However, all chickens used in the present study were euthanized by cervical dislocation and all efforts were made to minimize suffering. During the experiment, all chickens were reared in air-filtered isolators (Three-Shine, Korea) and feed and water were provided *ad libitum*.

Biochemical and antimicrobial susceptibility testing

Biochemical traits were tested as previously described [23] and the susceptibilities to 10 antimicrobial agents (ampicillin, chloramphenicol, enrofloxacin, erythromycin, gentamicin, nalidixic acid, norfloxacin, penicillin, streptomycin, and tetracycline) were tested by the disk diffusion assay according to the standard procedure [6].

Auto-agglutination test

SR2-N6 and SG002 (1×10^5 CFU per inoculum) were incubated in 200 μL of LB broth in U-bottom 96-well plates and incubated at 37°C overnight. Small, round margin precipitation spot and large, irregular margin precipitation spot were read to be autoagglutination negative and positive, respectively.

LPS analysis

LPS was extracted from 1×10^9 CFU of SR2-N6 and SG002 as previously described with slight modification [32]. We used RNeasy kit (iNtRON Biotechnology, Korea) for LPS extraction and proteinase K (30 mg/mL) was added into solubilized LPS to remove contaminated proteins. LPS electrophoresis was performed by using 4–12% gradient SDS-PAGE gel (Thermo Fisher Scientific, USA) and LPS was examined by silver staining as previously described [32].

Acid susceptibility test

SR2-N6, SG 9R, and SG002 strains were tested for their susceptibility to hydrogen chloride (HCl). Four milliliters of HCl (0.1 N, pH 3.0) and 1 mL of fresh cultured bacteria adjusted to an approximate density of 1×10^8 CFU/mL were mixed and incubated for 0, 0.5, 1, and 2 h. Each sample was diluted 10-fold and spread on two or three Mac Conkey agar plates for each dilution to calculate the CFU.

Vaccine efficacy test against *Salmonella* Gallinarum

The minimal protection titer of SR2-N6 was measured as follows. SR2-N6 was diluted from 2.8×10^8 CFU to 2.8×10^4 CFU in 10-fold steps. Each diluted SR2-N6 and 6.3×10^7 CFU of SG 9R was inoculated twice to 20 6- and 18-week-old commercial brown layer chickens via intramuscular route and challenged with 1.0×10^6 CFU of a virulent strain, SG0197 at 3 week post-vaccination. As a challenge control, 20 unvaccinated chickens were challenged at 21 weeks of age by SG0197 as above. The mortality was observed for 14 days.

To understand the effect of number and age of vaccination 2.8×10^7 CFU of SR2-N6 and 6.3×10^7 CFU of SG 9R were inoculated at 6- and 18-weeks-of-age. The mortality was observed for 14 days.

Vaccine efficacy test against *Salmonella* Enteritidis

SG 9R (6.3×10^7 CFU) and SR2-N6 (2.8×10^7 CFU) were inoculated into 40 1-day-old brown layer chicks (20 chicks for each vaccine) via subcutaneous route and challenged with SE38 (1.0×10^6 CFU) via subcutaneous route after 2 weeks. The mortality was observed for 14 days and surviving chicks were euthanized by cervical dislocation for observation of lesions in the livers and reisolation of bacteria. During 14 days of observation feed and drinking water were provided *ad libitum* for 11 days and feed was not provided during last 3 days to enrich *Salmonella* Enteritidis [13].

Potential pathogenicity test

Commercial 1-day-old male brown layer chicks ($n = 20$) were assigned to four groups, SG 9R challenge-no fasting, SG 9R challenge-fasting, SR2-N6 challenge-no fasting, and SR2-N6 challenge-fasting groups. SG 9R and SR2-N6 (1.0 ×

Table 1. Selection of avirulent and efficacious vaccine strain

Group	Inoculum (CFU)*	Number of chicks	Mortality† (%)	Mortality after challenge‡ (%)
Control	0	20	0	70
SR2-N6	1×10^7	20	0	0
SR2-R10	1×10^7	20	0	65

*Colony forming units. †Mortality caused for 7 days. ‡Mortality caused by challenge of wild virulent strain, SG120, for 10 days.

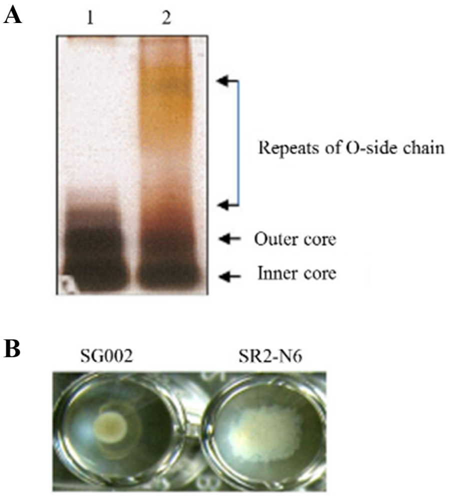

Fig. 1. LPS profiles (A) and autoagglutination test (B) of SR2-N6 (1-815). Lane 1, SR2-N6; Lane 2, SG002. LPS was extracted by using RNeasy kit and proteinase K (30 mg/mL) was added into solubilized LPS to remove contaminated proteins. LPS electrophoresis was performed by using 4-12% gradient SDS-PAGE gel and LPS was stained by using silver staining. SG002 and SR2-N6 (1×10^5 CFU/well) were inoculated into LB broth in a 96-well plate and incubated at 37°C overnight. SG002 grew as small and round margin precipitation spots, but SR2-N6 presented as large and irregular margin precipitation spots.

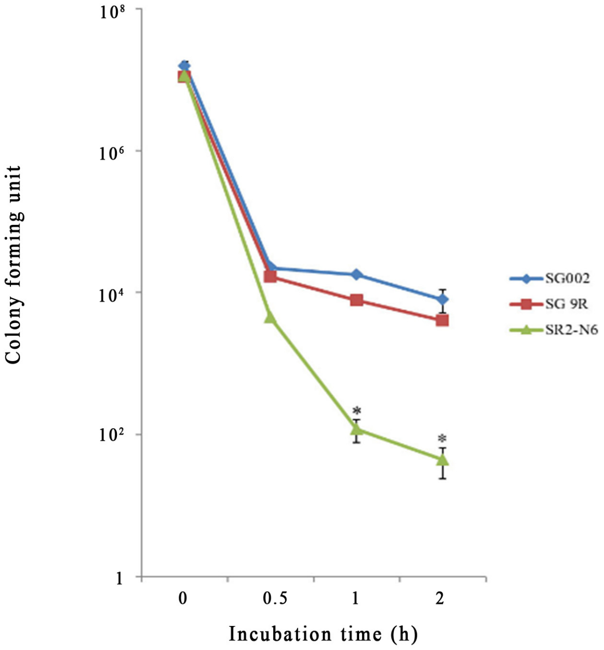

Fig. 2. Acid susceptibility test. Four milliliters of HCl (0.1 N, pH 3.0) and 1 mL of freshly cultured SR2-N6, SG 9R, and SG002 were mixed and incubated for 0, 0.5, 1, and 2 h. Each sample was diluted 10-fold and spread on MacConkey agar plate to calculate the CFU. Asterisk (*) represents a significant decrease of SR2-N6 compared to SG 9R and SG002 ($p < 0.05$).

10^7 CFU) were subcutaneously inoculated and fasting for 3 days with drinking water started on 14 day post inoculation of SG 9R and SR2-N6 [13]. Surviving chicks were euthanized by cervical dislocation for necropsy and the bacteria were isolated from cotton-bud samples of the livers.

Statistical analyses

The average CFUs of HCl-treated bacteria were evaluated for statistical significance using one-way analysis-of-variance and all data are expressed as the mean ± SD ($p < 0.05$). The mortality and bacterial re-isolation rate differences in the efficacy tests were assessed using Fisher's exact test (95% confidence intervals).

Results

Establishment of SR2-N6

Nineteen rough and Nal^r mutants were tested for their pathogenicity in 1-day-old chicks. Among them, SR2-N6 and SR2-R10 showed no mortality for 7 days. In contrast to SR2-R10 (65% mortality), SR2-N6 (0% mortality) showed perfect protection efficacy when challenged after 7 days with SG120. The avirulent and efficacious vaccine strain SR2-N6 was selected (Table 1).

Phenotypic characterization of SR2-N6

The LPS profile of SR2-N6 revealed only the inner and outer core of LPS due to loss of O-side chain repeats (Fig. 1A). SR2-N6 formed rough colonies with undulated margins and agglutinated precipitates when incubated overnight in U-bottom 96-well plates (Fig. 1B). SR2-N6 showed the same biochemical characteristics as *Salmonella* Gallinarum, except for negative H_2S production (data not shown). SR2-N6 was resistant to penicillin, erythromycin, nalidixic acid, and enrofloxacin (intermediate) but was susceptible to ampicillin, gentamicin, streptomycin, norfloxacin, tetracycline, and chloramphenicol. SR2-N6 was more susceptible to HCl than SG002 and SG 9R ($p < 0.05$; Fig. 2).

Table 2. Vaccine efficacy of SR2-N6 against *Salmonella* serovar Gallinarum biovar Gallinarum

Experiment	Group	CFU of vaccine/chick	Age (weeks) at		Mortality (%)
			Vaccination	Challenge	
1	SR2-N6	2.8×10^8	6/18	21	0/20 (0%)*
		2.8×10^7			0/20 (0%)*
		2.8×10^6			2/20 (10%)*
		2.8×10^5			8/20 (40%)
		2.8×10^4			9/20 (45%)
	SG 9R	6.3×10^7			0/20 (0%)*
	Control	0			12/20 (60%)
2	SR2-N6	2.8×10^7	6	21	3/20 (15%)*
			18		1/20 (5%)*
			6/18		0/20 (0%)*
	SG 9R	6.3×10^7	6		4/20 (20%)
			18		1/20 (5%)*
			6/18		0/20 (0%)*
	Control	0	–		10/20 (50%)

*Significant difference from the control ($p < 0.05$).

Table 3. Vaccine efficacy of SR2-N6 against *Salmonella* serovar Enteritidis (SE) infection

Group	Number of chickens	Positive rates (%)	
		Lesion of liver	Re-isolation of bacteria
SR2-N6	20	2/20 (10%)	1/20 (5%)*
SG 9R	20	8/20 (40%)	2/20 (10%, SE)*; 7/20 (35%, SG 9R)
Control	20	14/20 (70%)	11/20 (55%)

*, significant difference from the control ($p < 0.05$).

Table 4. Pathogenicity of SR2-N6 to chickens in the condition of protein-energy malnutrition

Group	Fasting*	Mortality	Lesion	Bacteria re-isolation
SG 9R	Yes	4/5	5/5	5/5
	No	0/5	1/5	1/5
SR2-N6	Yes	0/5	0/5	0/5
	No	0/5	0/5	0/5

*, no feed for 3 days with drinking water.

Vaccine efficacy of SR2-N6 against fowl typhoid

The minimal efficacious titer of SR2-N6 was determined to be 2.8×10^6 CFU/chick due to significant protection compared to the control group ($p < 0.05$). One or two rounds of vaccination of SR2-N6 at 6- and/or 18-weeks-of-age (0–15% mortalities) were significantly protective as compared to the negative control group (60% mortality), and the protection efficacies were similar to those of SG 9R (0–20% mortalities). Vaccination performed twice absolutely protected chickens from mortality (Table 2).

Vaccine efficacy of SR2-N6 against *Salmonella* Enteritidis infection

Vaccination of SR2-N6 significantly protected chickens in terms of liver lesions (10%) and bacterial growth (5%) compared to the control groups (70% and 55%, respectively) ($p < 0.05$). SG 9R vaccinated chickens showed relatively high rate of liver lesions (40%) and SG 9R (35%) was more frequently isolated than the challenge strain, SE38 (10%). However, the SG 9R vaccination protected significantly the growth of SE38 ($p < 0.05$; Table 3).

Potential pathogenicity of SR2-N6

Commercial 1-day-old male brown layer chicks were inoculated with SR2-N6 and SG 9R, and starved for the last 3 days during 14 days post-inoculation to reproduce fowl typhoid by SR2-N6 and SG 9R [13]. Four out of five SG 9R inoculated chicks starved for 3 days died, and all chicks were positive for severe liver necrotic foci and bacterial reisolation. However, SR2-N6 did not cause any mortality, lesion and bacterial growth in the starved chicks (Table 4).

Discussion

To date several live attenuated vaccine strains against fowl typhoid have been developed, but SG 9R has been used popularly in the field [18, 25]. Since the first outbreak of fowl typhoid in Korea in 1992, the disease has exacted huge economic losses in the Korean poultry industry [12, 23]. Nationwide vaccination of SG 9R has markedly reduced the frequency of fowl typhoid outbreaks in commercial layers. However, potential pathogenicity of SG 9R has been reported [13, 29]. SG 9R can be transmitted from hen to their progeny by transovarian route and its transmission to commercially sold eggs is possible [29]. Although the possibility of SG 9R transmission in table eggs has been denied in previous reports, it may depend on the health condition of layers [7, 13]. Single point mutation of *rfaJ* can convert the rough-type LPS of SG 9R to smooth-type LPS, which typically increases virulence [16]. Although a proteome and transcriptome study revealed multigenic changes of SG 9R, SG 9R has potential pathogenicity to be isolated from typical fowl typhoid cases [11, 13]. Thus, a safer but similarly efficacious vaccine strain has been demanded.

The immunity of humans and animals is severely reduced by PEM [3, 21]. Macrophages play important role in the protection and clearing of systemic *Salmonella* infection by oxidative killing and expression of the Th1-associated cytokine, IL18 [5, 30, 31]. PEM induces apoptosis and alteration of macrophage intracellular signaling [20, 26], and reduces T-lymphocyte number and function [4, 10]. Under the PEM condition SG 9R caused mortality and typical liver lesions but SR2-N6 caused no mortality and the lesions [13]. In addition, SG 9R persisted longer than SR2-N6 and was reisolated from 35% of chickens together with *Salmonella* Enteritidis. Thus, SR2-N6 was safer than SG 9R, but the protective efficacy of SR2-N6 against *Salmonella* Gallinarum and *Salmonella* Enteritidis was similar to SG 9R. Considering the fecal-oral transmission of *Salmonella* Gallinarum the higher susceptibility of SR2-N6 to low pH than SG 9R may reduce the chance of horizontal transmission of SR2-N6.

Recent frequent isolations of SG 9R from typical fowl typhoid cases encourage further differential diagnosis of field isolates from SG 9R. In the aspect of differential diagnosis SR2-N6 can be easily differentiated from field isolates and SG 9R by auto-agglutination, and nalidixic acid resistance and H_2S-negative traits, respectively. To eradicate *Salmonella* Enteritidis contamination in poultry products a serological method detecting O-side chain of LPS has been developed [22]. Because the antigenic structure of O-antigen of *Salmonella* Enteritidis is identical to *Salmonella* Gallinarum, therefore rough strain which is in defect of O-side chain may be preferable as a vaccine strain [8]. Thus, SR2-N6 may not induce antibody against O-antigen, and it may eliminate confusion to O-antigen-based *Salmonella* monitoring system in the field.

In conclusion SR2-N6 may be an efficacious and safe vaccine strain against both fowl typhoid and paratyphoid, and be apt for conventional *Salmonella* monitoring system.

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