Vaccine 29 (2011) 1311–1318

Contents lists available at ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

Pathogenicity of SG 9R, a rough vaccine strain against fowl typhoid

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ARTICLE INFO

Article history:
Received 26 April 2010
Received in revised form
16 November 2010
Accepted 22 November 2010
Available online 4 December 2010

Keywords:
Pathogenicity
Salmonella gallinarum
SG 9R
rfa
SPI-2
spv

ABSTRACT

SG 9R, a rough vaccine strain of *Salmonella gallinarum*, has been used for the prevention of fowl typhoid and paratyphoid in the world despite the presence of residual virulence. SG 9R-like rough strains have been recently isolated from fowl typhoid cases; however, molecular markers to differentiate SG 9R from field strains are not well-characterized and the molecular mechanisms of SG 9R residual virulence are unclear. Therefore, we analyzed LPS biosynthesis (*rfa* gene cluster) and virulence genes (*spv*, SPI-2) of both SG 9R and *S. gallinarum* rough field strains. SG 9R carried a unique nonsense mutation in *rfaJ* (TCA to TAA) and a shared *rfaZ* mutation (G-deletion) by rough and smooth *S. gallinarum* strains. SG 9R also carried intact SPI-2 and *spvC*, *B*, *A*, and *R* (except deleted *spvD*). SG 9R-like rough strains ($n = 10$) carried identical mutations in virulence-related genes to SG 9R. SG 9R and SG 9R-like rough strains did not demonstrate significant mortality or liver lesions under normal conditions. However, fowl typhoid was successfully reproduced in the present study by SG 9R inoculation to 1-day-old male brown layer chicks *per os* following starvation. Therefore, the LPS defect may be one of the major mechanisms of SG 9R attenuation, and the possession of intact SPI-2, *spvC*, *B*, *A*, and *R* virulence genes may be associated with residual SG 9R virulence.

1. Introduction

Fowl typhoid is primarily an acute septicemic disease of adult chickens, characterized by anemia, leukocytosis, and hemorrhage [1]. It is a disastrous disease in the poultry industry because it is extremely difficult to eradicate and results in enormous economic losses. *Salmonella gallinarum* is the causative agent; it is non-motile, host-adapted, and rarely induces food poisoning in humans [2,3].

Testing and slaughter policy has historically been the sole measure used to control fowl typhoid because of the eradication difficulty of *S. gallinarum*. *S. gallinarum* was originally identified in Korea during the 1992 fowl typhoid outbreak, and has been detected nationwide. Therefore, the rough vaccine strain SG 9R has been used in fowl typhoid prevention [4]. SG 9R is a rough strain and lacks O-side chain repeats [4,5]; however, the mutation of LPS biosynthesis genes and the molecular basis of SG 9R attenuation (including virulence gene expression) are unclear. The *spvB* and *spvC Salmonella* virulence plasmid (*spv*) genes (including *spvR*, *A*, *B*, *C* and *D*) on the large virulence plasmids of pathogenic *Salmonella* serotypes can replace the virulence of the entire plasmid [6,7]. *Salmonella typhimurium* can resist the bactericidal effects of complement through Rck (encoded by the *rck* gene, which confers resistance to complement killing) [8]. The *Salmonella* pathogenicity islands SPI-1 and SPI-2 are located in the *Salmonella* chromosome in two major gene clusters, and may be related to the virulence of *Salmonella* serotypes. SPI-2 in particular has been associated with *S. gallinarum* pathogenicity [8,9].

SG 9R has been used as vaccine for the control fowl typhoid and paratyphoid; however, vertical transmission via eggs and chicken virulence were suspected [10–13]. In addition, several SG 9R-like rough strains in Korea were isolated from fowl typhoid cases in chickens previously vaccinated with SG 9R. The mechanisms of attenuation and residual SG 9R virulence were characterized in the present study to assess virulence gene expression and mutation (*spv*, *rck*, *invA*, and SPI-2 genes) and nucleotide changes of the LPS biosynthesis gene (*rfa*) cluster. Further, we characterized SG 9R-like rough strains ($n = 10$) and examined residual SG 9R virulence through the reproduction of fowl typhoid by *per os* SG 9R inoculation following starvation.

2. Materials and methods

2.1. Bacteria, media and primers

The rough vaccine strain SG 9R (Intervet, Boxmeer, The Netherlands), the rough field strains SNU0005, SNU0098, SNU1014,

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doi:10.1016/j.vaccine.2010.11.067

Table 1
SG 9R-like rough strains of *Salmonella gallinarum*.

Strains	Year	Age at vaccination (1st/2nd; day-old)	Age at isolation (day-old)	Diagnosis
SNU0005	2000	nk[a]	113	FT (SG 9R)[c]
SNU0098	2000	nk	86	FT (SG 9R)
SNU1014	2001	nk	Pip embryo[b]	FT (SG 9R)
SNU1106	2001	42/107	137	FT (SG 9R)
SNU1108	2001	42/107	139	FT (SG 9R)
SNU2152	2002	48	95	FT (SG 9R)
SNU4014	2004	42/110	179	ND[d], LPAI[e], FT (SG 9R)
SNU5085	2005	60/115	113	ND, MD[f], FT (SG 9R)
SNU5094	2005	50/115	103	FT (SG 9R)
SNU5116	2005	70/115	159	FT (SG 9R)

[a] Not known.
[b] Dead embryo.
[c] Fowl typhoid (FT)-like lesions including SG 9R-associated hepatomegaly and necrotic hepatic foci.
[d] Newcastle disease.
[e] Low pathogenic avian influenza.
[f] Marek's disease.

SNU1106, SNU1108, SNU2152, SNU4014, SNU5085, SNU5094, and SNU5116 (Table 1), and virulent *S. gallinarum* strains SG16, SG52, SG60, SG101, and SG0197 [14] were cultured with Mac Conkey agar (DIFCO, Detroit, MI) plate and LB broth (DIFCO) at 37 °C. Primers were designed using nucleotide sequences of the *S. gallinarum* genome (NC_011274, LPS biosynthesis gene cluster, and SPI-2) and the *S. typhimurium* large virulence plasmid (CP001362, spv genes), as seen in Table 2.

2.2. Biochemical and serological test

Biochemical traits were tested as previously described [15] and *Salmonella* serotyping was conducted by the slide agglutination test using O group antisera (DIFCO).

2.3. Autoagglutination test

The SG 9R and field-isolated rough strains (1×10^5 cfu per inoculum) were incubated in 200 μl LB broth in V-bottom 96-well plates at 37 °C overnight.

2.4. LPS profile test

LPS was extracted from 1×10^9 cfu SG 9R as previously described [16]. Proteinase K (30 mg/ml) was added to remove contaminated proteins. LPS electrophoresis and staining were performed as previously described [16].

2.5. PCR and RT-PCR

S. gallinarum DNA was extracted with the G-spin for Bacteria kit (iNtRON Biotechnology Co., Seoul, Korea) per the manufacturer's instructions. *S. gallinarum* total RNA was extracted with the TRI reagent per the manufacturer's instructions with slight modifications. SG 9R (1×10^9 cfu) was resuspended in TRI reagent (1 ml) after centrifugation to separate aqueous and organic layers (200 μl of aqueous solution was added to 1 ml TRI reagent and RNA was extracted per the manufacturer's instructions). Primer sets used in *Salmonella* gene PCR and RT-PCR were summarized in Table 2. Primer sets and PCR and RT-PCR conditions were used as previously described for the diagnosis of Newcastle disease, low pathogenic avian influenza, and Marek's disease [17–19]. The PCR solution was composed of 10× buffer (2 ml), dNTPs (2.5 mM, 0.4 ml), forward and reverse primers (10 pmol/ml, 0.5 ml each), Taq DNA polymerase (5 U/ml, iNtRON Biotechnology Co., Seongnam, Korea, 0.2 ml), distilled water (15.4 ml), and template DNA (50 ng/ml, 1 ml). Cycling conditions were as follows: 94 °C, 5 min; 35 cycles at 94 °C, 30 s; 55 °C, 30 s; and 72 °C, 2.5 min; and a final extension step at 72 °C, 5 min. Amplicons were analyzed through electrophoresis on 1.0% agarose gels, and a 1 kb ladder was the molecular size marker (iNtRON Biotechnology Co.).

2.6. Sequencing, sequence analysis, and restriction enzyme analysis (REA)

The amplicons of the LPS biosynthesis gene cluster (15,414 nucleotides [nt] covering *kdtA*, *rfaQ*, *rfaG*, *rfaP*, *yibR*, *rfaB*, *rfaI*, *rfaJ*, *waaY*, *rfaZ*, *rfaK*, *rfaL*, *rfaC*, *rfaF*, and *rfaD*), the *spv* genes (6833 nt covering *spvR*, *spvA*, *spvB*, *spvC*, and *spvD*), and SPI-2 (27,863 nt covering *ssaU*, *ssaT*, *ssaS*, *ssaR*, *ssaQ*, *ssaP*, *ssaO*, *ssaN*, *ssaV*, *ssaM*, *ssaL*, *ssaK*, *ssaJ*, *ssaI*, *ssaH*, *ssaG*, *sseG*, *sseF*, *sscB*, *sseE*, *sseD*, *sseC*, *sscA*, *sseB*, *sseA*, *ssaE*, *ssaD*, *ssaC*, *ssaB*, *ssrA*, *ssrB*, *orf242*, *orf319*, and *orf70*) were purified by a PCR purification kit (iNtRON Biotechnology Co.) according to the manufacturer's instructions and were sequenced with an ABI3711 automatic sequencer (Macrogen Co., Seoul, Korea). Sequences were analyzed with the BioEdit program (T.A. Hall Software, version 5.0.9.1) and were compared to the GenBank database of the National Center for Biotechnology Information BLAST network [20]. Restriction enzyme analysis (REA) was used for the rapid and simple differentiation of SG 9R from virulent field strains. Amplicons generated by the primer set (rfaJF and rfaJR) were treated with *Mse*I (1 U for each sample, New England Biolabs Inc., MA) and electrophoresed on 2% agarose gels (Table 1).

2.7. Pathogenicity of field isolates of SG 9R

Commercial 1-day-old male brown layer chicks ($n = 50$) were assigned to challenge ($n = 4$) and negative groups ($n = 1$). Challenge groups were inoculated subcutaneously with rough field strains (SNU1014 and SNU5116), SG 9R, and a virulent field strain (SG0197) and observed for 14 days. A total of 1×10^7 cfu per chick was used for each strain. Surviving chicks were sacrificed for necropsy 14 days post-inoculation and bacteria were isolated from liver cotton-bud samples.

2.8. Reproduction of fowl typhoid by SG 9R

Commercial 1-day-old male brown layer chicks ($n = 15$) were assigned to 2 challenge groups and 1 negative control group. The challenge groups were orally inoculated with SG 9R and observed for 14 days. One challenge group was starved for 3 days with drinking water only after 14 days post-inoculation, and mortality was observed in this group. Surviving chicks were sacrificed for necropsy and the bacteria were isolated from cotton-bud samples of the livers.