Effect of 50 Kilobase-Plasmid, pKDSC50, of *Salmonella choleraesuis* RF-1 Strain on Pig Septicemia

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ABSTRACT. *Salmonella choleraesuis* strains with and without 50-kilobase plasmid (pKDSC50) were intravenously inoculated into Yorkshire pigs. By the inoculation of 7.2×10^5 - 3.5×10^7 cells, RF-1 strain with pKDSC50, but not 31N-1 strain without the plasmid, caused a septicemia. The inoculation of 8.7×10^9 RF-1 cells killed pigs at 2-4 day postinfection with severe hemorrhage on the whole body. Pigs with a similar number of 31N-1 cells (8.3×10^9 cells), showed milder hemorrhage, and they died at 6 day postinfection. These results indicated that pKDSC50 is required for RF-1 strain to express the full virulence causing a heavy cutaneous pig septicemia.—**KEY WORDS:** pig septicemia, *Salmonella choleraesuis*, virulence plasmid.

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We have previously shown that *Salmonella choleraesuis* strains isolated from septicemic pigs and humans harbored a cryptic 50-kilobase (kb) plasmid [3]. Using isogenic strains, we demonstrated that *S. choleraesuis* strain carrying the 50 kb plasmid, RF-1, was more virulent against mice infection than the plasmid-cured strain, 31N-1 [3]. Thus, the 50 kb plasmid (designated pKDSC50) of RF-1 was characterized to be the virulence plasmid in a mouse model [5]. *S. choleraesuis* is a pig-adapted bacterial agent to cause septicemia. Although experimental pig salmonellosis by a virulent or avirulent *S. choleraesuis* has been described [1, 6], the plasmid-mediated virulence of this organism for pig infection has not been reported. In the present study, the role of pKDSC50 of *S. choleraesuis* RF-1 strain for pig septicemia was examined.

Pigs (Yorkshire pig, 2-3 months old) were inoculated intravenously with two isogenic RF-1 and 31N-1 strains of *S. choleraesuis* with and without pKDSC50, respectively. As previously reported, RF-1 is a rifampicin resistant derivative of AH1-S44 strain isolated from septicemic pig in Japan. pKDSC50 of RF-1 is cured in 31N-1 strain, and RF-1 and 31N-1 have the same surface antigenicity (O7, Hc, Hi, H5), colony morphology (smooth), and susceptibility to various antibiotics [3]. Transient clinical signs such as inappetence and depression were observed in the pigs infected with 7.2×10^5 or 3.5×10^7 cells of RF-1. They showed a prolonged febrile state in rectal site (Fig. 1), but lived up to termination of the experiment. A similar number of 31N-1 cells did not cause the diseases except for a slight fever after the inoculation of 8.6×10^6 cells.

All four pigs inoculated with 8.7×10^9 cells of RF-1 developed inappetence, depression, and fever. These symptoms were observed immediately after the injection, and progressed rapidly. As shown in Fig. 2A, three of the four pigs died on day 2 postinfection with severe cutaneous hemorrhage on the whole body including ears, feet, tails, and ventral abdominal skin (3P-8 and 3P-9) or with purple ears (3P-10). The remaining pig (3P-7) lay buried in its bedding taking an abdominal position on day

Fig. 1. Fluctuation of body temperature of pigs infected with *S. choleraesuis* strains RF-1 and 31 N-1.

RF-1 and 31 N-1 grown on DHL-agar medium overnight were inoculated into L-broth medium, and incubated for 6 h with shaking. After washing and resuspension with phosphate-buffered saline (PBS), bacteria were intravenously inoculated into pigs. I; 7.2×10^5 (RF-1) and 7.4×10^5 (31N-1), II; 3.5×10^7 (RF-1) and 8.6×10^6 (31N-1), and III; 8.7×10^9 (RF-1) and 8.3×10^9 (31N-1) CFU cells. Pigs inoculated with RF-1; —●—●—, 31N-1; --○--○--, and PBS (control); —x—x—.

1, lateral position on day 2, dysstasis state on day 3, and died on day 4 postinfection with hemorrhage detected on the whole body as severe as that of 3P-8 and 3P-9. All four pigs inoculated with 8.3×10^9 cells of 31N-1 developed clinical signs such as inappetence, depression, and fever on day 2-5 and died on day 6 postinfection. As shown in Fig. 2B, the cutaneous changes were relatively mild in degree, but pig 3P-12 showed petechial hemorrhage on the whole body and had purple ears. These observations summarized in Table 1 demonstrated that RF-1 is more virulent than 31N-1 against pigs. It could also be pointed out that inoculation of about 10×10^9 cells of RF-1 and 31N-1 strains regardless to presence or absence of pKDSC50 leads to septicemia in pigs in our experimental condition, but inoculation of about 10^7 or less cells does not.

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Fig. 2. Appearance of pigs at death by the infection with *S. choleraesuis* strains RF-1 and 31N-1.

A; Pigs died on day 2 postinfection with 8.7×10^9 CFU of RF-1 cells. From top to bottom are pigs 3P-8, 3P-9, and 3P-10. B; Pigs died on day 6 postinfection with 8.3×10^9 CFU of 31N-1 cells. From top to bottom are pigs 3P-13, 3P-12, 3P-14, and 3P-11.

Table 1. Time of death and clinical signs of pigs infected with *S. choleraesuis* strain RF-1 or 31N-1

Exp.	Strain (pKDSC50)	No. of bacteria infected	Codes of pig	Time of death (day)	Clinical signs			
					fever	inap.	deprs.	hemor.
Exp. 1	RF-1(+)	7.2×10^5	1P-66	>21	41.5	+	+	—
			1P-72	>21	41.6	+	+	—
	31N-1(—)	7.4×10^5	1P-70	>21	40.5	—	—	—
			1P-71	>21	40.1	—	—	—
Exp. 2	RF-1(+)	3.5×10^7	2P-20	>7	41.6	+	+	—
			2P-21	>7	41.7	+	+	—
	31N-1(—)	8.6×10^6	2P-22	>7	41.1	—	—	—
			2P-23	>7	41.1	—	—	—
Exp. 3	RF-1(+)	8.7×10^9	3P-7	4	42.2	++	++	++
			3P-8	2	41.9	++	++	++
			3P-9	2	41.8	++	++	++
			3P-10	2	40.8	++	++	+
	31N-1(—)	8.3×10^9	3P-11	6	41.9	++	++	+
			3P-12	6	42.2	++	++	+
			3P-13	6	42.2	++	++	+
			3P-14	6	42.1	++	++	+

Fever is the maximum body temperature ($^{\circ}\text{C}$) shown during the observation periods. Inappetence (inap.), depression (deprs.), and hemorrhage (hemor.) were graded by —; negative, +; positive and mild, and ++; positive and severe.

Recovery of bacteria from the blood, organs, bile, and intestinal contents was examined chronologically in every experiment as shown in Tables 2 and 3. After the infection of 7.2×10^5 - 3.5×10^7 cells of RF-1, prolonged, but sporadic bacteremia was observed in all pigs, and a

significant number of bacteria was isolated from liver, spleen, kidney, lymph nodes, and bile. On the contrary, no bacteremic pig was found after the injection of 7.4×10^5 - 8.6×10^6 cells of 31N-1. These findings indicated that the injected RF-1 cells persisted in the pig organs longer than

Table 2. Recovery of bacteria from the blood of pigs inoculated with *S. choleraesuis* strain RF-1 or 31N-1

Exp.	Strains	Codes of pig	Number of bacteria isolated (log ₁₀ CFU/ml blood)									
			1	2	4	6	7	8	10	13	16	21 (day)
Exp. 1	RF-1	1P-66	+	—	+	—	nt	—	—	—	—	—
		1P-72	+	—	+	—	nt	—	—	+	—	—
	31N-1	1P-70	—	—	—	—	nt	—	—	—	—	—
		1P-71	—	—	—	—	nt	—	—	—	—	—
Exp. 2	RF-1	2P-20	+	—	—	—	—	—	—	—	—	—
		2P-21	2.0	1.0	3.0	+	—	—	—	—	—	—
	31N-1	2P-22	—	—	—	—	—	—	—	—	—	—
		2P-23	—	—	—	—	—	—	—	—	—	—
Exp. 3	RF-1	3P-7	3.5	4.5	6.0	—	—	—	—	—	—	—
		3P-8	5.0	6.4	—	—	—	—	—	—	—	—
		3P-9	5.8	6.2	—	—	—	—	—	—	—	—
		3P-10	6.0	—	—	—	—	—	—	—	—	—
	31N-1		(5.1)	(5.7)	(6.0)	—	—	—	—	—	—	—
		3P-11	3.5	3.5	4.3	5.5	—	—	—	—	—	—
		3P-12	3.0	3.9	4.6	6.4	—	—	—	—	—	—
		3P-13	3.0	3.3	4.5	5.4	—	—	—	—	—	—
		3P-14	3.7	3.8	4.8	7.3	—	—	—	—	—	—
			(3.3)	(3.6)	(4.6)	(6.2)	—	—	—	—	—	—

Juglar blood was spread on DHL-agar medium, and number of colonies grown on the medium was counted after incubation for overnight at 37°C. Limits of detection for Exp. 1 and Exp. 2 and 3 were 400 CFU and 10 CFU per ml of the blood, respectively. For selective enrichment, the blood was taken to Hajna-Tetrathionate medium and incubated for overnight at 37°C. +; bacteria were detected after selective enrichment, —; bacteria were not detected, nt; not tested. Figures in the parenthesis represent mean value.

Table 3. Recovery of bacteria from organs, bile, and intestinal contents of pig inoculated with *S. choleraesuis* strain RF-1 or 31N-1

Exp.	Strains	Codes of pig	Days examined	Number of bacteria isolated (log ₁₀ CFU/g samples)									
				liver	spleen	kidney	lymph node	bile	duodenum	ileum	cecum	colon	rectum
Exp. 1	RF-1	1P-66	21	—	—	—	—	—	—	nt	—	—	—
		1P-72	21	—	—	—	—	—	—	nt	—	—	—
	31N-1	1P-70	21	—	—	—	—	—	—	nt	—	—	—
		1P-71	21	—	—	—	—	—	—	nt	—	—	—
Exp. 2	RF-1	2P-20	7	2.8	3.3	+	3.1	—	+	+	+	—	+
		2P-21	7	2.7	3.1	+	3.7	3.6	—	+	—	—	—
	31N-1	2P-22	7	+	1.9	—	2.8	—	—	+	+	—	—
		2P-23	7	—	1.3	—	+	5.5	—	—	—	—	—
Exp. 3	RF-1	3P-7	4	7.6	8.1	6.5	6.5	7.4	5.5	4.2	5.5	+	2.6
		3P-8	2	7.2	8.2	7.8	5.2	nt	2.9	2.9	3.2	+	nt
		3P-9	2	7.5	8.7	7.3	7.7	4.4	3.8	4.1	3.5	3.5	3.5
		3P-10	2	8.1	8.7	7.7	8.0	4.1	3.2	4.3	3.8	+	+
	31N-1			(7.6)	(8.4)	(7.3)	(6.9)	(5.3)	(3.9)	(3.8)	(4.0)	(<1.6)	(<2.4)
		3P-11	6	8.8	8.5	6.4	7.4	8.8	6.1	6.2	6.6	7.1	nt
		3P-12	6	8.7	5.7	8.2	8.7	8.9	7.8	7.0	5.7	4.7	nt
		3P-13	6	9.1	8.9	8.0	8.0	8.6	6.2	5.6	6.1	5.5	6.5
		3P-14	6	8.7	8.7	8.2	8.7	9.1	nt	6.3	6.3	5.8	6.3
				(8.6)	(8.5)	(7.7)	(8.2)	(8.9)	(6.7)	(6.3)	(6.2)	(5.6)	(6.4)

A part of the emulsions prepared from 1 g of samples was spread on DHL-agar medium containing rifampicin at 50 µg/ml. The number of colonies grown after incubation for overnight at 37°C was counted. For selective enrichment, the emulsions were taken to Hajna-Tetrathionate medium and incubated for overnight at 37°C. +, —, nt, and figures in the parenthesis; see the footnote of Table 2.

the 31N-1 cells thus leading to the bacteremia. Pigs infected with either 8.7×10^9 cells of RF-1 or 8.3×10^9 cells of 31N-1 showed septicemia with about 10^6 bacterial cells/ml blood at the time of death. The time required to

attain this bacterial concentration was 2–4 days and 6 days postinfection for RF-1 and 31N-1, respectively, which demonstrates that RF-1 is more virulent than 31N-1.

At necropsy, pigs inoculated with 7.2×10^5 - 3.5×10^7

cells of RF-1 or 31N-1 showed relatively mild pathogenic lesions such as hepato- and splenomegaly, and swelling of mesenteric lymph nodes. Diseased and dead pigs on day 2–4 postinfection inoculated with 8.7×10^9 cells of RF-1 showed marked and cutaneous pathogenic lesions such as hepato- and splenomegaly, and swelling of mesenteric lymph nodes. They also showed congestion and hemorrhage in the lungs, heart, intestine, kidneys, spleen, liver and peritoneum, and insufficient blood coagulation. In the diseased and dead pigs on day 6 postinfection inoculated with 8.3×10^9 cells of 31N-1, retention of bloody ascites in the peritoneal-, pleural-, and pericardial cavities, hepato- and splenomegaly, and congestion or hemorrhage in internal organs or tissues were observed.

This paper reports a bacteriological and pathoanatomical study of the virulence of *S. choleraesuis* to pigs. In agreement with our previous reports on bacteriological and immunohistochemical experiments with *S. choleraesuis* in mice [3, 7], strain RF-1 with pKDSC50 plasmid showed persistent infection in pigs, but transient infection by strain 31N-1 without the plasmid. Our previous report showed that RF-1 and 31N-1 are chromosomally isogenic except for pKDSC50 [3, 4]. Consequently we concluded that the stronger virulence and severe septicemia in pig by RF-1 can be attributed to pKDSC50 plasmid. The clinical manifestations and pathological lesions produced by the infection of RF-1 were characteristic to septicemia cases reported previously [1, 2, 6, 8].

It is noteworthy that both RF-1 and 31N-1 strains caused pig septicemia by the inoculation of about 10^{10}

cells, though the former strain was more virulent than the latter. These results suggested that pKDSC50 is required for RF-1 strain to express a full virulence causing a heavy cutaneous pig septicemia.

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