SPI-2 of *Salmonella* Typhimurium is not necessary for long term colonization of pigs

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Abstract

Unravelling the role of *Salmonella* virulence factors in the porcine host could greatly contribute to the development of control measures such as vaccination. The virulence genes located on the *Salmonella* Pathogenicity Island 2 (SPI-2) are indispensable for the induction of systemic disease and persistence in BALB/c mice. The role of this pathogenicity island in the pathogenesis of *Salmonella* Typhimurium infections in pigs is not documented. Therefore, in the present study, the interactions of a porcine field strain of *Salmonella* Typhimurium and a non-polar isogenic SPI-2 (*ΔssrA*) deletion mutant were compared in both *in vitro* and *in vivo* models. The *ssrA* mutant strain displayed decreased SPI-2 expression levels *in vitro* and was attenuated in a mouse model after oral inoculation. No difference was seen in the expression of SPI-1 related virulence genes. Through flowcytometric analysis, the *ssrA* mutant strain was found to be moderately attenuated in intracellular replication in porcine macrophages *in vitro*. In an infection experiment, 2 groups of 10 piglets were orally inoculated with the wild type or the *ssrA* mutant strain. The infection of the animals inoculated with the *ssrA* mutant strain followed a similar course as the animals infected with the wild type strain. At days 5 and 28 post inoculation, the animals of both groups were infected to the same extent in the gut and gut-associated lymphoid tissue, as well as in the internal organs. These results suggest that SPI-2 of *Salmonella* Typhimurium may not contribute to the colonization of pigs to the same extent as it contributes to the colonization of BALB/c mice.

Introduction

Salmonella Typhimurium is the most frequently isolated serotype from pigs and pork (Botteldoorn et al, 2003). Carrier pigs can shed *Salmonella* for at least 28 weeks (Wood et al., 1989) and pose an important threat to animal and human health. The mechanism of this carrier state is not yet known. The virulence genes located on the *Salmonella* Pathogenicity Island 1 (SPI-1) are of great importance in the invasion of intestinal cells in various animal species (Zhou and Galan, 2001). The virulence genes located on the *Salmonella* Pathogenicity Island 2 (SPI-2) are indispensable for the induction of systemic disease and persistence in mice after both oral and intraperitoneal inoculation (Shea et al., 1999). The role of this pathogenicity island in the pathogenesis of *Salmonella* Typhimurium infections in pigs is not documented. It was the aim of the present study to determine the importance of SPI-2 in the colonization and persistence in pigs.

Material and Methods

Salmonella Typhimurium strain MB2486, isolated from a pig in Belgium, was used as the wild type strain (WT) to construct the *hilA* and *ssrA* deletion mutants, according to the one step inactivation method with lambda red for use in *Salmonella* Typhimurium (Boyen et al., 2006). The intracellular expression of the *ssrA* gene and of the SPI-2 effector gene *sifB* was quantified by real-time reverse transcription PCR, using SYBR Green, as described before (Botteldoorn et al., 2006). The SPI-1 expression level (*hilA*, coding for the major regulating protein of SPI-1; *sipA*, coding for a

SPI-1 effector protein) was measured in a late logarithmic culture in Luria-Bertani broth, also by real-time reverse transcrition PCR, using SYBR Green. *In vitro* invasion and cytotoxicity assays were conducted on porcine pulmonary alveolar macrophages (PAM), as described before (Boyen et al., 2006). BALB/c mice (6 weeks old, 2 groups of 8 animals) and conventional *Salmonella* negative piglets (5 weeks old, 2 groups of 10 animals) were inoculated orally with approximately 10^8 and 10^7 colony forming units respectively of the wild type strain or the *ssrA* deletion mutant. As a control group, 4 animals were sham-inoculated with PBS. Four mice of each *Salmonella* inoculated group were humanely euthanized at day 1 and day 4 post inoculation. Five piglets of each *Salmonella* inoculated group were humanely euthanized at day 5 and day 28 post inoculation. Control animals were euthanized together with the last group of *Salmonella* inoculated animals. Internal organs of mice and piglets as well as faecal samples collected daily from the piglets were examined for the presence of *Salmonella* by means of plating ten-fold dilutions on Brilliant Green Agar. To assess the intramacrophagal replication deficit of the *ssrA* mutant strain, PAM were inoculated with the wild type strain or the *ssrA* mutant strain carrying the green fluorescent protein (GFP) expressing plasmid pFPV25.1. After 30 min incubation at 37°C under 5% CO_2, the flasks were washed and fresh medium supplemented with 100 µg/ml gentamicin was added. After an additional 60 min incubation, cells were released using trypsin and maintained on ice, protected from light until use (T=0h). To assess intracellular growth, fresh medium supplemented with 15 µg/ml gentamicin was added and at 6 hours after inoculation, cells were released and handled as described (T=6h). Flow cytometry measurements were made using a FACScanto™ cytometer. Macrophages were discriminated from bacteria and debris based on forward (FSC) and side (SSC) light scatter. GFP fluorescence was recorded using the FL1 channel (emission wave length: 515-545 nm).

Results

The complete coding sequence of the *ssrA* gene was deleted, which was confirmed with PCR and sequencing of the surrounding area. The *ssrA* mutant strain displayed no intracellular *ssrA* expression and a decreased *sifB* expression level *in vitro*. The expression levels of 2 SPI-1 encoded proteins were not altered (Table 1).

Table 1: Relative expression levels of SPI-1 and SPI-2 related genes in the wild type, the ssrA *mutant and the* hilA *mutant strain*

Relative expression	**Wild type**	***ssrA* mutant**	***hilA* mutant**
Intracellular SPI-2 expression	*ssrA*: 1.0 *sifB*: 10.4	*ssrA*: 0.0 *sifB*: 2.6	Not Determined
SPI-1 expression in a logarithmic culture	*hilA* : 1.00 *sipA* : 0.10	*hilA* : 1.06 *sipA* : 0.07	*hilA* : 0.00 *sipA* : 0.01

In the murine *in vivo* model, the caeca of both groups of mice were colonized to the same extent 1 day post inoculation. Four days after inoculation, however, the internal organs of the mice inoculated with the *ssrA* mutant strain were colonized with a 100-fold reduction compared to the wild type strain (Table 2). The infection of the piglets inoculated with the *ssrA* mutant strain followed a similar course compared to the piglets infected with the wild type strain. The daily faecal excretion levels of both strains were not significantly different ($p>0.05$; non-parametric Kruskal-Wallis test; Table 3). At days 5 and 28 post inoculation, the animals of both groups were infected to the same extent in the gut and gut-associated lymphoid tissue, as well as in the internal organs (Table 3). All sham inoculated animals, mice as well as piglets, remained negative for *Salmonella* throughout the experiment.

Table 2: The colonization of different organs of BALB/c mice with the Salmonella *Typhimurium wild type or* ssrA *mutant strain ($log_{(10)}$ cfu/gram tissue +/- sd)*

Organ	Wild type	*ssrA* mutant
Caecum day 1	5.88 (+/- 1.13)	6.39 (+/- 1.20)
Caecum day 4	4.91 (+/- 1.43)	3.96 (+/- 0.39)
Liver day 4	3.02 (+/- 1.27)	1.03 (+/- 1.20)
Spleen day 4	3.79 (0.98)	1.37 (+/- 1.64)

Table 3: The colonization of different organs of piglets with the Salmonella *Typhimurium wild type and* ssrA *mutant strain (* frequency =* number of pigs positive/number of pigs inoculated)

		Wild type strain		*ssrA* mutant strain	
	Tissue	Frequency*	Log_{10} cfu g^{-1} ± sd	Frequency*	Log_{10} cfu g^{-1} ± sd
Day 5 pi	Tonsil	3/5	1.33 ± 1.57	4/5	3.14 ± 1.77
	Liver	4/5	0.8 ± 0.45	2/5	0.4 ± 0.55
	Spleen	2/5	0.58 ± 0.86	1/5	0.2 ± 0.45
	Ileocecal ln.	5/5	3.69 ± 0.62	5/5	4.04 ± 0.79
	Ileum	5/5	4.92 ± 0.52	5/5	5.17 ± 1.39
Day 28 pi	Tonsil	2/5	0.76 ± 1.23	4/5	1.88 ± 1.74
	Liver	0/5	0 ± 0	1/5	0.2 ± 0.45
	Spleen	0/5	0 ± 0	2/5	0.4 ± 0.55
	Ileocecal ln.	4/5	1.32 ± 1.34	4/5	0.98 ± 0.68
	Ileum	5/5	1.72 ± 1.61	5/5	1.18 ± 0.41

Six hours after inoculation, the mean and median green fluorescence of PAM inoculated with the GFP expressing *ssrA* mutant strain was significantly lower compared to PAM inoculated with the GFP expressing wild type strain (Table 4).

Table 4: Mean and median fluorescent values of infected porcine macrophages at 0 h and 6 h after inoculation. The average values of 3 independent experiments ± sem are shown. Both the mean and median fluorescent values of the PAM infected with the ssrA *mutant strain at 6 h pi were statistically significant lower (*; $p < 0.05$) than the values of the PAM infected with the wild type strain.*

	Mean fluorescence ± sd			Median fluorescent value ± sd	
	0h	6h		0h	6h
WT	1165 ± 110	3236 ± 488		831 ± 26	1862 ± 189
ΔssrA	1073 ± 124	1594 ± 298*		801 ± 57	1042 ± 166*

Discussion

In NRAMP$^{-/-}$ laboratory mice, SPI-2 has an important and highly documented impact on the pathogenesis of *Salmonella* Typhimurium infections, particularly on the systemic phase of the infection (Hensel et al., 1998; Shea et al., 1999), but also on the enteric phase (Coburn et al., 2005). Data describing the importance of SPI-2 in the systemic phase of infection obtained in food producing animals are less extensive. For host-restricted or host adapted serotypes (ex. Pullorum, Dublin, Choleraesuis) SPI-2 is a prerequisite for virulence and colonization in their respective hosts (Dunyak et al., 1997; Bispham et al., 2001; Jones et al., 2001; Wigley et al., 2002). However, for broad range serotypes, such as Enteritidis and Typhimurium, the role of SPI-2 in the pathogenesis of *Salmonella* infections in food producing animals is less described and less straightforward (Tsolis et al., 1999; Zhao et al., 2002; Morgan et al., 2004). In accordance, our results suggest that SPI-2 of *Salmonella* Typhimurium may not contribute to persistence in pigs to the same extend as it does in laboratory mice.

Conclusions

In conclusion, we have shown that a *ssrA* deletion mutant of a porcine field strain of *Salmonella* Typhimurium, that is attenuated *in vitro* and in an *in vivo* mouse model, is still capable of colonizing pigs and establishing a long term persistent infection. This work contributes to the recent insights in the serotype- and host-dependent pathogenesis of *Salmonella* infections in food producing animals.

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References

BISPHAM et al., 2000. *Salmonella* pathogenicity island 2 influences both systemic salmonellosis and *Salmonella*-induced enteritis in calves, Infection and Immunity, 69, 367-377.
BOTTELDOORN et al., 2003. *Salmonella* on pig carcasses: positive pigs and cross contamination in the slaughterhouse. Journal of Applied Microbiology, 95, 891-903.
BOTTELDOORN et al., 2006. Real-time reverse transcription PCR for the quantification of the *mntH* expression of *Salmonella enterica* as a function of growth phase and phagosome-like conditions. Journal of Microbiological Methods, 66, 125-135.
BOYEN et al., 2006. Role of SPI-1 in the interactions of *Salmonella* Typhimurium with porcine macrophages. Veterinary Microbiology, 113, 35-44.
COBURN et al.,2005. *Salmonella* Typhimurium pathogenicity island 2 is necessary for complete virulence in a mouse model of infectious enterocolitis. Infection and Immunity, 73, 3219-3227.
DUNYAK et al.,1997. Identification of *Salmonella* Pathogenicity Island 2 (SPI2) genes in Salmonella choleraesuis using signature-tagged mutagenesis. Abstr 97th ASM congress, 76.
HENSEL et al., 1998. Genes encoding putative effector proteins of the type III secretion system of *Salmonella* pathogenicity island 2 are required for bacterial virulence and proliferation in macrophages. Molecular Microbiology, 30, 163-174.
JONES et al., 2001. Salmonella enterica serovar Gallinarum requires the Salmonella pathogenicity island 2 type III secretion system but not the Salmonella pathogenicity island 1 type III secretion system for virulence in chickens. Infection and Immunity, 69, 5471-5476.
MORGAN et al., 2004. Identification of host-specific colonization factors of Salmonella enterica serovar Typhimurium, Molecular Microbiology, 54, 994-1010.
SHEA et al., 1999. Influence of the *Salmonella* typhimurium pathogenicity island 2 type III secretion system on bacterial growth in the mouse. Infection and Immunity, 67, 213-219.
TSOLIS et al., 1999. Contribution of *Salmonella* typhimurium virulence factors to diarrheal disease in calves. Infection and Immunity, 67, 4879-4885.
WIGLEY et al., 2002. Salmonella Pullorum requires the *Salmonella* pathogenicity island 2 type III secretion system for virulence and carriage in the chicken. Avian Pathology, 31, 501-506.

WOOD et al., 1989. Distribution of persistent *Salmonella* typhimurium infection in internal organs of swine. The American Journal of Veterinary Research, 50, 1015-1021.
ZHAO et al., 2002. Identification of genes affecting *Salmonella* enterica serovar enteritidis infection of chicken macrophages. Infection and Immunity, 70, 5319-5321.
ZHOU & GALAN, 2001. *Salmonella* entry into host cells: the work in concert of type III secreted effector proteins. Microbes and Infection, 3, 1293-1298.