Role of the *Salmonella abortusovis* virulence plasmid in the infection of BALB/c mice

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Received 29 February 2000; received in revised form 19 April 2000; accepted 1 May 2000

Abstract

Following oral inoculation of BALB/c mice, *Salmonella abortusovis* strain SS44 was recovered in lower numbers from the Peyer’s patches and mesenteric lymph nodes compared with *S. typhimurium* strain SL1344, whereas splenic infections were equivalent between the two serovars. SS44 was cured of its virulence plasmid or subjected to mutagenesis of the spv genes, and the Spv− derivatives were tested for virulence in mice. Plasmid-cured *S. abortusovis* SU40 retained virulence in BALB/c mice when inoculated intraperitoneally. On the other hand, mice infected orally with SU40 had greatly reduced splenic infection compared to those infected with wild-type SS44. Similar results were obtained after Tn5 insertion mutagenesis of the spvR gene or deletion of the spvABCD locus. These results suggest that in the gut-associated lymphoid tissues *S. abortusovis* may replicate less than *S. typhimurium* and that the *S. abortusovis* virulence plasmid primarily affects systemic infection after oral inoculation but not after intraperitoneal administration in the mouse model. ß 2000 Published by Elsevier Science B.V. on behalf of the Federation of European Microbiological Societies.

Keywords: *Salmonella abortusovis*; Virulence plasmid; spv gene; Mouse model; Peyer’s patch; Ovine salmonellosis

1. Introduction

*Salmonella enterica* serovar Abortusovis (*S. abortusovis*) is the leading cause of abortion in sheep among *Salmonella* serovars and has been mostly isolated in European countries where sheep herding is common. This serovar naturally infects ovises but not other animals of agricultural importance or humans. Infected animals present no signs of gastroenteritis, i.e., diarrhea, and the presence of the microorganism is generally investigated when several abortions occur in a flock.

Non-typhoid *Salmonella* serovars, including *S. abortusovis*, contain virulence plasmids that are required for the extra-intestinal phase of disease [1–3]. A common feature of the virulence plasmids is the presence of the spv genes, designated spvRABCD. The spv genes are involved in infection of extra-intestinal tissues, such as mesenteric lymph nodes, spleen, and liver, by increasing the growth rate of *Salmonella* in the intracellular compartment [4]. Analysis of 69 wild-type *S. abortusovis* isolates from different geographic areas showed the presence of a plasmid ranging in size from 50 to 67 kb in all strains [5]. Observations based on Southern blotting analysis of the spv locus suggest that the *S. abortusovis* virulence plasmid may be considered a virulence plasmid [5], but the function of the plasmid in *S. abortusovis* pathogenicity has not yet been determined.

Even though naturally occurring *S. abortusovis* infection appears to be limited to the ovine, infection of mice has been successfully used as a model to evaluate the virulence of wild-type and attenuated *S. abortusovis* strains [6,7]. Importantly, results from the mouse model have been confirmed in ovine infection [8]. In the present study, our objectives were: (1) to evaluate the invasion of Peyer’s patches, mesenteric lymph nodes, and spleen by *S. abortusovis* as compared to *S. typhimurium* in a murine model of oral infection; and (2) to examine the effect of *S. abortusovis* plasmid curing and mutagenesis after oral and intraperitoneal infection of BALB/c mice.
2. Materials and methods

2.1. Bacterial strains and plasmids

The strains and plasmids used in this study are described in Table 1. Wild-type \textit{S. abortusovis} SS44 [5] was isolated from the feral tissue of a sheep, mouse-passaged, and stored at $-70^\circ$C. TT2251 is \textit{S. typhimurium} carrying a Tn10 insertion in the virulence plasmid at an unknown position that does not affect virulence. Plasmid pLL6 is a temperature-sensitive (42°C) for replication, non-self-transferable, kanamycin-resistant plasmid of 55 kb previously used to construct plasmid-cured derivatives of \textit{S. typhimurium} LT2 [9].

2.2. Curing \textit{S. abortusovis} SS44 of the virulence plasmid and mutagenesis of the \textit{spv} genes

Plasmid pLL6 encoding kanamycin resistance was introduced into \textit{S. abortusovis} SS44 by electroporation. An isolated kanamycin-resistant colony was selected at random and subjected to three passages in broth culture with 50 µg ml$^{-1}$ of kanamycin at 30°C to enable displacement of the resident plasmid (the virulence plasmid and pLL6 are incompatible). Because the sizes of the two plasmids were nearly identical, we could not screen for loss of the resident plasmid by electrophoretic analysis. We therefore proceeded to eliminate pLL6 and examine for loss of both plasmids at the same time. After plating at 30°C on kanamycin, 10 colonies were selected at random and grown in broth without antibiotic at 42°C to cause the loss of the temperature-sensitive pLL6. A dilution was then plated out on non-selective plates at 42°C, and five isolate colonies were randomly chosen to carry out rapid mini-plasmid extractions. Four of the five colonies were kanamycin-sensitive and contained no plasmid DNA by electrophoretic analysis.

Table 1

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Description</th>
<th>Reference or source</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{S. abortusovis}</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SS44</td>
<td>wild-type</td>
<td>[5]</td>
</tr>
<tr>
<td>SU40</td>
<td>Plasmid-cured derivative of SS44</td>
<td>this study</td>
</tr>
<tr>
<td>SSM56</td>
<td>\textit{spvR}:Tn5 from UF006</td>
<td>this study</td>
</tr>
<tr>
<td>SU60</td>
<td>\textit{spvR}:Tn5 : tet from UF109</td>
<td>this study</td>
</tr>
<tr>
<td>\textit{S. typhimurium}</td>
<td></td>
<td></td>
</tr>
<tr>
<td>χ3456</td>
<td>SR-11, wild-type</td>
<td>[1]</td>
</tr>
<tr>
<td>TT2251</td>
<td>LT-2, \textit{zzc}69:Tn10, tet$^+$</td>
<td>B.A.D. Stocker</td>
</tr>
<tr>
<td>UF006</td>
<td>\textit{spvR}:Tn5, \textit{kan}$^+$</td>
<td>[10]</td>
</tr>
<tr>
<td>UF109</td>
<td>\textit{spvR}:Tn5, \textit{tet}$^+$</td>
<td>P.A. Gulig</td>
</tr>
<tr>
<td>Plasmids</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pLL6</td>
<td>temperature-sensitive, kan$^+$</td>
<td>[9]</td>
</tr>
<tr>
<td>pGTR061</td>
<td>carries \textit{S. typhimurium} \textit{spvR}:Tn5 and \textit{orfE}, \textit{amp}$^+$</td>
<td>[14]</td>
</tr>
</tbody>
</table>

DNA was transferred into smooth salmonellae by transduction with phage P22HTint. \textit{spvR}:Tn5 \textit{S. abortusovis} mutant SSM56 was obtained by transduction of the \textit{spvR}:Tn5 allele from \textit{S. typhimurium} UF006 [10] to \textit{S. abortusovis} SS44 and selecting for kanamycin-resistant colonies. Similarly, \textit{S. abortusovis} SU60 was constructed by transduction of the \textit{ΔspvR}:Tn5 : tet mutation from \textit{S. typhimurium} UF109 (Gulig, unpublished results), which is a precursor strain of \textit{S. typhimurium} UF110, into SS44 using P22 phage. The virulence plasmid of \textit{S. typhimurium} UF109 contains a deletion of a 6.3-kb 	extit{ColI} fragment encoding the \textit{spvR}:Tn5 genes replaced by a tetracycline marker (\textit{ΔspvR}:Tn5 : tet).

2.3. Mouse infections

Groups of five female BALB/c mice 6–8 weeks of age (specific-pathogen-free) were inoculated intraperitoneally (i.p.) or orally (p.o.) as previously described [1]. In specific experiments, following p.o. inoculation of mice, Peyer’s patches, mesenteric lymph nodes, and spleen were examined for colony-forming units (CFU) as described by Gulig and Doyle [4]. Briefly, mice were inoculated p.o. with $\sim 1 \times 10^8$ CFU of \textit{S. abortusovis}, and 5 days later, Peyer’s patches, mesenteric lymph nodes, and spleens were removed, homogenized in glass tissue homogenizers with phosphate-buffered saline, and plated to enumerate CFU.

3. Results and discussion

3.1. Tissue tropism of \textit{S. abortusovis} in infected BALB/c mice

BALB/c mice were infected orally with wild-type \textit{S. abortusovis} SS44, and the in vivo dissemination and survival of SS44 were compared to those of wild-type \textit{S. typhimurium} χ3456. Bacterial recovery from the Peyer’s patches and mesenteric lymph nodes of \textit{S. abortusovis}-infected mice was significantly lower than that observed in mice infected with \textit{S. typhimurium} (Table 2). In contrast, the splenic infections were very similar between the two

Table 2

<table>
<thead>
<tr>
<th>Strain</th>
<th>CFU (log$_{10}$±S.D.)</th>
<th>Peyer’s patches</th>
<th>Mesenteric lymph nodes</th>
<th>Spleen</th>
</tr>
</thead>
<tbody>
<tr>
<td>SS44</td>
<td>2.3±0.3$^*$</td>
<td>3.1±0.5$^*$</td>
<td>5.8±0.3$^{**}$</td>
<td></td>
</tr>
<tr>
<td>χ3456</td>
<td>4.9±0.2</td>
<td>4.9±0.5</td>
<td>5.6±0.8</td>
<td></td>
</tr>
<tr>
<td>SSM56</td>
<td>2.7±0.9</td>
<td>2.2±0.5</td>
<td>1.8±0.5</td>
<td></td>
</tr>
</tbody>
</table>

BALB/c mice were inoculated orally and killed 5 days later. Peyer’s patches, mesenteric lymph nodes, and spleens were aseptically removed, homogenized, and plated to enumerate CFU. P values for SS44 were calculated vs. χ3456: *$P<0.001$; **$P>0.5$. P values for SSM56 were calculated vs. SS44: $P>0.5$; $P=0.02$; $P<0.001$. 

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serovars. Therefore, it appears that *S. abortusovis* invades the Peyer’s patches and mesenteric lymph nodes before reaching the spleen, but replicates less in Peyer’s patches and mesenteric lymph nodes compared to *S. typhimurium* (Table 2).

Similar findings were obtained by Pascopella et al. [12] for *S. gallinarum*, the causative agent of the fowl typhoid syndrome, a disseminated infection that is also characterized by the absence of enteritis. These authors, while investigating the early steps of infection by *S. gallinarum* in the murine model, demonstrated that *S. gallinarum* has low tropism for the murine Peyer’s patch epithelium.

### 3.2. Association of the *S. abortusovis* virulence plasmid with virulence in BALB/c mice

Wild-type *S. abortusovis* SS44 was cured of its resident virulence plasmid by using an incompatible antibiotic-resistant plasmid, and the virulence of plasmid-cured mutant SU40 was tested by means of two routes of inoculation. Preliminary assays of LD<sub>50</sub> determinations showed that high doses of *S. abortusovis* are required to kill BALB/c mice both p.o. (LD<sub>50</sub> ≈ 5 x 10<sup>7</sup>) and i.p. (LD<sub>50</sub> ≈ 1 x 10<sup>8</sup>) (data not shown). Similar high values were previously found to be required for the subcutaneous and intravenous route of infection [6,7].

Mice infected p.o. with wild-type SS44 (1.5 x 10<sup>8</sup>) presented signs of disease after 5 days, whereas those infected with plasmid-cured SU40 (5.0 x 10<sup>8</sup>) appeared healthy. The SU40 splenic counts were significantly lower than those from SS44-infected mice (Table 3). As has been reported for other serovars, these results show that the *S. abortusovis* virulence plasmid is necessary for systemic infection of the reticuloendothelial organs in orally inoculated mice.

To evaluate the significance of the *S. abortusovis* virulence plasmid on the bacterial replication and survival following dissemination to the systemic sites, the virulence of *S. abortusovis* strain SS44 (3.1 x 10<sup>8</sup>) and its cured derivative, SU40 (5.4 x 10<sup>5</sup>), was also assessed by i.p. inoculation of BALB/c mice. Mice in both groups showed signs of disease 1 day post infection and were near death at 3 days when they were killed. The spleen counts obtained from SS44- and SU40-challenged animals did not differ significantly (Table 3). Similar results were observed by others with a plasmid-cured *S. pullorum* strain, which demonstrated a low, although significant, attenuation after parenteral inoculation, whereas orally inoculated cured salmonellae were completely avirulent [13]. It is possible that the i.p. route of infection initially results in less intracellular infection than oral inoculation. Because the plasmid-encoded *spv* genes increase the replication rate of salmonellae within host cells [4], there could be less dependence on the virulence plasmid for pathogenesis after i.p. inoculation of mice with *S. abortusovis*.

### 3.3. Effect of *spv* mutagenesis on *S. abortusovis* virulence in BALB/c mice

To assess the capability of the *spv* genes to restore virulence of SU40 in mice, we introduced the recombinant plasmid pGTR061 [14] carrying the *S. typhimurium* *spvRABCD* genes into SU40. The cloned *S. typhimurium* *spv* genes only partially complemented the lack of the entire virulence plasmid in SU40 for virulence (Table 3) since splenic CFU for SU40/pGTR061 were significantly higher than for SU40 alone, yet significantly lower than for SS44. This result is in contrast to the ability of pGTR061 to fully restore virulence to plasmid-cured *S. typhimurium* [14]. The observation that the cloned *S. typhimurium spv* genes on pGTR061 could not fully complement virulence of SU40 raises the possibilities that either the expression of *S. typhimurium spv* genes was not correctly regulated from pGTR061 in *S. abortusovis* or that other plasmid-encoded genes were involved in virulence of *S. abortusovis*. To examine these possibilities and to evaluate the role of *spv* genes in *S. abortusovis*, we constructed *spvR23::Tn5* (SSM56) and *ΔspvRABCD::tet* (SU60) *S. abortusovis* derivatives. The SSM56 and SU60 strains were then tested for virulence in mice by oral inoculation. Both strains were avirulent (Table 4). Recovery of both *spv* mutant strains from spleens was as low as that obtained with the plasmid-cured SU40 strain. In an attempt to complement the *ΔspvRABCD::tet* mutation, we introduced the *spv<sup>+</sup>* plasmid pGTR061 into SU60 and orally inoculated mice. Five days later, the spleen counts showed that pGTR061 was not able to fully restore virulence (Table 4). Interestingly, when pGTR061 was placed into wild-type SS44 the splenic CFU detected 5 days after infection were 10-fold lower than those of the wild-type parent, but the difference was not statistically significant (Table 4). These results are consistent with inappropriate expression of the *S. typhimurium spv* genes in *S. abortusovis* and are not consistent with other plasmid-encoded genes being involved with virulence in *S. abortusovis*.

To examine in more detail the differences in virulence between the wild-type strain and the *Spv<sup>-</sup>* derivatives,
SSM56 showed a 10^4-fold decrease in splenic CFU compared with SS44, and spleens were examined for CFU. The plasmid, SU60/pGTRO61, and 3.3 U for SU60, 2.4 U for SU60/pGTRO61, and 3.3 × 10^8 CFU for SS44/pGTRO61, and 3.3 × 10^8 CFU for SS44/pGTRO61. P values were calculated vs. SS44: *P = 0.009; **P = 0.116; P < 0.001.

Mice were infected orally with wild-type SS44 and spvR::Tn5 SSM56, and after 5 days Peyer’s patches, mesenteric lymph nodes, and spleens were examined for CFU. Inocula were 2.1 × 10^8 CFU for SS44, 3.0 × 10^8 CFU for SSM56, 1.1 × 10^8 CFU for SU60, 10^8 CFU for SSM56/pGTRO61, and 3.3 × 10^8 CFU for SU60/pGTRO61. P values were calculated vs. SS44: *P = 0.009; **P = 0.116; P < 0.001.

Mice were infected orally with wild-type SS44 and spvR::Tn5 SSM56, and after 5 days Peyer’s patches, mesenteric lymph nodes, and spleens were examined for CFU. Inocula were 2.1 × 10^8 CFU for SS44, 3.0 × 10^8 CFU for SSM56, 1.1 × 10^8 CFU for SU60, 2.4 × 10^8 CFU for SSM56/pGTRO61, and 3.3 × 10^8 CFU for SU60/pGTRO61. P values were calculated vs. SS44: *P = 0.009; **P = 0.116; P < 0.001.

In summary, S. abortusovis is capable of causing lethal systemic infection in BALB/c mice but, in contrast to S. typhimurium, does not rely on proliferation in the intestine as an initial step in the disease process. Furthermore, the S. abortusovis plasmid is not necessary for colonization of the intestine, but is essential for efficient infection of mesenteric lymph nodes before systemic dissemination. The possibility that systemic infection of the spleen mimics transplacental infection of fetuses in pregnant ewes suggests that the S. abortusovis virulence plasmid and spv genes could be essential in ewes for survival in the draining lymph nodes of the intestines and reaching the blood stream as early steps in infection of fetuses.

Acknowledgements

We thank M.P. Satta and P. Nicolussi for excellent technical assistance. This work was supported by NATO Grant 920838; by Regione Autonoma della Sardegna, Progetto Biotecnologie; by EU Grant AIR3-CT96-1743 to S.R.; and by NIH Grant AI24821 to P.A.G., who was an American Heart Association Established Investigator with funds contributed in part by the American Heart Association–Florida Affiliate.

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