

Salmonella enterica Serovars Typhi and Paratyphi A are avirulent in newborn and infant mice even when expressing virulence plasmid genes of Salmonella Typhimurium

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Abstract

Background: Salmonella enterica serovars Typhi and Paratyphi A are human host-restricted pathogens. Therefore, there is no small susceptible animal host that can be used to assess the virulence and safety of vaccine strains derived from these Salmonella serovars. However, infant mice have been used to evaluate virulence and colonization by another human host-restricted pathogen, Vibrio cholerae.

Methodology: The possibility that infant mice host could be adapted for Salmonella led us to investigate the susceptibility of newborn and infant mice to oral infection with S. Typhi and S. Paratyphi A. Salmonella enterica serovar Typhimurium causes enteric fever in adult mice and this system has been used as a model for human typhoid. The pSTV virulence plasmid, not present in S. Typhi and S. Paratyphi A, plays an essential role in S. Typhimurium colonization and systemic infection of mice. We also conjugated pSTV into S. Typhi and S. Paratyphi A serovars and evaluated these transconjugants in newborn and infant mice.

Results: We determined that the spv virulence genes from the S. Typhimurium virulence plasmid are expressed in S. Typhi and S. Paratyphi A in a RpoS dependent fashion. Also, we determined that S. Typhi and S. Paratyphi A with and without pSTV transiently colonize newborn and infant mice tissues.

Conclusion: Newborn and infant mice infected with S. Typhi and S. Paratyphi A do not succumb to the infection and that carriage of the S. Typhimurium virulence plasmid, pSTV, did not influence these results.

Key words: *Salmonella* Typhi; *Salmonella* Paratyphi A; newborn mice, infant mice; virulence plasmid

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Introduction

Typhoid and paratyphoid fever are severe human diseases caused by *S. Typhi* and *S. Paratyphi A* respectively, with an estimated 16 million cases resulting in more than 600,000 deaths annually [1,2]. Both *S. Typhi* and *S. Paratyphi A* are human host-restricted pathogens. The pathogenesis of typhoid and paratyphoid are poorly understood, in part due to the lack of a susceptible animal host that exhibits the same clinical signs as human infections. Attenuated *S. Typhi* strains have been used as live vectors to deliver foreign antigens either by expressing antigens or by delivering the antigen-encoding genes on eukaryotic expression plasmids [3,4,5]. The absence of an inexpensive, small animal host for pre-clinical evaluation of vaccine candidates is an obstacle to developing live attenuated *S. Typhi* vaccines. Chimpanzees infected with wild-type *S. Typhi* Ty2 produced a mild clinical illness that resembled human typhoid fever, but only when given in a high

dose (1×10^{11} CFU) [6]. This host is not convenient for high through-put analyses due to the high cost and scarcity of supply. In addition, since a high inoculum of wild-type is needed to cause clinical infection, this is not an ideal host for evaluating the virulence potential of attenuated strains [7].

S. Typhi and *S. Paratyphi A* are unable to induce progressive disease in adult BALB/c mice challenged orally or parenterally with high doses ($> 10^9$ orally; 10^7 to 10^8 parenterally) [8,9,10,11,12]. The current method for assessing the safety of *S. Typhi* vaccines consists of inoculating mice intraperitoneally with moderated doses ($> 10^3$ CFU) of *S. Typhi* suspended in hog gastric mucin [7]. However, death of the mice is believed to result from the toxic effects of endotoxin associated with the rapidly expanding peritoneal population of *S. Typhi* [13,14,15]. In addition, the attenuating effects of some mutations cannot be discerned by this method [16]. The use of other animal hosts, such as rabbits and pigs, has also

been explored, but these animals were found to be no more useful than mice [17, 18]. However, intranasal inoculation of mice has been used successfully to evaluate the immune responses to foreign antigens expressed by *S. Typhi* recombinant vaccines [19,20].

S. Typhimurium, the causative agent of enterocolitis infection in humans and cattle, causes a lethal systemic disease in susceptible mice that resembles human typhoid infection [21,22]. The mouse assay has been adopted and extensively used to study pathogenesis and immunity of typhoid fever. However, a shortcoming of this assay is the fact that *S. Typhimurium* does not cause typhoid fever in humans, suggesting that genetic differences between *S. Typhi* and *S. Typhimurium* are critically important for the disease outcome in both mice and humans. Whole-genome sequencing has revealed genome degradation in host-restricted *Salmonella* serotypes [23]. Therefore, the evolution from a broad host range serovar such as *Typhimurium*, to host-restricted serovars such as *Typhi* and *Paratyphi* A, may have occurred by genome degradation [21,23]. In addition, not all the information obtained using the *S. Typhimurium* mouse assay can be directly applied to improve understanding of typhoid fever since some of the virulence factors of *S. Typhimurium* such as *Salmonella* virulence plasmid pSTV, required for invasion of host tissues [24,25], are absent in *S. Typhi* and *S. Paratyphi* A [23,26,27,28].

Nevertheless, infant mice have been used to measure the median lethal dose (LD₅₀) as a parameter for disease production by *Vibrio cholerae* and other host-restricted bacterial strains [29,30,31] and therefore may be useful in developing a systemic infection model for *S. Typhi* and *S. Paratyphi* A, since adult mice are resistant to those pathogens [10]. In this study, the colonization and pathogenesis potential of *S. Typhi* and *S. Paratyphi* A in newborn and infant mice was evaluated. Newborn and infant mice were observed to be colonized by *S. Typhi* and *S. Paratyphi* A, but were tolerant of the infection. In addition, whether *S. Typhi* and *S. Paratyphi* A carrying and expressing the *S. Typhimurium* virulence plasmid were able to better infect and colonize newborn and infant mice was evaluated.

Methodology

Bacterial strains and culture conditions

The bacterial strains and plasmids used in this study are listed in Table 1. Bacteriological media and components were from Difco (Franklin Lakes, NJ). Antibiotics and reagents were from Sigma (St. Louis,

MO). *Salmonella* strains were grown at 37°C in either buffered magnesium minimal medium pH 5.5 (MgM) [32] or LB medium [33]. For plates, media was solidified with 1.5% (wt/vol) agar. When required, medium was supplemented with tetracycline (tet; 12.5 µg/ml), L-cysteine-HCl (cys; 22 µg/ml), DL-tryptophan (trp; 20 µg/ml) and L-histidine-HCl (his; 22 µg/ml). Buffered saline with gelatin (BSG) [34] was used as a diluent and to suspend bacteria prior to inoculation of mice.

Beta-galactosidase assays

Expression of *spvR-lacZ* and *spvA-lacZ* fusions in RpoS⁺ and RpoS⁻ *S. Typhi* was determined by β-galactosidase activity assay [35]. Strains were transformed with pGTR72 (*spvR-lacZ*, operon fusion), pGTR90 (*spvA-lacZ*, operon fusion) or pGTR75 (*tet-lacZ* operon fusion control) [36] and grown in MgM medium to stationary phase. The *spv* operon is up-regulated under conditions that mimic the *Salmonella* containing vacuole (SCV) [33]. MgM media was used for this experiment because it mimics the environment of SCV [37].

Construction and characterization of S. Typhi and S. Paratyphi A carry pSTV

S. Typhi and *S. Paratyphi* A strains harboring the *S. Typhimurium* virulence plasmid were constructed by conjugation on minimal media [38]. Plasmid pStSR101 is a Tn_{mini-tet}-labeled virulence plasmid derivative, which can restore the full virulence of pSTV-cured strains of *S. Typhimurium* [25]. Like the wild-type virulence plasmid, it is self-transmissible [38]. *S. Typhimurium* χ3351 SL1344 *hisG* carrying pStSR101 was used as the donor. Conjugation was performed in M9 minimal media agar [39], supplemented with cys, trp, and his. Selection was performed on M9 media supplemented with cys, trp and tet to select for *S. Typhi* and *S. Paratyphi* A transconjugants and against the histidine-requiring *S. Typhimurium* donor. Transconjugants were characterized for LPS, Vi antigen (*S. Typhi*), biochemical properties, and nutritional requirements as described [39] and were found to exhibit the expected phenotypes (Table 1). The stability of pStSR101 in *S. Typhi* and *S. Paratyphi* A was determined essentially as described by Konjufca *et al.* [40], except that strains were grown in the absence of tetracycline for fifty generations, at which point cells were plated onto LB agar and individual colonies were screened for tetracycline resistance.

Table 1. *Salmonella* strains and relevant characteristics

Strain	Relevant characteristics	Origin	Source or reference
χ 3744 <i>S. Typhi</i> ISP1820	Wild-type, RpoS ⁺ , Cys ⁻ , Trp ⁻ , OD ₁ :H _d : ⁻ :Vi, V & V/W form	Wild-type	41
χ 3744 <i>S. Typhi</i> ISP1820 (pStSR101)	Wild-type RpoS ⁺ , Cys ⁻ , Trp ⁻ , OD ₁ :H _d : ⁻ :Vi, V & V/W form, Tet ^r	χ 3744	This study
χ 3761 <i>S. Typhimurium</i> UK-1	RpoS ⁺ , pSTV, OB ₁ :H _i :H ₂ : ⁻	Wild-type	42
χ 3769 <i>S. Typhi</i> Ty2	Wild-type, RpoS ⁻ , Cys ⁻ , OD ₁ :H _d : ⁻ :Vi, V form	Wild-type	43
χ 3769 <i>S. Typhi</i> Ty2 (pStSR101)	Wild-type, RpoS ⁻ , Cys ⁻ , OD ₁ :H _d : ⁻ :Vi, V form, Tet ^r	χ 3769	This study
χ 8219 <i>S. Paratyphi</i> A	RpoS ⁺ , Cryptic plasmid pSPA1, OA:H _a : ⁻ : ⁻	Wild-type	ATCC 9281
χ 8387 <i>S. Paratyphi</i> A	RpoS ⁺ , Cryptic plasmid cured, OA:H _a : ⁻ : ⁻	χ 8219	This study
χ 8387 <i>S. Paratyphi</i> A (pStSR101)	RpoS ⁺ , OA:H _a : ⁻ : ⁻ , Tet ^r	χ 8387	This study
χ 8438 <i>S. Typhi</i> Ty2	RpoS ⁺ , Cys ⁻ , OD ₁ :H _d : ⁻ :Vi, V & V/W form	χ 3769	41
χ 8438 <i>S. Typhi</i> Ty2 (pStSR101)	RpoS ⁺ , Cys ⁻ , OD ₁ :H _d : ⁻ :Vi, V & V/W form, Tet ^r	χ 8438	This study
χ 8740 <i>S. Typhi</i> CT18	RpoS ⁺ , Vi ⁻ , OD ₁ :H _d : ⁻ : ⁻ , W form	Wild-type	27
χ 3337 <i>S. Typhimurium</i> SR-11	RpoS ⁺ , <i>gyrA1816</i> , pSTV ⁻ , OB ₁ :H _i :H ₂ : ⁻	χ 3306	25
χ 3351 <i>S. Typhimurium</i> SL-1344 (pStSR101)	RpoS ⁺ , <i>rpsL</i> , <i>hisG</i> , Tet ^r , OB ₁ :H _i :H ₂ : ⁻	χ 3340	25
Plasmids			
pGTR72	<i>spvR-lacZ, cat</i>		44
pGTR90	<i>spvA-lacZ, cat</i>		44
pGTR75	<i>spvR::lacZ, cat</i> cat, same as pGTR72, except the <i>lacZ, cat</i> is inserted in opposite orientation, under control of <i>tet</i> promoter of the plasmid		44
pSTV	<i>spvRABCD, pefBACD, rck</i>		25
pStSR101	<i>spvRABCD, pefBACD, rck, Tnmini-tet-</i>		25

Reverse Transcriptase-PCR

Expression of *spvR* and *spvA* in *S. Typhi* and *S. Paratyphi* A from pStSR101 was evaluated by RT-PCR. Total RNA extraction was performed by RNeasy QIAgene kit (Hilden, Germany) from strains grown in MgM media at 37°C to stationary phase [33,37]. Reverse transcription and PCR was performed using the one-step RT-PCR QIAgene kit. Specific primers were used for the *spvR* (5'-GGAAACAGGTTCTTCAGTATCGC-3' and 5'-TATTTGGCTGTAAACGGCTCTCCC-3') (size of the *spvR* amplified fragment: 349 bp) and *spvA* (5'-TTGTCCGTCAGACCCGTAACAGT-3' and 5'-TCTTCCAGCGACACATCGGTATT CAG-3') (size

of the *spvA* amplified fragment: 358 bp) genes. *16S rRNA* primers were used as control of expression (5'-ACTGGCAGGCTTGAGCTTGAGTA-3' and 5'-AAGGGCACAACCTCCAAGTA GACA-3') (size of the *16S rRNA* amplified fragment: 158 bp).

Animal experiments

BALB/c newborn and infant mice (Charles River Laboratories, Wilmington, MA) were bred and maintained at 22°C to 23°C with 12 hours of illumination daily. Mice older than two weeks were separated from their mothers 4 hours before infection and fed with regular food. Bacterial strains were grown overnight in standing cultures that were

diluted 1:100 in prewarmed LB broth and grown with mild aeration to an OD₆₀₀ of 0.8 to 0.9. Bacteria were sedimented by centrifugation at room temperature and resuspended in BSG to densities appropriate for the inoculation route and dose. Newborn mice (3 to 24 hours) and infant mice (48 hours to 3 weeks old) were challenged with ~10⁹ CFU of *S. Typhi*, *S. Paratyphi A*, or *S. Typhimurium*. Ten microliters of ~10⁹ CFU of the bacterial strain suspended in BSG were orally administered. Intranasal inoculations consisted in 5 µl of ~10⁹ CFU of the bacterial strain suspended in BSG, administered without anesthesia. Mice were euthanized via asphyxiation with CO₂ and necropsied at various times. The bacterial titers in

newborn mice inoculated orally were determined at 3, 7, 14 and 21 days post oral infection. Spleen, liver, and the intestines were collected and washed with BSG until homogenization. The homogenizer (Brinkman, Westburg, NY) was washed with 5% Amphyl, followed by a wash with 70% ethanol, followed by two washes with dH₂O. Homogenized tissues were plated onto MacConkey agar plates supplemented with 1% lactose to determine the number of viable bacteria. *Salmonella* colonies were white on the MacConkey plates. Isolated colonies were further identified by agglutination with *Salmonella* specific antiserum (Table 1) and antibiotic resistance marker.

Table 2. Infection of newborn and infant mice with *S. Typhi* and *S. Paratyphi A*.

Strain	Inoculating dose (CFU)	Mice age	Survivors/total	Route
χ3744 <i>S. Typhi</i> ISP1820	1.1 x 10 ⁹	3 weeks	5/5	oral
	1.1 x 10 ⁹	1 week	7/7	oral
	1.1 x 10 ⁹	<24 h	6/6	oral
	1.1 x 10 ⁹	1 week	7/7	intranasal
χ3769 <i>S. Typhi</i> Ty2 (RpoS ⁻)	1.1 x 10 ⁹	<24 h	5/5	oral
χ8438 <i>S. Typhi</i> Ty2 (RpoS ⁺)	1.5 x 10 ⁹	<24 h	4/4	oral
χ8740 <i>S. Typhi</i> CT18	1.0 x 10 ⁹	<24 h	8/9	oral
χ8387 <i>S. Paratyphi A</i>	1.1 x 10 ⁹	<24 h	8/8	oral
	1.1 x 10 ⁹	1 week	9/9	intranasal
χ3337 <i>S. Typhimurium</i> SR-11	2.1 x 10 ⁹	3 weeks	0/5	oral
	2.1 x 10 ⁹	<24 h	0/5	oral

Newborn: 3 h to 24 h after birth. Infant: 48 h to 3 weeks old. Mice were observed for 4-5 weeks. χ3337 *S. Typhimurium* pSTV-cured was used as positive control.

Statistics

Mann-Whitney U Test (version 5.0; GraphPad Software, Inc.) was used for comparing the expression of *spv-lacZ* fusions.

Results

Virulence and colonization of S. Typhi and S. Paratyphi A in newborn and infant mice

Newborn and infant mice infected orally with *S. Typhi* and *S. Paratyphi A* survived without any symptoms of disease, while the mice inoculated with a *S. Typhimurium* strain cured of plasmid pSTV succumbed to the infection (Table 2). Infant mice, one to three weeks of age, and newborn mice infected intranasally with *S. Typhi* and *S. Paratyphi A* strains

also survived without any symptoms of disease (Table 2). The bacterial titers in newborn mice inoculated orally were determined at 3, 7, 14 and 21 days post infection. Spleen, liver, and the intestines were collected and the number of viable bacteria was determined. *S. Typhi* RpoS⁺ and *S. Paratyphi A* RpoS⁺ were able to colonize the intestines for three weeks (Figure 1A). *S. Typhi* Ty2 RpoS⁻ was less able to persist in the intestines and cleared after one week. *S. Typhi* and *S. Paratyphi A* were able to colonize the spleen and liver of infected mice and the RpoS⁻ strain Ty2 was more effectively cleared than the RpoS⁺ strains (Figures 1B and 1C). These results are consistent with a report that *rpoS* *S. Typhimurium* mutants are less persistent in mice than their wild-

type RpoS⁺ parent strains [41, 45]. Taken together, these results show that although wild-type *S. Typhi* and *S. Paratyphi* A can transiently colonize young

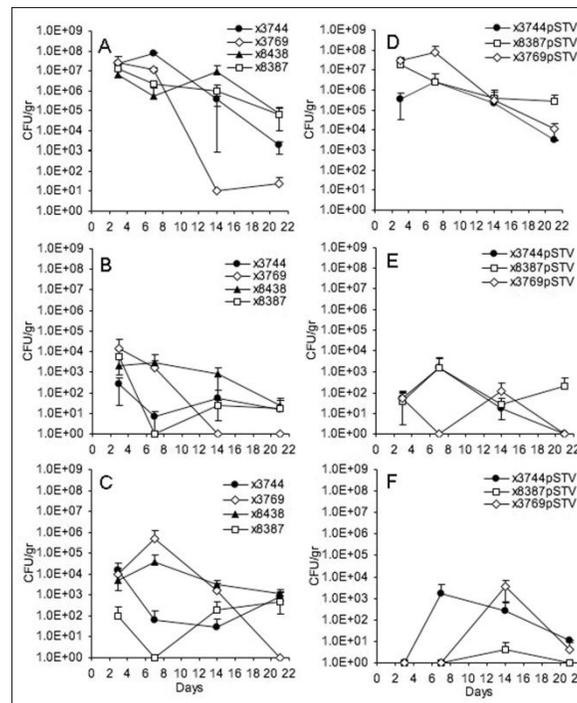
mice, they are not capable of establishing a disseminating infection.

Table 3. Infection of newborn and infant mice with *S. Typhi* pStSR101 and *S. Paratyphi* A pStSR101.

Strain	Inoculating dose (CFU)	Mice age	Survivors/total	Route
χ 3744 <i>S. Typhi</i> ISP1820 (pStSR101)	1.2 x 10 ⁹	<24 h	6/6	oral
	1.1 x 10 ⁹	<24 h	7/7	intranasal
χ 3769 <i>S. Typhi</i> Ty2 (pStSR101)	1.2 x 10 ⁹	<24 h	5/5	oral
χ 8387 <i>S. Paratyphi</i> A (pStSR101)	1.1 x 10 ⁹	<24 h	10/10	oral
	1.1 x 10 ⁹	<24 h	7/7	intranasal
χ 3351 <i>S. Typhimurium</i> SL-1344 (pStSR101)	1.8 x 10 ⁹	3 weeks	0/5	oral
	1.8 x 10 ⁹	<24 h	0/8	oral

Newborn: 3 h to 24 h after birth. Infant: 48 h to 3 weeks old. Mice were observed between 4-5 weeks. χ 3351 *S. Typhimurium* (pStSR101) was used as positive control.

Figure 1. Colonization of *S. Typhi* and *S. Paratyphi* A with and without pStSR101 virulence plasmid in newborn mice < 24 h old. **A-C.** Newborn mice orally infected with *Salmonella* without pStSR101; **D-F.** Newborn mice orally infected with *Salmonella* harboring pStSR101; **A, D.** Intestine colonization; **B, E.** Spleen colonization; **C, F.** Liver colonization. Each point represents the average between 4 animal tissues.



Expression of spv genes in S. Typhi RpoS⁺ and RpoS⁻

The *S. Typhimurium* virulence plasmid is required for colonization of mouse tissues [24]. An investigation into whether the addition of the virulence plasmid to *S. Typhi* and *S. Paratyphi* A

could enhance their ability to colonize young mice was conducted. One virulence plasmid operon that is critical for host invasion is encoded in the *spv* region [24,33,46]. The *spv* region consists of five genes, *spvRABCD* which are all transcribed in the same

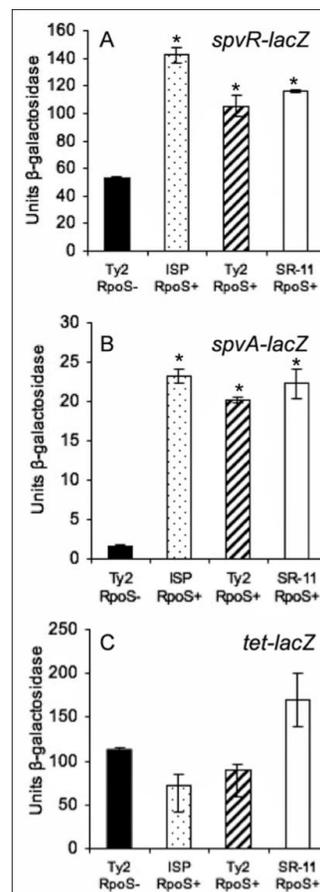
direction [46]. The *spvR* gene encodes SpvR, which activates the transcription of both the *spvR* and *spvABCD* transcriptional units [12,47,48]. In addition, the sigma factor RpoS is also required for maximum expression of the operon [49,50,51,52].

Because of the central role of *spv* in tissue colonization, expression of *spvR-lacZ* and *spvA-lacZ* fusions in RpoS⁺ and RpoS⁻ *S. Typhi* by β -galactosidase activity were compared to establish that these genes were able to be transcribed in *S. Typhi* and that transcription is dependent upon RpoS. [35]. The RpoS⁺ *S. Typhi* strains produced β -galactosidase levels comparable to the *S. Typhimurium* RpoS⁺ control for both fusions (Figures 2A and 2B). In contrast, reduced expression was observed for *S. Typhi* Ty2, which has a defective *rpoS* allele due to a frame-shift mutation at nucleotide 993 [53]. These results indicate that *S. Typhi* is able to transcribe the

spv genes to the same levels as *S. Typhimurium* and confirm that maximum *spv* expression requires RpoS. *Stability and expression of pSTV plasmid in S. Typhi and S. Paratyphi A*

A tetracycline-marked derivative of the *S. Typhimurium* virulence plasmid, pStSR101 [24], was moved into the *S. Typhi* and *S. Paratyphi A* strains. Because these host-restricted strains do not normally carry this plasmid, it is possible that the plasmid may not be maintained for enough generations to colonize a mouse. Therefore, the stability of pStSR101 in *S. Typhi* and *S. Paratyphi A* was examined. The pStSR101 virulence plasmid was stably maintained for 50 or more generations in all *S. Typhi* and *S. Paratyphi A* strains. In addition, whether the *spv* genes were transcribed from this plasmid was evaluated. RT-PCR products were detected in all strains harboring pStSR101 (Figure 3), indicating that the *spv* genes are transcribed in *S. Typhi* and *S. Paratyphi A*.

Figure 2. Evaluation of *spvR* and *spvA* expression in *S. Typhi* by β -galactosidase assay. **A.** Evaluation of *spvR*; **B.** Evaluation of *spvA*; **C.** Control. χ 3769 *S. Typhi* Ty2 RpoS⁻; χ 3744 *S. Typhi* ISP RpoS⁺; χ 3337 *S. Typhimurium* SR-11 RpoS⁺; strains harboring the respective plasmids; the strains were grown in MgM media to stationary growth phase. * $P < 0.01$ for the RpoS⁺ strains compared with RpoS⁻ strain, significant differences are indicated.



Virulence and colonization of *S. Typhi* and *S. Paratyphi A* harboring pSRSt101 in newborn and infant mice

The effect of the *S. Typhimurium* virulence plasmid on the ability of *S. Typhi* and *S. Paratyphi A* to cause disease in newborn and infant mice was evaluated. When newborn and infant mice were orally inoculated with *S. Typhi* and *S. Paratyphi A* harboring pStSR101, they survived without any disease symptoms, whereas all the mice inoculated with *S. Typhimurium* died (Table 3). Infant mice and newborn mice infected intranasally with *S. Typhi* or *S. Paratyphi A* carrying pStSR101 also survived without any symptoms of disease (Table 3).

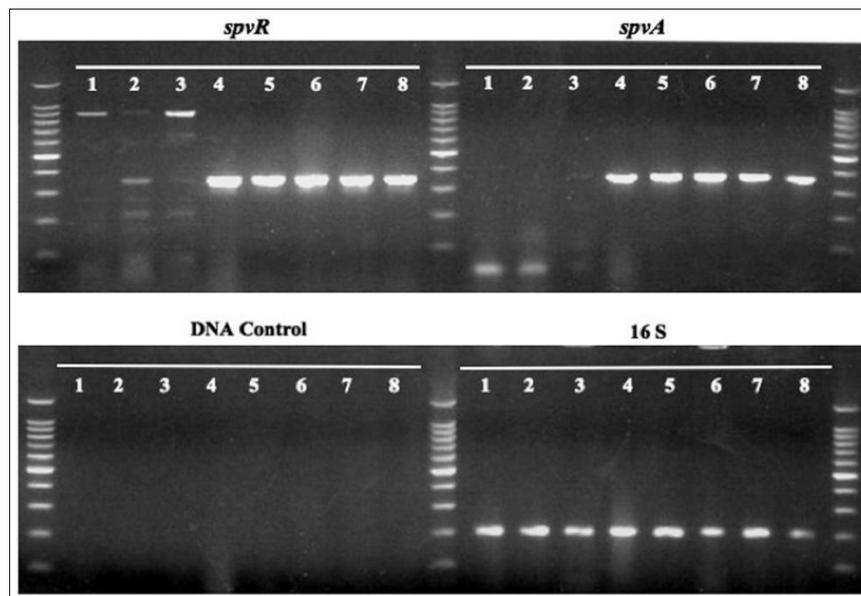
The presence of pStSR101 did not enhance the ability of *S. Typhi* and *S. Paratyphi A* to colonize infant mice (Figure 1). Unexpectedly, the presence of pStSR101 in *S. Typhi* Ty2 RpoS⁻ resulted in a slight increase in persistence in the intestines compared to the plasmid-free strain (Figures 1A and 1D), indicating that there may be a virulence plasmid gene(s) that can complement the defect in intestinal

colonization imparted by the RpoS⁻ phenotype. Recently, it has been reported that pP_{ST98}, a promiscuous R plasmid found in a multi-drug resistant isolate of *S. Typhi* from Asia, carries the *spv* region. This plasmid confers antibiotic resistance and increases virulence in mice when transferred to pSTV⁻ *S. Typhimurium* [54]. While it is possible that *spv* genes may confer an enhancement of virulence in human hosts, our results indicate that they have no effect on infant mouse colonization in wild-type *S. Typhi*.

Discussion

In summary, these results show that *S. Typhi* and *S. Paratyphi A* can transiently colonize young mice, but cannot establish a lethal infection. The *spv* genes are transcribed in *S. Typhi* and *S. Paratyphi A* and a functional *rpoS* gene is required for maximum expression. However, the virulence plasmid does not enable *S. Typhi* or *S. Paratyphi A* to establish a lethal infection in newborn or infant mice.

Figure 3. Expression of *spvR* and *spvA* evaluated by RT-PCR (agarose gel 1%). **1.** χ 3769 *S. Typhi* Ty2; **2.** χ 3744 *S. Typhi* ISP1820; **3.** χ 8387 *S. Paratyphi A*; **4.** χ 3769 *S. Typhi* Ty2 (pStSR101); **5.** χ 3744 *S. Typhi* ISP1820 (pStSR101); **6.** χ 8387 *S. Paratyphi A* (pStSR101); **7.** χ 3351 *S. Typhimurium* SL-1344 (pStSR101); **8.** χ 3761 *S. Typhimurium* UK-1 pSTV⁺; **16S:** 16S (*rRNA*) was used as positive control; **DNA control:** the RNA samples were used as templates in a PCR reaction to amplify the *16SrRNA* to detect DNA contamination.



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