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Construction and immunogenicity of recombinant swinepox virus expressing outer membrane protein L of *Salmonella*

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ABSTRACT

Salmonella are Gram-negative flagellated bacteria that cause a variety of diseases in humans and animals, ranging from mild gastroenteritis to severe systemic infection. To explore development of a potent vaccine against *Salmonella* infections, the gene encoding outer membrane protein L (*ompL*) was inserted into the swinepox virus (SPV) genome by homologous recombination. PCR, western blot and immunofluorescence assays were used to verify the recombinant swinepox virus rSPV-OmpL. Immune responses and protection efficacy of rSPV-OmpL were assessed in a mouse model. Forty mice were assigned to four groups, which were immunized with rSPV-OmpL, inactive *Salmonella* (positive control), wild-type SPV (wtSPV; negative control), or PBS (challenge control), respectively. The OmpL-specific antibody in the rSPV-OmpL immunized group increased dramatically and continuously over time post-vaccination, and was present at a significantly higher level than in positive control group ($P < 0.05$). The concentrations of IFN-γ and IL-4 which represent Th1-type and Th2-type cytokine responses, were significantly higher ($P < 0.05$) in the rSPV-OmpL-vaccinated group than in the other three groups. After intraperitoneal challenge with a lethal dose of *Salmonella typhimurium* CVCC542, eight out of ten mice in the rSPV-OmpL-vaccinated group were protected, whereas all the mice in the negative control and challenge control groups died within 3 days. Passive immune protection assays showed that hyperimmune sera against OmpL could provide mice with effective protection against challenge from *S. typhimurium*. The recombinant swinepox virus rSPV-OmpL might serve as a promising vaccine against *Salmonella* infection.

Keywords: Recombinant swinepox virus; *Salmonella*; Outer membrane protein L; Vaccine

Introduction

Salmonella are Gram-negative flagellated bacteria which include several very important serovars, including *Typhi*, *Paratyphi*, *Typhimurium*, *Enteritidis* and *Choleraesuis*. These bacteria cause a significant global burden of zoonosis, typically classified into enteric fever, gastroenteritis and, more recently, invasive non-typhoidal salmonellosis (iNTS) [26, 27, 35]. Epidemics of *Salmonella* infection cause great losses in animal production and are the main source of human food-borne diarrheal illness [15]. Antibiotics such as ampicillin, chloramphenicol and streptomycin are widely applied in the treatment of salmonellosis. However, as in many other bacteria, multidrug resistance of *Salmonella* is increasingly common and is a worldwide public health and economic problem. Multidrug resistant strains of *Salmonella* can pass to humans through the food chain via animals, posing a threat to human health and lead to human antibiotic resistance [19]. Vaccination is an effective and economic measure to prevent some infectious diseases and can effectively avoid multidrug resistance [1, 12, 23]. Therefore, it is necessary to develop a potent *Salmonella* vaccine to protect public health and safety, as well as healthy animal production.

The outer membrane proteins (OMPs) of *Salmonella* contain a family of pore-forming proteins called porins [28]. OMPs are immunologically important because of their accessibility to the host defense system [33]. Several *Salmonella* OMPs have been considered as potential candidates for conferring protection against *Salmonella* infection [9, 11, 20]. Outer membrane protein L (OmpL) is a transmembrane β-barrel (TMBB) protein of 230 amino acid residues, which has been proven to be an effective protective antigen against *Salmonella* infection [34]. Due to strong induction of immunity and large capacity for heterogeneous DNA insertion, pox viruses have attracted widespread attention as live virus carriers of human and animal vaccines, and are technologically suitable for the development of recombinant vaccines [8, 10, 21]. Swinepox virus (SPV), is known to infect porcine species only and manifests slight clinical symptoms with occasional localized skin lesions that

heals naturally [17]. Therefore, SPV has excellent features as a potential vaccine vector.

In this study, we constructed a recombinant SPV expressing *Salmonella* OmpL and characterized the replication and OmpL expression of the virus in PK-15 cells. In a variety of mouse trials, the recombinant swinepox virus rSPV-OmpL was proven to be a potential candidate vaccine against *Salmonella* infection.

Materials and methods

Cells and viruses

Porcine kidney PK-15 cells (CCL-33^{TM}) and SPV (VR-363^{TM}) used in this study were purchased from the American Type Culture Collection (ATCC). The cells were routinely cultured at 37 °C in 5% CO_2 in Eagle's Minimum Essential Medium, supplemented with 10% fetal bovine serum.

Animals and housing

Two-hundred and fifty 4-week-old female ICR mice were purchased from the Comparative Medicine Center of Yangzhou University. They were randomly divided into 25 groups. All experimental protocols involving mice were approved by the Laboratory Animal Monitoring Committee of Jiangsu Province and performed accordingly.

Construction and identification of the recombinant swinepox virus

The 633 bp *ompL* gene (NCBI Reference Sequence: NP_462896.1) was amplified from the *Salmonella typhimurium* CVCC542 genome using primers OmpL-F (5'-3': CAGGTCGACGGCGCTTATGTAGAAAACC) and OmpL-R (5'-3': CTAGGATCCTCAGAAGAAATACTTCGCCC), and then inserted into the

pUSG11/P28 plasmid to create the transfer vector pUSG11/P28OmpL (Fig. 1) [14]. The recombinant swinepox virus rSPV-OmpL was constructed by homologous recombination of wild type SPV with pUSG11/P28OmpL as previously described [14]. Briefly, PK-15 cells grown in a 6-well plate were infected with the SPV (m.o.i. of 0.05) for 1 h, and subsequently transfected with 4.0 μg of the pUSG11/P28OmpL plasmid using Exfect^{TM} Transfection Reagent (Vazyme Biotech Co., Ltd.). After 72 h, PK-15 cells were harvested and lysed by two rounds of freezing and thawing. The lysate was used to infect PK-15 cells grown in a 12-well plate for further purification of recombinant viruses. 1.5 ml of medium with 1% LMP agarose (TaKaRa) was added to each well and incubation was continued for six days until green fluorescence became visible. Recombinant viruses with green fluorescence was picked using fluorescent microscope, resuspended in 0.4 ml of medium and lysed by two rounds of freezing and thawing. Plaque isolation was repeated for 8-9 rounds until all plaques in a given well were green fluorescence. The recombinant SPV bearing OmpL of *Salmonella* was designated as rSPV-OmpL. The *ompL* gene and the expression of OmpL protein were analyzed by PCR, western blotting and indirect immunofluorescence. Polyclonal antibody of recombinant OmpL was used as primary antibody in western blotting and indirect immunofluorescence. Recombinant OmpL was expressed in Escherichia coli BL21 (DE3), purified by affinity chromatography, and utilized to raise polyclonal antibody in rabbit. The replication capacity and genetic stability of rSPV-OmpL were also evaluated as previously described [13].

Immunogenicity of rSPV-OmpL

Forty 4-week-old female ICR mice were randomly and equally assigned to four groups. Mice in group 1 were immunized intramuscularly with 4×10^7 plaque forming unit of rSPV-OmpL (0.2 ml); mice in group 2 were immunized intramuscularly with 4×10^7 plaque forming unit of wild-type SPV (0.2 ml) as negative controls; mice in group 3 were immunized with 4×10^6 colony forming unit (0.2 ml) inactive *Salmonella* as positive controls. The inactive *Salmonella* was produced by adding 0.8%

formaldehyde into *S. typhimurium* culture in log phase (OD600 = 0.6) for about 24h at 37°C, which was then centrifuged at 10000×*g* for 1 min and washed three times with PBS. The inactive *Salmonella* mixed equally with Freund's complete Adjuvant. Two booster inoculations were given to above three groups at biweekly intervals. Group 4 was the challenge control (treated with PBS). Two weeks after the last booster dose, all mice were challenged intraperitoneally (i.p.) with 0.2 ml *S. typhimurium* CVCC542 (2×10^6 colony forming unit; approximately 5× LD_{50}) of log phase bacteria (OD600 = 0.6). Signs of *Salmonella* infection (rough hair, diarrhea, decreased mobility or ataxia) and lethality were recorded daily for 10 d and animals showing signs of irreversible illnesses were humanely euthanized with 100% CO_2. The spleen and liver of dead animals were cultured to verify whether *Salmonella* was the cause of death. Experiments were repeated twice, total 20 mice per group.

Specific antibody titers

Forty 4-week-old female ICR mice were randomly and equally assigned to four groups; all groups of mice (groups 1 to 4) were treated as described in section 2.4. Blood was obtained for serum preparation on days 0, 7, 14, 21, 28 and 35. Two mice from each group were sampled on each date. Ten mice in each group were sampled in rotation to minimize the stress of blood loss. The sera were stored at −20°C. At the end of this process, all mice were humanely euthanized with 100% CO_2. ELISA plates (96-well; Corning) were coated with 0.2μg purified prokaryotic expression product of recombinant OmpL in 100 μl of 50 mM sodium carbonate buffer (pH 9.6) and incubated overnight at 4°C. The coated plates were washed three times with PBST and blocked with 5% skimmed milk in PBST at 37°C for 2 h. The plates were washed three times with PBST. The sera was serially diluted by two-fold (from 1:10 to 1:81,920), added to the wells and incubated for 1 h at 37 °C. The negative control (serum obtained from mice in the challenge control group) and the blank control (without sera) were set up at the same time. After three washes, 100 μl of horseradish peroxidase conjugated goat anti-mouse IgG (diluted 1:10,000 in PBST) was added to

each well, and the plates were incubated at room temperature in the dark for 30 minutes. After incubation, the plates were washed three times. The reaction products were developed using the TMB microwell peroxidase (TIANGEN; Beijing) substrate system for 20 minutes, and stopped with 100 μl of 2.0 M sulfuric acid per well. All assays were performed in duplicate. Reactions were measured using a Bio-Rad microplate reader at an absorbance of 450 nm.

The mean absorbance values for each set of duplicate samples were calculated. The S/N value of the ELISA results from each serum sample was calculated. The S/N value was: $[Abs_{sample} - Abs_{blank\ control}]/ [Abs_{negative\ control} - Abs_{blank\ control}]$. Sera with the S/N value >2.1 were considered to be positive. The antibody titers are expressed as the highest dilution of antibody producing 2.1 ratio values. Experiments were repeated twice, total 20 mice per group.

Cytokine assay

The levels of serum IFN-γ and IL-4 induced by rSPV-OmpL were investigated to evaluate the cellular immune response. Immune responses are mainly evoked by Th1 and Th2 T-cell subgroups. Th1 cells, which produce IFN-γ, IL-2 and TNF-β, evoke cell-mediated immunity and phagocyte-dependent inflammation. Th2 cells, which produce IL-4, IL-5, IL-6, IL-9, IL-10, and IL-13, evoke strong antibody responses and eosinophil accumulation. The immune response type was assessed indirectly by measuring the levels of IFN-γ and IL-4 in serum. They were detected using ELISA kits (ExCell Bio, China) according to the manufacturer's instructions. Standard curves were generated using control IFN-γ and IL-4 serially diluted twofold in PBS and coated onto ELISA plates overnight at 37° C. The levels of serum IFN-γ and IL-4 were calculated according to the corresponding standard curves.

Passive immune protection assays

Passive protection tests were performed as previously described [5]. Briefly, forty 4-week-old female ICR mice were randomly and equally assigned to four groups. Mice in group 1 were passively immunized with 200 μl hyperimmune sera derived from rSPV-OmpL immunized mice (antibodies titer of 9.25×10^4) by i.v.; mice in group 2 were passively immunized with 200 μl hyperimmune sera against *Salmonella* inactive vaccine (antibodies titer of 1.02×10^5) by i.v. as positive control; mice in group 3 were passively immunized with control sera obtained from *Salmonella* antibody-negative mice as negative control; group 4 was treated with PBS as challenge control. In the 24 h after immunization, all mice were challenged intraperitoneally with 0.2 ml (2×10^6 colony forming unit; $5\times LD_{50}$) of *S. typhimurium* CVCC542. Signs of *Salmonella* infection and lethality were recorded daily for 10 d and animals showing signs of irreversible illnesses were humanely euthanized with 100% CO_2. Experiments were repeated twice, total 20 mice per group.

Statistical analysis

All data were analyzed using one-way ANOVA. *P* values < 0.05 were considered statistically significant.

Results

Construction of the transfer plasmid

The transfer plasmid pUSG11/P28O, which includes the SPV flanking sequences, the modified promoter P28 with the downstream *ompL* gene, and the P11-GFP gene expression cassette, was constructed to generate the recombinant SPV (Fig. 1). The gene *ompL* were inserted into the SPV genome by homologous recombination and the recombinant SPV was screened using the GFP reporter.

Characterization of the recombinant swinepox virus

An approximately 633 bp *ompL* gene fragment was amplified by using specific ompL primers and was present in the recombinant virus but not in wild-type (wt) SPV (Fig. 2A). Western blot analysis that using polyclonal antibody of recombinant OmpL as primary antibody showed a specific protein band of 28 kDa in the cell lysates infected with rSPV-OmpL, in accordance with the predicted size of the *Salmonella* OmpL protein (Fig. 2B). In the indirect immunofluorescence assays which using polyclonal antibody of recombinant OmpL as primary antibody, a significant red fluorescence was observed in rSPV-OmpL infected PK-15 cells (Fig. 2C), whereas no specific red fluorescence was detected in wtSPV infected PK-15 cells (Fig. 2D). Therefore, we conclude that the rSPV-OmpL virus was generated and efficiently expressed *Salmonella* OmpL.

rSPV-OmpL induces specific antibody response in mice

The OmpL-specific antibody response elicited after immunization with rSPV-OmpL was monitored by detecting the serum antibody titers in mice. From 7 d post-vaccination, the OmpL-specific antibody titers dramatically increased and reached a peak after the third vaccination (35 d post the initial vaccination). The OmpL-specific antibody titers of mice vaccinated with inactive *Salmonella* were significantly lower at all time points post-vaccination than those of mice vaccinated with rSPV-OmpL ($P < 0.05$) (Fig. 3).

rSPV-OmpL induces Th1-type and Th2-type cytokine responses in mice

Changes in serum IL-4 and IFN-γ levels in immunized mice were analyzed using ELISA kits. The concentrations of IL-4 and IFN-γ in the rSPV-OmpL group were significantly higher than those in the control groups at all post-infection time points ($P < 0.05$) (Figs.4 and 5). These results suggest that rSPV-OmpL elicits potent Th1-type and Th2-type cytokine responses in mice.

rSPV-OmpL mediates immunoprotection against *Salmonella* lethal challenge

After challenge with a lethal dose of *S. typhimurium* CVCC542, all mice in the wtSPV group (negative control) and PBS group (challenge control) showed severe clinical symptoms including rough hair, diarrhea, decreased mobility, severe lethargy, severe ataxia, and died within 3 d (Fig. 6). All the twenty mice in the inactive *Salmonella*-immunization group (positive control) exhibited slight diarrhea but these symptoms diminished within 3 d with the exception of two mice that died on day 2. Four mice in the rSPV-OmpL immunized group showed severe symptoms of disease and died on day 2, while the remaining mice only showed slight clinical symptoms and recovered gradually. The results indicate that rSPV-OmpL provided mice with strong protection against *Salmonella* challenge.

Passive immune protection

Mice passively immunized with hyperimmune sera against OmpL (group 1) showed mild symptoms and recovered within 3 d after challenge with *S. typhimurium* CVCC542, with the exception of four mice that died. Hyperimmune sera against inactivated *Salmonella* (group 2) provided 100% protection against *Salmonella* infection. In contrast, mice in the negative control group and challenge control group (groups 3 and 4) all died. These results confirmed that the antibody against OmpL could provide effective protection against *Salmonella* infection (Fig. 7).

Discussion

Salmonella are group of common pathogenic bacteria in animals and humans with global distribution that adversely affected animal health, human public safety and food safety. Swine salmonellosis, also known as swine paratyphoid, is characterized by acute sepsis and chronic necrotizing enteritis which makes epidemic

prevention difficult [4]. The short course of the disease, rapid transmission, and high mortality rate cause serious economic losses [18]. The use of antibiotics against *Salmonella* infection can lead to antibiotic resistance, flora imbalance in the host, and toxin release from bacterial cell lysis. Vaccine immunization is an important measure in prevention and control of swine salmonellosis, and effective vaccines are needed to raise swine specific resistance to ensure the safety of public health and the development of the swine industry. Thus, the need for a vaccine against swine salmonellosis is increasingly urgent, while only rarely effective vaccines have been developed [7, 25]. *Salmonella* contains 2 species, 7 subspecies and approximately 2500 serovars [27]. Dozens of *Salmonella* serovars are relatively common in animals, and it is hard to develop vaccines that are effective against all serovars. Analysis of the amino acid sequence of OmpL indicates that this protein is widely distributed in *Salmonella* spp. and conserved among different *Salmonella* serovars (Fig. 8), which raises the possibility that OmpL could be a promising target for the development of a general candidate vaccine against *Salmonella* infection.

Swinepox virus (SPV) as a live virus vector is currently widely used for recombinant vaccines [2]. SPV has many advantages as the carrier. First, its replication occurs in the cytoplasm which avoids the possibility of viral genome integration into host cell chromosomes, thereby eliminating the potential threat to humans and other animals of application of a recombinant virus [31]. Second, exogenous genes can be readily accommodated due to the large packaging capacity for recombinant DNA of the virus genome [24]. Third, proteins expressed by recombinant SPV usually possesses satisfactory immunogenicity [30]. Moreover, SPV has the advantages of low production cost, easy administration and strict host range restriction, and thus has real potential as a safe and effective vaccine carrier for wide use in the expression of exogenous genes [3, 10, 29]. Although SPV does not naturally infect non swine species, SPV can enter human, monkey, mouse, rabbit and feline cells to serve as a vector for the expression plasmid [2, 3, 22, 32]. The mouse model is used widely in *Salmonella* infection studies aimed toward understanding the

basis of mucosal immune responses and diseases such as gastroenteritis and typhoid in mice [16]. These conditions set the stage for using mice as the preliminary research animal.

In this study, we evaluated the feasibility of using SPV as a live vector for a *Salmonella* vaccine. The recombinant SPV we developed, rSPV-OmpL, was genetically stable in PK-15 cells and expressed OmpL correctly. Mice immunized with rSPV-OmpL generated a remarkably high level of specific antibody, as well as Th1-type and Th2-type cytokines. We monitored OmpL-specific antibody titer by indirect ELISA, and the 96-well ELISA plates were coated with 0.2μg purified prokaryotic expression product of recombinant OmpL in 100μl of 50 mM sodium carbonate buffer (pH 9.6) and incubated overnight at 4 °C. rSPV-OmpL (recombinant vaccine) is able to express foreign protein OmpL exclusively and efficiently. As a live vector, recombinant SPV continue to replicate, proliferate and express OmpL. Meanwhile inactive *Salmonella* (positive control) contains plenty of protein antigens, which distracted the OmpL-specific antibody response.

A novel approach to vaccine development was reported recently[6]. Gas vesicle nanoparticles (GVNPs) produced by extremophilic *Halobacterium* sp. NRC-1, bioengineered to display highly conserved *Salmonella enterica* antigen SopB, were being used to develop an improved vaccine against *Salmonella* pathogens. Proinflammatory cytokines IFN-γ, IL-2, and IL-9 were significantly induced in mice boosted with this vaccine (SopB-GVNPs), consistent with a robust Th1 response. The animals boosted with SopB-GVNPs resulted in reduced bacterial load in key organs. Nevertheless, this vaccine delayed the death of animals challenged with lethal doses of *S. Typhimurium*, instead of preventing it (0% survival after pathogen challenge). By contrast with these results, it shows that our attempt to use SPV to deliver OmpL had a better immune effect with 80% protection against *Salmonella* challenge. rSPV-OmpL (recombinant vaccine) elicited stronger humoral immune responses through a remarkably high level of OmpL-specific antibody as well as Th2-type cytokine IL-4, which did not mention in SopB-GVNPs assay. Additionally, passive

immune protection confirmed that hyperimmune sera against rSPV-OmpL provide effective protection against *Salmonella* infection. Taken together, better protective efficiency, low cost in production, the potential for further development by inserting more exogenous genes into the swinepox virus and low immune doses make the recombinant swinepox virus rSPV-OmpL more competitive than SopB-GVNPs.

Unlike inactive *Salmonella*, rSPV-OmpL as a live virus mainly evokes cell-mediated immunity. IFN-γ represents Th1-type cytokine responses and is positively correlated with cell-mediated immune response. At 14 day and 28 day post primary inoculation, the serum was collected for evaluating the level of IFN-γ, than booster inoculation were given respectively. The concentration of IFN-γ decreased slightly at 28 day post primary inoculation as a portion of rSPV-OmpL has been eliminated by the body. IFN-γ in the rSPV-OmpL-vaccinated group was re-increased at 35 day after vaccination because of the second booster inoculation. But wtSPV and inactive Salmonella had little influence on cell-mediated immune response compare second booster inoculation with the first one.

rSPV-OmpL can express *Salmonella* protective antigen OmpL continuously and efficiently and elicit high level of OmpL-specific antibody titer. However, for bacterial pathogens, various kinds of virulence factors have critical roles in complicated pathogenesis. It is unlikely that choosing a single virulence factor as protective antigen can confer complete protection. Inactive *Salmonella* (positive control) retains good antigenicity and contains various kinds of virulence factors. Thus vaccination with inactive *Salmonella* was more effective vaccination with rSPV-OmpL both in active immune protection assay and passive immune protection assay.

Taken together, our data indicate that rSPV-OmpL is a promising and attractive vaccine candidate for the prevention and control of *Salmonella* infection. However, for bacterial pathogens, various kinds of virulence factors have critical roles in complicated pathogenesis. It is unlikely that choosing a single virulence factor as protective antigen can confer complete protection. In future work coexpressing other

Salmonella virulence factors will be undertaken in order to develop vaccines that confer better immunoprotection against salmonellosis.

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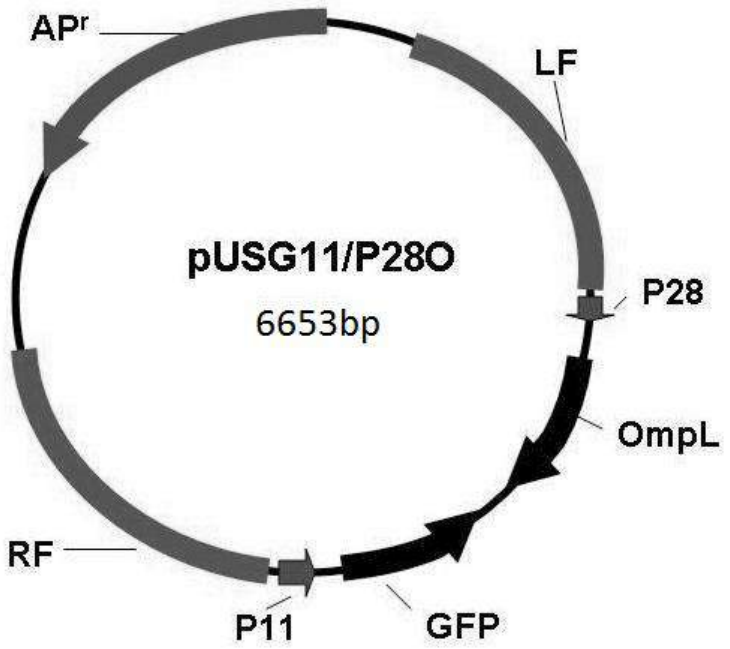

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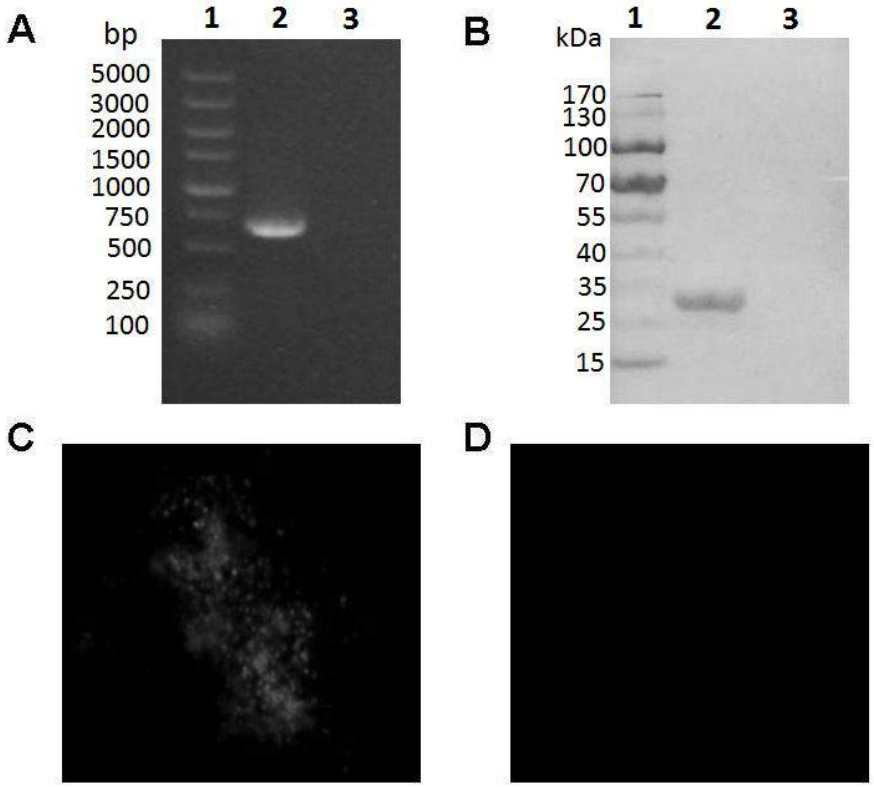

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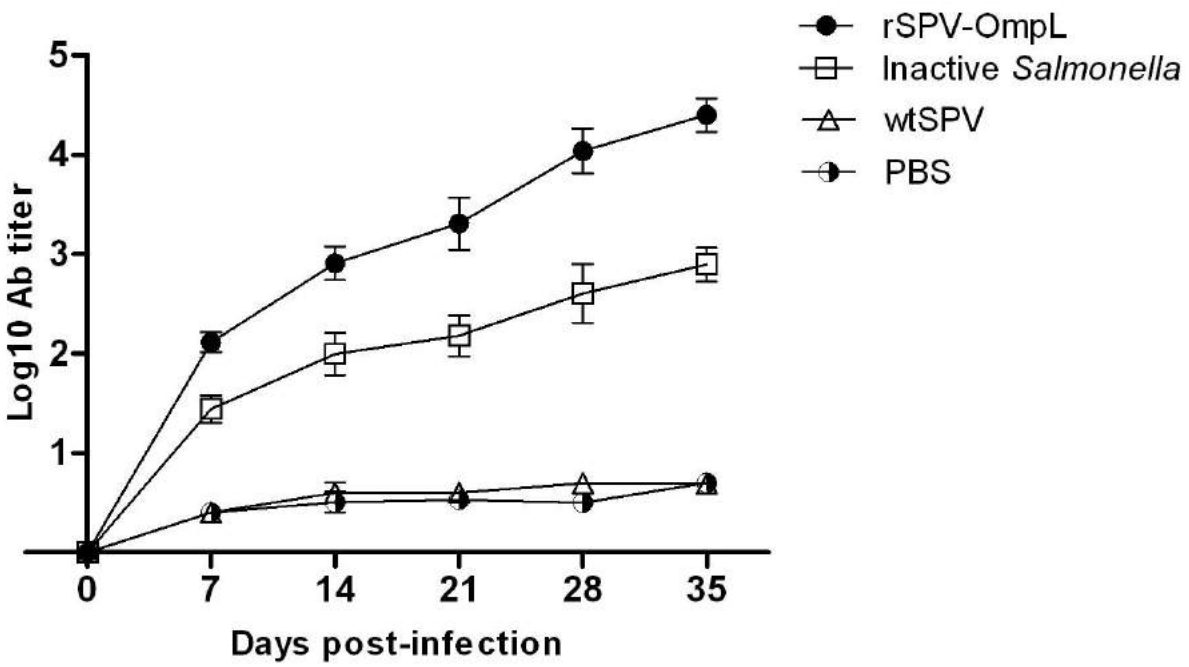

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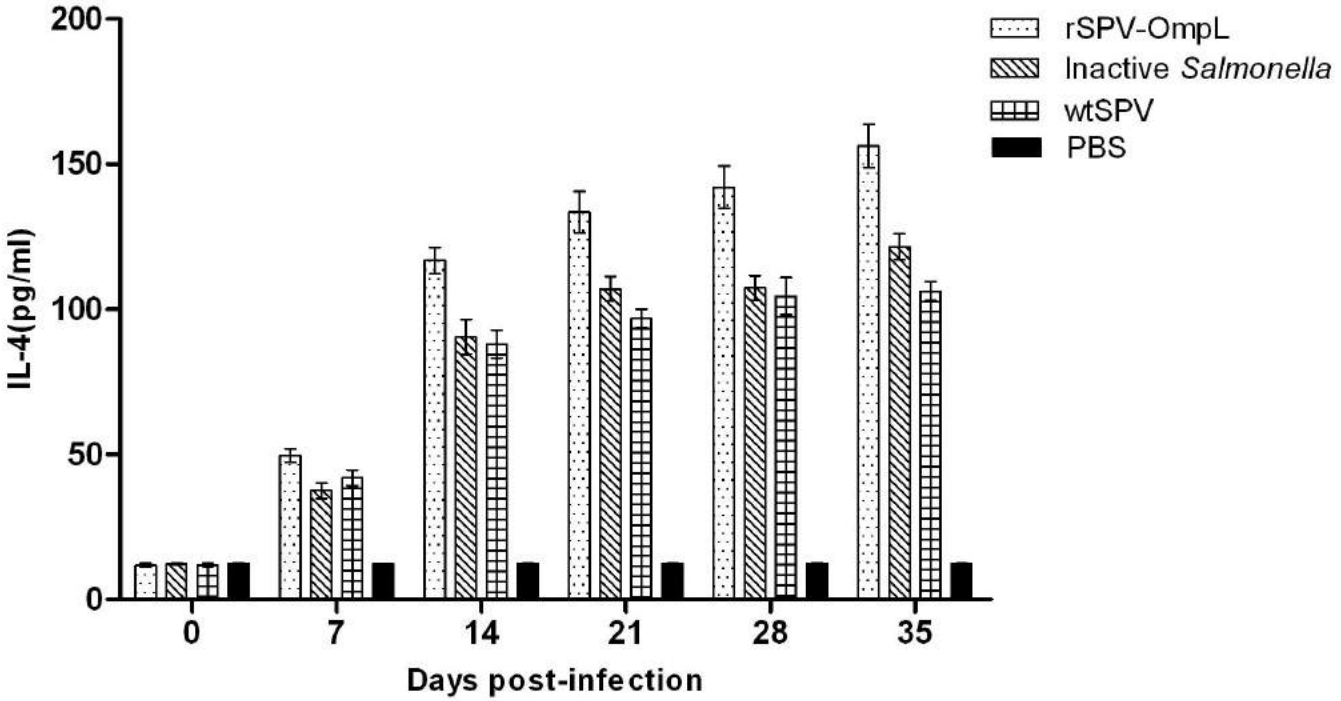

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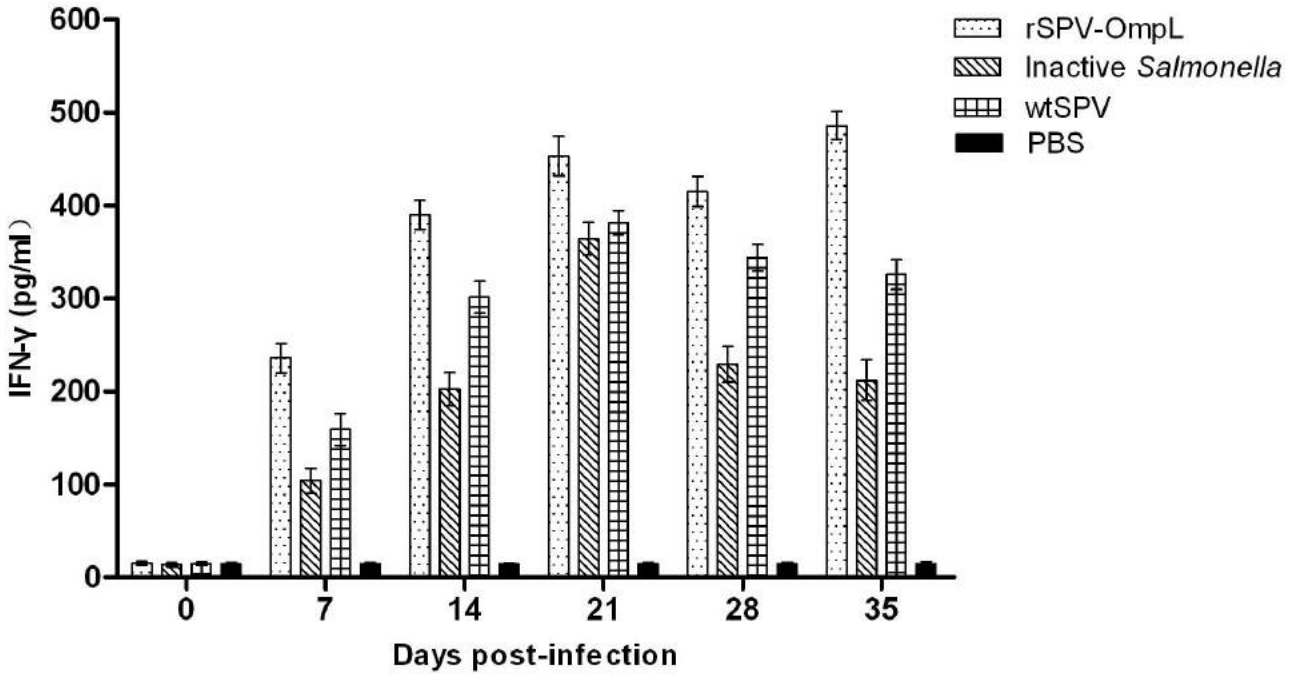

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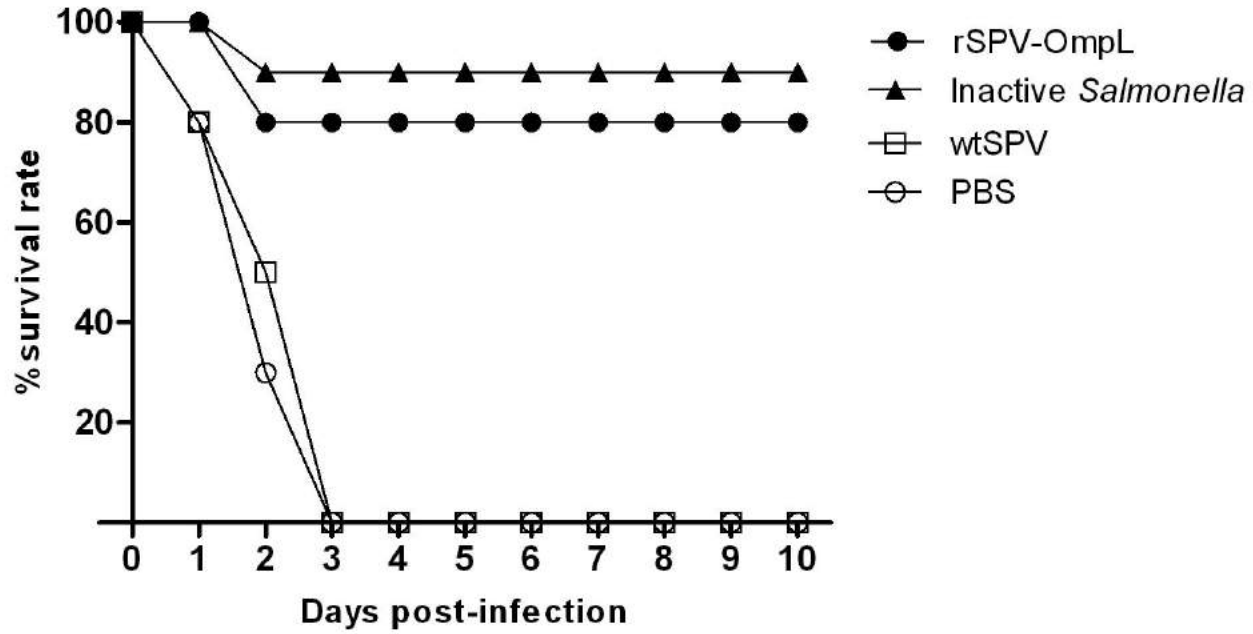

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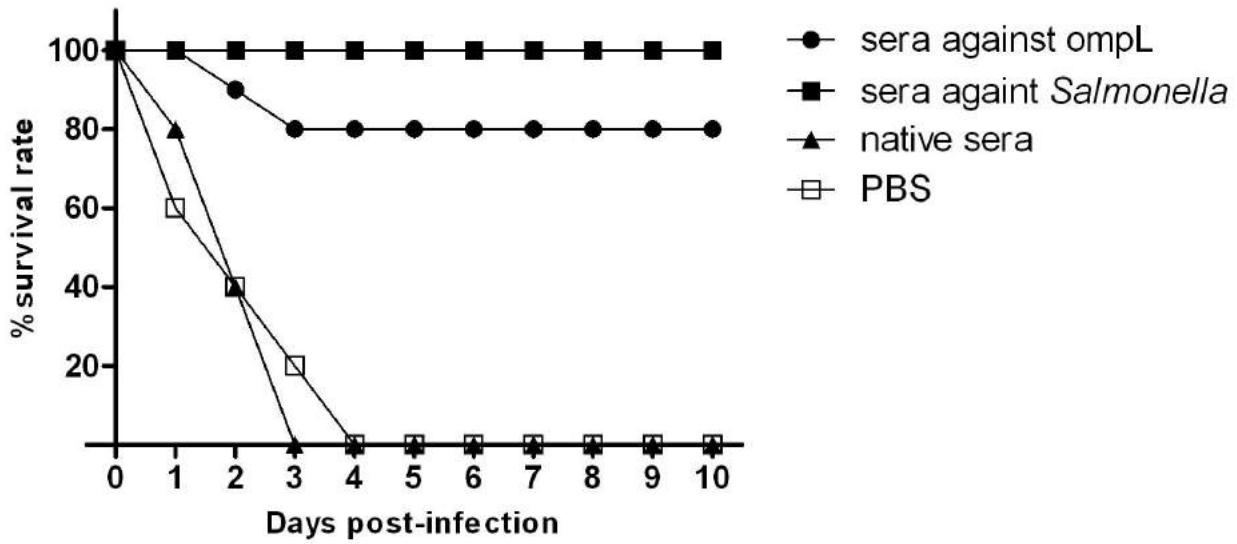

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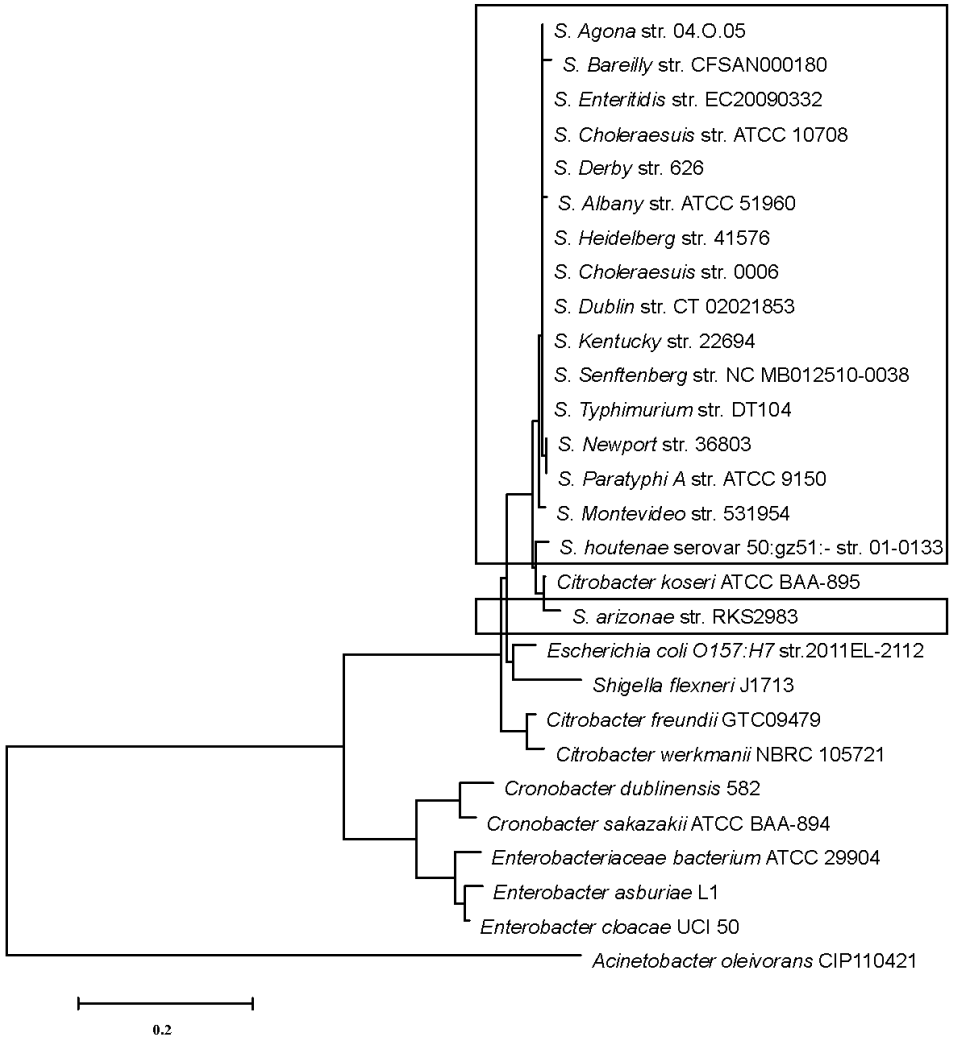

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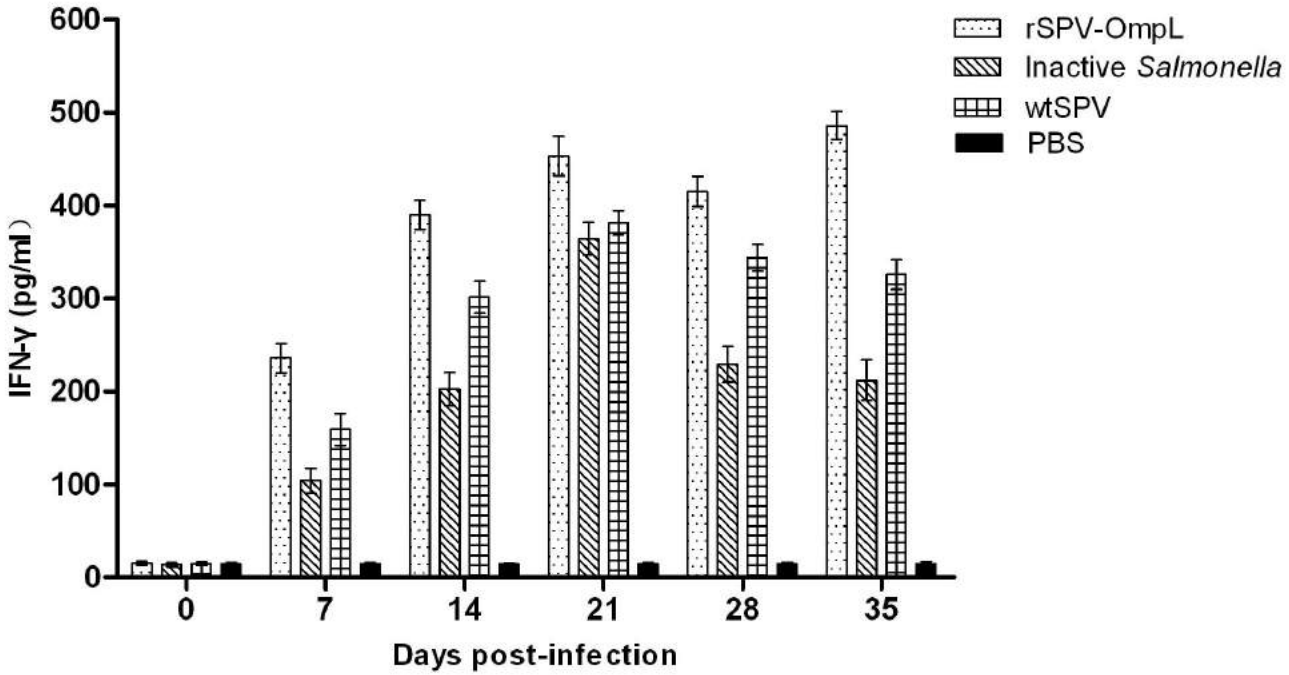

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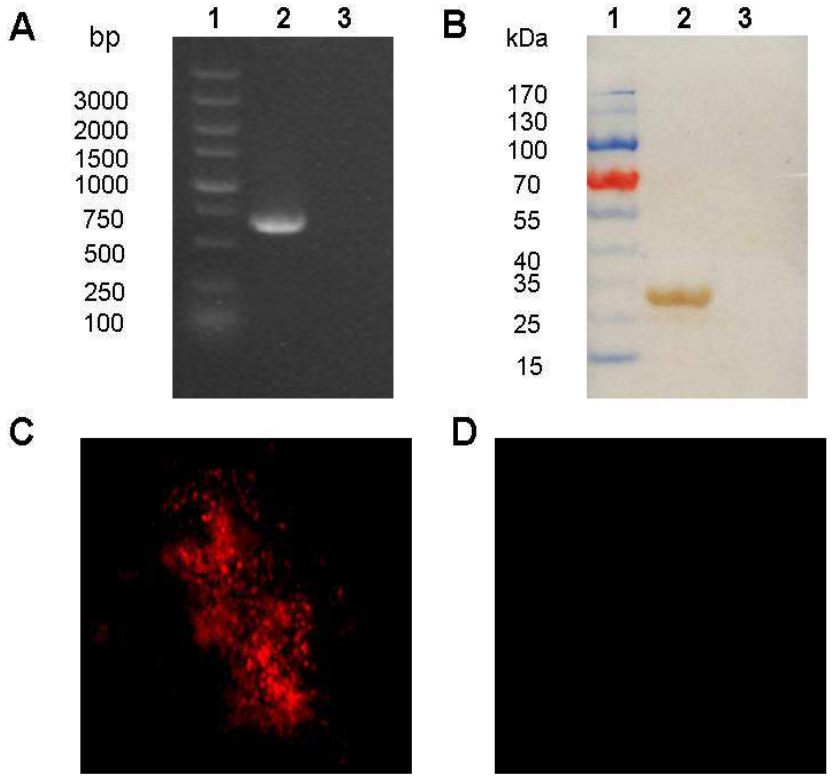

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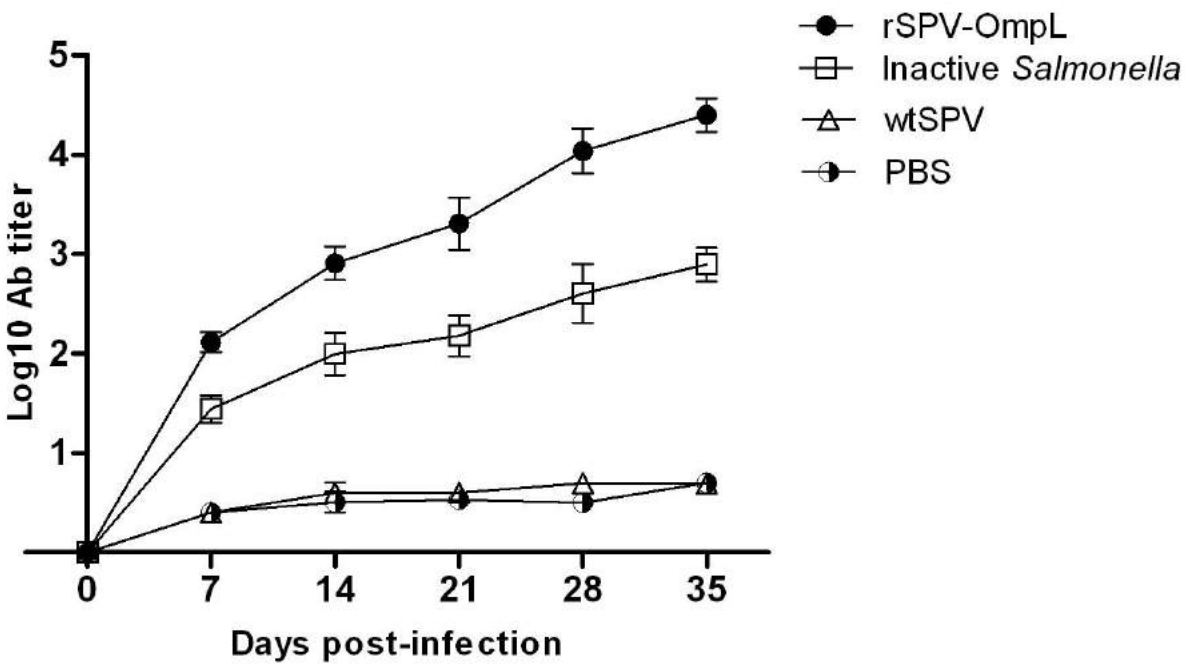

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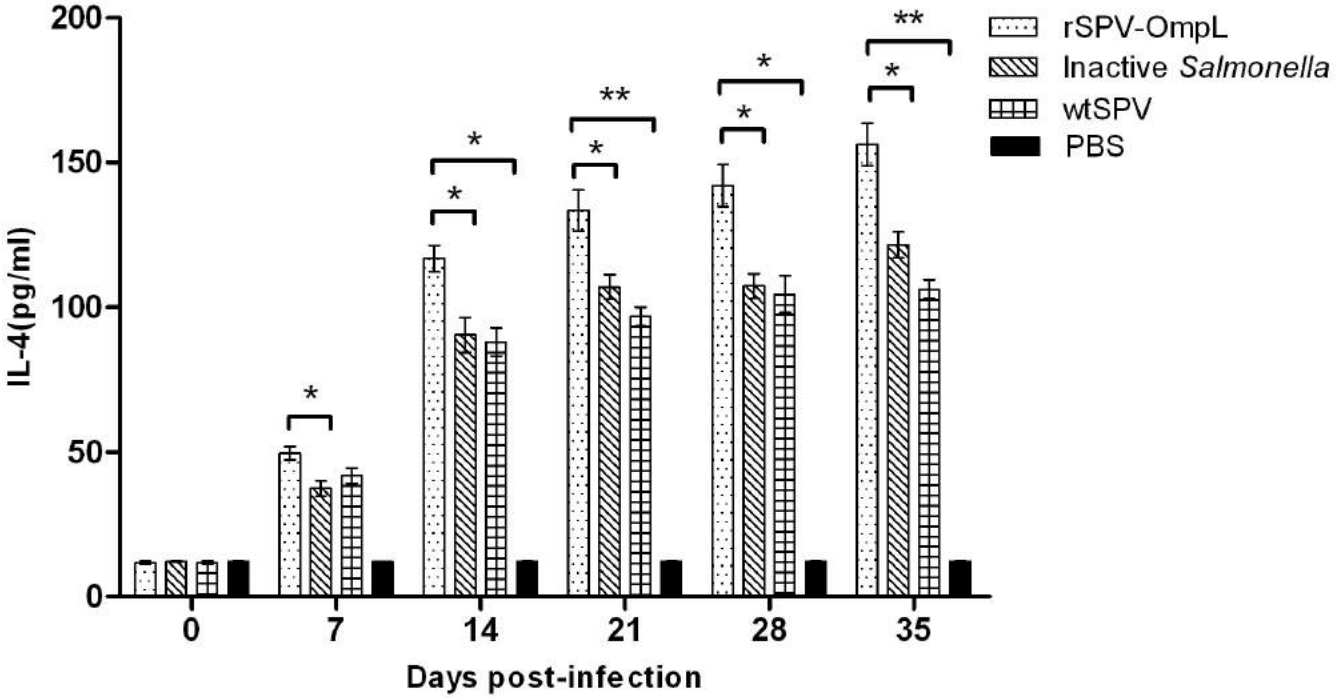

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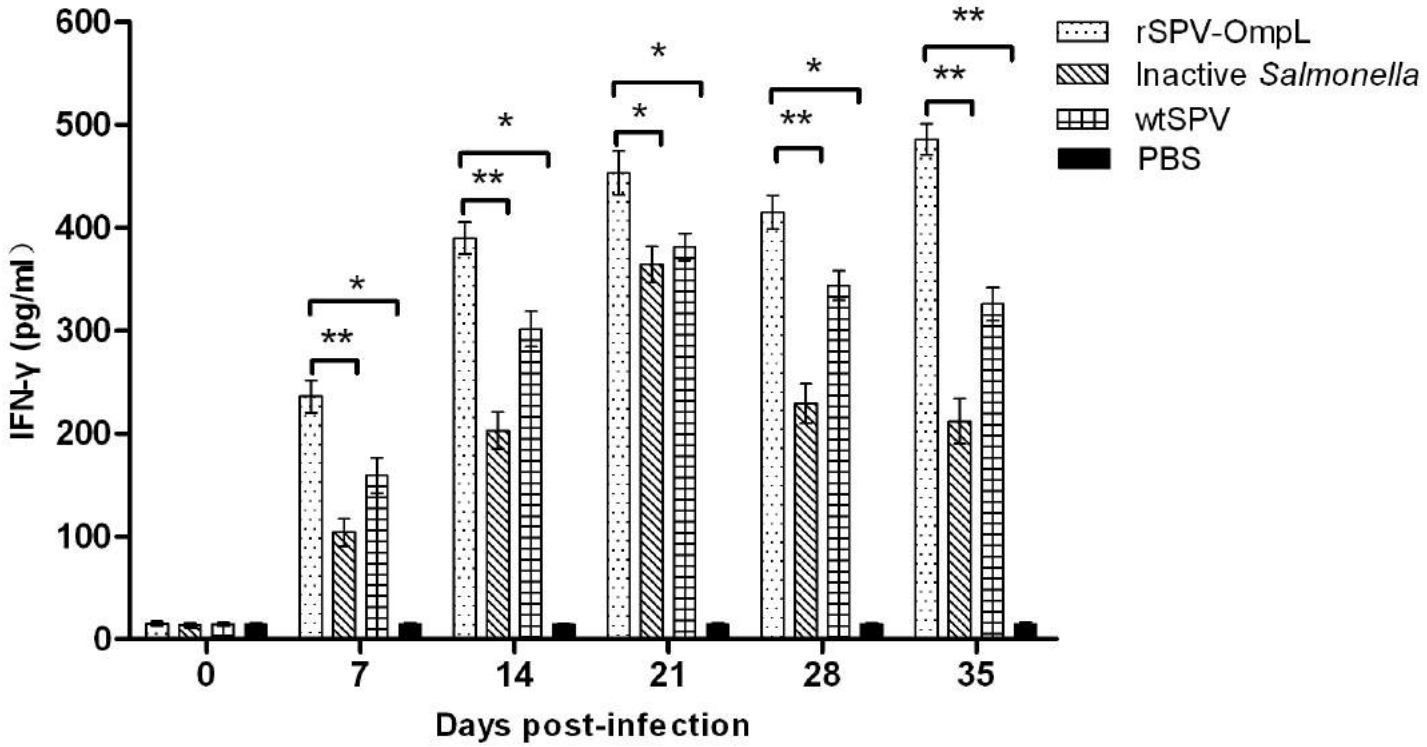

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