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Attenuated Virulence and Protective Efficacy of a Vaccine with Multiple Mutated *Salmonella enterica* Serovar Typhimurium in Mouse

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***Salmonella typhimurium* is the causative agent of bacterial gastroenteritis in human via livestock products. *S. typhimurium* mutants (STΔ4 and STΔ5) deleted the *hmpA*, *rpoS*, *ssrAB*, *aroA* and *ppk* genes were constructed and, attenuated virulence and protective efficacy of the multiple mutants were evaluated in mice as a novel vaccine candidate. In the virulence test, CFUs of STΔ4 (*hmpA*, *rpoS*, *ssrAB*, and *aroA*) and STΔ5 recovered from the liver and spleen of the mice were highly decreased at the range of 2.40 to 5.51 logs for 15 days postinfection. The changes of organ/body weight ratios in mice were also showed same trends as the changes of CFUs in virulence test. In the protection test, CFUs of STΔ4 and STΔ5 recovered from the organs in mice were highly decreased compared to non-vaccination group. The Polyphospate kinase (*ppk*) gene was not associated with protective efficacy but attenuated virulence in *S. typhimurium* mutant. Our results indicated that STΔ4 and STΔ5 firstly developed in this study showed highly attenuated virulence and protective efficacy. This work shows that *S. typhimurium* mutant deleted the multiple genes provide a basis for further development of a novel vaccine.**

Key words: Live attenuated vaccine, protective efficacy, *Salmonella* Typhimurium.

Even though similar to *Escherichia coli* morphologically and physiologically, the genus *Salmonella* was classified as a separate genus using bacterial antigen composition due to clinical convenience by F. Kauffmann in 1934 [1]. *Salmonella* has been primarily reported more than 2,500 serotypes up to date and falls into two serotypes of host-specific (*S*. Typhi, *S*. Dublin, *S*. Choleraesuis, *S*. Pullorum, and *S*. Gallinarum) and non host-specific (*S*. Typhimurim, *S*. Enteriditis, and *S*. Newport)[2, 3]. *Salmonella* strains which can cause food poisoning in human are belong to the latter[4]. Especially *Salmonella enteric* serovar Typhimurium (ST) is a leading cause of bacterial gastroenteritis in human via meat of cattle, pigs and chickens or their by-products[5, 6]. To improve the safety of livestock products, vaccination can be better protection than antibiotic treatment. Inactivated vaccine and live attenuated vaccine are currently used in the vaccine industry. Live attenuated vaccine has been mainly researched for *Salmonella* vaccination since cell-mediated immunity induced by live attenuated vaccines is more efficient in protection of *Salmonella* species. However, inactivated vaccine can be used as the adjuvant after vaccination with live attenuated vaccine, since it can protect the invasion of wild-

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type pathogenic *Salmonella* into the organ. WHO has recommended effective attenuated vaccine treatment to animals to prevent the food poisoning in humans caused by *Salmonella* since 1988.

To construct live attenuated vaccine, various genes from different loci have been applied with single or multiple deletions[7-9]. The flavohaemoglobin (*hmp*) gene is required to persistent infectivity in host during *Salmonella* infections[10]. This gene detoxifies nitric oxide which inhibits overall expression of *Salmonella* Pathogenicity Island (SPI)-2 effectors of *Salmonella*[11]. The *ssrAB* gene is the secretion system regulator and required to persistent infectivity[12]. The *rpoS* gene encodes an alternative sigma factor in *Salmonella* and is important for persistent virulence in the spleen and liver of murine Peyer's patches[13,14]. The *ppk* gene encodes polyphosphate kinase responsible for the synthesis of inorganic polyphosphate from ATP[15]. Polyphosphate regulates the expression of *rpoS*[16]. The *aroA* gene is associated with the biosynthesis of aromatic amino acid to make auxotroph vaccine.

Although *Salmonella* mutants with single deletions of various genes were well characterized in the vaccine study, few studies have been carried out on the behavior of *Salmonella* mutants with multiple deletions of the genes in mice model. In this study, ST mutants were constructed by quadruple or quintuple mutations and tested for attenuation of virulence and protection. This study firstly aimed to investigate the contribution of the highly attenuated gene variations to the virulence of ST. Our results showed that intraperitoneal and oral immunization with ST mutant provides excellent protection against virulent ST and against challenge with the pathogen ST.

MATERIALS AND METHODS

Bacteria culture preparation

Two mutant challenge strains ST and the parental wild-type challenge strain isolated from ileocecal lymph nodes of pigs in Korea were used in this study (Table 1). Bacteria were growing in Luria-Bertani (LB) broth at 37°C for 18 h. Subsequently, they were centrifuged at 13,000 rpm for 20 min, washed with phosphate buffered saline (PBS) twice, and the cell density was adjusted to final concentration, 1×10^3 CFU/100 µl.

Salmonella *live attenuated vaccine*

STΔ4 (*ssrAB, rpoS, hmp,* and *ppk*) and STΔ5 (*ssrAB, rpoS, hmp, ppk,* and *aroA*) mutants were constructed using the phage λ red recombinase system[17]. The method was homologous method which inserted genes using transvector such as pkD3 (chloramphenicol resistance, Cm^R) and pkD4 (kanamycin resistance, Km^R). After recombination, inserted gene was deleted by replacing antibiotic cassette. Finally, the cassette was deleted with pcp20 vector. To confirm production of mutant, bacteria were grown in selective medium with appropriate antibiotics. Subsequently, the colonies were amplified with PCR to investigate the size of PCR product[17].

Attenuated virulence of mutants from mice

Female 8-week-old BALB/c mice was used for virulence study. One group of 5 specific pathogen-free mice (control group, 3 mice) was housed in IVC cage and fed sterile food and provided tap water *ad libitum*. All mice were grown at the condition of 22°C, 40 ~ 70 % air humidity, and 12 h light/12 h dark cycle. Animal studies were carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals and protocols were approved by the Kangwon University Animal care and use Committee. At 8 weeks of age, mice were challenged by the dose of approximately 1×10^3 CFU/100µl per one mouse through intraperitoneal injection. After post injection (PI), mice were euthanized at day 3, 6, 9, 12, and 15 days by cervical dislocation. At each time point, the liver and spleen were separated, weighed and homogenized with peptone buffered saline by tissue lyser (Qiagen, USA, 30Hz, 3 min., 3 times). Lysed organs were inoculated on *Salmonella Shigella* (SS) agar (BD) and incubated at 37°C for 24 h. Cultured bacteria were counted, respectively.

Efficacy studies

Female 8-week-old BALB/c mice were used for protection test and all mice were grown at the same condition as virulence test. One group of 7 specific pathogen-free mice was used to evaluate efficacies of ST4 and ST5 mutants against subsequent virulent infection. At 8 weeks of age, groups of vaccinated mice were infected by the dose of 1×10^3 CFU/100µl per one mouse through intraperitoneal injection. And, second vaccination (1×10^3 CFU) was proceed 7 days later. One week

after the second vaccination, 1×10^4 CFU/100µl of wild type strain was challenged to each group of mice. Then, mice were euthanized at day 7 day by cervical dislocation. The liver and spleen were treated, and the bacteria was counted as same method as attenuated virulence test.

Statistical analysis

Data were analyzed using the General Linear Model (GLM) and the least significant difference (LSD) procedures of the Statistical Analysis System (SAS). Significant mean differences among treatments were compared by Fisher's LSD at $p<0.05$.

RESULTS

Salmonella *attenuated live vaccine*

Two mutants of ST were constructed from the wild type using the phage λ red recombinase system (Table 1). To confirm the mutant constructed, selective medium with appropriate antibiotics was used and, the size of PCR product was assessed. In this study, all the mutants inserted pKD3 (cm^R) and pKD4 (km^R) were achieved by the recombination process (data not shown).

Attenuated virulence of ST mutants

Infection of mice with ST mutants (STΔ4 and STΔ5) deleted multiple genes (*rpoS*, *hmp*, *ssrAB*, *aroA*, and *ppk*) was used to evaluate attenuated virulence of the vaccine strain (Fig. 1). Intraperitoneal infection doses were 6.9×10^3, 4.8×10^3, and 1.5×10^4 CFUs for wild type, STΔ4, and STΔ5, respectively. Compared to wild type strain, CFUs of STΔ4 recovered from the liver were dramatically dropped by 2.46, 2.94, 2.40, 5.29, and 5.51 logs at 3, 6, 9, 12, and 15 days postinfection, respectively (Fig. 1a). CFUs of STΔ5 recovered from the liver also showed similar trend as magnificent decrease at the range of 2.28 to 6.57 log for 15 days postinfection. CFUs of STΔ5 slightly increased or decreased compared to those of STΔ4 with the range from -0.12 to 1.19. Compared to wild type strain, CFUs of STΔ4 recovered from the spleen were highly decreased by 1.89, 2.74, 1.38, 4.6, and 5.01 logs at 3, 6, 9, 12, and 15 days postinfection, respectively (Fig. 1b). CFUs of STΔ5 recovered from the spleen also showed similar trend as magnificent decrease by 2.53, 3.25, 1.60, 4.80, and 5.49 log at 3, 6, 9, 12, and 15 days postinfection, respectively. CFUs of STΔ5 slightly decreased compared to those of STΔ4 with the range from 0.15 to 0.63. However, comparison of CFUs between wild-type and mutant strains in the liver and spleen didn't show statistical significance. The levels of STΔ4 and STΔ5 in the liver remained unchanged or slightly reduced for 15 days postinfection.

The average of the organ/body weight ratio of mice was also estimated in the liver and spleen to evaluate the attenuated virulence of STΔ4 and STΔ5 (Fig. 2). No trend was shown in the average of liver/body weight ratio of mice during day 3 to 9 (Fig. 2a). However, at day 12 and 15, STΔ4 and STΔ5 groups showed slightly lower liver weights in the mice compared to wild type group. STΔ5 group showed slightly lower liver weights in the mice than STΔ4 group at day 12 and 15. Statistical significance wasn't shown between the averages of the organ/body weight ratios in all the mice groups for experiment period. Compared to wild type strain, the averages of the spleen/body weight ratios in the STΔ4 group were significantly decreased by 0.04, 0.18 ($P<0.01$), 0.20 ($P<0.001$), 0.35 ($P<0.001$), and 0.40 g ($P<0.001$) at 3, 6, 9, 12, and 15 days postinfection, respectively (Fig. 2b). The averages of the spleen/body weight ratios in the STΔ5 group also showed similar trend of decrease compared to wild type strain by 0.06, 0.18 ($P<0.001$), 0.22 ($P<0.001$), 0.38 ($P<0.001$), and 0.43 g ($P<0.001$) at 3, 6, 9, 12, and 15 days postinfection, respectively.

Table 1. *Salmonella* Typhimurium wild type and mutant used in this study

S. enteric serovar Typhimurium or mutant	Relevant characteristics[1]
IVKB010198	Wild type (isolate from pig)
STΔ4	Δ*rpoS*::Δ*ssrAB*::Δ*hmpA*::Δ*aroA*;Km^r
STΔ5	Δ*rpoS*::Δ*ssrAB*::Δ*hmpA*::Δ*aroA*::Δ*ppk*;Km^r

[1]Km^r, kanamycin resistance

Efficacy of ST mutants against ST wild type challenge infection

To evaluate the protection of mice vaccinated with STΔ4 and STΔ5 groups against intraperitoneal challenge with non-vaccination group, the systemic infection of challenge strain of mice were estimated in the liver and spleen (Fig. 3). During the 7 days of observation, no mouse died in STΔ4 and STΔ5 groups. Also, non-vaccinated mouse group showed any death. Seven days after challenge, CFUs in the liver of mice in STΔ4 and STΔ5 groups were magnificently decreased by 0.81 and 1.0 log compared to non-vaccination group, respectively (Fig. 3a). However, comparison of CFUs in the liver didn't show statistical significance in both groups. Seven days after challenge, CFUs in the spleen of mice in STΔ4 and STΔ5 groups were significantly decreased by 0.80 and 0.65 log compared to non-vaccination group, respectively. However, comparison of CFUs between STΔ4 and STΔ5 groups didn't show statistical significance.

The average of the organ/body weight ratio of mice was also estimated in the liver and spleen to evaluate the protection efficacy of mice (Fig. 4). Seven days after challenge, the average of the liver/body weight ratio of mice in STΔ4 and STΔ5 groups were, respectively, 0.072 ± 0.006 g and 0.065 ± 0.015 g. The ratio of liver/body weight in ST4 (P<0.05) and ST5 (P<0.01) groups were

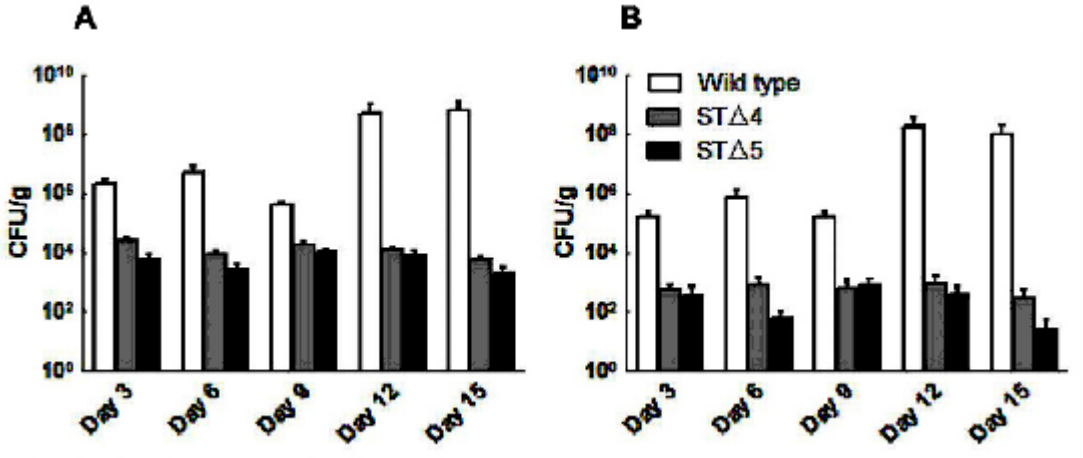

Fig. 1. Mean colony forming unit of ST recovered from the (a) liver and (b) spleen in mice infected by STΔ4 and STΔ5. Female 8-week-old BALB/c mice were intraperitoneally infected with STΔ4, STΔ5 and wild-type-infected control

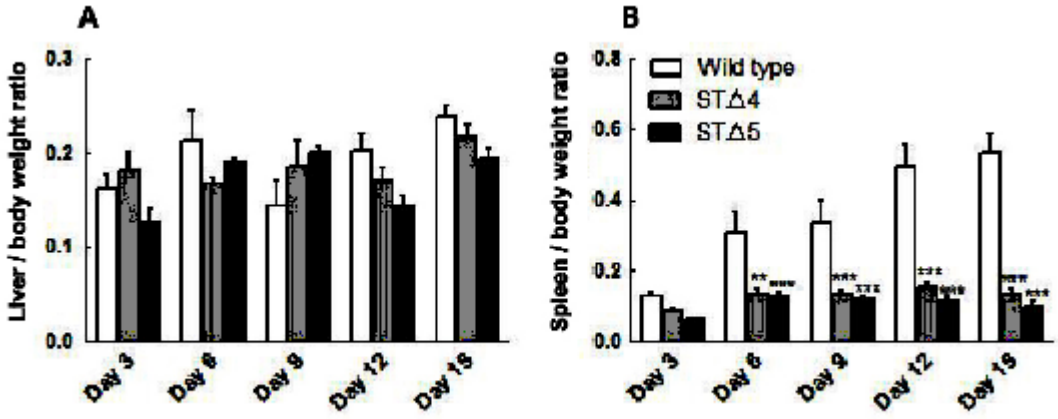

Fig. 2. Mean ratio of the (a) liver/body and (b) spleen/body in mice infected by STΔ4 and STΔ5. Asterisks (****, *****) indicate the significant difference between strains at $P < 0.01$ and $P < 0.001$

statistically significantly lower than that in no vaccination group (0.088 ± 0.007 g). The average of the spleen/body weight ratio in the mice in STΔ4 and STΔ5 groups were, respectively, 0.011 ± 0.001 g and 0.011 ± 0.004 g. The ratio of spleen/body weight ratio of mice in STΔ4 and STΔ5 groups ($P<0.01$) were statistically significantly lower than that in no vaccination group (0.017 ± 0.004 g).

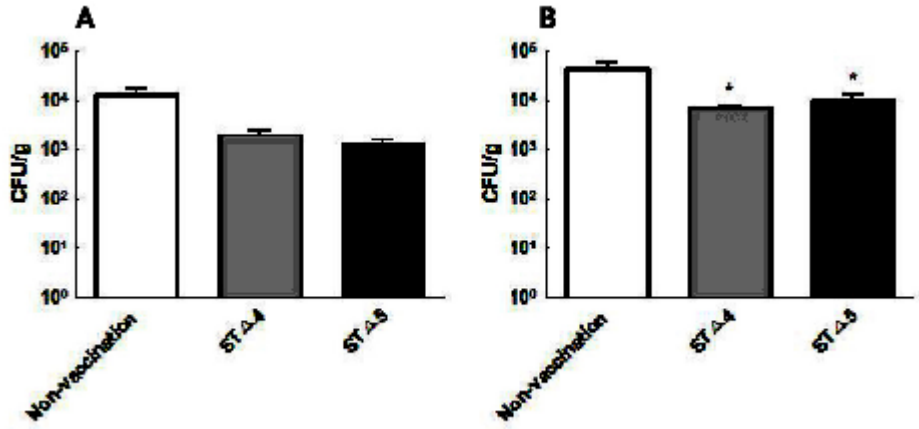

Fig. 3. Mean colony forming unit of ST recovered from the (a) liver and (b) spleen in mice challenged by ST wild type. Female 8-week-old BALB/c mice were intraperitoneally immunized with STΔ4, STΔ5 and noninfected negative control. A challenge by intraperitoneal infection with ST wild type was performed 1 week after the second immunization. Asterisk (*****) indicates the significant difference between strains at $p < 0.05$

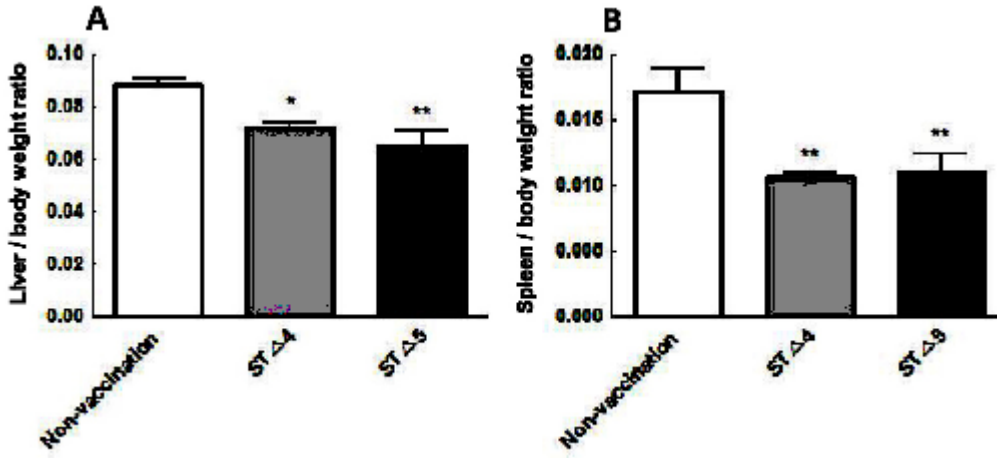

Fig. 4 Mean ratio of the (a) liver/body and (b) spleen/body in mouse challenged by ST wild type. Asterisks (****, *****) indicate the significant difference between strains at $P < 0.01$ and $p < 0.001$

DISCUSSION

The primary objective of this study was to investigate the attenuated virulence and protective efficacy of the ST vaccine with deletions of the genes (*hmp, ssrAB*, and *rpoS*) required to persistent infectivity, the *aro* gene associated with the biosynthesis of aromatic amino acid, and the *ppk* gene encoding polyphosphate kinase responsible for the synthesis of inorganic polyphosphate from ATP. This study firstly constructed the ST vaccine with multiple deletions of aro and ppk genes and assessed the deletion effect on protective efficacy.

The virulence of ST mutant deleted with the *hmp, ssrAB*, and *rpoS* genes was evaluated in mouse model. The virulence of this mutant recovered from the liver of mouse showed

magnificent attenuation at the range of 3.03 to 3.97 log for 12 days, compared with that of wild type strain (unpublished results). From the ST mutant with deletion of three genes, the *aro* and *ppk* genes were deleted consecutively to construct STΔ4 and STΔ5.

In the virulence test, intraperitoneal infection doses were slightly different for each group despite advanced adjustment of dose concentration. It, however, seems that didn't significantly affect the trends of virulence of each group. CFUs of STΔ4 and STΔ5 recovered from the liver and spleen were highly attenuated while wild type strain retained full virulence and slightly changed level of attenuation for 15 days (Fig. 1). The weight ratios of liver/body and spleen/body in mice infected by ST wild type corresponded with highly increased CFUs of ST wild type (Fig. 1a and 2a). This observation is in good agreement with previous report that mice were orally inoculated with *hmp* mutant *Salmonella* and wild type strain, and the *hmp* mutant showed a 100 fold decrease of virulence compared to wild type while it didn't show significantly different virulence at day 5 [10]. These results demonstrated that the flavohemoglobin *Hmp* promotes *Salmonella* virulence during chronic infection. This observation was also in good agreement with previous report that ST *rpoS* single mutant were highly attenuated in mouse compared to virulent ST strains, and the mutant retained significant ability to protect mice against salmonellosis[9, 13]. ST mutant with deletion with *rpoS* and *aroA* was also demonstrated to be more attenuated and retain higher protective efficacy than corresponding single *aroA* or *rpoS* mutants after oral or intraperitoneal vaccination[9,13]. CFUs of STΔ5 showed slightly lower level than those of STΔ4 for 15 days which indicates the effect of the *ppk* gene encoding polyphosphate kinase responsible for the synthesis of inorganic polyphosphate from ATP[15]. Previous report also demonstrated that inactivation of the *ppk* gene in ST resulted in important defects in growth and survival *in vitro*, and virulence *in vivo*[16]. This reduced virulence of the ST *ppk* mutant indicates the crucial importance of maintaining stable intracellular ATP during infection and nutritional stress[16]. The results in this study assessed how the gene deletion affected phenotype of bacterial virulence, while previous reports focused on single or double deletions.

In the virulence test, it can be concluded that ST mutants used in this study are safer than wild type in female 8-week-old BALB/C mice after inoculation by intraperitoneal route. And, ST mutants were able to invade the organs of the host while mutant strain was attenuated. In the protection test, CFUs of STΔ4 and STΔ5 recovered from the liver and spleen were highly decreased while wild type strain retained full virulence for 7 days (Fig. 3). This experiment indicated that STΔ4 and STΔ5 vaccinated mice shed the ST challenge strain at lower levels than did unvaccinated control mice. There was evidence that liver and spleen invasion by the challenge bacteria was reduced in vaccinated mice. CFUs of STΔ5 recovered from the liver or spleen of mice showed slightly lower or higher level than those of STΔ4, respectively. This result implies that the *ppk* gene was not associated with protective efficacy but attenuated virulence in *S. typhimurium* mutant.

CONCLUSION

Our ultimate goal is to develop an attenuated vaccine candidate with multiple deletions due to the side effect of whole bacteria vaccine. The most consistent effect of vaccination is the significant reduction of shedding by vaccinates upon challenge. Although the results obtained in this study are not enough to make a novel *Salmonella* vaccine, this study sheds new light on our understating of important characteristics of a live attenuated vaccine with multiple deletions.

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REFERENCES

1. Rabsch, W., Andrews, H.L., Kingsley, R.A., Prager, R., Tschape, H., Adams, L.G., Baumler, A.J. *Salmonella enterica* serotype Typhimurium and its host-adapted variants. *Infect. Immun.*,

2002; **70**: 2249-55.

2. Grassl, G.A., Finlay, B.B. Pathogenesis of enteric *Salmonella* infections. *Curr. Opin. Gastroenterol.* 2008; **24**: 22-6.
3. Blondel, C.J., Yang, H.J., Castro, B., Chiang, S., Toro, C.S., Zaldivar, M., Contreras, I., Andrews-Polymenis, H.L., Santiviago, C.A. Contribution of the type VI secretion system encoded in SPI-19 to chicken colonization by *Salmonella enterica* serotypes Gallinarum and Enteritidis. *PLoS One,* 2010; **5**: e11724.
4. Barrow, P.A., Page, K., Lovell, M.A. The virulence for gnotobiotic pigs of live attenuated vaccine strains of *Salmonella enterica* serovars Typhimurium and Enteritidis. *Vaccine,* 2001; **19**: 3432-6.
5. Leyman, B., Boyen, F., Verbrugghe, E., Parys, A.V., Haesebrouck, F., Pasmans, F. Vaccination of pigs reduces *Salmonella* Typhimurium numbers in a model mimicking pre-slaughter stress. *Vet. J.,* 2012; **194**: 250-252.
6. Boyen, F., Haesebrouck, F., Maes, D., Van Immerseel, F., Ducatelle, R., Pasmans, F. Non-typhoidal *Salmonella* infections in pigs: a closer look at epidemiology, pathogenesis and control. *Vet. Microbiol.*, 2008; **130**: 1-19.
7. Kim, S.J., Han, Y.W., Rahman, M.M., Kim, S.B., Uyangaa, E., Lee, B.M., Kim, J.H., Roh, Y.S., Kang, S.H., Kim, K., Lee, J.H., Kim, B., Park, K.I., Eo, S.K. Live attenuated *Salmonella enterica* serovar Typhimurium expressing swine interferon-alpha has antiviral activity and alleviates clinical signs induced by infection with transmissible gastroenteritis virus in piglets. *Vaccine,* 2010; **28**: 5031-7.
8. Lee, H.Y., Cho, S.A., Lee, I.S., Park, J.H., Seok, S.H., Baek, M.W., Kim, D.J., Lee, S.H., Hur, S.J., Ban, S.J., Lee, Y.K., Han, Y.K., Cho, Y.K. Evaluation of *phoP* and *rpoS* mutants of *Salmonella enterica* serovar Typhi as attenuated typhoid vaccine candidates: virulence and protective immune responses in intranasally immunized mice. *FEMS Immunol. Med. Microbiol.,* 2007; **51**: 310-8.
9. Coynault, C., Norel, F. Comparison of the abilities of *Salmonella typhimurium rpoS, aroA* and *rpoS aroA* strains to elicit humoral immune responses in BALB/c mice and to cause lethal infection in athymic BALB/c mice. *Microb. Pathog.,* 1999; **26**: 299-305.
10. Bang, I.S., Liu, L., Vazquez-Torres, A., Crouch, M.L., Stamler, J.S., Fang, F.C. Maintenance of nitric oxide and redox homeostasis by the *Salmonella* flavohemoglobin hmp. *J. Biol. Chem.,* 2006; **281**: 28039-47.
11. Haveri, M., Suominen, S., Rantala, L., Honkanen-Buzalski, T., Pyorala, S. Comparison of phenotypic and genotypic detection of penicillin G resistance of *Staphylococcus aureus* isolated from bovine intramammary infection. *Vet. Microbiol.,* 2005; **106**: 97-102.
12. Kuhle, V., Hensel, M. Cellular microbiology of intracellular *Salmonella enterica*: functions of the type III secretion system encoded by *Salmonella* pathogenicity island 2. *Cell. Mol. Life Sci.,* 2004; **61**: 2812-26.
13. Coynault, C., Robbe-Saule, V., Norel, F. Virulence and vaccine potential of *Salmonella typhimurium* mutants deficient in the expression of the RpoS (sigma S) regulon. *Mol. Microbiol.,* 1996; 22: 149-60.
14. Nickerson, C.A., Curtiss, R. 3rd. Role of sigma factor RpoS in initial stages of *Salmonella typhimurium* infection. *Infect. Immun.*, 1997; **65**: 1814-23.
15. Rashid, M.H., Rao, N.N., Kornberg, A. Inorganic polyphosphate is required for motility of bacterial pathogens. *J. Bacteriol.,* 2000; **182**: 225-7.
16. McMeechan, A., Lovell, M.A., Cogan, T.A., Marston, K.L., Humphrey, T.J., Barrow, P.A. Inactivation of *ppk* differentially affects virulence and disrupts ATP homeostasis in *Salmonella enterica* serovars Typhimurium and Gallinarum. *Res. Microbiol.,* 2007; **158**: 79-85.
17. Datsenko, K.A., Wanner, B.L. One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc. Natl. Acad. Sci. USA.,* 2000; **97**: 6640-5.