Acta Veterinaria Hungarica 63 (4), pp. 401–412 (2015)
DOI: 10.1556/004.2015.038

EVALUATION OF *SALMONELLA* GALLINARUM GHOST FORMULATED WITH MONTANIDE™ ISA 70 VG ADJUVANT AS A VACCINE AGAINST FOWL TYPHOID

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(Received 2 February 2015; accepted 22 June 2015)

Escherichia coli heat-labile enterotoxin B subunit (LTB) protein is a potent adjuvant. *Salmonella* Gallinarum ghosts carrying LTB (*S.* Gallinarum-LTB ghosts) were genetically constructed using a plasmid, pJHL187-LTB, designed for the co-expression of the LTB and E lysis proteins. This study evaluates the immunopotentiating effects of Montanide™ ISA 70 VG on *S.* Gallinarum-LTB ghost vaccination against fowl typhoid. Five-week-old layer chickens were injected intramuscularly with sterile PBS (non-immunised control, Group A), *S.* Gallinarum-LTB ghost (Group B) or *S.* Gallinarum-LTB ghost emulsified with Montanide™ ISA 70 VG adjuvant (Group C). Chickens from both Groups B and C showed significant induction of antigen-specific systemic IgG response compared to controls; in addition, Group C showed enhanced induction of systemic IgG response compared to Group B. We observed significant induction of antigen-specific lymphocyte proliferative response and increased mRNA levels of Th1 cytokines (IFN-γ and IL2) in both Groups B and C. Furthermore, in the challenge experiment with a virulent strain of *S.* Gallinarum, Group C showed higher survival rates compared with other groups. These results indicate that vaccination with the *S.* Gallinarum-LTB ghost in combination with Montanide™ ISA 70 VG may enhance the protective immunity against fowl typhoid.

Key words: Fowl typhoid, bacterial ghost, Montanide™ adjuvant, immunity, protection

Fowl typhoid (FT) is a septicaemic disease of domestic birds caused by the Gram-negative intracellular bacterial pathogen, *Salmonella enterica* serovar Gallinarum (Pomeroy and Nagaraja, 1991). Vaccination is the most effective and practical intervention for the eradication of fowl typhoid and lacks the risks and challenges associated with other control measures. Various vaccines to prevent fowl typhoid have been tested in fowl models (Bouzoubaa et al., 1989; Zhang-Barber et al., 1998; Rosu et al., 2007; Chaudhari et al., 2011; Desin et al., 2013).

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Bacterial ghosts are generated by temperature-controlled expression of the lysis *E* gene of bacteriophage PhiX174, which forms transmembrane holes through the bacterial cellular envelope, cloned in Gram-negative bacteria (Witte et al., 1992). More recently, we have demonstrated the vaccine potential of *S.* Gallinarum ghosts for protection against fowl typhoid (Chaudhari et al., 2012; Jawale and Lee, 2014*b*). The vaccine construction strategy incorporates a foreign immunomodulator protein such as *Escherichia coli* heat-labile enterotoxin B subunit (LTB) into the architecture of the ghost membrane, which may improve the immunogenic potential of the vaccine (Jawale and Lee, 2014*a*).

Although bacterial ghosts possess conserved surface antigenic structures and are capable of inducing humoral and cell-mediated immune responses, their nonreplicating nature may be responsible for the rapid destruction and elimination of antigens from the host (Desin et al., 2013). The combination of bacterial ghost-based vaccine with suitable adjuvant material might trigger long-lasting immune responses and improved protection against pathogens. In particular, Montanide™ ISA 70 VG is a water-in-oil emulsion adjuvant containing a highly refined mannitol/oleic acid emulsifier (Dupuis et al., 2006). Montanide™ ISA 70 VG has already been demonstrated to be safe and efficient in numerous poultry disease models (Aucouturier et al., 2006; Jang et al., 2010; Liu et al., 2011).

In the present study we constructed the *S.* Gallinarum ghost carrying LTB protein and an animal study was designed to evaluate the efficacy of Montanide™ ISA 70 VG when combined with *S.* Gallinarum-LTB ghost in protecting against fowl typhoid infection. To assess the adjuvant effect, chickens were immunised with either *S.* Gallinarum-LTB ghosts combined with Montanide™ ISA 70 VG or *S.* Gallinarum-LTB ghosts alone. To assess the protective effect of vaccination, we challenged chickens with a lethal dose of wild-type *S*. Gallinarum.

Materials and methods

Bacterial strains and plasmids

The bacterial strains and plasmids used in this study are listed in Table 1. The *S.* Gallinarum bacteria with deleted aspartate β-semialdehyde dehydrogenase *(asd)* gene were grown at 37 °C in LB broth containing 50 μg/ml diaminopimelic acid (DAP).

Generation of S. *Gallinarum ghost carrying LTB protein*

The JOL1253 strain was transformed with plasmid pJHL187-LTB by means of electroporation and the resultant strain was named JOL1367. The bacterial ghost cells were generated from the JOL1367 strain as described previously (Jawale and Lee, 2014*a*). The induction of gene *E*-mediated bacteriolysis

was monitored by measuring the optical density at 600-nm wavelength (OD600) and the colony-forming units (CFU) of viable cells at different lysis time-points. The vector control (JOL1366) strain was constructed by transforming JOL1253 with plasmid pMMP187.

Table 1

Bacterial strains, plasmids and primers utilised in this study

Strain/plasmid	Description	Reference
Bacterial strains		
JOL1253	*Salmonella* Gallinarum, *asd* gene knockout strain	Chaudhari et al., 2012
JOL420	*Salmonella* Gallinarum wild type	Chaudhari et al., 2012
JOL422	*Salmonella* Gallinarum wild type, challenge strain	Chaudhari et al., 2012
JOL1366	JOL1253 containing pJHL187	This study
JOL1367	JOL1253 containing pJHL187-LTB	This study
Plasmids		
pJHL187	Asd+ p15A ori plasmid carrying ghost cassette	Jawale and Lee, 2014*a*
pJHL187-LTB	pJHL187 containing *eltB* gene	Jawale and Lee, 2014*a*
Primers		
GAPDH-F	5'-AGAACATCATCCCAGCGTCC-3'	This study
GAPDH-R	5'-CGGCAGGTCAGGTCAACA-3'	
IFN-γ-F	5'-CAAAGCCGCACATCAAACA-3'	This study
IFN-γ-R	5'-TTTCACCTTCTTCACGCCATC-3'	
IL2-F	5-'ATCTTTGGCTGTATTTCGGTAG-3'	This study
IL2-R	5'-TGGGTCTCAGTTGGTGTGTAG-3'	

Confirmation of LTB protein production in the S. *Gallinarum ghost*

The presence of LTB protein in the outer membrane of *S.* Gallinarum-LTB ghost envelopes was validated by Western blot analysis. Outer membrane protein fraction samples from *S.* Gallinarum-LTB ghosts and vector control strain ghost cells were prepared as described previously (Jawale and Lee, 2014*a*). Briefly, the *S.* Gallinarum-LTB ghost cells were resuspended in sterile PBS and subjected to sonication for 30 min. The sonicated suspension was centrifuged at 20,000 rpm for 30 min. The pellet was dissolved in Tris-Sarkosyl buffer (20 mM Tris containing 1% sarkosyl, pH 8.6) and incubated on ice for 30 min. The suspension was centrifuged with 132,000 *g* for 1 h at 4 °C to obtain the supernatant containing the outer membrane protein fraction, and the samples were stored at –20 °C. A similar procedure was used to prepare outer membrane protein fractions of *S.* Gallinarum vector control ghost cells. Protein extracts of *S.* Gallinarum-LTB ghosts were heated at 95 °C for 5 min and then resolved by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) on 15% gels. The resolved proteins were then electrophoretically transferred to polyvinylidene fluo-

ride membranes (Millipore, Billerica, MA) for immunoblotting. The membranes were blocked overnight at 4 °C with 3% bovine serum albumin (BSA) in PBS with 0.01% Tween 20, and they were subsequently incubated with polyclonal anti-LTB rabbit serum and horseradish peroxidase- (HRP-) conjugated goat anti-rabbit IgG antibodies. Immunoreactive bands were detected with chemiluminescent dye and the WEST-one Western blot detection system (iNtRON, Seongnam, South Korea). Signals were detected using the multi-wavelength illumination system on a Kodak Image Station 4000MM (Kodak, New Haven, CT, USA).

Immunisation and challenge regimen

The oil-adjuvanted ghost vaccine was prepared by emulsifying the *S.* Gallinarum-LTB ghost suspension with Montanide™ ISA 70 VG (Seppic, Puteaux, France) at a ratio of 30:70 (w/w). The final dose of the inactivated oil-emulsion *S.* Gallinarum-LTB ghost vaccine was 1×10^9 ghost cells per bird. All experimental work involving animals was approved (CBU 2014-1-0038) by the Chonbuk National University Animal Ethics Committee in accordance with the guidelines of the Korean Council on Animal Care. A total of 60 *Salmonella*-free female Brown Nick layer chickens were randomly divided into Groups A, B, and C (n = 20). All birds were housed in the same room and given *ad libitum* access to water and antibiotic-free feed. Chickens were intramuscularly primed at 5 weeks of age and subsequently boosted at 8 weeks of age. Group A chickens were used as non-immunised controls and were injected intramuscularly with sterile phosphate buffered saline (PBS, pH 7.4). Group B chickens were primed and boosted with 0.3 mL of suspension containing 1×10^9 *S.* Gallinarum-LTB ghost cells. Group C chickens were primed and boosted with inactivated oil-emulsion *S.* Gallinarum-LTB ghost vaccine. At 11 weeks of age, all chickens were orally challenged with 5×10^6 CFU of virulent *S.* Gallinarum (JOL422).

Sample collection and humoral immune response

Plasma samples were obtained by centrifuging the blood samples at 13,000 rpm for 5 min. Indirect ELISA was performed with an outer membrane protein (OMP) fraction that was extracted from the wild-type *S.* Gallinarum strain JOL420. Evaluation of plasma IgG concentrations was performed as previously described using chicken IgG and IgA ELISA Quantitation Kits (Bethyl Laboratories, TX, USA) (Chaudhari et al., 2012).

Antigen-specific lymphocyte proliferation assay

The lymphocyte proliferation assay (LPA) was performed with soluble antigens prepared from the wild-type JOL420 *S.* Gallinarum strain as previously described (Chaudhari et al., 2012). Briefly, a suspension of 5×10^5 viable peripheral blood mononuclear cells (PBMCs) was incubated in triplicate in wells of

96-well tissue culture plates with 50 μL of complete RPMI medium alone or containing 4 μg/mL of soluble antigen at 40 °C in a humidified 5% CO_2 atmosphere for 72 h. The proliferation of stimulated lymphocytes was measured and the mean stimulation index (SI) was determined as described previously (Chaudhari et al., 2012).

Quantitative analysis of cytokine mRNA by real-time PCR

At 3 weeks after booster vaccination, the *in vitro* stimulated PBMCs from immunised and non-immunised chickens were assayed to determine cytokine gene expression. Briefly, 1×10^6 viable PBMCs were incubated in 6-well tissue culture plates with 4 μg/mL of soluble antigen extracted from JOL420. The plates were incubated at 40 °C in a humidified 5% CO_2 atmosphere for 72 h. Total RNA from cultured cells was treated with DNAse I and converted into cDNA using a high-capacity cDNA reverse transcription kit (Applied Biosystems, USA). The primers used for quantification of cytokine mRNA are listed in Table 1. Relative quantification of cytokines IFN-γ and IL-2 was performed using the comparative Ct method. The endogenous control GAPDH was used to normalise expression of target cytokines.

Protection against virulent efficacy challenge

We challenged 20 chickens from each group with JOL422. Chickens from all groups were observed for fowl typhoid symptoms for 2 weeks post challenge. The birds that died during the post-challenge observation period were necropsied immediately, and survivors were euthanised 14 days post challenge, with necropsies performed at that time. Gross hepatic and splenic lesions were scored as 0, 1, 2 or 3, using a scoring system described previously (Chaudhari et al., 2012). Detection of the challenge strain in the liver and spleen tissues was carried out as described previously (Nandre and Lee, 2014).

Statistical analysis

Statistical analysis was performed with SPSS 16.0 (SPSS Inc., Chicago, IL, USA). All results are expressed as the mean ± standard deviation of the mean (SD) unless otherwise specified. The data regarding plasma IgG concentration, SI, cytokine gene expression and organ lesion score were analysed using one-way ANOVA with Bonferroni correction. In addition, the number of birds positive for the challenge strain and bird mortality were analysed by Chi-square test. Differences were considered to be statistically significant when P values were ≤ 0.05.

Results

Production and characterisation of the S. *Gallinarum-LTB ghost*

The OD of the JOL1367 culture decreased during the first few hours after induction of gene *E* expression as a function of cell lysis and remained almost constant until the ghost cells were harvested. The CFU count decreased rapidly after the temperature was increased, and no viable cells were detected at 48 h post induction. The *eltB* gene was cloned in the antigen delivery cassette of the plasmid pMMP187-LTB under the control of *λpR* promoter. The presence of the LTB protein was detected by Western blot assay. The fusion protein comprising OmpA-TM and LTB is approximately 26.9 kDa in size, which was observed in the outer membrane protein preparation extracted from *S.* Gallinarum-LTB ghost cells (Fig. 1). No corresponding band of similar size was detected in wells loaded with the outer membrane protein preparation extracted from ghosts of the vector control JOL1366 strain.

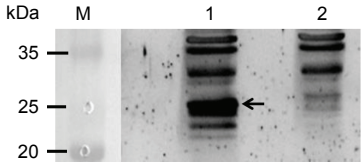

Fig. 1. Confirmation of *Escherichia coli* heat-labile enterotoxin B subunit (LTB) protein production in *Salmonella* Gallinarum LTB-ghosts by Western blot analysis. *Salmonella* Gallinarum ghost prepared from the vector control strain (JOL1366) was used as negative control. Lane M, size marker; lane 1, *S.* Gallinarum-LTB ghost showing the 26.9-kDa OmpA-LTB fusion protein band (indicated by arrowhead); lane 2, *S.* Gallinarum-ghost prepared from the vector control strain

Development of systemic antibody response after vaccination

The mean plasma IgG titres in Groups B and C were significantly elevated (1.64- and 3.06-fold, respectively), compared to the non-immunised control Group A in the second week post immunisation ($P < 0.05$) (Fig. 2). The booster dose of immunisation enhanced the significant induction of plasma IgG titres in both immunised groups. Comparison of IgG titres between immunised groups revealed that the titres of Group C chickens were significantly higher than those of Group B chickens in the second, third, fifth and sixth weeks after prime immunisation ($P < 0.05$) (Fig. 2).

Lymphocyte proliferation assay

The PBMCs of immunised Group B and C chickens exhibited significantly greater proliferation upon antigen-specific stimulation than those of non-immunised Group A chickens ($P \leq 0.05$) (Fig. 3). Stimulation index (SI) values (mean ± SD) of Groups A, B and C were 1.2 ± 0.12, 2.08 ± 0.58 and 2.47 ± 0.56, respectively.

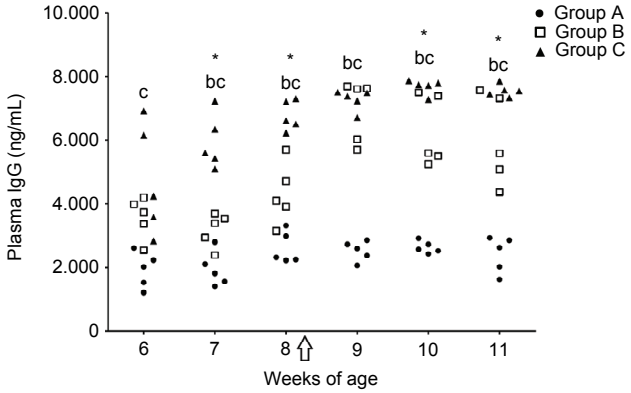

Fig. 2. Systemic antibody response. Antigen-specific plasma IgG responses in the non-immunised control group (A), the group immunised with *S.* Gallinarum-LTB ghost (B) and the group immunised with *S.* Gallinarum-LTB ghost plus Montanide^{TM} ISA 70 VG (C) were determined in the weeks after immunisation. Arrowhead indicates the time-point for booster immunisation. The plasma IgG titres of each analysed sample are indicated by a group-specific symbol. Lowercase letters indicate significant differences in the IgG values of the immunised groups compared to those of the control group: b, immunised Group B chickens compared to the control group; c, immunised Group C chickens compared to the control group. Significant differences between the vaccinated groups are denoted by asterisks

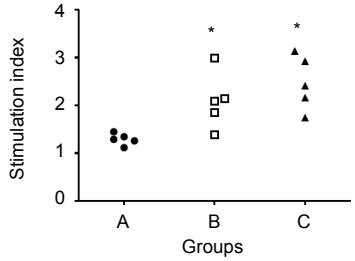

Fig. 3. Lymphocyte proliferative response: *S.* Gallinarum antigen-specific lymphocyte proliferative responses in the immunised and non-immunised chickens in the 3rd week after booster vaccination. The asterisks indicate significant differences between the stimulation index (SI) values of the immunised and non-immunised groups. Groups A, B, and C refer to Table 1 and Fig. 2

Cytokine gene expression after in vitro *antigenic stimulation*

The mRNA levels of IFN-γ and IL-2 were significantly increased in the total RNA isolated from stimulated PBMCs of immunised Group B and C chickens compared those from non-immunised chickens ($P \leq 0.05$) (Fig. 4). These data indicate the immunostimulatory effects of vaccination with bacterial ghosts that result in the induction of Th1 cytokine response, which is involved in protective immunity against intracellular bacterial pathogens.

Protective efficacy after wild-type challenge

On the 5th day after challenge with the virulent strain, 90% of the control Group A chickens started to show distinctive clinical signs of fowl typhoid such as anorexia, depression and diarrhoea. The clinical signs of fowl typhoid were also observed in 50% of Group B chickens and 20% of Group C chickens. At 14 days post challenge, the immunised Groups B and C exhibited 65% ($P = 0.01$) and 80% ($P = 0.001$) survival, respectively, compared with the 20% survival found in the control Group A. The immunised and challenged Group B and C chickens showed significantly lower gross lesion scores of liver and splenic tissue ($P \leq 0.05$) (Table 2). Bacterial recovery results indicated that a significantly lower number of birds in the immunised Groups B and C exhibited challenge infection compared to the control group ($P \leq 0.05$) (Table 2). Among the immunised groups, Group C chickens were better protected against challenge.

Table 2

Survival rate, gross lesion score and bacterial recovery after virulent challenge

Group[#]	N	Survival[a] (%)	n/N	Gross lesion score[b]	
				Liver	Spleen
A	20	4 (20%)	20/20	2.7 ± 0.73^{c}	2.65 ± 0.74
B	20	13 (65%)**	13/20**	$1.6 \pm 1.5^{*}$	$1.35 \pm 1.42^{**}$
C	20	16 (80%)***	13/20**	$1.25 \pm 1.44^{**}$	$1.1 \pm 1.16^{***}$

[#]Groups: A, non-immunised control; B, immunised with *S.* Gallinarum-LTB ghost; C, immunised with *S.* Gallinarum-LTB ghost plus Montanide™ ISA 70 VG; N = number of birds used in each group; n/N = number of *S.* Gallinarum challenge strain positive birds per total number of birds; [a]number of birds surviving after virulent *S.* Gallinarum challenge; [b]gross lesions scores observed after necropsy of challenged birds; [c]group lesion score (mean ± SD). All values were considered to be significant if $P \leq 0.05$ or 0.01. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. the non-immunised Group A

Discussion

Adequate balance between safety and immunogenicity is crucial for designing effective vaccines against fowl typhoid. Live attenuated vaccines are frequently used for effective protection against fowl typhoid. However, safety is a major concern when using live vaccines due to their residual virulence (Kwon and Cho, 2011) and long persistence in the internal organs of immunised chickens, which may cause adverse effects in immunised hosts and contaminate the environment via shedding of live attenuated strains through faeces (Nandre and Lee, 2014). Thus, we have directed efforts towards the development of genetically inactivated bacterial ghost-based vaccines.

Gene *E*-mediated lysis of a mid-logarithmic phase-grown culture of JOL1367 was induced with no further increase in OD600, and the number of viable cells decreased dramatically. Protein E acts by inhibiting peptidoglycan synthesis and thereby prevents cell wall synthesis, which accounts for the reduced OD and number of viable cells during the lysis procedure (Bernhardt et al., 2000). In the present study we made an attempt to incorporate the LTB molecule in the outer membrane portion of *S.* Gallinarum ghost cells. The ability of LTB to bind to the GM1 receptor of eukaryotic cells also facilitates the increased uptake of antigens coupled with LTB by antigen-presenting cells (Freytag and Clements, 2005). The shifting of JOL1367 cultures from 28 °C to 42 °C also triggered λpR-driven expression of *eltB* and the presence of LTB protein in the ghost preparation was confirmed by Western blot (Fig. 1). The idea behind the utilisation of Montanide™ ISA 70 VG in combination with bacterial ghost was to prolong the presence of the bacterial ghost antigens in the immunised birds. The mechanism of action of Montanide™ ISA adjuvant includes the formation of a depot at the injection site, which enables the slow release of the antigen, and formulated antigens are concentrated and protected against degradation while phagocytosis is stimulated (Levast et al., 2014).

A number of studies have demonstrated that the expression of acquired resistance to fowl typhoid depends upon the combined humoral and cell-mediated immune response of the infected host (Rana and Kulshreshtha, 2006; Park et al., 2010; Matsuda et al., 2011). In the present study, we observed a significant induction of systemic IgG responses in the immunised groups, especially in chickens immunised with a combination of ghost cells with Montanide™ ISA 70 VG (Fig. 2). Montanide™ ISA series adjuvants are known to enhance systemic antibody responses against combined antigens (Jang et al., 2010). The systemic antibodies clear *Salmonella* from the blood, and also promote phagocytosis of *Salmonella* via opsonisation (Mastroeni et al., 2011). Anti-*Salmonella* IgG plays a primary role during the early clearance phase of the infection, but once the organisms become established in an intracellular environment within the liver and spleen, their subsequent elimination depends upon cell-mediated immune response (Rana and Kulshreshtha, 2006; Lee et al., 2007). In the present study, significant cell-mediated immune responses were shown by LPA in both immunised groups (Fig. 3). The present study shows that Th1 cytokines are produced in high numbers by vaccinated chickens. The levels of IL-2 and IFN-γ cytokine mRNA produced *in vitro* by antigen-stimulated PBMCs from the vaccinated chickens were significantly higher than those produced by cells from chickens of the non-vaccinated control group (Fig. 4). Without a doubt, the Th1-cell immunity developed by the vaccinated chickens fits with the protection conferred against fowl typhoid (Chappell et al., 2009; Park et al., 2010).

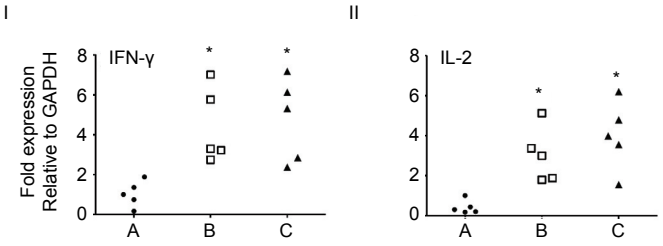

Fig. 4. Cytokine profile of *in vitro* stimulated PBMC cultures. Groups A, B, and C refer to Table 2 and Fig. 2. The significant differences in cytokine expression between the control and immunised groups are indicated by asterisks (*indicates $P \leq 0.05$)

In the current experimental design we used 5×10^6 CFU of challenge strain per bird administered via an oral route. In spite of this large challenge strain dose, both immunised groups showed significant protection, evidenced by significantly higher survival rates and lower gross lesions in the liver and spleen after virulent *S.* Gallinarum challenge infection. The challenge strain remained in the liver and spleen of some of the vaccinated chickens on the 14th day post challenge, but it is likely to be cleared via acquired immunity (Wigley et al., 2005). Among the immunised groups, the chickens vaccinated with the ghost plus Montanide ISA^{TM} 70 VG showed higher survival rates compared to those from chickens vaccinated with the ghost alone. Until recently, very few efforts have been directed toward the production of safe and immunogenic inactivated vaccines against fowl typhoid (Bouzoubaa et al., 1987; Bouzoubaa et al., 1989; Chaudhari et al., 2012; Jawale et al., 2014; Jawale and Lee, 2014*b*). The results of the current study highlight further progress in the development of inactivated *S.* Gallinarum vaccines. It stands very clear that the vaccination of chickens with a combination of *S.* Gallinarum-LTB ghost with Montanide ISA^{TM} 70 VG may be a useful and effective tool to prevent fowl typhoid.

Acknowledgement

This work was supported by a National Research Foundation of Korea (NRF) grant funded by the Korean government (MISP) (No. 2013R1A4A1069486).

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