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Immunization Procedures of Δcrp Δasd Double Deleted Attenuated Salmonella Enterica Serovar Typhimurium delivering Somatostatin Antigen in Mice

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Abstract: In order to estimate the optimal immunization procedures for a novel somatostatin vaccine carried by Δcrp Δasd double deleted attenuated Salmonella enterica serovar Typhimurium. Mice were vaccinated with somatostatin vaccine strain (C500/pVGS/2SS-asd) by using three immunization dosages and three alternative schedules. The specific antibody response was evaluated by indirect ELISA Method. The results showed that 0.2×10^{10} CFU per mouse was the optimal immunization dosage to induce specific IgG response. Interestingly, the booster groups either 2 weeks interval or 4 weeks interval after the first immunization were not improving the level of specific IgG response as expected compared with only injection one dose groups. Furthermore, the different interval period of booster injections also affected the immune response, antibody levels of booster given 4 weeks interval was much higher than that booster given 2 weeks interval. Similar results were obtained on body weight gains analysis. The results of body weight gains of immunized mice also showed that the most effective immunization dosage is 0.2×10^{10} CFU per mouse and the optimal immunization number is a single dose. These observations have a very important application in the field of animal production.

Key words: Somatostatin, Salmonella, dose, interval, mice

INTRODUCTION

Somatostatin (SS) exerts an inhibitory effect on the synthesis and secretion of Growth Hormone (GH) from the anterior pituitary gland. Experimentally, all known regulation of growth hormone is under the control of at least two hypothalamic peptides, a stimulatory GH-Releasing Hormone (GHRH) found in the Arcuate Nucleus (Arc) and an inhibitory hormone, Somatostatin (SS), synthesized in the Periventricular nucleus (PeV) (Wagner *et al*., 1998; Muller *et al*., 1999; Mullis, 2005). Recently, it has been reported immuno-neutralization of somatostatin could increase blood concentrations of growth hormone as do animals that are genetically engineered to disrupt the secretion of endogenous somatostatin (Spencer *et al*., 1986; Elsaesser and Drath, 1995; Han *et al*., 2008; Liang *et al*., 2008; Xue *et al*., 2010).

With the development and widespread use of DNA vaccine an attenuated strain of salmonella typhimurium has been considered a promising and Versatile Oral Delivery System, applicable to the large-scale vaccination of a range of antigens (Redman *et al*., 1996; Flo *et al*., 2001; Schoen *et al*., 2004). There are some reports indicating that the oral immunization with attenuated salmonella typhimurium carrying plasmid encoding the fusion gene of somatostatin and HBsAg under eukaryotic promoter elicit a strong cellular immune response and humoral response and show a more effective result for improving the growth in mice and pigs than intramuscular immunization (Bai *et al*., 2011; Liu *et al*., 2011). However, these studies also point out the drawback of these vaccines for the presence of corresponding antibiotic resistance genes.

In the laboratory, plasmids can be stably maintained in bacteria through the use of antibiotic selection genes encoded on the plasmid. However, antibiotics and their resistance genes are not desirable for *in vivo* DNA vaccine delivery due to biosafety and regulatory concerns. Recent studies have demonstrated that the asd based host-plasmid balanced lethal system could overcome the problem of the use of antibiotic resistance gene (Curtiss *et al*., 1990; Galan *et al*., 1990; Zhao *et al*.,

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2008). In this stability system, the plasmid encoding an essential gene *asd* (aspartate-semialdehyde dehydrogenase) as a selective marker in place of antibiotic-resistance markers and bacterial has been deleted asd gene using suicide vector technology. DAP is a key constituent of peptidoglycan in the Gram-negative cell wall so that asd mutants lyse in a growth medium deprived of DAP. However, cells deleted for the chromosomal *asd* gene but containing complementing asd^+ plasmids are restored to normal growth. As a result only plasmid carrying cells could grow, making the strain totally dependent on the maintenance of the plasmid. Since, DAP is not prevalent in the animal host, essentially 100% of the surviving avirulent salmonella recovered from an immunized animal host still contain the recombinant plasmid and express the foreign antigen.

Based on these informations, a balanced lethal system was introduced into somatostatin oral DNA vaccine. In the current study an asd-complementing plasmid expressing GMCSF and hybrid hepatitis B virus S particles and *SS fusion* gene was constructed and then transformed into Δasd Δcrp double deletion salmonella C500 strains (Liang *et al*., 2009). The aims of this study were to estimate the levels of neutralizing antibodies induced by this novel system by using three alternative immunization procedures and to determine the optimal immunization design.

MATERIALS AND METHODS

Plasmids and bacterial strains: The pVGS/2SS-asd plasmid encoding two copies of *somatostatin* genes presented by the Hepatitis B surface Antigen (HBsAg) particle and Granulocyte/Macrophage Colony-Stimulating Factor gene (*GM-CSF*) was described in Fig. 1. The pVAX-asd vector was the same as pVAX1 vector

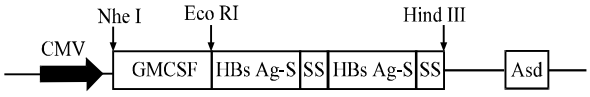

Fig. 1: Schematic diagram for the construction of plasmid pVGS/2SS-asd. The GS/2SS fusion gene encoding two copies of somatostatin genes presented by the Hepatitis B surface Antigen (HBsAg) particle and Granulocyte/Macrophage Colony-Stimulating Factor gene (*GM-CSF*) was shown with NheI and HindIII enzymes. In addition, pVGS/2SS-asd plasmid contained *asd* gene used to instead the kanamycin resistance gene

(Invitrogen) with the exception that kanamycin resistance cassette was replaced with the asd cassette from plasmid pYA3493. pYA3493 plasmid and strain of χ6097 (Kang *et al*., 2002) was kindly provided by Curtiss R 3rd at Department of Biology, Washington University. *Salmonella enterica* sv. *Choleraesuis* C500 strain with Δcrp Δasd double deletion was offered from Dr. Guo Aizhen (Xu *et al*., 2006).

Preparation of bacterial strains for vaccination: C500 (pVGS/2SS-asd) is referred to here as the vaccine strain, asd^- strains C500 (DAP was added at 50 μg mL^{-1}) and vaccine strains were inoculated into Luria-Bertani (LB) broth and grown to an optical density at 650 nm (OD_{650}) of 0.5. Then, cells were harvested by centrifugation at 3000×g for 10 min at 4°C and resuspended in Phosphate Buffered Saline (PBS) for oral vaccination. Prior to administration to animals, bacterial cells were adjusted spectrophotometrically and plated on LB agar to determine viable cell counts.

Oral immunization: Female KunMing mice, 4-5 weeks of age were purchased from Medical Laboratory Animal Center of Hubei province and were divided into 11 groups Table 1; n = 10/group. A total 30 min prior to oral inoculation, mice were administered 200 μL of 7.5% sodium bicarbonate to neutralise stomach acidity in mice. Mice were then fed 200 μL of either PBS or C500 strains and vaccine strains (1×10^{10}, 1×10^{9} and 1×10^{8} CFU mL^{-1}) by intragastric gavage with a 20/25 mm feeding needle. Three different immunization procedures were performed in this experiment. Groups T1, T4, T7 received only one vaccine dose at day 0, groups T2, T5, T8 received the first dose at day zero and then another dose at 2 weeks (early booster) and groups T3, T6, T9 were also immunized with two doses; however, the booster was postponed until 4 weeks after the initial immunization (delayed booster).

Weight and serum collection: Animals were deprived of food and water for 4 h before body weighing and they were weighed before the primary immunization, 1, 2, 4, 6 and 8 weeks after the primary immunization, respectively. Blood samples were collected from the tail vein of each mouse at 0 day, 2, 4, 6 and 8 weeks after the beginning of immunization. The samples were allowed to stand at room temperature for 4 h and then were incubated overnight at 4°C. Serum was collected by centrifugation at 2000×g for 10 min and then was stored at -20°C until use.

Indirect ELISA: To evaluate the levels of specific IgG in serum, standard indirect ELISA was employed using

SS-14 (S9129, Sigma-Aldrich) as the coating antigen. Briefly, 96 well immunoplates (Greiner bio-one, Germany) were coated with 100 ng of SS diluted in bicarbonate buffer (pH 9.6, 0.05 mol L^{-1}) overnight at 4°C. Following blocking with 1% (w/v) of BSA in PBS (pH 7.2) for 1 h at 37°C, serum samples diluted by 2, 50, 100, 200, 400, 800, 1600 and 3200 times in PBST (0.05% Tween-20 in PBS) were added to the wells and the plates were incubated for 1 h at 37°C. Goat anti-mouse IgG HRP-conjugated (Boster, Wuhan, China) diluted 1:2500 in PBS were added for another 1 h at 37°C. To develop the ELISA result, 10 mg of 3, 3', 5, 5'-tetramethylbenzidine (TMB tablets, Sigma-Aldrich) was dissolved in 0.025 mol L^{-1} phosphate-citrate buffer with H_2O_2 as the substrate then incubated for 40 min at RT. Reactions were ended by 2 mol L^{-1} H_2SO_4 and the resulting Optical Density (OD) was measured at 450 nm of wavelength using an ELISA reader (thermo electron corporation). Titres were expressed as the reciprocal of the highest serum dilution to give a positive reaction (Tsang *et al*., 2007).

Statistical analysis: The data of weight gains are presented in mean±SE. All analyses were performed by use of SPSS System 11.5 Software (SPSS Inc). SS-specific antibodies and the body weight gains were compared by ANOVA. If data indicated significant differences among the groups, pairwise comparisons of groups were made and the probability values were adjusted for multiple comparisons. A two-tailed $p<0.05$ was considered to be statistically significant.

RESULTS AND DISCUSSION

Identification of recombinant plasmids: A schematic representation of the different cloning steps for construction pVGS/2SS-asd plasmid was given in Fig. 1. The recombinant plasmid pVGS/2SS-asd was identified by digestion methods and sequence analysis. The digestions were carried out with NheI/HindIII and EcoRI/HindIII restriction enzymes, respectively. The results of the electrophorogram showed that there were two bands of 780 and 4208 bp segments with EcoRI and HindIII digestions (Lanes 1-2, Fig. 2) 1230 and 3758 bp with NheI and HindIII digestions (Lanes 3-4, Fig. 2).

Antibody responses of the vaccine strain immunized against mice: Serum antibody activity to somatostatin was detected after orally immunized vaccine strain with protocols against mice. The IgG antibody titers were shown and compared among three doses groups (Fig. 3). The serum IgG response to somatostatin in high dose groups was significantly greater at weeks of different 2 and 4 than that in moderate dose groups ($p<0.01$) and low dose groups ($p<0.01$). While there was no significant differences among three dose groups at 6th week after the primary vaccination. At the termination of experiment, statistical difference between the high dose groups and the low dose groups was also observed ($p<0.05$). These results indicate that dose-dependent relationship was presented in this study.

For the high dose groups (Fig. 4), mice in once immunized group (T1) showed high serum IgG titers compared to twice immunized groups with 2 weeks (T2) or 4 weeks intervals (T3) among three different time points except 6 weeks time point. The similar trend of antibody responses in moderate dose immunized groups (Fig. 5)

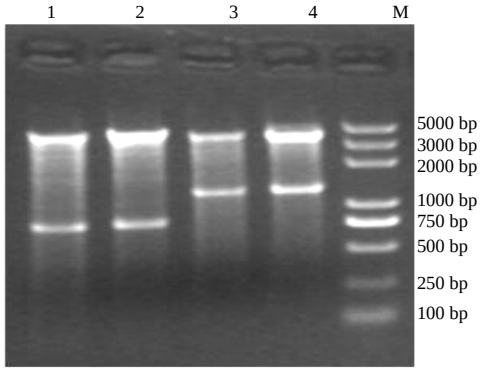

Fig. 2: Agarose gel electrophoresis of pVGS/2SS-asd plasmid digested by double restriction endonucleases. Lanes 1 and 2 show two bands of 780 and 4208 bp with EcoRI and HindIII digestion; Lanes 3 and 4 show two bands of 1230 and 3758 bp with NheI and HindIII digestion; M: 250 bp ladder DNA marker (5000 bp)

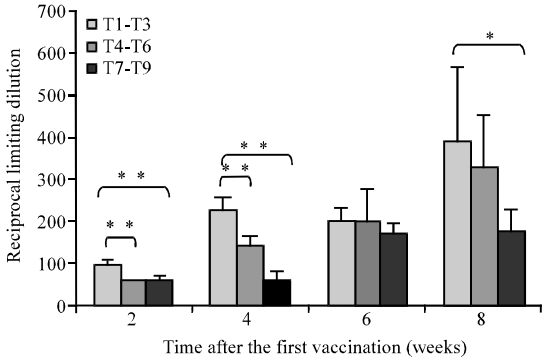

Fig. 3: IgG antibody titers of mice immunized with various doses of C500 (pVGS/ 2SS -asd) vaccine strain. At weeks of 2, 4, 6 and 8 after the first immunization, serum antibody activity to somatostatin was detected after orally immunized vaccine strain with different protocols against mice. *$p<0.05$, **$p<0.01$

was also observed. In contrast, IgG antibody titers in once immunized group (T7) began to augment at 6 weeks after primary immunization and this response continued to increase at 8 weeks after primary immunization (Fig. 6).

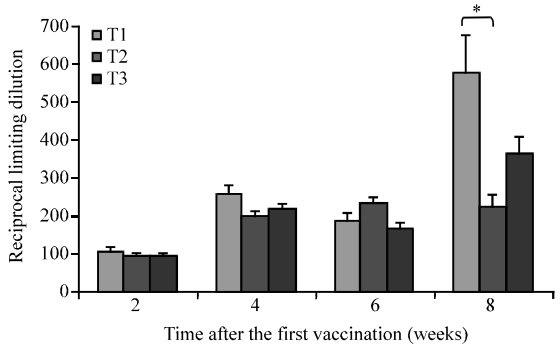

Fig. 4: IgG antibody titers of mice immunized with high doses of different immunization strategy. For the high dose groups, the induced IgG levels in once immunized group (T1) and twice immunized groups with 2 weeks (T2) and 4 weeks intervals (T3) were analyzed. *p<0.05

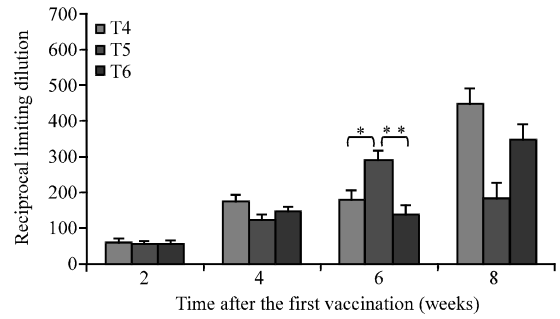

Fig. 5: IgG antibody titers of mice immunized with medium doses of different immunization strategy. For the middle dose groups, the antibodies of mice in once immunized group (T4) and twice immunized groups with 2 weeks (T5) and 4 weeks intervals (T6) were detected. *p<0.05; **p<0.01

There was statistically significant difference between once immunized group (T1) and twice immunized group with 2 weeks intervals (T2) at 8th week after primary immunization (p<0.05) (Table 1).

Weight gains of the vaccine strain immunized against mice: Weight gains of animals were monitored at different time point in this experiment. Mice immunized with high dose strain (T1-T3) showed statistically significant difference compared to moderate dose group (T4-T6) and control strain C500 group (p<0.01) at 1 week after primary immunization (Table 2). There was no statistically significant difference found between the immunized groups and control groups at 2, 4 and 6 weeks after initial immunization (Table 2). At the termination of experiment (8 weeks after primary immunization), mice of high dose groups on weight gain was significantly higher than mice of control groups (p<0.05). These results suggest that mice immunized with high dose vaccine strain shows potential advantages on body weight. For the high dose groups (Table 3), mice in once immunized group (T1)

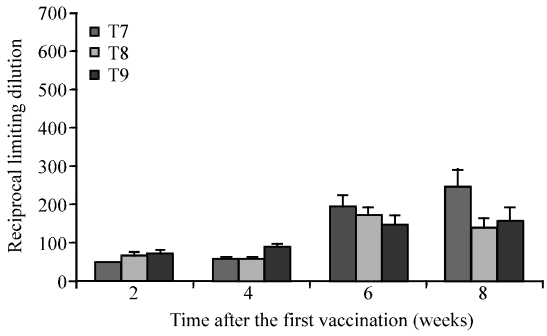

Fig. 6: IgG antibody titers of mice immunized with low doses of different immunization strategy. The antibody levels in once immunized group (T7) and twice immunized groups with 2 weeks (T8) and 4 weeks (T9) intervals were compared and there was no statistically significant differences among immunized groups

Table 1: Experimental design and vaccination protocol

		Primary immunization		Secondary immunization		
Groups	n	Dose (CFU/mice)	Vaccine	Dose (CFU mice)	Vaccine	Intervals (weeks)
T1	10	0.2×10^{10}	C500(pVGS/2SS-asd)	-	-	-
T2	10	0.2×10^{10}	C500(pVGS/2SS-asd)	0.2×10^{10}	C500(pVGS/2SS-asd)	2
T3	10	0.2×10^{10}	C500(pVGS/2SS-asd)	0.2×10^{10}	C500(pVGS/2SS-asd)	4
T4	10	0.2×10^{9}	C500(pVGS/2SS-asd)	-	-	-
T5	10	0.2×10^{9}	C500(pVGS/2SS-asd)	0.2×10^{9}	C500(pVGS/2SS-asd)	2
T6	10	0.2×10^{9}	C500(pVGS/2SS-asd)	0.2×10^{9}	C500(pVGS/2SS-asd)	4
T7	10	0.2×10^{8}	C500(pVGS/2SS-asd)	-	-	-
T8	10	0.2×10^{8}	C500(pVGS/2SS-asd)	0.2×10^{8}	-	2
T9	10	0.2×10^{8}	C500(pVGS/2SS-asd)	0.2×10^{8}	-	4
C500	10	0.2×10^{9}	C500	-	-	-
PBS	10	200 mL	PBS	-	-	-

showed significant differences on weight gain compared control groups with C500 strain ($p<0.01$) and PBS ($p<0.05$) at 1 week after initial vaccination. The same results between T1 and control groups were observed at 8 weeks after primary vaccination. In addition, weight gains of mice in twice immunized group with 4 weeks intervals (T3) was significant higher than that of C500 control group ($p<0.05$) at 1 week after initial vaccination. For the moderate dose groups (Table 3), statistically significant differences were found among the groups immunized with vaccine strains at 2 weeks after primary vaccination. For the low dose groups (Table 3), weight gain of mice in T9 group increased higher than that of T7 group ($p<0.05$) at 4 weeks time point.

Salmonella is a promising and versatile carrier for DNA vaccine, applicable to the large-scale vaccination of a range of antigens (Redman *et al*., 1996; Flo *et al*., 2001; Schoen *et al*., 2004). However, loss of heterologousgene encoded antigen is problematic and antibiotic resistance genes are not desirable for DNA vaccine delivery due to bio-safety and regulatory concerns. To solve these problems, researchers describe here an Asd+balanced-lethal Host-Vector System. In this system, strain C500 with crp^- asd^- double deleted was used as a delivery system for an asd + plasmid (pVGS/2SS-asd) encoding two copies somatostatin genes. The introduction of an asd + plasmid (pVGS/2SS-asd) into asd mutants makes the bacterial strain (asd^- C500) plasmid-dependent and this dependence on the asd + plasmid vector created a balanced-lethal complementation between the asd^- C500 strain and the pVGS/2SS-asd recombinant plasmid. In addition, the antigenicity of pVGS/2SS-asd plasmid was checked before the evaluation on the effect of vaccine strain (Liang *et al*., 2009).

Table 2: Weight gains of mice immunized with various doses of vaccine strain (g)

	Weeks				
Groups	PM 1	PM 2	PM 4	PM 6	PM 8
T1-T3	5.15±0.23^{A}	1.56±0.19	3.20±0.24	3.46±0.33	3.07±0.32^{a}
T4-T6	4.06±0.22^{B}	1.97±0.26	3.65±0.25	2.95±0.26	2.38±0.22^{ab}
T7-T9	4.80±0.25^{AB}	1.70±0.18	3.03±0.30	2.92±0.27	2.38±0.27^{ab}
PBS	4.33±0.43^{AB}	1.75±0.33	2.59±0.51	2.70±0.38	1.49±0.37^{b}
C500	3.45±0.24^{B}	1.52±0.36	3.93±0.53	3.95±0.59	1.57±0.43^{b}

In the vertical row, highly significance level was $p<0.01$ between values scripted with completely different capital letters, and $p>0.01$ with same letters. Significance level was $p<0.05$ between values scripted with completely different small letters and $p>0.05$ with same letters

The main aim of this study was to estimate the optimal immunization program of a balanced-lethal complementation vaccine strain. In this respect, researchers compared the immunization effect of vaccine strain (C500/pVGS/2SS-asd) by using three different immunization doses: groups T1, T2, T3 received vaccine at the high dose of 1×10^{10} CFU mL^{-1}, groups T4, T5, T6 received vaccine at the moderate dose of 1×10^{9} CFU mL^{-1} and groups T7, T8, T9 received vaccine at the low dose of 1×10^{8} CFU mL^{-1}. Researchers could easily find that the protocol used to immunize with high dose of vaccine strain was clearly more efficient. In these high dose groups (T1-T3), antibody levels were higher in comparison the other two dose groups during almost all period of investigation. Additionally, the data also showed that high dose groups obtain potential advantages on body weight. However, there was no statistically significant difference among different dose groups at the final week. This result suggests that the dose of somatostatin vaccine strain having a good correlation with the level of elicited IgG response and the optimal effective dose of vaccine strain is 0.2×10^{10} CFU in mice (De Rose *et al*., 2006; Liu *et al*., 2007).

In order to evaluate the optimal immunization protocol of vaccine strain, researchers also investigated whether the booster was necessary for the vaccine

Table 3: Weight gains of mice immunized with high doses, moderate doses, low doses by different immunization strategy (g)

Groups	PM 1 weeks	PM 2 weeks	PM 4 weeks	PM 6 weeks	PM 8 weeks
T1	5.72±0.33^{Aa}	1.92±0.40	3.13±0.54	4.10±0.71	4.38±0.28^{Aa}
T2	4.74±0.46^{AaBbc}	1.61±0.27	2.77±0.38	2.69±0.38	2.65±0.54^{AaBb}
T3	5.07±0.39 ^{AaBb}	1.14±0.34	3.72±0.29	3.50±0.51	2.83±0.62^{AaBb}
PBS	4.33±0.43^{ABbc}	1.75±0.33	2.59±0.51	2.70±0.38	1.49±0.37^{ABb}
C500	3.45±0.24^{Bc}	1.52±0.36	3.93±0.53	3.95±0.59	1.57±0.43^{Bb}
T4	4.29±0.44	1.40±0.33^{b}	3.56±0.41	2.51±0.36	1.35±0.18
T5	3.52±0.51	3.14±0.59^{a}	3.60±0.41	3.50±0.56	2.80±0.54
T6	4.25±0.21	1.50±0.22^{b}	3.76±0.53	2.92±0.43	2.53±0.20
PBS	4.33±0.43	1.75±0.33^{ab}	2.59±0.51	2.70±0.38	1.49±0.37
C500	3.45±0.24	1.52±0.36^{ab}	3.93±0.53	3.95±0.59	1.57±0.43
T7	5.46±0.32	1.99±0.33	2.22±0.49^{b}	3.70±0.55	2.17±0.41
T8	4.46±0.47	1.74±0.34	2.76±0.41^{ab}	2.63±0.34	2.12±0.55
T9	4.49±0.44	1.40±0.25	3.96±0.56^{a}	2.45±0.44	2.87±0.45
PBS	4.33±0.43	1.75±0.33	2.59±0.51^{ab}	2.70±0.38	1.49±0.37
C500	3.45±0.24	1.52±0.36	3.93±0.53^{ab}	3.95±0.59	1.57±0.43

In the vertical row, highly significance level was $p<0.01$ between values scripted with completely different capital letters and $p>0.01$ with same letters. Significance level was $p<0.05$ between values scripted with completely different small letters and $p>0.05$ with same letters

strain. The three alternative procedures was performed as follows: groups T1, T4, T7 received only one vaccine dose at day zero, groups T2, T5, T8 received the first dose at day zero and then another dose at 2 week (early booster), groups T3, T6, T9 were also immunized with two doses of 4 weeks interval. Interestingly, the booster groups either 2 weeks interval or 4 weeks interval after the first immunization were not improving the humoral immune response as expected compared with only injection one dose groups.

The results are contrary to conventional views that the more boosters were given, the higher was the response level of specific IgG (Tims *et al*., 2000; Mizuno *et al*., 2007). Different immune response seen in different papers may be due to the strain of somatostatin vaccine used. In addition, antibody levels of booster given 4 weeks group after first dose was much higher than the values detected in booster given 2 weeks group at the termination of experiment. Generally, there are two possibilities to explain these observations. One possible is that at booster days the amount of antigen from the first dose was still enough to induce a good immune response. (Griffin *et al*., 1999; Albas *et al*., 2006).

In this case, an extra antigen supply could not stimulate a better response and the suitable intervals between the first dose and booster was important for antibody response. Another possibility that does not exclude the first one is that immune memory cells were not fully developed after booster immunization followed the first vaccine dose (Tsang *et al*., 2007). In this case, the antigen would not interact with memory cells and consequently would not induce an anamnestic immune response. However, whether the booster induced higher cellular immune response has not been studied more.

CONCLUSION

In the current study, researchers have investigated the immunization procedures of Δcrp Δasd double deleted attenuated Salmonella enterica serovar Typhimurium delivering Somatostatin (SS) antigen. The investigation clearly showed the optimal effective dose of somatostatin vaccine strain is 0.2×10^{10} CFU and the optimal immunization number is a single dose. This observation is very interesting not only because it is original but also because it has an important application in the field of animal production.

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