Development of Live Attenuated Salmonella Typhimurium Vaccine Strain Using Radiation Mutation Enhancement Technology (R-MET)

Hyun Jung Ji1,2, A-Yeung Jang3, Joon Young Song3, Ki Bum Ahn1, Seung Hyun Han2, Seok Jin Bang4, Ho Kyong Jung4, Jin Hur5 and Ho Seong Seo1,6*

1 Research Division for Radiation Science, Korea Atomic Energy Research Institute, Jeongeup, South Korea, 2 Department of Oral Microbiology and Immunology, and Dental Research Institute (DRI), School of Dentistry, Seoul National University, Seoul, South Korea, 3 Department of Internal Medicine, Korea University College of Medicine, Seoul, South Korea, 4 Research and Development Center, HONGCHEON CTCVAC Co., Ltd., Hongcheon, South Korea, 5 Department of Veterinary Public Health, College of Veterinary Medicine, Jeonbuk National University, Iksan, South Korea, 6 Department of Radiation Science, University of Science and Technology, Daejeon, South Korea

Salmonella enterica is a leading cause of food-borne diseases in humans worldwide, resulting in severe morbidity and mortality. They are carried asymptomatically in the intestine or gallbladder of livestock, and are transmitted predominantly from animals to humans via the fecal-oral route. Thus, the best preventive strategy is to preemptively prevent transmission to humans by vaccinating livestock. Live attenuated vaccines have been mostly favored because they elicit both cellular and humoral immunity and provide long-term protective immunity. However, developing these vaccines is a laborious and time-consuming process. Therefore, most live attenuated vaccines have been mainly used for phenotypic screening using the auxotrophic replica plate method, and new types of vaccines have not been sufficiently explored. In this study, we used Radiation-Mutation Enhancement Technology (R-MET) to introduce a wide variety of mutations and attenuate the virulence of Salmonella spp. to develop live vaccine strains. The Salmonella Typhimurium, ST454 strain (ST WT) was irradiated with Cobalt60 gamma-irradiator at 1.5 kGy for 1 h to maximize the mutation rate, and attenuated daughter colonies were screened using in vitro macrophage replication capacity and in vivo mouse infection assays. Among 30 candidates, ATOMSal-L6, with 9,961-fold lower virulence than the parent strain (ST454) in the mouse LD50 model, was chosen. This vaccine candidate was mutated at 71 sites, and in particular, lost one bacteriophage. As a vaccine, ATOMSal-L6 induced a Salmonella-specific IgG response to provide effective protective immunity upon intramuscular vaccination of mice. Furthermore, when mice and sows were orally immunized with ATOMSal-L6, we found a strong protective immune response, including multifunctional cellular immunity. These results indicate that ATOMSal-L6 is the first live vaccine candidate to be developed using R-MET, to the best of our knowledge. R-MET can be used as a fast and effective live vaccine development technology that can be used to develop vaccine strains against emerging or serotype-shifting pathogens.

Keywords: live vaccine, radiation, R-MET, Salmonella typhimurium, mutation- genetics, attenuation
INTRODUCTION

Invasive non-typhoidal Salmonella (iNTS) is a leading cause of bacterial bloodstream infections in both humans and animals (1). Salmonella infections commonly result in self-limiting diarrheal illness that rarely leads to death; however, the case fatality rate increases to 20–25% in infants, elderly, and immunocompromised individuals (2–5). Recent systematic analysis reported that iNTS caused an estimated 535,000 illnesses and 77,500 deaths in 2017 (6), particularly in sub-Saharan Africa, where iNTS is a leading cause of community-onset bloodstream infection (7–9). In that region, iNTS was the second most common invasive bacterial disease, following Streptococcus pneumoniae infection (3, 7, 10). Although Salmonella can be controlled using antibiotics, an increased prevalence of multidrug-resistant strains has been reported over recent decades (11–13). Vaccines can potentially control the prevalence of Salmonella in both humans and animals (14–16). There are two possible vaccination strategies (4); vaccinating high risk groups among humans, such as elderly and/or immunocompromised adults and (5) mass vaccination to poultry and pig to prevent transmission of Salmonella to human via the consumption of Salmonella-contaminated meat.

Approximately 20–36% of Salmonella cases in humans were linked to the consumption of eggs, poultry meat, and red meats contaminated with Salmonella (17). At present, there is no vaccine that directly targets S. Typhimurium in humans, but several types of vaccines have been introduced to pigs and chickens (18–20). Surprisingly, the mass poultry vaccinations carried out in the United Kingdom, which were introduced to combat Salmonella infections, has dramatically decreased transmitted illness from 1.6 cases per 1000 persons in 1993 to 0.2 cases per 1,000 persons in 2009 (21). Therefore, vaccinating economically important animals might be the safest and most effective strategy to prevent the spread of Salmonella infection in humans.

Primarily, live attenuated vaccines have been favored because they elicit both cellular and humoral immunity, which provide long-term protective immunity (22). To date, several live attenuated vaccines are available worldwide for use in pig and poultry. In Australia, there is only one registered, commercially available live attenuated S. Typhimurium vaccine; it was produced by disrupting the aroA gene by inserting the Tn10 transposon (23). IDT Biologika licensed Salmovac440 for chickens and Salmoporc for pigs, which are auxotrophic Salmonella vaccine strains of both histidine and adenine (24, 25). Recently, whole genome sequencing (WGS) results showed that Salmovac440 was attenuated by only 6 SNPs, and these mutations dramatically reduced Salmonella virulence (26). However, the mutations caused by SNPs easily revert and regain original virulence. To overcome this, a Salmonella vaccine strain using LMO (Living Modified Organism) is being developed. CVD1921, which is mutated in both the guaBA genes that are involved in the biosynthesis of guanine nucleotides, and the clpP gene affecting flagella expression, was shown to be significantly attenuated with decreased shedding, systemic spread, and clinical disease manifestations in the digestive tract of a primate model (rhesus macaque) (27). Nevertheless, LMO vaccines have not been approved for use in the farm in many countries due to environmental contamination risks and transmission of modified genes to environmental microorganisms.

Spontaneous mutations have been extensively used as sources of novel genetic diversity for selecting new, improved organisms (28, 29). However, the appearance of new mutations is a very rare event in bacteria, because the mutation rate of Escherichia coli is only 10<sup>−3</sup> per genome per generation (30). After Hermann Joseph Muller first discovered that exposure to high-energy radiation induces a variety of genetic mutations and can transmit these new mutations to offspring (31, 32), radiation-induced mutation breeding is being widely used to generate genetic variability in various organisms (33). Radiation-induced mutagenesis can be caused by direct or indirect action on the DNA. In the direct action method, the radiation penetrates the cell and hits the DNA causing single-stranded or double-stranded DNA breaks (34). In the indirect action method, the radiation hits the water molecules, the major constituent of the cell, and other organic molecules in the cell, whereby free radicals such as hydroxyl (HO·) and alkoxy (RO·) are produced. Free radicals are characterized by an unpaired electron in the structure, which is highly reactive and reacts with DNA molecules to cause molecular structural damage (35–37). Chemical mutagens and ultraviolet rays have been widely used to accelerate the onset of mutations and develop live attenuated vaccine strains, but SNPs are the major type of mutations and deletions, and insertions are limitedly introduced in the genome (37–39). However, radiation can cause spontaneous DNA mutations including deletions, insertions, and point mutations. In fact, we first introduced radiation mutation enhancement techniques (R-MET) to induce various mutations in cancer targeting Salmonella in our previous study (40). However, R-MET has not yet been applied to vaccine development.

In this study, we developed a hyper-attenuated, but immunologically active Salmonella vaccine strain ATOMSal-L6 by accelerating mutation using gamma irradiation. ATOMSal-L6 is at least 9,961-fold less virulent than its parent strain, but can enhance both humoral and cellular immune responses, and was found to confer protective immunity in both mice and porcine models. In addition, WGS analysis showed that ATOMSal-L6 introduced many SNPs and deletion/insertion mutations. This newly developed attenuated vaccine strain is a genetically stable vaccine strain that can potentially overcome the shortcomings of existing vaccines and can be easily and quickly developed into bacterial vaccines using radiation.

MATERIALS AND METHODS

Ethics Statement

This study was performed in strict accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. All animal experiments were approved by the Committee on the Use and Care of Animals at the Korea Atomic Energy Research Institute (KAERI; approval no. KAERI-IACUC-2020-004,
KAERI-IACUC-2021-003) according to accepted veterinary
standards set by the KAERI animal care center. Mice were
euthanized by CO₂ inhalation, as specified by the KAERI
Institutional Animal Care and Use Committee guidelines.

Bacterial Strains
S. Typhimurium ST454 (ST WT) was obtained from the Korea
Veterinary Culture Collection (Kimchun, Republic of Korea),
ATOMSal-L6 was derived from ST WT, and gene mutation was
induced by gamma-radiation. Their genome was sequenced
using the PacBio RS II platform (Pacific Biosciences, Menlo
Park, CA, USA) and Illumina Hiseq platform at Macrogen Co.,
Ltd. (Seoul, Republic of Korea). The assembled genome of ST
WT contained two contigs, one circular genome (4,823,318 bp)
and one plasmid (109,428 bp). After complete genome assembly,
the genome of ST WT was analyzed using the API ZYM (enzyme activities), API
20NE, and API 50CH (utilization of carbohydrate) kits
(bioMérieux, Inc.; Marcy L’Etoile, France) according to
manufacturer’s instructions. In brief, the cultured bacteria
were diluted using the provided medium until adequate turbidity was
attained. Diluted samples were added into the cupsules, and
incubated for 48 h at 37°C.

Mutation Rate Analysis
ST WT was grown in Luria-Bertani (LB; Difco, BD Biosciences,
Franklin Lakes, NJ, USA) broth at 37°C and 200 rpm under
aerobic conditions. The stain attained an optical density (OD₆₀₀)
of 0.5, exposed to 0.5–3.5 kGy for 1 h at room temperature using a
⁶⁰Co-gamma irradiator (point source AECL, IR-79, MDS
Nordion International Co., Ottawa, Canada) at the Advanced
Radiation Technology Institute of KAERI (Jeongeup, Republic of
Korea). The irradiated samples were concentrated and plated on
LB agar plates with 10 μg/mL of kanamycin to select for mutants
that acquired kanamycin resistance, as has been described
previously (41). The overall mutation rate of the population
was calculated using the mean number of mutants.

Macrophage Invasion and
Replication Assay
RAW 264.7 cells were purchased from the American Type
Culture Collection (ATCC, Manassas, VA, USA), and
were grown in high-glucose Dulbecco’s modified Eagle’s medium
(DMEM; Sigma-Aldrich; St. Louis, MO, USA) supplemented
with 10% fetal bovine serum (FBS; Biowest, Nuaille
France), and 1% antibiotics (100 U/mL penicillin and 100 μg/mL
streptomycin; Gibco; Waltham, MA, USA) at 37°C in the
presence of 5% CO₂. RAW 264.7 cell (3 x 10⁴ cells per well)
was seeded on 48-well plates (SPL Life Sciences, Pocheon,
Republic of Korea) and incubated for 16 h. Attenuated
Salmonella candidates were cultured in LB at OD₆₆₀ of 1.0
and harvested. The strains were treated to RAW 264.7 cells
with 3-fold diluted antibiotics.

Scanning Electron Microscope
Assay
ST WT and ATOMSal-L6 were fixed with 4% glutaraldehyde
solution at 4°C and kept overnight. After centrifuging, the fixed
samples were washed thrice with PBS and dehydrated using 30,
50, and 70% ethanol sequentially, following which the samples
were dried and coated with gold sputter. The plate was observed
using a JEOL-JSM-840 Scanning Microscope (Tokyo, Japan) at the
Seoul National University.

Motility Assay
Motility medium, which was composed of LB supplemented
with 0.4% agar (BD) and 1% triphenyltetrazolium chloride (TTC;
Sigma) was poured into the 14 mL round bottom tube (SPL).
Overnight cultured ST WT and ATOMSal-L6 were re-inoculated
into 3 mL LB medium at an OD₆₆₀ of 1.0. The cultured samples
were pierced deeply into the motility medium using the loop
(SPL). The tubes were incubated for 3 days at 37°C.
with 825.98-fold coverage. The total length of read bases was 4,088,887,030 bp, which covered 99.98% length of the ST WT strain. The raw reads from the ST WT genome were mapped and aligned to the reference genome sequence using Burrows-Wheeler aligner (BWA-0.7.12) and Picard. Next, the genetic variants were detected using SAMTools (ver. 1.2). All coding variants were identified based on the open reading frames of ST WT. The whole-genome sequences of ST WT (ST454) and ATOMSal-L6 has been deposited in DDBJ/EMBL/GenBank under the accession number CP098438-CP098439. The BioProject accession numbers are PRJNA844490, and PRJNA841760 and the BioSample accession number are SAMN28818465 and SAMN28614156, respectively.

Mouse and Pig Experiments
The animal housing conditions, which were designed for specific pathogen-free animals, and the animal experimental design were approved by the Committee on the Use and Care of Animals at the KAERI and implemented according to the ethical standards accepted by the National Health Institute. The ventilated housing cage (Orient Bio Inc., Seoul, Republic of Korea) was maintained in an animal biological safety level 2 facility at 22–23°C. Irradiated rodent diet food (5053; Orient Bio Inc.) and sterile bedding (Beta Chip; Orient Bio Inc.) in a static airflow environment. Bedding (Beta Chip; Orient Bio Inc.) at an approximate depth of 1.0 cm was changed weekly. Irradiated rodent diet food (5053; Orient Bio Inc.) and sterile water were provided ad libitum through a wire cage top. Flow cytometry

Splenocytes Analysis by Flow Cytometry
Two weeks after the final immunization, spleens from mice immunized with either the PBS or ATOMSal-L6 vaccine were isolated and filtered through a cell strainer (70 µm; SPL). Red blood cells (RBCs) were lysed with RBC lysis buffer (Sigma-Aldrich) and washed with RPMI-1640 medium containing 10% FBS. The cell suspension was seeded into a 48-well plate (2 × 10^6 cells/well) and stimulated with 10 µg/mL ST WT lysate, 0.5 µg/mL GolgiStop (BD Bioscience, San Diego, CA, USA), and 0.5 µg/mL GolgiPlug (BD Bioscience) at 37°C for 12 h. To analyse Help T cells, the cells were washed with PBS and stained with a Live/Dead Staining Kit (L/D; Invitrogen, San Diego, CA, USA), anti-CD8-FITC (BD Bioscience), and anti-CD4-BV421 (BD Biosciences) for 20 min at 37°C to stain T cell surface markers. Cells were fixed and permeabilized using a Cytofix/Cytoperm kit (BD Bioscience) for 20 min at 4°C, and the intracellular cytokines were stained with anti-IFN-γ-PE (BD Biosciences), anti-IL-5-APC (BD Biosciences), and anti-IL-17A-PE-Cy7 (BD Bioscience) for 20 min at 23°C. After staining, the cells were analyzed using a MACS Quant Flow cytometer (Miltenyi Biotec, San Diego, CA, USA) and FlowJo software (TreeStar, Ashland, OR, USA). For
further analysis of the multifunctional T cells, the staining was performed in the same method as was described above. Briefly, the T cells surface staining antibodies were used with 7-AAD (7-Aminoactinomycin D; Sigma), anti-CD3e-Alexa Fluor 488 (BD Biosciences), anti-CD4-BV421 (BD Biosciences), and anti-CD8-V500 (BD Biosciences) and intracellular cytokines staining antibodies were stained with anti-IFN-γ-PE (BD Biosciences), anti-TNF-α-APC (BD Bioscience), and anti-IL-2-PE-Cy7 (BD Bioscience).

Adoptive Transfer of Sera, CD4+ or CD8+ T Cells

Individual mouse sera, prepared as described above, were mixed and 100 µL of pooled sera were administered i.p. to naïve C57BL/6 mice (n = 5). Mouse spleen cells were prepared by passing spleen specimens through a cell strainer (70 µm; SPL), and red blood cells were lysed with RBC lysis buffer (Sigma-Aldrich). Splenic CD4+ and CD8+ T cells were separated using Miltenyi MACS microbeads conjugated with anti-CD4 and anti-CD8 monoclonal antibodies (Miltenyi Biotec) and a MACS LS column (Miltenyi Biotec). Isolated CD4+ or CD8+ T cells (5 × 10^6 cells or 5 × 10^5 cells/mouse) were administered i.p. to naïve C57BL/6 mice (n = 5). After 12 h, mice were challenged i.p. with ST WT (5 × 10^5 CFU/mouse) and mouse survival was monitored for 14 days.

Statistical Analysis

Data are expressed as the mean ± standard deviation (SD). Data in the bar and dot graphs between groups were compared using an unpaired Student’s t-test for normal data distribution or the Mann–Whitney non-parametric test for abnormal data distribution using GraphPad Prism (version 7.0; GraphPad Software, Inc., La Jolla, CA, USA). The survival of mice was determined using Kaplan–Meier survival analysis, and the significance of the difference was analyzed using a log-rank test with GraphPad Prism software. P < 0.05 was considered statistically significant.

RESULTS

Construction of the Attenuated Salmonella Strain (ATOMSal-L6) Using R-MET

Radiation mutatin rate was calculated as the rate of generation of antibiotic resistant before and after irradiation (43). To optimize the R-MET condition, ST WT (10^9 - 10^10 CFU, A_600 = 1.0) was irradiated with the indicated dose of gamma ray and then plated on LB agar with or without kanamycin. As shown in Figure 1A, the number of ST WT on the LB agar plate gradually decreased after irradiation, and no colonies were detected above a radiation dose of 2.5 kGy. In contrast, kanamycin-resistant mutations were not detected before irradiation, but were predominantly present at doses between 0.5–1.5 kGy. We compared the ratio of survived viable and mutated bacteria and selected 1.5 kGy as the optimal radiation dose, because it gave rise to 0.88 ± 0.18 mutants/10^10 CFU. A schematic procedure for the development of an attenuated Salmonella vaccine strain is presented in Figure 1B. To construct an attenuated vaccine strain, the ST WT strain was exposed to 1.5 kGy γ-irradiation for 1 h followed by plating on LB agar. After incubation for at least 2 days at 28°C, unusual shaped colonies were picked and inoculated into LB broth. This process was repeated 3 or more times to enrich the mutated strains. Finally, 30 colonies were selected as the mutant candidates of ST WT.

The ability of Salmonella to invade and replicate in the intracellular vacuoles is crucial for the initial stage of an invasive disease (44). Therefore, we examined the attenuation of mutant candidates by performing cell invasion and replication assays and compared them to the ST WT. RAW264.7 monolayers were infected with each mutant strain (ST WT-IR #) and invasion (2h) and replication (18h) rates were compared to the ST WT strain (Figure 1C). Most of the selected mutants showed at least 50% lower levels of invasion and replication capacity than the parent strain (ST WT). We selected five mutants. The mutants #8 and #16 had lower levels of invasiveness (<1%), but higher levels of replication (>25%). Mutants #17 and #18 had high levels of invasiveness (>40%), but low levels of replication (<25%). Mutant #29 was chosen as the control mutant.

To compare virulence, mice (BALB/c; n=5/group) were orally inoculated with the candidates (ST WT-IR #8, #16, #17, #18, #29) and their colonization in cecum and invasion into the spleen and mesenteric lymph node (mLN) were counted 1 day post infection (d.p.i.). Compared to the ST WT, most of the mutants, except #18, had similar levels of colonization in the caecum, spleen, and mLN. Mutant #18 did not show significant change in the level of colonization in the cecum, but showed a significant reduction in organ invasiveness compared to the ST WT (Figure 1D). No bacteria were detected in the blood, liver, and lungs (data not shown). Thus, #18 was possibly the most attenuated mutant among the selected candidates. To analyze the lethal dose 50 (LD50), mice (n=3/group) were injected with an increasing dose of #18 or ST WT i.p. LD50 was calculated using “Quest Graph LD50 calculator”, ST WT was 2.71 × 10^6 CFU/mouse, while #18 was approximately 2.69 × 10^8 CFU/mouse, making #18 about 9,961 times less virulent than its parent strain (ST WT); therefore, #18 was designated ATOMSal-L6 in this study.

Genetic and Biochemical Characterization of ATOMSal-L6

To confirm the phenotypical stabilization of ATOMSal-L6 strain, it was sequentially cultured 10 times in LB broth and re-examined for virulence. When growth rates were compared with ST WT, ATOMSal-L6 showed a similar growth pattern to ST WT at 37°C and 42°C, but no growth at 45°C (Figure 2A). Next, we examined its biochemical characteristics using Analytical Profile Index (API) analysis (Tables S1–3). The biochemical profiling of Gram-negative identification (API 20NE) showed no differences; however, esterase (C4) and several carbohydrates utilization profiled (API ZYM, 50CH) were slightly different compared to ST WT. For example,
ATOMSal-L6 fully utilized esterase and carbon sources (L-arabinose and D-mannose) and showed weak signal at D-ribose, L-rhamnose, and melibiose, but ST WT did not. We tested the antibiotic susceptibility of ST WT and ATOMSal-L6 with MIC (Table S4). The MIC of ATOMSal-L6 against KAN, CFR, TMP, and TOB were same with ST WT. The MIC of ATOMSal-L6 against TET, ERM, GEN, AMC, AMK, and STR were 3-fold lower than ST WT. The MIC of ATOMSal-L6 against AMP, SPT, LIN, and CLI were more than 9-fold lower than ST WT. These data indicated that ATOMSal-L6 likely loses its ability to resist antibiotic stress during R-MET process.

To directly visualize the extracellular structure of ATOMSal-L6, Scanning Electron Microscopy (SEM) was performed (Figure 2B). Compared with ST WT, ATOMSal-L6 showed no significant difference in size and shape; however, a higher level of flagellin was expressed (Figure 2B). To examine whether higher expression of flagellin affected the motility of ATOMSal-L6, we performed swarming assay (Surprisingly, even though ATOMSal-L6 expressed higher flagellin than ST WT, its motility on semi-solid swarming agar media was generally lower than that of ST WT (Figure 2C).

The virulence attenuation of ATOMSal-L6 was re-examined in vitro and in vivo. ATOMSal-L6 or ST WT was added onto RAW 264.7 monolayers at MOIs of approximately 1, 10, or 100 and their invasion and replication abilities were compared as above (Figure 2D). As expected, ATOMSal-L6 showed dramatically reduced invasiveness and replication capacity compared to ST WT. When mice (n=5/group) were injected i.p. with ST WT or ATOMSal-L6, all mice infected with ST WT (10^5 CFU/mice) died within 5 days post-infection and only 20% of mice were survived by infecting with extremely high number of ATOMSal-L6 (10^8 CFU/mouse), whereas all mice infected with 1,000-fold higher numbers of ATOMSal-L6 (10^8 CFU/mouse) exhibited 100% survival for more than 14 days (Figure 2E).

To analyze the location of mutations in ATOMSal-L6, the complete genome of ATOMSal-L6 was sequenced and compared to ST WT as a reference genome. As shown in Figure S1A, we
found 137 mutations in ATOMSal-L6 genome, including 6.56% (n=9) of point mutation (transition and transversion), 90.51% (n=124) of insertion, and 2.92% (n=4) of deletion. Mutation sites were designated to the circular form of ATOMSal-L6 genome (Figure S1B and Table S5). Surprisingly, only 9 mutations were occurred in A or T nucleotides and the others (n=126) were all mutated in G and T. Of note, we found that ATOMSal-L6 lost one bacteriophage located at 3,440,538 bp - 3,481,579 bp encoded by IS1595 transposase phage genes (gene bank number = CP098438-CP098439). All these data suggested that R-MET introduced many mutations and that these mutations could attenuate its virulence in vitro and in vivo.

High Immune Response by Immunizing I.M. With ATOMSal-L6 Vaccine in Mice

To determine whether the ATOMSal-L6 could be used as a live attenuated vaccine, the vaccine efficacy of ATOMSal-L6 was examined using a mouse model. Mice (n=5/group) were immunized intramuscularly i.m. with 10⁵, 10⁶, or 10⁷ CFU of ATOMSal-L6 and Salmonella-specific humoral, cellular, and protective immune response were measured. At 2 weeks after the last immunization, Salmonella-specific IgM and IgG were measured with ELISA. As shown in Figure 3A, Salmonella-specific IgM was significantly increased in all groups, whereas Salmonella-specific IgG was significantly increased only in the group immunized with 10⁷ CFU compared to unvaccinated (NT) group. Furthermore, we found that Th2 response (IgG1) was the dominant immune response over Th1 (IgG2a) in the group immunized with 10⁷ CFU (Figure 3B).

Next, since both CD4⁺ and CD8⁺ T cells are crucial for protection against Salmonella infection (45, 46), we evaluated T cell subtypes induced by ATOMSal-L6 vaccination. Mice (n=5/group) were immunized i.m. thrice at 2-week intervals, and single cell splenocytes were re-stimulated with 10 µg of ST lysate, followed by analyzing Th1 (IFN-γ-producing CD4⁺ T cells), Th2 (IL-5-producing CD4⁺ T cells), Th17 (IL-17A-producing CD4⁺ T cells), and activated CD8⁺ T cells (IFN-γ-producing CD8⁺ T cells) using flow cytometry gating, as shown in Figure S2. The population of Th2 and Th17 cells was not changed after immunization (data not shown), but significant...
enhancement of Th1 (IFN-γ CD4+ T cells) and CD8+ T cells (IFN-γ CD8+ T cells) were detected when immunized with 10^6 or 10^7 CFU ATOMSal-L6 vaccination compared to the NT group (Figure 3C).

To investigate whether humoral and cellular immunity induced by ATOMSal-L6 vaccination could provide a protective immune response, ATOMSal-L6 (10^6 CFU) vaccinated mice (n=5/group) were infected i.p. with ST WT (5 × 10^5 CFU/mouse) and their survival monitored for 14 days. As shown in Figure 4A, all unvaccinated mice died at 7 d.p.i, whereas all vaccinated mice survived more than 14 days. In addition, ST WT that invaded the spleen or liver were counted at 1 d.p.i (Figure 4B). More than 10^6 CFU/g of invasive bacteria were detected in the spleen and liver from unvaccinated mice, whereas significantly lower number of ST WT were detected in ones from ATOMSal-L6 vaccinated mice.

To test whether the protective immune response was due to humoral or cellular immune responses, sera (100 μL/mouse), CD4+ T cells (5 × 10^6 cells/mouse), or CD8+ T cells (5 × 10^6 cells/mouse) were collected from ATOMSal-L6 vaccinated, or unvaccinated mice followed by adoptive transfer to naïve mice (n=5/group). After infecting i.p. with ST WT (5 × 10^5 CFU), all mice transferred with sera or T cells from unvaccinated mice had died at 6–7 d.p.i, whereas all mice transferred with sera from ATOMSal-L6 vaccinated mice survived for more than 14 d.p.i (Figure 4C). Although only 40% of the mice that were provided with CD8+ T cells from ATOMSal-L6 vaccinated mice survived, it was not significant, but still marginally higher (p=0.1338) than that of mice transferred with CD8+ T cells from unvaccinated mice (Figure 4D). We did not observe a significant difference between CD4+ T cells adopted transferred from different groups (data not shown). All these data suggested that ATOMSal-L6 provided an effective immune response to protect from Salmonella infection by activating both humoral and cellular immune responses.

High Immune Response by Immunizing Orally With ATOMSal-L6 Vaccine in Mice

Because oral vaccination of live attenuated Salmonella vaccine is recommended for adult pigs and humans (47, 48), we next investigated whether ATOMSal-L6 could be used as an oral vaccine. To examine the virulence of ATOMSal-L6 via oral vaccination, mice were immunized orally with ST WT or ATOMSal-L6. No mice died even after oral administration of 10^7 CFU of ST WT or ATOMSal-L6 (data not shown). When intestinal inflammation after ST WT infection, we observed substantial infiltration of immune cells in both the small and large intestine in the ST WT-immunized group (Figure 5A). No significant inflammation or damage were observed in the intestinal tissues of ATOMSal-L6 immunized mice, which

![Figure 3](https://example.com/figure3.png)

**Figure 3** | Humoral and cellular immune responses induced by i.m. immunization of ATOMSal-L6. C57BL/6 mice (n = 5 per group) were immunized i.m. with 10^5, 10^6, or 10^7 CFU of ATOMSal-L6 thrice at two-week intervals. (A, B) Humoral immune response. Serum levels of Salmonella-specific IgG and IgM were analyzed at 7 days following the last immunization (A). Subclass levels of Salmonella-specific IgG1 and IgG2a were analyzed at 7 days subsequent to the last immunization (B). Data are representative of three independent experiments and are presented as the mean ± standard deviation. (C) Cellular immune response. Single cell suspensions of spleen were re-stimulated with 10 μg/mL ST WT lysate for 12 h and ST-specific CD4+ and CD8+ T cells were analyzed. Percentages of activated CD4+ and CD8+ T cells in spleens of vaccinated mice. Data were presented as mean ± standard deviation. *P < 0.05, **P < 0.01, ***P < 0.001, compared to unvaccinated mice.
showed similar results to those observed in the NT group (Figure 5A). To evaluate whether oral immunization of ATOMSal-L6 elicited Salmonella-specific immune response, mice (n=5/group) were immunized orally thrice with 10^6, 10^7, or 10^8 CFU of ATOMSal-L6, and the humoral and cellular immune responses were evaluated. Oral ATOMSal-L6 vaccination resulted in an increase in serum Salmonella-specific IgG, and a slight increase in Salmonella-specific IgM (Figure 5B).

We next analyzed the functional composition of Salmonella-specific single- or multi-functional cellular immune responses (49). Mice (n=5/group) were immunized orally thrice at two-weeks interval, following which they were analyzed for Salmonella-specific splenic CD4^+ T cells and CD8^+ T cells using cytometric gating, as shown in Figure S3. Only the 10^8 CFU ATOMSal-L6 were found to have significantly increased frequencies of IFN-γ CD4^+ (compared to NT group; up to 7.6-fold, p<0.001) and TNF-α CD4^+ (compared to NT group; up to 2.05-fold, p=0.005), but no changes were found from IL-2^+ CD4^+ T cells. In addition, we found that multifunctional IFN-γ^+ TNF-α^+ CD4^+ (compared to NT group; up to 18.15-fold, p=0.007) and IFN-γ^+ IL-2^+ CD4^+ (compared to NT group; up to 6.06-fold, p=0.004) were significantly increased upon oral vaccination. Similarly, single- and multi-functional CD8^+ T cells were significantly increased in the 10^8 CFU ATOMSal-L6 (Figure 5C). All these data indicated that oral immunization of live ATOMSal-L6 could induce Salmonella-specific humoral and cellular immunities.

To evaluate the protective immunity of ATOMSal-L6 oral vaccination, mice (n=5/group) were immunized orally thrice with ATOMSal-L6 (10^8 CFU) at two-weeks interval, followed by injecting i.p. ST WT (ST454; 5 × 10^5 CFU). As shown in Figure 5D, all unvaccinated mice died at 14 d.p.i, but 60% of the vaccinated mice survived for more than 14 d.p.i. To examine whether the protective immune response was due to humoral or cellular immune responses, sera (100 µL/mouse), CD4^+ T cells (5 × 10^6 cells/mouse), or CD8^+ T cells (5 × 10^5 cells/mouse) were collected from ATOMSal-L6 vaccinated or unvaccinated mice, followed by adoptive transfer to naïve mice (n=5/group). After infecting with ST WT (5 × 10^5 CFU), all mice transferred with sera or T cells from unvaccinated mice died at 6 - 7 d.p.i whereas 80%, 40%, and 20% of the mice transferred with sera (Figure 5E), CD4^+ T cells, and CD8^+ T cells (Figure 5F), respectively. All these data suggested that oral live ATOMSal-L6 vaccine provided effective immune response to protect from Salmonella infection by activating both humoral and cellular immune responses.

High Protective Immune Response by Immunizing I.M. With ATOMSal-L6 Vaccine in Pig

To examine the efficacy of ATOMSal-L6 vaccine (2 × 10^9 CFU/pig) in pig model, pregnant sows were immunized orally with live ATOMSal-L6 twice at three-week intervals. Sera were collected at 3, 6, and 8 weeks, and Salmonella-specific IgG antibodies were measured using ELISA. As shown in Figure 6A, the vaccinated group showed the increase IgG levels compared to the unvaccinated group. We also collected colostrum on the day of delivery and observed that Salmonella-specific IgG and IgA levels were enhanced in the vaccinated group (Figure 6B).
To measure the protective response of ATOMSal-L6 vaccine, vaccinated sows were orally infected with ST WT (5 × 10<sup>8</sup> CFU/pig) and their diarrheic symptoms were monitored for 14 days. All the vaccinated sows (n=10) were free of diarrheic symptoms, whereas all the unvaccinated sows had severe diarrhea (Figure 6C). To determine whether *Salmonella*-specific protective antibodies were delivered from the gilt to the piglet, piglets (n=10) born from vaccinated gilts were infected orally
with ST WT (5 × 10⁸ CFU/pig) and their survival rates were monitored. As shown in Figure 6D, all piglets from the unvaccinated gilts had severe diarrhea and died at 7 d.p.i, but only 30% of piglets born from vaccinated gilts showed lethal and severe diarrhea at 14 d.p.i. We monitored the surviving piglets for more than 21 d.p.i and found no severe diarrheic symptoms. All these data indicated that ATOMSal-L6 could be a safe and effective live attenuated vaccine in pig.

DISCUSSION

The Salmonella vaccine program in poultry has been successfully implemented to control the prevalence of human Salmonellosis in the UK (50, 51), and mass vaccine administration to economically important animals is considered the best strategy to prevent transmission of Salmonella to animals and humans. However, due to the emergence of new serotypes and multi-drug resistant Salmonella worldwide (12, 52–54), more effective and broad-spectrum Salmonella vaccines are being developed. Unlike conventional inactivated vaccines, live attenuated vaccines could induce life-long immunity through one or two doses by activating multifunctional cellular immune responses (22, 55, 56). Nevertheless, this type of vaccine has not been widely used against bacteria because it could cause diseases in immunocompromised individuals and the vaccine could potentially re-acquire its pathogenicity by reverting the mutation (57, 58). In addition, rapidly developing the vaccines against newly emerging serotypes or new pathogens has proven difficult. In this study, we introduced a technology to rapidly develop a live attenuated Salmonella vaccine, ATOMSal-L6, using R-MET that can be attenuated by accelerating mutation. In addition, because R-MET technology can introduce various forms of mutations (deletion, insertion, SNP), it will be possible to solve the problem of current live vaccines that re-acquired pathogenicity by genetic revertant (40, 59, 60). Due to these mutations, ATOMSal-L6 differed in biochemical properties from its parent strain. For example, it produces more flagellin but less mobility, and cannot be grown at high temperatures (45°C). And it was confirmed that resistance to specific antibiotic resistance was reduced compared to ST WT. The resistance to the aminoglycoside antibiotics did not change significantly, but the resistance to the macrolides antibiotics was reduced more than 3 times compared to the ST WT. This change in antibiotic resistance will be a good standard for separating and analyzing wild-type and vaccine strain in the clinical samples. All these genetic and biochemical changes might have contributed to the attenuation and immunological properties of ATOMSal-L6.

To the best of our knowledge, compared to UV radiation and chemical mutagens, γ-radiation has not been widely used to induce mutations in vaccine industries because it requires a high-dose radiation facility, and all mutations must be detected and selected painstakingly at the phenotypic level. However, in recent years, new and re-emerging infectious diseases have become prevalent. Using R-MET, which can rapidly and effectively develop vaccines, might be more attractive (40). In addition, recent advances in large-scale genomic analysis techniques have enabled easy analysis of the effects of radiation and the location of mutations in the bacterial genome. In this study, we screened only 30 colonies after irradiation using R-MET and found several attenuated candidates with significantly reduced screening times compared to UV or chemical mutagens. Overall, it took about 4 weeks to develop ATOMSal-L6, as the colony selection process took about 2–3 days and the in vitro and in vivo virulence examination took about 2–3 weeks. However,
the whole genome sequence with comparative genomics and genetic stabilization tests are time-consuming, often requiring several months to complete. Therefore, a systematic process to speed up these genomic analysis processes must be developed.

Live attenuated Salmonella vaccines must balance attenuation with immunogenicity. In particular, both CD4+ and CD8+ T cells are highly associated with protection against early infection of Salmonella (61). CD4+ T cells might play a central role in acquired immunity against Salmonella infection and make an additional important contribution to both CD8+ T cell- and B cell-immunities. Therefore, live attenuated Salmonella vaccines are preferred over inactivated vaccines that do not have high T-cell immunity. Since ATOMSal-L6 induced protection against ST WT infection by activation of CD4+ and CD8+ T cells in mice, it is a good vaccine candidate with the balance between high immunogenicity to enable cellular and humoral immune response and sufficiently high attenuation of its virulence. Our previous study showed that inactivated S. Gallinarum activated moderate CD4+ and CD8+ T cell response, but higher Th17 responses (62). It is known that IL-17, increased by Salmonella infection, stimulates intestinal epithelial cells to enhance the production of antimicrobial proteins and chemokines, thereby inhibiting the early invasion of Salmonella bacteria (63, 64). In contrast, ATOMSal-L6 shows no induction of Salmonella-specific Th17 response (data not shown) but does high expression of Salmonella-specific CD4+ and CD8+ T cells. We therefore speculate that ATOMSal-L6 may have mutated genes involved in the expression of IL-17-induced antigens during the R-MET process. Therefore, immunization with inactivated Salmonella vaccine together or sequentially is another option to increase ATOMSal-L6 efficacy.

ATOMSal-L6 is the first attenuated Salmonella vaccine strain developed using R-MET. It is more sensitive to high temperature and showed lower motility compared to its parent strain. In addition, we found 8 SNPs, 3 deletions, 60 insertions, and loss of one bacteriophage upon comparing its genome with its parent strain. Compared to licensed Salmonella vaccine strains, its genomic mutations are wide and variable. Although there is no parent strain for comparison with Salmonella enterica Serovar Choleraesuis vaccine strain C500 attenuated by chemical mutation, when compared to another WT SC-B67 strain, it was deficient in the rpoS gene, a vital transcriptional regulator playing an important role in Salmonella infection (65). Salmovac440 developed by IDT Biologika has only 6 SNPs, but lacks the pathogenic plasmid that encodes a number of virulence factors (26, 65, 66). Therefore, the attenuation of Salmovac440 may be due to the amino acid biosynthetic system and other virulence mechanisms involving the lost pathogenic plasmid. In this study, we did not investigate on the degree to which each of these mutations in ATOMSal-L6 affected the virulence attenuation. Thus, to use it as a vaccine strain, accurate biochemical information of ATOMSal-L6 must be acquired, and in particular, additional research on the relationship with the mutation and the virulence must be performed.

In the absence of overt disease, the vaccine strain attenuated in metabolic gene(s) must be metabolically active to reach immune inductive sites and elicit a biologically relevant protective immunity. However, the hyper-attenuation of vaccine strains may result in lower virulence and less effective protective immune responses. Thus, it is necessary to develop a strain that can moderately reduce virulence and induce immunity at a level that does not cause disease. For example, WT05 is the attenuated S. Typhimurium vaccine in which the aroC gene, involved in aromatic amino acid biosynthesis, and the ssaV gene, a component of a Type 3 secretion system (T3SS) apparatus of Salmonella pathogenicity island 2 (SPI-2), are deleted. However, this vaccine strain was eliminated through prolonged defecation in healthy volunteers immunized with WT05, thereby failing the phase 1 clinical trial (67, 68).

Another advantage of R-MET is that it can be applied to strains that are not sufficiently attenuated to further reduce pathogenicity, allowing it to be used as a vaccine strain. Therefore, further attenuation with R-MET can be attempted in the event of clinically significant safety issues such as those resulting from the use of WT05 and LH1160. In addition, if ATOMSal-L6 has not been sufficiently attenuated, R-MET may be additionally applied. Thus, R-MET will be a very effective and attractive method for live bacterial vaccine development.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: NCBI SRA Biproject accession no for HJJ22 (ST WT): PRJNA844490; accession no for ATOMSal-L6: PRJNA841760.

ETHICS STATEMENT

The animal study was reviewed and approved by Korea Atomic Energy Research Institute.

AUTHOR CONTRIBUTIONS

HJJ, AYJ, KBA, SHH, HKJ, JH, and HSS were responsible for conceptualization of the study. HJJ, AYJ, SJB, and JH performed the experiments and analyzed the data. HJJ, KBA, SHH, JYS, and HSS wrote the manuscript. HSS supervised the work. HSS was
responsible for funding acquisition. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported in part by the Internal R&D program of KAERI (523210) funded by Ministry of Science and ICT (MIST) and the National Research Foundation of Korea grants 2017M2A2A6A02020925, NRF-2018K2A206023828, and NRF-2020M2A206023828 to HS and NRF-2019M2D3A2060217 to KA.

ACKNOWLEDGMENTS

We would like to thank Lima Seo (Kwangju Foreign School, South Korea) for drawing Figure 1B.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.931052/full#supplementary-material

REFERENCES

1. Albert MJ, Bulach D, Alifouzan W, Izumiya H, Carter G, Alobaid K, et al. Multi-Drug Resistant Salmonella Newport Outbreak of the Centers for Disease Control and Prevention. Outbreak of Multi-Drug Resistant Salmonella Newport–United States, January–April 2002. JAMA (2002) 288(8):951–3. doi: 10.1001/jama.288.8.951-JW0828-2-1

2. Haselbeck AH, Panzner U, Im J, Baker S, Meyer CG, Marks F. Current Perspectives on Invasive Nontyphoidal Salmonella Disease. Curr Opin Infect Dis (2017) 30(5):498–503. doi: 10.1097/QCO.0000000000000398

3. Feasey NA, Dougan G, Kingsley RA, Heyderman RS, Gordon MA. Invasive non-Typhoidal Salmonella Disease: An Emerging and Neglected Tropical Disease in Africa. Lancet (2012) 379(9835):2489–99. doi: 10.1016/S0140-6736 (11)61752-2

4. Wiedemann A, Virologeux-Payant I, Chausse AM, Schikora A, Velge P. Interactions of Salmonella With Animals and Plants. Front Microbiol (2014)5:791. doi: 10.3389/fmicb.2014.00791

5. Majowicz SE, Musto J, Scallan E, Angola FJ, Kirk M, O’Brien SJ, et al. International Collaboration on Enteric Disease ‘Burden of Illness: The Global Burden of Nontyphoidal Salmonella Gastroenteritis. Clin Infect Dis (2010) 50 (6):882–9. doi: 10.1086/650733

6. Marchello CS, Birkhold M, Crump JAC. C. Vacci-NITS. Complications and Mortality of non-Typhoidal Salmonella Infection: A Global Systematic Review and Meta-Analysis. Lancet Infect Dis (2022) 22(5):692–705. doi: 10.1016/S1473-3099(21)00615-0

7. Reddy EA, Shaw AV, Crump JA. Community-Acquired Bloodstream Infections in Africa: A Systematic Review and Meta-Analysis. Lancet Infect Dis (2010) 10(6):417–32. doi: 10.1016/S1473-3099(10)70072-4

8. Uche IV, MacLennan CA, Saul A. A Systematic Review of the Incidence, Risk Factors and Case Fatality Rates of Invasive Nontyphoidal Salmonella Infections in Sub-Saharan Africa: A Systematic Review. Frontiers in Immunology | www.frontiersin.org 13 July 2022 | Volume 13 | Article 931052

9. Doshi PR, Langley JM, Rajasekaran S, Doshi PM. Salmonella Invasive Disease: A Systematic Analysis for the Global Burden of Disease Study 2017. Lancet Infect Dis (2019) 19(12):1312–24. doi: 10.1016/S1473-3099(19)30418-9

10. Majowicz SE, Musto J, Scallan E, Angola FJ, Kirk M, O’Brien SJ, et al. International Collaboration on Enteric Disease ‘Burden of Illness: The Global Burden of Nontyphoidal Salmonella Gastroenteritis. Clin Infect Dis (2010) 50 (6):882–9. doi: 10.1086/650733

11. Tack B, Vanaenrode J, Verbakel JY, Toelen J, Jacobs J. Invasive non-Typhoidal Salmonella Disease: Current Status of Vaccine Research and Development. Vaccine (2016) 34(26):2907–10. doi: 10.1016/j.vaccine.2016.03.072

12. Strugnell RA, Scott TA, Wang N, Yang C, Peres N, Bedoui S, et al. Salmonella Vaccines: Lessons From the Mouse Model or Bad Teaching? Curr Opin Microbiol (2014) 17:99–105. doi: 10.1016/j.mib.2013.12.004

13. Westens K, Rip D, Gouws P. Salmonella in Chicken Meat: Consumption, Outbreaks, Characteristics, Current Control Methods and the Potential of Bacteriophage Use. Foods (2021) 10(8):1742. doi: 10.3978/j.10018018742

14. Baliban SM, Lu YJ, Malley R. Overview of the Nontyphoidal and Paratyphoidal Salmonella Vaccine Pipeline: Current Status and Future Prospects. Clin Infect Dis (2020) 71(Suppl 2):S151–4. doi: 10.1093/cid/caa314

15. Walters AD, Davies RE. Salmonella Vaccination in Pigs: A Review. Zoonoses Public Health (2017) 64(1):1–13. doi: 10.1111/zph.12256

16. Desin TS, Koster W, Potter AA. Salmonella Vaccines in Poultry: Past, Present and Future. Expert Rev Vaccines (2013) 12(1):87–96. doi: 10.1586/erv.12.138

17. O’Brien SJ. The “Decline and Fall” of Nontyphoidal Salmonella in the United Kingdom. Clin Infect Dis (2013) 56(5):705–10. doi: 10.1093/cid/cis967

18. Tennant SM, Levine MM. Live Attenuated Vaccines for Invasive Salmonella Infections. Vaccine (2015) 33 Suppl 3:C36–41. doi: 10.1016/j.vaccine.2015.04.029

19. Alderton MR, Fahey KJ, Coloe PJ. Humoral Responses and Salmonellosis Protection in Chickens Given a Vitamin-Dependent Salmonella Typhimurium Mutant. Avian Dis (1991) 35(3):435–42. doi: 10.2307/1591205

20. Springer S, Theuss T, Toth I, Szogenyi Z. Invasion Inhibition Effects and Immunogenicity After Vaccination of SPF Chicks With a Salmonella Enteritidis Live Vaccine. Tierarztl Prax Ausg G Grosstiere Nutztiere (2019) 49(4):249–55. doi: 10.1055/a-1520-1369

21. Schmidt S, Kreutzmann H, Stadler M, Mair KH, Stas MR, Koch M, et al. T-Cell Cytokine Response in Salmonella Typhimurium-Vaccinated Versus Infected Pigs. Vaccines (Basel) (2021) 9(8):845. doi: 10.3978/j.1999-80845

22. Tang Y, Davies R, Petrovska L. Identification of Genetic Features for Attenuation of Two Salmonella Enteritidis Vaccine Strains and Differentiation of These From Wildtype Isolates Using Whole Genome Sequencing. Front Vet Sci (2019) 6:447. doi: 10.3389/fvets.2019.00447

23. Ault A, Tennant SM, Gorres JP, Eckhaus M, Sandler NG, Roque A, et al. Safety and Tolerability of a Live Oral Salmonella Typhimurium Vaccine Candidate in SIV-Infected Nonhuman Primates. Vaccine (2013) 31(49):5879–88. doi: 10.1016/j.vaccine.2013.09.041

24. de Serres FJ, Webber BB. Quantitative and Qualitative Comparisons of Spontaneous and Radiation-Induced Specific-Locus Mutation in the Ad-3 Region of Heterokaryon 12 of Neurospora Crassa. Mutat Res (1997) 375 (1):37–52. doi: 10.1016/S0027-5107(96)00253-9

25. Santhanarayanan K. Ionizing Radiation and Genetic Risks IX. Estimates of the Frequencies of Mendelian Diseases and Spontaneous Mutation Rates in Human Populations: A 1998 Perspective. Mutat Res (1998) 411(2):129–78. doi: 10.1016/S0168-7777(00)80012-X

26. Foster PL, Lee H, Popodi E, Townes JP, Tang H. Determinants of Spontaneous Mutation in the Bacterium Escherichia Coli as Revealed by Whole-Genome Sequencing. Proc Natl Acad Sci USA (2015) 112(44):E5990–9. doi: 10.1073/pnas.1512361132
Mutations by Immunization of Healthy Volunteers. *Infect Immun* (2002) 70 (7):3457–67. doi: 10.1128/IAI.70.7.3457-3467.2002

69. MacLennan CA, Martin LB, Micoli F. Vaccines Against Invasive Salmonella Disease: Current Status and Future Directions. *Hum Vaccin Immunother* (2014) 10(6):1478–93. doi: 10.4161/hv.29054

70. Sears KT, Galen JE, Tennant SM. Advances in the Development of Salmonella-Based Vaccine Strategies for Protection Against Salmonellosis in Humans. *J Appl Microbiol* (2021) 131(6):2640–58. doi: 10.1111/jam.15055

71. Tennant SM, Schmidlein P, Simon R, Pasetti MF, Galen JE, Levine MM. Refined Live Attenuated Salmonella Enterica Serovar Typhimurium and Enteritidis Vaccines Mediate Homologous and Heterologous Serogroup Protection in Mice. *Infect Immun* (2015) 83(12):4504–12. doi: 10.1128/IAI.00924-15

**Conflict of Interest:** Authors SJ B and HKJ are employed by CTCVAC.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

**Publisher’s Note:** All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Copyright © 2022 Ji, Jang, Song, Ahn, Han, Bang, Jung, Hur and Seo. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.