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Immunization with *Salmonella* Enteritidis secreting mucosal adjuvant labile toxin confers protection against wild type challenge via augmentation of $CD3^+CD4^+$ T-cell proliferation and enhancement of IFN-γ, IL-6 and IL-10 expressions in chicken

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ABSTRACT

The protective efficacy and immunological profiles of chickens immunized with an attenuated *Salmonella* Enteritidis (SE) constitutively secreting double mutant heat labile enterotoxin (dmLT) were investigated. The dmLT is a detoxified variant of *Escherichia coli* heat labile toxin and is a potent mucosal adjuvant capable of inducing both humoral and cell-mediated immunity. In this study, four-week-old chickens were inoculated with SE-dmLT strain JOL1641, parental SE strain JOL1087 or phosphate buffered saline control. Peripheral blood mononuclear cells of SE-dmLT inoculated birds showed significant proliferation upon stimulation with SE antigens as compared to the control and JOL1087 groups ($P \leqslant 0.05$). One week post-challenge, the ratio of $CD3^+CD4^+$ to $CD3^+CD8^+$ T-cells showed a significant increase in the immunized groups. Significant increases in IFN-γ levels were observed in JOL1641 birds immunized via oral and intramuscular routes. While immunizations with the JOL1087 strain via the intramuscular route also induced significant increases in IFN-γ, immunization via the oral route did not trigger significant changes. Pro-inflammatory cytokine IL-6 was also elevated significantly in immunized birds; a significant elevation of IL-10 was observed only in oral immunization with JOL1641 ($P \leqslant 0.05$). JOL1641 immunized birds showed significant reduction of challenge bacterial-organ recovery as compared to JOL1087 and non-immunized birds. Collectively, our results revealed that immunization with the adjuvant-secreting *S.* Enteritidis confers protection against wild type SE challenge via induction of strong cell proliferative response, augmentation of $CD3^+CD4^+$: $CD3^+CD8^+$ T-cells ratio and enhancement of IFN-γ, IL-6 and IL-10 cytokine secretion.

1. Introduction

Salmonella enterica serovar Enteritidis is one of the most frequently isolated bacteria from human infections worldwide [1]. Livestock products are the most common transmission vehicles of *Salmonella* infections [2]. Poultry products and by products such as meat, eggs, feather etc. plays an important role in *Salmonella* transmissions. Enforcing control measures to curb the organism remains expensive option due to the highly ubiquitous presence and rapid spread of *Salmonellae* in poultry premises. Control strategies, such as culling, antibiotic interventions, and *Salmonella*-free feed approaches, are being deployed with varying success to control the *Salmonella* transmission cycle [3]. With the case *Salmonella* Enteritidis (SE), poultry vaccination is the suggested ideal strategy for controlling infections on poultry farms and thereby reducing food contamination [4,5].

Vaccines capable of inducing mucosal immunity are pragmatic, as most infections are initiated from the mucosal surfaces of the respiratory, gastrointestinal, and genital tracts. There is an enormous challenge in developing vaccines that can either prevent the infectious agent from colonizing, penetrating and replicating in the mucosa, and/or that can block microbial toxins from binding to and affecting epithelial and other target cells. The effectiveness of mucosal vaccines and adjuvants in eliciting mucosal immune responses against infectious diseases is primarily studied by determining the secretory antibody production in the target host [6]. However, information related to the cellular components and cytokine profiles of the host may not be overlooked, considering the importance of cell-mediated immunity in controlling invading pathogens, particularly with intracellular *Salmonella*. It is necessary

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to have a holistic approach while investigating immunological processes pertaining to mucosal adjuvants. The heat-labile enterotoxin (LT) of *Escherichia coli* is a potent adjuvant, which boosts both humoral and cellular immune responses when co-administered with antigens [7]. However, the inherent toxicity of LT has limited its uses. To bypass the adverse effects, detoxified variants of LT have been generated, and among these was the double mutant LT, or dmLT [8,9]. While the native *E. coli* LT of chicken origin is identical to that *E. coli* LT obtained from human or porcine sources [10,11], information on the cellular and cytokine profiles induced by detoxified LT and also its uses in combination with live attenuated vaccines in a chicken model is limited. In our previous study, we have reported the development of live attenuated SE JOL1641 strain capable of constitutively secreting mucosal adjuvant-dmLT [12]. In this study, we investigated the effect of JOL1641 immunization on interferon gamma (IFN-γ), interleukin (IL-6), and IL-10 cytokines expression and also on cell mediated response. IFN-γ is crucial for immunity against intracellular pathogens and for tumor control. The cytokine is produced mainly by natural killer and natural killer T cells and by Th1 $CD4^+$ and $CD8^+$ cytotoxic T lymphocyte effector T cells after the development of antigen-specific immunity [13]. *Salmonella* infection, in particular flagellin protein activates splenic dendritic cells, induced rapid IL-6 production, increased expression of activation markers, and dendritic cell redistribution in the spleen [14]. Production of IL-10 by B-cells and plasma cells exert immuno-inhibitory effects, which otherwise protects mice from autoimmunity but impair resistance to infection [15]. It has also been reported that cholera toxin, another closely related enterotoxin synergizes with LPS to induce IL-6 and IL-1β, in addition to IL-10 production by immature DC [16]. The objective of the study was to investigate the relationship between the selected cytokines expression, CMI response, in particular T-cells response and the protection efficacy of the mucosal adjuvanted SE strain.

2. Materials and methods

2.1. Experimental animals

All experimental works involving birds were 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. All chickens used in the study were housed and maintained humanely, they were provided water and antibiotic-free food *ad libitum*.

2.2. Bacterial strains, plasmids, media, and growth condition

The bacterial strains and plasmids used in this study are listed in Table 1. Live attenuated SE strain constitutively secreting dmLT was constructed via stable host-plasmid complementation [12]. Briefly, codon-optimized dmLT cassette containing the desired mutations (R192G/L211A) was cloned into pJHL65 and was designated pJHL65-dmLT. The pJHL65 contained a pBR ori and SE *asd* gene, which facilitates the antibiotic-free stable maintenance of the plasmid in an auxotrophic *asd* deleted mutant. JOL1087 was transformed with pJHL65-dmLT, and the resultant strain was designated JOL1641. The secretion of dmLT protein was directed by the *bla* secretion signal and was expressed under the constitutive promoter Ptrc. The presence of dmLT subunits A and B in the secretions of JOL1641 was validated by immunoblot assay [12]. For comparative study, JOL1087 was transformed with pJHL65 without dmLT cassette. All *E. coli* and *Salmonella* Enteritidis strains were grown in Luria-Bertani (LB) broth. For bacterial counting, *Salmonella* strains were grown on brilliant green agar (BGA) at 37 °C, for enrichment and recovery they were grown in Rappaport-Vassiliadis (RV) broth at 42 °C.

Table 1
Bacterial strains, plasmids and primers used in the study.

Strain/plasmid/primers	Description	Reference
Salmonella Enteritidis		
JOL1182	Wild type isolate from chicken, challenge strain	Lab stock
JOL860	Wild type isolate from chicken for antigen preparations	Lab stock
JOL1087	Δ*lon*Δ*cpxR*Δ*asd*, used as base vaccine strain	[35]
JOL1641	JOL1087 containing pJHL65-dmLT	[12]
Plasmids		
pJHL65	*asd* + vector, pBR ori, b-lactamase signal sequence-based periplasmic secretion plasmid, 6xHis, high copy number	[36]
pJHL65-dmLT	pJHL65 containing dmLT constitutively express under Ptrc promoter, secreted under *bla* secretory system	[12]
Salmonella genus specific primers		[37]
OMPC-Forward	5-ATCGCTGACTTATGCAATCG-3	
OMPC-Reverse	5-CGGGTTGCGTTATAGGTCTG-3	
S. Enteritidis specific primers		[37]
ENT-F	5-TGTGTTTTATCTGATGCAAGAGG-3	
ENT-R	5-TGAACTACGTTCGTTCTTCTGG-3	

2.3. Immunization, virulent-wild type challenge and bacterial recovery

Sixty white leghorn *Salmonella*-free chickens were divided equally into five groups (n = 12) and were immunized orally with PBS (Control; Group A); orally or intramuscularly with JOL1087 (parental strain SE-pJHL65 only; Group B) and JOL1641 (SE-dmLT; Group C). At the fourth week of age, birds were inoculated with 1×10^8 cells in 200 μL PBS orally or 1×10^7 cells in 200 μL PBS intramuscularly (IM). These birds were simultaneously utilized for PBMC sample collections and protective efficacy studies. The birds were orally challenged with 1×10^9 of a wild type SE challenge strain, JOL1182, at the fifth week post-immunization. A total of 6 birds each were euthanized at days 7 and 14 post-challenge. Organ bacterial recovery and post-mortem examination were performed as per protocol described earlier [12]. Briefly, to determine bacterial loads from organs, the samples were weighed and then homogenized in 2 mL buffered peptone water. Hundred μL of the homogenate sample was plated on BGA for direct culture. After incubation at 37 °C for 16 h, the resulting colonies were enumerated. In parallel, 1 mL homogenate was enriched with 4 mL of RV broth and then incubated at 42 °C for 48 h. A loop of the enrichment broth was streaked onto BGA and incubated at 37 °C for 16 h. *Salmonella*-like colonies were further confirmed using specific PCR primers. The number of bacterial colonies obtained via direct culturing were determined and expressed as the mean $\log_{10}$ CFU/g of samples.

2.4. Lymphocyte proliferation assay

The lymphocyte proliferation assay (LPA) was performed at the third week post-immunization. The proliferative capability of peripheral blood mononuclear cells (PBMCs) was evaluated based on a previously described protocol with minor modifications [17]. PBMC was collected from ten birds from each groups, viable cells from each individual bird was seeded in triplicate in 96-well plates at 1×10^5 cells/well. Cells were treated with 4 μg/ml of soluble bacterial protein suspension prepared from a wild type SE JOL860 or 10 μg/ml of concanavalin A or RPMI alone and incubated at 40 °C with a humidified 5% CO_2 atmosphere for 72 h. The cells proliferative response against a specific antigen was mea-