Construction of Phix174 gene *E* mediated lysis system for generation of *Salmonella* Enteritidis ghost, and its evaluation as a vaccine candidate for the immunogenicity and protective efficacy against avian salmonellosis

Chetan Vilas Jawale

Post-doctoral Research Associate,

College of Veterinary Medicine,

Chonbuk National University, Jeonju, South Korea

Bacterial food poisoning is a major world-wide health problem.
Salmonellae remain among the leading sources of food-borne illness

***Salmonella* Enteritidis is the human illness-causing serovar most commonly associated with contaminated poultry meat and eggs.**

-Major disease complications: Gastroenteritis and diarrhoea-abdominal pain, vomiting, nausea, blood in stools.

- Minor disease complications: Focal infection, Septicemia-Neurological complication, Osteomyelitis.

- Morbidity and mortality is approximately 17% of all human salmonellosis cases.

- In South Korea, prevalence rate of *Salmonella* Enteritidis is about 18.5% in food products.

Despite the on-going implementation of targeted control and prevention measures, *Salmonella* Enteritidis is responsible for 93.8 million illnesses and 155,000 deaths worldwide each year.

Bacterial Ghost Technology

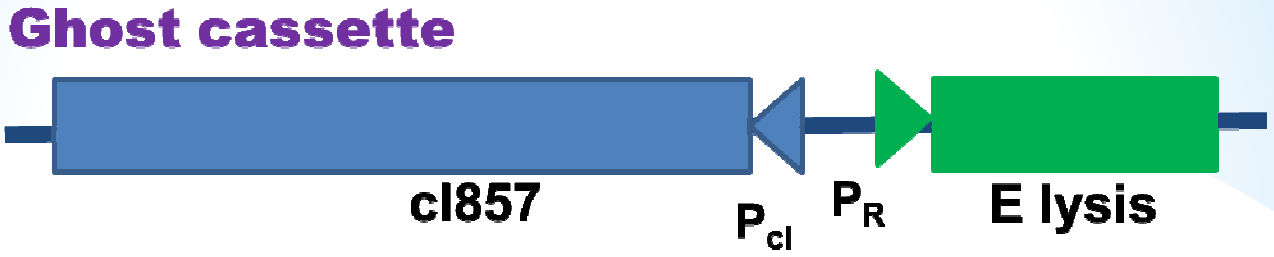
- Genetically inactivating pathogenic Gram-negative bacteria by controlled expression of the cloned bacteriophage PhiX174 lysis gene *E* offers a promising new approach in non-live vaccine technology for protection against infectious diseases.

- These are produced by controlled expression of the cloned bacteriophage PhiX174 gene *E*.

- Gene *E* codes for a 91-amino-acid polypeptide that assembles and penetrates the inner and outer membranes of Gram-negative bacteria, leading to the formation of a transmembrane tunnel structure through the cell envelope, resulting in the loss of the cytoplasmic contents.

- Bacterial ghosts (BG) are empty cell envelopes which possess intact bacterial surface structures and integrated antigen proteins.

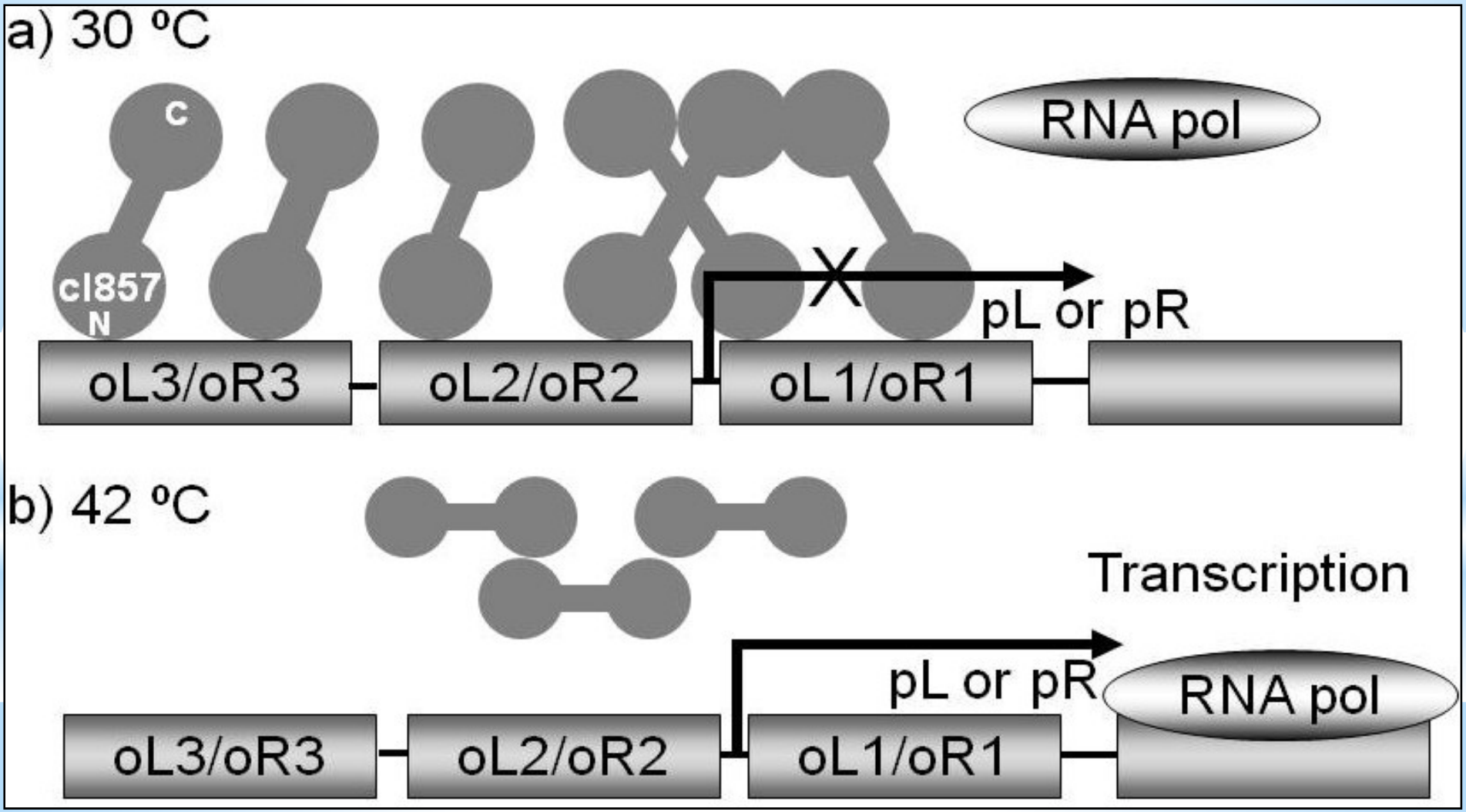
The components of the ghost cassette



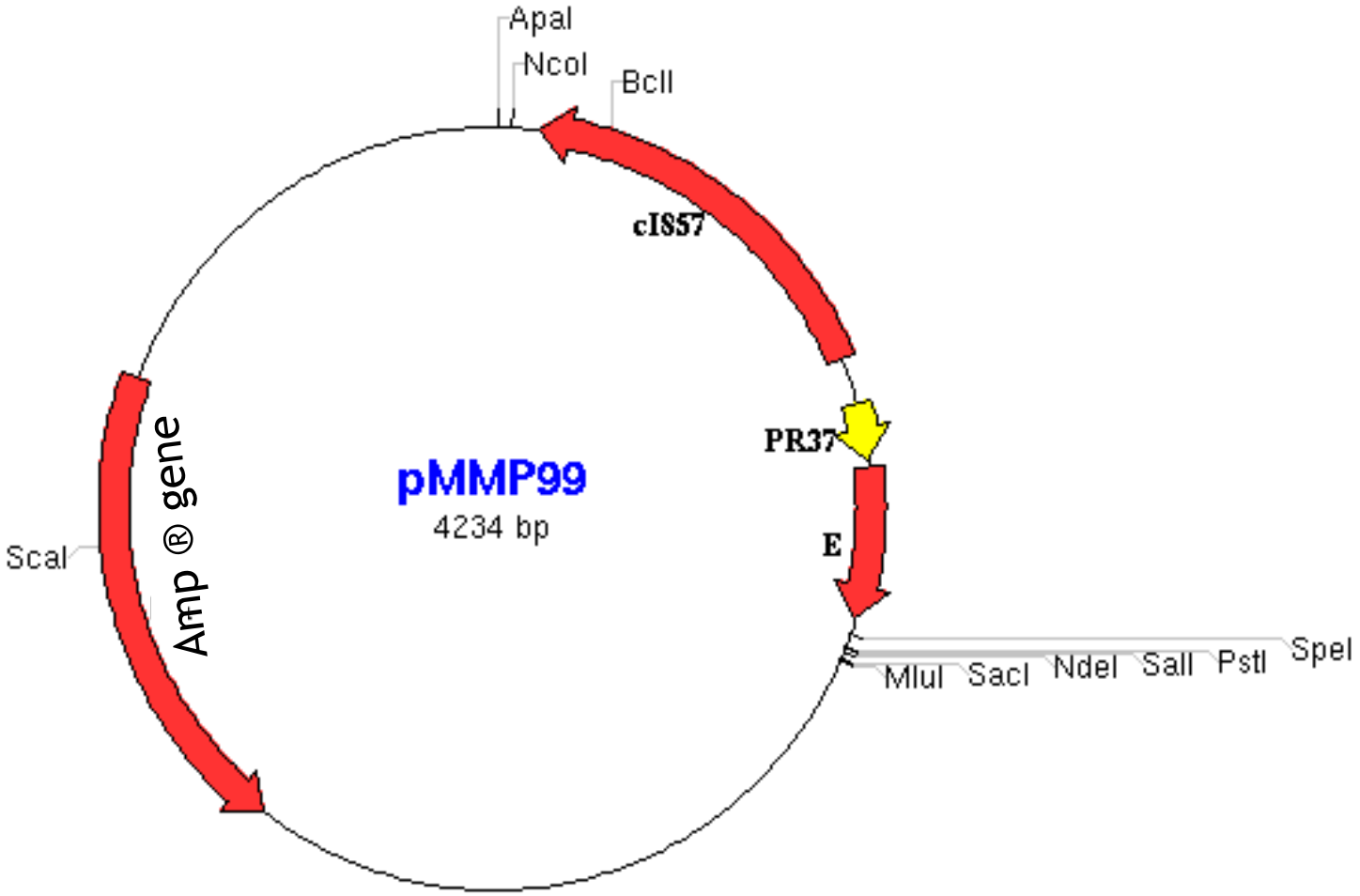
➢The regulatory system is derived from the phage Lambda right promoter/operator system, and expression is controlled by the thermo-sensitive cI857 repressor, which enables the growth of bacteria at lower temperature, and E-mediated lysis at 42°C

E-lysis: Lysis gene *E* of the PhiX174 phage
λP_R : Rightward Lambda Promoter
cI857: Thermolabile cI857 repressor of bacteriophage λ

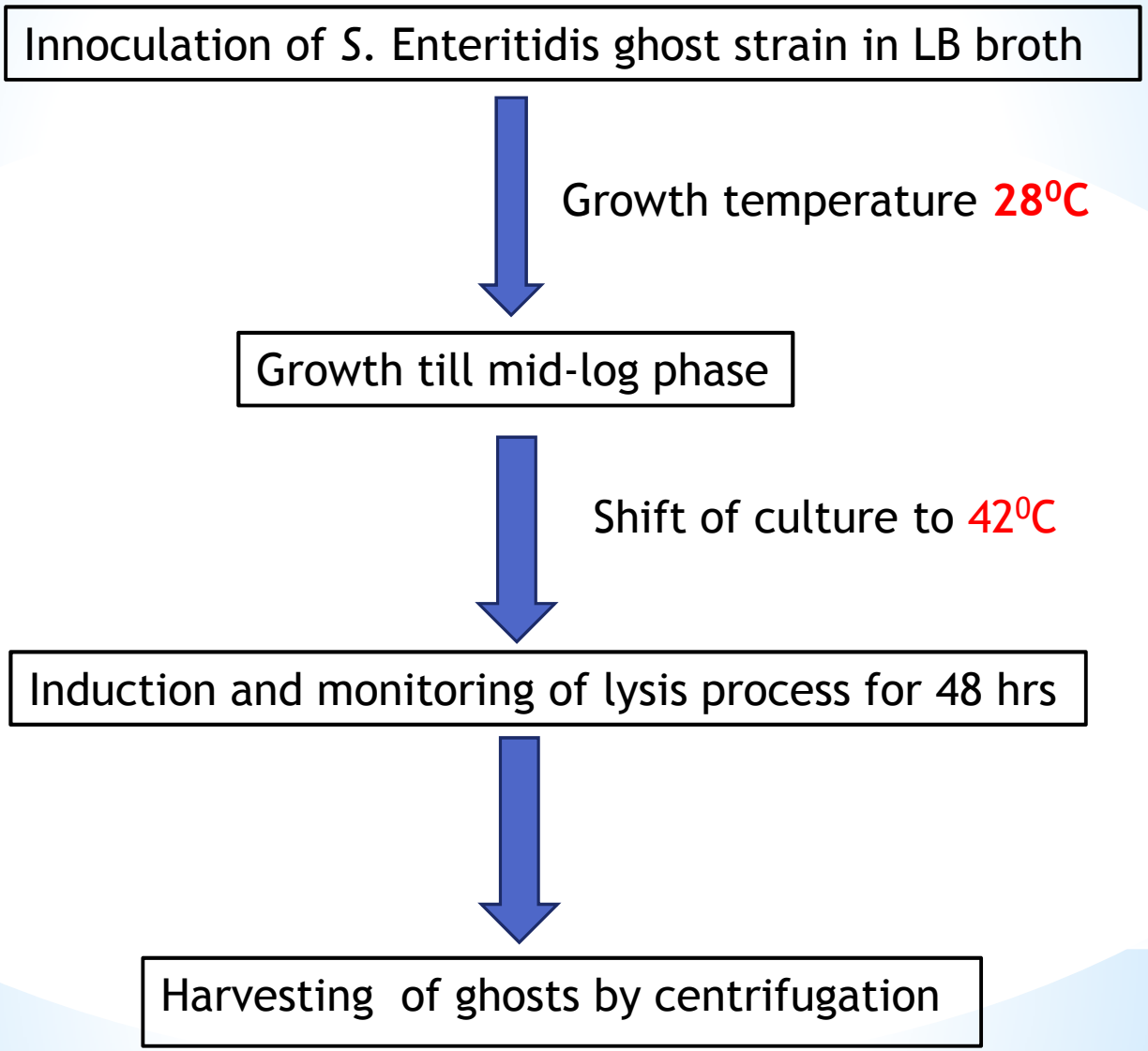
Representation of the lambda pL/pR promoters controlled by the cI857 repressor.



The ghost cassette is carried in the multi-copy plasmid (T-easy vector)



Work flow for production of ghost cells



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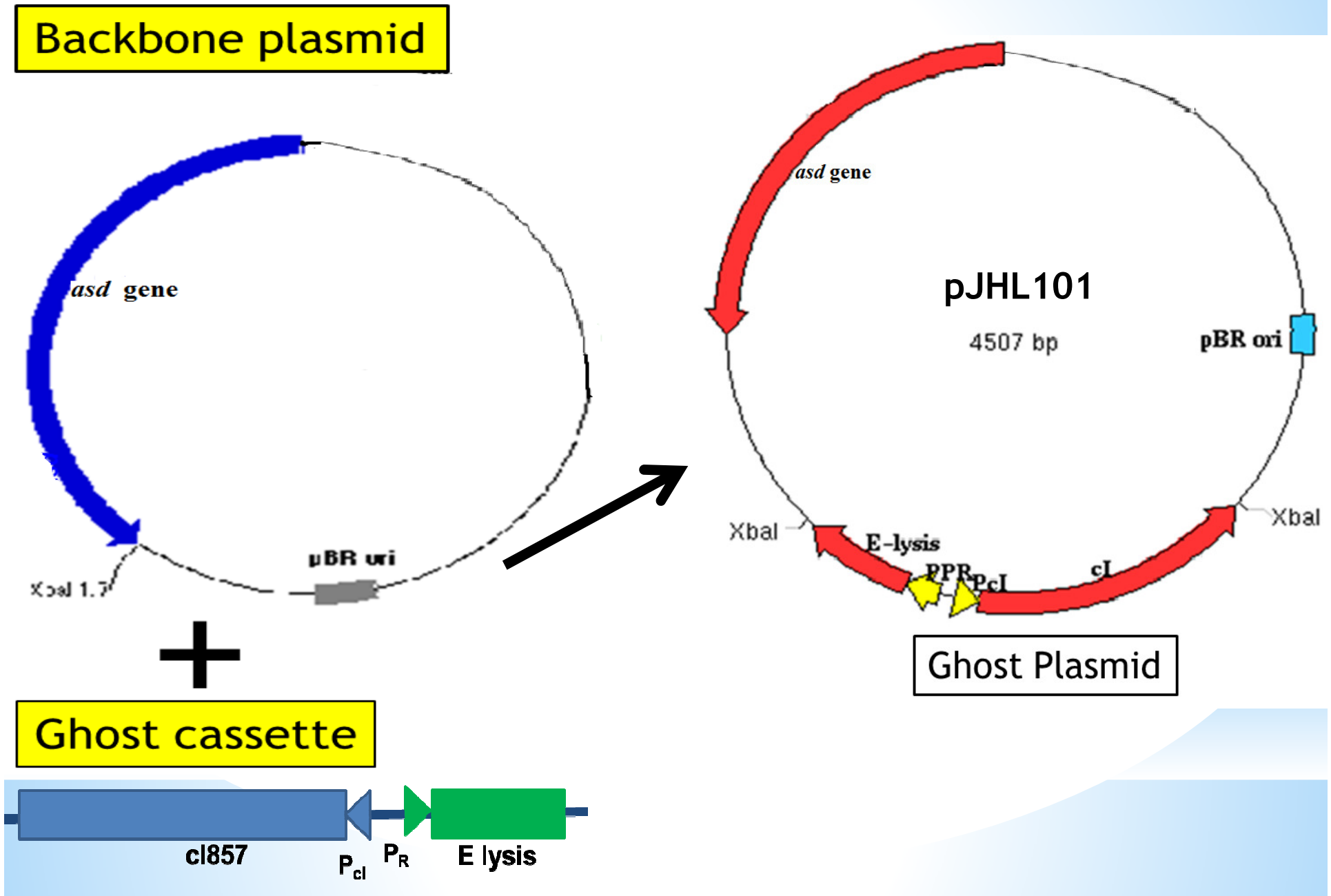
Characterization of a Novel Inactivated *Salmonella enterica* Serovar Enteritidis Vaccine Candidate Generated Using a Modified cI857/λ P_R/Gene *E* Expression System

Chetan V. Jawale, Atul A. Chaudhari, Byung Woo Jeon, Rahul M. Nandre, and John Hwa Lee

College of Veterinary Medicine, Chonbuk National University, Jeonju, Republic of Korea

A new strategy to develop an effective vaccine is essential to control food-borne *Salmonella enterica* serovar Enteritidis infections. Bacterial ghosts (BGs), which are nonliving, Gram-negative bacterial cell envelopes, are generated by expulsion of the cytoplasmic contents from bacterial cells through controlled expression using the modified cI857/λ P_R/gene *E* expression system. In the present study, the pJHL99 lysis plasmid carrying the mutated lambda pR37-cI857 repressor and PhiX174 lysis gene *E* was constructed and transformed in *S.* Enteritidis to produce a BG. Temperature induction of the lysis gene cassette at 42°C revealed quantitative killing of *S.* Enteritidis. The *S.* Enteritidis ghost was characterized using scanning and transmission electron microscopy to visualize the transmembrane tunnel structure and loss of cytoplasmic materials, respectively. The efficacy of the BG as a vaccine candidate was evaluated in a chicken model using 60 10-day-old chickens, which were divided into four groups ($n = 15$), A, B, C, and D. Group A was designated as the nonimmunized control group, whereas the birds in groups B, C, and D were immunized via the intramuscular, subcutaneous, and oral routes, respectively. The chickens from all immunized groups showed significant increases in plasma IgG and intestinal secretory IgA levels. The lymphocyte proliferation response and $CD3^+$ $CD4^+$ and $CD3^+$ $CD8^+$ T cell subpopulations were also significantly increased in all immunized groups. The data indicate that both humoral and cell-mediated immune responses are robustly stimulated. Based on an examination of the protection efficacy measured by observations of gross lesions in the organs and bacterial recovery, the candidate vaccine can provide efficient protection against virulent challenge.

Construction of Antibiotic resistance gene free Ghost Plasmid



Balanced-Lethal Host-Vector System

S. Enteritidis host with chromosomal Asd gene deletion

+

Asd+ ghost plasmid



S. Enteritidis ghost Strain

For overcoming the issue of antibiotic resistance gene marker, we used the auxotrophic *asd* gene based balanced-lethal host-vector system for carrying the E lysis cassette

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Development of a Biosafety Enhanced and Immunogeni *Salmonella* Enteritidis Ghost Using an Antibiotic Resistance Gene Free Plasmid Carrying a Bacteriophag Lysis System

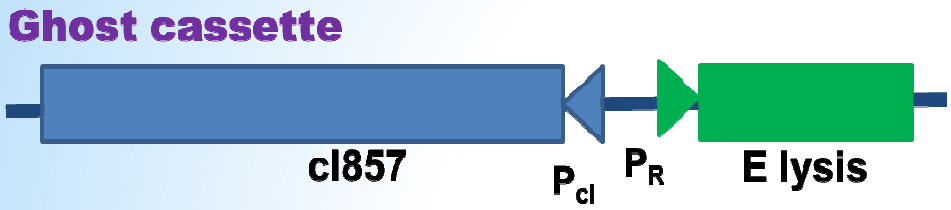
Chetan V. Jawale, John Hwa Lee*

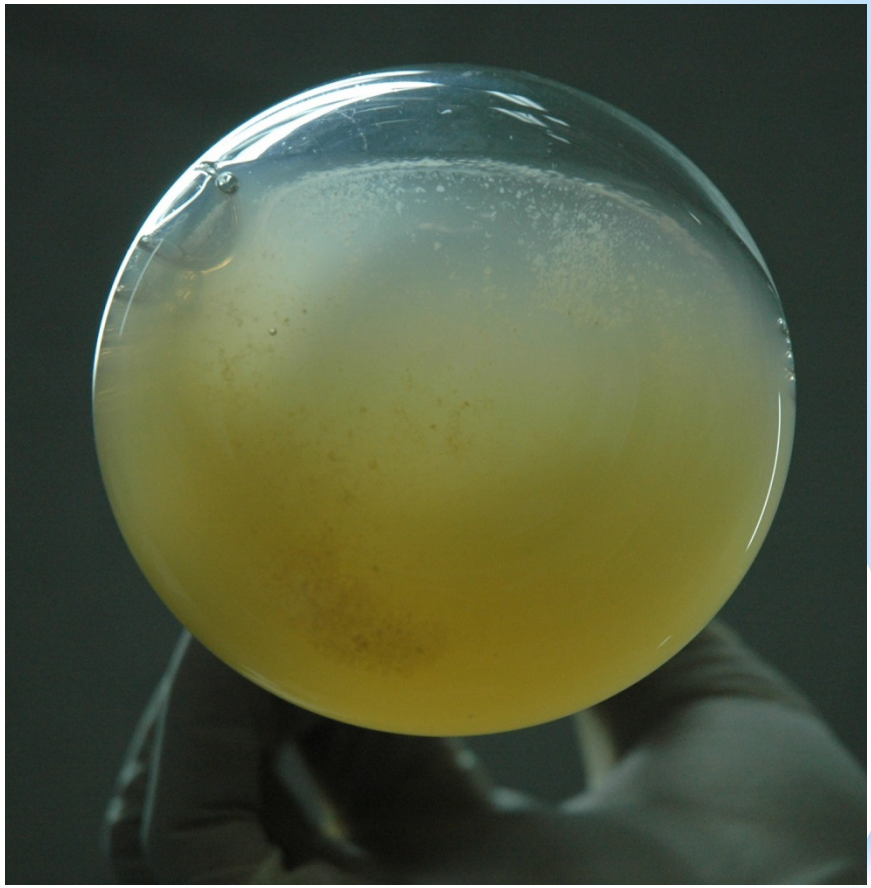
College of Veterinary Medicine, Chonbuk National University, Jeonju, Republic of Korea

Abstract

In the development of genetically inactivated bacterial vaccines, plasmid retention often requires the antibioti resistance gene markers, the presence of which can cause the potential biosafety hazards such as the horizonta spread of resistance genes. The new lysis plasmid was constructed by utilizing the approach of balanced-letha systems based on auxotrophic gene Aspartate semialdehyde dehydrogenase (*asd*). The PhiX174 lysis gene *E* an λPR37-cI857 temperature-sensitive regulatory system was cloned in the *asd* gene positive plasmid and this nove approach allowed the production of antibiotic resistance marker free *Salmonella* Enteritidis (*S*. Enteritidis) ghost. Th immunogenic potential of the biosafety enhanced antibiotic resistance gene free *S*. Enteritidis ghost was evaluated i chickens by employing the prime-boost vaccination strategy using a combination of oral and intramuscular routes. A total of 75 two-week-old chickens were equally divided into five groups: group A (non-immunized control), group B (intramuscularly primed and boosted), group C (primed intramuscularly and boosted orally), group D (primed an boosted orally), and group E (primed orally and boosted intramuscularly). Chickens from all immunized group demonstrated significant increases in plasma IgG, intestinal secretory IgA levels, and antigen-specific lymphocyt proliferative response. After a virulent *S*. Enteritidis challenge, all immunized groups showed fewer gross lesions an decreased bacterial recovery from organs in comparison with the non-immunized control group. Among th immunized chickens, groups B and D chickens showed optimized protection, indicating that the prime-booste immunization with the ghost via intramuscular or oral route is efficient. Taken together, our results demonstrate tha an antibiotic resistance gene free lysis plasmid was successfully constructed and utilized for production of safet

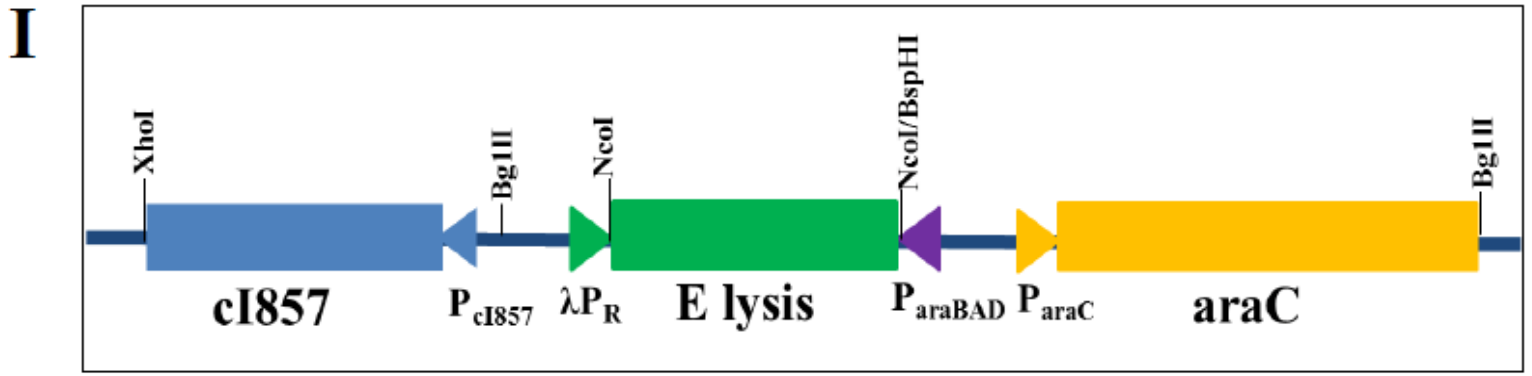
The leaky expression of lysis gene E from λP_R





The leaky expression of lysis gene *E* induced the cell death at normal growth temperature of 28°C

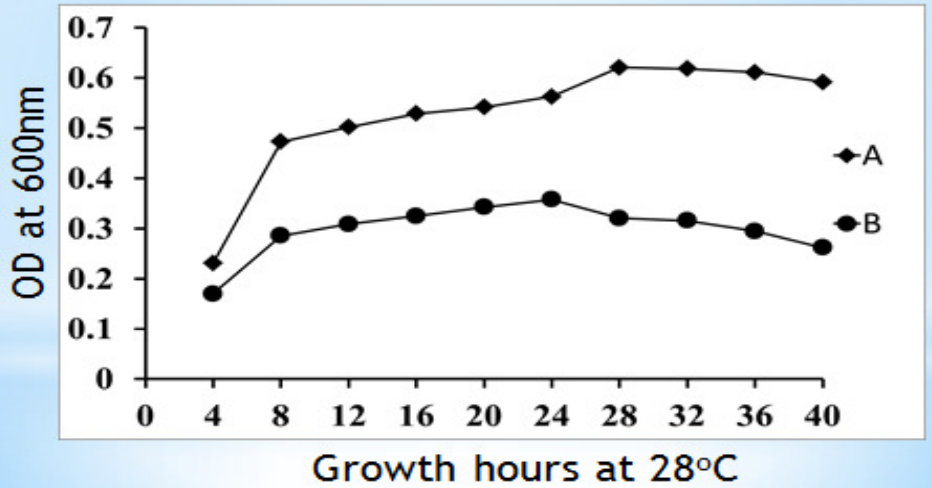
Ghost cassette with tight regulatory system for expression of PhiX174 gene *E*



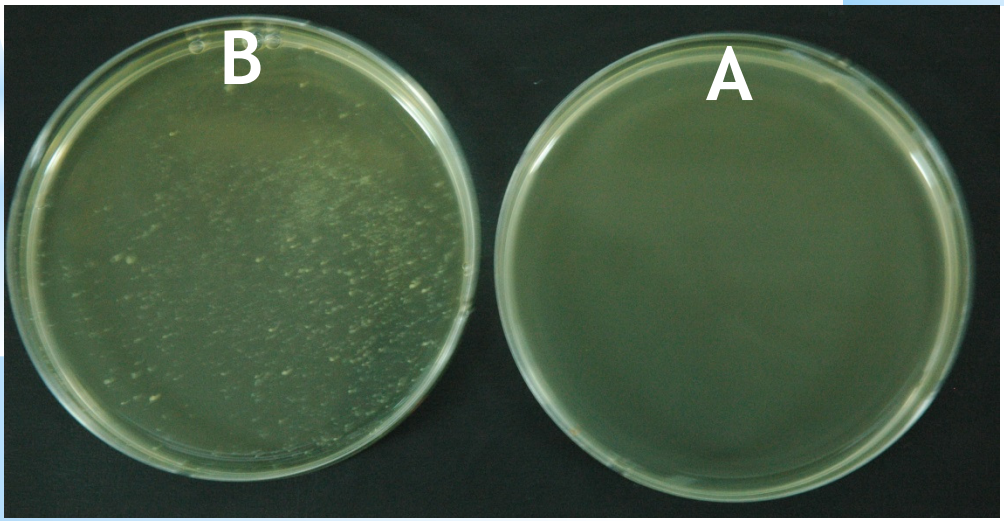
Lysis gene *E* is located between the sense *λpR* promoter with the *cI857* regulator and antisense P_{araBAD} promoter with the *araC* regulator.

In the presence of L-arabinose, the leaky transcription of lysis gene *E* at 28°C from the sense *λpR* promoter was repressed by the simultaneous transcription event from P_{araBAD} promoter through the anti-sense RNA mediated inhibition.

I



II



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Research article

Tightly regulated bacteriolysis for production of empty Salmonella Enteritidis envelope

Chetan V. Jawale [a], Sam Woong Kim [b], John Hwa Lee [a,*]

[a] College of Veterinary Medicine, Chonbuk National University, Jeonju 561-756, Republic of Korea
[b] Swine Science and Technology Center, Gyeongnam National University of Science and Technology, 150 Chilam-dong, Jinju, Gyeongnam, 660-758, Republic of Korea

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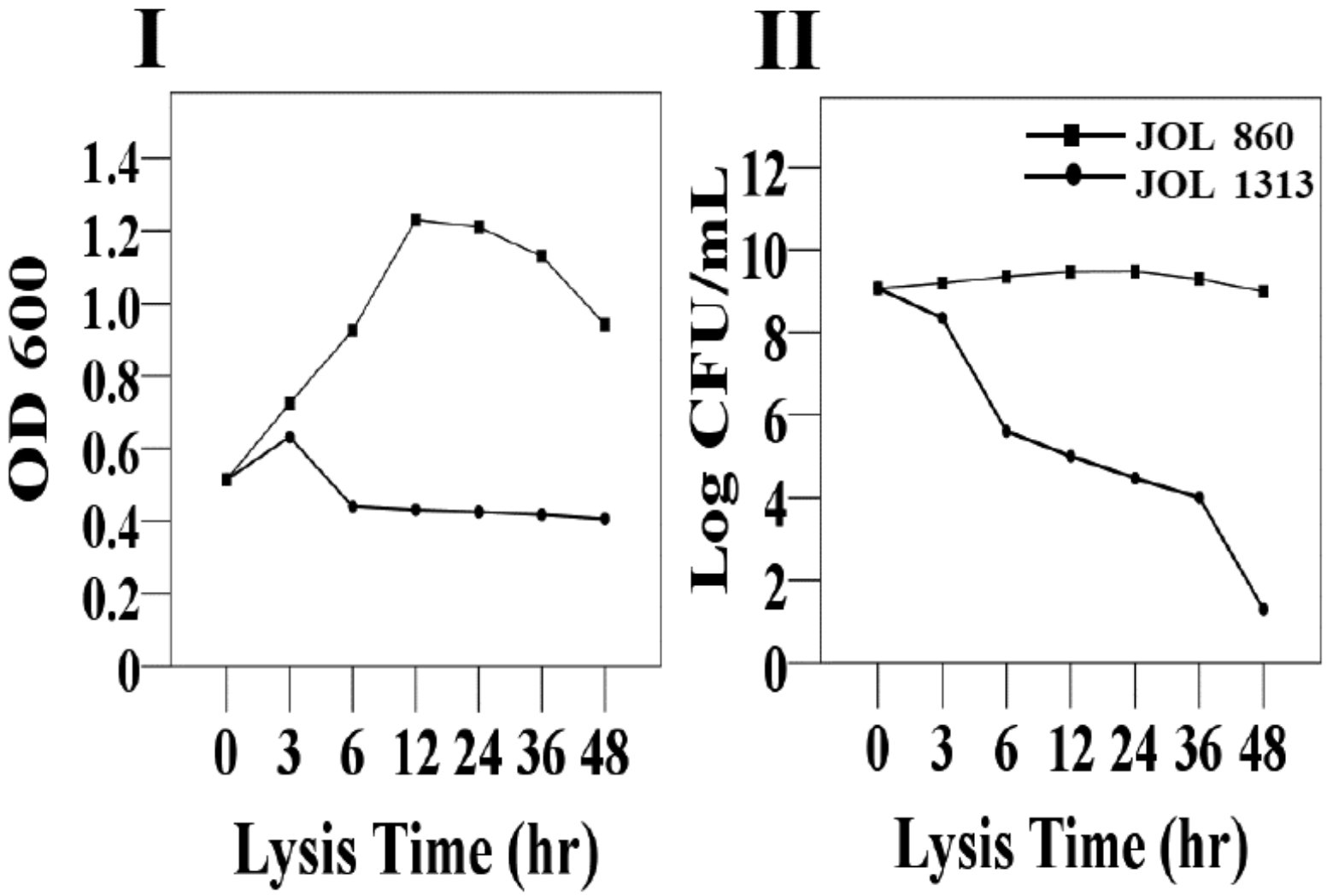
Keywords:
Tight regulation
Leaky transcription
Lysis gene *E*
Convergent promoter
Salmonella Enteritidis
Bacterial ghost

ABSTRACT

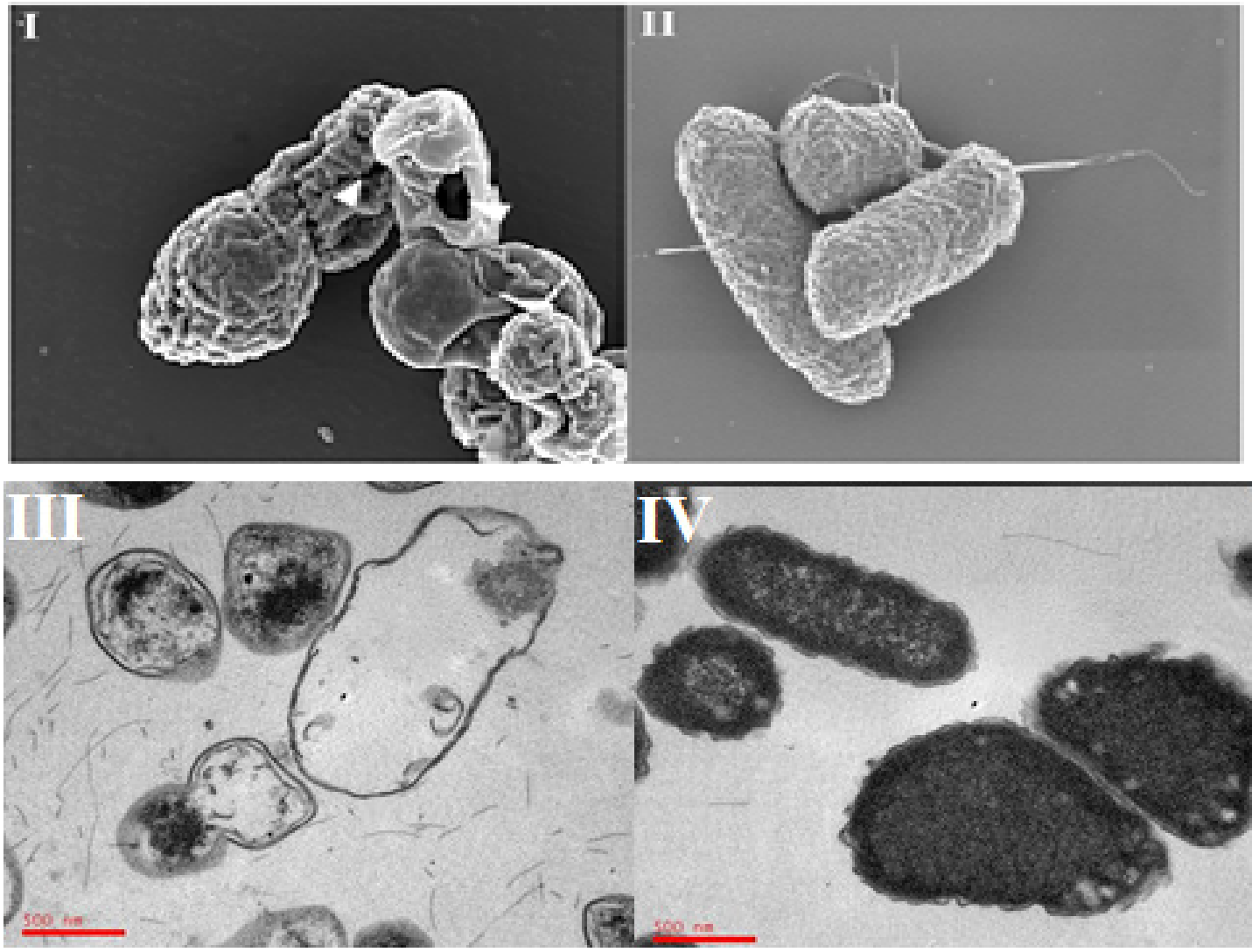
To avoid leaky expression of the bacterial host-toxic PhiX174 lysis gene *E* from the λ*pR* promoter, a convergent promoter construct was made in which gene *E* was placed between a sense λ*pR* promoter and an anti-sense P_{araBAD} promoter. In the presence of L-arabinose, leaky transcription of lysis gene *E* at 28 °C from the sense λ*pR* promoter was repressed by an anti-sense RNA simultaneously expressed from the P_{araBAD} promoter. The stringent repression of lysis gene *E* in the absence of induction temperature resulted into higher concentration of bacteria in culture suspension, and consequently higher and stable production of a *Salmonella* Enteritidis (*S.* Enteritidis) ghost. The immunogenicity of the *S.* Enteritidis ghost was evaluated by immunizing chickens. Chickens from the immunized group demonstrated a significant increase in the levels of *S.* Enteritidis-specific plasma IgG, intestinal sIgA, and lymphocyte proliferative response. After virulent *S.* Enteritidis challenge, the immunized group exhibited decreased bacterial recovery from organs compared with the non-immunized group. Together, these results demonstrate that the stringent molecular control over leaky transcription of lysis gene *E* enabled the stable production of *S.* Enteritidis ghost, and immunization with the *S.* Enteritidis ghost can protect chickens by inducing robust humoral and cellular immune responses.

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Shifting of optimally grown *S.* Enteritidis cultures at 42^{0}C induced the lysis



Electron Microscopic characterization of SE ghost



The single lysis pore appear either in the middle or polar regions of the cell, areas of the envelope actively involved in cell division

A- Non-immunized control

B- Intramuscularly immunized

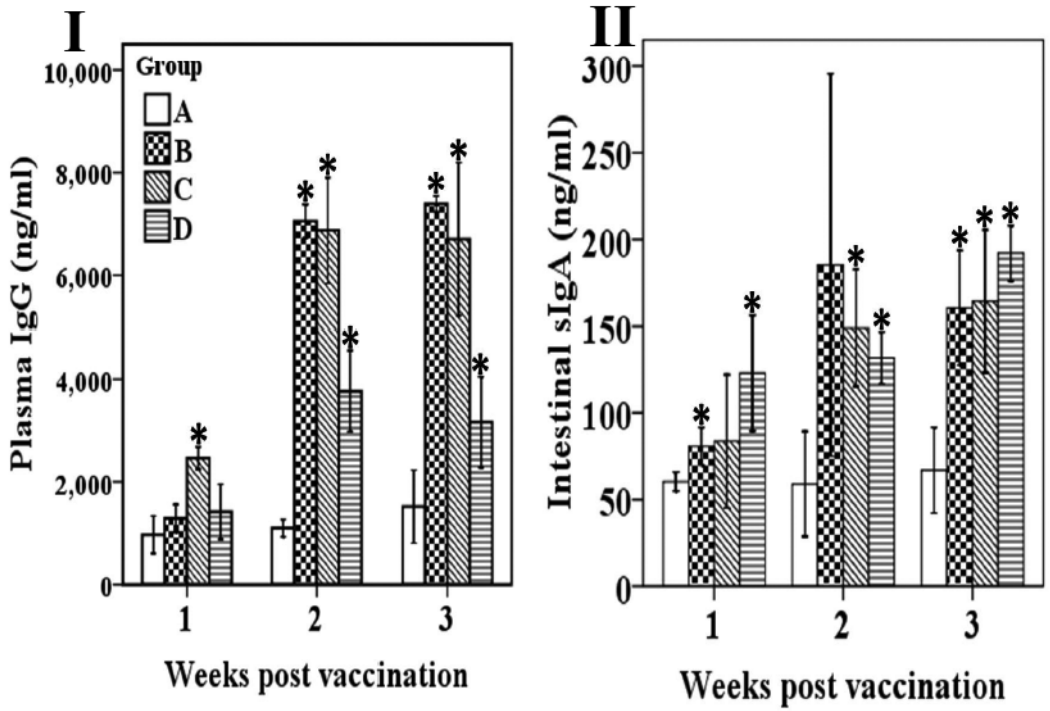
C-Subcutaneously immunized

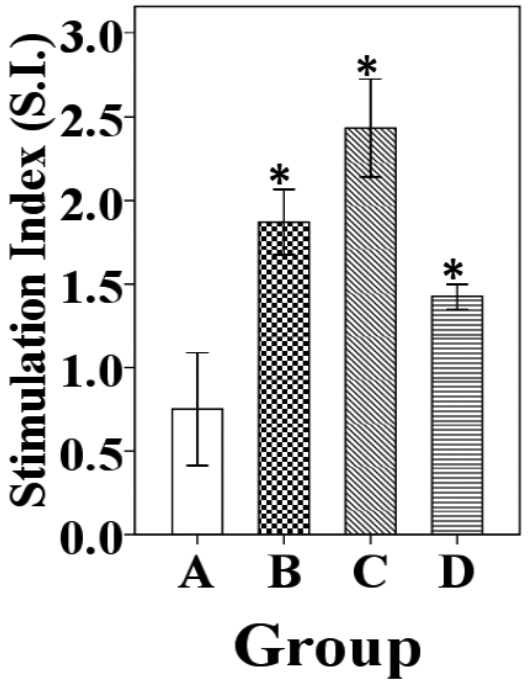
D-Orally immunized

Dosage of SE ghost for vaccination

Parenteral - 1x 10^8 cells

Oral- 1x 10^{10} cells



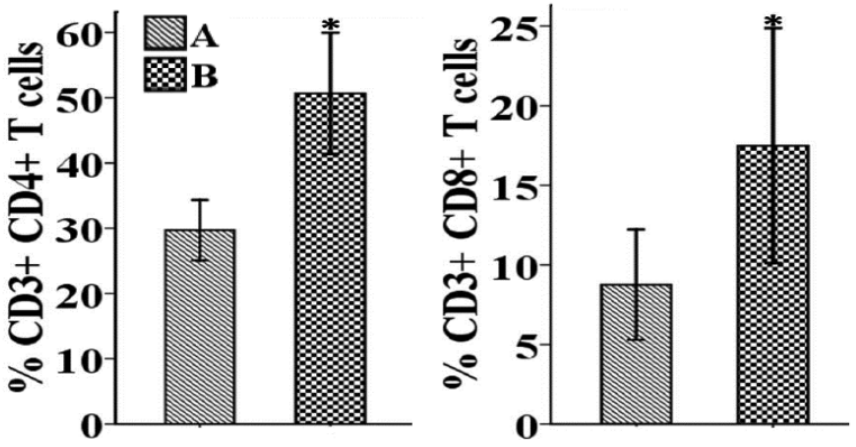


A- Non-immunized control

B- Intramuscularly immunized

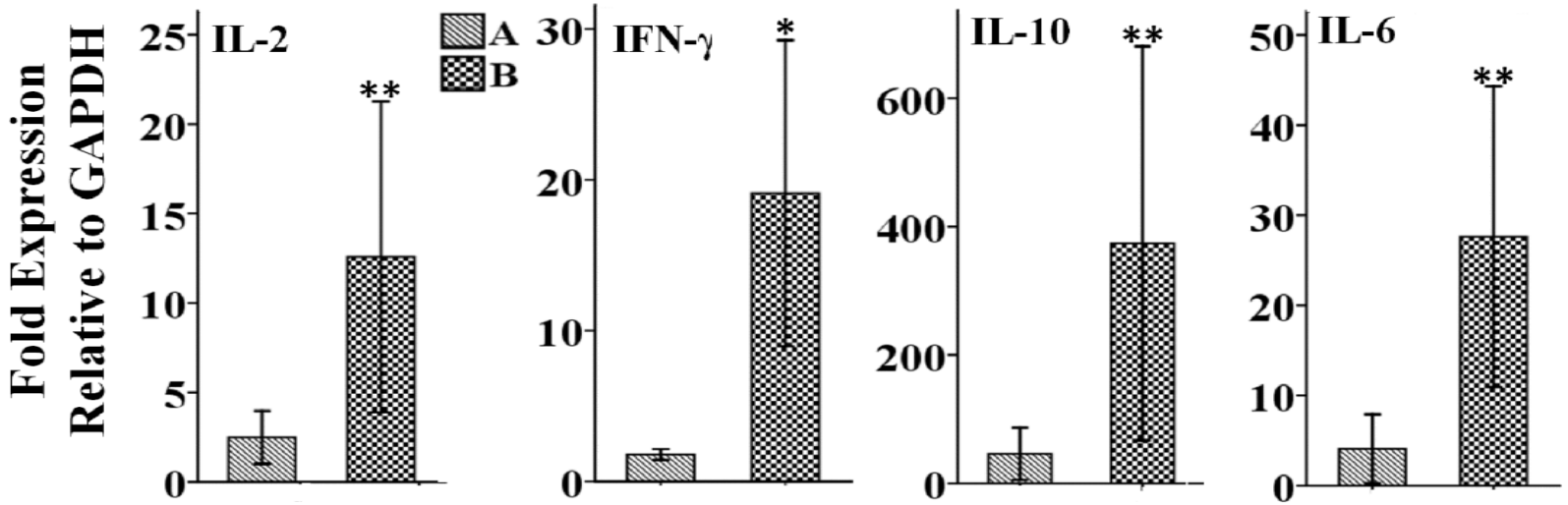
Population of CD4 and CD8+ T cells after vaccination

I



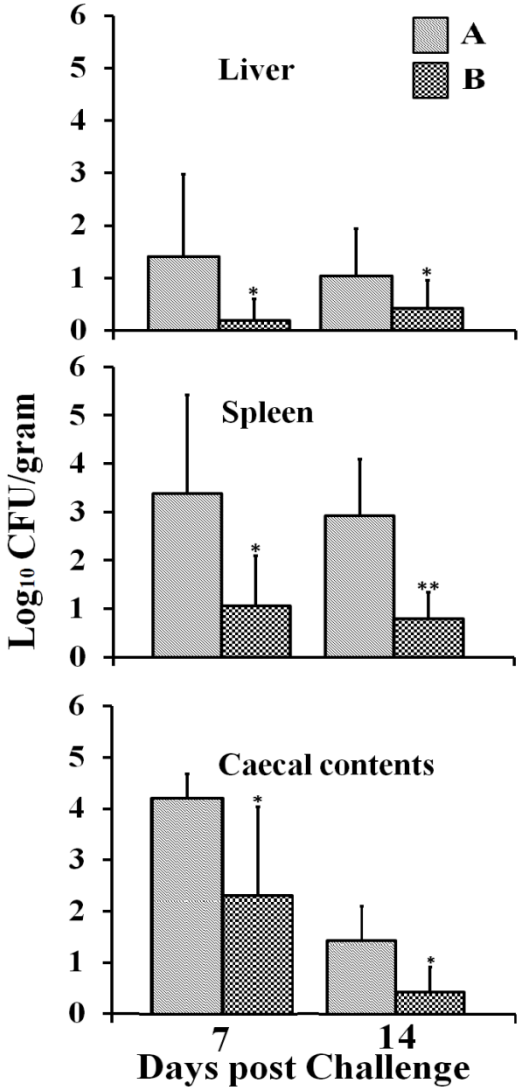
Cytokine profile of PBMC after in-vitro stimulation with SE antigen

II



Challenge study:

Recovery of challenge strain from the internal organs of chickens



Chickens from all the groups were challenged virulent SE at 4 weeks post-vaccination.

Challenge dose: $1x10^9$ CFU/bird via Oral route

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Characterization of adaptive immune responses induced by a new genetically inactivated *Salmonella* Enteritidis vaccine

Chetan V. Jawale, John Hwa Lee*

College of Veterinary Medicine, Chonbuk National University, Jeonju 561-756, Republic of Korea

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ABSTRACT

The superior conservation of antigenic determinants on the surface of genetically inactivated bacterial ghosts makes them attractive immunogenic inactivated vaccine candidates. The efficacy of *Salmonella* Enteritidis (SE) ghost vaccination was evaluated in chickens by characterizing the nature of the adaptive immune response. Chickens from the immunized group demonstrated significant increases in SE-specific plasma IgG, intestinal secretory IgA, and lymphocyte proliferative response. The populations of CD4, CD8, and TCR γδ T-cells in immunized chickens were significantly greater than in the controls. Increased levels of IFN-γ, IL-2, IL-6 and IL-10 were observed in peripheral blood mononuclear cells stimulated with SE specific antigen. After virulent SE challenge, the immune system of immunized chickens was rapidly stimulated, as indicated by significantly increased population of CD4 and CD8 T-cells. Furthermore, the immunized group exhibited decreased challenge strain recovery of the internal organs compared to the non-immunized group. Together, these data indicate that the immunization induced humoral and cell-mediated immunity might be responsible for significant reduction of the virulent challenge strain load in the internal organs of immunized chickens.

Vaccination and challenge study in adult layer chickens

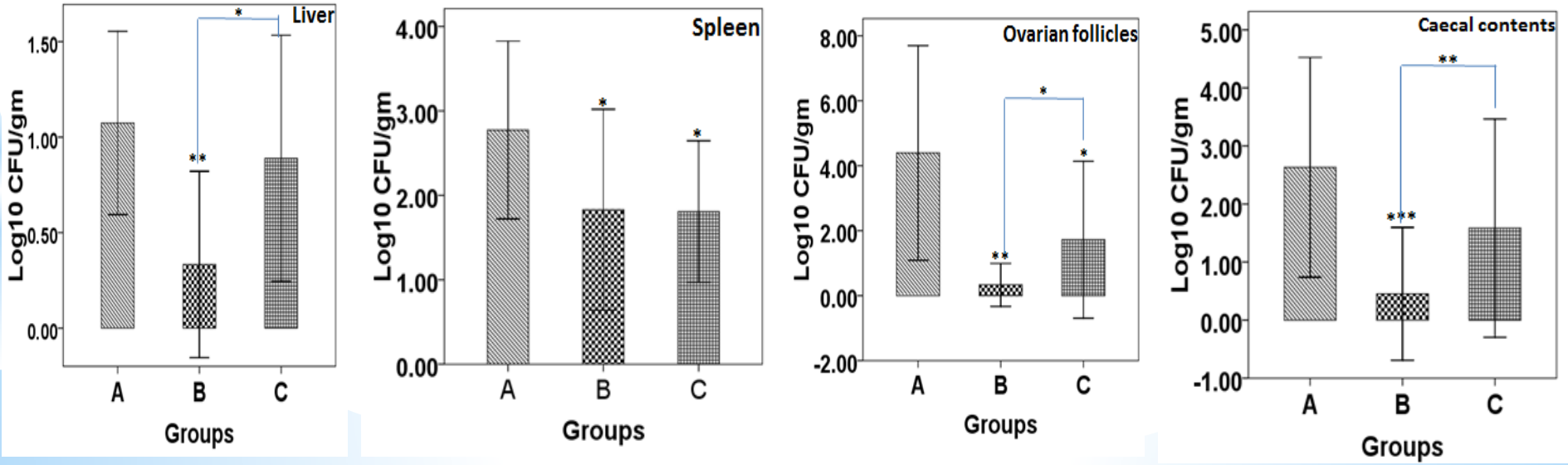
Group A: Non-immunized challenge control

Group B: Two time vaccination-challenge (Vaccination at 8th and 16th week of age)

Group C: One time vaccination-challenge (Vaccination at 16th week of age)

Chickens from all the groups were challenged virulent SE at 23rd week of age

Challenge dose: $1x10^7$ CFU/bird via intra-venous route



Effect of Vaccination on contamination of eggs with *Salmonella* Enteritidis

Group[a]	Contaminated eggs per week (%)[b]			Total number of contaminated eggs (%)[c]
	1st week	2nd week	3rd week	
A	23.8	19.4	9.3	15.7
B	12.0	3.8 *	2.0 *	4.9 ***
C	10.4	6.0	3.3	5.8 **

Conclusions

1) The stable system for production of bacterial ghosts was established

2) The production of bacterial ghosts is relatively simple approach for production of highly immunogenic genetically activated vaccines.

3) The S. Enteritidis ghosts are capable of inducing both the cell mediated and humoral immune responses after vaccination and are capable of protecting chickens against virulent S. Enteritidis chickens

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