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Generation of a safety enhanced *Salmonella* Gallinarum ghost using antibiotic resistance free plasmid and its potential as an effective inactivated vaccine candidate against fowl typhoid

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ABSTRACT

A safety enhanced *Salmonella* Gallinarum (SG) ghost was constructed using an antibiotic resistance gene free plasmid and evaluated its potential as fowl typhoid (FT) vaccine candidate. The antibiotic resistance free pYA3342 plasmid possesses aspartate semialdehyde dehydrogenase gene which is complimentary to the deletion of the chromosomal *asd* gene in the bacterial host. This plasmid was incorporated with a ghost cassette containing the bacteriophage PhiX174 lysis gene *E*, designated as pJHL101. The plasmid pJHL101 was transformed into a two virulence genes-deleted SG. The SG ghosts with tunnel formation and loss of cytoplasmic contents were observed by scanning electron microscopy and transmission electron microscopy. The cell viability of the culture solution was decreased to 0% at 24 h after the induction of gene *E* expression by an increase in temperature from 37 °C to 42 °C. The safety and protective efficacy of the SG ghost vaccine was further examined in chickens which were divided into three groups: group A (non-immunized control), group B (orally immunized), and group C (intramuscularly immunized). The birds were immunized at 7 d of age. No clinical symptoms associated with FT such as anorexia, depression and greenish diarrhea were observed in the immunized chickens. Upon challenge with a virulent SG strain at 3 week post-immunization, the chickens immunized with the SG ghost via various routes were efficiently protected, as shown by significantly lower mortality and post-mortem lesions in comparison with control group. In addition, all the immunized chickens showed significantly higher antibody responses accompanied by a potent antigen-specific lymphocyte proliferative response along with significantly increased numbers of $CD4^+$ and $CD8^+$ T lymphocytes. Overall, our results provide a promising approach of generating SG ghosts using the antibiotic resistance free plasmid in order to prepare a non-living bacterial vaccine candidate which could be environmentally safe yet efficient to prevent FT in chickens.

1. Introduction

Fowl typhoid (FT), a septicemic disease of poultry, causes acute mortality and induces severe inflammation of internal organs such as liver and spleen, caecum and yolk sac which results in significant economic losses to the poultry industry worldwide. FT is caused by facultative intracellular Gram-negative bacterium, *Salmonella enterica* serovar Gallinarum (SG), which also rarely causes illness in humans [1–3].

Prevention of FT by means of vaccination has been demonstrated earlier [4–8]. However, the commercially available live vaccine strain has several limitations such as its only limited use for layer breeds older than 6 weeks, low body weight in vaccinated poultry [4], and residual virulence that can cause hepatitis and splenic lesions in chicks [9]. The live *Salmonella* vaccine shares the disadvantages of the safety concerns for the animals and cause environmental contamination through fecal shedding [10–12]. The use of inactivated or killed vaccines could be an alternative for these live vaccines. However, conventionally produced formalin or heat inactivated vaccine formulations can alter the physiochemical/structural properties of the antigens thereby negatively affecting the development of protective immunity [13,14].

Bacterial ghost (BG) technology has been a new and progressive approach to construct the safe and immunogenic inactivated vaccines against wide variety of infectious diseases [14–18]. BGs are formed by protein E mediated lysis followed by expulsion of cytoplasmic contents [15]. The most sensitive and fragile bacterial surface structures are highly preserved following the ghost formation and thus capable of inducing cellular and humoral immune

Abbreviations: SG, *Salmonella* Gallinarum; FT, Fowl Typhoid; BG, Bacterial Ghost; sbcp, sonicated bacterial cell protein.

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responses [13,19–23]. In the recent few years we have shown that use of bacterial ghosts is an effective measure to prevent salmonellosis in chickens [17,18].

Despite its significance in disease prevention, BG technology still remains debatable as most of the plasmids used for the development of ghost vaccines include an antibiotic resistance gene used for the selection and maintenance in the host cell [14,17,18,24]. The continued use of such plasmids is undesirable in the vaccine production due to the risk of spreading antibiotic resistance traits to environmental microbes [25].

In our previous study, we generated the SG using the genetic repression/expression system containing repressor cI857 and lysis gene *E*; however the lysis plasmid contained the ampicillin resistance gene as a selectable marker and virulent SG wild-type strain was used as host bacterium [17]. Although we could observe the complete lysis in the ghost preparation of the previously published report [17], but we cannot rule out the possibility of fewer cell populations which may remain partially lysed or live or may lead to the formation of the E-lysis resistant mutant. Although there is the least possibility, but in the event that a ghost strain containing an antibiotic-resistant gene bearing plasmid is not efficiently lysed and still used as a vaccine candidate, then the chicken may become infected with virulent SG and the external environment might be at higher risk due to a possible fecal shedding of such antibiotic-resistant gene bearing strains virulent through animal body. Such strains could be a cause to generate new antibiotic resistant pathogenic strains due to acquisition of antibiotic resistant plasmids through horizontal transfer. Although the possibility of presence of few live SG wild-type cells in ghost preparation leading infection in immunized birds and shedding antibiotic resistance gene carrying strain is less likely to happen, this fact cannot be overlooked. The intact plasmid DNA bearing antibiotic resistance genes are reported even in the completely lysed bacterial ghost preparations [24,26]. In order to minimize or avoid the theses potential hazards, the non-antibiotic marker-based Asd^+ (aspartate-semialdehyde dehydrogenase) plasmid can be utilized for carrying E-lysis system and the live attenuated non-pathogenic *asd* gene deleted SG strain can be used as host bacterium. The selection of the Asd+ ghost plasmid containing SG ghost strain can be based on the balanced lethal host-vector system wherein the plasmid contains *asd* gene that complements a host bacterial chromosomal *asd* gene deletion [27,28].

The reports are described in the literature where the efforts were directed for minimization or the removal of genomic DNA content and antibiotic resistance gene containing plasmid DNA from the ghost preparation by utilizing the host bacterial strain transformed with dual vector carrying staphylococcal nuclease A (SNA) gene and lysis gene *E* [26,29], or two plasmids carrying lysis *E* lysis and SNA gene separately [23,30]. Although this approach was successful for removal of DNA content of ghost preparation, but still it needs the addition of antibiotics and chemical inducers in the culture [23,30].

The present study was carried out to develop a safety enhanced SG ghost vaccine, in which an antibiotic resistance free plasmid containing the *phi*X174 lysis gene *E* was constructed and introduced into a two virulence genes-deleted SG mutant. The successful production of the SG ghost and its immune-potential was evaluated.

2. Materials and methods

2.1. Construction of SG ghost

Plasmid pHCE GAPDH ghost 37SDM carrying the parent ghost cassette was constructed as previously described [31]. PCR amplification of the ghost cassette was performed using pHCE GAPDH ghost 37SDM as a template and the primers ghost-F-XbaI (5′-TCTAGAGACCAGAACACCTTGCCGATC-3′) and ghost-R-XbaI (5′-TCTAGAACATTACATCACTCCTTCCG-3′). The amplified DNA segment was cloned onto the pGEM-T easy vector (Promega, Madison, WI, USA) and was designated pJHL99. To construct the antibiotic gene free plasmid containing the ghost cassette, pYA3342 plasmid containing *asd* gene was used as *asd*-based balanced lethal host vector system [32]. The plasmid pJHL99 was cut by the restriction endonuclease *Xba*I, and a 1.2 kb DNA-fragment of the ghost cassette was isolated. The ghost cassette was comprised of the PhiX174 lysis gene *E* and the lambda PR_{37}-cI857 regulatory system. Then the antibiotic resistance gene free backbone plasmid pYA3342 was linearized by *Xba*I digestion, and the DNA fragment containing the ghost cassette was inserted. The constructed antibiotic resistance gene free ghost plasmid was designated as pJHL101. For the construction of the safety enhanced SG ghost vaccine, the *lon, cpxR* and *asdA16* deleted SG mutant strain JOL967 [28] was used. Transformation of the plasmid pJHL101 into JOL967 cells was performed by electroporation and transformants were selected on LB agar plates in deficiency of Diaminopimelic acid (DAP) (Sigma, St Louis, MO, USA). The resultant SG ghost strain was denoted as JOL1291. The vector control strain was constructed by transforming JOL967 with backbone plasmid pYA3342 (without lysis gene E).

2.2. Lysis pattern and characterization of SG ghost strain

A single colony of JOL1291 harboring pJHL101 was inoculated into 40 mL of LB broth. When the culture reached an optical density (OD_{600}) of 0.5–0.6, 20 mL of the culture was shifted from 37 °C to 42 °C temperature to induce gene *E*-mediated lysis. To compare the viable cell count of the culture at 37 °C and 42 °C, the remaining 20 mL culture was further grown at 37 °C. The lysis was monitored by performing the viable cell counts as colony forming units (CFU) at different time points (0 h, 3 h, 6 h, 18 h and 24 h). The vector control strain was also grown in the same manner for comparison of viable cell count at 37 °C and 42 °C. The morphological features of the SG ghost were characterized by scanning electron microscopy (SEM) and transmission electron microscopy (TEM) as previously described with minor modifications [17].

2.3. Immunization and observation of general condition in chickens after vaccination

The animal experiment described in this study was conducted with approval (CBU 2011-0017) from the Chonbuk National University Animal Ethics Committee in accordance with the guidelines of the Korean Council on Animal Care. One-day old female Brown Nick chickens were divided into three groups ($n = 15$ per group), and provided with water and antibiotic-free food *ad libitum*. Chickens were immunized with the SG ghosts at 7-d of age. The dose and route of the vaccination were decided based on the data from our previous study [17]. In the control group A, chickens were orally inoculated with PBS. In group B, the birds were orally inoculated with the SG ghosts at a concentration of 1×10^{10} cells/0.1 mL/chicken. Group C birds were injected intramuscularly with 0.1 mL of the SG ghost suspension containing 1×10^8 cells/chicken.

2.4. Collection of plasma and intestinal wash samples for antibody response assessment

For weekly determination of plasma IgG and intestinal secretory IgA (sIgA), five birds per each group were used. The plasma samples were obtained by centrifugation of the peripheral blood. The intestinal wash samples were collected as described earlier [33]. The samples were stored at −20 °C until use.