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Evaluation of protective immune response against fowl typhoid in chickens vaccinated with the attenuated strain *Salmonella* Gallinarum Δ*cob*SΔ*cbi*A

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ABSTRACT

Salmonella enterica serovar Gallinarum biovar Gallinarum (SG) causes fowl typhoid in chickens, a septicemic infection which results in high mortality rates. This disease causes high economic impact to the poultry industry worldwide because of the mortality or elimination of positive flocks to control bacterial dissemination. Live vaccines are used in the fields, however the characterization of immune mechanisms important for protection are being studied to improve the efficacy of vaccination schemes. In this study, we evaluated the immune response in brown layer-hens, vaccinated or not, during the most critical period of infection. Cellular and humoral immunity were extensively evaluated until 7 days post-infection (DPI), by flow cytometry and ELISA, respectively. Furthermore, we evaluated the expression of important pro-inflammatory cytokines after infection of bone marrow derived macrophages (BMDMs) with the live attenuated SG vaccine and with the wild SG strain. The results showed an increasing production of IgG and IgM during the first week post-infection, in vaccinated layer-hens, which was absent in unvaccinated birds. The population of $CD8^+CD44^+$ and $CD4^+CD44^+$ T cells in spleen and cecal tonsils constantly decreased in unvaccinated birds in comparison with vaccinated layers. The expression of IFN-γ and TNF-α in BMDMs was induced by both SG strains (attenuated and wild) at similar levels ($p>0.05$). Vaccination with live SG vaccine reduced systemic infection by challenge strain of SG and prevented the mortality rate of 85% that occurred in unvaccinated layer-hens during 30 dpi. Furthermore, the immunization enhanced the proliferation of effector $CD4^+$ and $CD8^+$ T cells after challenge.

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1. Introduction

Salmonella enterica serovar Gallinarum biovar Gallinarum (SG) is an avian host-specific serovar that causes fowl typhoid (Barrow and Freitas Neto, 2011). Despite biosecurity measures, this disease still affects commercial poultry flocks worldwide (O.I.E., 2015). The outcome of the infection is marked by high morbidity and up to 80% mortality of naturally infected birds (Shivaprasad, 2000). During outbreaks, severe protocols are followed to restrain and eliminate the spread of SG, and in case of infections of breeders, the complete removal of the flock and quarantine are required by strict legislation (Barrow and Freitas Neto, 2011; BRASIL, 2003).

The control of infections by biosecurity measures and vaccination is adopted in many countries to prevent outbreaks in commercial flocks (Feberwee et al., 2001; Meunier et al., 2016; Penha Filho et al., 2009). Currently the live vaccines have shown better efficacy to protect chickens against fowl typhoid in comparison with the killed vaccines available, however the mechanisms involved in the protective immune response against this pathogen are still being clarified (Wigley et al., 2005). The infection occurs by oral route, however SG quickly invades the epithelial barrier, the gut associated lymphoid tissue (GALT) or can be passively captured by M cells, consequently spreading to internal organs, leading to a severe septicemic infection (Shivaprasad, 2000; Tahoun et al., 2012). The bacterial colonization of organs and multiplication in these tissues can cause acute death of the host after a short incubation period of 4 days-post infections, marked by a profound hepatosplenomegaly (Chappell et al., 2009). Immunity to protect against SG requires significant participation from both the innate and acquired branches of the immune system (McSorley, 2014; Santos et

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al., 2001). Due to the capability to survive and replicate in the intracellular micro-environment especially inside macrophages, the cellular mediated immunity (CMI) is important for the control of *Salmonella* infection (Chappell et al., 2009). The relevance of humoral immune response is not completely elucidated, but evidences point out an important involvement of B cells for immunity against this pathogen, independently from the production of antibodies (Nanton et al., 2012). Although brown layer-hens and broilers (meat type chickens) are not capable to control bacterial proliferation and are highly susceptible to mortality caused by SG, which has high capacity to inhibit and evade the innate immune response during infection of these varieties of chickens (Barrow and Freitas Neto, 2011; Shivaprasad, 2000). The vaccination and priming of B and T cells with attenuated live vaccines has shown to be capable to stimulate a protective immune response which can control bacterial dissemination and mortality of brown layer-hens, thus this animal model is frequently used to evaluate SG infection (Wigley et al., 2005).

The first vaccine against fowl typhoid was introduced in the late 1950s with the rough strain of SG (SG9R) obtained by chemical mutagenesis (Smith, 1956). Since then, the efforts of many researchers to improve the safety and obtain novel vaccines resulted in different live vaccines that are being tested experimentally (Jawale and Lee, 2014; Mitra et al., 2013; Shehata et al., 2013). It is essential that vaccines for invasive host-specific *Salmonella* serovars stimulates development of antigen-specific memory and effector $CD4^+$ T cells and $CD8^+$ T cells (Wahid et al., 2015). T cells express CD44 on the membrane after activation in response to invading pathogens and the expression remains at high levels in effector and memory T cells after activation (Baaten et al., 2010).

The strain SGΔ*cob*SΔ*cbi*A is a mutant strain of SG 287/91, with deletion on genes *cob*S and *cbi*A, previously shown to be attenuated and capable to colonize internal organs, in reduced numbers in comparison with wild SG strain (de Paiva et al., 2009). Previous studies have also shown that this strain is immunogenic and one vaccine dose elicited protection against SG and cross protection against *S.* Enteritidis (Penha Filho et al., 2010). A higher local influx of $CD8^+$ T cells in the cecal tonsils of white layer-hens and faster reduction of *S.* Enteritidis intestinal burden were noticed in the group vaccinated with SGΔ*cob*SΔ*cbi*A (Penha Filho et al., 2010). However little is known about the immunological mechanisms involved in the control of mortality and systemic infection by SG in vaccinated chickens. Thus, the present work was designed considering that experimental development of fowl typhoid starts during the first week, and the onset of the disease clinical signs and mortality occurs after 5 days post-infection (DPI). We aimed to study the elements of acquired immune response that are effective to protect susceptible chickens against SG during this critical period of onset of the disease, in comparison with unvaccinated susceptible chickens. For this, an extensive evaluation of the cellular and humoral immune responses was performed, before and after the challenge with wild SG.

2. Materials and methods

2.1. Experimental birds

Two hundred and forty commercial Brown layer-hens, free of immunizations against *Salmonella*, were purchased at day of hatch of eggs from grandparents breeder flocks with high biosecurity standard. Birds were reared in SPF facilities with controlled ambient conditions with water and food supplied *ad libitum* during the experiments. At arrival, pools of spleen samples from 10 extra birds and feces on the transport boxes were analyzed by bacteriological culture and PCR for *inv*A gene (Dobhal et al., 2014) for confirmation of the *Salmonella sp.* free status of the chicken flock. Animal experimentation was approved by the Brazilian Committee of Animal Welfare and Ethics (permit number 00155813).

2.2. Bacterial strains and vaccines

The live vaccine (LV) consisted of the attenuated SGΔ*cob*SΔ*cbi*A (de Paiva et al., 2009; Penha Filho et al., 2010), resistant to kanamycin. An invasive highly pathogenic SG strain (SG 287/91), kindly donated by Prof. Paul Barrow (University of Nottingham, UK) was used to challenge birds. Bacterial cultures were prepared in Luria-Bertani (LB) broth (Invitrogen, USA) at 100 rpm in a shaking incubator at 37ºC/Overnight. The inocula for vaccination and challenge were previously counted by serial dilutions method to obtain the OD_{600} value corresponding to 10^8CFU/mL.

2.3. Experimental design

For each experiment three groups containing 30 birds each were formed. Chickens in vaccinated group (VAC) were immunized with the LV at 25 days of age and challenged at 45 days of age. Chickens in infected group (INF) were not vaccinated and were challenged at 45 days of age with wild SG287/91. Another group of 30 chickens was kept as negative control (unvaccinated and unchallenged) for the normalization of cytokine quantification. Twenty additional chickens in groups VAC and INF were used to record the mortality rates during 30 DPI. The vaccine and challenge inocula were administered orally, into the crop and consisted of 10^8 CFU of the corresponding bacteria, eluted on 1mL of Phosphate Buffer Saline (PBS) pH 7.4 (Merck, Germany). The same experimental designs were repeated to evaluate vaccine efficacy against SG, analyzing the bacterial numbers, clinical signs and mortality.

2.4. Sampling and bacteriology

At day 1 and 15 before infection (DBI) and 1, 3 and 7 DPI, five birds from each group were euthanized for sampling. Spleen and cecal tonsil samples were harvested, snap-frozen in liquid nitrogen and stored at -80ºC for cytokine quantification or used fresh for flow cytometry analysis and bacterial enumeration as described previously (Wigley et al., 2005). Blood was harvested and the sera was used for IgG and IgM quantification. SG numbers were expressed as Log_{10} per gram of tissue. Enriched positive samples ($\leq 10^2$ CFU/g) were expressed as 2 (Log_{10} of CFU/g) for calculations. To confirm the absence of SGΔ*cob*SΔ*cbi*A in internal organs at 1 DBI and 1 DPI, pools of liver, spleen and cecal tonsil samples from group VAC were enriched on Rappaport broth for 24h at 37C and plated in Brilliant Green agar containing kanamycin (30μg/mL), for selective growth of the LV strain. Additionally, a PCR to detect SGΔ*cob*SΔ*cbi*A was carried out with the enriched samples, according to methods previously described (Penha Filho et al., 2010).

2.5. Antibody production

Indirect Enzyme-linked immunosorbent assay (ELISA) using SG soluble protein antigen was applied to quantify IgG (IgY) and IgM in the sera of five birds at each moment, as previously described (Penha Filho et al., 2012). Briefly, SG was cultured in LB broth until OD600 reached 1, bacterial pellet was obtained after centrifugation for 10 min at 10000 *g* and washed in PBS pH 7.4, the bacterial pellet was suspended in 10 mL of PBS and submitted to sonication (Branson Sonifier 250, USA), using 8 cycles of 85 watts with 30s intervals. After sonication and centrifugation at 13000 *g* for 30 min, the supernatant containing SG soluble proteins was used as the soluble antigen diluted at 1:10000 for detection of IgG or IgM. Conjugated antibodies anti-chicken IgG and anti-chicken IgM were used at 1:1000 (Bethyl Laboratories, USA). The optical density values (OD) were used to calculate the adjusted *E* values using the following formula:

$$E\ value = (OD\ sample - OD\ negative\ control)/OD\ positive\ control - OD\ negative\ control)$$

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