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Oral vaccination with a live *Salmonella* Enteritidis/Typhimurium bivalent vaccine in layers induces cross-protection against caecal and internal organ colonization by a *Salmonella* Infantis strain

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ABSTRACT

Salmonella is an important zoonotic agent, and poultry products remain one of the main sources of infection for humans. *Salmonella* Infantis is an emerging serotype in poultry worldwide, reflected by an increased prevalence in poultry flocks, on broiler meat and in human foodborne illness cases. In the current study, the efficacy of oral administration of a live monovalent *Salmonella* Enteritidis and a live bivalent *Salmonella* Enteritidis/Typhimurium vaccine, against a *Salmonella* Enteritidis and Infantis infection, was determined. Oral administration of the live vaccines to day-old chickens caused a decrease in caecal colonization by *Salmonella* Enteritidis, but not Infantis, at day 7, when challenged at day 2. Vaccination with the bivalent vaccine at day 1 resulted in a decreased spleen colonization by both *Salmonella* Infantis and Enteritidis. Twice (at day 1 and week 6) and thrice vaccination (at day 1, week 6 and 16) of laying hens with the bivalent vaccine resulted in a decreased caecal colonization by *Salmonella* Enteritidis and Infantis, and significantly lower oviduct colonization levels by *Salmonella* Enteritidis. These data show cross-protection against *Salmonella* Infantis by oral administration of live vaccine strains belonging to other serogroups.

1. Introduction

Salmonella is one of the most important food-borne zoonotic agents worldwide. More than 99% of all isolated *Salmonella* strains belong to *Salmonella enterica* subspecies *enterica* (Fuche et al., 2016), further subdivided into serogroups on the basis of the structural variation in lipopolysaccharide structure, named O-antigens (Wang et al., 2002). The most common serogroups are A, B, C and D and are responsible for approximately 70% of all *Salmonella* infections in man and in animals (Zhou et al., 2014). Epidemiologic evidence shows that poultry-derived food, mainly eggs and egg products, but also chicken meat, are a very common source of human *Salmonella* infections (Barrow and Page, 2000). Serotypes such as *Salmonella* Enteritidis (serogroup D) and *Salmonella* Typhimurium (serogroup B) have been associated frequently with poultry product related outbreaks (Shah et al., 2017). Due to the implementation of monitoring and control programs in the poultry industry, including vaccination, there is a declining trend in the prevalence of *Salmonella* Enteritidis and *Salmonella* Typhimurium (O'Brien, 2013). A variety of other *Salmonella* serotypes also have been associated with human infections, albeit at lower frequencies. Serogroup C serotypes such as *Salmonella* Infantis have gained importance in layer-type chickens (Gole et al., 2014; Im et al., 2015; Iwabuchi et al., 2010; Murase et al., 2006) and broilers (EFSA, 2015) and have been contributing significantly to the numbers of multi-drug resistant (MDR) *Salmonella* (Gal-Mor et al., 2010; Liebana et al., 2004; Nogrady et al., 2012; Shah et al., 2017). The spread of closely related MDR clones of *Salmonella* Infantis throughout the food chain and then ultimately into humans is highly worrisome and require the necessity of strategies to reduce the prevalence of *Salmonella* Infantis within the food producing industry (Hindermann et al., 2017).

There are several vaccine types for *Salmonella* immunization of poultry ranging from vaccines containing live attenuated strains to inactivated (killed) vaccines and subunit vaccines. While live attenuated vaccine strains are widely used in layers, inactivated vaccines are more common in breeder flocks. Broiler flocks are not commonly vaccinated against *Salmonella*. Poultry vaccines available on the market are mostly based on *Salmonella* Enteritidis and Typhimurium strains. Vaccines against serogroup C strains are limited in use (Fuche et al., 2016). When chicks are given live vaccines early post-hatch, they are protected within 24 h against intestinal colonization by strains from the same serotype, a process that is called colonization-inhibition. This early protection can have value in all poultry production types, including

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broilers (Bohez et al., 2007; De Cort et al., 2013). Booster immunizations are used to decrease gut colonization, shedding, internal organ colonization and egg contamination in layers and breeders, based on stimulation of cell-mediated and humoral immune responses (Nandre et al., 2014; Nandre et al., 2011; Tran et al., 2010). The number of studies that evaluate cross-protection between serotypes, i.e. protection conferred by vaccines derived from specific serotypes, against challenge with strains from other serotypes, is limited (Deguchi et al., 2009; Pavic et al., 2010; Varmuzova et al., 2016). Assessing cross-protection is, however, crucial before making a decision to either develop serotype-specific vaccines or use current vaccines as cross-protective vaccines. The outcome can depend on the serotype that is targeted and the serotype or serogroup the vaccine is produced from.

In the present study we evaluated whether a live vaccine based on an attenuated strain of *Salmonella* Enteritidis and a live vaccine consisting of attenuated strains from the serotypes Enteritidis and Typhimurium are able to confer cross-protection against *Salmonella* Infantis. Different experimental setups were used, aiming to evaluate both early protection due to colonization-inhibition after single vaccination of day-old chicks and protection after double and triple vaccination regimens in layers.

2. Methods and techniques

2.1. Chickens

Newly hatched day-old Lohmann Brown laying type chicks were obtained from a local hatchery (De Biest, Kruishoutem, Belgium) and housed in containers on wood shavings. Commercial feed and drinking water were provided *ad libitum*. All the animals were confirmed to be *Salmonella*-free by bacteriological analysis of cloacal swabs. Experiments were performed with the permission of the Ethical Committee of the Faculty of Veterinary Medicine, Ghent University, Belgium (experiment authorization number EC2016/34)

2.2. Bacterial strains

The commercially available live, rifampicin resistant *Salmonella* Enteritidis vaccine strain Sm24/Rif12/Ssq (AviPro® *Salmonella* VacE, Lohmann Animal Health, Cuxhaven, Germany) and combined, rifampicin resistant *Salmonella* Enteritidis/Typhimurium vaccine strains Sm24/Rif12/Ssq and Nal2/Rif9/Rtt (AviPro® *Salmonella* Duo, Lohmann Animal Health, Cuxhaven, Germany), used for oral immunization, were suspended in sterile Hank's balanced salt solution (Invitrogen, Paisley, U.K.) according to the manufacturer's protocol to render a final concentration between 1×10^8 and maximal 6×10^8 colony forming units (cfu) of each strain per dose. Spontaneous nalidixic acid-resistant strains of *Salmonella enterica* subspecies *enterica* from different serotypes of the epidemiologically important serogroups C (Infantis SI1490) and D (Enteritidis SE147) were selected to facilitate enumeration. The bacteria used for infection of the birds were cultivated in Luria Bertani medium (Sigma, St. Louis, MO) at 37 °C. After overnight incubation, 10-fold dilutions were plated on brilliant green agar (BGA, Oxoid, Hampshire, UK) and incubated overnight to determine the titer. The cultures were in the meanwhile kept at 4 °C and afterwards diluted in phosphate-buffered saline (Sigma, St. Louis, MO, USA) to the desired cfu/ml.

3. Study design

Two experimental studies were set up in order to evaluate either the colonization-inhibition potential of the vaccines early post-hatch, or protection conferred by double or triple immunization regimens, against experimental infection with strains from two different *Salmonella* serotypes. In all experimental infection studies, chickens were orally administered 1 dose of the vaccine that was reconstituted in 200 µl for administration on day 1 and in 500 µl for administration at week 6 and week 16. The *Salmonella* challenge strains were diluted to the appropriate concentration and administered on day 2 at 200 µl and at week 7 and 17 at 500 µl per animal via gavage into the crop.

3.1. Early protection induced by AviPro®Salmonella VacE and AviPro® Salmonella Duo against infection with Salmonella Enteritidis and Infantis

Sixty one-day-old chicks were randomly divided into 6 groups of 10 animals. On day 1, two groups (20 animals) were given 1 dose of the AviPro® *Salmonella* Duo, two groups (20 animals) received 1 dose of the AviPro® *Salmonella* VacE while the animals from the last two groups (20 animals) were given water by oral gavage in the crop. Twenty-four hours later, one untreated group, one group treated with AviPro® *Salmonella* Duo and one group treated with AviPro® *Salmonella* VacE were challenged with 4.70×10^5 cfu *Salmonella* Enteritidis. Similarly, one untreated group, one group treated with AviPro® *Salmonella* Duo and one group treated with AviPro® *Salmonella* VacE were challenged with 2.37×10^5 cfu *Salmonella* Infantis. Cloacal swabs were taken before vaccination, before challenge and 24 hours after challenge to monitor shedding of both vaccine and challenge strains. Six days after the bacterial challenge, all chicks were euthanized and caecum and spleen were sampled for bacteriological analysis.

3.2. Protection induced by AviPro®Salmonella Duo against infection with Salmonella Enteritidis and Salmonella Infantis, after repeated administration of the vaccine strains

One hundred and sixty one-day-old chicks were randomly divided into 2 groups of 80 animals. On the first day, all chickens from group 1 were vaccinated with AviPro® *Salmonella* Duo. The chickens from the second group were kept as non-vaccinated controls.

At 6 weeks of age the vaccinated group (n = 80) received a booster treatment by vaccinating them with AviPro® *Salmonella* Duo. Seven days post vaccination, the chickens of the vaccinated and these of the non-vaccinated control group were each randomly split in 4 new groups of 20 animals, bringing the total number of groups to 8 with each 20 chickens. One vaccinated (n = 20) and 1 non-vaccinated control group (n = 20) were then challenged with 5×10^8 cfu *Salmonella* Enteritidis per bird. The other vaccinated (n = 20) and non-vaccinated control group (n = 20) were challenged with 4.6×10^8 cfu *Salmonella* Infantis. The other animals were left untreated. At 7 days post infection, the 40 challenged animals were euthanized and samples from caecum, spleen and liver were taken for bacteriological analysis. At 16 weeks, the 2 groups of 20 chickens that were vaccinated twice but remained uninfected were vaccinated a 3rd time with AviPro® *Salmonella* Duo. At seven days post vaccination, 20 animals of the triple vaccinated and 20 animals of the non-vaccinated group were challenged with 1×10^9 cfu *Salmonella* Enteritidis per bird. The other 20 birds of the triple vaccinated group and the animals from the non-vaccinated group were inoculated with 7×10^9 cfu *Salmonella* Infantis. As the *Salmonella* susceptibility decreases with age, the infection dose in this experiment was higher as compared to the first trial. At seven days post infection all animals were euthanized and samples from caecum, spleen, ovary and oviduct were taken for bacteriological analysis. Experimental design is summarised in Table 1.

3.3. Bacteriological analysis

The cloacal swabs, taken at the start of the experiment, were inoculated on BGA plates to evaluate the *Salmonella* status of the chickens. The cloacal swabs of the vaccinated and challenged animals were plated on BGA supplemented with 100 µg/ml rifampicin (Sigma, St. Louis, MO) to evaluate the shedding of the vaccine strains and on Xylose Lysine Deoxycholate agar (XLD, Oxoid, Basingstoke, UK) plates with 30 µg/ml nalidixic acid (Sigma, St. Louis, MO, USA) to quantify