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Combination of competitive exclusion and immunisation with a live *Salmonella* vaccine in newly hatched chickens: Immunological and microbiological effects

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ABSTRACT

In addition to evaluating the efficacy potential of a combined use of vaccination and competitive exclusion (CE) against *Salmonella* exposure in chicks at 3-days of age, a live *Salmonella* Enteritidis vaccine (SE-LV) and a CE culture were tested for their ability to induce parameters of the innate immunity.
Whereas the invasive SE-LV induced an influx of granulocytes and macrophages as well as an increased transcription of several cytokines in the caecal mucosa, the CE culture did not demonstrate any differences in these parameters compared to controls. It is therefore highly probable that the effects observed with CE cultures are not due to the rapid stimulation of the immune system. The combined use of both preparations did not result in an additive intestinal exclusion effect of the challenge strain more pronounced than that after single administration of the CE culture. The combined use of the *Salmonella* live vaccine and the CE culture resulted in an additive protective effect and prevented completely the systemic dissemination of the *Salmonella* challenge strain. To exploit the potential of combined use of CE and vaccination further and most effectively, live *Salmonella* vaccines are needed that are despite their attenuation in virulence still capable to induce both intestinal colonisation- and invasion-inhibition effects against *Salmonella* exposure.

1. Introduction

Poultry meat and eggs are considered to be the major source of *Salmonella* infection for humans (EFSA, 2013). In addition to efficient hygiene regimes at all stages of production, immunisation with both live and inactivated *Salmonella* vaccines and competitive exclusion (CE) represent the most important methods to increase the resistance of both very young and adult chickens (EFSA, 2004). CE cultures are effective against different serovars (Mead, 2000); however, the mechanisms of protection are not fully understood. Effects like the creation of a restrictive physiological environment, the competition for enteric receptor sites or with other microbes for nutrients, the production of antimicrobial compounds and the stimulation of the immune system are discussed (La Ragione and Mead, 2013).

Protective effects induced by vaccination of birds include the reduced intestinal colonisation and the diminished systemic invasion of *Salmonella* wild-type organisms by mechanisms of the adaptive immunity (EFSA, 2004). In general it is accepted that live *Salmonella* vaccines are more effective against intestinal and systemic infection than are inactivated vaccine preparations (Lillehoj et al., 2000). Moreover, live *Salmonella* vaccines are also capable of inducing protective mechanisms effective during the 'immunity gap', the time between administration of the vaccine and development of the adaptive immune response. The i) intestinal colonisation-inhibition effect (Barrow et al., 1987; Methner et al., 2011) and the ii) invasion-inhibition effect (Methner et al., 2010) have not been considered until now in the development of live *Salmonella* vaccines for chickens. The combined use of a CE culture and a live *Salmonella* vaccine which is able to induce both a colonisation-inhibition and an invasion-inhibition effect, may produce an additive protective impact more pronounced than the single use of either of these methods.

A registered live *Salmonella* Enteritidis vaccine is capable to stimulate a protective adaptive immune response in juvenile chickens (Springer et al., 2000); however, the vaccine has not yet been tested for its potential to induce colonisation- or invasion-inhibition effects. Apart from evaluating the possible protective effects after single and

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combined use of this live *Salmonella* Enteritidis vaccine and a commercial CE culture against *Salmonella* Enteritidis exposure in newly hatched chicks, the study also aims to identify parameters of the innate immune response which might bring about the effects observed.

2. Materials and methods

2.1. Chickens

Specific pathogen-free White Leghorn chickens were hatched at the facilities of the Friedrich-Loeffler-Institute from eggs obtained from Charles River Deutschland GmbH. Experimental and control groups were kept in cages in separate negative pressure rooms. Commercial feed (coarse meal without antibacterial additives) and public drinking water were both available ad libitum. The single groups were managed separately (including cleaning and feeding regimes) to prevent cross-contamination between the groups effectively throughout the trials. Animal experiments were performed in accordance with the German Animal Protection Act and approved by an ethical committee (Animal Ethics approval number: 04-005/11- 01 December 2011).

2.2. Bacterial strains and culture

Salmovac SE (IDT Biologika), a registered *Salmonella* Enteritidis live vaccine (SE-LV) strain (phage type PT4) was used for oral immunisation of the chickens on day 1 of life with or without combined use of a commercial CE culture (Aviguard, Microbial Developments Ltd.). To facilitate accurate enumeration of the vaccine strain in caecal contents and liver, a spontaneous nalidixic acid-resistant (N) mutant was produced for immunisation (Smith and Tucker, 1980). The resistance has no perceptible impact on the in vivo results (Barrow et al., 1987; Methner et al., 2010, 2011). To confirm this assumption, the original non-resistant SE-LV and the nalidixic acid-resistant SE-LV were compared using in vitro experiments for both, their adhesion and invasion in a cell-culture model as well as for their ability to inhibit the growth of other *Salmonella* organisms in nutrient broth (Methner and Barrow, 1997). Both variants of the SE-LV did not differ in these characteristics (data not shown). The viable count of the attenuated SE-LV administered PO via crop instillation was 2×10^8 colony forming units (cfu) per bird. The CE culture was dissolved in accordance with the manufacturer's instruction and administered via crop instillation. Oral infection of the chicken was carried out with a rifampicin (R) resistant variant (Methner et al., 2011) of the comprehensively characterised strain *Salmonella enterica* subspecies *enterica* serovar Enteritids 147 (SE 147R, phage type PT4) (Methner et al., 2010, 2011) at a dose of 2×10^5 cfu/bird. All strains had been stored in a Cryobank system (Mast Diagnostica) at −20 °C.

2.3. Experimental design and bacteriology

Caecal colonisation and systemic invasion of the attenuated SE-LV after single administration on day 1 of life as well as in combination with a CE culture (SE-LV on day 1 followed by the CE culture on day 2 of age) without *Salmonella* challenge was studied in experiment 1 (Table 1). SE-LV was enumerated in caecal contents and in liver at days 3, 4, 7, 8, 9, 10, 11, and 14 of life from 4 birds/group, respectively, by a standard plating method (Methner et al., 2001, 2010). Homogenised organ samples were diluted and plated on brilliant-green phenol red agar (SIFIN) with sodium nalidixate (50 μg/mL) and incubated at 37 °C for 18–24 h. Caecal contents and liver samples from all birds in groups A–D (Table 1) were also pre-enriched in buffered peptone water (SIFIN), incubated at 37 °C for 18–24 h and streaked onto brilliant-green phenol red agar with sodium nalidixate (SIFIN). Additionally, caeca from each animal of the groups administered the SE-LV alone or in combination with the CE culture, a control group and a group given the CE culture only were taken and frozen in liquid nitrogen or stored in RNAlater (Qiagen) until use for immunohistochemistry or studying mRNA expression of selected cytokines, respectively.

Table 1
Number (mean $\log_{10}$ cfu/g of 4 birds) of *Salmonella* Enteritidis live vaccine (SE-LV) in liver and caecal contents of chickens after oral administration of 2×10^8 cfu/bird at 1 day of age without or with subsequent application of a competitive exclusion (CE) culture at day 2 of age (experiment 1).

Day of age	Group A	Group B	Group C	Group D
1	CE culture	SE-LV	SE-LV	–
2	–	–	CE culture	–

Day of age	Group A		Group B		Group C		Group D	
	Liver	Caecal contents	Liver	Caecal contents	Liver	Caecal contents	Liver	Caecal contents
3	–	–	2.5	8.5	2.7	8.9	–	–
4	–	–	3.3	8.7	3.0	8.4	–	–
7	–	–	3.0	7.9	2.8	7.6	–	–
8	–	–	2.8	8.4	2.8	6.9[b]	–	–
9	–	–	2.5	7.9	2.7	6.1[b]	–	–
10	–	–	1.6	6.7	1.8	4.7[b]	–	–
11	–	–	1.6	6.6	1.7	4.9[b]	–	–
14	–	–	1.3	7.3	1.3	5.7[b]	–	–

Standard error: liver: 0.199; caeca: 0.357.
[b] Significantly lower than group B.
[c] Significantly lower than C.

In experiment 2 the protective effect induced by the CE culture and the SE-LV alone or after combination of both (SE-LV on day 1 followed by the CE culture on day 2 of age) against challenge with SE 147R administered on day 3 of life was compared with an untreated control group (Table 2). The challenge strain was enumerated in caecal contents and liver from 4 birds/group at days 4, 5, 8, 9, 10, 11, 12, and 15 of age using a standard method described above.

2.4. Immunohistochemistry

Using immunohistochemistry, the invasion of the SE-LV into lower regions of the caecal mucosa as well as the influx of granulocytes and macrophages into the caecum was examined in experiment 1. Cryostat sections of 7 μm thickness of every chicken caecum were prepared and

Table 2
Number (mean $\log_{10}$ cfu/g of 4 birds) of *Salmonella* Enteritidis 147R (SE 147R; 2×10^5 cfu/bird PO at 3 days of age) in liver and caecal contents of chickens after pre-treatment with a competitive exclusion (CE) culture or a *Salmonella* Enteritidis live vaccine (SE-LV) (2×10^8 cfu/bird PO at 1 day of age) without or with subsequent application of a CE culture at day 2 of age (experiment 2).

Day of age	Group A	Group B	Group C	Group D
1	CE culture	SE-LV	SE-LV	–
2			CE culture	–
3	SE 147 R	SE 147 R	SE 147 R	SE 147 R

Day of age	Group A		Group B		Group C		Group D	
	Liver	Caecal contents	Liver	Caecal contents	Liver	Caecal contents	Liver	Caecal contents
4	0	2.9[b,d]	0	4.7[d]	0	3.5[d]	0.3	7.7
5	0	3.8[d]	0	4.2[d]	0	2.8[d]	0.3	6.5
8	0.3[d]	4.3[d]	0[d]	6.4	0[d]	4.6	1.8	6.9
9	0.5[d]	4.2[b,d]	0[d]	7.0	0[d]	4.1[b,d]	1.9	7.0
10	0[d]	3.6[b,d]	0[d]	6.5	0[d]	4.6[d]	1.6	7.0
11	0.6[d]	4.1[d]	0.5[d]	5.3	0[d]	3.8[d]	2.2	6.9
12	0.7[d]	3.8[b,d]	0.4[d]	6.4	0[d]	3.6[b,d]	1.9	7.4
15	0.3[d]	4.0[d]	0.3[d]	5.7	0[a,d]	3.3[d]	1.5	6.6

[a] Significantly lower than group A.
[b] Significantly lower than group B.
[c] Significantly lower than group C.
[d] Significantly lower than group D.