Invasion inhibition effects and immunogenicity after vaccination of SPF chicks with a *Salmonella Enteritidis* live vaccine

Nachweis von Invasionshemmeffekten und der Immunogenität nach Impfung von SPF-Küken mit einer *Salmonella-Enteritidis*-Lebendvakzine

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**ZUSAMMENFASSUNG**

**Gegenstand und Ziel** Für den Eintrag von Salmonellen in die Lebensmittelkette des Menschen haben Infektionen der Hühner mit *Salmonella Enteritidis*, *Salmonella Typhimurium* und *Salmonella Infantis* große Bedeutung. Zur Bekämpfung von Infektionen werden, zusätzlich zu veterinärhygienischen Maßnahmen, inaktivierte Impfstoffe und Lebendimpfstoffe auf der Basis attenuierter Impfstämme eingesetzt. Neben einer adaptiven Immunantwort induzieren bestimmte Impfstämme angeborene Immunmechanismen, die eine Invasion des Erregers in innere Organe verhindern bzw. behindern. Es ist ungeklärt, ob sich diese Hemmungseffekte auch gegen andere *Salmonella*-Serovare richten. Weiterhin ist nicht bekannt, ob sich die adaptive Immunantwort nach Impfung mit einem *Salmonella-Enteritidis*-Phagentyp-4-Lebendimpfstoff auch auf andere Lysotypen von *Salmonella Enteritidis* und *Typhimurium* erstreckt.

**Material und Methode** Eintagsküken wurden mit einer *Salmonella-Enteritidis*-Lebendvakzine oral vakziniert. Zur Prüfung des Auftretens der Invasionshemmung wurden die Tiere am Tag darauf mit einem markierten *Salmonella-Typhimurium*- bzw. *Salmonella-Infantis*-Stamm infiziert. Zum Nachweis der adaptiven Immunantwort erfolgte nach der Impfung eine orale Infektionsbelastung mit markierten *Salmonella-Enteritidis*-Stämmen der Phagentypen 1, 8 und 21 am 63., 42. und 51. Lebenstag sowie einem monophasischen *Salmonella-Typhimurium*-Stamm („Definitive Type“ 193) am 17. Lebenstag. Nach der Infektion wurde zu verschiedenen Zeitpunkten der Infektionsstammgehalt in Leber und Blinddarmgewebe ermittelt und mit den Werten einer nicht geimpften Kontrollgruppe verglichen.

**Ergebnisse** Die Impfung mit dem *Salmonella-Enteritidis*-Lebendimpfstoff führte zu einer signifikanten Reduzierung der Invasion in die Blinddarmschleimhaut und Leber nach früher Infektion mit *Salmonella Typhimurium* und *Salmonella Infantis*. Weiterhin induzierte die Impfung eine adaptive Immunität gegen die getesteten „Non-Phagentyp-4“-*Salmonella-Enteritidis*-Stämme und den monophasischen *Salmonella-Typhimurium*-Stamm.
Introduction

Despite intensive control measures to reduce Salmonella infection in poultry and humans in the last decades, 91,857 confirmed cases of salmonellosis in humans were reported in the EU countries in 2018 [1]. Although the prevalence of Salmonella in eggs and egg products has been markedly reduced, these are still the most common causes of Salmonella cases in humans. The 3 most commonly reported Salmonella serovars in humans were Salmonella Enteritidis, Salmonella Typhimurium, and Salmonella Infantis [1]. These serovars have been associated with broiler meat [1].

Historically, human infections with S. Enteritidis were predominantly phage type 4 (PT4) [2]. However, other phage types like PT1, PT8, PT14b and PT21 (non-PT4 strains) have played an important role [2][3], as well.

In addition to hygiene measures at all levels of production, live attenuated inactivated and attenuated vaccines against S. Enteritidis and S. Typhimurium have proven to be effective in chicken flocks for many years. It is generally accepted that live attenuated vaccines are not only directed against other phage types of Salmonella Typhimurium, but also directed against other phage types of Salmonella Enteritidis and Typhimurium. In contrast, the occurrence of an adaptive immune response has been shown several times for different vaccines by different authors [6][7][8][9][10].

The aim of our study was to test whether the homologous invasion-inhibition effect described for the SE-LV [5] can also be demonstrated against other serovars (S. Typhimurium and S. Infantis). In addition, we examined whether the SE-LV based on PT4 also induces immunogenicity against other non-PT4 strains and a monophasic S. Typhimurium strain.

Materials and methods

Chickens

Specific pathogen-free day-old White Leghorn chicks were hatched from eggs, which were obtained from VALO BioMedia GmbH (Osterholz-Scharmbeck, Germany). The vaccinated and control groups were kept in cages in separate rooms to avoid the spread of the vaccine strain to the control groups. During the rearing period, commercial feed for hens without feed additives (ssnif Special diets GmbH, Soest, Germany) and drinking water were available ad libitum. The experiments were performed in accordance with the German law of animal welfare (registration number IDT-A042-2015).

Bacterial strains and culture

Vaccine strain

In all trials, SPF chickens were orally vaccinated using a buttoned cannula with the minimum effective dose (1 × 10⁸ cfu per animal)
of the attenuated and auxotrophic (adenine, histidine) S. Enteritidis PT4 strain no. 441/114 (Salmovac 440 [Salmovac SE], Ceva Santé Animale, Libourne, France) (SE-LV).

Challenge strains
To examine the heterologous invasion inhibition effect of the SE-LV, a nalidixic acid resistant S. Typhimurium strain Definitive Type (DT) 204 (FLI no. 421) and a multidrug-resistant S. Infantis strain (RKI no. 12–2357) were used.

The immunogenicity of the SE-LV against certain non-PT4 strains was examined by using spontaneously occurring nalidixic acid resistant S. Enteritidis strains: PT1 (RKI no. 06–7328), PT8 (RKI no. 04–7681) and PT21 (RKI no. 04–2049).

The monophasic S. Typhimurium strain (4,[5],12:i:-) DT 193 resistant to ampicillin, streptomycin, sulfamerazine, and oxytetracycline was used to demonstrate cross immunogenicity of the SE-LV against a monophasic S. Typhimurium strain. This strain was described in detail by Trüpschuch et al. [11].

Cultivation of the infection strains was carried out over 2 pre-cultures in STM 6/83 Medium (IDT Biologika GmbH, Dessau-Roßlau, Germany). After cultivation the strains were washed, concentrated and stored at –20°C until use.

Experimental design
All trials were designed as randomized, blinded and controlled studies.

Invasion inhibition trials
A total of 12 day-old chicks per group were vaccinated orally with the SE-LV at day 1 of age. A corresponding control group remained unvaccinated (► Table 1). On day 2 of age the chickens were challenged orally at a dose of $5 \times 10^5$ cfu S. Typhimurium no. 421/bird or at a dose of $5 \times 10^4$ cfu S. Infantis no. 12–2357/bird using a buttoned cannula. On days 6 and 9 (10) of age, the animals were euthanized and the number of challenge strain bacteria in liver and cecal tissue (complete cecum without ingesta) was determined.

Immunogenicity trials
For testing of the immunogenicity against S. Enteritidis non-PT4 strains 8 birds/group were vaccinated orally with the SE-LV on days

| Trial | Group | Animals (n) | Vaccination | Challenge | Quantitative determination of challenge bacteria |
|-------|-------|-------------|-------------|-----------|-----------------------------------------------|
| 1     | Vaccinated | 12 | Day 1 | Day 2 (STm) $5 \times 10^5$ cfu/chicken | Days 6 and 10 |
|       | Control   | 12 | –     | Day 2 (SI) $5 \times 10^4$ cfu/chicken | Days 6 and 9 |
| 2     | Vaccinated | 12 | Day 1 | Day 2 (SI) $5 \times 10^4$ cfu/chicken | Days 6 and 9 |
|       | Control   | 12 | –     | Day 2 (SI) $5 \times 10^4$ cfu/chicken | Days 6 and 9 |

STm = S. Typhimurium, SI = S. Infantis, Day = day of age

| Trial | Group | Animals (n) | Vaccination | Challenge | Quantitative determination of challenge bacteria |
|-------|-------|-------------|-------------|-----------|-----------------------------------------------|
| 3     | Vaccinated | 8 | Days 2 and 15 | Day 42 (SE PT8) $1 \times 10^9$ cfu/chicken | Day 49 |
|       | Control   | 8 | –     | Day 51 (SE PT21) $1 \times 10^9$ cfu/chicken | Day 58 |
| 4     | Vaccinated | 8 | Days 2 and 15 | Day 51 (SE PT21) $1 \times 10^9$ cfu/chicken | Day 58 |
|       | Control   | 8 | –     | Day 63 (SE PT1) $1 \times 10^9$ cfu/chicken | Day 58 |
| 5     | Vaccinated | 8 | Days 2 and 15 | Day 63 (SE PT1) $1 \times 10^9$ cfu/chicken | Day 58 |
|       | Control   | 8 | –     | Day 63 (SE PT1) $1 \times 10^9$ cfu/chicken | Day 58 |
| 6     | Vaccinated | 16 | Day 1 | Day 17 (mSTm) $1 \times 10^9$ cfu/chicken | Days 24 and 31 |
|       | Control   | 16 | –     | Day 17 (mSTm) $1 \times 10^9$ cfu/chicken | Days 24 and 31 |

SE = S. Enteritidis, PT = phage type, mSTm = monophasic S. Typhimurium strain, Day = day of age
2 and 15 of age. Corresponding control groups were not vaccinated (Table 2). Both the vaccinated and non-vaccinated control groups were challenged orally using a buttoned cannula with Salmonella Typhimurium (DT 193), with S. Enteritidis PT21 on day 51 and with S. Enteritidis PT1 on day 63 of age. The infection dose was $1 \times 10^9$ cfu per animal on day 17 of age (trial 6). The animals of the vaccinated group (n = 16) and the unvaccinated control group (n = 16) were orally infected with $1 \times 10^9$ cfu per animal. The animals were stunned by head impact followed by exsanguination 7 days (trials 3–5) post challenge (dpc). Liver and cecal mucosa were taken from each animal for determination of the number of the challenge strain bacteria. Blood samples were taken for serological testing for antibodies against S. Enteritidis on the day of challenge and necropsy, respectively.

To prove the immunogenicity against monophasic S. Typhimurium (DT 193), only a single oral vaccination was carried out on day 1 of age (trial 6). The animals of the vaccinated group (n = 16) and an unvaccinated control group (n = 16) were orally infected with $1 \times 10^9$ cfu per animal on day 17 of age (Table 2). The quantification of the Salmonella challenge strain in the liver and cecal tissue (complete cecum without ingesta) was performed on days 24 and 31.

Quantitative determination of the challenge strain content in liver and cecal mucosa

The quantification of the Salmonella challenge strains ($\log_{10}$ cfu/g) in the liver and cecal tissue was carried out using the plate counting method as previously described by Methner et al. [12]. In brief, the intestinal content was removed from the ceca. The livers, ceca (trials 1, 2 and 6) or cecal mucosa (trials 3–5) were respectively weighed and subsequently homogenized (Ultra-Turrax T25, Janke & Kunkel, IKA®-Labortecnik, Staufen, Germany). A 10-fold dilution series was prepared for enumeration of the Salmonella challenge strain of the processed organs. The number of bacteria was then estimated by spreading out the homogenate on deoxycholate citrate agar according to Leifson (Merck, Darmstadt, Germany) supplemented with antibiotics (50 μg/l nalidixic acid [S. Typhimurium 421, S. Enteritidis PT8, 1 and 21]; 500 μg/l streptomycin [S. Infantis]; 50 mg/l ampicillin, 500 μg/l streptomycin, 100 mg/l sulfamerazine and 10 mg oxytetracycline [monophasic S. Typhimurium]). The incubation was performed at 37 ± 1 °C for 24 hours under aerobic conditions. Samples which failed to grow were enriched in Rappaport Vassiliadis Broth (Merck, Darmstadt, Germany) at 37 ± 1 °C for 24 hours and spread on the above-mentioned agar. Samples that were only positive after enrichment received a $\log_{10}$ = 1. Samples that showed no growth received a $\log_{10}$ = 0. The mean and standard deviation were calculated for each group. Statistical evaluation was performed using the Mann-Whitney U-test (level of significance $p < 0.05$) (GraphPad Prism version 8.4.3 for Windows, GraphPad Software, San Diego, California USA, www.graphpad.com).

Serological testing

Serum was obtained after centrifugation of blood samples for 10 minutes and 3500 × g at 2–8 °C. For the detection of Salmonella-specific antibodies (group D), serum samples were tested using a commercially available ELISA kit (Chicken Salmonella Antibody Test Kit Group D, BioChek, Reeuwijk, The Netherlands) according to the manufacturer’s instructions. Results were recorded as sample to positive (S/P) ratios determined by the ratio between the mean optical density (OD) of each sample and the mean OD of the positive control.

Results

Invasion inhibition effect

Post infection, the challenge strains were isolated in the liver and cecal tissue of the control groups. In the vaccinated groups, the corresponding challenge strain was only detected in the cecal tissue. However, clinical signs were not observed in any chicken. The results of quantitative bacteriological examination of the liver and cecal tissue of the vaccine and control group animals are shown in Table 3. After infection with S. Typhimurium, significantly lower bacterial counts were found in the livers of the vaccine group animals compared to the control group chicks (8 dpc, $p = 0.0011$) in addition to the cecal tissue (4 dpc, $p = 0.0465$; 8 dpc,

| Trial | Group | Challenge strain | Day post challenge | Challenge strain content (mean ± SD) in $\log_{10}$ cfu/g |
|-------|-------|------------------|-------------------|-----------------------------------------------------|
|       |       |                  |                   | Liver                                                |
|       |       |                  |                   | Cecal tissue                                        |
| 1     | Vaccinated | STm            | 4                 | 0                                                    |
|       | Control       |                | 4                 | $1.09 \pm 1.74$                                       |
|       | Vaccinated       |                | 8                 | $0^*$                                                |
|       | Control       |                | 8                 | $2.30 \pm 0.76$                                       |
| 2     | Vaccinated | SI              | 4                 | 0                                                    |
|       | Control       |                | 4                 | $0.43 \pm 0.78$                                       |
|       | Vaccinated |                | 7                 | $0^*$                                                |
|       | Control       |                | 7                 | $0.67 \pm 0.67$                                       |

$^* \text{Mann-Whitney test (one sided)} \ p < 0.05, \ STm = S. \ Typhimurium, \ SI = S. \ Infantis$
Significantly lower invasion of the cecal tissue was also observed after infection with *S. enteritidis* on 4 dpc (p = 0.0055) and in both the liver and cecal tissue on 7 dpc (liver: p = 0.0350, ceca: p = 0.0006).

**Immunity against non-PT4 *S. Enteritidis* strains and monophasic *S. Typhimurium***

After infection with the different phage types of *S. Enteritidis* and the monophasic *S. Typhimurium* strain, no symptoms associated with the infection were detected in the chickens.

The results of the mean log_{10} cfu/g of the challenge strain in liver and cecal tissue of the vaccine groups compared to the control groups after infection with the different *S. Enteritidis* phage types and the monophasic *S. Typhimurium* strain are shown in Table 4.

The mean log_{10} cfu/g of the challenge strain in liver and cecal tissue of the vaccinated animals was significantly lower after infection with *S. Enteritidis* PT8 (liver: reduction 1.65 log_{10}, p = 0.0001, cecal tissue: reduction 0.91 log_{10}, p = 0.0003), PT21 (liver: reduction 1.02 log_{10}, p = 0.0048, cecal tissue: reduction 0.85 log_{10}, p = 0.0190) and PT1 (liver: reduction 1.40 log_{10}, p = 0.0001, cecal tissue: reduction 0.77 log_{10}, p = 0.0325) when compared to the controls.

After infection with the monophasic *S. Typhimurium* strain, significant differences in the number of the challenge strain bacteria in liver and cecal tissue were found in vaccinated chickens compared to the controls on both 7 dpc (liver: reduction 0.87 log_{10}, p < 0.0001, cecal tissue: reduction 0.49 log_{10}, p = 0.023) and 14 dpc (liver: reduction 0.63 log_{10}, p = 0.020, cecal tissue: reduction 0.60 log_{10}, p = 0.032).

The results of the serological examination after vaccination and challenge with *S. Enteritidis* PTs 1, 8 and 21 are shown in Fig. 1. Immediately before the challenge and on day 7 after the challenge (day of necropsy), higher antibody titers occurred in the vaccine groups than in the corresponding control groups. Before challenge with *S. Enteritidis* PT1 (trial 5), the difference was found to be significant (p = 0.0065). At necropsy significant differences (p = 0.0047) occurred after challenge with *S. Enteritidis* PT8 (trial 3). Overall, the vaccination only resulted in a negligible increase in antibody titers. A total of only 2 out of 48 vaccinated animals (all 3 trials together) showed antibodies above the cut off (SP ≥ 50 %) before the challenge.

**Discussion**

After infection with *Salmonella* wild-type strains, phagocytes of the intestinal mucosa release reactive oxygen intermediates (ROI). According to Sterzenbach et al. [13] these processes are directed against the invasion of the pathogen and are an expression of innate immunity. Optimally, live vaccines should also induce ROI, which are directed against invasion of infectious strains after early infection. However, studies confirming these assumptions are currently lacking.

Braukmann et al. [5] showed an influx of neutrophils and macrophages into the lamina propria of the cecal mucosa after vaccination of day-old chicks with an attenuated SE-LV (Salmovac SE, Ceva Santé Animale, Libourne, France). This was associated with a strong elevated transcription of the pro-inflammatory mediators iNOS, IL-1β, LITAF, and IL12 which led to an inhibition of invasion and short-term inhibition of colonization after challenge with a *S. Enteritidis* wild-type strain.

In the present study, the invasion-inhibition effect could also be demonstrated after infection with *S. Typhimurium* and *S. Infantis*. Eeckhaut et al. [14] showed an invasion-inhibition effect against *S. Enteritidis* and *S. Infantis* after vaccination with a combined *S. Enteritidis*/*S. Typhimurium* live vaccine. After vaccination with a *S. Enteritidis* live vaccine alone, this effect was not present. Only both vaccines in combination showed a significant reduction of colonization of ceca (tissue and content) after infection with *S. Enteritidis* but not after infection with *S. Infantis*. In comparison to the re-
The present study demonstrates that a reduction of invasion of the cecal mucosa by

$S. Typhimurium$ and $S. Infantis$ wild-type strains occurs also after immunization with SE-LV. The challenge strain used [16] and certainly the vaccination and infection doses can influence these effects. To compare our results with those of Braukmann et al. [5], how long these effects last and what significance they have for the field should be further examined.

The evidence of an adaptive immune response against $S. Enteritidis$ PT4 after vaccination with a SE-LV (Salmova SE, Salmo- vac 440, both Ceva Santé Animale, Libourne, France) was demonstrated by Springer et al. [17], Springer et al. [9][18], Atterbury et al. [19] and Methner [7]. Furthermore, Theuß et al. [10] showed the efficacy of the vaccine after challenge with a $S. Enteritidis$ PT4 strain according to the qualitative method described in the European Pharmacopoeia monograph 04/2013:2520.

In this study, immunity after vaccination with the SE-LV was also shown against PT1, 8 and 21 of $S. Enteritidis$. The differences in the quantification of the challenge strain in liver and cecal tis-

sue between the vaccinated and the control group correspond to the results of Springer et al. [18] after infection with virulent $S. Enteritidis$ PT4 strains.

Effects of cross-protection against $S. Typhimurium$ have been demonstrated by Springer et al. [9] and for another SE-LV by Nandre et al. [20]. Results of this study show that a significant reduc-
tion of invasion of cecal tissue and liver also occurred after infec-
tion with a monophasic $S. Typhimurium$ strain (DT 193). A direct

comparison with the above-mentioned examinations is not possible, as the trials differ in the number of vaccinations and/or the infection model used.

Arnold et al. [21] demonstrated a reduction of eggshell contamination after infection with $S. Enteritidis$ (PT14b), a biphasic $S. Typhimurium$ strain (DT 8) and monophasic $S. Typhimurium$ strains after vaccination with inactivated and live vaccines based on $S. Enteritidis$ and $S. Typhimurium$, as well as by combinations of live and inactivated vaccines. However, they could not show a significantly reduced excretion of the challenge strains, measured by the number of $Salmonella$ positive cloacal swabs, independently from the different vaccination regimen. This contrasts with the results of Theuß et al. [10], showing a significant and relevant reduction of the number of positive cloacal swabs after threefold vaccination with a SE-LV.

An unequivocal conclusion to this question is not possible because so many factors can influence the excretion rate including the vaccine and vaccination schedule, the challenge strain, the challenge dose, and individual animal factors. Nevertheless, Arnold et al. [21] recommend the use of vaccination against $S. Enteritidis$, $S. Typhimurium$ and monophasic $S. Typhimurium$ strains. As the contamination of the eggshells also depends on other factors [22] the use of vaccines should always be combined with veterinary hygiene measures like cleaning and disinfection in addition to rodent control.

The animals used to demonstrate immunogenicity against the $S. Enteritidis$ non-PT strains in this trial were $Salmonella$ negative and kept under isolated conditions. Thus it can be assumed that the $Salmonella$ group D specific antibodies detected are antibodies against $S. Enteritidis$. The increase of the specific antibody titers after vaccination with the SE-LV and subsequent challenge with virulent $S. Enteritidis$ strains corresponds to reports from Methner [7], Springer et al. [9] and Theuß et al. [10]. The increase after vaccination is usually much lower than after infection. This is most likely related to the attenuation of the vaccine strain.
CONCLUSION FOR PRACTICE
As chicks are hatched without any substantial gut flora, they are very susceptible to Salmonella infections in their first few days of life. The results of this study demonstrate that vaccination with a Salmonella Enteritidis live vaccine provides early and not exclusively homologous protective effects. As Salmonella Enteritidis non phage type 4 strains and monophasic Salmonella Typhimurium strains are increasingly important in laying hen farms, the demonstration of immunogenicity against these variants after vaccination with a live Salmonella Enteritidis phage type 4 vaccine is of increased relevance for the field.

Conflict of interest statement
The authors are employed by Ceva Santé Animale, which distributes the S. Enteritidis live vaccines Salmovac SE and Salmovac 440. The content or opinions expressed in the manuscript have not been influenced by any proprietary, financial, professional or other personal interests.

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