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Case report

Reduction of *Salmonella* prevalence at slaughter in *Lawsonia intracellularis* co-infected swine herds by Enterisol® Ileitis vaccination

J. Meschede [a], S. Holtrup [b], R. Deitmer [b], A.P. Mesu [b], C. Kraft [c,*]

[a] *Vivet Schweinegesundheit GmbH, Kahrweg 33, 59590 Geseke, Germany*
[b] *Boehringer Ingelheim Vetmedica GmbH, Binger Str. 173, 55216 Ingelheim, Germany*
[c] *Boehringer Ingelheim Veterinary Research Center GmbH & Co. KG, Bemeroder Str. 31, 30559 Hannover, Germany*

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ABSTRACT

Salmonella spp. remains a wide-spread pathogen among pig herds and its control has major impact on food borne *Salmonella* infections in humans. The objective of the study was to investigate the influence of an Enterisol® Ileitis vaccination on *Salmonella* seroprevalence in *Lawsonia intracellularis* (*L. intracellularis*) and *Salmonella* spp. co-infected pig herds under field conditions.

This study compared vaccinated and non-vaccinated pigs of consecutive piglet batches, housed on four different finishing units. Prior to study start, endemic field infections of *L. intracellularis* and *Salmonella* spp. were confirmed by serology in the nursery and in all finishing units. Field infection of *L. intracellularis* occurred at the middle of the nursery phase.

In total twenty-five batches of finishing pigs were included in the study, pigs were investigated for four (non-vaccinated group, n = 9) or six months (vaccinated group, n = 16). The primary outcome parameter was *Salmonella* serology (antibody titers) at the end of fattening. Secondary parameters comprised serology for *L. intracellularis* and performance parameters, including average daily weight gain (g), duration of fattening period (days), feed conversion (kg/kg) and mortality (%).

A total of 709 blood samples were assessed, deriving from vaccinated (n = 439) and non-vaccinated finishing pigs (n = 270). Evaluation of the antibody titers demonstrated that vaccination significantly reduced the *Salmonella* seroprevalence in the finishing pigs on all four farms. The average OD% values were reduced from 32.7% to 13.4% in addition to a reduced variability in the vaccinated pigs compared to the control group. The *Salmonella* category of all finishing farms improved by at least one category in accordance with the German *Salmonella* monitoring program. In addition, vaccination had a positive tendency on the average daily weight gain, fattening duration and reduced mortality.

In conclusion, this study demonstrates that vaccination with Enterisol® Ileitis has a positive and direct impact on reduction of *Salmonella* infection in co-infected herds.

1. Introduction

Salmonellosis, caused by different serovars of *Salmonella enterica*, is a common zoonosis worldwide and an important cause of food-borne illness. Therefore, effective control measures are important to reduce the incidence of infected herds and by that the economic burden through infection. The mean *Salmonella* prevalence in slaughter pigs is calculated to be 10.3 % with a wide variation among European states (0–29 %) with *S. Typhimurium* (90.7 %) and *S. Derby* (5.4%) among the most common serovars isolated from pork and pig farms (EFSA, 2013) (Powell et al., 2016). Current interventions have had little effects on the prevalence of this pathogen in swine or on the incidence of diseases in humans (Majowicz et al., 2010) thus, improved strategies to reduce the prevalence of *S. enterica* in swine are needed.

Risk factors for *Salmonella* shedding by pigs at the end of the finishing period have been identified, including concurrent infections with other pathogens such as *L. intracellularis* (Beloeil et al., 2004). It has been shown previously that co-infection of *Salmonella* spp. and *L. intracellularis* is commonly found in swine (Moller et al., 1998; Stege et al., 2000) as both pathogens share a similar infection dynamic within a herd (Brandt et al., 2010; Kranker et al., 2003). In addition, *L. intracellularis* infection has been found to promote prolonged shedding of *S. Typhimurium* and

* Corresponding author.
E-mail address: chrkraft@gmx.de (C. Kraft).

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contributing to the development of a carrier state of *S. enterica* in pigs (Patterson et al., 2016). This significant association between infections with *L. intracellularis* and carriage of *S. enterica* has led to the hypothesis that *L. intracellularis* infection of pigs increases the risk of salmonellosis in humans (EFSA, 2012). Recent studies determined the effect of vaccination against *L. intracellularis* on shedding of *S. enterica* in co-infected pigs. Enterisol® Ileitis is a licensed oral, live attenuated vaccine that confers reduction of intestinal lesions caused by *L. intracellularis* as well as loss of weight gain associated with the infection. Under experimental conditions, vaccination against *L. intracellularis* significantly reduces the shedding of *S. enterica* in co-infected pigs, reduces the number of shedders and the spread to pen-mates (Visscher et al., 2018). Furthermore, vaccination with live attenuated *L. intracellularis* modulates the gut microbiota in order to reduce the ability of *Salmonella* to colonize in the acute phase of the infection (Leite et al., 2018; Visscher et al., 2018).

The objective of the study was to investigate the efficacy of Enterisol® Ileitis vaccination to reduce the *Salmonella* seroprevalence in *L. intracellularis* and *Salmonella* spp. co-infected pig herds under normal husbandry conditions.

2. Methods

2.1. Selection of animals

The serological *Salmonella* status between vaccinated and non-vaccinated pigs from four different finishing units was compared at the end of the finishing period. All four finishing farms received the piglets at approx. 28 kg bodyweight, from the same nursery unit comingling piglets from three different breeders. At the finisher farms A to D, animals were raised until slaughter. The finishing farms had a history of *Salmonella* infection with an increasing percentage of *Salmonella* positive rated animals ahead of the start of the study. During the course of the study no confounding changes were introduced on the farms and all animals were raised under the same conditions.

A total of 709 animals from 25 batches (approx. 28 animals/batch) were analyzed for their *Salmonella* status at slaughter. Depending on the finisher farm a batch size varied between 216 and 500 animals. Vaccination with Enterisol® Ileitis (Boehringer Ingelheim Vetmedica GmbH, Germany) was performed via drinking water at 4 days post weaning (approximately 27 days of age) in the nursery unit according to the manufacturer's recommendation followed by antibiotic treatment (n = 16 finishing batches, 439 animals). The control group of non-vaccinated pigs came from previous finishing batches of the respective farms over a representative length of time (n = 9 finishing batches, 270 animals). All samples were collected from consecutive batches over a time period of ten month. Study animals were subject to slaughter at the same time than all batch-mates, hence the study animals were sampled at the commercial end of their lifespan (end of fattening) and not slaughtered premature due to their participation in the study. To be precise, study animals were only identified and included in the study on their pre-set day of slaughter. Prior to study start, natural co-infection with *L. intracellularis* and *Salmonella* spp. was confirmed in all four finishing units. In all farms, high serological titers for *Salmonella* spp. were detected and *L. intracellularis* field infection was observed at the middle of the nursery period by seroconversion and positive fecal samples. *S. Typhimurium* was isolated from fecal samples of the respective breeding sows, the nursery and finishing pigs.

2.2. Blood sampling

For pre-screening prior to study start, blood sampling via venous puncture was performed according to good veterinary practice at the farm of origin as part of routine veterinary herd care. Blood sampling at slaughter was done during exsanguination for animal welfare reasons by a trained veterinarian. Whole blood was allowed to clot at room temperature. Serum was separated, aliquoted and stored at 4 °C until further use.

2.3. ELISA testing

Serum samples were analyzed by ELISA for the presence of *Salmonella* spp. antibodies using a validated, commercially available test kit (IDEXX Swine *Salmonella* Ab Test) according to the manufacturer's instructions. The test kit is approved by the German QS scheme for *Salmonella* monitoring. Results of ELISA tests were reported as optical density (OD) values in respect to an integrated positive control (OD%). The limit of detection is set to 0.1x of the positive control (OD10%).

Serum samples were assayed by ELISA for the presence of *L. intracellularis* antibodies using a validated, commercially available test kit (Svanovir *L. intracellularis*/Ileitis-AB, Boehringer Ingelheim Svanova) used according to the manufacturer's instructions.

2.4. Risk class assessment

Salmonella monitoring by the QS scheme aims to identify farms with high risk of *Salmonella*, which increase the risk of contaminating meats at the time of processing (www.q-s.de/en). The QS scheme rates samples tested with an accredited ELISA assay as positive or negative. The cut-off value used for this purpose is OD40%, giving all samples tested with an OD% value less than 40 a negative and above 40 a positive rating. The percentage of samples assessed as positive categorizes each herd in one out of three categories with economic impact:

Category I: low *Salmonella* risk, if $\leq$ 20% of samples are positive,
Category II: medium *Salmonella* risk, if $>$ 20 and $\leq$40 % of samples are positive,
Category III: high *Salmonella* risk, if $\geq$ 40% of samples are positive.

2.5. Production data

Production parameter data were recorded at time of transfer to the respective finishing farm and at slaughter. Data captured included individual weight to calculate the average daily weight gain (ADWG), the time span of fattening and feed consumption.

2.6. Statistical evaluation

The study was performed as a randomized, negative controlled study under field conditions with two treatment (study) groups at four sites. The individual animal was used as the experimental unit for the statistical analyses of *Salmonella* ELISA data while for the production data the batch was used as the statistical unit.

Descriptive statistics were calculated per site and treatment group and pooled over sites per treatment group.

Differences of OD value between groups (vaccinated vs. non-vaccinated) were analyzed using t-test (Mann-Whitney). Frequency tables were generated per site and pooled over sites with respect to animals with a 'positive' sample. Data of *Salmonella* ELISA were evaluated (a) using a cut-off OD% = 10 and (b) using a cut-off OD% = 40 and tested on differences between the treatment groups by Fisher's exact test. The production data were compared using t-test.

3. Results

3.1. Exposure of study animals to L. intracellularis and Salmonella spp.

The scientific outcome of the study is considered valid as vaccinated and control animals have been exposed to *L. intracellularis* and *Salmonella* spp. during the study.

Concerning *L. intracellularis*, antibodies and fecal shedding prior to study start showed that natural infection occurred at the middle of the

nursery period. In addition, farm A suffered from an acute episode of Proliferative Haemorrhagic Enteropathy (PHE), demonstrating the circulation of the pathogen. Concerning *Salmonella* spp., serology data showed a strong infection pressure in all sites with more than 70% animals tested positive in the non-vaccinated group.

3.2. Seroprevalence of Salmonella at time of slaughter is reduced in vaccinated animals

Blood samples used in this trial were taken at slaughter. *Salmonella* serology was assessed qualitatively, as well as by frequency of positive ratio according to the cut-off value of the QS-scheme (cut-off: OD40%) and the manufacturer's detection limit (cut-off: OD10%).

On each individual site the median *Salmonella* ELISA OD% values were significantly reduced in vaccinated groups compared to their respective non-vaccinated counterparts ($p < 0.0144$, Table 1). Additionally, the distribution of individual values was more consolidated in the vaccinated group compared to the non-vaccinated group. Figure 1 shows boxes, including 50% of values (25^{th} to 75^{th} percentile), were reduced in span in all farms although the single maximum values are comparable between groups and sites (Figure 1).

According to the requirement of the QS scheme a threshold of ≥40 OD%-value is considered positive. The cumulative analysis of all sites demonstrates that vaccination resulted in a highly significant reduction of the frequency of positive rated animals in the vaccinated group (59 out of 439, 13.4%) compared to the unvaccinated group (88 out of 270, 32.6%; $p < 0.0001$). Individually, in each of the four finishing units the percentage of positive pigs was significantly reduced in vaccinated pigs compared to non-vaccinated pigs ($p < 0.0212$; Table 2a).

Using the more stringent manufacturer's threshold of the OD10%-value, among all sites, vaccination resulted in a highly significant reduction of positive animals in the vaccinated group (285 out of 439, 64,9%) compared to the unvaccinated group (212 out of 270, 78.5%; $p = 0.0001$). In detail, all sites reported a reduction of positive animals, but not all reached statistical significance (one out of four, Table 2b).

3.3. Vaccination improves the risk class assignment

According to the categorization of the *Salmonella* monitoring system, all finishing units are assigned to a certain category of low, moderate or high *Salmonella* risk, depending on the percentage of positive serological samples.

In accordance with the requirements of the *Salmonella* monitoring program, which is in place in Germany, the cut-off value of OD40% was applied for data analysis. Vaccination with Enterisol® Ileitis reduced the proportion of positive samples significantly in all finishing units (Table 2a). In more detail, three out of four farms improved their category by one (Cat. II to Cat. I, Cat. III to Cat. II) and one farm even enhanced by two categories (Cat. III to Cat. I).

3.4. Production parameters

Production data routinely collected by the producer was assessed from periods before and after vaccination, including average daily weight gain (g), duration of fattening period (days), feed conversion (kg/kg) and mortality (%) as secondary parameters (Table 3).

All data show a beneficial effect in the vaccinated groups across all farms, although not reaching statistical significance. The average daily weight gain increased by 19g with no change on the feed conversion rate and the average time to slaughter was reduced by two days. In addition, the mortality was slightly reduced in the vaccinated animals compared to their non-vaccinated counterparts.

4. Discussion

In Germany, serological monitoring of *Salmonella* in herds is routinely performed in the field to categorize farms according to their risk of *Salmonella* infection (QS scheme). Similar monitoring systems are in place in other European countries to track the status and spread of *Salmonella* in food producing animals (Snary et al., 2010; Sorensen et al., 2004). Thus, antibody levels of *Salmonella* form the basis to evaluate the potential risk of *Salmonella* infection in the field to reduce the risk of food borne pathogen transmission at processing as well as to reduce economic losses to producers.

This field study demonstrates the efficacy of Enterisol® Ileitis in the target species under field condition and on a large scale, using the product through its drinking water application. It included the challenge of the animals by exposure to natural infections for both *L. intracellularis* and *Salmonella* spp. while infection with *L. intracellularis* occurred approx. in the middle of the nursery period. The *Salmonella* prevalence was assessed using an authorized *in-vitro* test system according to QS-scheme. *Salmonella* infection was detected by serology instead of bacterial detection from feces as most carrier animals require specific stresses to cause *S. enterica* shedding, and most naturally infected pigs only shed

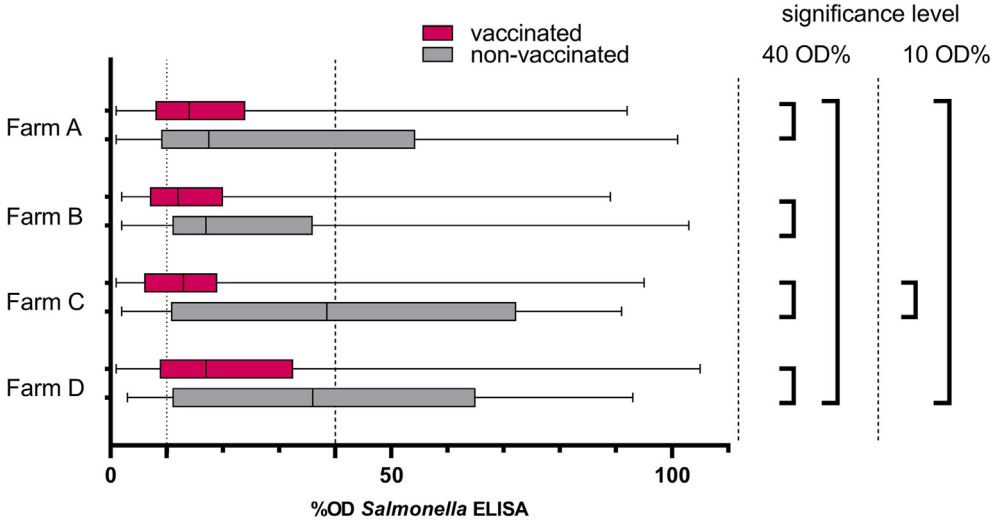

Figure 1. Box-plot (Min to Max) of OD values of farms A to D. At a cut-off level of OD40% (dotted line) all individual farms show significant differences between groups as well as in a summary of all farms. Taken the more stringent manufacturer's cut-off level of OD10% (light dotted line) into account the total number of animals in a group was still showed statistical significance, however, only one out of four individual farms reached a statistical relevant reduction.

Table 2a. Proportion of *Salmonella* positive animals at slaughter based on ELISA cut-off OD40%.

Farm	Treatment group	N_{total}	$N_{positive}$	$\%_{positive}$	95% CI		*P*
A	Vaccination	109	11	10.1	5.15	17.34	0.0016
	Non-vaccination	90	25	27.8	18.85	38.22	
B	Vaccination	110	12	10.9	5.77	18.28	0.0212
	Non-vaccination	89	21	23.6	15.24	33.78	
C	Vaccination	110	11	10.0	5.10	17.19	<0.0001
	Non-vaccination	30	15	50.0	31.30	68.70	
D	Vaccination	110	25	22.7	15.28	31.70	0.0052
	Non-vaccination	61	27	44.3	31.55	57.55	
All	Vaccination	439	59	13.4	10.39	16.99	<0.0001
	Non-vaccination	270	88	32.6	27.04	38.54	

Table 2b. Proportion of *Salmonella* positive animals at slaughter based on ELISA cut-off OD10%.

Farm	Treatment group	N_{total}	$N_{positive}$	$\%_{positive}$	95% CI		*P*
A	Vaccination	109	71	65.1	55.42	74.01	0.1677
	Non-vaccination	90	67	74.4	64.16	83.06	
B	Vaccination	110	67	60.9	51.14	70.07	0.0088
	Non-vaccination	89	70	78.7	68.69	86.63	
C	Vaccination	110	66	60.0	50.22	69.22	0.0532
	Non-vaccination	30	24	80.0	61.43	92.29	
D	Vaccination	110	81	73.6	64.38	81.58	0.1828
	Non-vaccination	61	51	83.6	71.91	91.85	
All	Vaccination	439	285	64.9	60.25	69.38	0.0001
	Non-vaccination	270	212	78.5	73.13	83.27	

Table 1. Quantitative Mean and Median ELISA OD% values.

Farm	Treatment group	$N_{batches}$	N_{total}	Median	Mean	*P*
A	Vaccination	4	109	14.0	19.22	0.0144
	Non-vaccination	3	90	17.5	30.53	
B	Vaccination	4	110	12.0	17.13	0.0007
	Non-vaccination	3	89	17.0	28.22	
C	Vaccination	4	110	13.0	17.55	0.0005
	Non-vaccination	1	30	38.5	42.03	
D	Vaccination	4	110	17.0	27.38	0.0074
	Non-vaccination	2	61	36.0	37.89	

S. enterica intermittently and usually in low numbers (Isaacson et al., 1999). Thus, detection of most infected animals will only be achieved by routine monitoring of either blood serum or meat juice at slaughter to assess the prevalence of *Salmonella* infection. Regarding the study design, a group of vaccinated animals was compared to an equivalent group of unvaccinated control pigs. The treatment groups consisted of cohorts of exclusively vaccinated animals or controls in direct timely coherence; thus, groups were as contemporaneous as possible. To this end, animals of all treatment groups were raised and housed under the same conditions. Even the natural exposure to *L. intracellularis* was as similar as possible, as infections took place during nursery when all animals were commingled, before the pigs were moved to the four different finishing farms. This is likely also true for *Salmonella* spp. infections, as the breeding sows supplying these herds were tested positive for

Table 3. Production data.

Farm	Treatment group	N	ADWG (g/day)	Fattening (days)	Feed conversion (kg/kg)	Mortality (%)
All	Vaccination	16	809.2	117.2	2.83	1.36
	Non-vaccination	9	790.9	119	2.83	1.66
	P		0.1513	0.3378	0.9537	0.1757

N, number of batches; ADWG, average daily weight gain.

S. Typhimurium. Clearly, serology demonstrated the exposure of the animals to both pathogens prior and at the end of the trial on all four farms. The starting point for this study was the increase of *Salmonella* prevalence observed in all four finishing farms at the slaughterhouse together with *L. intracellularis* exposure. Thus vaccination of piglets against *L. intracellularis* was the best approach in regard to animal welfare as performance parameters show an improvement of general health of the vaccinated animals in agreement with previous studies (Kroll et al., 2004; Walter et al., 2004).

The four finishing units revealed different average OD% values in both treatment groups, which reflects the different assignments of the four finishing farms to *Salmonella* risk categories ranging from I to III. In each of the four farms, vaccination with Enterisol® Ileitis resulted in a reduction of the average OD%-value compared to unvaccinated pigs and in total a reduction of animals considered positive in the QS scheme by 59% (32.7%–13.4%). This shows that vaccination against *L. intracellularis* reduces the average *Salmonella* prevalence on herd level in finishing units independent of the present infection pressure, moreover, each finishing unit improved its *Salmonella* risk category by vaccination of at least on category according to the German *Salmonella* monitoring system. This treatment effect across different sites allows extrapolation of data to other finishing units. This finding is supported by studies that showed a reduced risk of *Salmonella* infection in a shed-spread model (Visscher et al., 2018). Furthermore, a significant reduction of *Salmonella* burden in the acute phase of infection after oral *Lawsonia* vaccination in an artificial challenge model through modulation of the gut microbiome was observed (Leite et al., 2018). It was demonstrated that the application of attenuated live *L. intracellularis* had an immediate beneficial effect on the composition of the gut microbiota (e.g. favorable for short-chain fatty acid producing bacteria). Shifts in the bacterial composition supported an environment with enhanced resistance to be colonized by *Salmonella*, eventually reducing the number of animals tested positive for shedding.

In addition to the benefits of *L. intracellularis* vaccination and its influence on *Salmonella* prevalence, secondary production parameters investigated in this study showed positive trends. In particular, the average daily weight gain reflects the general health status of the animal and a positive effect on this parameter indicates the efficacy of a vaccine under typical field study conditions. Furthermore, the vaccination was associated with a reduced duration of finishing period and mortality, thus confirming previous studies (Bak and Rathkjen, 2009). All production data show a beneficial effect in the vaccinated groups across all farms, without reaching statistical significance, probably due to an insufficient sample size.

In conclusion, this study demonstrated that vaccination with Enterisol® Ileitis significantly reduces the seroprevalence of *Salmonella* in herds co-infected with *L. intracellularis* and *Salmonella* spp. compared to non-vaccinated animals at time of slaughter.

Declarations

Author contribution statement

All authors listed have significantly contributed to the investigation, development and writing of this article.

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Data availability statement

Data will be made available on request.

Declaration of interests statement

The authors declare the following conflict of interests: Holtrup, S., Deitmer R., Mesu A.P., Kraft C. are employees of Boehringer Ingelheim Vetmedica.

Additional information

No additional information is available for this paper.

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