Effects of Fluoroquinolone Treatment Acidified Feed, and Improved Hygiene Measures on the Occurrence of Salmonella Typhimurium DT104 in an Integrated Pig Breeding Herd


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Summary
Worldwide, the use of antimicrobials in food production has been associated with drug resistance in foodborne pathogens such as Salmonella. However, little is known about the efficaciousness of fluoroquinolone treatment on Salmonella Typhimurium T104 infections in pig breeding herds. A combined eradication procedure with enrofloxacin application on sows and piglets, feeding of encapsulated organic acids to sows, disinfection with peracetic acid, separation of the growers from the sows and serological discrimination using a new whole-cell-based enzyme-linked immnosorbent assay (ELISA) was evaluated for the suitability to eradicate and to control endemic S. Typhimurium DT104 infections in a closed herd. Thirty-seven sows and their piglets were treated everyday from day 14 ante partum until the day of weaning. Eighteen sows and their piglets served as controls. From the first day of life until day 168 after birth, faecal samples (n = 1671) of all piglets were analysed for Salmonella shedding. In parallel, systemic antibody responses were monitored by whole-cell-based isotype-specific ELISA systems. From birth to weaning the prevalence in both groups was between 2% and 9%. After weaning, intermittent shedding could be observed in both groups, and salmonellae could be found in up to 7.7% of the faecal samples. As a result, a dramatic increase in Salmonella-infected growers was observed, as of day 115 after birth, 47.4% of the animals of the treated group were tested positive for S. Typhimurium. Our results indicate that despite long-term antibiotic treatment and optimized hygiene measures, shedding of S. Typhimurium by the sows and the subsequent infection of their offspring could not be effectively prevented. Although it could not be shown that elimination of S. Typhimurium DT104 infection was achieved, the disinfection procedures described and the diagnostic test used are effective instruments to decrease the Salmonella load and to identify individual infected animals. Both of these are important factors for an improved consumer protection.

Introduction
Worldwide, the use of antimicrobials in food production has been associated with drug resistance in foodborne pathogens such as Salmonella. Salmonellosis is one of the most important enteric infections in humans as well as in livestock. Salmonellae of various serotypes can cause a variety of clinical and subclinical infections, mainly self-limiting gastroenteritis or systemic disease. Besides Salmonella Enteritidis, Salmonella Typhimurium is the most frequent and most important cause of food poisoning. Although pigs usually do not develop clinical salmonellosis, they become carriers and shedders, resulting in a substantial disease-causing potential for humans (via meat and faeces) (Poppe et al., 1998; Kranker et al., 2001; Nielsen et al., 2001; Hurd et al., 2002).

Diagnosis of Salmonella infections can be conducted directly in the piggery or at the slaughterhouse by isolating salmonellae with various established cultural methods (Imberechts et al., 1998; Baloda et al., 2001; Christensen et al., 2002; Chang et al., 2003). Additionally, pigs can be controlled serologically using lipopolysaccharide (LPS)-based enzyme-linked immnosorbent assay (ELISA) systems (Nielsen et al., 1995, 2001; Steige et al., 2000; Wüff et al., 2002). For practical purposes, these serological results are used to classify pig herds in one of three categories. Category 3 has the highest prevalence of Salmonella infection, defined as at least 40% of the pigs being seropositive. The LPS-based ELISA systems are useful diagnostic instruments for serological examination and categorization of Salmonella-infected herds. Individual Salmonella-infected pigs, however, can be more reliably detected by using whole-cell-based isotype-specific ELISA systems, which are also suitable for control of vaccination measures (Lehmann et al., 2004).

In category 3 stables, a significant reduction in Salmonella prevalence could be achieved by improved keeping and feeding hygiene and vaccination with attenuated vaccines (Lumsden and Wilkie, 1992; Letellier et al., 2001; Beal et al., 2002; Springer et al., 2001).

As porcine salmonellosis is mostly a persistent and clinically inapparent infection, attempts of Salmonella eradication on herd level are rare and they succeed only in few cases (Dahl et al., 1997; Osterberg et al., 2001). These eradication programmes were performed by using the spatial separation of the different ages combined with a serological monitoring. Nevertheless, one unresolved problem in pig breeding is the
Salmonella shedding by infected sows and the subsequent infection of their piglets.

Treatments with antibiotics were generally limited to herds showing clinical salmonellosis. Although such antibiotic treatment of sows is widely discussed as being a possible and effective means to eliminate salmonellae from infected herds, very few reports are available that explicitly demonstrate a positive effect (Fenwick and Olander, 1987). In contrast, there are some reports, which show the failure of an antibiotic treatment with tetracyclines, or of other antibiotics in salmonellae reduction on herd level (Guttmann et al., 1976; Jacks et al., 1988; Ebner and Mathew, 2000). Furthermore, it was recently also shown that a 5-day enrofloxacin treatment failed to eradicate S. Typhimurium DT104 from experimentally infected growers (Delsol et al., 2004).

There has been inadequate evaluation of an antibiotic for the eradication of non-typhoidal salmonellae (NTS) in asymptomatic carriers. For over a decade, fluoroquinolones have been the standard treatment of NTS infections. They are also reported to reduce the occurrence or duration of convalescent carriage (Sammalkorpi et al., 1987; Raymond et al., 1994) and consequently to reduce the risk of relapse or dissemination (Ahmad et al., 1991).

However, given the extensive use of these drugs in veterinary and human practice, both in vitro resistance and clinical failures in human practice occur, especially with some serotypes (S. Typhimurium, Salmonella Hadar, Salmonella Choleraesuis) (Threlfall et al., 1997; Molbak et al., 1999; Chiu et al., 2002; Angulo et al., 2004).

Hitherto, no alternative antibiotics have been available to clear Salmonella carriage in cases of fluoroquinolone resistance. This convalescent carriage is well documented in Salmonella infections and reaches rates of 20% in NTS strains (Buchwald and Blaser, 1984).

The aim of the study was to prevent S. Typhimurium infection of the piglets and subsequently of weaned pigs in the growing units. Therefore, based on serological monitoring with a newly developed whole-cell-based ELISA system, we have evaluated the efficacies of a long-term treatment with enrofloxacin on the kinetics of prevalence and carriage of S. Typhimurium DT104 in an integrated pig-breeding herd combined with feeding with encapsulated acids and disinfecting routines with peracetic acid.

Material and Methods

Herd description

The investigated herd was a nucleus herd of 80 pure-bred Large White sows with specific-pathogen-free (SPF) status for endo- and ecto-parasites, for porcine respiratory and reproductive syndrome virus (PRRSV), rhinitis atrophicans, Actinobacillus pleuropneumoniae and for Brachyspira hyodysenteriae. Boars for artificial insemination and breeding sows (gilts) were selected from the herd. The stable was run as an integrated system with segregated growing units each 10 km in distance. All piglets are weaned at day 28 and transferred to the growing units, which are managed with the same hygiene measures as the sow herd. At day 160, selected gilts are returned to the sow herd for mating. Before this study, the gilts as well as the growers in the growing units were tested repeatedly for S. Typhimurium DT104 by bacteriological investigation. Thereby, clinical signs of salmonellosis (e.g. diarrhoea) could be observed on the growers of the segregated growing units.

Eradication measures

Based on the management system, the SPF status, and based on the results of antibiotic susceptibility tests of the isolated S. Typhimurium DT104 strains, the eradication programme was designed and implemented. The eradication measures of this programme were applied to 37 randomly selected sows and to their piglets. As controls, 18 randomly selected sows and their piglets in the same herd were included in this study. With the exception of enrofloxacin treatment (no treatment), the controls were handled and treated like the test group.

Before treatment, 51.4% of the faecal samples from the sows of the treated group were tested positive for S. Typhimurium, faecal samples were positive from 28.8% of the control sows. The additionally performed serological investigation of all sows revealed positive results in each case.

In antibiotic susceptibility tests, the isolated S. Typhimurium DT104 displayed the typical penta-resistance, and all isolates were sensitive to enrofloxacin. To prevent shedding of S. Typhimurium with faeces, all sows were treated daily with 2.5 mg/kg enrofloxacin (Baytril® 10%; Bayer AG, Leverkusen, Germany) starting at day 7 before farrowing. Additionally, to prevent absorption and subsequent invasion caused by S. Typhimurium, all piglets were also treated daily with 1.7 mg/kg enrofloxacin per os (Baytril® 0.5% Pigdoser) starting from birth. The treatment of sows and piglets ended at weaning.

All farrowing units and growing units were thoroughly whitewashed and disinfected with peracetic acid (Wofasteril®, E400, Kesla Pharma GmbH, Wolfen, Germany). The disinfectant used was a 2-fold increase of the recommended concentration (1% v/v). Before farrowing, the body surface of all sows (particularly the claws and the teats) were also disinfected with peracetic acid (Venno-Oxygen®, Menno Chemic GmbH, Norderstedt, Germany). To control these disinfection measures, swabs from the well-drained concrete floors of the farrowing units and from the claws of the sows were tested pre- and post-disinfection for S. Typhimurium.

In addition to the above-mentioned measures, the sows were fed 30 g/kg feed/sow/day Formyl® (SODA, Monaco), which is an encapsulated mixture of formiate 66% and citrate 33%. This treatment had the potential to acidify the colon content, resulting in an inhibition of salmonellae reproduction (Fenwick and Olander, 1987; Hjoberg et al., 2003; Anderson et al., 2004). The Formyl® feed was started at day 14 antepartum and ended at weaning.

Bacteriological examination

All piglets were examined for Salmonella spp. at days 7, 28, 40, 60, 87, 115, 142 and 168 after birth using bacteriological and serological tests. To investigate shedding of salmonellae in faeces, all faecal samples (n = 1671) and samples from the disinfected well-drained concrete floors of the farrowing units (pre- and post-disinfection, each with n = 37) and claws of the sows (pre- and post-disinfection, each with n = 37) were examined and processed in standard procedures in accordance to ISO 6579.

In brief, specimens (5 g) were homogenized in 45 ml of buffered peptone-water and subsequently aerobically incuba-
Serological examination

In order to control the isotype-specific systemic anti-S. Typhimurium antibody responses during the study, a slightly modified whole-cell-extract-based ELISA system was used (SalmoSoft® Pig STM-WCE ELISA; Labordiagnostik GmbH Leipzig, Leipzig, Germany) (Lehmann et al., 2004). All blocking incubations, blocking and washing steps were carried out as recommended by the supplier. As a positive-reference standard, a serum was used, obtained from a pig experimentally infected with a highly virulent, penta-resistant isolate of S. Typhimurium DT104 originally isolated from a diseased pig. Serum from a pig tested bacteriologically negative in 15 samples of tissues and inner organs, which originated from a herd without any history of salmonellosis, served as a negative control. Enzymatic reactions were developed with a TMB-based chromogen (Boehringer GmbH, Mannheim, Germany) and measured by a computer-controlled photometer at 490 nm (Multiscan MCC-340; Flow Lab, Inc., McLean, VA). Antibody activities were expressed as ELISA units/ml (EU) using the positive-reference standard method (Butler et al., 1978).

Data were calculated using a computer-based program developed for ELISA evaluation (SalmoSoft™, Labordiagnostik). The values of the positive reference standards were set to 1000 EU.

ELISA performance and statistical analysis

The activities of isotype-specific antibodies and the results of the quantitative Salmonella detection were calculated and plotted as notch boxes. The median (internal horizontal line), upper and lower quartiles (the upper and lower horizontal margins of the boxes), the 95% confidence limits (the oblique margins of the boxes) and the extreme values are shown (McGill et al., 1978). Significant differences between both groups were tested by Student’s t-tests for unpaired observations or by Welch’s test. P-values of < 0.05 were considered significant.

Results

Bacteriological examinations

The Salmonella isolation rates of the enrofloxacin-treated group and of the negative control group are summarized in Fig. 1. These results show that longtime antibiotic combined with optimized hygiene and a special diet did not succeed in preventing a S. Typhimurium infection of piglets, which stemmed from Salmonella infected sows.

However, the disinfection measures were very effective, as no salmonellae were detectable in the farrowing units or on the claws after disinfection, whereas before disinfection all of the units (100%) and 10.8% of the samples from the claws were tested positive for S. Typhimurium DT104.

Long-term investigation of the weaned pigs, however, showed that the growers of both treated and untreated groups were infected by S. Typhimurium with a very high prevalence. From birth to weaning, the infection rates of both groups were determined to be between 2% and 9%. Intermittent shedding could be observed in both groups after weaning until day 87 and salmonellae could be found in 0.0–7.7% of the faecal samples. After day 87, a strong significant (P < 0.05) increase of Salmonella-infected growers was observed at both groups. At day 115 after birth, 47.4% of the enrofloxacin-treated group was tested positive for S. Typhimurium (Fig. 1). The growers of the control group also showed a drastically increasing Salmonella prevalence, which was highest at day 142 with 25.8%. At the post-mortem examination (tonsils,
cecum and ileocolic lymphnode) of fallen piglets from both groups, S. Typhimurium DT104 could be recovered from all animals investigated. At the end of the study, none of the growing units examined (from both groups) were Salmonella-negative. Therefore, it is highly likely that a high percentage of the pigs were infected, which is supported by the serological results of the ELISA.

Using micro-agglutination, all isolated strains were identified as S. Typhimurium. The subsequent determination of the phage-type revealed consistently the determination type (DT) 104. The weekly performed resistance tests by agar gel diffusion showed that there was no increasing resistance of the circulating S. Typhimurium DT104 strain to enrofloxacin.

Serological examinations
The serological results of the reference standard ELISA corresponded very well with the bacteriological findings (Fig. 2).

Starting from birth, the activities of the maternal anti-Salmonella antibodies progressively decreased in all piglets of the treated group to a minimum after weaning (between day 28 and day 40). However, at this time, strongly increasing IgA-antibody activities were determined in some piglets (3.6% of all) indicative of a recent infection in these animals.

Starting at day 87 after birth, the activities of specific anti-Salmonella antibody activities (IgG and IgA) increased constantly. At the end of the study (at day 168), all growers (100%) showed significantly increased (P < 0.05) Salmonella-specific antibody activities of specific IgG and IgA in serum, supporting the bacteriological findings, that nearly all animals were infected with S. Typhimurium DT104. The activities of the Salmonella-specific antibodies of the controls increased in a similar way as at the enrofloxacin group (data not given).

Discussion
Prevalence and incidence of S. Typhimurium in pig herds significantly depend on hygienic and feeding routines as well as on antimicrobial intervention measures. The intention of this study was to examine the efficiency and the practicability of a combination of antibiotics/Formyl® treatment and optimized hygiene conditions to prevent new infections with S. Typhimurium in piglets from infected sows.

In the course of the study, it soon became obvious that this aim could not be achieved. However, the detection rates of Salmonella were low, when the piglets were tested around weaning. At the growing units (and after the end of the antibiotic application), a significant rise in Salmonella shedding and subsequently also in the Salmonella-specific antibody activities were observed. Thus, the study demonstrated a missing effect of the antibiotic treatment on the prevalence of S. Typhimurium infections in pigs. The reason for the delayed increase of Salmonella shedding in the controls, compared with the enrofloxacin-treated group, is equivocal. At first, this delayed increase could be a result of the different percentages of Salmonella-infected sows in both groups. Furthermore, it can be assumed that the persisting salmonellae in the lymphoid tissues had better growth conditions because of lack of competing bacteria after the antibiotic treatment.

Quinolons (including enrofloxacin) are known to be powerful antibiotics to treat clinical salmonellosis in humans, pigs, cattle and poultry. In contrast, it was recently shown that a 5-day enrofloxacin treatment failed to eradicate S. Typhimurium DT104 from experimentally infected growers (Delsol et al., 2004). Our study demonstrates that the long-term treatment with enrofloxacin is also insufficient to prevent shedding of salmonellae and colonization in piglets. Additionally, the observed drastically increasing antibody activities in the gilts will also have a negative...
impact on the classification of pig herds by meat juice ELISA at slaughtering.

Beside these practical aspects, long-term antibiotic treatment bears the risk of inducing antibiotic resistances in salmonellae which are very often resistant against many antibiotics (Poppe et al., 2001; Gebreyes et al., 2004). In Germany, the percentage of multiresistant Salmonella isolates from pigs reached 76.7% in 2002. Thereby, resistance to quinolone nalidixic acid was detected in 10% of the Salmonella strains isolated (Schroeter et al., 2004). Quinolone-resistant salmonellae are an increasing problem for public health and it was shown that ciprofloxacin-resistant salmonellae from poultry or pigs can induce severe systemic salmonellosis in humans (Molbak et al., 1999; Piddock, 2002; Molbak, 2004). Additionally, a long-term medication with daily application of enrofloxacin is a very expensive attempt to prevent Salmonella infection. Considering these results, alternatives have to be elucidated in further studies.

Such possible alternative strategies for protection of piglets from Salmonella-infected breeding sows could be the vaccination of sows antepartum with live attenuated Salmonella vaccines (Lumsden and Wilkie, 1992; Springer et al., 2001), or with inactivated Salmonella vaccines based on herd-specific isolates (Roesser et al., 2004).

As the disinfection measures performed were shown to effectively decrease the number of salmonellae in the environment and on animal body surfaces, these measures contribute in lowering the infection pressure in stables with a high incidence of Salmonella infection.

The employed reference standard ELISA was shown to be a suitable tool for detecting endemic S. Typhimurium infections. Identification of intermittent shedders is a primary goal of Salmonella serology and the detection of these animals is essential for the assessment of the true Salmonella prevalence. The commonly used LPS-based ELISA systems may allow the assessment of the Salmonella prevalence on a herd basis, but not of individual infected pigs (van der Heijden, 2001). Our results indicate that the used whole-cell-based reference standard ELISA is capable of identifying these animals. Furthermore, the detection of isotype-specific antibodies allows discriminating ancient from chronic infections and Salmonella-vaccinated from Salmonella-infected pigs (Lehmann et al., 2004). The fact that at the end of this study, all growers (100%) showed significantly increased antibody concentration or affinity. Immunochemistry 15, 131–136. Chang, V. P., E. W. Mills, and C. N. Cutter, 2003: Comparison of recovery methods for freeze-injured Listeria monocytogenes, Salmonella Typhimurium, and Campylobacter coli in cell suspensions and associated with pork surfaces. J. Food Prot. 66, 798–803.

References


