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Evaluation of the efficacy of a *Clostridium perfringens* type A toxoid vaccine for pigs using a toxin challenge model and under field conditions

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Summary The objective of the trials was to test the efficacy of a *Clostridium perfringens* type A toxoid vaccine with a toxin challenge model and under field conditions according to Regulation (EU) 2019/6.
15 gilts were vaccinated twice at 5 and 2 weeks before the 1st and once more 2 weeks before the 2nd parturition. Antibodies against the alpha and beta2 toxins were determined in the serum and colostrum of gilts and in the serum of piglets. At 2 days of age (after 1st parturition), piglets were challenged intra-abdominally with an alpha and beta2 toxin-containing supernatant of a heterologous *Clostridium perfringens* type A strain. The piglets were examined clinically and euthanized animals pathologically after the challenge.
In a field trial, 18 gilts were vaccinated twice with the same vaccine. Antibodies against the toxins in serum and colostrum of gilts were determined and mortality and incidence of diarrhea in piglets up to 26 days of age were monitored.
The vaccination elicited antibodies against the alpha and beta2 toxins, which were transferred to the offspring by the uptake of colostrum. Piglets were significantly protected from clinical signs and mortality by the antibodies after receiving an intra-abdominal toxin challenge. A 3rd vaccination at 2 weeks before 2nd farrowing led to a further significant increase of antibodies in colostrum of the second parity sows and in serum of the piglets compared to basic vaccination. Vaccination under field conditions resulted in a significant increase of antibodies and a significant reduction in the incidence of diarrhea in piglets.

Keywords alpha toxin, beta2 toxin, challenge model, vaccination, neonatal diarrhea

Untersuchung der Wirksamkeit eines Clostridium perfringens-Typ-A-Toxoid-Impfstoffes für Schweine mit einem Toxin-Challenge-Modell und unter Feldbedingungen

Zusammenfassung Ziel der Arbeit war die Prüfung der Wirksamkeit eines *Clostridium perfringens*-Typ-A-Toxoidimpfstoffes in einem Toxin-Challenge-Modell und unter Feldbedingungen gemäß der Verordnung (EU) 2019/6.
Fünfzehn Jungsauen wurden zweimal fünf und zwei Wochen vor der ersten und ein weiteres Mal zwei Wochen vor der zweiten Geburt geimpft. Es wurden Antikörper gegen das Alpha- und Beta2-Toxin im Serum und Kolostrum der Jungsauen und im Serum der Ferkel bestimmt. Zur Überprüfung der Wirksamkeit erfolgte am zweiten Tag nach der ersten Geburt eine intra-abdominale Belastung von jeweils zwei Ferkeln pro Sau mit einem Alpha- und Beta2-Toxin-haltigen Überstand eines heterologen *Clostridium perfringens*-Typ-A-Stammes. Die Ferkel wurden nach der Belastung klinisch und euthanasierte Tiere pathologisch untersucht.
In einem Feldversuch wurden 18 Jungsauen zweimal fünf und zwei Wochen vor der Geburt geimpft. Es wurden ebenfalls die Antikörper gegen die Toxine im Serum und Kolostrum der Jungsauen bestimmt. Im Anschluss wurden die Mortalität und das Auftreten von Durchfall bei den Ferkeln bis zum Alter von 26 Tagen überwacht.
Die Impfung führte zur Bildung von Antikörpern gegen das Alpha- und Beta2-Toxin, die über das Kolostrum auf die Nachkommen übertragen wurden. Die Antikörper schützten Ferkel sicher vor klinischen Symptomen und Mortalität nach intra-abdominaler Belastung mit dem toxinhaltigen *Clostridium perfringens*-Type-A Überstand. Eine dritte Impfung zwei Wochen vor der zweiten Geburt führte im Vergleich zur Grundimmunisierung zu einem weiteren signifikanten Anstieg der Antikörper im Kolostrum der Sauen und im Serum der Ferkel. Die Impfung unter Feldbedingungen hatte ebenfalls einen signifikanten Anstieg der Antikörper zur Folge und verringerte das Auftreten von Durchfall bei den Ferkeln signifikant.

Schlüsselwörter Alpha-Toxin, Beta2-Toxin, Challenge-Modell, Impfung, Saugferkeldurchfall

Introduction

Clostridium (*C.*) *perfringens* is an obligate anaerobic, gram-positive, spore-forming rod-shaped bacterium which is classified into the toxovars A-E based on the formation of the toxins alpha, beta, epsilon and iota. In addition to these toxins the pathogen can produce a number of other toxins, e.g. perfringolysin O (teta toxin), enterotoxin, necrotic enteritis toxin B (NetB), TpeL and beta2 toxin, as well as the enzymes collagenase and sialidase (Kiu and Hall 2018). All of these toxins have been described as virulence factors in various diseases. Due to the importance of enterotoxin forming strains for humans and NetB-forming strains for chicken, the toxovars F (alpha toxin and enterotoxin) and G (alpha toxin and NetB) were proposed to be included in the toxin-based typing (Rood et al. 2018).

The toxovar C of *C. perfringens* is of major importance for pigs. It forms the alpha and beta toxin and causes necrotizing enteritis of suckling piglets (NE), which can lead to high losses of suckling piglets in affected herds (Posthaus et al. 2020). Beta toxin plays a key role in the pathogenesis of NE (Johannsen et al. 1986, Sayeed et al. 2007, Miclard et al. 2009, Richard et al. 2019) .

Toxovar A has also been described as a cause of diarrhea in suckling piglets (Garmory et al. 2000, Bueschel et al. 2003, Jost et al. 2005, Springer et al. 2012, Kiu and Hall 2018). Since *C. perfringens* type A is part of the physiological gut flora of piglets during the first days of age (Melin et al. 1997, Songer and Uzal 2005) it is often difficult to relate *Clostridium perfringens* type A to occurrence of diarrhea. Suckling piglets usually develop serious diarrhea within the first 5 days of age, which may be associated with transient toxemic general disturbances (Nabuurs et al. 1983, Johannsen et al. 1993). Morbidity is high but mortality tends to be low. Severe outbreaks have been described in association with the birth of weak piglets as a result of infection with PRRSV and after mixed infections with Rotaviruses, enterotoxic *E. coli* and *Cystoisospora suis* as part of neonatal diarrhea complex (Gresham 1997, Holmgren et al. 2004). The pathogenesis of the disease is not fully understood, yet. All *C. perfringens* strains, including the toxovar A, form the alpha toxin (zinc metallophospholipase C). Alpha toxin leads to cell lysis by degradation of membrane phospholipids, tissue necrosis, hemolysis, platelet aggregation, contraction of blood vessels, superoxide generation, cytokine storm and ultimately death (Uzal et al. 2010, Li et al. 2013, Oda et al. 2015). The alpha toxin also plays a major role as a virulence factor in bovine necro-hemorrhagic enteritis (Goossens et al. 2017).

In addition to alpha toxin, beta2 toxin was described as a virulence factor for swine (Gibert et al. 1997). It has a molecular weight of 28 kDa and thus differs significantly from the beta toxin (molecular weight 35 kDa) (Uzal et al. 2010). Based on genetic studies of the beta2 toxin gene (*cpb2*) of various *C. perfringens* strains of different origin, a differentiation is made between the consensus beta2 toxin gene (*ccpb2*) and the atypical beta2 toxin gene (*acpb2*). *C. perfringens* isolates from pigs in particular harbor the *ccpb2* variant (Jost et al. 2005). The mode of action of the toxin is not fully understood (van Asten et al. 2010). A pore-forming effect of the toxin leading to a change in cell membrane permeability which finally results in membrane disruption and cell death is suggested (Smedley et al. 2004, Fisher 2006). Benz et al. (2022) were able to show that consensus beta2 toxin has a pore-forming component with an extremely high activity in lipid bilayers, although it does not have the typical structure of a pore-forming protein. Zeng et al. (2016) demonstrated moderate cytotoxicity of a recombinant beta2 toxin on NC460 human epithelial cells. This cytotoxicity was neutralized by monoclonal antibodies. Luo et al. (2020) indicated that beta2 toxin can cause a dose-dependent inhibition of growth of a porcine jejunum epithelial cell line (IPEC-J2) and results in cell inflammation. Beta2 toxin simultaneously reduces a tight junction protein that leads to barrier dysfunction of the intestinal epithelium. Furthermore, beta2 toxin increases the accumulation of Reactive Oxygen Species (ROS), the reduction of mitochondrial membrane potential and the expression of apoptosis-related genes. These processes lead to apoptosis of IPEC-J2 cells. In contrast, attempts to demonstrate cytotoxic activity on porcine IPI-21 and Caco-2 cells in vitro failed (Allaart et al. 2014). Springer et al. (2012) speculated an influence of beta2 toxin on the effect of alpha toxin or its resorption in the gut.

Basically, it can be assumed that other factors such as the histotoxic gas production and the formation of the enzymes microbial collagenase (kappa toxin) and sialidase may also have an influence on the pathogenesis of the disease (Kiu and Hall 2018).

For prophylaxis of *Clostridium perfringens* type A associated diarrhea, autogenous vaccines are commonly used. Furthermore, a vaccine containing alpha, beta and beta2 toxoids (Enteroporc AC, Ceva Santé Animale, Libourne, France), a vaccine containing alpha toxoid (*C. perfringens* type A) and TcdA and TcdB toxoids of *Clostridioides difficile* (Suiseng® Diff/A, Hipra) and a combined vaccine containing the alpha, beta and beta2 toxoid of *C. perfringens* types A and C and the fimbrial antigens (F4ab, F4ac, F5, F6) of enterotoxic *E. coli* (Enteroporc COLI AC, Ceva Santé Animale, Libourne, France) are licensed in the EU.

All of the mentioned maternal vaccines are administered two times during the last third of gestation. This ensures high antibody titers in the sows' colostrum at farrowing. Some vaccines are also approved for threefold vaccination. In this case, gilts are vaccinated twice before insemination and a third time two weeks before parturition.

The safety and efficacy testing of these vaccines is required by Regulation (EU) 2019/6. In this context, proof of efficacy must be provided under controlled laboratory conditions by a provocation test after administration of the vaccine under the recommended conditions of use. Results from the laboratory trials must be supplemented by data from field trials. Because *C. perfringens* type A associated diarrhea is a multifactorial disease, no infection model has been described that induces diarrhea in suckling piglets, yet. However, Johannsen et al. (1993) were able to induce transient general symptoms after application of bacteria as well as toxin-containing supernatant of *C. perfringens* type A. Based on this observation, a toxin challenge model that showed differences between vaccinated and non-vaccinated sows after intra-abdominal toxin challenge of piglets was developed (Springer et al. 2012).

The objective of the presented trials was the combined testing of the *C. perfringens* type A component of a *C. perfringens* type A/C toxoid vaccine using the described toxin challenge model and in a field trial in

accordance with the requirements of the Regulation (EU) 2019/6.

Material and Methods

Animals

Both studies were conducted as blinded studies. The toxin challenge trial was conducted according to the German law of Animal Welfare and was registered under the animal experiment announcement nos. IDT-A-03b-2012 and IDT-A-04b-2012. Thirty pregnant gilts (German Landrace x German Large White, 8–10 months old) were included in the study. The animals were seronegative against alpha toxin (<0.02 antibody units (AU) per ml) and showed no antibodies against the beta2 toxin before the first vaccination. Gilts were randomly assigned to the vaccine and control groups based on sera antibody content (B0) against the alpha toxin. If gilts did not show antibodies against the alpha toxin, random assignment was based on ear tag number. The gilts were housed in groups on concrete slatted floor. One week before farrowing the gilts were transferred to the farrowing pens (single housing) with slatted floor and a heated area (piglet nest). The gilts were fed a commercial gestation feed (Gravisan fit, Deuka GmbH, Könnern) and after farrowing a commercial lactation feed (Lactosan, Deuka GmbH, Könnern) according to nutritional requirements. The water supply was ad libitum via drinking water.

In the field trial (registered under animal experiment application No. 42502-3-834 IDT) 35 pregnant gilts (Dan breed, approximately 340 days old) of a farrowing group in a sow breeding facility with 2000 sows were used. The sows farrowed in a weekly rhythm and the suckling period was 4 weeks. The farm was selected because of diarrhea in piglets during the first week of age. Prior to the field trial, *C. perfringens* type A was detected in fecal samples of the first days of age. Enterotoxic or enteropathogenic *E. coli, C. perfringens* type C, Rota- and Coronaviruses were not detected. The selected gilts showed no antibodies against alpha and beta2 toxin in serum before the first vaccination (B0). The allocation of the animals to the vaccination and control group was randomized and the study performed as blinded study. After farrowing a total of 474 suckling piglets (mother: see above, father: Duroc, weight of the piglets > 800 g) deriving from both treatment groups were included in the study.

Vaccine

In both, the laboratory and field study the *C. perfringens* type A/C toxoid vaccine Enteroporc AC (batch 001 07 14, Ceva Santé Animale, Libourne, France) was used. The alpha and beta2 toxoids of this vaccine are also part of the vaccine Enteroporc COLI AC (Ceva Santé Animale, Libourne, France).

Challenge material (testing of the efficacy in the toxin challenge model)

The alpha and beta2 toxin containing *Clostridium perfringens* type A strain no. 178 was cultivated in TVS medium (Ceva Santé Animale, Libourne, France). After separation of the biomass by centrifugation (10,000 x g) the supernatant was concentrated by ultrafiltration. The concentrated toxin was sterile filtered, freeze dried and stored at 2 to 8°C. For toxin administration, one vial of the toxin was dissolved in 10.0 ml sterile water for injection (WFI). One toxin dose (2.0 ml per piglet) contained 9563 relative units (rU) alpha toxin and 6114 rU beta2 toxin.

Experimental design of the testing of the efficacy in the toxin challenge model

In group 1 (vaccine group), 15 pregnant gilts were immunized with the vaccine Enteroporc AC. Fifteen pregnant gilts of group 2 served as control group and received physiological saline solution (placebo). As a basic vaccination gilts received two separate injections of 2.0 ml of the vaccine or placebo intramuscularly (i.m.) at 5 and 2 weeks before 1st farrowing. For a follow up study 12 out of 15 basic immunized animals were inseminated again and received a booster vaccination with 2.0 ml of the vaccine i.m. at two weeks before 2nd farrowing. Animals of group 2 (n=11) received 2.0 ml of the placebo at the same time. At the age of 3 to 5 days all piglets received an iron supplement (Ursoferran 200 mg/ml, Serumwerk Bernburg AG, Germany).

Blood samples from the jugular vein from each gilt were collected one week before 1st vaccination (B0), immediately before 2nd vaccination (B1) and 4 days after farrowing (B2). During the 2nd pregnancy blood samples were taken immediately before booster vaccination (B3) and at 4 days after farrowing (B4). Colostrum was collected at both times of farrowing. For this purpose, carbetocin (Depotocin 70 µg/ml, Veyx Pharma GmbH, Germany) was administered to the gilts after the birth of the 3rd to 4th piglet. Approximately 40 ml colostrum per sow was milked from the anterior 4 mammary complexes.

In addition, blood samples were collected from the jugular vein of two randomly selected piglets per litter at 1 (PB1), 2 (PB2), 3 (PB3) and 4 weeks (PB4) post farrowing. Always the same piglets were sampled. The serum and colostrum samples were tested for antibodies against the alpha and beta2 toxins. Colostrum and serum samples of the piglets (PB1-4) were further tested for the presence of neutralizing antibodies against the alpha toxin by the Lecithinase Neutralization Test (LNT). Until testing the samples were stored at –20°C.

For animal welfare reasons a challenge was carried out on piglets from only 10 gilts per group after 1st farrowing. The gilts were randomly selected according to their farrowing date. Two piglets per gilt received a challenge by intra-abdominal administration of the toxin (2.0 ml) on day 2 of age.

On the day of challenge the clinical parameters of general condition, behavior and milk uptake were assessed using a scoring system (Table 1). In case of

TABLE 1: *Evaluation of clinical symptoms after intoxication of the piglets (score) under laboratory conditions*

Parameter	Score
General condition (overall clinical impression)	0 = undisturbed 1 = disturbed
Posture	0 = normal, body supported equally by all four limbs, animal moves physiologically 1 = unsteady gait, kyphosis 2 = animal unable to get up
Behavior	0 = normal, animal is attentive 1 = listless 2 = somnolent
Milk uptake*	0 = yes 1 = no

* Milk intake was assessed visually and by weight gain.

severe disturbance of the general condition (somnolence, body temperature <35°C, no milk intake), the animals were euthanized. Animals that died or had to be euthanized during the observation period were examined gross-pathologically. When lesions typical for the challenge occurred (accumulation of serosanguineous fluid in the thoracic and abdominal cavities), the animal received the highest score of 6. Piglets were weighed and clinically examined daily before and until day 3 post challenge.

Experimental design of the testing of the efficacy under field conditions

Group 1 (18 gilts) was vaccinated at 5 and 2 weeks before farrowing with 2.0 ml Enteroporc AC. Group 2 (17 gilts) received 2.0 ml of physiological saline solution at the same times. All sows were vaccinated simultaneously (with a 2-day offset) with a vaccine against neonatal *E. coli* diarrhea (Coliporc Plus, Ceva Santé Animale, Libourne, France). Blood samples were taken at 2 weeks before 1st vaccination (B0) and at 4 days after farrowing (B1). A colostrum sample was taken from each sow at farrowing (see testing of the efficacy in the toxin challenge model). The samples were tested for antibodies against alpha and beta2 toxins (ELISA). Serum and colostrum samples were further tested for the presence of neutralizing antibodies against the alpha toxin (LNT).

Three to 5 days after farrowing the piglets of both groups were given an iron supplement (Ursoferran 200 mg/ml, Serumwerk Bernburg AG, Germany) and a coccidiostat (Baycox 50mg/l, Elanco, US). Treatment with antibiotics was not carried out. If piglets had to be treated for other reasons (i.e. arthritis), they were removed from the study. Cross-fostering of piglets (litter compensation) took place on the 2nd day of age but only within the treatment groups. The suckling piglets were clinically (behavior, diarrhea, skin turgor) examined daily for the individual first 5 days of age (individual examination). Further clinical examinations were performed at the average age of 7, 10, 12, 14, 21 and 26 days. Piglet losses and the occurrence of diarrhea in both groups were recorded. In case of diarrhea of the litter rectal swabs were taken from two piglets per pen and examined bacteriologically for *C. perfringens*. If *C. perfringens* was detected, typing was performed by multiplex PCR (Polymerase Chain Reaction).

ELISA (alpha- and beta2 toxin antibodies)

Serum from blood samples and skimmed milk from colostrum samples were generated by centrifugation (blood samples 3,500 x g for 10 minutes, colostrum samples 23,000 x g for 20 minutes). The ELISA was performed as a sandwich-ELISA. Microtiter plates (MaxiSorp®, Nunc GmbH, Wiesbaden, Germany) were coated with a 1:1700 dilution of a monoclonal anti-alpha toxin antibody (clone 6H7, Ceva Santé Animale, Libourne, France) or a 1:170 dilution of a monoclonal anti-beta2 toxin antibody (clone 4A5, Ceva Santé Animale, Libourne, France). Unspecific binding sites were blocked with milk powder supplemented phosphate buffered saline (PBS). Subsequently, the primary antibody was saturated with alpha toxin (>0.15 mg/ml in house preparation) or with recombinant (r) beta2 toxin (>80 µg/ml in house preparation). One well without addition of alpha toxin or beta2 (r) toxin on every microtiter plate served as negative control for subtraction of the unspecific binding capacity of the serum. This was followed by a washing step with PBS supplemented with Tween 20 (PBST) (Merck, Germany). The sera and a standard serum (positive control; hyper immune serum of a pig) were then added in 2-fold dilution series (incubation for 1 h at 37°C). After a further washing step in PBST a goat anti-pig horseradish peroxidase conjugated antibody (1:8000 for alpha and 1:3000 for beta2, Bethyl Laboratories, USA) was added. SeramunBlau (Seramun Diagnostica GmbH, Wolzig, Germany) was used as chromogen according to the manufacturer's instruction. The reaction was stopped with 0.5 M sulfuric acid. The plates were subsequently measured at a wavelength of 450 and 620 nm using a plate reader (Sunrise, Tecan Group Ltd., Männedorf, Switzerland). The antibody titer of the samples is given in antibody units per ml (AU/ml).

Lecithinase Neutralization Test (LNT, alpha toxin neutralizing antibodies)

The test principle is based on the ability of the alpha toxin of *C. perfringens* (phospholipase C) to cleave lecithin contained in yolk lactose agar. In this process the lecithin loses its emulsifying effect and a turbidity court is formed. The enzymatic effect of phospholipase C can be neutralized by antibodies directed against alpha toxin (neutralizing antibodies).

Dilution series from serum or colostrum samples were prepared with phosphate buffered saline (PBS) and incubated with sterile filtered pre-diluted alpha toxin from a supernatant of a *Clostridium perfringens* type A culture. 10 µl of each sample and control preparation were then incubated in stamped-out wells of yolk lactose agar and further incubated for 16 to 24 hours at 35–38.5°C.

The turbidity of the area around the wells was estimated visually. If the turbidity court of the first dilution of a sample was clearly reduced or not visible, the sample contained neutralizing antibodies and was evaluated as positive. The last dilution at which neutralization was detected was subsequently given as the reciprocal titer (e.g., 2 for a titer of 1:2). Samples without neutralizing antibodies do not affect the turbidity and were evaluated as negative (0).

Bacteriological examination of the swabs in the field trial

Fecal swabs were spread out on Columbia blood agar (Merck KGaA, Darmstadt, Germany) coated with 0.1 ml of a 1% neomycin solution (Merck KGaA, Darmstadt, Germany) and incubated anaerobically at 37 °C. Colonies suspicious for *C. perfringens* were analyzed for the presence of the toxin genes *cpa, cpb, etx, iap, cpe*, and *cpb2* using a multiplex PCR (Baums et al. 2004).

Statistics

The statistical evaluation was done by SPSS (SPSS for Windows, Version 15.0, IBM). The mean antibody titers in sera and colostrum (challenge and field trial) were compared by Mann-Whitney U test (level of significance $p<0.05$). In the clinical evaluation of the results of the toxin challenge model, the score values for each animal were summed. Animals that died or had to be euthanized received the highest score of 6. Subsequently, the mean values and standard deviations

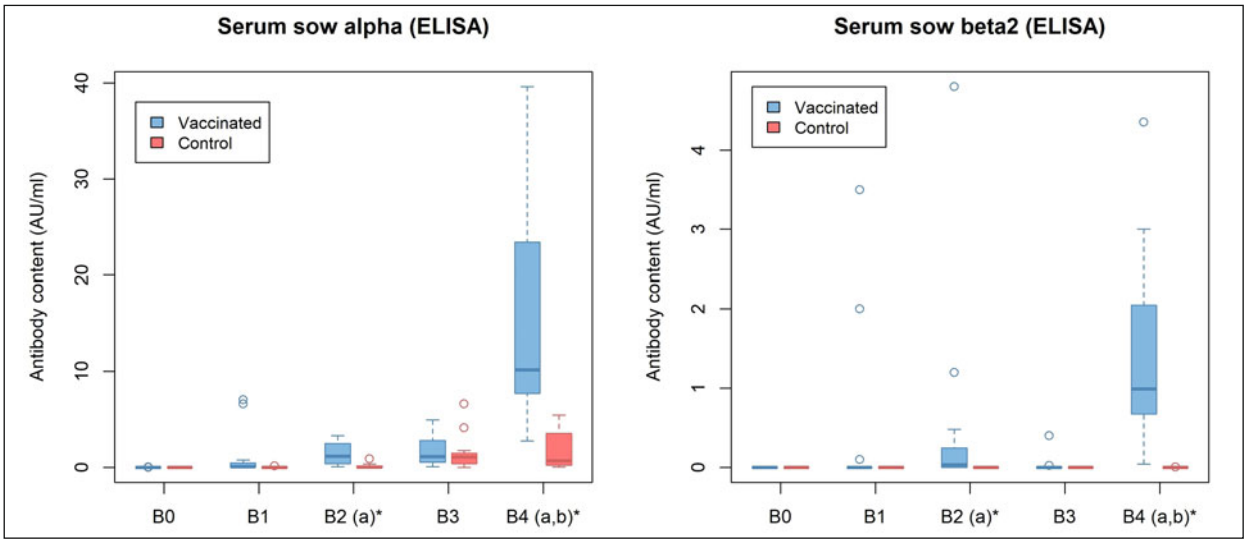
were calculated and the vaccinated group was compared to the non-vaccinated group by Mann-Whitney U test (level of significance $p<0.05$). Fisher's exact test (level of significance $p<0.05$) was used to evaluate the mortality (toxin challenge trial), losses of piglets (field trial), incidence of diarrhea and the detection of *C. perfringens* type A (field trial).

Results

Efficacy under laboratory conditions

The antibody titers against the alpha and beta2 toxins determined in sera and colostrum of the gilts are shown in Figures 1 and 2. Significantly higher (Mann-Whitney U test, $p<0.05$) antibody titers in the vaccinated group compared to the control group for both, alpha and beta2 toxins, were found in the serum at B2 as well as in the colostrum (colostrum 1). All vaccinated gilts showed neutralizing antibodies against alpha toxin (LNT) in the colostrum (titer from 4 to 256) at 1st farrowing. In contrast, only 6 out of 15 control gilts showed neutralizing antibodies against the alpha toxin (maximum titer 4) in the colostrum.

Piglets from priming-immunized (5 and 2 weeks before 1st farrowing) gilts showed significantly ($p<0.05$) higher antibody titers against alpha and beta2 toxins compared to the control group until four weeks after 1st farrowing (Figures 3). Neutralizing antibodies against the alpha toxin were detected in the majority of piglets from vaccinated gilts until 4 weeks post farrowing (PB1: 90.0%, PB2: 83.3%, PB3: 73.3%, PB4: 56.7%). In contrast, only 2 out of 30 piglets (6.7%) of the control gilts showed low titers of neutralizing antibodies (maximum



Source: Sven Springer

FIGURE 1: Antibodies against alpha and beta2 toxin (ELISA) detected in serum of gilts under laboratory conditions (B0 = before 1st vaccination, B1 = before 2nd vaccination, B2 = at 1st farrowing, B3 = before 3rd vaccination, B4 = at 2nd farrowing, blue bars = vaccinated animals, red bars = control animals, the line in the bars indicates the median, (a) = significant differences between vaccinated and control group, (b)* = significant difference between 1st and 2nd farrowing, p<0.05).*

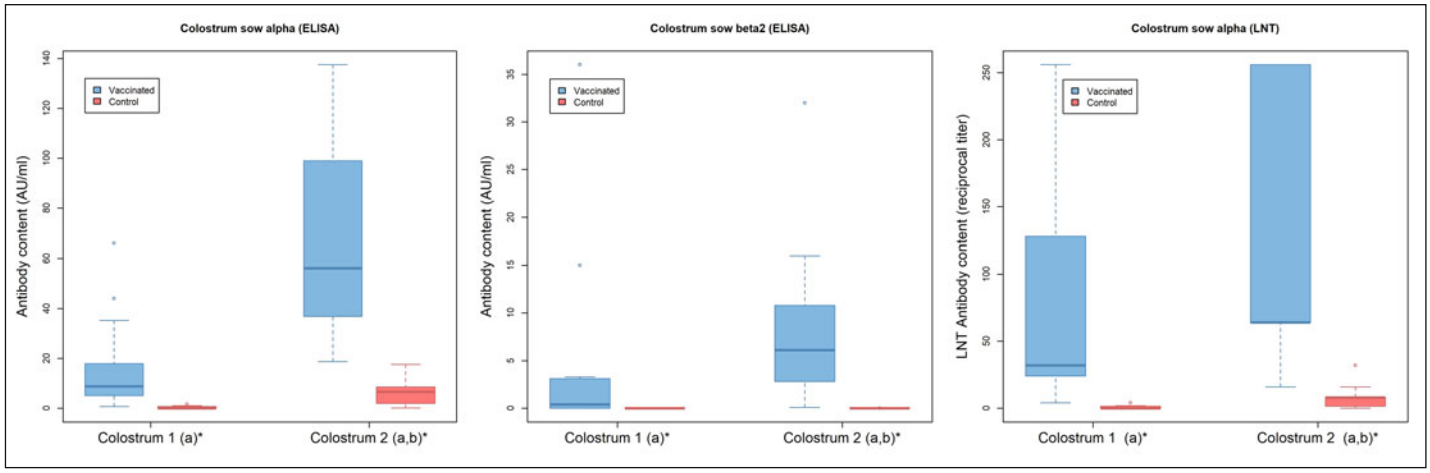


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FIGURE 2: Antibodies against alpha toxin (ELISA, LNT) and beta2 toxin (ELISA) detected in colostrum of gilts under laboratory conditions (Colostrum 1 = at 1st farrowing, Colostrum 2 = at 2nd farrowing, blue bars = vaccinated animals, red bars = control animals, the line in the bars indicates the median, (a) = significant differences between vaccinated and control group, (b)* = significant difference between colostrum 1 and 2, p<0.05).*

titer 2) against the alpha toxin up to 2 weeks after birth (PB2).

Before booster vaccination (B3) during 2nd gestation the antibody titers in serum against alpha and beta2 toxins had decreased when compared to the titers determined directly after 1st farrowing (B2) (Figures 1). However, booster vaccination significantly gained both, the serum (B4) and colostrum (colostrum 2) antibody titers against alpha and beta2 toxins compared to the first pregnancy (Figures 1 and 2).

All sows vaccinated three times showed high titers of neutralizing antibodies against the alpha toxin in the colostrum (32 to 256) at 2nd farrowing (Figure 2). Neutralizing antibodies were also detected in the colostrum of control sows (maximum titer 32, 9 out of 11 animals).

In addition, the piglets of the 2nd gestation had significantly higher serum antibody titers against alpha and beta2 toxins compared to the piglets of the 1st gestation of the same sows. Similar to the piglets from the 1st gestation the antibody titers decreased over the period of 4 weeks after farrowing (Figure 3).

All piglets of 3 times vaccinated sows showed neutralizing antibodies against the alpha toxin until 3 weeks (PB3) post 2nd farrowing (maximum titer 16). At 4 weeks post farrowing 21 out of 22 piglets had neutralizing antibodies. In the control group 13 (PB1), 10 (PB2), 7 (PB3) and 4 piglets (PB4) out of 22, respectively had neutralizing antibodies against alpha toxin. The maximum titer was 4.

The results of clinical symptoms (clinical score) and the mortality after intra-abdominal toxin challenge performed after the 1st farrowing are given in Table 2. None of the piglets from vaccinated gilts showed clinical symptoms, died or had to be euthanized as a result of the toxin administration. In contrast, most piglets of the control group showed severe clinical symptoms (complete recumbency, somnolence, no milk intake). The mean of the clinical score in the vaccinated group was significantly lower (Mann-Whitney U test, $p<0.001$) than in the control group. Following administration of the challenge toxin altogether 11 out of 20 piglets of the control group died or had to be euthanized due to severe clinical symptoms. Overall, the difference between the vaccinated group and the control group was significant (Fisher's exact test, $p<0.001$).

Efficacy under field conditions

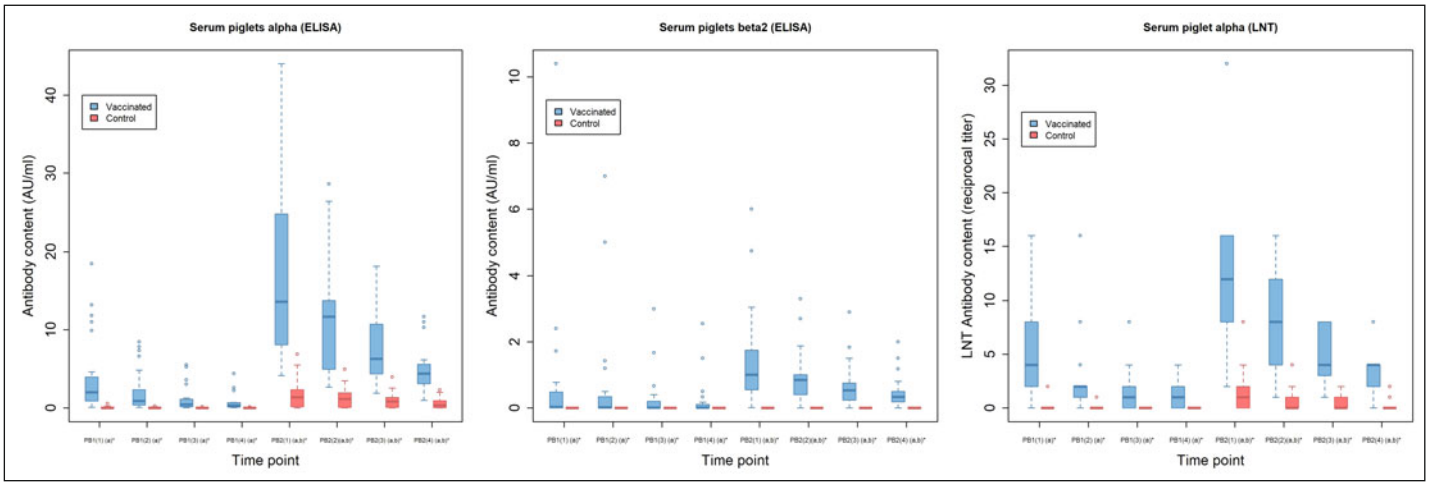
The results regarding antibodies against the alpha and beta2 toxins in serum and colostrum are shown in Figure 4 and 5. The vaccinated gilts showed a significant increase (Mann-Whitney U test, $p<0.05$) in antibodies against the alpha and beta2 toxins in serum (B1) and colostrum compared to control gilts. Before vaccination, neutralizing antibodies against the alpha toxin could not be detected in the serum of any gilts in the vaccine and control group. At farrowing all vaccinated gilts showed neutralizing antibodies against the alpha toxin in serum (titers 1 to 16) and colostrum (titers 2 to 512). No neutralizing antibodies against the alpha toxin could be detected in the serum and colostrum of the control gilts.

In the vaccinated group, 13 out of 217 piglets (6%) died until the age of 26 days. In the control group, 16 out of 257 piglets (6.2%) died during the same period. Significant differences between the groups were not present. In both groups most of the losses of piglets occurred during the 1st week of age. Most of the piglets died during

TABLE 2: *Results of clinical examination (clinical score) and mortality after vaccination of the sows with Enteroporc AC and challenge of the piglets with an alpha and beta2 toxin containing supernatant of a heterologous C. perfringens type A strain under laboratory conditions*

Group	Gilts (n)	Piglets (n)	Score (Mean ± SD)	Dead piglets (n)	Mortality (%)
Vaccinated	10	20	0[a]	0[b]	0[b]
Placebo	10	20	4.50 ± 1.28	11	55.00

[a] $p<0.001$ (Mann-Whitney U test), [b] $p<0.001$ (Fisher's exact test)



Source: Sven Springer

FIGURE 3: Antibodies against alpha toxin (ELISA, LNT) and beta2 toxin (ELISA) detected in serum of piglets under laboratory conditions (PB1(1) = 1 week after 1st farrowing, PB2 (1) = 2 weeks after 1st farrowing, PB3 (1) = 3 weeks after 1st farrowing, PB4 (1) = 4 weeks after 1st farrowing, PB1(2) = 1 week after 2nd farrowing, PB2 (2) = 2 weeks after 2nd farrowing, PB3 (2) = 3 weeks after 2nd farrowing, PB4 (2) = 4 weeks after 2nd farrowing, blue bars = vaccinated animals, red bars = control animals, the line in the bars indicates the median, (a) = significant differences between vaccinated and control group, (b)* = significant difference between 1st and 2nd farrowing, p<0.05).*

this time because of being crushed by the mother sow, because of being a runt or due to aspiration of amniotic fluid and subsequent pneumonia.

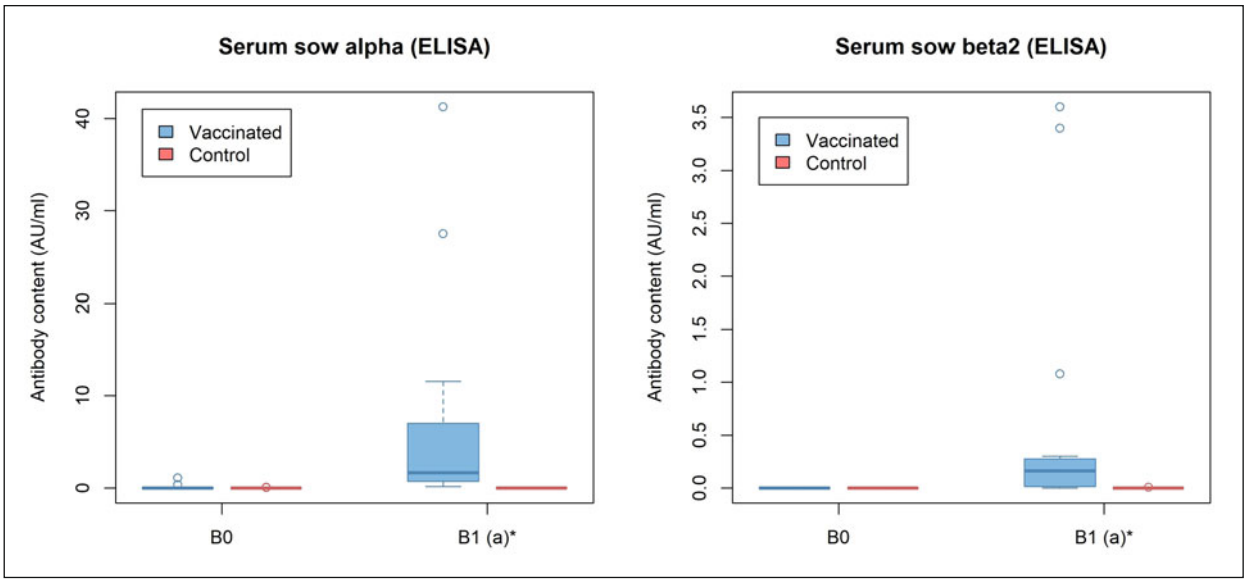
The incidence of diarrhea (1 to 26 days of life) in the piglets of the vaccine and the control group are shown in Table 3. Diarrhea occurred most frequently in piglets up to the age of 5 days. Between the age of 2 to 5 days and at the average age of 7 days these differences between the groups were significant (Fisher's exact test, $p<0.05$).

Within the entire suckling period there were in total 38.7% of piglets (84 out of 217 piglets) from vaccinated gilts with diarrhea compared to 62.6% piglets (161 out of 257 piglets) from control gilts. This difference was also significant (Fisher's exact test, $p<0.001$).

Bacteriological examination of 69 rectal swabs of diarrheal piglets from the vaccinated and control group revealed *C. perfringens* type A (*cpa, cpb2*) in 40 and *C. perfringens* type A (*cpa*) in 5 of the cases. In the vaccinated group, *C. perfringens* type A (*cpa, cpb2*) was detected 17 times and *C. perfringens* type A (*cpa*) three times. In the control group *C. perfringens* type A (*cpa, cpb2*) occurred 23 times and *C. perfringens* type A (*cpa*) occurred twice. Significant differences were not detectable between the groups. *Clostridium perfringens* type C or enterotoxin producing *C. perfringens* type A strains were not detected.

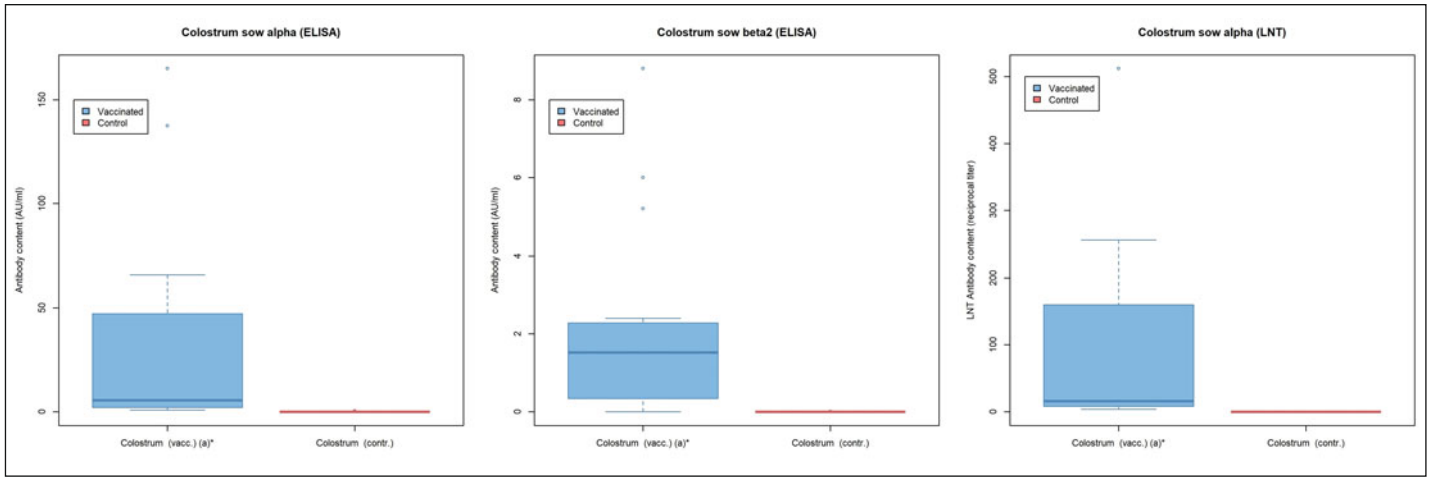
Discussion

Both, in the toxin challenge model and under field conditions, the vaccinated gilts developed significantly higher antibody titers against alpha and beta2 toxins in serum and colostrum compared to placebo-treated gilts. Antibodies against the alpha toxin showed neu-



Source: Sven Springer

FIGURE 4: Antibodies against alpha and beta2 toxin (ELISA) detected in serum of gilts under field conditions (B0 = before 1st vaccination, B1 = at farrowing, blue bars = vaccinated animals, red bars = control animals, the line in the bars indicates the median, (a) = significant differences between vaccinated and control group, p<0.05).*



Source: Sven Springer

FIGURE 5: Antibodies against alpha toxin (ELISA, LNT) and beta2 toxin detected in colostrum of gilts under field conditions (blue bars = vaccinated animals, red bars = control animals, the line in the bars indicates the median, (a) significant differences between vaccinated and control group, p<0.05).*

tralizing activity, which is in line with reports from Springer et al. (2012) after vaccination of gilts with an alpha and beta2 toxoid containing *Clostridium perfringens* type A vaccine.

When sows were boostered during their 2nd gestation the mean colostrum antibody titer and the mean piglet serum antibody titer were also significantly higher at 2nd farrowing compared to the 1st farrowing. These results are in line with previous reports, that also showed a significant increase of antibodies against beta toxin (*C. perfringens* type C) and transmission to the piglets after basic and booster vaccination of gilts with a *C. perfringens* type A/C toxoid vaccine (Richard et al. 2019). The results also support the current vaccination scheme with one booster vaccination prior to every following farrowing (Hogh 1976). However, the results of the determination of antibodies in colostrum and serum of the piglets also show that significantly higher antibody levels occur after three times vaccination than after two times vaccination. To guarantee an adequate supply of antibodies to large litters via colostrum, vaccination three times, e.g., twice at an interval of 3 weeks before insemination and once before the 1st farrowing, can be recommended.

As already shown by Springer et al. (2012), piglets originating from vaccinated sows were significantly better protected after intra-abdominal administration of an alpha and beta2 toxin containing supernatant of a *Clostridium perfringens* type A strain compared to piglets from non-vaccinated sows. Restrictively, it should be noted that the intra-abdominal toxin administration model applied in this study is not capable to reproduce the major clinical symptoms (diarrhea) that occur under natural conditions. However, Johannsen et al. (1993) were also able to induce transient signs of systemic toxin effects after oral administration of vegetative *Clostridium perfringens* type A bacteria and intragastric application of toxin containing CpA supernatants.

The intra-abdominal toxin administration model clearly shows that severe clinical symptoms occur when the toxins are absorbed and can be considered as the worst-case scenario of the outcome of the clinical infection caused by toxins producing *Clostridium perfringens* type A strains. This may explain the sudden death of young piglets and increased piglet mortality described after infection with *Clostridium perfringens* type A (Gresham 1997, Holmgren et al. 2004).

In the field trial presented, Rota- and Coronaviruses (examination during selection of the herd), enterotoxin producing *Clostridium perfringens* type A strains and *C. perfringens* type C were ruled out by laboratory evaluation before and during the study. Accompanying vaccination of gilts with a vaccine against *E. coli* diarrhea and metaphylactic treatment of piglets with a coccidiostat further excluded the involvement of enterotoxic *E. coli* and coccidia. Thus, the study demonstrated that *Clostridium perfringens* type A was able to cause severe diarrhea in suckling piglets from non-vaccinated animals and could be effectively controlled by vaccination. The occurrence of *Clostridium perfringens* type A as a causative agent of diarrhea is in accordance with previous communications (Garmory et al. 2000, Bueschel et al. 2003, Jost et al. 2005, Springer et al. 2012) but are contrary to results from Kongsted et al. (2018), who questioned the involvement of *Clostridium perfringens* type A in diarrhea. However, the occurrence of clinical signs, positive culture, detection of toxins or toxin genes (alpha and beta2 toxin) and exclusion of other relevant infectious agents are required for a confirmed diagnosis. At the same time, it is important to note that *Clostridium perfringens* type A strains can differ significantly in their in vitro toxin production capacity (Springer et al. 2012).

TABLE 3: *Incidence of diarrhea in piglets of vaccinated sows and control sows (proof of efficacy under field conditions)*

Age (days)	Group	N	No diarrhea	Diarrhea	Diarrhea %	P-value*
1	1	217	190	27	12.4	0.356
	2	257	217	40	15.6	
2	1	214	193	21	9.8	<0.001
	2	252	181	71	28.2	
3	1	212	193	19	9.0	<0.001
	2	250	199	51	20.4	
4	1	210	183	27	12.9	0.005
	2	249	191	58	23.3	
5	1	209	193	16	7.7	<0.001
	2	249	192	57	22.9	
Ø 7	1	209	205	4	1.9	0.013
	2	247	230	17	6.9	
Ø 10	1	209	206	3	1.4	0.156
	2	245	235	10	4.1	
Ø 12	1	208	207	1	0.5	0.130
	2	242	236	6	2.5	
Ø 14	1	208	207	1	0.5	1.000
	2	242	241	1	0.4	
Ø 21	1	205	203	2	1.0	0.212
	2	240	240	0	0.0	
Ø 26	1	204	202	2	1.0	0.459
	2	238	233	5	2.1	

N = Number of piglets, Ø = Average age of the piglets, *Fisher's exact test

In the presented field trial vaccination with a *C. perfringens* type A/C vaccine had no effect on piglet mortality and detection of *C. perfringens* type A. Nevertheless, mortality is not a typical outcome of *Clostridium perfringens* type A infection compared to infection with *C. perfringens* type C, especially in clinical cases, where other enteric pathogens were ruled out. At the same time a reduction of the detection of *C. perfringens* type A in fecal samples after basic immunization is not expected because the antibodies are primarily directed against the toxins. However, the incidence of diarrhea was significantly reduced by vaccination compared to the control group in piglets between 2 to 5 days of age as well as throughout the entire study period. Including the results of the intra-abdominal toxin administration model, it can be assumed that vaccination can also prevent systemic clinical courses that may occur after resorption of the toxins during mixed infections with other pathogens. The results of the trials also show evidence of the efficacy of the vaccine under laboratory (toxin challenge model) and field conditions, which is required according to Regulation 2019/6. Because *C. perfringens* belongs to the spore-forming bacteria and has a shorter generation time compared to other bacteria (Kiu and Hall 2018), vaccination should always be accompanied by a check of hygiene measures. Since colostrum uptake is a prerequisite for the efficacy of a maternal vaccination, it should also be verified whether piglets are able to take up sufficient amounts of colostrum.

Ethical approval

The authors assure that they have followed the generally accepted rules of good scientific practice during the development of the present paper. All relevant international, national and/or institutional ethical guidelines for the handling of animals used in the study were observed. Details of the laboratory animal application and its approval can be found in the published text.

Conflict of interest

The authors, with the exception of MB, are employed by Ceva Santé Animale, which distributes the vaccine. The content or opinions expressed in the manuscript have not been influenced by any proprietary, financial, professional or other personal interests.

Autors contribution

SSp, RF and OB developed the idea and designed the studies. MB, RF, OB and SSp organized the animal trials, performed the clinical examinations and collected the samples. TT carried out the pathological examination. JF and VF developed the tests to detect the specified antibodies and examined the samples. TL performed the statistical analysis. SSp and RF analyzed and interpreted the data and wrote the manuscript. TT, OB, JF, and TL helped with the interpretation of the data. All authors reviewed the manuscript and approved the final version.

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