Retrospective study on necrotizing enteritis in piglets in Switzerland

M. Jäggi¹, N. Wollschläger², C. Abril³, S. Albini³, C. Brachelente⁴, M. Wyder¹, H. Posthaus¹

¹Institute of Animal Pathology, ²Swine Clinic and ³Institute of Veterinary Bacteriology, University of Bern, Switzerland, ⁴Department of Biopathological Sciences, University of Perugia, Italy

Abstract

The re-emergence of necrotizing enteritis (NE) in Swiss pig breeding farms raised concern that, besides C. perfringens type C strains, additional C. perfringens toxinotypes might cause this disease. Therefore we retrospectively investigated the association of NE with C. perfringens type C or different C. perfringens toxinotypes. We evaluated pathological lesions, routine diagnostic bacteriology results, and multiplex real-time PCR analyses from DNA extracts of archived intestinal samples of 199 piglets from our diagnostic case load. 96.5% of NE cases and 100% of herds affected by NE were positive for C. perfringens type C genotypes. Animals without necrotizing enteritis revealed a significantly lower detection rate of type C genotypes. Non affected piglets showed a high prevalence for beta-2-toxin positive C. perfringens type A strains. Collectively, our data indicate that outbreaks of NE in piglets in Switzerland cannot be attributed to newly emerging pathogenic toxinotypes, but are due to a spread of pathogenic C. perfringens type C strains.

Keywords: Clostridium perfringens type C, necrotizing enteritis, beta-toxin, porcine

Introduction

Clostridium perfringens are gram-positive, anaerobic, endospore-forming bacteria which are wide-spread in the environment. They are normal inhabitants of the intestinal tract of mammals, but certain toxigenic strains frequently cause severe enteric diseases in animals and humans (Songer, 1996). A large array of exotoxins and other virulence factors have been discovered (Petit et al., 1999; Smedley et al., 2004). Individual pathogenic isolates, however, only secrete a fraction of this toxin repertoire. The production of 4 major toxins α, β, ε, and τ allows the categorization into 5 toxinotypes (A–E). The cpb gene encoding α-toxin is located on the bacterial chromosome, whereas those encoding β- (cpb), ε- (ctx) and τ-toxin (iap/iab) are located on plasmids (Petit et al., 1999). Individual strains can secrete a variety of additional toxins such as enterotoxin (cpe) or β-toxin (cpb2). The β-toxin has recently been associated with gastrointestinal diseases in animals and humans (Schotte et al., 2004; Fisher et al., 2005). Two alleles of the cpb2 gene have been described (Gibert et al., 1997; Fisher et al., 2005; Jost et al., 2005). Whereas the consensus cpb2 gene is found in porcine C. perfringens isolates, an atypical gene, which has 62.3% identity and 80.4% similarity to the former one, is mainly present in non-porcine isolates (Bueschel et al., 2003; Jost et al., 2005). Clostridium perfringens types A and C are principally involved in enteric disease in swine (Songer and Uzal,
2005). Type A strains produce α-toxin, type C strains α- and β-toxin. Forcine isolates belonging to both toxino-types are frequently β-toxin (cpb2) positive (Bueschel et al., 2003; Waters et al., 2003; Jost et al., 2006). C. perfringens type C strains are known to cause necrotizing enteritis (NE) in neonatal piglets. This disease occurs worldwide and represents an economically important problem (Songer and Uzal, 2005). Typically, neonatal piglets from their first day of life until approximately 3 weeks of age are affected. Peracute to acute disease affects piglets within the first days post partum. Depression is followed by rapid death and in a proportion of cases hemorrhagic diarrhea. Piglets showing signs at 1–3 weeks of age often have a more protracted clinical course with non-hemorrhagic diarrhea. The hallmark lesion is a deep, segmental mucosal necrosis of the small intestine, mainly the jejunum, with massive hemorrhage in the intestinal wall. More protracted cases may show a segmental, fibrino-necrotizing enteritis (Songer and Uzal, 2005).

Immunization of pregnant sows with C. perfringens type C toxoid based vaccines and antibiotic treatment of newborn piglets reduces the incidence of fatal NE (Springer and Selbitz, 1999). However, vaccination does not always protect from disease (Springer and Selbitz, 1999; Lugrin-bühl, 2002). Failure of piglets to receive adequate amounts of protective colostral antibodies, trypsin secretion deficiencies, centroal protease inhibitors and the involvement of further toxins in the pathogenesis of the disease are possible factors which contribute to disease outbreaks (Songer and Uzal, 2005). C. perfringens type A strains are generally considered to be normal, non-pathogenic inhabitants of the intestinal tract in pigs (Songer, 1996). Since the discovery of β-toxin (Gilbert et al., 1997), an association of β-toxigenic type A strains with diarrhea and enteritis in piglets was however reported in pigs and other species (Herholz et al., 1999; Klaassen et al., 1999; Waters et al., 2003; Schotte et al., 2004). Frequent isolations of such strains from porcine fecal samples in the Institute of Veterinary Bacteriology, Bern, raised questions whether they could contribute to a re-emergence of necrotizing enteritis in Switzerland.

To address this we investigated whether outbreaks of NE in piglets in Switzerland were solely associated with a C. perfringens type C infection or whether β-toxigenic C. perfringens type A strains could have played a role in this disease. We thus retrospectively evaluated our routine diagnostic case material from piglets submitted to the Institute of Pathology from January 2000 to July 2006. For a limited number of animals, which were necropsied in parallel to an epidemiological study by Wollschräger (2007), available herd management data allowed us to evaluate the status of C. perfringens type C infections on herd level. All animals were examined macroscopically. Confirmation of the diagnosis of NE was achieved by histopathology and/or routine diagnostic bacteriology which included genotyping of isolated cultures. In cases where these examinations were inconclusive or incomplete, multiplex real time PCR analyses of intestinal tissue DNA extracts was performed to identify clostridial infection.

**Animals, Material and Methods**

**Animals**

199 one day to four weeks old suckling piglets submitted for routine diagnostic necropsy to the Institute of Animal Pathology from January 2000 to July 2006 were included in this study. 142 of these were classified as NE cases based on macroscopical findings. 57 animals, which died of other causes, were not affected by NE and served as reference cases. In the following these cases are referred to as NE cases and reference cases, respectively.

**Herds**

57 of the 199 piglets, necropsied during 2005 and 2006, derived from herds which took part in a parallel epidemiological study at the Swine Clinic, Vetsuisse Faculty, University of Bern (Wollschräger, 2007). Thus reliable information about the occurrence of NE within the herd was available.

**Histopathology**

Formalin-fixed, paraffin-embedded small intestinal tissue samples were available from 104 NE and 57 reference cases. In the remaining animals a conclusive diagnosis was achieved through the combination of macroscopical and bacteriological findings. Small intestinal tissue samples were routinely processed for histology and stained with hematoxylin and eosin (H & E).

**Routine bacteriological examination and genotyping**

Routine bacteriological culturing for C. perfringens was performed on intestinal samples of 125 NE and 53 reference cases at the Institute of Veterinary Bacteriology, Vetsuisse Faculty, Bern. C. perfringens culture growth was achieved from 118 necrotizing enteritis and 53 reference animals. Genotyping data achieved by a conventional PCR based genotyping protocol (Albini et al., 2008; Herholz et al., 1999) were available from 105 NE and 38 reference cases. Briefly, 5 individual bacterial colonies, which showed the typical morphology of C. perfringens, were pooled. Template DNA was obtained by direct lysis and PCR using specific primers for cpa, cpb, and cpb2 was performed.

**DNA extraction and multiplex real-time-PCR from tissue samples**

C. perfringens culture genotyping results were not available in 37 cases of NE and 19 reference animals. These cases were thus examined by multiplex-real-time-PCR on
intestinal tissue DNA extracts. Additionally, all cases negative for either \( cpb \) or \( cpb2 \) were re-evaluated using this method to exclude false negative results due to limitation of culture genotyping to 5 colonies. Paraffin embedded tissue blocks or small intestinal tissue samples, frozen at necropsy, were investigated by direct multiplex real time PCR. Three 20 μm thick slices of paraffin-embedded small intestinal tissue blocks were cut and transferred to 1.5 ml microcentrifuge tubes. To avoid cross contamination during sample processing, microtome blades were changed after cutting each tissue block. Additional, control samples from empty paraffin blocks (empty controls) were cut before each new tissue block was processed. DNA was extracted using the Qiagen DNeasy Kit (Qiagen, Basel, Switzerland). Additionally, during necropsies in 2005 and 2006 samples of the small and large intestine of each animal were separately frozen at –20 °C. After thawing, DNA was extracted from each individual sample using the Quiagen mini stool kit (Quiagen; Basel, Switzerland). 2.5 μl of template DNA of either paraffin embedded or frozen tissues was used for gene amplification applying a recently described multiplex real-time PCR protocol for detection of \( cpa \), \( cpb \), \( cpb2 \), \( etx \) and \( cpe \) (Albini et al., 2008). Finally, the results of culture genotyping and direct gene amplification from the intestinal samples were combined. The combination of individual toxin genes thus indicated presence (\( cpb \) positivity in the absence of \( etx \)) or absence (\( cpb \) negative) of \( C.\ perfringens \) type C strains.

**Statistical analysis**

Animals were defined as affected or non-affected by NE according to the morphological findings. Statistical analysis was done by cross tabulation of the disease status to the final bacterial genotype. The respective frequencies were compared using Chi-Square and Fisher’s Exact Tests. All statistical analyses were run in the statistical software package NCSS 2007 (www.ncss.com). The level for statistical significance was set to \( p < 0.05 \).

**Results**

**NE cases**

Macroscopically, 142 piglets showed a typical segmental, necro-hemorrhagic jejunitis (Fig. 1). The histological evaluation of 104 of these cases revealed acute lesions in 60 cases. These were characterized by deep coagulation necrosis of the mucosa, vascular necrosis and massive hemorrhage in the lamina propria, submucosa and occasionally the muscular layers (Fig. 2). Inflammation was mostly absent and large numbers of gram positive rods were visible in the necrotic zones. Subacute lesions were present in 44 cases and were characterized by an additi-

Figure 1: Small intestine of a 2 day old piglet showing the characteristic segmental necro-hemorrhagic enteritis (arrowheads).

Figure 2: Histopathology of acute NE in a 2 day old piglet. Deep necrosis of the lamina mucosae (arrows), underlying hemorrhage (arrowheads) without inflammatory reaction (H&E stain, bar 100 μm).

Figure 3: Histopathology of subacute NE in a 2 week old piglet. Deep necrosis of the lamina mucosae (arrow) and demarcation by a rim of large numbers of mainly neutrophilic granulocytes (arrowheads) (H&E stain, bar 100 μm).
onal moderate to massive infiltration with neutrophilic granulocytes at the margin of the necrotic zone (Fig. 3). Colonies of small, gram-negative, coccoid bacteria were present in necrotic areas and clostridium-like organisms were visible in smaller numbers. The acute form of the disease was mainly present in neonatal piglets, which died during the first 3 days of life. Subacute lesions predominated in older animals (Fig. 4).

Retrospective evaluation of routine diagnostic genotyping data revealed the genotypes $cpa/cpb/cpb2$ in 95/105 (90.4 %), $cpa/cpb2$ in 5/105 (4.8 %), and $cpa$ in 5/105 (4.8 %) cases. 50 cases of NE, where either no culture genotyping results were available or where these were negative for $cpb$ or $cpb2$, were investigated. No gene amplification was achieved from empty control blocks which excluded contamination during tissue processing. All intestinal samples tested positive for $cpa$, confirming the presence of $C. perfringens$, even in culture negative cases.

Amplification of $cpe$ and $etx$ was not achieved in any of the samples, indicating that neither $C. perfringens$ type B or D strains were present in detectable quantities. Genotyping revealed $cpa/cpb/cpb2$ in 42 (84 %), $cpa/cpb$ in 4 (8 %), $cpa/cpb2$ in 2 (4 %), and $cpa$ in 2 (4 %) cases. Thus, compared to culture genotyping results we were able to detect $C. perfringens$ type C genotypes in 40 additional animals. The combined results of both methods on the individual animal level are depicted in Figure 5.

Figure 4: Age distribution of piglets with necrotizing enteritis.

Reference cases

Fifty-seven animals neither had macroscopical nor histological lesions of NE. Retrospective evaluation of necropsy reports revealed that the cause of death was $E. coli$ diarrhea in 15, coccidiosis in 3, inanition in 9, accidental crushing by the mother in 11, bacterial septicaemia in 2 and unresolved in 17 cases.

Routine diagnostic genotyping revealed the genotypes $cpa/cpb/cpb2$ in 4/38 (10.5 %), $cpa/cpb2$ in 29/38 (76.3 %), and $cpa$ in 5/38 (13.2 %) cases. 52 samples were tested. Genotyping revealed $cpa/cpb/cpb2$ in 11 (21.2 %), $cpa/cpb2$ in 28 (53.8 %), and $cpa$ in 13 (25 %) cases. The combined results of culture genotyping and direct real-time PCR on the individual animal level are depicted in Figure 5. The predominant gene combination detected was $cpa/cpb2$ (61.4 %), which corresponds to the presence of $cpb$ positive type A strains.

Overall, 96.5 % of animals with NE were positive for a genotype indicative of $C. perfringens$ type C infection ($cpa/cpb/cpb2$ or $cpa/cpb$), in contrast to 26.3 % of reference animals without NE.

Figure 5: Association of necrotizing enteritis with $cpb$ positive $C. perfringens$. Combination of culture genotyping and multiplex real-time PCR analyses revealed that necrotizing enteritis cases (NE) were significantly associated (*) with detection type C genotypes ($cpa/cpb/cpb2$; $cpa/cpb$). In contrast to this, the frequency of type C genotype detection was significantly lower (**) in animals without necrotizing enteritis (reference animals) (Fisher’s exact test, $p < 0.001$).

Figure 6. Genotyping results depicted on the herd level. 100 % of herds diagnosed with necrotizing enteritis (NE) were positive for $cpb$ ($C. perfringens$ type C). Most herds not affected by necrotizing enteritis (no NE) were positive for $cpb2$ positive $C. perfringens$ type A strains ($cpa/cpb2$).
animals (Fig.5). This difference was highly significant (p < 0.0001). Exclusive detection of \textit{C. perfringens} type A genotypes (genotype \textit{cpa} or \textit{cpa/cpb2}) was significantly higher in reference cases.

\textbf{Herd level data}

Data on 57 animals which derived from 35 farms allowed us to evaluate \textit{C. perfringens} type C detection on a herd level (Fig.6). All herds in which NE was diagnosed in at least one animal, also tested positive for \textit{cpb}. In contrast to this, in herds not-affected by NE we mainly detected \textit{cpa/cpb2} positive \textit{C. perfringens} type A.

\textbf{Discussion}

\textit{C. perfringens} type C strains are known to cause severe necrotizing enteritis in suckling piglets. In Switzerland, this disease remains to be a problem in swine production despite the availability of commercial \textit{C. perfringens} type C vaccines (Gut et al., 2002; Luginiuhl, 2002). Emerging reports of \textalpha–toxigenic \textit{C. perfringens} as potential additional cause of enteric disease in piglets (Klaassen et al., 1999; Garmory et al., 2000; Bueschel et al., 2003), increasing concern about the spread of the disease to and also from Swiss stock breeding farms led us to re-evaluate the association of necrotizing enteritis with \textit{C. perfringens} type C in Switzerland. To retrospectively evaluate cases where initial diagnostic culture genotyping results were not available we made use of a recently established multiplex-real-time PCR analysis (Albini et al., 2008) on intestinal DNA extracts. By combining these results with available data from the initial diagnostic bacteriology, we were able to evaluate which toxin genes were present in \textit{C. perfringens} from the intestinal tract of each individual animal. Our results demonstrate a significant association between the detection of \textit{C. perfringens} type C strains and NE in piglets. The gene combinations \textit{cpa/cpb/cpb2} and \textit{cpa/cpb}, which in case of absence of \textit{ext} indicate \textit{C. perfringens} type C infection, were detected in 96.5\% of all NE cases. These results correspond to published reports from other countries (Soner and Uzal, 2005). Interestingly, all but 1 \textit{cpb} positive cases were also \textit{cpb2} positive. Due to the experimental setup chosen in our study (DNA extracts of intestinal samples or pooled colonies of \textit{C. perfringens}) it was not possible to determine whether the \textit{cpb2} gene derives from the same \textit{cpa/cpb/cpb2} positive type C, or a combination of \textit{cpa/cpb} positive type \textit{C} and \textit{cpa/cpb2} positive type A strains. Several studies (Bueschel et al., 2003; Waters et al., 2003; Jost et al., 2006) have shown that porcine type C strains are frequently \textit{cpb2} positive. It is also known, that in acute cases, which represent more than half of our case material, \textit{C. perfringens} type C can be isolated almost in pure culture (Soner and Uzal, 2005). It is thus conceivable that in our study most of the \textit{C. perfringens} type C strains harbour the \textit{cpb2} gene and account for a large proportion of \textit{cpa/cpb/cpb2} positive results. Additional presence of \textit{cpb2} positive type A strains can however not be excluded. Two studies (Sayed et al., 2008; Vidal et al., 2008) recently demonstrated that \textit{C. perfringens} \textalpha–toxin is both required and sufficient to induce experimental necrotizing enteritis in the rabbit ileal loop model. Although not proven experimentally for the pig intestine, it is conceivable that \textit{C. perfringens} type C strains alone are the causative agent of NE in our cases. Data on the herd disease status were available through a parallel clinical study (Wollschläger, 2007) for 57 animals. When all data from one herd were combined, \textit{C. perfringens} type C infection were detected in 100\% of herd affected by recent outbreaks of NE.

We also detected high prevalence of \textit{cpb2} positive \textit{C. perfringens} type A strains in piglets which were not affected by NE. This supports the conclusion that these strains are not associated with NE in Swiss pig herds but may be found frequently in our case material. Several reports indicate, that \textalpha–toxigenic type A strains might induce milder forms of enteritis in piglets, however these strains are also frequently isolated from healthy pigs (Soner and Uzal, 2005). Detailed studies on the prevalence of these strains in healthy pigs have however been lacking so far, which hampers statistical evaluations of suspected increased prevalence of these strains in clinically diseased animals.

The age distribution of necrotizing enteritis in our study corresponds to published data (Soner and Uzal, 2005). NE mainly affected animals within the first three days post partum. These animals showed acute lesions with large numbers of clostridium-like organisms. \textit{C. perfringens} type C, when ingested early post-partum, rapidly outcompetes the developing intestinal flora and thus leads to extensive damage of the mucosa by secretion of \textbeta–toxin (Soner and Uzal, 2005). Because, \textbeta–toxin is rapidly degraded by trypsin, high trypsin inhibitor levels in the bowel wall leads to extensive damage of the mucosa by secretion of \textbeta–toxin.
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Corresponding address

Dr. H. Posthaus
Institute of Animal Pathology, Vetsuisse Faculty
University of Bern
PO-Box 8466
CH-3001 Bern, Switzerland
E-Mail: horst.posthaus@itpa.unibe.ch

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