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Clonal Relationship and Antimicrobial Susceptibility of Porcine Clostridium perfringens type C Isolates from Switzerland

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SUMMARY

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Summary

Clostridium perfringens type C causes fatal necrotizing enteritis (NE) in newborn animals and humans. Outbreaks of NE are still recorded in Swiss pig breeding farms, however the clonal relationship of different outbreak isolates and their antibiotic resistances are unknown. We evaluated 38 Swiss *C. perfringens* type C isolates obtained during outbreaks of NE from 1999 to 2011 and 6 isolates from a Belgian pig breeding farm. Clonal relationship of isolates was investigated by pulsed-field gel electrophoresis (PFGE) and the resistance to 15 antibiotics was determined using minimal inhibitory concentrations (MICs). We identified 12 different PFGE types in Swiss isolates, and the Belgian isolates clustered in three additional PFGE types. On several farms, isolates obtained from the same outbreak showed different PFGE types. All isolates were resistant to tetracycline. Additionally, resistance against cefotetan, clindamycin, metronidazole and penicillin were detected. Our study shows that NE in Switzerland is caused by more than one clonal lineage of *C. perfringens* type C and resistance to clinically important antibiotics occurs in porcine *C. perfringens* isolates. The use of antibiotics as metaphylactic treatment should be avoided by using prophylactic vaccination in order to avoid selecting resistant *C. perfringens* type C in pig populations.

Keywords***:*** *Clostridium perfringens* type C, necrotizing enteritis, PFGE, clonality, antibiotic resistance

OVERVIEW AND AIM OF THE THESIS

Clostridium perfringens is a gram positive, widely distributed enteric pathogen in livestock and humans. Different types of *C. perfringens* (A, B, C, D and E) have been described and their pathogenic mechanisms are incompletely characterized. *C. perfringens* type C strains induce a rapidly fatal necrotizing enteritis (NE) in animals (mainly pigs) and humans. One essential virulence factor of these strains is the beta-toxin (CPB), a plasmid borne beta-barrel pore-forming toxin.

The first outbreak of NE in Switzerland were three decades ago. The disease is still present with a decreased incidence. In affected litters the mortality is high and can reach 100%. While passive immunisation of sows with the *C. perfringens* type C toxoid vaccines is protective against NE it is not generally implemented to protect pig herds in Switzerland. Thus antibiotics are still used metaphylactically in affected pig herds to reduce mortality. However, the intensive use of antibiotics in pig herds increases the risk of selecting antibiotic resistance amongst pathogenic clostridia. Furthermore there was no knowledge about whether one or few predominant clones of *C. perfringens* type C were circulating in Swiss pig farms.

The goal of our study was to investigate, whether specific clones were responsible for the NE outbreaks in Switzerland and to determine the antibiotic resistance profiles of porcine *C. perfringens* type C isolates from different outbreaks of NE in Switzerland.

1.) INTRODUCTION

Clostridium perfringens, a Gram-positive, anaerobic, rod-shaped bacterium, is a part of the normal gut microbiata of humans and animals [1]. Certain pathogenic strains however can cause wound infections, food poisoning, enteritis, or enterotoxemias in animals and humans. Pathogenicity is associated with the ability of strains to produce potent exotoxins. *C. perfringens* strains are divided into five toxinotypes (A to E) depending on the production of the four major toxins, α (CPA), β (CPB), ε (ETX) and ι (ITX), (Table 1). CPA is produced by all types of *C. perfringens,* and is a metallophospholipase C that has both phospholipase C (PLC) and sphingomyelinase activity and activates host cell signaling [2]. It causes hemolysis, dermonecrosis, and is lethal for the cells [3]. CPB is a beta-barrel pore-forming, cell specific toxin leading to vascular damage, and is encoded on a conjugative plasmid in *C. perfringens* types B and C [4, 5]. ETX is a plasmid born, pore-forming and highly potent toxin produced by *C. perfringens* types B andD [6]. ETX causes entertoxemia and has an ability to cross the blood-brain barrier and targets glutamatergic neurons [7]. ITX is composed of two separate proteins IA and IB encoded on a plasmid in *C. perfringens* type E. It has an ADP-ribosylating action causing cell death [8].

Table 1. Toxinotypes of *Clostridium perfringens.*

	Major toxins			
Toxinotype	α	β	ε	ι
A	+	-	-	-
B	+	+	+	-
C	+	+	-	-
D	+	-	+	-
E	+	-	-	+

All strains can produce additional toxins (e.g., CPE, CPB2, PFO, TpeL, kappa-toxin, delta-toxin, mu-toxin, lambda-toxin, μ-toxin) not used for typing and several enzymes (e.g., clostripain, cysteine protease, sialidase, DNase, urease) [9]. CPE is a small pore-forming toxin produced by all types of *C. perfringens* except type B and is either encoded on the chromosome or plasmid and acts enterotoxic [5]. CPB2 is a plasmid born toxin which acts necrotizing but its pathophysiological activitiy is still unknown [9]. PFO is a chromosomal toxin and can be produced by all *C. perfringens* types and is a member of the cholesterol-dependent cytolysin (CDC) family of pore forming toxins [10, 11]. TpeL is plasmid born and is the largest known clostridial toxin belonging to the clostridial glycosilating toxin (CGT) family produced by *C. perfringens* types A, B and C strains [12]. Other toxins either plasmid (delta-toxin, lambda-toxin) or chromosomal encoded (kappa-toxin, mu-toxin, μ-toxin) and enzymes (clostripain, cysteine protease, sialidase, DNase and urease) act as additional virulence factors with different modes of actions [9] (Table 2).

Table 2. Currently known *C. perfringens* toxins and their proven or hypothetical role in diseases.

Toxin (Abbreviation)	Gene	Gene locus	Activity	Proven Disease Association *Putative Disease Association (italics)*
Enterotoxin (CPE)	*cpe*	chromosome plasmid	PFT	Humans: food intoxication, sporadic diarrhea *Pigs: diarrhea* *Dogs: diarrhea*
β-toxin (CPB)	*cpb*	plasmid	PFT	Humans: enteritis necroticans/pigbel Pigs, foals, sheep, goats: necrotizing enteritis
ε-toxin (ETX)	*etx*	plasmid	PFT	Ruminants: enterotoxemia
NetB	*netb*	plasmid	PFT	Chicken: necrotic enteritis
β2-toxin (CPB2)	*cpb2*	plasmid	PFT	*Pigs: enteritis* *Horses: colitis*
ι-toxin (ITX)	*iap* *ibp*	plasmid plasmid	Binary toxin	*Calves, sheep, rabbits: enterotoxemia, enteritis*
α-toxin (CPA)	*cpa*	chromosome	Phospho-lipase	Human: gas gangrene
Perfringolysin (PFO)	*pfo*	chromosome	PFT (CDC)	Human: gas gangrene

C. perfringens type C causes a fatal, necrotizing enteritis (NE) in newborn animals and humans [1]. Piglets are most frequently affected and mortality rates can reach 100%. The disease can be peracute, acute and subacute-chronic. Peracute to acute forms occur in piglets within the first days postpartum with abdominal pain and bloody diarrhea. The subacute-chronic form affects piglets of 1-3 weeks of age with a more protracted clinical course and non-hemorrhagic diarrhea [13]. Clinically, the acute and peracute disease is characterized by abdominal pain, depression and bloody diarrhea, which starts after 8 to 22 hours after exposure to the pathogen [14]. At necropsy, findings are mainly in the small intestine but the large intestine can also be affected. Grossly, there is a segmental, hemorrhagic and necrotizing enteritis, while in the subacute disease, a fibrinohemorrhagic enteritis with pseudomembranes is observed [10, 15]. The histological hallmark of the disease is a segmental hemorrhagic necrosis of all layers of the small intestine, especially of the jejunum [16].

C. perfringens type C induces enteritis necroticans in malnourished humans and was firstly described in Germany after the second World War as Darmbrand [17]. In Papua New Guinea the disease was endemic and known as pigbel [18]. The main dietary item in Papua New Guinea used to be sweet potato, containing heatstable trypsin inhibitors. The consumption of large amounts of potentially highly contaminated meat (especially pork) together with a background of a generally low protein diet caused a predisposition to the disease [19]. In humans, clinical abdominal distention, pain, vomiting, diarrhea and hypotension is present [20]. The main autopsy finding is a hemorrhagic and necrotizing enteritis affecting mainly the proximal Jejunum [21]. Histologically, a transmural hemorrhagic necrosis of the intestinal wall with fibrin thrombi in the capillaries is found [21]. *C. perfringens* type C isolates produce at least CPA and CPB and may secrete several other toxins including *β2* (CPB2), enterotoxin (CPE), perfringolysin O (PFO) and

TpeL [9, 10]. CPB is the essential virulence factor of these strains [22]. It induces rapid cytopathic effects and programmed necrosis in endothelial cells, while epithelial cells do not seem to be affected by the toxin. [4, 23]. CPB is highly sensitive to trypsin which partly explains the susceptibility of neonates, due to low trypsin levels in the intestine and trypsin inhibitors in the colostrum [24].

CPB is encoded by the *cpb* gene, carried on a large uncharacterized virulence plasmid [25], and the size ranges from ~65 to ~110 kb [5]. *C. perfringens* type C isolates can contain several toxin encoding plasmids for beta 2 toxin, enterotoxin and TpeL production. The *cpb2*-carrying plasmids are distinct from the *cpb*-carrying plasmids [5]. The *tpeL* gene can be located on a different plasmid but some cpb-carrying plasmids also carry the *tpeL* gene 3 kb upstream of the *cpb* gene [20]. Non of the *tpeL* gene positive isolates were found to carry the *cpe* gene [20]. Most of the toxin and resistance plasmids share a region called *tcp* (transfer of a clostridial plasmid) which mediates a highly efficient conjugation of toxin and resistance plasmids [6, 20]. It is recognized that conjugative plasmids contain both virulence and antibiotic resistance genes [20, 26].

Antibiotic resistance genes are widely disseminated in bacteria [27]. However, several resistance genes are more prevelant and transferable in gram-positive bacteria [28]. Resistance genes for tetracycline, chloramphenicol, bacytracin and lincomycin are common in *C. perfringens* [1, 26, 29]. Tetracycline resistance is the most common resistance phenotype in *C. perfringens* and is encoded by *tetA(P)*, *tetB(P)* and *tet(M)*-like genes, which are located on conjugative and non-conjugative plasmids and on the chromosome [30]. The genes *catD* and *catP* may be responsible for chloramphenicol resistance in *C. perfringens*, and the latter is located on transposon Tn*4451*, found on a conjugative plasmid [31]. The *catQ* gene can also cause chloramphenicol

resistance but is located on the chromosome [32]. The gene *bcrABDR* is a chromosomally located gene causing bacytracin resistance especially in poultry isolates [33]. Lincomycin resistance is mostly caused by the *erm(B)* gene [34]. The recently found *InuP* gene, located on the transposon tIS*Cpe8*, is found on a conjugative plasmid and causes lincomycin resistance [26].

Resistances against antimicrobial substances are increasing in all bacteria including *Clostridium* spp. [34]. Resistant clostridia carry most of these resistance genes on conjugative plasmids, which are capable to transfer these genes to susceptible bacteria [26, 35, 36]. Any use of antibiotics in veterinary or human medicine will induce a selective pressure on bacteria to develop or acquire antibiotic resistance. Therefore, improper or excessive use of antibiotics in veterinary medicine can lead to spread of resistances to clinically important antibiotics, not only used in the veterinary field but also in human medicine [37].

In Switzerland NE was first reported in 1983 [38]. Since then, NE has been regularly diagnosed in our diagnostic service at the Vetsuisse Faculty in Bern [15]. Due to its spore forming ability, *C. perfringens* can persist in the environment for extended periods of time. Therefore, movement of healthy carrier animals to unaffected farms is considered to play a major role in distributing the pathogen [1]. The implementation of *C. perfringens* type C toxoid vaccines in sows reduced the disease incidences worldwide [39, 40]. Piglets receive passive immunisation through antibodies taken up from the colostrum and are efficiently protected against disease, but not against infection by *C. perfringens* type C. Recent work from our group in collabortion with the porcine clinic showed, that outbreaks of NE in Switzerland mainly occured in non-vaccinated herds [15, 16]. Vaccinated herds were mostly protected and ”vaccination failures” were mainly due to improper vaccination of individual sows (Posthaus, personal observation). Additionally,

we could demonstrate the presence of the pathogen even several years after initial disease outbreaks on the affected farms and a subsequent disease-free period due to vaccination [41]. We also found indications, that a combination of vaccination together with adequat hygiene and herd health management could significantly reduce the prevalence of the pathogen in an infected herd [42]. Nevertheless, vaccination with *C. perfringens* type C toxoid vaccines is not generally used to protect pig herds in Switzerland [15, 40]. The Swiss porcine health service recommends vaccinations only in farms with high risk situations while A-R farms and replacement herds can only vaccinate after an outbreak. Although incidences of NE have decreased over the last decade, we have still experienced several outbreaks of NE in Switzerland in recent years. During an outbreak of NE, mortality can only be reduced by metaphylactic antibiotic therapy of all newborn piglets [15]. However, the metaphylactic use of antibiotics in pig husbandry once again increases the risk of selecting antibiotic resistance amongst pathogenic clostridia [37]. We were therefore interested in investigating antibiotic resistance profiles in all porcine *C. perfringens* type C strains available in our strain collection. Additionally we were interested in whether one or few predominant clones of *C. perfringens* type C were circulating in Swiss pig farms. The genetic relationship of *C. perfringens* strains can be analysed by several methods [43]. PFGE has been used successfully to subtype over 50 different bacterial species [44], and still remains a meaningful tool for molecular typing [45]. Isolates originating from humans, animals and food were investigated by pulsed-field gel electrophoresis (PFGE) [46]. Results from previous studies showed that isolates belonging to the same outbreak show similar patterns while in isolates from non-outbreaks the genetic diversity is high [47]. However, data about genetic diversity of porcine *C. perfringens* isolates are sparse and there is no study about the clonal relationship focussing on porcine *C. perfringens* type C isolates. Therefore we did not know the molecular epidemiology of *C. perfringens* type C strains circulating in swiss pig herds.

The goal of our study was to investigate, whether specific clones were responsible for the NE outbreaks in Switzerland and to determine the antibiotic resistance profiles of porcine *C. perfringens* type C isolates from different outbreaks of NE in Switzerland from 1999 to 2011. Six Belgian isolates were included to compare Swiss *C. perfringens* type C isolates to those from a geographically distinct region.

2.) MATERIALS AND METHODS

2.1. Bacterial strains and growth conditions

A total of 44 *C. perfringens* type C isolates were obtained from 15 newborn piglets with typical post-mortem lesions of NE during outbreaks in their herds between 1999 and 2011. Isolates derived from 9 different Swiss pig breeding farms (A-I) and one Belgian farm (J) (Table. 3). Isolates from farm A-H were stock cultures of the strain collection of the Institute of Veterinary Bacteriology of the University of Berne and were obtained from previous research studies [15, 16, 48, 49]. Isolates from farm I and J were isolated from outbreaks in 2010 and 2011. One isolate per farm was available for farm A, B, C, E, F and G, whereas several isolates were obtained from farms D, H, I and J. Isolates from farm I (n=20) derived from 2 individual piglets. Ten *C. perfringens* colonies, showing typical colony morphology on *Clostridium perfringens* agar plates (mCP; Oxoid, Basel, Switzerland), from each piglet were subcultured, tested by multiplex PCR for *C. perfringens* toxin genes as described previously [50, 51] and frozen as separate stock cultures for further analyses. The six Belgian isolates derived from 2 piglets from one outbreak of NE on a commercial breeding farm and were isolated as described [52, 53] and frozen as stock cultures. Isolates were routinely cultivated on blood agar and in brain heart

infusion (BHI) broth (Difco) supplemented with 0.05% L-cysteine (Sigma-Aldrich) under anaerobic conditions at 37 ˚C for 18 to 24 hours.

Table 3. Description of the *C. perfringens* type C isolates collected from 9 Swiss farms and one Belgian farm.

Farm	Outbreak year	Piglet	Number of isolates	Isolate
A	1999	1	1	JF 2256
B	2000	1	1	JF 2519
C	2003	1	1	MLP 101
D	2005	1	2	659-4, 659-10
		2	2	691, 697
		3	2	JF 3719, JF 3720
E	2005	1	1	JF 3716
F	2005	1	1	JF 3721
G	2006	1	1	JF 3717
H	2006	1	5	144
		2	1	JF 3718
I	2011	1	10	871
		2	10	872
J	2010	1	5	2252, 2253, 2254, 2255, 2256
		2	1	2258

2.2. PCR genotyping of isolates

Total DNA was extracted from a 4ml overnight culture in BHI using guanidium thiocyanate as described previously [54]. The presence of toxin genes, *cpa*, *cpb*, *cpb2*, *cpe*, *etx*, *itx*, *pfo*, *tpeL* and *netB* was determined by polymerase chain reaction (PCR) as described previously [4, 51].

2.3. Pulsed-field-gel-electrophoresis (PFGE)

Isolates were grown in 5ml BHI medium overnight at 37 ˚C under anaerobic conditions. Four ml of the culture were centrifuged at 14,000 rpm, resuspended in 500µl TE-buffer (10mM Tris, 100mM EDTA, p.H 8.0) and fixed with 4% formaldehyde (vol/vol) to reduce DNase activity as described previously [55]. The bacteria were washed twice by resuspension in TE-buffer and

centrifugation at 14,000 rpm, cell pellets were weighted and resuspended in a proportional volume of TE-buffer to obtain the same cell density for all samples. 300µl of the bacterial suspension was mixed with 300 µl melted 1.5% agarose (Seakam Gold agarose, Lonza) and the samples were put on ice to solidify. The plugs were incubated in 1.2ml TE-buffer containing lysozyme (2mg/ml) at 37 ˚C for 5 hours. The plugs were further incubated overnight in 0.5M EDTA, 1% N- laurosylsarcosin, proteinase K (2mg/ml), p.H 8.0 at 50 ˚C. Plugs were washed five times in TE-buffer for 30min. DNA was digested using 50 U of *SmaI* (Roche) and the digested DNA fragments were separated in a 1% agarose gel (Pulsed Field Certified Agarose; Bio-Rad) in 0.5 x TBE buffer containing thiourea (9mg/l) using a CHEF-DR III system (Bio-Rad) for 20 hours at 6 V/cm and with pulse time ramping from 0.5 to 40 sec at 12°C. Gels were stained with ethidium bromide for 45min, destained 2 x 20min with distilled water, and photographed with a Gel Doc 2000 system (Bio-Rad). DNA banding patterns were analyzed using the BioNumerics software (version 6.6, Applied Maths, Saint-Martens-Latem, Belgium). Dice coefficient was used for the level of similarity between isolates and the cluster analysis was performed using the unweighted pair-group method with arithmetic averages (UPGMA) with settings of optimization 1.5% and position tolerance 1.5% [56]. PFGE types were created with a similarity of more than 95% [53].

2.4. Plasmid profiles

Plasmid DNA isolation was performed using Plasmid DNA Purification NucleoBond PC100 kit (Macherey-Nagel) according to the manufacturer`s protocol and was analyzed in 0.7% agarose gel. Electrophoresis was performed at voltage of 40V for 10 min and followed by 70V for 3 hours. The agarose gels were stained with ethidium bromide and photographed with Gel-Doc 2000 system.

2.5. Determination of antimicrobial resistance

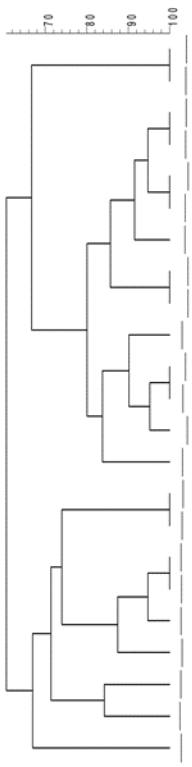
Minimal inhibitory concentrations (MICs) were determined in supplemented brucella broth containing horse blood (Trek Diagnostics Systems, East Grinstead, UK) using customized standard susceptibility microtiter plates ANO2B (Trek Diagnostics Systems, East Grinstead, UK) for anaerobic bacteria following the manufacturer`s instructions. 10µl of cell suspension with a density of 0.5 Mc Farland were mixed into 10ml of supplemented brucella broth with blood (Trek Diagnostics Systems, East Grinstead, UK) to obtain a final concentration of 10^6 cells/ml and inoculated for 24 hours at 37 °C under anaerobic conditions. Breakpoints used were those for gram positive anaerobes defined by the European Committee of Antimicrobial Susceptibility Testing (EUCAST) except for cefotetan, tetracycline, cefoxitin and mezlocillin for which breakpoints of Clinical and Laboratory Standards Institute M100-S21 [57] were used. The following antibiotics and resistance breakpoints were used: ampicillin/sulbactam (> 8 mg/L), amoxicillin/clavulanic acid (> 8 mg/L), ampicillin (> 8 mg/L), cefotetan (≥ 64 mg/L), cefoxitin (≥ 64 mg/L), chloramphenicol (> 8 mg/L), clindamycin (> 4 mg/L), imipenem (> 8 mg/L), meropenem (> 8 mg/L), metronidazole (> 4 mg/L), mezlocillin (≥ 128 mg/L), penicillin (> 0.5 mg/L), piperacillin (> 16 mg/L), piperacillin/tazobactam (> 16 mg/L) and tetracycline (≥ 16 mg/L). *Bacteroides fragilis* (ATCC 25285) and *Bacteroides thetaiotaomicron* (ATCC 29741) were used for quality control and MIC values were within acceptable ranges. *Clostridium perfringens* ATCC 13124 was used as a control. Isolates displaying MIC for penicillin >0.25 were tested for β-lactamase production using DrySlide Nitrocefin test (Becton Dickinson). Isolates displaying antimicrobial resistance and increased minimal inhibition concentration (MIC) values were further analyzed by PCR for the presence of resistance genes *tetA(P), tetB(P), erm(B), erm(Q), catD, catP, catS* and *catQ* as described [28]. PCR was performed using taq

polymerase with the following conditions: 1 cycle of 94°C for 3min and 25 cycles of 94°C for 30 s, 50°C for 30 s, and 72°C for 1min, followed by a final single extension of 3 min at 72°C.

3.) RESULTS

3.1. Genotyping and clonal relationship among C. perfringens type C isolates

All 44 porcine *C. perfringens* type C isolates contained the *cpa, cpb*, *cpb2*, *pfo* and *tpeL* genes and were negative for *cpe*, *etx*, *itx*, and *netB*. They could be subdivided into 15 pulsed field gel electrophoresis types (I-XV; Fig. 1). Twelve PFGE types were identified in Swiss isolates and another three in Belgian isolates independently from farm or individual piglet origin. Distinct PFGE types were isolated from each farm except for farms E and G, where isolates displaying identical PFGE profiles were found (Fig.1, isolates JF3716 and JF3717). In farms D, I and J, where several isolates from different animals were available, more than one PFGE type was found (Table 4 and Fig. 1). Isolates obtained from the same animal (Table 4; Farm D, H, I and J) exhibited the same PFGE type except in two cases (Table 4; Farm D piglet nr. 3, Belgium, piglet nr. 1) where isolates displaying different PFGE types (XII, XIII and VII, VIII, IX; Fig. 1) were isolated from one animal. On farm H, *C. perfringens* type C isolates exhibiting two different PFGE types (I and XV; Fig. 1) were detected in pigs from two separate outbreaks in the same year. The Belgian isolates clustered in 3 different PFGE-types (VII, VIII, IX; Fig. 1) and showed a markedly different restriction pattern compared to the Swiss isolates, with the exception of one Swiss isolate (JF2519), whose PFGE profile exhibited 90% similarity to the Belgian isolates.



Isolate	Farm	Piglet	Outbreak Year	PFGE type	Plasmid type	Resistance genes	Resistance profile
144-1	H	1	2006	I	X	*tet A(P), erm(Q)*	TET, CLI
144-2	H	1	2006	I	X	*tet A(P), erm(Q)*	TET, CLI, MTZ
871-1	I	1	2011	II	I	*tet A(P)*	TET
872-1	I	2	2011	II	I	*tet A(P)*	TET
659-10	D	1	2005	III	IV	*tet A(P)*	TET
659-4	D	1	2005	III	IV	*tet A(P)*	TET
JF2256	A	1	1999	IV	VI	*tet A(P)*	TET
691	D	2	2005	V	V	*tet A(P)*	TET, CLI, PEN, CTT, MTZ
697	D	2	2005	V	IV	*tet A(P)*	TET
JF2519	B	1	2000	VI	III	*tet A(P)*	TET, PEN
2252	J	1	2010	VII	I	*tet A(P)*	TET, PEN
2256	J	1	2010	VII	I	*tet A(P)*	TET, PEN
2253	J	1	2010	VII	I	*tet A(P)*	TET, PEN
2254	J	1	2010	VIII	II	*tet A(P)*	TET, PEN
2255	J	1	2010	IX	III	*tet A(P)*	TET, PEN
2258	J	2	2010	IX	III	*tet A(P)*	TET, PEN, MTZ
JF3716	E	1	2005	X	IV	*tet A(P)*	TET
JF3717	G	1	2006	X	VII	*tet A(P)*	TET
MLP101	C	1	2003	XI	VII	*tet A(P)*	TET
JF3719	D	3	2005	XII	IX	*tet A(P)*	TET
JF3720	D	3	2005	XIII	IX	*tet A(P)*	TET
JF3721	F	1	2005	XIV	IV	*tet A(P)*	TET
JF3718	H	2	2006	XV	VIII	*tet A(P), erm(Q)*	TET, CLI

Figure 1. Dendrogram constructed from PFGE-*SmaI* patterns from porcine *C. perfringens* type C isolates generated by UPGMA and Dice coefficient (Comparison settings: optimization 1.5 %, position tolerance 1.5 %). CLI, clindamycin; MTZ, metronidazole; PEN, penicillin; CTT, cefotetans; TET, tetracycline.

3.2 Plasmid profiles

Genetic diversity was also observed by determining plasmid profiles (Appendix, pp. 37-38). Ten different plasmid profiles were determined among the 44 *C. perfringens* type C isolates (Fig. 1). Isolates with the same PFGE pattern were found to have distinct plasmid profiles and vice versa. Interestingly, in five separate isolates from one piglet on farm J three different plasmid profiles were detected (Table. 4).

Table 4. PFGE and plasmid types of porcine *C. perfringens* type C isolates.

Farm	Outbreak year	Piglet	Number of isolates	Isolate	PFGE type	Plasmid type
A	1999	1	1	JF 2256	IV	VI
B	2000	1	1	JF 2519	VI	III
C	2003	1	1	MLP 101	XI	VII
D	2005	1	2	659-4, 659-10	III	IV, IV
		2	2	691, 697	V	V, IV
		3	2	JF 3719, JF 3720	XII, XIII	IX, IX
E	2005	1	1	JF 3716	X	IV
F	2005	1	1	JF 3721	XIV	IV
G	2006	1	1	JF 3717	X	VII
H	2006	1	5	144	I	X
		2	1	JF 3718	XV	VIII
I	2011	1	10	871	II	I
		2	10	872	II	I
J	2010	1	5	2252, 2253, 2254, 2255, 2256	VII, VIII, IX	I, I, II, III, I,
		2	1	2258	IX	III

3.3. Antimicrobial resistance profile

Minimal inhibitory concentrations and the MIC50 and MIC90 values of all antibiotics tested for all *C. perfringens* isolates are shown in Table 5. All 44 isolates were resistant to tetracycline and contained the *tetA(P)* gene (Fig.1). Resistance to cefotetans (n=1), clindamycin (n=7), metronidazole (n=6) and penicillin (n=8) was also detected. Additionally, several isolates showed MICs of 4µg/ml for metronidazole (n=14), 4 µg/ml for ampicillin (n=4), 16 µg/ml for piperacillin-tazobactam (n=2), 16 µg/ml for piperacillin (n=3) and 8 µg/ml for chloramphenicol (n=5) which are situated one two-fold dilution below the resistance breakpoints of EUCAST. During an outbreak in one Swiss farm (D), two isolates (691 and 697) which had distinct plasmid profiles, but shared the same PFGE pattern, were found to exhibit different resistance profiles (Fig. 1). Two isolates exhibiting resistance to penicillin (2252 and 2254) and showed increased MICs to cephalosporins (MIC= 16 µg/ml) were negative for β-lactamase production. All but one

isolate (691) with an MIC of >8 for clindamycin contained the *erm(Q) gene*. Genes for chloramphenicol resistance were not detected in the isolates even if a strain showed decreased susceptibility to this drug. Belgian isolates showed distinct resistance profiles compared to Swiss isolates (Fig. 1). In particular, penicillin resistance was found in all isolates.

Table 5. Minimal inhibitory concentration (MIC) of 15 antibiotics for porcine *Clostridium perfringens* type C isolates.

		Number of strains with MIC (µg/ml)													
		≤0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	128	MIC50	MIC90
Antimicrobial	**ATCC 13124***														
Ampicillin/sulbactam	≤0.5/0.25		41				1	2						≤0.5/0.25	≤0.5/0.25
Amoxicillin/clavulanic acid	≤0.5/0.25		41				2	1						≤0.5/0.25	≤0.5/0.25
Ampicillin	≤0.5			39		1		4						≤0.5	≤0.5
Cefotetan	≤4						43						1	≤4	≤4
Cefoxitin	≤1				26		5	5	3	5				≤1	16
Chloramphenicol	4					23		16	5					≤2	8
Clindamycin	≤0.25		29			6	2			7				≤0.25	>8
Imipenem	≤0.12	36			6	1	1							≤0.12	0.5
Meropenem	≤0.5			43				1						≤0.5	≤0.5
Metronidazole	4					1	23	14	5		1			2	8
Mezlocillin	≤4						41		1	2				≤4	≤4
Penicillin	≤0.06	24	8	4		1	4	1	2					≤0.06	2
Piperacillin	≤4						41			3				≤4	≤4
Piperacillin/tazobactam	≤0.25/4		36			1	2	2	1	2				≤0.25/4	4
Tetracycline	≤0.25							6	30	8				8	>8

The dilution ranges tested for each antibiotic are those contained within the white area. Resistance breakpoints for anaerobes (vertical lines) were obtained from the European Committee of Antimicrobial Susceptibility Testing (EUCAST) and the Clinical and Laboratory Standards Institute (CLSI) (see Determination of antimicrobial resistance, Material and Methods). * *Clostridium perfringens* ATCC 13124

4.) DISCUSSION

Our study demonstrates that during the past 12 years in Switzerland, NE was caused by *C. perfringens* type C isolates belonging to different clonal lineages. Distinct PFGE types were not only observed in isolates from different farms, but also from *C. perfringens* type C isolates derived from the same farm. Similarly, several clonal lineages were also observed within one farm in Belgium. This indicates, that more than one pathogenic clone of *C. perfringens* type C is associated with NE on Swiss and Belgian pig farms. Isolates from the Belgian outbreak formed a specific PFGE cluster and were markedly distinct from Swiss isolates. The overall genetic diversity determined by PFGE was high between farms among Swiss and Belgian porcine *C. perfringens* type C isolates.

In Switzerland NE caused by *C. perfringens* type C was first described in 1983 and the disease spread rapidly [38, 58]. Especially some geographic regions with a high swine population were predisposed and breeding farms were more severely affected [15]. One explanation for the rapid spread in these regions could be the increasing animal transport and translocation of healthy carrier animals [15]. The Swiss porcine health service updated their regulations with new vaccination protocols. Vaccination in farms with a high risk was recommended, whereas A-R farms and replacement herds can only vaccinate after an outbreak and get an additional status of ”Vaccinating against Clostridia spp.”. Outbreaks of NE decreased over time but are still present in Switzerland and cause death in piglets [41]. Before the onset of our study, there was no knowledge about the clonal relationship of *C. perfringens* type C isolates causing NE in Switzerland. Typing of different bacterial isolates from the same species is important and is required for epidemiological surveys [44]. Clonal relationship of *Clostridium perfingens* in outbreaks from humans, animals and from different food sources have been determined by

PFGE, multilocus sequence typing (MLST) and other methods [43, 46]. PFGE is defined as the gold standard among molecular typing methods [59]. As PFGE uses variably expressed genes, e.g virulence genes, additionally to conserved genes, it is more discriminating for characterization of clonal relationships between different isolates than MLST is [60]. However, not all clostridial isolates are typeable by PFGE due to DNA degradation and smearing [20, 55]. In order to prevent DNA degradation and smearing, an additional step during DNA extraction was performed. Formaldehyde was added to fix the DNA and thiourea was added to the running buffer [55]. After this treatment all isolates in our study were typeable by PFGE. Previous studies on *C. perfringens* in poultry using PFGE have shown a high genetic diversity among isolates from healthy poultry while in diseased flocks mainly one or two distinct clones were found [46, 53, 61]. Our results are therefore similar to studies on avian *C. perfringens* isolates, which showed similar PFGE patterns with one to three distinct PFGE types per outbreak. Plasmids can contribute to this diversity because large plasmids (> 50kb) lead to a single band difference in the PFGE pattern [45]. In addition to the differences in PFGE patterns, our isolates showed distinct plasmid types. This could also contribute to the differences in the resistance profiles observed between several isolates.

Antibiotics have improved the health and life expectancy since they have been used for treating infectious diseases in humans and animals [37]. However, the increased use of antimicrobials causes selective resistances in bacteria, which is a worldwide problem [62]. Among anaerobic bacteria, such as in *Clostridium* spp., antimicrobial resistances increased and furthermore the susceptibility to these, have decreased erratically over the last years [34]. Both penicillin and lincomycin/spectinomycin are used for metaphylactic treatment in NE outbreaks in Switzerland while enrofloxacin, gentamicin, amoxicillin/clavulanic acid and ampicillin are used until the

diagnosis of NE has been proven [58]. *C. perfringens* carry resistance genes on mobile elements like plasmids [37]. Plasmids are important in *C. perfringens* as they carry genes for virulence and resistance factors [63]. Many plasmids are known to be conjugative as they harbor the transfer of a clostridial plasmid (*tcp*) locus [5, 35] and therefore, virulence and antibiotic resistance can potentially be disseminated via conjugation [20]. Moreover, localization of antibiotic resistance genes on conjugative clostridial plasmids can enable a transfer to other clostridial strains, including human pathogens [17, 26, 35].

We determined different antibiotic resistance profiles of isolates from different farms but also of isolates from the same farm and detected *tetA(P)* in all strains. Tetracycline was once a drug of choice in anaerobic infections until widespread resistance occurred [34]. The *tetA(P)* gene is the most common gene for tetracycline resistance and is located on the conjugative plasmid pCW3 [30, 64, 65].

Clindamycin and lincomycin belong to the macrolide-lincosamide-streptogramin (MLS) family and resistances have been described in porcine *C. perfringens* isolates [66]. These resistances are likely to be caused by the *erm(Q)* gene encoding (MLS)-type 23S methylase [66, 67]. We found the *erm(Q)gene* in all but one clindamycin resistant strain. Also lincomycin resistance is known to be encoded on a conjugative plasmid [26].

Metronidazole is the most prescribed antimicrobial worldwide and is the first choice of drugs for diarrhea therapy in dogs [34, 68]. The resistance is due to the *nim* gene which is mostly found on mobilizable plasmids or on the chromosome [69]. A previous study showed that *C. perfringens* isolates obtained from dogs showed decreased susceptibility against metronidazole [50]. Our results are similar and in our porcine *C. perfringens* type C isolates resistances against metronidazole were found.

β-lactam antibiotics are one of the largest and most important antibiotic classes including penicillins, cephalosporins, carbapenems and monobactams [70]. Resistances are common and anaerobic bacteria use three mechanisms to gain resistancy: inactivating enzymes, mainly beta-lactamases, low-affinity penicillin-binding proteins (PBPs), and decreased permeability through alterations in the porin channel. In some *Clostridium spp*. β-lactam resistances are due to expression of β-lactamases and resistances due to changes in the outer membrane protein (OMP)/porin channels, decreased PBP affinity, and efflux pumps are not well known [34].

C. perfringens is known to be susceptible to cephalosporins [1], and cefoxitin is inactive against most species of *Clostridium* except *C. perfringens* [34]. Interestingly, we detected increased MICs for cefoxitin (n=18) in some isolates but the mechanism of resistance to cephalosporins in *C. perfringens* is currently unknown. Resistance due to β-lactamase production has been described in some *Clostridium* species (except *C. perfringens*) [71]. In neurotoxigenic *Clostridium butyricum* type E strains, linear megaplasmids were found to be the hosts for β-lactamase genes [72]. Further studies are ongoing to investigate its basis in our isolates.

Previous studies described no penicillin resistance in *C. perfringens* isolates [1, 64]. Our results show that penicillin resistance and decreased susceptibility occurs in porcine *C. perfingens* type C isolates. This is of particular importance, because these antibiotics are used for metaphylactic and prophylactic treatment in cases of outbreaks of NE in Switzerland [15, 58]. In order to avoid the selection of such resistant clones, and with regard to the potential long term persistence of *C. perfringens* in once affected herds [41], metaphylactic use of antibiotics should be avoided by using vaccination as a suitable prophylaxis. A previous study by Schäfer et al [41] showed, that once affected herds should always be regarded as *C. perfringens* type C carriers. Despite disease free periods of several years, the pathogen can still be detected in very low quantities in once

affected and now vaccinated herds. Because of the low quantity in healthy and vaccinated pig herds, and the potential long term persistence of spores in the environment, it is not possible to declare a vaccinated, disease-free herd also free of the pathogen. Therefore, a continuous vaccination program together with an appropriate herd and hygiene management should be implemented in once affected herds.

In summary our results show, that recent outbreaks of NE in Switzerland were caused by different clones of *C. perfringens* type C. Resistances to tetracycline, penicillins, clindamycin, and decreased susceptibility to cephalosporins, metronidazole and chloramphenicol are common in these isolates and should be monitored in future.

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Characterization of virulence plasmids in porcine *Clostridium perfringens* type C

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Introduction

Clostridium perfringens is a gram positive, widely distributed enteric pathogen in livestock and humans. *C. perfringens* type C strains induce a rapidly fatal necrotizing enteritis in animals (mainly pigs) and humans. One essential virulence factor of these strains is the beta-toxin (CPB), a plasmid borne beta-barrel pore-forming toxin. Many of the virulence factors are plasmid borne in *C. perfringens* and potentially essential virulence factors may be encoded on the same plasmid. Especially the co-localization of toxin genes and antibiotic resistance genes could provide pathogenic strains with a selective advantage. The aim of our study is to determine whether additional virulence-associated as well as antibiotic-resistance genes are present on the *cpb*-containing plasmid of porcine *C. perfringens* type C strains that could confer selective growth advantages for these strains.

Material and Methods

39 Swiss and 6 Belgian *C. perfringens* type C isolates from several outbreaks between 1999 and 2011 were used. The genetic relationship between the isolates was investigated by PFGE. The antibiotic susceptibility was determined in one isolate representative of each PFGE profile by Minimal Inhibitory Concentrations (MIC) using customized standard susceptibility plates (ANO2B; TREK Diagnostic Systems, East Grinstead, West Sussex, United Kingdom). MICs were determined following the guidelines of the European Committee of Antimicrobial Susceptibility Testing (EUCAST) and breakpoints used were those for gram positive anaerobes defined by EUCAST if available (www.eucast.org). Southern Blots on plasmid DNA were performed to localise *cpb*, *cpb2* and *tet(A)*.

Results

Southern Blot

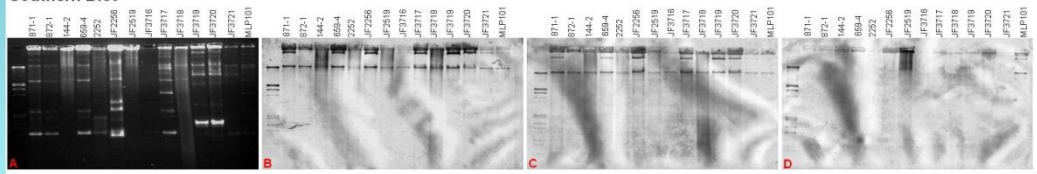

Figure 1. DNA-DNA hybridisation of plasmid DNA . Plasmid profile of ethidium bromide stained agarose gel (A) and hybridisation with a digoxigenin-labeled *cpb* (B), *cpb2* (C) and *tet(A)* (D) probe.

Genetic diversity of *Clostridium perfringens* Type C

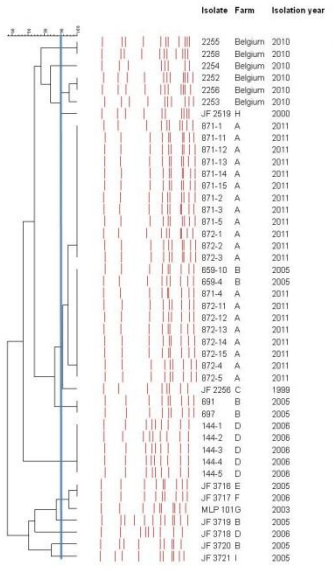

Fig 1:
Phylogenetic tree constructed from pulsed-field gel electrophoresis (PFGE) pattern of *Clostridium perfringens* Type C. *Cluster analysis was generated by Bionumerics 6.6; Applied Maths, Kortrijk, Belgium. Comparison settings: Dice, UPGMA, optimization 1.5 %, position tolerance 1.5 %.*

The blue line indicates the cut off value of ≥ 90 % determing clonality between the isolates.

Antibiotic resistance profile

Table 1. Minimal inhibitory concentration (MIC) of 15 antibiotics.

Isolate	A/ S2	TANS	IMI	CLI	MRD	AMP	TET	P/T4	PEN	AUG2	FOX	CHL	PIP	MEZ	MERO
EUCAST/ CLSI*	>8	>=64*	>8	>4	>4	>8	>=16*	>16	>0.5	>8	>=64*	>8	>16	>=128*	>8
871-1	<=0.5/0.25	<=4	<=0.12	<=0.25	4	<=0.5	8	<=0.25/4	<=0.06	<=0.5/0.25	<=1	4	<=4	<=4	<=0.5
872-1	<=0.5/0.25	<=4	<=0.12	<=0.25	4	<=0.5	8	<=0.25/4	<=0.06	<=0.5/0.25	<=1	<=2	<=4	<=4	<=0.5
144-2	<=0.5/0.25	<=4	<=0.12	>8	8	<=0.5	4	<=0.25/4	0.25	<=0.5/0.25	2	8	<=4	<=4	<=0.5
659-4	<=0.5/0.25	<=4	<=0.12	<=0.25	2	<=0.5	8	<=0.25/4	0.12	<=0.5/0.25	2	4	<=4	<=4	<=0.5
697	<=0.5/0.25	<=4	<=0.12	<=0.25	4	<=0.5	8	<=0.25/4	<=0.06	<=0.5/0.25	<=1	4	<=4	<=4	<=0.5
JF 2256	<=0.5/0.25	<=4	<=0.12	<=0.25	2	<=0.5	8	<=0.25/4	0.12	<=0.5/0.25	<=1	<=2	<=4	<=4	<=0.5
JF 2519	<=0.5/0.25	<=4	0.5	1	2	1	>8	2/4	2	<=0.5/0.25	8	<=2	<=4	<=4	<=0.5
JF 3716	<=0.5/0.25	<=4	<=0.12	<=0.25	4	<=0.5	8	<=0.25/4	<=0.06	<=0.5/0.25	<=1	4	<=4	<=4	<=0.5
JF 3717	<=0.5/0.25	<=4	<=0.12	<=0.25	4	<=0.5	8	<=0.25/4	0.12	<=0.5/0.25	<=1	4	<=4	<=4	<=0.5
JF 3718	<=0.5/0.25	<=4	<=0.12	>8	4	<=0.5	4	<=0.25/4	0.12	<=0.5/0.25	4	4	<=4	<=4	<=0.5
JF 3719	<=0.5/0.25	<=4	<=0.12	<=0.25	2	<=0.5	>8	<=0.25/4	<=0.06	<=0.5/0.25	<=1	4	<=4	<=4	<=0.5
JF 3720	<=0.5/0.25	<=4	<=0.12	<=0.25	4	<=0.5	8	<=0.25/4	<=0.06	<=0.5/0.25	2	4	<=4	<=4	<=0.5
JF 3721	<=0.5/0.25	<=4	<=0.12	1	2	<=0.5	>8	<=0.25/4	<=0.06	<=0.5/0.25	<=1	<=2	<=4	<=4	<=0.5
MLP 101	<=0.5/0.25	<=4	<=0.12	<=0.25	4	<=0.5	>8	<=0.25/4	<=0.06	<=0.5/0.25	<=1	4	<=4	<=4	<=0.5
2252 Belgium	<=0.5/0.25	<=4	<=0.12	2	2	4	>8	4/4	>4	<=0.5/0.25	16	4	<=4	<=4	<=0.5
2254 Belgium	2/1	<=4	0.5	1	4	4	>8	8/4	>4	2/1	16	<=2	16	16	<=0.5
2255 Belgium	4/2	<=4	0.5	1	4	4	8	16/4	2	2/1	16	4	16	8	<=0.5

Conclusion

- Different clonal lineages caused outbreaks in Switzerland.
- More than one clonal lineage was found per farm.
- Belgian isolates showed a distinct antibiotic resistance profile than Swiss isolates especially with β-lactam antibiotics.
- All isolates were positive for *cpb and cpb2* while *cpb2* is on the same plasmid or on a plasmid with the same size.
- *tet(A)* could be located on the chromosome except in MLP101.

Outlook

- Isolates with different antibiotic resistance profile and belonging to different clonal lineages will be selected to determine co-localisation of virulence factors on plasmids.
- We will attend to sequence the *cpb* plasmid of one porcine *C. perfringens* type C strain.

Clonal Relationship and Antimicrobial Susceptibility of Porcine *Clostridium perfringens* type C Isolates

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Poster presented at the ClostPath 2013, Tropical North Queensland, Australia, 22-26 October 2013

Clonal Relationship and Antimicrobial Susceptibility of Porcine *Clostridium perfringens* type C Isolates from Switzerland

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Introduction

Clostridium perfringens is a widely distributed enteric pathogen in livestock and humans. *C. perfringens* type C strains induce a rapidly fatal necrotizing enteritis (NE) in newborn animals and humans. Piglets are frequently affected and mortality rates can reach 100%. During an outbreak of NE, mortality can only be reduced by metaphylactic antibiotic therapy, however the frequent use of antibiotics in pig husbandry increases the risk of selecting antibiotic resistance amongst pathogenic clostridia. Prophylactic vaccination of sows has been shown to be effective in prevention of the disease but was not systematically implemented on breeding farms in Switzerland. This has lead to several outbreaks of NE in Switzerland in recent years and repeated metaphylactic antibiotic treatments using lincomycin and penicillin as first choice antibiotic. Whether outbreaks were caused by one or more clonal lineages of *C. perfringens* type C was unknown. The goal of our study was to investigate whether specific clones were responsible for the outbreaks and to determine the antibiotic resistance profiles of porcine *C. perfringens* type C isolates from different outbreaks of NE in Switzerland from 1999 to 2011. Six Belgian isolates were included to compare Swiss *C. perfringens* type C isolates to those from a geographically distinct region.

Material and Methods

39 Swiss and 6 Belgian *C. perfringens* type C isolates from several outbreaks between 1999 and 2011 were used. The genetic relationship between the isolates was investigated by PFGE. The antibiotic susceptibility was determined in all isolates by Minimal Inhibitory Concentrations (MIC) using customized standard susceptibility plates (ANO2B; TREK Diagnostic Systems, East Grinstead, West Sussex, United Kingdom). MICs were determined following the guidelines of the European Committee of Antimicrobial Susceptibility Testing (EUCAST) and breakpoints used were those for gram positive anaerobes defined by EUCAST if available (www.eucast.org) except for cefotetan, tetracycline, cefoxitin and mezlocillin for which breakpoints of Clinical and Laboratory Standards Institute (CLSI) were used.

Results

Genetic diversity of *Clostridium perfringens* Type C

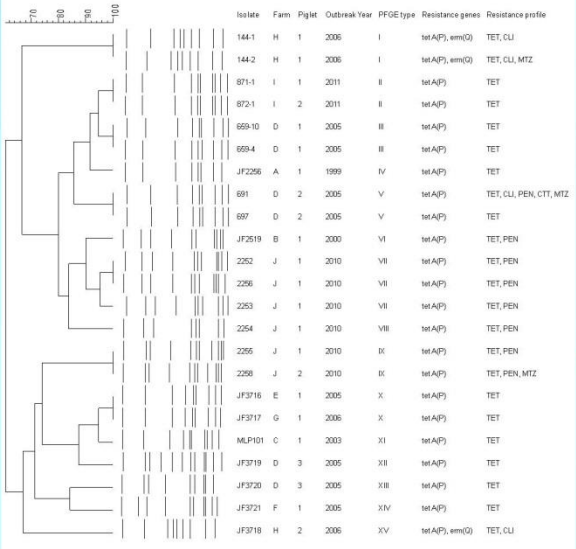


Fig 1:
Dendrogram constructed from PFGE-*Sma*I patterns from porcine *C. perfringens* type C isolates generated by Bionumerics 6.6; Applied Maths, Kortrijk, Belgium. Comparison settings: Dice, UPGMA, optimization 1.5 %, position tolerance 1.5 %. CLI, clindamycin; MTZ, metronidazole; PEN, penicillin; CTT, cefotetans; TET, tetracycline.

Antibiotic resistance profile

Table 1. Minimal inhibitory concentration (MIC) of 15 antibiotics.

		Number of strains with MIC (µg/ml)														
		≤0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	128	MIC50	MIC90
Antimicrobial	**ATCC 13124***														
Ampicillin/sulbactam	≤0.5/0.25		41				1	2						≤0.5/0.25	≤0.5/0.25
Amoxicillin/clavulanic acid	≤0.5/0.25		41				2	1						≤0.5/0.25	≤0.5/0.25
Ampicillin	≤0.5			39		1		4						≤0.5	≤0.5
Cefotetan	≤4						43						1	≤4	≤4
Cefoxitin	≤1					26		5	5	3	5			≤1	16
Chloramphenicol	4						23		16	5				≤2	8
Clindamycin	≤0.25		29				6	2			7			≤0.25	>8
Imipenem	≤0.12		36			6	1	1						≤0.12	0.5
Meropenem	≤0.5			43				1						≤0.5	≤0.5
Metronidazole	4						1	23	14	5		1		2	8
Mezlocillin	≤4							41		1	2			≤4	≤4
Penicillin	≤0.06	24		8	4		1	4	1	2				≤0.06	2
Piperacillin	≤4							41			3			≤4	≤4
Piperacillin/tazobactam	≤0.25/4		36				1	2	2	1	2			≤0.25/4	4
Tetracycline	≤0.25								6	30	8			8	>8

The dilution ranges tested for each antibiotic are those contained within the white area. Resistance breakpoints for anaerobes (vertical lines) were obtained from the European Committee of Antimicrobial Susceptibility Testing (EUCAST) and the Clinical and Laboratory Standards Institute (CLSI).

* *Clostridium perfringens* ATCC 13124

Conclusion

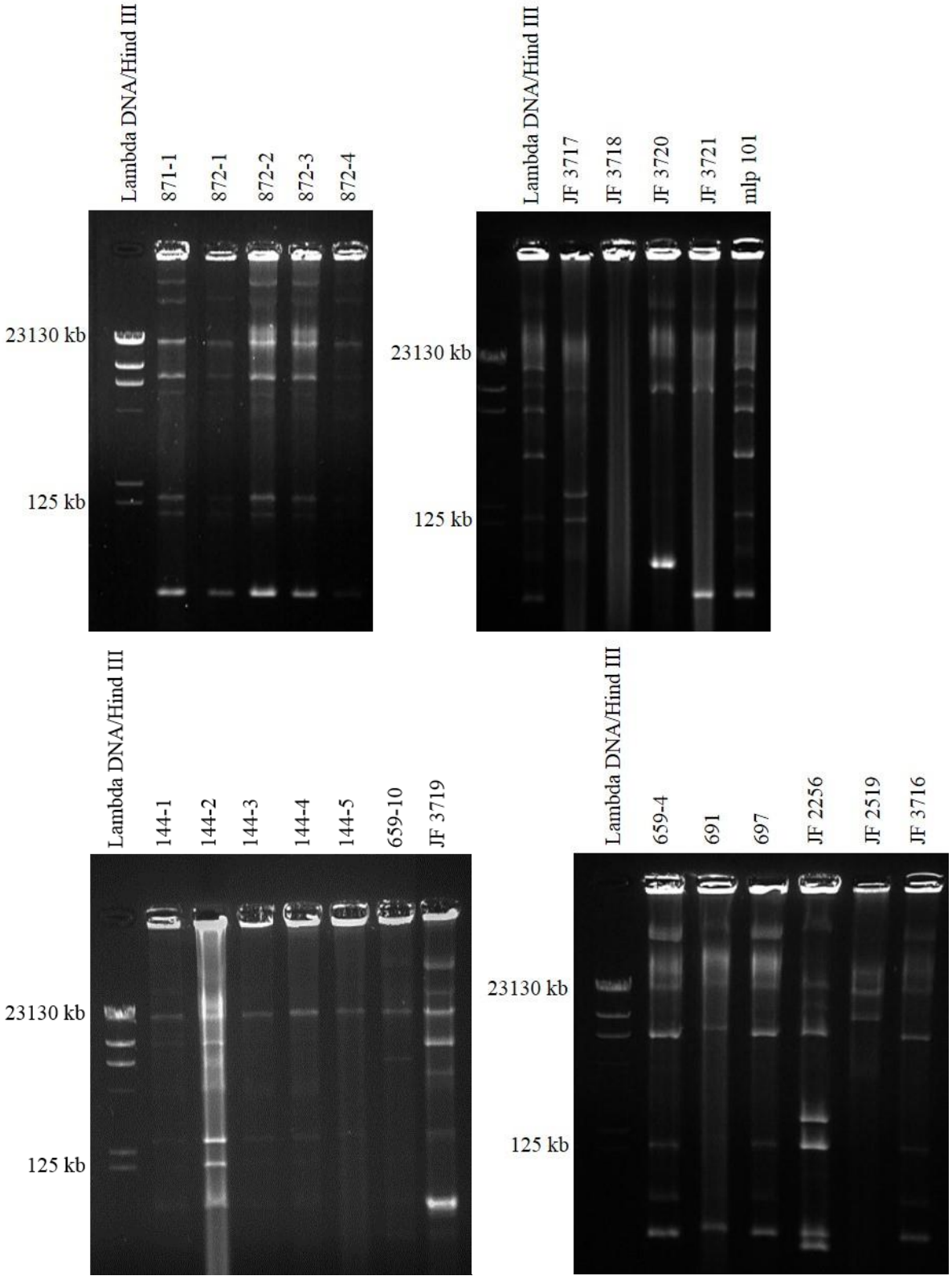
- More than one pathogenic clone of *C. perfringens* type C caused NE in Swiss pig farms.
- Different clonal lineages were isolated per farm.
- Resistances to tetracycline, penicillins, clindamycin, and decreased susceptibility to cephalosporins are common in these isolates.
- Vaccinations should be regularly applied to avoid metaphylactic antibiotic treatments, especially because resistance to regularly used antibiotics are present in Swiss isolates.

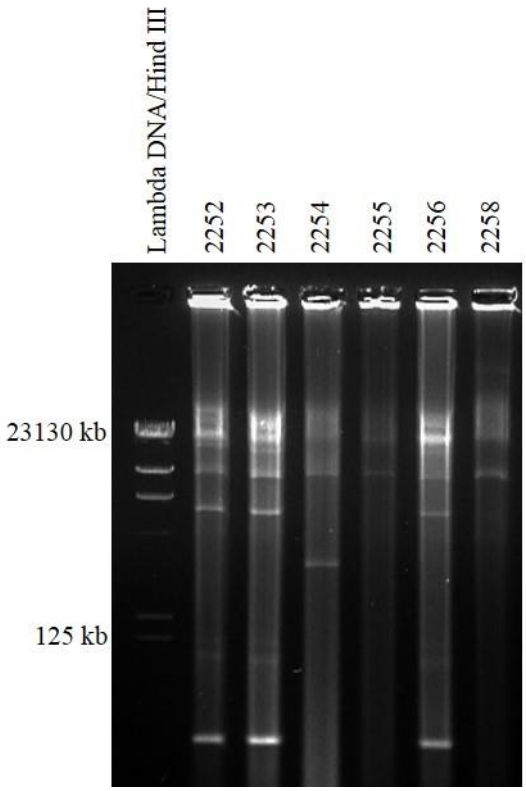
Outlook

- Isolates with different antibiotic resistance profiles and belonging to different clonal lineages will be selected to determine co-localisation of virulence factors on plasmids.
- We will attempt to sequence the *cpb* plasmid of porcine *C. perfringens* type C strains.

APPENDIX

Plasmid profiles of selected isolates




Lambda DNA/Hind III
2252
2253
2254
2255
2256
2258
23130 kb
125 kb

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