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Research paper

NetF-producing *Clostridium perfringens*: Clonality and plasmid pathogenicity loci analysis

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ABSTRACT

Clostridium perfringens is an important cause of foal necrotizing enteritis and canine acute hemorrhagic diarrhea. A major virulence determinant of the strains associated with these diseases appears to be a beta-sheet pore-forming toxin, NetF, encoded within a pathogenicity locus (NetF locus) on a large *tcp*-conjugative plasmid. Strains producing NetF also produce the putative toxin NetE, encoded within the same pathogenicity locus, as well as CPE enterotoxin and CPB2 on a second plasmid, and sometimes the putative toxin NetG within a pathogenicity locus (NetG locus) on another separate large conjugative plasmid. Previous genome sequences of two *netF*-positive *C. perfringens* showed that they both shared three similar plasmids, including the NetF/NetE and CPE/CPB2 toxins-encoding plasmids mentioned above and a putative bacteriocin-encoding plasmid. The main purpose of this study was to determine whether all NetF-producing strains share this common plasmid profile and whether their distinct NetF and CPE pathogenicity loci are conserved. To answer this question, 15 equine and 15 canine *netF*-positive isolates of *C. perfringens* were sequenced using Illumina Hiseq2000 technology. In addition, the clonal relationships among the NetF-producing strains were evaluated by core genome multilocus sequence typing (cgMLST).

The data obtained showed that all NetF-producing strains have a common plasmid profile and that the defined pathogenicity loci on the plasmids are conserved in all these strains. cgMLST analysis showed that the NetF-producing *C. perfringens* strains belong to two distinct clonal complexes. The pNetG plasmid was absent from isolates of one of the clonal complexes, and there were minor but consistent differences in the NetF/NetE and CPE/CPB2 plasmids between the two clonal complexes.

1. Introduction

Clostridium perfringens is one the best-known pathogenic and toxin-producing species of the genus *Clostridium* (Songer, 1996). This bacterium is a ubiquitous inhabitant of the gastrointestinal tract of vertebrates, and is also present in soil and water (Songer, 1996). *Clostridium perfringens* can cause a wide range of diseases, including histotoxic, neurotoxic, and enteric diseases in both human and animals when relevant toxins and extracellular enzymes are expressed (Rood, 1998 and Songer, 1996).

The pathogenesis of *C. perfringens* diseases is directly associated with its prolific toxin-producing ability (Rood, 1998, Rood and Cole, 1991 and Songer, 1996). The *C. perfringens* exotoxins and exoenzymes are classified into two categories, major and minor toxins (Cavalcanti et al., 2004). Traditionally, the major toxins (CPA, CPB, ETX, and ITX) provide a basis for classification of the individual strains into five toxinotypes (A–E).

Recently, our group described toxigenic type A *C. perfringens* isolates which produced three putative pore-forming toxins (NetE, NetF and NetG) (Mehdizadeh Gohari et al., 2015). A series of mutations, complementation and conjugation assays indicated that NetF was responsible for their marked *in vitro* cytotoxicity (Mehdizadeh Gohari et al., 2015). Although *netF* and *netE* were always found together in these toxigenic *C. perfringens* isolates, *netG* was present in only about half of them (Mehdizadeh Gohari et al., 2015).

There is a highly significant association between the presence of NetF-positive isolates of *C. perfringens* and severe canine hemorrhagic diarrhea syndrome (hemorrhagic enteritis/hemorrhagic gastroenteritis) (Unterer et al., 2014) or from necrotizing enteritis in foals (Mehdizadeh Gohari et al., 2015), suggesting that NetF plays a crucial role in pathogenesis of these infections. In addition, NetF-producing *C. perfringens* isolates were not found in clinically normal animals (Mehdizadeh Gohari et al., 2015 and Finley et al., 2016). A previous PFGE study suggested that NetF-producing strains associated with foal

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and canine necrotizing enteritis belong to a single clonal lineage and found that some canine and equine strains shared identical PFGE profiles (Mehdizadeh Gohari et al., 2015). Although the discovery of NetF may improve our understanding of the role of type A *C. perfringens* in foal and canine enteritis, further work is required to fully understand the pathogenesis of NetF-producing type A *C. perfringens* in these diseases.

More recently, whole genome sequencing of two *netF*-positive strains (JFP55, JFP838), recovered from a fatal case of foal necrotizing enteritis and a fatal case of canine hemorrhagic enteritis, respectively, revealed that these strains carry three plasmids in common (Mehdizadeh Gohari et al., 2016). These shared plasmids were two *tcp*-conjugative plasmids carrying either *netF*/*netE* or *cpe*/*cpb2*, and a bacteriocin-encoding plasmid. Apart from the shared plasmids, each strain harbored two unique plasmids, one of which encoded *netG* (Mehdizadeh Gohari et al., 2016). Comparative analysis of toxin-encoding plasmids of NetF strains with other *tcp*-conjugative plasmids of *C. perfringens* available in NCBI showed that *netF*/*netE* and *netG* toxin genes are located on two relatively large pathogenicity loci within pNetF/NetE and pNetG, respectively. These were designated the "NetF pathogenicity locus" and the "NetG pathogenicity locus" (Mehdizadeh Gohari et al., 2016).

In this study, we used whole genome sequencing to examine the distribution of the common and unique plasmids described earlier (Mehdizadeh Gohari et al., 2016) as well as similarities between NetF, NetG, and CPE pathogenicity loci, respectively, in a set of 30 *netF*-positive strains derived from either foal necrotizing enteritis or canine hemorrhagic diarrhea. In addition, the genetic relatedness of these strains was assessed using cgMLST to provide greater insight into evolutionary relationships of *netF*-positive *C. perfringens* strains.

2. Materials and methods

2.1. Bacterial strains and genomic DNA isolation

The 30 *netF*-positive type A *C. perfringens* strains used in this study were epidemiologically unrelated clinical isolates from foals with necrotizing enteritis (n = 15) and dogs with acute hemorrhagic diarrhea (n = 15) in Canada, the USA and Switzerland.

These isolates were selected based on the results of a previous PFGE study that identified 19 individual pulsotypes for *netF*-positive isolates (Mehdizadeh Gohari et al., 2015). Isolates were selected to represent all different pulsotypes. If multiple isolates from different species had the same pulsotype, both an equine and canine isolate were selected. Furthermore, an additional set of newly available isolates recovered from different geographic regions was included in this study. A summary of sources and origins of these isolates is presented in Table 1.

Genomic DNA was extracted from 20 ml of overnight culture in TPG broth (5% Tryptone, 0.5% Protease Peptone, 0.4% Glucose, 0.1% Thioglycolic acid [Difco Laboratories, Detroit, MI]) at 37 °C under anaerobic conditions using the Qiagen bacterial DNA extraction kit (Qiagen, Mississauga, ON). The quality of the genomic DNA was evaluated by standard agarose gel electrophoresis and the virulence genotype of DNA samples confirmed by PCR amplification of *cpa*, *cpe*, *netE*, *netF*, and *netG* (Mehdizadeh Gohari et al., 2015).

2.2. Whole genome sequencing, assembly and analysis

The genome sequencing was performed using Illumina Hiseq2000 paired-end read technology, generating reads of 100 bp in length. Raw

Table 1
Clostridium perfringens strains used in this study.

Strain	Origin	Country of isolation	Source	Year of isolation
JFP55[a]	Equine/FNE[b]	Canada	D. Weese	1999
JFP718	Canine/AHD[b]	Canada	D. Slavic	2011
JFP727	Equine/FNE	Canada	D. Slavic	2011
JFP728	Equine/FNE	Canada	D. Slavic	2011
JFP771	Canine/AHD	Canada	D. Slavic	2011
JFP774	Canine/AHD	Canada	D. Slavic	2011
JFP795	Canine/AHD	Canada	D. Slavic	2012
JFP796	Canine/AHD	Canada	D. Slavic	2012
JFP801	Equine/FNE	USA	T. E. Besser	2002
JFP804	Equine/FNE	USA	T. E. Besser	2010
JFP810	Canine/AHD	Canada	D. Slavic	2012
JFP826	Canine/AHD	USA	T. E. Besser	2012
JFP828	Equine/FNE	USA	T. E. Besser	2011
JFP829	Equine/FNE	USA	T. E. Besser	2010
JFP833	Equine/FNE	USA	T. E. Besser	2000
JFP834	Equine/FNE	USA	T. E. Besser	2002
JFP836	Canine/AHD	USA	T. E. Besser	2008
JFP838[a]	Canine/AHD	USA	T. E. Besser	2009
JFP914	Canine/AHD	Switzerland	V. Perreten	2009
JFP916	Canine/AHD	Switzerland	V. Perreten	2009
JFP921	Canine/AHD	USA	R. J. Carman	2007
JFP922	Canine/AHD	USA	R. J. Carman	2006
JFP923	Canine/AHD	USA	R. J. Carman	2006
JFP941	Canine/AHD	Canada	D. Slavic	2013
JFP961	Canine/AHD	Canada	D. Slavic	2014
JFP978	Equine/FNE	USA	J. F. Timoney	2011
JFP980	Equine/FNE	USA	J. F. Timoney	2006
JFP981	Equine/FNE	USA	J. F. Timoney	2004
JFP982	Equine/FNE	USA	J. F. Timoney	2001
JFP983	Equine/FNE	USA	J. F. Timoney	2004
JFP986	Equine/FNE	USA	J. F. Timoney	2011
JFP992	Equine/FNE	USA	J. F. Timoney	2008

Bold: strains belong to the clonal complex II. Italic: strains used for the PFGE study in the previous publication (Mehdizadeh Gohari et al., 2015).
[a] Reference strains from the previous sequence publication (Mehdizadeh Gohari et al., 2016).
[b] FNE: foal necrotizing enteritis; AHD: acute canine hemorrhagic diarrhea.