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Expansion of the *Clostridium perfringens* toxin-based typing scheme

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Abstract

Clostridium perfringens causes many different histotoxic and enterotoxic diseases in humans and animals as a result of its ability to produce potent protein toxins, many of which are extracellular. The current scheme for the classification of isolates was finalized in the 1960s and is based on their ability to produce a combination of four typing toxins - α-toxin, β-toxin, ε-toxin and ι-toxin – to divide *C. perfringens* strains into toxinotypes A to E. However, this scheme is now outdated since it does not take into account the discovery of other toxins that have been shown to be required for specific *C. perfringens*-mediated diseases. We present a long overdue revision of this toxinotyping scheme. The principles for the expansion of the typing system are described, as is a

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mechanism by which new toxinotypes can be proposed and subsequently approved. Based on these criteria two new toxinotypes have been established. *C. perfringens* type F consists of isolates that produce *C. perfringens* enterotoxin (CPE), but not β-toxin, ε-toxin or ι-toxin. Type F strains will include strains responsible for *C. perfringens*-mediated human food poisoning and antibiotic associated diarrhea. *C. perfringens* type G comprises isolates that produce NetB toxin and thereby cause necrotic enteritis in chickens. There are at least two candidates for future *C. perfringens* toxinotypes, but further experimental work is required before these toxinotypes can formally be proposed and accepted.

1. Introduction and historical perspective

Clostridium perfringens is a Gram-positive spore-forming anaerobe that is the causative agent of many histotoxic and enterotoxic diseases in humans and animals [1]. The key feature of these diseases is that they are mediated by the production of potent protein toxins, most of which are extracellular. These toxins generally are involved in specific disease syndromes. For example, α-toxin is essential for human clostridial myonecrosis or gas gangrene [2], *C. perfringens* enterotoxin (CPE) is required for human food poisoning [3], β-toxin is essential for specific enteric *C. perfringens* infections in several species [4, 5], ε-toxin is the key toxin in many enterotoxemic *C. perfringens* infections in sheep and goats [6] and NetB toxin is essential for necrotic enteritis in chickens [7].

C. perfringens was first isolated at the end of the nineteenth century from a case of acute articular rheumatism [8] and from a cadaver of a person who died of an aortic aneurism [9]. Welch and Nuttal named the organism as *Bacillus aerogenes* capsulatus nov. spec. [9] and Fränkel subsequently called it *Bacillus phlegmonis emphysematosae* [10]. It was subsequently called *Bacillus perfringens*, from the Latin name *perfringere*, to break, since the culture in deep agar medium disrupts the agar by abundant production of gas [11], and *Bacillus welchii*, in honour of William H. Welch [12]. The genus *Clostridium*, from the Greek name *kloster* latinized into *Clostridium*, meaning spindle-shaped [13, 14], was formally proposed in 1920 and both *Clostridium perfringens* and *Clostridium welchii* were listed in the Society for American Bacteriologists report on bacterial classification [15].

Pribram [16] distinguished the genus *Clostridium* (motile and non-capsulated bacteria) and the genus *Welchia* (non-motile and capsulated bacteria). This classification was used by Prévot and French bacteriologists from 1933 [17], with two species recognized in the genus *Welchia*, *W. perfringens* and *W. agni* on the basis of different toxicity [18, 19]. The older species name, *perfringens*, was used in 1931 by the Permanent Standards Commission of the Health Organization of the League of Nations (as reported in [20]) and the current name of *C. perfringens* adopted thereafter [21]. However, for many years the organism was called *Clostridium welchii* in English-speaking countries, *C. perfringens* in French-speaking countries and even Fränkel's bacillus in Germany. *C. perfringens* was one of the approved codified bacterial names published in 1980 [22, 23]. In this article the organism will be referred to as *C. perfringens* irrespective of which name was used in the original cited paper.

Initially, *C. perfringens* was classified into subgroups based on the ability of individual strains to produce acid and gas from the fermentation of inulin and glycerine and to produce

spores in media containing these carbohydrates [24–26]. Subsequently, Bull and Pritchard demonstrated that *C. perfringens* produced lethal exotoxins [27, 28] and Wilsdon developed a typing scheme based upon toxin production ([29] as cited in [30]). He used antisera produced against culture supernatants in toxin-antitoxin neutralisation tests to classify strains into four types based on their ability to produce three toxins, which were called W toxin (now known as α-toxin), X toxin (ε-toxin) and Z toxin (β-toxin). He described type A strains as classical gas gangrene strains that only produced W toxin, type B strains as isolates that produced W, X and Z toxins and caused lamb dysentery, type C strains as ovine *Bacillus plaudis* strains that produced W and Z toxins and type D strains as W and X toxin-producing *Clostridium ovitotoxicus* isolates from sheep. Other workers confirmed these observations in 1933 and first used the terms α-toxin, β-toxin and ε-toxin [30]. Wilsdon's system forms the foundation for the current *C. perfringens* toxinotyping scheme and toxinotypes A to D are virtually the same as his initial definition, apart from the obvious differences in nomenclature. In 1941, MacFarland and Knight [31] discovered that α-toxin is a phospholipase C that is produced by all *C. perfringens* strains; it was the first bacterial toxin shown to act as an enzyme.

For epidemiological investigations of *C. perfringens* strains, notably those involved in food poisoning, a serological typing was developed [32]. This procedure eventually required more than 91 antisera and most of the strains were not typable [33]. Therefore, toxinotyping, and more recently genetic characterization, were preferred to serotyping.

Wilsdon's toxinotyping scheme has been modified three times since it was introduced; we are now making a fourth modification. In 1943 a strain was isolated from a calf and shown to produce α-toxin and an additional toxin, ι-toxin, which was not neutralized by antiserum against α-, β- or ε-toxins [34]. Such strains were designated as belonging to a new toxin type, *C. perfringens* type E. Several years later strains that produced α-toxin and β-toxin were isolated from cases of human necrotic enteritis in Germany [35] and based on their spore properties were designated as type F [35, 36]. This type A to F scheme was reported in MacLennan's classic review on histotoxic clostridial infections of man, which was published in 1962 [37]. Finally, in 1964 it was realised that type F was simply a variant of type C and type F was dropped from the scheme [23, 38]. The toxinotyping scheme has not been altered subsequently and has been reproduced in various formats in almost every major review of the field published since that time [39–44]. In virtually all of these reviews it was pointed out that most *C. perfringens* isolates, including type A strains, produce numerous other toxins and extracellular hydrolytic enzymes, the latest count is 20 such toxins and putative hydrolytic virulence factors [1, 45].

Finally, it has been known for many years that the genes encoding three of the four typing toxins (β-toxin, ε-toxin and ι-toxin) are encoded on large plasmids [43, 46]. The *C. perfringens* toxinotyping scheme therefore is fundamentally plasmid-based. More recently these plasmids have been shown to carry the *C. perfringens* Tcp conjugation locus and hence are highly likely to be conjugative [47–50]. Indeed, conjugative transfer of epsilon toxin plasmids from type D strains to a type A strain has been demonstrated [51], technically leading to the laboratory conversion of a type A strain to type D. Genes encoding other currently non-typing toxins such as CPE [52–54], β2-toxin [55], δ-toxin [56], BEC [57],

NetB [58–61], NetE, NetF and NetG [62, 63] also have been shown to be plasmid determined and in several instances conjugative transfer has been demonstrated [51, 54, 60, 62]. Recent studies have provided evidence for *in vivo* conjugative transfer of the NetB plasmid, within the gastrointestinal tract of chickens [64].

2. The current toxinotyping scheme is outdated

The *C. perfringens* toxinotyping scheme in its various forms has been valuable for the diagnosis of *C. perfringens* infections in both humans and animals, but it is outdated and currently does not always serve its original diagnostic and epidemiological purpose. For example, CPE-producing strains of *C. perfringens* have been recognized for over fifty years and represent one of the world's major causes of human food poisoning [65]. More recently, NetB toxin was identified and shown to be essential for necrotic enteritis in chickens [7]. These strains both produce very different toxins and cause very different diseases yet they are currently both classified as *C. perfringens* type A along with gas gangrene-causing strains of *C. perfringens*. Therefore, we conclude that the current toxinotyping scheme needs to be updated to improve its epidemiological and diagnostic value.

3. Principles for the expansion of the toxinotyping scheme

The expansion of the typing system has been the subject of discussion between many of the authors for several years and was presented and approved in principle at the 10th International Conference on the Molecular Biology and Pathogenesis of the Clostridia held in Ann Arbor, U.S.A. in August 2017. The major principle that has been agreed upon is that what is required is an expansion that builds upon the existing toxin-based typing system rather than a completely new scheme. It is considered that an expanded scheme will be more readily accepted and more widely used by both diagnostic and research focussed laboratories.

How are new toxinotypes to be determined?

The major principle here is that a new toxinotype needs to be unique; it must involve a new typing toxin that is not part of the existing toxinotyping scheme. It is not considered relevant to the typing scheme whether that toxin is encoded on a plasmid or on the chromosome. The established toxinotypes B to E have priority; that is, strains belonging to new toxinotypes cannot produce β-toxin, ε-toxin or ι-toxin. Most importantly, new toxinotypes must be disease based. The new toxinotype strains must have been clearly demonstrated to be associated with a specific disease syndrome, in humans or animals, by either fulfilling molecular Koch's postulates for the toxin associated with the disease, or by extensive epidemiological analysis if the former is not technically feasible.

How are new toxinotypes to be approved?

A three-stage process is proposed for delineation of a new toxinotype. First, there should be extensive and open discussion between relevant researchers in the *C. perfringens* field. Second, the new toxinotype should be formally presented for comment and ratification at the biennial clostridial pathogenesis meeting. Third, the formal establishment of the new

toxinotype should be published in a relevant peer reviewed journal in an article dedicated to that task. Appropriate leading researchers in the field should be authors of that article so that the new toxinotype is readily accepted by the clostridial community.

4. Two new toxinotypes

To initiate this process we hereby propose the establishment of two new toxinotypes, *C. perfringens* type F and *C. perfringens* type G. These new toxinotypes are proposed in accordance with the guidelines established in the previous section. A summary of the latest toxinotyping scheme is presented in Table 1.

C. perfringens type F

Strains belonging to *C. perfringens* type F are defined as isolates that carry the α-toxin gene and the *cpe* gene and produce CPE upon sporulation, but do not carry the structural genes for β-toxin, ε-toxin or ι-toxin. These strains have been shown to be responsible for human food-poisoning and non-foodborne *C. perfringens*-mediated diarrhea, including some instances of antibiotic-associated diarrhea [66, 67]. The epidemiological evidence for the association of these strains with *C. perfringens*-meditated food poisoning and some cases of non-foodborne diarrhea is very clear and well documented [66, 68]. In addition, molecular Koch's postulates have been demonstrated for CPE [3]. It was shown that concentrated culture supernatants from two sporulating wild-type strains of what is now designated as *C. perfringens* type F caused fluid accumulation and mucosal damage in a rabbit intestinal loop model of disease, unlike isogenic *cpe* mutants isolated by allelic exchange in these different *C. perfringens* strains. Complementation of the mutants with the wild-type *cpe* gene restored the ability to cause fluid accumulation and mucosal damage, which provided clear evidence that CPE was essential for disease in a relevant animal model that mimics the human disease. Currently, these strains are referred to as CPE-positive strains of *C. perfringens* type A. Their designation as *C. perfringens* type F will provide a sound basis for the clinical and epidemiological analysis of these distinct strains. We suggest that for the next few years researchers mention in their publications that the *C. perfringens* type F strains were formerly called CPE-positive strains of *C. perfringens* type A. A consequence of this nomenclature change is that *C. perfringens* type A food poisoning will be renamed as *C. perfringens* type F food poisoning, again cross-referencing to the earlier nomenclature would be valuable. As discussed earlier, it is noted that there is a group of type C strains that was previously designated as type F. However, since the original type F nomenclature has not been used for over 50 years we do not consider that there will be any diagnostic confusion.

C. perfringens type G

Strains belonging to *C. perfringens* type G are defined as isolates that produce α-toxin and NetB toxin, but do not produce β-toxin, ε-toxin or ι-toxin. Note that *C. perfringens* type A strains are now defined as strains that produce α-toxin, but do not produce β-toxin, ε-toxin, ι-toxin, CPE or NetB.

C. perfringens type G strains have been shown to be responsible for necrotic enteritis in chickens [7]. Once more the genetic evidence for the essential role of NetB in this disease is

clear and it is supported by very strong epidemiological evidence [69–71]. Molecular Koch's postulates again have been fulfilled [7]. NetB-producing strains of *C. perfringens* cause lesions in a chicken model of subclinical necrotic enteritis, the economically most important form of the disease. Specific mutation of the *netB* gene eliminates the ability to cause these lesions, which is restored by complementation *in trans* with the wild-type *netB* gene. Currently, these strains are referred to as avian necrotic enteritis strains of *C. perfringens* type A. Their designation as *C. perfringens* type G isolates again will provide a sound basis for the diagnosis and epidemiological analysis of these distinct isolates.

5. Multiplex PCR toxinotyping test

We propose that the designation of unknown *C. perfringens* isolates as belonging to toxinotypes A to G should be based on the molecular analysis of their DNA; that is, it should involve the detection of the structural genes encoding the specific typing toxins. Given the rapidly changing nature of molecular methods of genomic analysis we will not specify the method that should be used. However, to assist researchers new to the field we have presented the results of a simple multiplex PCR test carried out on sample isolates from toxinotypes A to G (Fig. 1). This test was done using PCR primers (Table 2) specific for the α-toxin (*plc* or *cpa*), β-toxin (*cpb*), ε-toxin (*etx*), ι-toxin (*iap*), CPE (*cpe*) and NetB (*netB*) genes and designed so that the PCR products would have different sizes. The value of this approach is shown in Fig. 1, where the distinct toxinotypes, including the new types F and G can readily be distinguished. Note that the *C. perfringens* type D and E strains that were analysed here also carried the *cpe* gene.

6. Lead candidates for future toxinotypes

C. perfringens produces at least 20 extracellular toxins and hydrolytic enzymes [1, 45], of which only six are typing toxins in the expanded scheme presented here. Although none of the other toxins meet the criteria described earlier for inclusion as typing toxins, two toxins are obvious candidates for inclusion in a future expansion of the scheme, once critical experiments have been carried out. These toxins are NetF [62] and BEC [57, 72].

NetF is an extracellular β-pore-forming toxin that belongs to the same toxin superfamily as NetB, β-toxin and *C. perfringens* δ-toxin [62]. The *netF* gene is encoded on a conjugative plasmid that also carries another putative toxin gene, *netE*. NetEF-positive strains also carry a plasmid that encodes CPE and β2-toxin and a proportion also carry a NetG plasmid. These plasmids all carry the Tcp conjugation locus. The *netF* gene is preferentially found in *C. perfringens* strains from cases of acute canine hemorrhagic enteritis and necrotizing enteritis in neonatal foals; these isolates appear to be clonal in origin [62, 73].

Several epidemiological studies have been carried out [62, 74–76] and have shown that the *netEF* plasmid is associated with isolates from these syndromes. A *netF* insertional inactivation mutant has been constructed and shown to be no longer toxic for an equine ovarian (EO) cell line [62]. EO toxicity was restored by complementation *in trans* with the wild-type *netF* gene. These data, together with conjugation and transformation experiments on these plasmids, clearly showed that NetF was responsible for the cytotoxicity for EO

cells. However, molecular Koch's postulates have not been proven yet for either the canine or equine disease syndromes. The exact role of NetE, NetF and CPE in these diseases remains to be determined [73]. What is required for NetF-positive strains of *C. perfringens* to be considered as a separate toxinotype is the analysis of isogenic wild-type, *netF* mutant and complemented strains in an animal model whose pathology resembles that of the canine or equine diseases. If such studies showed that NetF toxin was required for disease causation in this model then the establishment of a new NetF-positive toxinotype clearly would be justified.

Two independent studies have identified a novel binary toxin that appears to be associated with cases of acute foodborne gastroenteritis in Japan [57, 72]. Unfortunately, this toxin has been given two separate names, BEC [57] and CPILE [72]. This situation should not be allowed to continue as inevitably it has caused confusion in the literature. BEC signifies binary enterotoxin of *C. perfringens* and the two genes encoding the components of the binary toxin are referred to as *becA* and *becB* [57]. The term CPILE signifies *C. perfringens* iota-like enterotoxin. The genes are referred to as *cpile-a* and *cpile-b* [72], which does not conform to usual genetic conventions. We have concluded that the BEC terminology provides a simpler and more acceptable nomenclature for the clostridial community. We recommend that all researchers refer to the toxin as BEC, to the individual components as BECa and BECb and to the genes as *becA* and *becB*. However, for the next few years it would be helpful if researchers cross-referenced to the CPILE terminology in the abstract and introduction of their papers.

BEC toxin was identified in strains of *C. perfringens* isolated from several large outbreaks of acute foodborne gastroenteritis in Japan. Since these strains do not produce CPE and do not carry the *cpe* gene a novel toxin was suspected and subsequently identified as the binary toxin BEC (or CPILE) [57, 72]. A survey of Japanese *C. perfringens* isolates using a *becAB*-specific PCR indicates that BEC-positive strains are rare [77]. The *becA* and *becB* genes are plasmid determined and encode separate binary toxin components that are distantly related to the components of the clostridial binary toxin family, which includes *C. perfringens* ι-toxin (43-44% amino acid sequence identity to the individual components). Supernatants from sporulating cultures of these strains cause fluid accumulation in rabbit ileal loop and suckling mouse assays [57], as does purified recombinant toxin [72], and BEC also causes rounding of Vero and L929 cells [72]. Purified BECa has been shown to have ADP-ribosyltransferase activity on purified actin [57, 72], as expected for the enzymatic component of a clostridial binary toxin. Insertional inactivation of the *becB* gene abrogated the ability of the resultant strain to cause fluid accumulation in the suckling mouse assay, but unfortunately this mutant was not complemented. Therefore, it cannot be said for certain that the loss of fluid accumulation was the direct result of mutation of the *becB* gene. For this reason, we consider that it would be premature to designate BEC-positive strains as a separate toxinotype at this time.

7. Conclusions

In summary, we have proposed an updated toxinotyping scheme that incorporates two new toxinotypes. *C. perfringens* type F strains consist of isolates that produce CPE, but do not

produce β-toxin, ε-toxin or ι-toxin. These strains are responsible for *C. perfringens*-mediated human food poisoning and antibiotic associated diarrhea. *C. perfringens* type G strains comprise isolates that produce NetB toxin and thereby cause necrotic enteritis in chickens. In addition, we describe a mechanism by which new toxinotypes can be formally proposed and subsequently approved.

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Highlights

- An expanded *C. perfringens* toxinotyping scheme is presented.
- Two new toxinotypes are proposed.
- *C. perfringens* type F strains produce CPE, but not β, ε or ι toxins.
- *C. perfringens* type G strains produce NetB.
- A mechanism for the introduction of new toxinotypes is presented.

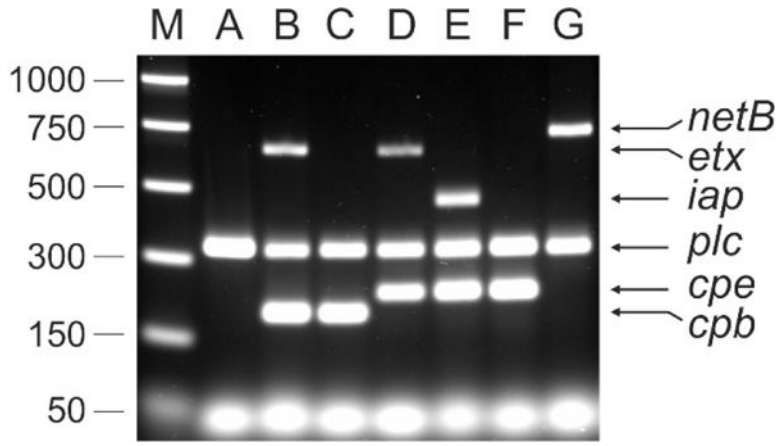

Fig. 1. Multiplex PCR analysis of representative *C. perfringens* type A to G strains

The strains were grown in TGY broth [79] to a turbidity at 600nm between 1.0 and 1.5. Genomic DNA (equivalent to 5mL of culture) was prepared as described previously [80]. DNA preparations were diluted 1 in 50 in sterile distilled water and used as templates in the multiplex toxin PCR based on a previous method [81]. The oligonucleotide primers and their concentrations are listed in Table 2. PCR reactions were prepared using 0.1 units/μL Taq DNA polymerase (Roche) in 1 x supplied buffer (Roche), 2 mM $MgSO_4$ and 0.4 mM dNTPs. The template constituted 0.1 volumes of the final reaction. PCR was performed with an initial denaturation at 95°C for five minutes, followed by 35 cycles of 95°C for 1 minute, 55°C for 1 minute and 72°C for 1 minute. Amplified products were resolved by electrophoresis through a 1.5% (w/v) TAE agarose gel. The multiplex PCR profiles of the following *C. perfringens* strains are shown: JIR325 (type A)[82], JGS1984 (type B)[83], CN3717 (type C)[84], JGS4138 (type D)[85], ATCC27324 (type E), SM101 (type F)[86], EHE-NE18 (type G)[87]. Size standards were PCR Markers (Promega).

Table 1

The 2018 *C. perfringens* toxin-based typing scheme[a]

Toxinotype	α-toxin (*plc or cpa*)	β-toxin (*cpb*)	ε-toxin (*etx*)	ι-toxin (*iap* and *ibp*)	CPE (*cpe*)	NetB (*netB*)
A	+	−	−	−	−	−
B	+	+	+	−	−	−
C	+	+	−	−	+/−	−
D	+	−	+	−	+/−	−
E	+	−	−	+	+/−	−
F	+	−	−	−	+	−
G	+	−	−	−	−	+

[a] the names of toxin structural genes are shown in parentheses.

Table 2

Oligonucleotide primers for multiplex toxin PCR

Gene	Primers	Sequence (5′-3′)	size	Conc.*	Reference
netB	JRP6656 JRP6655	CTTCTAGTGATACCGCTTCAC CGTTATATTCACTTGTTGACGAAAG	738 bp	0.6 0.6	V. Adams & J. Rood
etx	JRP4234 JRP4235	CCACTTACTTGTCCTACTAAC GCGGTGATATCCATCTATTC	656 bp	0.44 0.44	[78]
iap	JRP5507 JRP5508	GGAAAAGAAAATTATAGTGATTGG CCTGCATAACCTGGAATGGC	461 bp	0.5 0.5	V. Adams & J. Rood
plc	JRP4232 JRP4233	GCTAATGTTACTGCCGTTGACC CCTCTGATACATCGTGTAAG	324 bp	0.4 0.4	Based on [78]
cpe	JRP5179 JRP5180	GGAGATGGTTGGATATTAGG GGACCAGCAGTTGTAGATA	233 bp	0.3 0.3	[78]
cpb	JRP5181 JRP5182	GCGAATATGCTGAATCATCTA GCAGGAACATTAGTATATCTTC	196 bp	0.3 0.3	[78]

*Concentration of oligonucleotides in final reaction (μM)