# A molecular approach to the characterization and evaluation of *Clostridium perfringens* type A field strains

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*Clostridium perfringens* has long been recognized as an important pathogen for both man and animals. It is classified into five types (A, B, C, D, and E) according to the existence of the four major toxin genes (*cpa*, *cpb*, *etx*, *iap/ibp*). In addition to the four major toxins, most strains of *C perfringens* produce a wide variety of extracellular toxins and enzymes. To date, as many as 17 exotoxins of *C perfringens* have been described. These include alpha toxin (*cpa* is the gene encoding this toxin), beta toxin (*cpb*), ipsilon toxin (*etx*), iota toxin (*iap/ibp*), beta2 toxin (*cpb2*), enterotoxin (*cpe*), perfringolysin (*pfoA*), collagenase (*colA*), lamda toxin (*lam*), hyaluronidase (*nagH*), DNase (*cadA*), neuraminidase (*nanH, I*), and urease (*urea-C*). But the role each toxin plays in the pathogenesis of porcine enteritis is not totally understood.[1,2]

During the past decade, it has become evident that pathogenic strains of *C perfringens* type A can cause serious enteric diseases in suckling and feeder pigs. A recent study at Iowa State University indicated that *C perfringens* type A enteritis is the most common enteric disease in piglets less than 10 days of age.[3] Some strains of *C perfringens* type A can exist as normal flora in the intestinal tract of pigs while other strains can cause severe enteritis in young pigs. There must exist an erratic distribution of the toxin/enzyme genes within *C perfringens* type A population.[4]

*C perfringens* type A strains may differ in their genetic composition and mode of gene expression. The routine *C perfringens* typing being used at veterinary diagnostic laboratories is a multiplex PCR that can detect the presence of the four major toxin genes. Simply relying on the major toxin gene typing scheme is not sufficient for making an intra-species differentiation of *C perfringens* type A strains isolated from the field. In response to the growing number of enteric diseases caused by *C perfringens* type A strains in young pigs, Tailor-Made™ vaccines that contain the herd-specific strains are being used more often in pig farms than ever before. The first step leading to the success of pig protection with Tailor-Made™ vaccines begins with an in depth characterization and selection of field isolates as well as an in vitro evaluation of toxin levels in culture supernatants.

In this study, 200 field strains of *C perfringens* type A isolated from pigs with enteric diseases were examined using a PCR assay to detect *cpa*, *cpb2*, *cpe*, *colA*, and *pfoS* toxin genes. Briefly, the PCR reaction mix for a test sample contains 2.5 µl of 10 × PCR buffer, 1.2 µl of dNTP (each at a concentration of 40 µM), 2.0 µl of 25 mM magnesium chloride, 0.5 µl of Taq DNA polymerase (5U/µl), 5 µl of DNA template, an appropriate amount of primers, and an appropriate amount of deionized water to make a final volume of 25 µl. The oligonucleotide primers for this PCR assay are listed in Table 1. The PCR conditions are as follows: Thirty five cycles at 94°C for one minute, 53°C for one minute, and 72°C for two minutes. Then one cycle at 72°C for ten minutes. The PCR amplicons are analyzed by electrophoresis in 1.5% agarose gel stained with ethidium bromide and recorded by using UV transillumination and Polaroid film (Table 1).

In addition to PCR toxin gene assay, in vitro toxin production evaluation is another important tool for the characterization and evaluation of *C perfringens* type A strains. To determine the alpha toxin (phospholipase C, PLC) activity of each *C perfringens* type A strain, the test strain was cultured in a medium that had been proved to be ideal for toxin production. Briefly, one milliliter of the culture was centrifuged at 13,000 X g for three minutes. The cell pellet was washed with PBS and centrifuged again. The pellet was suspended in 200 µl of PBS. Equal volumes of the test material and the enzyme substrate [80 mM p-nitrophenylphosphorylcholine (Sigma Chemical Co. St. Louis, MO) in 0.1M Tris buffer (pH 7.4)] were incubated at 37°C in a micro-centrifuge tube for three hours and then centrifuged to get the supernatants. In the presence of PLC activity, p-nitrophenol will be released into the supernatants and can be measured at 405 nm using an ELISA reader. The negative control containing substrate only

**Table 1:** Oligonucleotide primers for PCR amplification of *cpa*, *cpb*, *etx*, *iA*, *cpe*, *cpb2*, *pfoS*, and *colA* toxin genes of *C perfringens*

| Toxin gene | | Oligonucleotide sequence |
|---|---|---|
| *cpa* | CPAF | 5′-GCT AAT GTT ACT GCC GTT GA-3′ |
| | CPAR | 5′-CCT CTG ATA CAT CGT GTA AG-3′ |
| *cpb* | CPBF | 5′-GCG AAT ATG CTG AAT CAT CTA-3′ |
| | CPBR | 5′-GCA GGA ACA TTA GTA TAT CTT C-3′ |
| *etx* | ETXF | 5′-GCG GTG ATA TCC ATC TAT TC-3′ |
| | ETXR | 5′-CCA CTT ACT TGT CCT ACT AAC-3′ |
| *iA* | IAF | 5′-ACT ACT CTC AGA CAA GAC AG-3′ |
| | IAR | 5′-CTT TCC TTC TAT TAC TAT ACG-3′ |
| *cpe* | CPEF | 5′-GGA GAT GGT TGG ATA TTA GG-3′ |
| | CPER | 5′-GGA CCA GCA GTT GTA GAT A-3′ |
| *cpb2* | CPB2F | 5′-AGA TTT TAA ATA TGA TCC TAA CC-3′ |
| | CPB2R | 5′-CAA TAC CCT CCT TCA CCA AAT ACT C-3′ |
| *pfoS* | PFOSF | 5′-CGG GTA TAG GCA TAC AAA AGG A-3′ |
| | PFOSR | 5′-GTG CAG TTG CAA CCA CTG TT-3′ |
| *colA* | COLAF | 5′-TAG GAA CAA AGG CGC AAG AT-3′ |
| | COLAR | 5′-TTC TCC TTG TCC CCA CAT TC-3′ |

and a positive control containing *C perfringens* type A toxin (IRP446, obtained from Center for Veterinary Biologics) was included in the test. A standard curve was prepared with serial dilution of p-nitrophenol (Sigma Chemical Co.). To determine the enzyme activity of PLC in each test sample, optical density (OD) value for negative control was subtracted from OD value of the test sample and compared with a standard curve.[5]

Beta2 toxin (CPB2), encoded by *cpb*2 toxin gene, has recently been linked to porcine enteritis. Most evidence implicating CPB2 in pathogenesis of porcine enteritis is based on the finding of a strong correlation between the prevalence of *cpb*2 gene in isolates from pigs with enteritis and the absence of *cpb*2 gene in isolates from healthy pigs.[6] The specific role CPB2 may play in pathogenesis of *C perfringens* type A enteritis is not known, but its strong association with cases of enteritis suggests that it could be a marker of virulence. Due to a lack of specific activity assay for CPB2, an immunoblotting assay using rabbit antiserum against CPB2 has been adopted for the detection and semi-quantification of CPB2 in bacterial culture supernatants prepared from *cpb*2-positive genotype A isolates as described before.[7] Briefly, undiluted culture supernatants either separated from late log phase culture or from adjuvanted vaccines were mixed 1:1 with 2 × SDS-PAGE loading buffer and boiled for 5 minutes, and then electrophoresed on 4-20% SDS-PAGE gel (BioRad). After transfer to nitrocellulose, the blots were probed with rabbit polyclonal anti-CPB2 antibodies, followed by peroxidase-conjugated Protein G (Sigma Chemical Co.) and metal enhanced DAB substrate (Pierce Biotechnology). If a test sample was found to be positive, a series of 10 fold dilution of the test sample was tested again to determine its endpoint.

An in vitro test to identify neuraminidase activity in culture supernatants was also done as described before.[8] Briefly, the substrate for neuraminidase was prepared at 0.63 mM of BCIN (Sigma Chemical Co.) in 150 mM sodium acetate, 25 mM calcium chloride and 1 mM sodium chloride and was used to dampen a piece of Whatmann No. 1 filter paper. 15 ul of the culture supernatant was added onto the center of the dampened filter paper and placed in a covered Petri dish. The Petri dish was then incubated at 37°C for 30 minutes. In the absence of neuraminidase activity in the test sample, the filter paper will remain colorless. In the presence of neuraminidase activity, the filter paper will have a blue dot.

Previous epidemiological studies suggested that *cpb*2-positive *C perfringens* isolates are highly associated with enteritis in piglets.[6] In this study (Table 2), some 78 field strains of *C perfringens* type A isolated at our laboratories during 2002 to 2004 and 194 strains isolated during 2005 to 2007 were screened using a multiplex PCR. Of these, 50% were PCR positive for *cpb*2 toxin gene among 78 strains isolated between 2002 and 2004, while 78.4% were positive for pcb2 toxin gene among 194 strains isolated between 2005 and 2007. Only 7.7% of the 194 strains isolated between 2005 and 2007 were *cpe*-positive. It appeared that more field strains isolated during 2005 to 2007 carried *cpb*2 toxin genes than those isolated during 2002 to 2004.

In this study we also tried to examine the presence of two potential virulence genes in *C perfringens* type A isolates using PCR assay. One is the *colA* gene, which encodes *k*-toxin (a collagenase) that facilitates tissue necrosis. The other is the *pfoS* gene, which is located immediately upstream of *pfoA* and thought to encode a *pfoA* activator. The structural gene, *pfoA*, and other pfo genes encode a lethal hemolysin called perfringolysin O. Although there is no direct evidence to show any involvement of these two genes in virulence in *C perfringens*, PCR results of these toxin genes can be used for strain differentiation (Table 3).

Figure 1 shows the Western blot quantification of CPB2 in *C perfringens* type A culture supernatants. Genotype A isolates were grown in a known growth medium to the late log phase at 37°C and centrifuged at 13000 × G to obtain the culture supernatants. In order to obtain the endpoint titer, a ten fold dilution of each supernatant was made before electrophoresis and Western blot. Although the endpoint titer for each *cpb*2-positive isolate could be different, a 28 kD CPB2 can be detected in the culture supernatants of all of the *cpb*2-positive porcine isolates surveyed. This finding is consistent with a recent report.[9]

Figure 2 shows the detection of neuraminidase (sialidase) activity of each type A isolate using BCIN as an enzyme substrate. In this study, some 70 percent of the

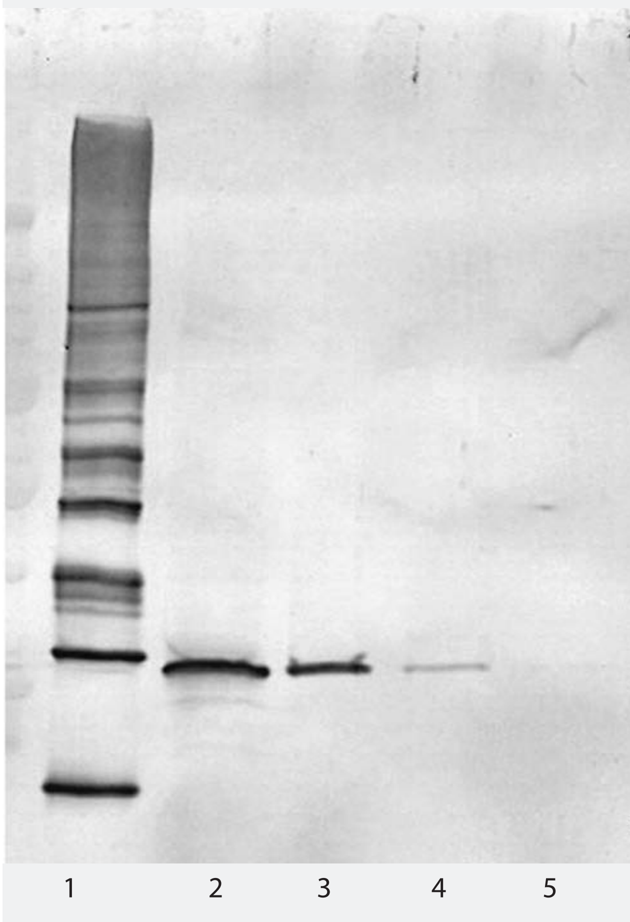
**Table 2:** Prevalence of *cpa*, *cpb2*, and *cpe* toxin genes of *C perfringens* type A among 78 field isolates obtained from pigs with enteric diseases during 2002 to 2004 and among 194 isolates obtained during 2005 to 2007.

| **Major toxin gene pattern** | | | **Number of isolates** | |
|---|---|---|---|---|
| ***cpa*** | ***cpb2*** | ***cpe*** | **2002 to 2004** | **2005 to 2007** |
| + | + | + | 6 (8.3%) | 10 (5.2%) |
| + | + | -- | 33 (45.8%) | 142 (73.2%) |
| + | -- | + | 6 (8.3%) | 5 (2.6%) |
| + | -- | -- | 33 (45.8%) | 37 (19.0%) |
| **Number of isolates carrying *cpb2* gene** | | | 39 (50.0%) | 152 (78.4%) |
| **Number of isolates carrying *cpe* gene** | | | 12 (15.4%) | 15 (7.7%) |

**Table 3:** Prevalence of the other two virulence genes (*colA* and *pfoS*) of *C perfringens* among field strains isolated between 2005 and 2007.

| **Other toxin gene** | | **Number of isolates** |
|---|---|---|
| ***colA*** | ***pfoS*** | **2005 to 2007** |
| + | + | 52 (68.4%) |
| + | -- | 10 (13.2%) |
| -- | + | 2 (2.6%) |
| -- | -- | 12 (15.8%) |
| **Number of isolates carrying *colA* gene** | | 62 (81.6%) |
| **Number of isolates carrying *pfoS* gene** | | 54 (71.1%) |

**Figure 1:** Detection and quantification of CPB2 in *cpb2*-positive genotype A culture supernatants using Western blotting. Lane 1: Molecular weight markers (Invitrogen's MagicMark XP Western Standard, 220, 120, 100, 80, 60, 50, 40, 30, and 20 kD); lane 2: undiluted culture supernatant of *cpb2*-positive isolate; lane 3: 1:10 diluted supernatant; lane 4: 1:100 diluted supernatant; lane 5: 1:1000 diluted supernatant.




type A isolates have been found to be able to secrete the neuraminidase to the culture supernatant during the late log phase. Neuraminidase may play a role in disease by destroying receptor molecules on cell surfaces or by disrupting connective tissue.

During the past few years, infection of young pigs with *C perfringens* type A has become a frustrating experience in the pig industry. Taylor-Made™ vaccines are being used to meet the demand for disease control. However, anecdotal reports are mixed from no effect to very effective.[10] The selection of inappropriate isolates for vaccine production can cause vaccine failure. In this study, we developed an analysis scheme for performing the intra-species differentiation and evaluation of *C perfringens* type A field isolates. Table 4 shows an example of a molecular characterization of *C perfringens* type A field isolates using PCR assay, Western blot assay, and toxin/enzyme activity assays. This analysis scheme provides more insight into the virulence profile of each field isolate and offers better disease control in younger pigs through vaccination with a Taylor-Made™ vaccine containing the appropriate field isolates (Table 4).

**Figure 2:** The neuraminidase activity of each *C perfringens* type A isolate was detected using BCIN as an enzyme substrate. In the presence of neuraminidase activity, the filter paper will have a blue dot. In the absence of neuraminidase activity, the paper will remain colorless.

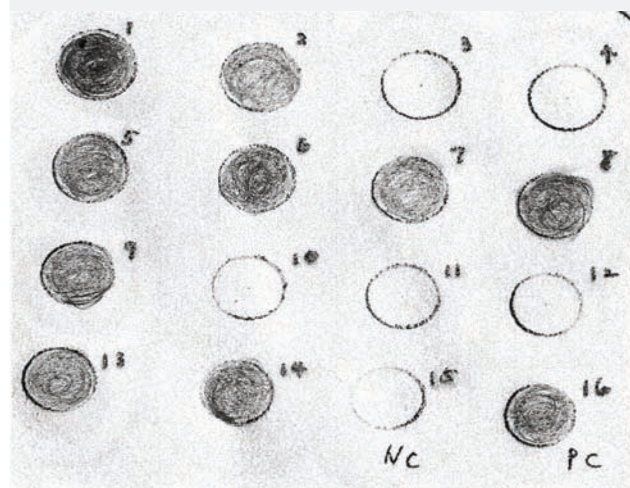


**Table 4:** Characterization and differentiation of *C perfringens* type A field strains.

| Sample ID | Toxin genotype | | | | | In vitro evaluation of toxin/enzyme activity | | |
|---|---|---|---|---|---|---|---|---|
| | *cpa* | *cpb2* | *cpe* | *colA* | *pfoS* | PLC (n mole/ml) | CPB2 (titer) | Neuraminidase |
| 076706-0 | + | + | -- | + | + | 2700 | + (1:100) | + |
| 076299-1 | + | -- | -- | + | + | 800 | -- | + |
| 076299-2 | + | + | -- | + | + | 1200 | + (1:10) | -- |
| 076134-1 | + | -- | + | -- | -- | 3600 | -- | + |
| 076134-2 | + | + | -- | + | -- | 900 | + (1:1000) | -- |
| 076693-0 | + | -- | -- | + | + | 8600 | -- | + |
| **Positive control (IRP446)** | | | | | | 25000 | | |

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