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1 A new PCR followed by *Mbol* digestion for the detection of all
2 variants of the *Clostridium perfringens cpb2* gene

3

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17

18 Abstract

19 *Clostridium perfringens* which is a causative agent of several diseases in
20 animals and humans is capable of producing a variety of toxins. Isolates are
21 typed into five types on the basis of the presence of one or more of the four
22 major toxins genes i.e. *cpa*, *cpb*, *etx*, and *iap*. A decade ago another toxin
23 termed beta2 (β 2) and its gene (*cpb2*) were identified. Two alleles of *cpb2*
24 are known and a possible link between differences in gene expression and
25 allelic variation has been reported. A correlation between the level of
26 expression and the origin of the isolates has also been suggested. The
27 demonstration and typing of the *cpb2* gene in the genome of isolates can be
28 seen as a vital part of research on the role of the beta2 toxin in the
29 pathogenesis of disease. This study describes a PCR with a single primer
30 set which in contrast to published primer sets recognizes both alleles.
31 Subsequent restriction enzyme analysis of the PCR product enables typing of
32 the alleles. Applying this protocol on a total of 102 isolates, a sub-variant was
33 found which occurred only in *C. perfringens* isolates from pigs and appeared
34 to be the predominant variant found in *C. perfringens* isolates from this
35 species.

36

37 1. Introduction

38 The Gram-positive anaerobic bacterium *Clostridium perfringens* is part of the
39 normal intestinal flora and is ubiquitous in the environment. However, it is also
40 an important cause of enteric diseases in animals and humans. Type and
41 severity of the disease are correlated with the toxin(s) produced by the
42 bacterium (Songer, 1996). Based on the four major toxins (alpha, beta,
43 epsilon and iota) the strains of *C. perfringens* are classified into five toxin-
44 types: type A-E, e.g. by demonstrating the presence of the encoding genes by
45 PCR.

46 Another toxin produced by a *C. perfringens* strain was described a decade
47 ago (Gibert et al., 1997). This toxin was designated beta2 since its biological
48 activities were comparable to those of the beta toxin. The gene encoding the
49 beta2 toxin was named *cpb2* and shown to be located on a plasmid (Gibert et
50 al., 1997). The *cpb2* gene has been demonstrated in isolates originating from
51 a variety of animals i.e. pigs, horses (Gibert et al., 1997), lambs, calves
52 (Garmory et al., 2000), chickens (Siragasa et al., 2006), dogs (Thiede et al.,
53 2001), and from humans (Fisher et al., 2005). It was shown that 88.5% of the
54 *cpb2*-positive nonporcine isolates (n=78) and 2 out of 76 (2.6%) *cpb2*-positive
55 porcine isolates examined carried a variant of *cpb2*. This variant had a
56 sequence-identity of only 70.2 to 70.7% with the original *cpb2* (GenBank
57 accession number L77965) resulting in a 62.3% identity and a 80.4% similarity
58 at amino-acid level (Jost et al., 2005). The original *cpb2* was termed the
59 consensus gene/allele whereas the variant was termed the atypical gene or
60 atypical allele (Jost et al., 2005; Jost et al. 2006). Up to now all known *cpb2*

61 sequences can be assigned to one of the two variants with some ambiguities
62 within each variant. In order to demonstrate the presence of *cpb2*, several
63 sets of primers have been used. However using BLAST (Basic Local
64 Alignment Search Tool; <http://www.ncbi.nlm.nih.gov/BLAST/>) none of the
65 published primer combinations recognized all *cpb2* sequences in the
66 Genbank/EMBL/DDBJ databases. The aim of this study was to design primers
67 recognizing all available *cpb2* sequences and to find ways to discriminate
68 between the PCR products originating from either the atypical or the
69 consensus gene (allele).

70

71

72 2. Materials and methods

73 2.1 Bacteria

74 Sixty *C. perfringens* strains used in the development of the PCR were from
75 the collection of the Veterinary Microbiological Diagnostic Center (VMDC),
76 Division of Clinical Infectiology, Department of Infectious Diseases and
77 Immunology, Faculty of Veterinary Medicine, Utrecht University. Fecal
78 samples used in the evaluation of the protocol for the demonstration and
79 typing of *C. perfringens* in clinical samples originated from pigs, sheep, cows,
80 horses, dogs and cats as well as from a variety of other (exotic) species.
81 Isolation of *C. perfringens* from these samples was performed using standard
82 procedures.

83

84 *2.2 Primers and PCR conditions*

85 Primers used are given in table 1. For sample preparation one *C. perfringens*
86 colony was resuspended in 25-50 µl of distilled water and heated for 10 min at
87 95°C. 50 µl of PCR reaction mix consisted of 1 µl of this heated bacterial
88 suspension, 100 ng of each primer (Isogen, The Netherlands), 0.2 mM of
89 each dNTP (Fermentas, Germany), 1 U recombinant *Taq* DNA polymerase
90 (Fermentas), 5 µl of 10 X *Taq* buffer, 1.5 mM MgCl₂ and distilled water. The
91 PCR program consisted of 3 min at 95 °C followed by 40 cycles of 30 sec at
92 95 °C, 30 sec at the annealing temp (Table 1) and amplification at 72 °C
93 during the appropriate time (1kb/min). A final amplification step of 7 min at 72
94 °C completed the PCR reaction. PCR was performed using either the
95 GeneAmp PCR system 2400 (Perkin Elmer, USA) or the iCycler (BioRad,
96 England). As a sample preparation control, all samples were subjected to a
97 PCR for the *cpa* gene which is present in all *C. perfringens* strains (Titball et
98 al., 1999). After performing the PCR reaction, the total reaction volume was
99 subjected to gel electrophoresis.

100

101 *2.3 Sequence and restriction enzyme analysis*

102 PCR products were extracted from gel using the QIAEX II Gel Extraction Kit
103 (Qiagen) and subsequently sequenced (BaseClear, The Netherlands). The
104 resulting sequences were mapped for restriction sites using the NEBcutter
105 software (www.neb.com).

106

107 **3. Results**

108 *3.1 PCR*

109 Sixty strains of the collection of the VMDC were tested with the consensus-
110 primer set, the atypical-primer set, and the new total-primer set for the
111 presence of the *cpb2* gene. A total of 43 strains were negative with all primer
112 sets. Eight strains were positive with the consensus-primer set but negative
113 with the atypical-primer set. Nine strains were positive with the atypical-primer
114 set but negative with the consensus-primer set. However, these seventeen
115 strains were all positive with the newly designed total-primer set.

116

117 *3.2 Sequence results and restriction enzyme analysis*

118 A PCR product of each variant generated by the total-primer set was
119 sequenced. Alignment of the sequence obtained from the consensus primers
120 positive strain (accession number EU085382) using the BLAST software
121 showed a 98-100% identity with known sequences of this variant. Analyzing
122 the sequence of the PCR product from the atypical-primer set positive strain
123 (accession number EU085383) resulted in a 94-99% identity with the available
124 sequences of the atypical variant. Next, both sequences were subjected to
125 restriction enzyme analysis which showed that digestion with the enzyme
126 *Mbol* would result in two clearly different restriction patterns; the consensus
127 strain product would result in fragments with sizes of respectively 6 bp, 114
128 bp, 164 bp and 264 bp whereas the atypical strain product would generate
129 two 6 bp fragments, one fragment of 193 bp and one of 343 bp. Subsequently
130 all PCR products obtained with the total-primer set were purified from gel
131 using the QIAEX II kit (Qiagen) and digested with (FastDigest) *Mbol*

132 (Fermentas) according to the manufacturers instructions. Indeed *Mbol*
133 digestion and subsequent electrophoresis of all nine PCR products from the
134 atypical-primer set positive strains showed bands of the predicted sizes (Fig.
135 1). However, *Mbol* digestion of only four out of the eight PCR products from
136 consensus-primer set positive strains resulted in fragments of the expected
137 sizes (con3 pattern, Fig. 1). The other four PCR products showed only two
138 visible bands: one band of 164 bp and one of approximately 390 bp (con2
139 pattern, Fig. 1). Partly sequencing of one of these PCR products (accession
140 number EU085384) revealed a difference in nucleotide 270 (TAATC versus
141 GAATC) between this sequence and seq EU085382 resulting in a difference in
142 the number of *Mbol* restriction sites. Indeed in silica restriction analysis
143 indicated three fragments: one 6 bp fragment, one fragment of 164 bp and
144 one fragment of 378 bp. Strikingly the PCR products with a con2 pattern were
145 generated from strains originating from pigs whereas the PCR products
146 resulting in one of the two other *Mbol* patterns were from nonporcine origin. In
147 order to study the possible correlation between *Mbol* pattern and allelic variant
148 as well as the possible correlation between *Mbol* pattern and sample origin,
149 36 atypical sequences and 37 *cpb2* consensus sequences in the
150 Genbank/EMBL/ DDBJ databases were analyzed for the *Mbol* restriction
151 pattern. The atypical sequences all originating from nonporcine sources
152 showed the predicted atypical pattern. Analysis of 24 consensus sequences
153 originating from sheep (n=4), cattle (n=7), horses (n=6), humans (n=5) and
154 other sources (n=2) resulted in the con3 pattern. Thirteen consensus
155 sequences all originating from pig isolates (including the firstly described *cpb2*
156 gene with accession number L77965) resulted in the con2 pattern. Analyzing

157 19 submitted partial consensus-sequences showed that 17 harbored the *Mbol*
158 site at position 270 downstream the first nucleotide of the CpB2total F2
159 primer. These sequences all originated from nonporcine sources like food,
160 poultry, etc., whereas 2 sequences which were devoid of this *Mbol* site were
161 of porcine origin.

162

163 3.3. Use of the new total-primer set and *Mbol* digestion in routine screening

164 Next, a protocol for the demonstration and typing of the *cpb2* containing *C.*
165 *perfringens* in clinical samples was formulated. At the VMDC, probable *C.*
166 *perfringens* colonies from clinical samples were streaked on blood agar for
167 single colonies. Subsequently, one colony was used in a PCR with the new
168 total-primer set and a PCR with the CPA-primer set. PCR products obtained
169 with the total-primers were digested with FastDigest *Mbol* (Fermentas) without
170 any purification and the resulting digest was electrophoresed on a 3 %
171 agarose gel. Forty two isolates were typed accordingly: all were *cpa* positive,
172 twenty four were positive for *cpb2*. *Mbol* digestion patterns of PCR products
173 obtained from isolates from all types of animals other than pig resulted in
174 either the atypical pattern (17) or in the con3 pattern (5). Both products
175 amplified from isolates from pigs showed a con2 pattern. To confirm these
176 findings all positive strains were also tested with the corresponding primer set.
177 Results with the consensus-primer set were in agreement with the *Mbol*
178 restriction patterns. However, 3 isolates which were typed atypical with the
179 total-primer set did not result in a PCR product when using the atypical-primer
180 set and the advised protocol (Jost et al., 2005). Changing PCR conditions like
181 annealing temperature or annealing time did result in a PCR product from only

182 one of these isolates. The remaining two isolates were also negative with the
183 consensus-primer set.

184

185 4. Discussion

186 The difference in pathogenicity between *C. perfringens* strains is thought to be
187 largely correlated with the toxins produced. The role of some of these toxins in
188 the pathogenicity of disease has not been clarified yet. One of the toxins with
189 an unresolved role is the beta2 toxin. *C. perfringens* strains harboring *cpb2*
190 have frequently been isolated from or associated with enteritis in pigs (Gibert
191 et al., 1997; Johansson et al., 2006), enterocolitis in horses (Gibert et al.,
192 1997; Johansson et al., 2006), enterotoxaemia in calves (Lebrun et al. 2007)
193 and diarrhea in dogs (Thiede et al., 2001). The actual presence of the toxin in
194 affected tissue suggesting an association between the beta2 toxin and the
195 post mortem findings has been demonstrated by immunohistochemistry in an
196 African elephant (*Loxodonta africana*) with ulcerative enteritis (Bacciarini,
197 2001) and in horses that died from typhlocolitis (Bacciarini et al., 2003; Vilei et
198 al, 2005).

199 In order to study the role of the beta2 toxin in various animals, a rapid
200 screening method for the presence of the beta2 toxin would be helpful.

201 Although the presence of *cpb2* in the genome of the bacterium does not
202 necessarily mean that the toxin is produced, a PCR for the toxin gene can be
203 seen as such a first screening.

204 Several sets of primers for the demonstration of *cpb2* have been published,
205 however when using the BLAST software none of these primers (fully)
206 aligned with all complete *cpb2* sequences in the Genbank/EMBL/DDBJ

207 databases. Therefore primers that recognize all known (complete) *cpb2*
208 sequences were designed and tested.

209 Recent studies indicate that there might be a difference in the toxin production
210 between the consensus and atypical variants (Bueschel et al, 2003; Jost et al,
211 2005; Lebrun et al., 2007). Furthermore the level of expression of the
212 consensus *cpb2* gene seems to be correlated with the origin of the isolates,
213 with the genotype – phenotype correlation being the highest in porcine
214 isolates (Bueschel et al.2003; Jost et al, 2005; Vilei et al, 2005). The *Mbol*
215 restriction pattern of the PCR product generated with our total-primer set
216 discriminates between atypical and consensus variants and between porcine
217 and nonporcine origin and might therefore correlate with the level of beta2
218 toxin production.

219 In conclusion, the described combination of a PCR using the newly designed
220 total-primer set with subsequent *Mbol* digestion is a practical tool in the
221 diagnostics, and may be of value in the research on the role, of the beta2
222 toxin. Research should firstly focus on the correlation between *cpb2* type and
223 beta2 toxin production.

224

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227 their skilful technical assistance.

228

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280 Figure caption

281

282 Figure 1: *Mbol* digest of PCR products obtained with the total-primer set and

283 an atypical or a consensus strain.

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284 Legend to the figure

285

286 **Figure 1:**

287 Fig. 1. Agarose gel (3 %) electrophoresis of *Mbol* digests of PCR products
288 obtained with the total-primer set on an atypical sample (lane 2) or consensus
289 samples (lane 3,4). Lane 1 and 5: size marker (MassRuler Express DNA
290 ladder Mix, Fermentas). The presence of the 6 bp bands can not be
291 demonstrated in this gel under given conditions.

Figure 1

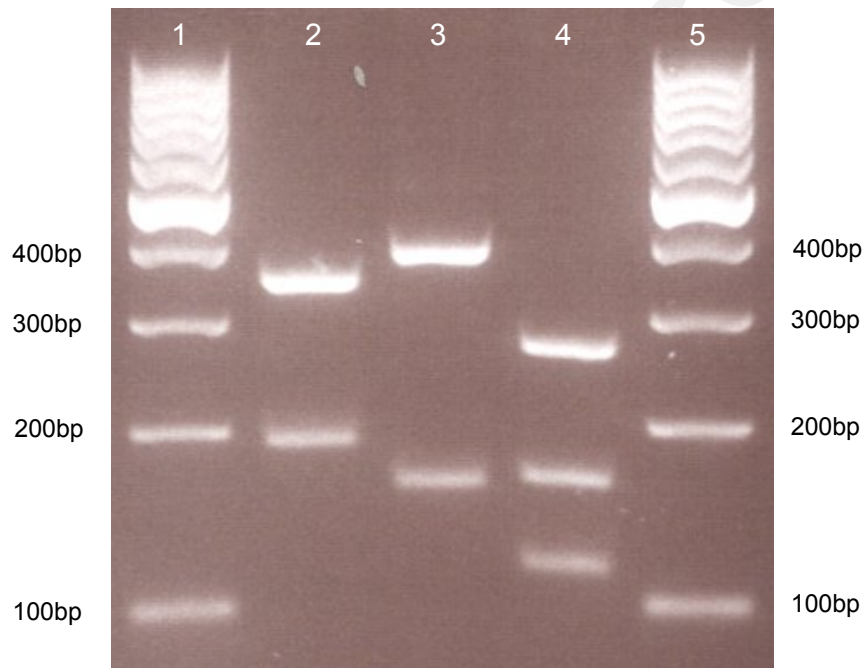


Table 1

Primers used

name primer	target gene	sequence (5'-3')	T _{an}	product length	name primer set
Cpbeta2F ^a	consensus <i>cpb2</i>	CAAGCAATTGGGGGAGTTTA	53°C	200bp	consensus-primer set
Cpbeta2R ^a	consensus <i>cpb2</i>	GCAGAATCAGGATTTTGACCA	53°C		
CpEEB2F ^b	atypical <i>cpb2</i>	AACATAATAAATCCTATAACCC	50°C	1239bp	atypical-primer set
CpEEB2R ^b	atypical <i>cpb2</i>	ATAAATATAATTCTCTAAAACC	50°C		
CpB2totalF2 ^c	consensus/atypical <i>cpb2</i>	AAATATGATCCTAACCAAM ^e AA	48°C	548bp	total-primer set
CpB2totalR ^c	consensus/atypical <i>cpb2</i>	CCAAATACTY ^f TAATYGATGC	48°C		
CPAF ^d	<i>cpa</i>	GCTAATGTTACTGCCGTTGA	53°C	325bp	CPA-primer set
CPAR ^d	<i>cpa</i>	CCTCTGATACATCGTGAAG	53°C		

^a: Baums et al, ^b: Jost et al., 2005, ^c: this paper, ^d: Meer et al.

^e: M = A or C

^f: Y = C or T