



**HAL**  
open science

## Development of PCR protocols for specific identification of and detection of and genes

Ilenia Drigo, C. Bacchin, M. Cocchi, L. Bano, F. Agnoletti

► **To cite this version:**

Ilenia Drigo, C. Bacchin, M. Cocchi, L. Bano, F. Agnoletti. Development of PCR protocols for specific identification of and detection of and genes. *Veterinary Microbiology*, 2008, 131 (3-4), pp.414. 10.1016/j.vetmic.2008.04.013 . hal-00532413

**HAL Id: hal-00532413**

**<https://hal.science/hal-00532413>**

Submitted on 4 Nov 2010

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

## Accepted Manuscript

Title: Development of PCR protocols for specific identification of *Clostridium spiroforme* and detection of *sas* and *sbs* genes

Authors: Ilenia Drigo, C. Bacchin, M. Cocchi, L. Bano, F. Agnoletti



PII: S0378-1135(08)00148-X  
DOI: doi:10.1016/j.vetmic.2008.04.013  
Reference: VETMIC 4011

To appear in: *VETMIC*

Received date: 14-2-2008  
Revised date: 27-3-2008  
Accepted date: 10-4-2008

Please cite this article as: Drigo, I., Bacchin, C., Cocchi, M., Bano, L., Agnoletti, F., Development of PCR protocols for specific identification of *Clostridium spiroforme* and detection of *sas* and *sbs* genes, *Veterinary Microbiology* (2007), doi:10.1016/j.vetmic.2008.04.013

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.



## 21 Abstract

22 Rabbit diarrhoea caused by toxigenic *Clostridium spiroforme* is responsible for  
23 significant losses in commercial rabbitries but the accurate identification of this micro-  
24 organism is difficult due to the absence of both a commercial biochemical panel and  
25 biomolecular methods. The aim of this study was therefore to develop PCR protocols  
26 for specific detection of *C. spiroforme* and its binary toxin encoding genes. The *C.*  
27 *spiroforme* specie-specific primers were designed based on its 16S rDNA published  
28 sequences and the specificity of these primers was tested with DNA extracted from  
29 closely related *Clostridium* species. The sa/bs\_F and sa/bs\_R *C. spiroforme* binary toxin  
30 specific primers were designed to be complementary respectively to a sequence of 21  
31 bases on the 3' end of *sas* gene and on the 5' of the *sbs* gene. The detection limits of in  
32 house developed PCR protocols were 25 CFU/ml of bacterial suspension and  $1.38 \times 10^4$   
33 CFU/g caecal content for specie-specific primers and 80 CFU/ml of bacterial  
34 suspension and  $2.8 \times 10^4$  CFU/g of caecal content in case of sa/bs primers. These result  
35 indicated that the described PCR assays enable specific identification of *C. spiroforme*  
36 and its binary toxin genes and can therefore be considered a rapid, reliable tool for the  
37 diagnosis of *C. spiroforme*-related enterotoxaemia.

38

39 Keywords: *Clostridium spiroforme*, binary toxin, PCR, rabbit.

40

## 41 1. Introduction

42 Enteric pathologies are the main cause of disease, mortality and economic loss in  
43 intensive rabbitries. Among the bacterial agents known to be involved in rabbit enteric

44 pathologies, *Clostridium spiroforme* plays a particularly important role (Borriello et al.,  
45 1983; Songer, 1996)

46 The pathogenicity of this micro-organism is linked to the production of a binary toxin  
47 that is antigenically and structurally similar to the iota toxin produced by *C. perfringens*  
48 type E (Perelle et al., 1993; Gibert et al., 1997). The toxin produced by *C. spiroforme* is  
49 formed by two independent subunits, Sa and Sb, which are similar to subunits Ia and Ib  
50 of *Clostridium perfringens* iota toxin and which, in association, present marked  
51 cytolytic activity (Barth et al., 2004).

52 Currently, diagnosis of *C. spiroforme* enterotoxaemia is based on clinical symptoms,  
53 necropsy, visualization of characteristic bacteria by means of microscopic examination  
54 of the caecal content (Boriello et al., 1986) and subsequent isolation in selective media.  
55 Lastly, diagnosis should be confirmed by detection of the binary toxin in the intestinal  
56 contents. However, precise identification of the isolates is hindered by the lack of both  
57 biomolecular techniques and rapid commercial kits designed to detect this micro-  
58 organism. The aim of this study was therefore to develop PCR protocols for the rapid  
59 detection of *C. spiroforme* and its binary toxin encoding genes (*sas* and *sbs*) directly in  
60 caecal content specimens and bacterial culture.

## 61 **2. Materials and methods**

### 62 *2.1. Strains and growth conditions*

63 A total of 29 strains were tested in this study; 15 of these were *C. spiroforme* field  
64 strains and 14 were reference strains.

65 Reference strains included one type strain of *C. spiroforme* (ATCC 29900) and 9 other  
66 reference strains belonging to the genus *Clostridium* (*C. cocleatum* CCUG 1551, *C.*  
67 *ramosum* CCUG 1402, *C. innocuum* CCUG 1286, *C. tertium* ATCC 19405, *C. sordellii*

68 ATCC 9714, *C. perfringens* CCUG 2037 and ATCC 27324, *C. difficile* ATCC 51695,  
69 *C. colinum* ATCC 27770). *S. aureus* (ATCC 29213), *B. fragilis* (ATCC 25285), *K.*  
70 *pneumoniae* (ATCC 700603) and *E. coli* (ATCC 25922) strains were also tested.

71 All strains were grown in Columbia Agar Base (Oxoid) at 37°C in an anaerobic  
72 chamber for 48 hrs with the exception of *E. coli* and *K. pneumoniae*, which were  
73 incubated in aerobic conditions. In order to determine PCR sensitivity, *C. spiroforme*  
74 strains were cultured in Reinforced Clostridial Medium (RCM, Oxoid) and plated on  
75 Wilkins Chalgren Anaerobe Agar (WCA, Oxoid).

#### 76 2.2. DNA extraction

77 The chromosomal DNA of cultured bacteria were extracted with GeneElute Bacterial  
78 Genomic DNA kit (Sigma), according to the manufacturer's instructions, while the  
79 DNA from intestinal content samples were obtained using QIAamp DNA Stool Mini  
80 Kit (QIAGEN).

#### 81 2.3. Sequence data and primers design

82 The nucleotide sequences were obtained from the Gene-Bank database. The multiple  
83 alignment of these sequences were performed with the ClustalW programme  
84 ([www.ebi.ac.uk/clustalw/](http://www.ebi.ac.uk/clustalw/)), whereas *C. spiroforme* specific oligonucleotide primers  
85 were designed using the Web-Primer programme ([seq.yeastgenome.org/cgi-bin/web-](http://seq.yeastgenome.org/cgi-bin/web-primer)  
86 primer).

#### 87 2.4. PCR amplification conditions

88 The amplifications were performed in a total volume of 25 µl containing 1.5 mM  
89 MgCl<sub>2</sub>, 0.05 U/µl FastStart Taq DNA Polymerase (Roche), 200 µM of each dNTPs  
90 (Applied Biosystems), 0.5 µM of each primer and 5 µl of DNA. The amplification  
91 protocols were performed with an Eppendorf Mastercycler Ep Gradient S (Eppendorf).

92 The thermal cycling conditions for specific amplification of *C. spiroforme* 16S rDNA  
93 were as follows: initial denaturation at 95 °C for 4 min, 8 cycles at 95 °C for 30 s, 70 °C  
94 for 30 s with a decrease of 1 °C/cycle and 72 °C for 30 s, followed by 30 cycles at 95 °C  
95 for 30 s, 62 °C for 30 s, 72 °C for 30 s and a final step of 3 min at 72 °C. The PCR  
96 conditions for specific detection of *C. spiroforme* binary toxin were instead: 95 °C for 5  
97 min followed by 30 cycles at 95 °C for 30 s, 60 °C for 30 s, 72 °C for 30 s and a final  
98 elongation step of 3 min at 72 °C. After amplification, 10 µl of PCR products were  
99 subjected to electrophoresis on 2% agarose gel (Sigma) added with 0.5 µg/ml ethidium  
100 bromide (Sigma), visualized under UV light and photographed. The PCR 100 bp Low  
101 Ladder (Sigma) was used as a DNA size marker. The *C. spiroforme* specie-specific  
102 primers Spiro1\_F (3'-CAA TGC TCG AGT GGC GAA-5') and Spiro4\_R (3'-TCT  
103 GAG GTC TTT ATC CGG ATG-5') designed based on 16S rDNA sequence are able to  
104 amplify a fragment of 916 bp. The amplicon size of sa/bs\_F (3'-CGT GAA CCT ATT  
105 CCC GCT AAT-5') and sa/bs\_R (3'-GCA ATT TCA CCT TCA CCT TCA GCA TTT-  
106 5') *C. spiroforme* binary toxin specific primers is 824 bp.

### 107 2.5. Sequencing

108 PCR products were cleaned up with ExoSAP-IT (USB) and the amplicons were  
109 sequenced using the ABI-Prism Dye Terminator Cycle Sequencing kit (Applied  
110 Biosystems) and ABI PRISM 3130 Genetic Analyzer (Applied Biosystems).

### 111 2.6. PCR specificity and sensitivity

112 PCR assay specificity was tested with the amplification protocol described above using,  
113 as a template, DNA extracted from all field and reference strains and from intestinal  
114 contents positive and negative for *C. spiroforme* at microscopic and cultural  
115 examination.

116 In order to test the sensitivity of the PCR assays, *C. spiroforme* suspension (optical  
117 density at 600 nm of approximately 0.8-0.9) was serially 10-fold diluted in RCM and  
118 the bacterial concentration was determined by plating 1 ml of each dilution onto WCA.  
119 Simultaneously the DNA was extracted from 1 ml of the same bacterial suspension and  
120 samples were amplified with the PCR protocol. To investigate the effect of caecal  
121 contents on PCR sensitivity, the experiment was also performed with DNA extracted  
122 from caecal contents infected with the same *C. spiroforme* dilutions. Sensitivity was  
123 expressed as the lowest CFU number detectable with the PCR protocol.

### 124 **3. Results**

#### 125 *3.1. Clostridium spiroforme* specie-specific primer development

126 The BLAST search for sequences similar to 16S rDNA of *C. spiroforme* (accession  
127 number X73441 and X75908) showed that this gene has high sequence similarity with  
128 16S rDNA of *C. ramosum* (accession number X73440), *C. cocleatum* (accession  
129 number Y1818) and *C. innocuum* (accession number M23732). The alignment studies  
130 of these sequences revealed small but consistent sequence differences among the  
131 species investigated and then specie-specific primers were designed on hypervariable  
132 regions of the *C. spiroforme* 16S rRNA gene. The oligonucleotide sequences were used  
133 in a BLAST search of the Gene-Bank to verify that they were unique to *C. spiroforme*.  
134 The PCR assay applied to DNA extracted from *C. spiroforme* type strain and other  
135 reference strains showed that the selected PCR primer pair amplify, with a single and  
136 specific amplification product of 912 bp, only DNA extracted from *C. spiroforme*  
137 reference and field strains and from intestinal contents positive for this micro-organism  
138 (Figure 1). Sequencing of the amplified product confirmed that the targeted gene was  
139 amplified with this PCR assay. The detection limit of the specie-specific primers under

140 the above-described PCR conditions was 25 CFU/ml of bacterial suspension in the  
141 absence of caecal content, while the presence of intestinal content in the bacterial  
142 solution decreased the sensitivity of the assay to  $1.38 \times 10^4$  CFU/g of caecal content.

### 143 3.2. Development of specific primers for *Clostridium spiroforme* binary toxin

144 Alignment studies of published sequences of *C. spiroforme* and *C. difficile* binary toxins  
145 and *C. perfringens* iota toxin (accession numbers: X97969, L76081, X73562) have  
146 shown that these genes have a high degree of similarity. Primers sa/bs\_F and sa/bs\_R  
147 were then developed selecting two fragments specific for the *C. spiroforme* binary toxin  
148 gene. The forward primer was designed to be complementary to a sequence of 21 bases  
149 at the 3' end of the *sas* gene while the reverse primers were designed into a sequence of  
150 21 bases on the 5' end of the *sbs* gene. The DNA extracted from *C. spiroforme*, *C.*  
151 *perfringens* type E strains and the *C. difficile* field strain (positive for binary toxin genes  
152 using the PCR protocol described by Stubs et al. [2000]), in addition to the DNA  
153 extracted from intestinal contents negative or positive for *C. spiroforme*, were amplified  
154 with the above-described primers. The expected band of 825 bp was obtained only for  
155 *C. spiroforme* strains and for caecal contents positive for this micro-organism (Figure  
156 2). In this case too, amplicon sequencing analysis confirmed the specificity of the  
157 primers. Sensitivity experiments showed that the maximum detection limit of this PCR  
158 was 80 CFU/ml of bacterial suspension in the absence and  $2.8 \times 10^4$  CFU/g in the  
159 presence of caecal content.

160 The PCR protocols developed in this study, applied on 15 *C. spiroforme* field strains,  
161 demonstrated that all strains were positive for identification of *C. spiroforme* and for the  
162 presence of the binary toxin genes.

163

164 **4. Discussion**

165 *C. spiroforme* is recognized as one of the main causes of enterotoxaemia in farmed  
166 rabbits (Marlier et al., 2003). At present, this pathology is diagnosed by microscopic  
167 examination of caecal content followed by isolation of the bacteria in selective culture  
168 media. However, the cultural characteristics of this bacteria and the lack of commercial  
169 kits for its identification make diagnosis a laborious process. A further complication is  
170 that *C. cocleatum*, frequently isolated in the human intestine, is not only genetically  
171 closely related to *C. spiroforme*, but also has similar morphology and can be  
172 biochemically differentiated from this only by the galactose fermentation test (Kaneuchi  
173 et al., 1979; Euzéby, 1998).

174 This scenario prompted the need to develop a rapid, sensitive diagnostic test to identify  
175 *C. spiroforme* either in bacterial culture or directly in the caecal contents. The resulting  
176 specie-specific primers, designed on 16S rDNA of *C. spiroforme*, proved to be specific  
177 for this micro-organism and the sensitivity tests showed that the highest bacterial  
178 dilution at which the band was visible was 25 CFU/ml bacterial suspension, while the  
179 presence of caecal content, which contain numerous polymerase inhibitors, greatly  
180 decreased the sensitivity of the assay to  $1.38 \times 10^4$  CFU/g. Although it is rather high, this  
181 value does have diagnostic value, considering that the mean concentration reported in  
182 the literature of *C. spiroforme* isolated in rabbits with enteritis is approximately  $10^6$   
183 cells/g of caecal content (Borriello et al., 1983).

184 Unlike *Clostridium perfringens* iota toxin and *C. difficile* binary toxin, there are no  
185 reports in the literature of specific primers for the toxin produced by *C. spiroforme* nor  
186 epidemiological studies on the distribution of potentially toxigenous strains.

187 The sa/bs primers, designed in this study and specific for *C. spiroforme* binary toxin  
188 proved to have a sensitivity of 80 CFU/ml bacterial suspension in the absence and  
189  $2.8 \times 10^4$  CFU/g in the presence of caecal content.

190 Application of the PCR protocols developed in this study on DNA extracted from 15  
191 field strains revealed that all tested strains were positive for the identification of *C.*  
192 *spiroforme* and presented the binary toxin encoding genes.

193 Our results thus indicate that our PCR protocols are able to identify *C. spiroforme* and  
194 the binary toxin genes *sas* and *sbs* in both bacterial culture and intestinal content  
195 specimens and can therefore be considered a rapid, reliable tool for the diagnosis of *C.*  
196 *spiroforme*-related enterotoxaemia.

197

#### 198 **Acknowledgements**

199 This study was supported by grants from Italian Ministry of Health in the contest of the  
200 IZS VE 11/04 RC project.

201 We wish to thank Salomoni Angela and Pertile Marcella, Istituto Zooprofilattico  
202 Sperimentale delle Venezie, Struttura Complessa Ricerca e Sviluppo, Legnaro (PD) for  
203 DNA sequencing.

204

#### 205 **References**

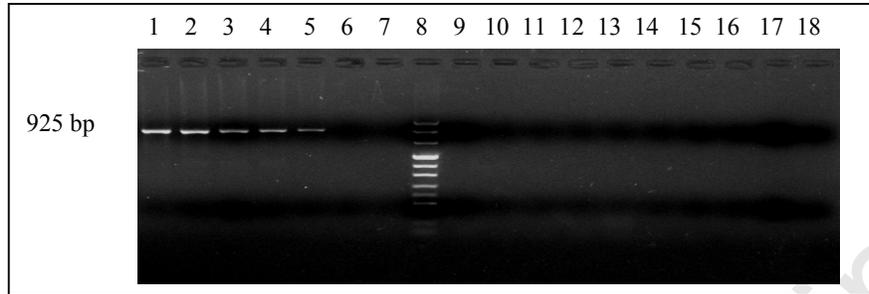
206 Barth, H., Aktories, K, Popoff, M.R., Stiles, B.G., 2004. Binary bacterial toxins:  
207 biochemistry, biology, and applications of common *Clostridium* and *Bacillus*  
208 proteins. Microbiol. Mol. Biol. Rev. 68, 373-394.

- 209 Borriello, S.P., Carman, R.J., 1983. Association of iota-like toxin and *Clostridium*  
210 *spiroforme* with both spontaneous and antibiotic associated diarrhoea and colitis  
211 in rabbits. J. Clin. Microbiol. 17, 414-418.
- 212 Euzéby, J.P., 1998. Dictionnaire de Bactériologie Vétérinaire.  
213 <http://www.bacterio.cict.fr/bacdicto/cc/spiroforme.html>.
- 214 Gibert, M, Perelle, S., Daube, G., Popoff, M.R., 1997. *Clostridium spiroforme* toxin  
215 genes are related to *C. perfringens* iota toxin genes but have a different genomic  
216 localization. Syst. Appl. Microbiol. 41, 337-347.
- 217 Kaneuchi, C., Toshimitsu, M., Toshiharu, S., Tomotari, M., 1979. Taxonomic study of  
218 helically coiled, spore forming anaerobes isolated from the intestine of humans  
219 and other Animals: *Clostridium cocleatum* sp. nov. and *Clostridium spiroforme*  
220 sp. nov. Int. J. Syst. Bacteriol. 29, 1-12.
- 221 Marlier, D., Dewrée, R., Delleur, V., Licois, D., Lassence, C., Poulipoilis, A.,  
222 Vindevogel, H., 2003. Description des principales étiologies des maladies  
223 digestives chez le lapin européen (*Oryctolagus cuniculus*). Ann. Med. Vét. 147,  
224 385-392.
- 225 Perelle, S., Gibert, M., Boquete, P., Popoff, M.R., 1993. Characterization of *Clostridium*  
226 *spiroforme* iota-toxin genes and expression in *Escherichia coli*. Infect. Immun.  
227 63, 5147-5156.
- 228 Songer, J.G., 1996. Clostridial enteric diseases of domestic animals. Clin. Microbiol.  
229 Rev. 9, 216-234.
- 230 Stubbs, S., Rupnik, M., Gibert, M., Brazier, J., Duerden, B., Popoff, M., 2000.  
231 Production of actin-specific ADP-ribosyltransferase (binary toxin) by strains of  
232 *Clostridium difficile*. FEMS Microbiol. Lett. 186, 307-312.

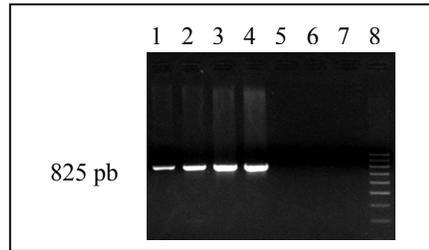
233 Fig. 1. Specificity of 16S rDNA based PCR for *C. spiroforme*. (1) *C. spiroforme* ATCC  
234 29900, (2-3) *C. spiroforme* field strains isolated in our laboratory, (4-5) intestinal  
235 contents positives for *C. spiroforme* at the microscopic examination, (6) *C. coleatum*  
236 CCUG 1551, (7) *C. ramosum* CCUG 1402 (8) 100 bp ladder, (9) *C. innocuum* CCUG  
237 1286, (10) *C. tertium* ATCC 19405, (11) *C. sordellii* ATCC 9714, (12) *C. perfringens*  
238 CCUG 2037, (13) *C. difficile* ATCC 51695, (14) *S. aureus* ATCC 29213, (15) *B.*  
239 *fragilis* ATCC 25285, (16) *K. pneumoniae* ATCC 700603, (17) *E. coli* ATCC 25922,  
240 (18) intestinal content negative for *C. spiroforme* at the microscopic examination.

241 Fig. 2. Specificity of sa/bs primers. (1) Intestinal content positive for *C. spiroforme* at  
242 the microscopic examination, (2-3-4) *C. spiroforme* field strains isolated in our  
243 laboratory, (5) type E *C. perfringens* ATCC 27324, (6) *C. difficile* field strain isolated  
244 in our laboratory and positive for binary toxin genes, (7) intestinal content negative for  
245 *C. spiroforme* at the microscopic examination, (8) 100bp ladder.

Accepted Manuscript



Accepted Manuscript



Accepted Manuscript