PCR IDENTIFICATION AND TOXIN-TYPING OF *CLOSTRIDIUM SPIROFORME* FIELD STRAINS

Drigo I.*, Bacchin C., Cocchi M., Bano L., Agnoletti F.

Istituto Zooprofilattico Sperimentale delle Venezie, Viale Brigata Treviso 13/a, 31100 Treviso, Italy
*Corresponding author: idrigo@izsvenezie.it

ABSTRACT

Rabbit diarrhoea caused by toxigenic *Clostridium spiroforme* is responsible for significant losses in commercial rabbitries. This micro-organism usually affects rabbits during weaning and its pathogenicity is related to the production of a strong cytotoxic binary toxin that is similar in function and structure to *Clostridium perfringens* type E iota toxin. The definitive diagnosis of *C. spiroforme* enterotoxaemia is currently difficult due to the absence of both biochemical commercial panel and bio-molecular methods for specific identification.
The aim of this study was therefore initially to develop PCR protocols for the specific detection of *C. spiroforme* and its binary toxin coding genes and then apply them in a field study. A total of 80 *C. spiroforme* field strains isolated from rabbits with intestinal disorders were analysed; 30 isolates were obtained from the caecal content of rabbits with anatomopathologic evidence of enteritis caused by *C. spiroforme*, 19 from rabbits for which *C. spiroforme* was a co-agent of infection, and 31 from rabbits with intestinal diseases not caused by *C. spiroforme*. All strains were positive for species-specific identification and the presence of the genes coding for *C. spiroforme* binary toxin.
In conclusion, we developed a useful and reliable biomolecular tool for specific identification of *C. spiroforme* and detection of genes coding for its binary toxin. When subsequently applied in a field study, these PCR protocols demonstrated all the strains analysed to be potentially toxigenic.

Key words: *Clostridium spiroforme*, Binary toxin, PCR, Rabbit.

INTRODUCTION

Diseases associated with diarrhoea, mainly after weaning, are a major cause of economic losses in commercial rabbit breeding. The etiology of these diseases is complex and includes dietary and environmental factors, viral or bacterial infections, and coccidiosis. Bacterial agents known to play a role in enteric pathologies are *Escherichia coli*, *Klebsiella pneumonie*, *Salmonella spp*. and some strains belonging to the genus *Clostridium* (Peeters *et al*., 1984; Percy *et al*., 1993; Agnoletti *et al*., 2006). Among the genus *Clostridium*, *C. spiroforme* is one of the most common pathogens isolated in rabbits with enteric diseases. The pathogenicity of this micro-organism is caused by a production of a binary toxin composed of two components, Sa and Sb, respectively coded by *sas* and *sbs* chromosomal genes. Sa and Sb, as first determined by crossed immunoelectrophoresis and neutralization studies with *Clostridium perfringens* type E antiserum, are analogous to the Ia and Ib components of *C. perfringens* iota toxin (Borriello *et al*., 1983; Popoff *et al*., 1988; Songer *et al*., 1996). These two toxins are also similar in function; both Sa and Ia mediate ADP-rybosilation of monomeric actin, whereas Sb and Ib are responsible for binding and internalization of enzymatic components in target cells (Simpson *et al*., 1989; Songer, 1996; Barth *et al*., 2004).

C. spiroforme is not a normal inhabitant of the rabbit bowel and is acquired from the environment. Anyway, there is some evidence, that the destabilization of caecal microflora may be important in initiating disease. Diarrhoea caused by *C. spiroforme* develops rapidly and death ensues soon after. At necropsy, the caecum appear dilated with watery contents, and the necrosis of the surface of epithelium surface and pronounced inflammatory infiltration of the *lamina propria* are observed (Songer, 1996; Agnoletti *et al*., 2006).

Diagnosis of *C. spiroforme* enterotoxaemia is currently based on necropsy and the visualization of semicircular gram-positive bacteria in the caecal contents of affected rabbits. The definitive identification of this micro-organism is difficult due to the absence of both biochemical commercial panel and bio-molecular methods for *C. spiroforme* specific identification. The aim of this study was therefore to initially develop PCR protocols for the rapid and reliable identification of *C. spiroforme* and its binary toxin genes and then apply these protocols on *C. spiroforme* strains isolated from rabbits affected by enteric disease.

MATERIALS AND METHODS

Strains, growth condition, and DNA extraction.

A total of 80 *C. spiroforme* field strains were tested in this study, all of which were isolated by streaking caecum content of diseased rabbit with sterile swabs onto selective medium (Agnoletti *et al*., 2004). Fourteen reference strains were also tested, included one type strain of C. *spiroforme* (ATCC 29900) and 8 other reference strains belonging to the genus *Clostridium* (*C. cocleatum* CCUG 1551, *C. ramosum* CCUG 1402, *C. innocuum* CCUG 1286, *C. tertium* ATCC 19405, *C. sordellii* ATCC 9714, *C. perfringens* CCUG 2037 and ATCC 27324, *C. difficile* ATCC 51695, *C. colinum* ATCC). Strains of *S. aureus* (ATCC 29213), *B. fragilis* (ATCC 25285), *K. pneumoniae* (ATCC 700603) and *E. coli* (ATCC 25922) were also tested. All strains were grown in Columbia Agar Base (Oxoid) at 37°C in anaerobic chamber for 48 hrs with the exception of *E. coli* and *K. pneumoniae,* which were incubated in aerobic condition. In order to determine PCR assay sensitivity, *C. spiroforme* strains were cultured in Reinforced Clostridial Medium (RCM, Oxoid) and plated on Wilkins Chalgren Agar (WCA, Oxoid).

The DNA of cultured bacteria were extracted using a GeneElute Bacterial Genomic DNA kit (Sigma) according to the manufacturer's instructions.

Primer design and PCR amplification conditions

The primers used in the PCR assays were designed in our laboratory using Web-primers program (seq.yeastgenome.org/cgi-bin/web-primer). Species-specific primers were developed selecting two segments of 18 and 21 bp in the 16S rDNA of *C. spiroforme,* whereas the primers for the specific amplification of *C. spiroforme* binary toxin were designed onto non-conserved regions of *sas* and *sbs* genes. In both cases, the amplifications were performed in a total volume of 25 µl containing 1.5 mM $MgCl_2$, 0.05 U/µl FastStart *Taq* DNA Polimerase (Roche), 200 µM of each dNTPs (Applied Biosystems), 0.5 µM of each primer and 5 µl of DNA. The amplification protocols were performed with an Eppendorf Mastercycler Ep Gradient S (Eppendorf).

The thermal cycling conditions for specific amplification of *C. spiroforme* 16S rDNA were as follows: initial denaturation at 95°C for 4 min, 8 cycles at 95°C for 30 s, 70°C for 30 s with a decrease of 1°C/cycle and 72°C for 30 s, followed by 30 cycles at 95°C 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 conditions for specific detection of *C. spiroforme* binary toxin were 95°C for 5 min followed by 30 cycles at 95°C for 30 s, 60°C for 30 s, 72°C for 30 s and a final elongation step of 3 min at 72°C. After amplification, 10 µl of PCR products were subjected to electrophoresis on 2% agarose gel (Sigma) added with 0.5 µg/ml ethidium bromide (Sigma) visualized under UV light and photographed. The PCR 100 bp Low Ladder (Sigma) was used as a DNA size marker.

PCR specificity and sensitivity

PCR assay specificity was tested with the amplification protocol described above using DNA extracted from all field and reference strains as a template. Primer specificity was also demonstrated

by sequencing the amplified product. In order to test the sensitivity of the PCR assays, bacterial suspensions were serially 10-fold diluted in RCM. One ml of each dilution was plated on WCA and the DNA was extracted from 1 ml of the same bacterial suspension at the same time. All samples were amplified with the PCR protocol, and sensitivity was expressed as the lowest CFU number detectable with the PCR protocol.

RESULTS AND DISCUSSION

The BLAST search for sequences similar to 16S rDNA of *C. spiroforme* (accession number X73441) showed that this gene has high sequence similarity with 16S rDNA of *C. ramosum*, *C. cocleatum* and *C. innocuum* (accession numbers respectively X73440, Y1818, M23732). Species-specific primers were therefore designed on hypervariable regions of the *C. spiroforme* 16S rDNA gene. The PCR assay applied to DNA extracted from *C. spiroforme* type strain and other reference strains showed that the selected PCR primer pairs amplify with a single and specific amplification product (925 pb) only DNA extracted from *C. spiroforme* strains (Figure 1). The sequencing of the amplified product confirmed that the targeted gene was amplified with the PCR assay. The detection limit of the species–specific primers under the PCR conditions described above was 25 CFU/ml of bacteria suspension.

Alignment studies of *C. spiroforme* and *C. difficile* binary toxins and *C. perfringens* ι toxin (accession numbers: X97969, L76081, X73562) showed that these genes have high degree of similarity. Primers for specific detection were then developed selecting two fragments specific for *C. spiroforme* binary toxin gene. The forward primer was designed to be complementary to a sequence of 21 bases at the 3' end of the *sas* gene, while the reverse primer was designed into a sequence of 21 bases on the 5' of the *sbs* gene. The DNA extracted from *C. spiroforme*, *C. difficile* and *C. perfringens* type E strains were amplified with these primers, and the expected band of 825 bp was obtained only for *C. Spiroforme* (Figure 2). In this case as well, the amplicon sequencing analysis confirmed the specificity of the primers. Sensitivity experiments showed the maximum detection limit of this PCR to be 80 CFU/ml of bacteria suspension.

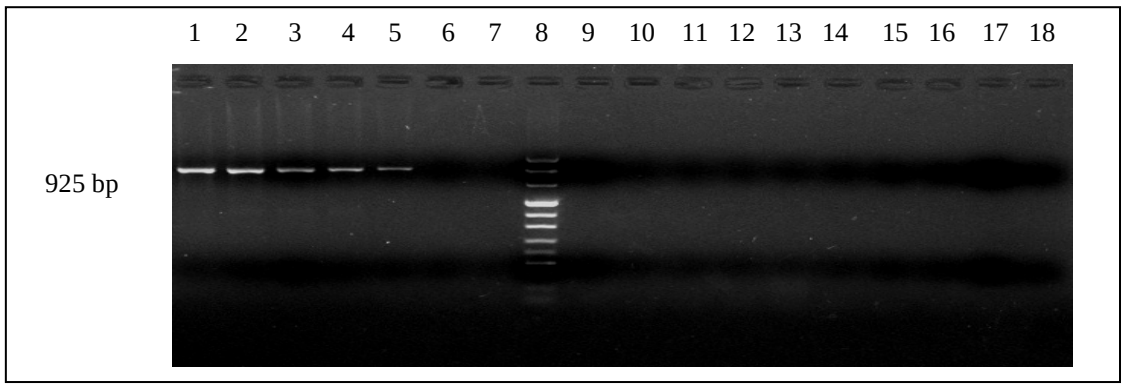

Figure 1: Specificity of 16S rDNA based PCR for *C. spiroforme*. (1) *C. spiroforme* ATCC 29900, (2-3-4-5) *C. spiroforme* field strains isolated in our laboratory, (6) *C. coleatum* CCUG 1551, (7) *C. ramosum* CCUG 1402 (8) 100 bp ladder (9) *C. innocuum* CCUG 1286, (10) *C. tertium* ATCC 19405, (11) *C. sordellii* ATCC 9714, (12) *C. perfringens* CCUG 2037, (13) *C. difficile* ATCC 51695, (14) *S. aureus* ATCC 29213, (15) *B. fragilis* ATCC 25285, (16) *K. pneumonite* ATCC 700603 (17) *E.coli* ATCC 25922 (18) negative control

These PCR protocols were subsequently applied in a field study in which 80 *C. spiroforme* strains identified by bacterioscopic examination were isolated as follows: 30 from the caecal content of rabbits with anathomopathologic evidence of enteritis caused by *C. spiroforme*, 19 from rabbits for which *C. spiroforme* was a causative agent of enteritis in concomitance with enteropathogenic *E. coli*, and 31 from rabbits with intestinal diseases not caused by *C. spiroforme*. The strains were initially analyzed using species-specific primers for *C. spiroforme* identification and subsequently for the

presence of the *sas* and *sbs* genes as well. All the field strains resulted positive for the identification of *C. spiroforme* and the presence of the genes coding for binary toxin.

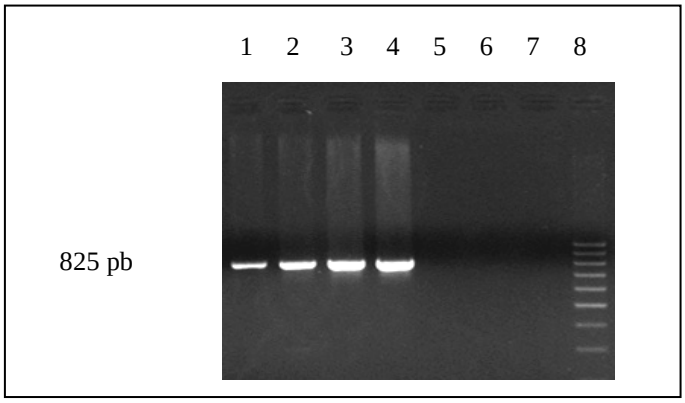

Figure 2: Specificity of primers specific for *C. spiroforme* binary toxin. (1-2-3-4) *C. spiroforme* field strains isolated in our laboratory, (5) *C. perfringens* type E ATCC 27324, (6) *C. difficile* field strain positive for binary toxin, (7) negative control (8) 100bp ladder.

CONCLUSIONS

We developed a rapid and useful tool for both the specific identification of *C. spiroforme* and the detection of genes coding for its binary toxin. When applied in a field study, these PCR protocols showed all the 80 *C. spiroforme* strains analysed in this study and isolated from rabbits affected by enteric diseases to be positive for the presence of the genes coding for binary toxin. The results also demonstrated that there were no differences in binary toxin genes positivity between strains isolated from rabbits with enteric disease caused by *C. spiroforme* and rabbits with enteritis not caused by this micro-organism.

REFERENCES

Agnoletti F., Bano L., Cocchi M., Mazzolini E. 2006. Aggiornamenti sulle enteriti batteriche del coniglio. *Riv. Zoot. Vet., 34, 29-38.*

Agnoletti F., Bano L., Comin D., Parenti E., Marcati M., Bertolin M., Mazzolini E. 2004. Selective culture medium to isolate *Clostridium spiroforme* from rabbit gut. *In: Proc. 8th World Rabbit Congress, 2004 September, Puebla, Mexico, 410-415.*

Barth H., Aktories K., Popoff M.R., Stiles B.G. 2004. Binary bacterial toxins: biochemistry, biology, and applications of common *Clostridium* and *Bacillus* proteins. *Microbiol. Mol. Biol. Rev., 68, 373-402.*

Borriello S.P., Carman R.J. 1983. Association of iota–like toxin and *Clostridium spiroforme* with both spontaneous and antibiotic associated diarrhoea and colitis in rabbits. *J. Clin. Microbiol., 17, 414-418.*

Peeters J.E., Pohl P., Charlier G. 1984. Infectious agents associated with diarrhoea in commercial rabbits: a field study. *Ann. Rech. Vet., 15, 335-340.*

Percy D.H., Muckle C.A., Hampson R.J., Brash M.L. 1993. The enteritis complex in domestic rabbits: a field study. *Can. Vet. J., 34, 95-102.*

Popoff M.R., Boquet P. 1988. *Clostridium spiroforme* toxin is a binary toxin which ADP-ribosylates cellular actin. *Biochem Biophys. Res. Commun., 152, 1361-1368.*

Simpson L.L., Stiles B.G., Zepeda H., Wilkins T.D. 1989. Production by *Clostridium spiroforme* of an iotalike toxin that possesses Mono (ADP-ribosiyl) Trensferase Activity: identification of a novel class of ADP-ribosyltransferases. *Infect. Immun., 57, 255-261.*

Songer J.L. 1996. Clostridial enteric diseases of domestic animals. *Clin. Microbiol. Rev., 9, 216-234.*