

Development of a polymerase chain reaction assay for specific identification of *Clostridium colinum*

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Clostridium colinum is the causative agent of ulcerative enteritis, a serious disease of the bobwhite quail (*Colinus virginianus*) and sporadically of young chickens. The aim of the present study was to develop a polymerase chain reaction (PCR) assay specific for *C. colinum* identification. The 16S rDNA sequence of *C. colinum* was analysed and two species-specific primers were designed. The specificity of these primers was tested with closely related *Clostridium* species and the expected amplified product (935 base pairs) was observed only with DNA from samples containing *C. colinum*. Results from performing PCR assays on faecal samples from quails spiked with different concentrations of *C. colinum*, showed that the detection limit of the assay was 1.6×10^4 colony-forming units per gram of faecal material. This PCR assay can be used in diagnostic laboratories to confirm the presence of *C. colinum* in pure cultures and could be used to screen enriched samples or faecal samples for the presence of this pathogen.

Introduction

Ulcerative enteritis (UE) or “quail disease” is a severe bacterial disease produced by *Clostridium colinum* that occurs with epidemic proportions in bobwhite quails (*Colinus virginianus*) (Berkhoff, 1975). In addition to quails, UE has been found in other avian species, including chickens, turkeys, pheasants, grouse, pigeons, robins and lories (Bullis & Van Roekel, 1944; Glover, 1951; Buss *et al.*, 1958; Berkhoff, 1975; Winterfield & Berkhoff, 1977; Ononiwu *et al.*, 1978; Kondo *et al.*, 1988; Perelman *et al.*, 1991; Pizarro *et al.*, 2005). In chickens, UE appears typically between 4 and 20 weeks of age with a mortality rate of up to 50% in untreated flocks (Berkhoff, 1975; Ononiwu *et al.*, 1978; Kondo *et al.*, 1988; Perelman *et al.*, 1991). Diseases such as coccidiosis, aplastic anaemia, Gumboro disease and stress conditions can lead to an increase in mortality (Peckham, 1960; Davis, 1973). The intestinal lesions can vary from haemorrhagic enteritis to ulcerations of the intestinal mucosa, frequently accompanied by liver necrosis and occasionally by spleen infarcts (Berkhoff, 1975; Ononiwu *et al.*, 1978).

C. colinum is a Gram-positive anaerobic rod and, based on 16S rRNA sequencing, it is closely related to *Clostridium piliforme*, the causative agent for Tyzzer's disease (Feldman *et al.*, 2006). Identification of *C. colinum* using standard biochemical tests is difficult and time-consuming as a routine diagnostic activity. The aim of the present study was to develop a PCR assay for rapid identification of *C. colinum* from bacterial cultures and to determine the sensitivity of this assay on artificially contaminated faecal material.

Materials and Methods

Bacterial strains and DNA extraction. Type strains of *C. colinum* (ATCC 27769, ATCC 27770), *Clostridium sordellii* (ATCC 9714), *Clostridium perfringens* (CCUG 2037), *Clostridium cocleatum* (CCUG 1551), *Clostridium ramosum* (CCUG 1286), *Clostridium innocuum* (CCUG 1286), *Clostridium tertium* (ATCC 19405), *Clostridium spiroforme* (ATCC 29900), *Clostridium difficile* (ATCC 51695), and *Bacteroides fragilis* (ATCC 25285) were inoculated in Cooked Meat Broth (Difco), incubated in anaerobic conditions (90% N₂, 5% CO₂, 5% H₂) at 37°C for 24 h, and plated on pre-reduced Tryptic Soy Agar (Difco) supplemented with 5% defibrinated sheep blood under anaerobic conditions. *Staphylococcus aureus* (DSMZ 11729) and *Escherichia coli* (field strain) were plated on Columbia agar base (Difco) supplemented with 5% of defibrinated sheep blood and incubated in aerobic conditions. The bacterial DNA was extracted with the Bacterial Genomic DNA kit (Sigma-Aldrich), while the EX-WAX DNA extraction kit (Chemicon International) was used to extract DNA from four paraffin rabbit liver samples with histological signs of Tyzzer's disease.

Primer design. Sequences of the 16S rRNA of *C. colinum*, *C. piliforme* and *Clostridium disporicum* were retrieved from GenBank and aligned using MultAlin (<http://bioinfo.genopole-toulouse.prd.fr/multalin/multalin.html>). Primers were designed using hypervariable regions of the gene. Forward primer CcF (5'-GTCGAGCGGAGTTTTATGGG-3') and reverse primer CcR (5'-CATTACACAGATTGTCATCGGG-3') were used to amplify a segment of 936 base pairs of the *C. colinum* gene.

Polymerase chain reaction protocol. Polymerase chain reaction (PCR) assays were performed in 25 µl aliquots in a PTC-100 Programmable Thermal Controller (MJ Research, Inc.). The temperatures for the cycles were 94°C for 4 min to denature DNA followed by 25 cycles of 94°C for 1 min, 60°C for 1 min and 72°C for 1 min. Final extension was performed at 72°C for 4 min. Amplicons were detected by standard gel electrophoresis in 1.5% agarose (Ultra Pure DNA Grade Agarose;

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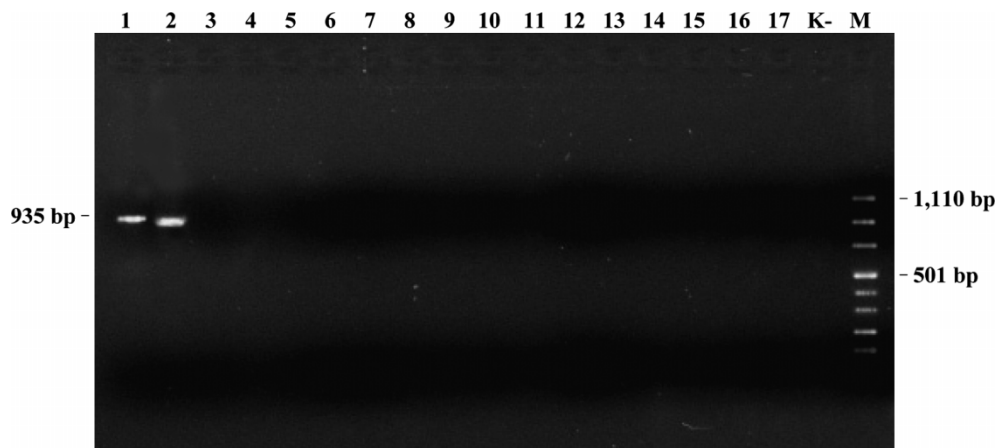


Figure 1. Agarose gel electrophoresis of PCR products from reference strains, field strains and Tyzzer's disease positive liver samples. Lane 1, *C. colinum* ATCC 27770; lane 2, *C. colinum* ATCC 27769; lane 3, *C. cocleatum* CCUG 1551; lane 4, *C. ramosum* CCUG 1286; lane 5, *C. innocuum* CCUG 1286; lane 6, *C. tertium* ATCC 19405; lane 7, *C. sordellii* ATCC 9714; lane 8, *C. spiroforme* ATCC 29900; lane 9, *C. perfringens* CCUG 2037; lane 10, *C. difficile* ATCC 51695; lane 11, *E. coli* field strain; lane 12, *S. aureus* DSMZ 11729; lanes 13 to 16, paraffin rabbit liver samples with histological signs of Tyzzer's disease (*C. piliforme*); lane 17, *B. fragilis* ATCC 25285; lane K-, negative control; lane M, DNA molecular weight marker VIII (Roche).

Bio-Rad Laboratories). Gels were stained with ethidium bromide and visualized using an ultraviolet transilluminator (Gel-Doc System).

PCR amplicons were cleaned up with the Minielute PCR Purification Kit (Qiagen) according to the manufacturer's instructions and sequenced using an ABI Genetic Analyzer (Applied Biosystems). Sequences were then confirmed by performing BLAST analysis.

Detection limit of *C. colinum* by PCR in faecal material. To test the sensitivity of the PCR assays, bacterial suspensions of *C. colinum* (ATCC 27770) were serially diluted in PBS and 0.1 ml of each dilution was plated on blood agar. DNA was extracted simultaneously from each dilution tube. Bacterial enumeration was performed after 48 h of incubation.

To investigate the effect of faecal inhibitors on the PCR assay, 1 g of fresh faecal material collected from healthy breeder quails was added to each 1 ml of the bacterial suspension and DNA extracted using the QIAamp DNA Stool Mini Kit (Qiagen). The extracted DNA was amplified using the described PCR protocol. Before inoculation with *C. colinum*, faeces were tested with the same PCR assay to exclude a pre-existing contamination with this microorganism.

Results and Discussion

The alignment of published 16S rRNA of *C. colinum*, *C. disporicum* and *C. piliforme* showed that the hyper-variable areas could be suitable for the design of specific PCR primers for *C. colinum* identification. The designed primers showed specificity to the two ATCC strains of *C. colinum* (Figure 1). The sequencing of the amplified product confirmed that the target gene was indeed amplified with this PCR assay. The diagnosis of UE has been based historically on macroscopic lesions and histopathology. However, after the recognition of the aetiological agent, the confirmation of the causative agent is still not pursued systematically by diagnostic laboratories probably because identification of this organism based on biochemical tests is time-consuming and sometimes of questionable interpretation. Furthermore, the low incidence of UE could be due to the wide use of effective anticlostridial drugs of new generation. These reasons explain the difficulties in finding more *C. colinum* strains to expand the test panel for the PCR assay.

The detection limit of the described PCR protocol was of 10 colony-forming units/ml in the absence of faeces,

while the presence of faeces decreased the sensitivity of the assay to a detection limit of 1.6×10^4 colony-forming units/g faeces. The presence of PCR inhibitors in faeces has been traditionally associated with this lower sensitivity (Lou *et al.*, 1997; Rådström *et al.*, 2004).

The results obtained from the direct application of PCR on the faecal samples encouraged us to believe that this PCR assay could also be used for the direct identification of *C. colinum* from enriched samples. The described PCR assay provides a rapid and accurate tool that will be helpful for monitoring the health of susceptible avian species and will therefore result in a better control of UE produced by *C. colinum*.

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