Detection of toxigenic *Clostridium difficile* in pig feces by PCR

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ABSTRACT: *Clostridium difficile* is considered an important uncontrolled cause of neonatal diarrhea. Also, the presence of bacteria in the feces of the animal could represent a zoonosic risk for the contamination of meat products. Therefore, it is necessary to have procedures available for the early detection of *C. difficile* in animals. The current study describes a new semi-automated procedure for the recovery of *C. difficile* DNA from pig feces and subsequent amplification by polymerase chain reaction (PCR) of three different sequences: the triose phosphate isomerase gene *tpi*, specific for this bacterial species, and the *tcdA* and *tcdB* genes, which code for the A and B toxins of *C. difficile*, respectively. Twenty-two fecal samples microbiologically positive for *C. difficile* were used. The *tpi* and *tcdA* genes were amplified in all of them. The internal fragment of *tcdB* was detected from 21 of these extracts; the negative sample gave a positive result when a different primer pair was used. None of the 10 DNA extracts obtained from culture-negative samples gave a positive result. The method presented in this article eliminates the interference caused by the possible presence of PCR inhibitors. To the authors' knowledge, this is the first description of a PCR procedure for detection of *C. difficile* DNA from domestic animal feces.

Keywords: *Clostridium difficile*; DNA isolation; feces; molecular diagnostic; pig; toxin

Clostridium difficile is a Gram-positive anaerobic spore-forming bacterium that has emerged recently as a pathogen of humans and animals (Yaeger et al., 2002; Bartlett and Perl, 2005; Songer and Uzal, 2005; Songer and Anderson, 2006; Rupnik, 2007) Infections caused by *C. difficile* not only threaten human health and animal welfare, but also have a great economic impact on health care (Kuijper et al., 2006) and animal production systems (Kiss and Bilkei, 2005; Songer and Uzal, 2005).

Among pigs, the relative importance of *C. difficile* as a cause of neonatal diarrhea is increasing (Yaeger et al., 2002). In fact, some authors have suggested that *C. difficile* may now be the most important uncontrolled cause of neonatal diarrhea in this species (Songer, 2004; Songer and Anderson, 2006). Although *C. difficile*-associated infection affects mainly 1–7-day-old piglets (Songer et al., 2000; Songer and Uzal, 2005), outbreaks in adult animals have also been described (Kiss and Bilkei, 2005).

An important aspect that is discussed frequently is the possible role of animals as a reservoir for *C. difficile* and its possible zoonotic transmission (Borriello et al., 1983; Arroyo et al., 2005; Rodriguez-Palacios et al., 2006; Rupnik, 2007). Related to this, we have recently reported a high prevalence of toxigenic *C. difficile* in 1–7-day-old nondiarrheic, normal appearing piglets (Alvarez-Perez et al., 2009). The presence of *C. difficile* spores in the feces of production animals represents a risk for contamination of meat products (Rodriguez-Palacios et al., 2007). Therefore, there is a need for new procedures for the early detection of *C. difficile* in animal husbandry, aimed at controlling carriers and preventing the entry of this pathogen into the food chain. In such cases, the detection of *C. difficile* in feces by polymerase chain reaction (PCR)

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is an interesting alternative to consider. PCR is a rapid and sensitive method to detect the presence of the bacteria, and then, to classify the animal in suspected and non-suspected ones.

The major advantages of PCR are its rapidity, sensitivity and the possibility of using it to detect unculturable or difficult to culture microorganisms. However, the difficulty to obtain DNA extracts free of PCR inhibitors limits the application of this technique for the analysis of fecal samples (Lou et al., 1997). Nevertheless, in recent years several procedures to detect *C. difficile* in human feces by conventional or real-time PCR have been proposed (Gumerlock et al., 1993; Wolfhagen et al., 1994; Alonso et al., 1999; Guilbault et al., 2002; Belanger et al., 2003; van den Berg et al., 2005, 2006, 2007; Sloan et al., 2008). Different PCR-based techniques have also been applied to the detection of other pathogenic clostridia in animal feces, such as *C. perfringens* (Uzal et al. 1997; Kanakaraj et al., 1998; Fujita and Kageyama, 2007; Gurjar et al., 2008) and *C. botulinum* (Dahlenborg et al., 2001, 2003; Myllykosky et al., 2006). However, there are no reports about the application of such techniques to detect *C. difficile* in fecal samples of animal origin.

The aim of this work was to develop a new semi-automated procedure to recover *C. difficile* DNA from pig feces that would allow subsequent PCR detection of three species-specific sequences: an internal fragment of the triose phosphate isomerase housekeeping gene (*tpi*), the non-repeating portion of the *C. difficile* toxin A gene (*tcdA*) and an internal fragment of the toxin B gene (*tcdB*).

MATERIAL AND METHODS

Samples

Thirty-two fecal samples from different pigs (13 diarrheic and 19 non-diarrheic animals) were selected for this study. Samples were stored at –20°C until they were processed. From 22 of these (10 from diarrheic and 12 from non-diarrheic pigs), *C. difficile* had been isolated previously by the usual procedure of ethanol shock, culture in selective medium (*C. difficile* agar, BioMeriéux, Marcy l'Etoile, France) and anaerobic incubation at 37°C for 48 h (Alvarez-Perez et al., 2009). The remaining 10 fecal samples (three from diarrheic and seven from non-diarrheic pigs), processed following the same procedure, yielded a negative result for *C. difficile* culture (Alvarez-Perez et al., 2009).

DNA extraction

DNA extraction from fecal samples was carried out using the semi-automated system QuickGene-810 (Fujifilm, Tokyo, Japan). All reagents used in this procedure are contained in a commercial kit (QuickGene DNA Tissue Kit S, Fujifilm). Approximately 20 mg of solid feces or 20 µl of diarrheic feces were placed in a 1.5 ml microfuge tube with 180 µl of MDT lysis buffer (Fujifilm) and 20 µl of proteinase K (Fujifilm). After 3 h incubation at 55°C, the lysates were centrifuged at 8 000 g for 3 min to precipitate non-digested remains and fecal debris. The supernatants were transferred to new tubes and 180 µl of LDT buffer (Fujifilm) was added, then the samples were vortexed and incubated at 70°C for 10 min. After the addition of 240 µl of absolute ethanol (Panreac, Barcelona, Spain), the tubes were vortexed and the lysates were transferred into the cartridges provided with the kit. The semi-automated system was prepared for DNA extraction following the manufacturer's instructions, using the recommended quantities of elution (CDT, Fujifilm) and washing (WDT, Fujifilm) buffers. DNA extracts were eluted in a final volume of 50 µl.

A pure culture of a strain of *C. difficile* producing both toxins A and B (A^+B^+) was used as a positive control for DNA extraction. A loopful of this culture was resuspended in a 1.5 ml microfuge tube containing 180 µl of MDT lysis buffer. After the addition of 20 µl of proteinase K, DNA extraction was continued as with fecal samples (see above).

PCR

Two PCR reactions were separately used: one duplex PCR to detect fragments of *tpi* and *tcdB* genes, and one simple PCR to detect *tcdA* gene.

A duplex PCR was performed to detect the species-specific fragments of both *tpi* and *tcdB* genes. The following primers were used (Lemee et al., 2004): tpi-F (5′-AAAGAAGCTACTAAGGGTACAAA-3′) and tpi-R (5′-CATAATATTGGGTCTATTCCTAC-3′) (Isogen Life Science, Maarssen, The Netherlands), to amplify the internal fragment of the *tpi* gene; and tcdB-F (5′-GGAAAAGAGAATGGTTTTATTAA-3′)

and tcdB-R (5′-ATCTTTAGTTATAACTTTGACATCTTT-3′) (Isogen Life Science), for *tcdB* detection. One microlitre of DNA extract was added to each reaction mixture, which consisted of 2.5mM $MgCl_2$, 50mM KCl, 10mM Tris-HCl (pH 8.3), 250μM of each dNTP, 17.5 pmol of primers tpi-F and tpi-R, 35 pmol of primers tcdB-F and tcdB-R and 1.5 IU of AmpliTaq Gold DNA Polymerase (Applied Biosystems, Madrid, Spain) in a final volume of 25 μl. Amplifications were carried out in a GeneAmp PCR System 9700 thermocycler (Applied Biosystems, Foster City, USA) as follows. After an initial denaturation step for 3 min at 95°C, in the first 11 cycles, a touchdown procedure was applied: 30 s of denaturation at 95°C, 30 s of annealing at 65°C, with the temperature for each successive cycle lowered by 1°C, and 30 s of extension at 72°C. Cycling was then continued for a further 34 cycles with an annealing temperature of 55°C. After completion of the cycles, the reaction mixtures were cooled to room temperature.

The detection of the non-repeating portion of the *C. difficile* toxin A gene was achieved by a second PCR, using primers NK3 (5′-GGAAGAAAAGAACTTCTGGCTCACTCAGGT-3′) and NK2 (5′-CCCAATAGAAGATTCAATATTAAGCTT-3′) (Isogen Life Science) (Kato et al., 1998). One microlitre of DNA extract was added to each reaction mixture, which consisted of 2.5mM $MgCl_2$, 50mM KCl, 10mM Tris-HCl (pH 8.3), 250μM of each dNTP, 10 pmol of primer NK2, 10 pmol of primer NK3 and 1.5 IU of AmpliTaq Gold DNA Polymerase (Applied Biosystems). In all cases, the final volume was adjusted to 30 μl. PCR amplifications consisted of a 5-min initial denaturation at 95°C and 45 cycles of 15 s at 95°C, 20 s at 50°C and 40 s at 72°C.

Positive (A^+B^+ *C. difficile* DNA) and negative (sterilized distilled water) controls were included in all reactions. Ten microlitres of each PCR product was electrophoresed on 1.6% agarose gels and stained with ethidium bromide (Sigma-Aldrich, Madrid, Spain). The presence of specific amplicons of 230 bp and 160 bp, for *tpi* and *tcdB* fragments, respectively, or 252 bp for *tcdA*, was assessed under UV illumination.

RESULTS

An example of the results obtained in the two PCR assays used in this work can be seen in Figures 1 and 2.

When DNA extracts from the 22 fecal samples that cultured positive for *C. difficile* were used in the duplex PCR, the *tpi* fragment was amplified in all cases. Moreover, the internal fragment of *tcdB* was also detected from 21 of these extracts. The sample negative for *tcdB* in the duplex PCR gave a positive result when a different primer pair described by Fluit et al. (1991) was used. This might have been caused by a mutation in the priming site of primers tcdB-F or tcdB-R. The alternative PCR assay was as follows: 2 μl of DNA extract was added to a PCR mixture that contained 1.5mM $MgCl_2$, 50mM KCl, 10mM Tris-

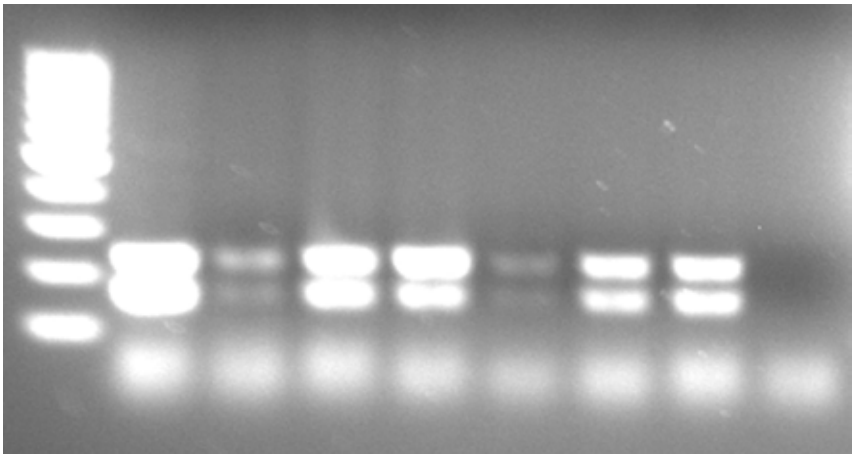

Figure 1. Duplex PCR detection of *C. difficile* triose phosphate isomerase (*tpi*) and toxin B (*tcdB*) gene fragments. Lane 1 = molecular weight marker (GeneRuler 100 bp DNA Ladder, Fermentas Life Science); lane 2 = positive control (DNA isolated from a culture of an A^+B^+ *C. difficile* strain); lanes 3–8 = *C. difficile* DNA recovered from pig faeces by the QuickGene-810 system; lane 9 = negative control. Specific bands corresponding to *tpi* and *tcdB* fragments – 230 bp and 160 bp, respectively – can be seen in lanes 2–8

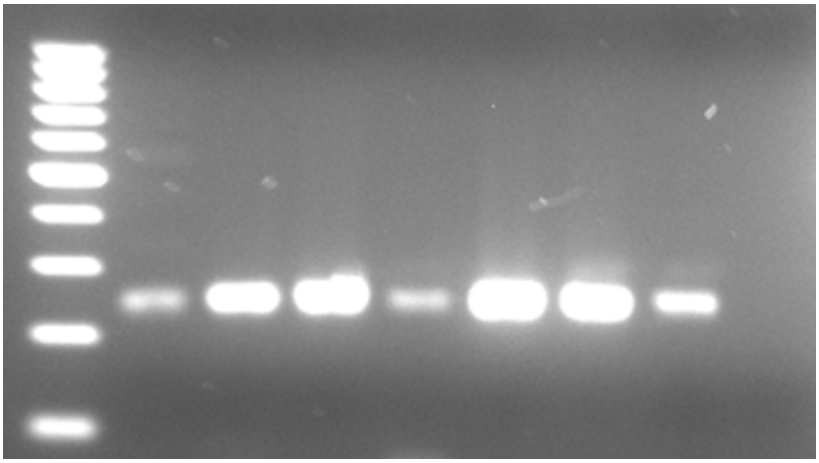

Figure 2. PCR detection of the non-repeating portion of *C. difficile* toxin A gene (*tcdA*). Lane 1 = molecular weight marker (GeneRuler 100 bp DNA Ladder, Fermentas Life Science); lane 2 = positive control (DNA isolated from a culture of an A^+B^+ *C. difficile* strain); lanes 3–8 = *C. difficile* DNA recovered from pig faeces by the QuickGene-810 system; lane 9 = negative control. Specific bands corresponding to the non-repeating portion of the *tcdA* gene (252 bp) can be seen in lanes 2–8

HCl (pH 8.3), 100μM of each dNTP, 50 pmol of each primer (5′-TAATAGAAAACAGTTAGAAA-3′ and 5′-TCCAATCCAAACAAAATGTA-3′) (Isogen Life Science) (Fluit et al., 1991) and 2.5 IU of AmpliTaq Gold DNA Polymerase in a final volume of 50 μl. Amplifications were carried out under the following conditions: 5 min denaturation at 94°C followed by 40 cycles of 1 min at 94°C, 1 min at 50°C and 1 min at 72°C.

All DNA extracts from culture-positive samples yielded a specific band corresponding to the non-repeating portion of the *tcdA* gene in the second PCR cycle. On the contrary, none of the 10 DNA extracts obtained from samples that cultured negative for *C. difficile* gave a positive result for any of the three specific fragments in the two PCR assays, suggesting the absence of both toxigenic and non-toxigenic strains in those samples.

DISCUSSION

In a previous work, we carried out the screening of 780 pig fecal samples for the detection of *C. difficile* by microbiological culturing methods (Alvarez-Perez et al., 2009). Such study is a laborious and time-consuming task, as highly selective media and incubation for a minimum of 48 h under anaerobic conditions is required for *C. difficile* recovery. Moreover, the ethanolic shock performed to select for bacterial spores not only reduces the number of contaminants, but also the number of vegetative forms of *C. difficile* (Aspinall and Hutchinson, 1992). Therefore, new procedures for the early detection of *C. difficile* in animal feces are urgently needed.

Nowadays, different molecular techniques are available for the diagnosis of the most common microbial pathogens of animals. In this article, we present an application of these techniques to the detection of *C. difficile* in pig feces, which consists in a semi-automated procedure for the recovery of bacterial DNA from fecal samples and the subsequent amplification of three species-specific sequences by PCR. As stated above, the results obtained using this procedure were in agreement to those obtained by traditional microbiological culturing.

Toxins A and B are considered the main virulence factors of *C. difficile* (Songer et al., 2000; Keel and Songer, 2006). Although most *C. difficile* strains produce both toxins, some strains produce only toxin B, or no toxins at all (Songer and Uzal, 2005). In this work, fecal samples positive for *C. difficile* by PCR also yielded specific bands for *tcdA* and *tcdB*. Thus the 22 samples positive for *C. difficile* by both culture and PCR harboured toxigenic strains.

DNA isolation methods are usually labour intensive, time-consuming and sensitive to contamination (Widjojoatmodjo et al., 1992; Wang et al., 1996), especially when working with complex clinical samples. Therefore, for routine diagnostics,

simpler methods that are amenable to automation are preferred. This is particularly true when numerous samples have to be analyzed or time is limited. Although other authors have developed automated procedures to isolate *C. difficile* DNA from human feces (van den Berg et al., 2005, 2006, 2007; Sloan et al., 2008), this is the first time that this kind of procedure has been applied on domestic animal feces. The procedure described in this article for the recovery of *C. difficile* DNA from feces is not completely automated, but it reduces considerably the time for which the direct intervention of a technician is required.

It is known that the presence of some substances in feces, such as bilirubin or bile salts, even at low concentrations, can inhibit PCR amplification (Widjojoatmodjo et al.,1992; Lou et al., 1997). To reduce this inhibition, DNA extracts can be diluted, but this is accompanied by a loss in sensitivity proportional to the dilution factor (Hopwood et al., 1996). Furthermore, many procedures used to remove or inactivate PCR inhibitors in fecal specimens are either laborious or inefficient (Lou et al., 1997). However, the semi-automated procedure described in the present study eliminates or decreases the interference caused by such inhibitors, resulting in a high correspondence between PCR results and isolation of *C. difficile* in culture.

In epidemiological studies, it would be very interesting the isolation of the bacterial strains to complete the molecular study. Nevertheless, in herds with a wide number of animals, detailed epidemiological studies are usually not feasible. In these cases, the PCR could be an interesting alternative to consider, not only to identify the bacteria, but also to know the toxigenic characteristics of the isolates.

In conclusion, *C. difficile* detection in fecal samples by PCR appears to be a useful tool for the early diagnosis of infections caused by this microorganism in pigs and the detection of asymptomatic carriers. This information might be of great assistance when studying the prevalence of *C. difficile* in different herds and to implement measures to prevent the possible transmission of this emerging pathogen.

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