Short Paper

Isolation of Clostridium difficile and molecular detection of binary and A/B toxins in faeces of dogs

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Summary

The aim of this study was to isolate Clostridium difficile from dogs’ faeces, and to study the frequency of its virulence genes. A total of 151 samples of dogs’ faeces were collected. The isolation of C. difficile was performed by using the bacterial culture methods followed by DNA extraction using boiling method. Multiplex PCR method was performed for identification of tcdA, tcdB, cdtA and cdtB genes and single method was carried out for detection of tcdC. Twelve samples (7.9%) were positive in bacteriological assay and based on molecular assay, 66.7% of the isolates (8 of 12 C. difficile isolated) had shown tcdA², tcdB² profile. This is the first investigation on molecular assay of C. difficile in Iran’s dog population.

Key words: Clostridium difficile, Dog, Molecular detection

Introduction

Clostridium difficile is a Gram-positive spore-forming anaerobic bacillus which has been identified as a main bacterial pathogen in both human and animals’ intestine. It is a common cause of enteritis in a variety of animal species (Doosti and Mokhtari-Farsani, 2014). In addition, some reports have recently raised the importance of wild animals as a reservoir of C. difficile for humans and domestic animals.

A number of bacterial organisms commonly associated with diarrhea in dogs and cats include Salmonella, Campylobacter, Clostridium perfringens and C. difficile (Marks et al., 2011).

Two large clostridial toxins A and B (TcdA and TcdB) were among the main virulence factors. TcdA and TcdB are strong cytotoxic enzymes damaging the human colonic mucosa (Deneve et al., 2009).

TcdA and TcdB in the pathogenicity locus are controlled by two regulators, TcdR and TcdC. TcdR is an alternative sigma factor which positively regulates transcription of tcdA and tcdB while TcdC may function as an anti-sigma factor impeding the activity of TcdR, although some researches have reported that TcdC does not influence toxin production (McKee et al., 2013).

The C. difficile ADP-ribosyltransferase was a binary toxin consisting of two independently coded protein components: a binding component (CDTb) and an enzymatic component (CDTa) which catalyzes the ADP-ribosylation of monomeric action, inducing alterations in the cytoskeleton (Marks and Kather, 2003).

In dogs, pathogenicity and the importance of C. difficile is not fully understood as yet. Clinical signs that have been associated with canine C. difficile infection range from asymptomatic carrier to a potentially fatal acute hemorrhagic diarrheal syndrome (Marks et al., 2011).

A simple and quick method is required in order to distinguish toxigenic and non-toxigenic strains of C. difficile in dogs. The objective of the current study was to investigate the molecular characteristics of various isolates of C. difficile isolated from diarrheic and non-diarrheic dogs, through the use of toxin gene profiling.

Materials and Methods

A total of 151 faecal samples was collected from 151 dogs, 131 of which were apparently healthy and 20 were diarrheic. The samples from diarrheic dogs were obtained directly from the rectum, in a veterinary teaching clinic at the time of consultation and were only collected from dogs in which the main motivation for the consultation was the occurrence of diarrhea. There were 60 male and 91 female dogs, aged from 3 months to 11 years. Isolation and identification of C. difficile were performed according to standard procedures (Fedorko et al., 1997; Pituch et al., 2002). Two or three passages were done to obtain the pure culture. Reference strains of C. difficile were used as positive controls.

A single colony of each strain was suspended in 100 μL distilled water, boiled for 10 min and then centrifuged at 10,000 x g for 10 min. The supernatants were collected carefully and used as template DNA for PCR (Aldous et al., 2005).

A 5-plex PCR was performed for the detection of
Table 1: Multiplex PCR primer and single PCR primer in this study

| Analysis   | Gene target | Primer name       | Primer concentration (µM) | Amplicon size (bp) | Reference |
|------------|-------------|-------------------|---------------------------|--------------------|-----------|
| 5-plex PCR | tcdA        | tcdA-F3345         | 0.6                       | 629                | Persson et al. (2008) |
|            | tcdA-R3969  |                   |                           |                    |           |
|            | tcdB        | tcdB-F5670         | 0.4                       | 410                | Persson et al. (2008) |
|            | tcdB-R6079A |                   | 0.2                       |                    |           |
|            | cdtA        | cdtA-F739A         | 0.05                      | 221                |           |
|            | cdtA-R958   |                   | 0.1                       |                    |           |
|            | cdtB        | cdtB-F617          | 0.1                       | 262                |           |
|            | cdtB-R878   |                   |                           |                    |           |
|            | 16S rDNA    | PS13               | 0.05                      | 1062               |           |
|            |             | PS14               | 0.05                      |                    |           |
| tcdC analysis | tcdC     | tcdC-121-F         | 0.15                      | 139 (intact)       | Antikainen et al. (2009) |
|            | tcdC-121-R  |                   | 0.15                      |                    |           |

Table 2: Identification of *C. difficile* isolated form canine faecal samples complemented with other data

| Dogs         | 16S RND tcdA* | tcdB* | tcdC* (139 bp) | 16S RND tcdA* | tcdB* | tcdC (58 bp), tcdAB* | 16S RND tcdA* | tcdB* | tcdC (85 bp) | 16S RND tcdA* | tcdB* | tcdC |
|--------------|---------------|-------|----------------|---------------|-------|---------------------|---------------|-------|---------------|---------------|-------|------|
| Diarrheic    | 4/4 (100%)    |       |                | 4/4 (100%)    |       |                     | 4/4 (100%)    |       |               | 4/4 (100%)    |       |
|              | 4/20 (20%)    |       |                |               |       |                     |               |       |               | 4/20 (20%)    |       |
| Non-diarrheic| 3/8 (37.5%)   | 1/8   | (12.5%)        | 2/8 (25%)     | 1/2   | (16.7%)             | 2/12 (16.7%)  |       | 12/151 (7.9%) |               |       |
|              | 3/131 (2.2%)  | 1/131 | (0.8%)         | 2/211 (1.5%)  | 1/2   | (16.7%)             | 2/151 (1.3%)  |       | 12/151 (7.9%) |               |       |
| Total        | 7/12 (58.3%)  | 1/12  | (8.3%)         | 2/212 (16.7%) | 1/2   | (16.7%)             | 2/151 (1.3%)  |       | 12/151 (7.9%) |               |       |
|              | 7/151 (4.6%)  | 1/151 | (0.7%)         |               |       |                     |               |       |               |               |       |
| Female       | 6/10 (60%)    |       |                | 2/10 (20%)    |       |                     | 2/10 (20%)    |       | 10/10 (100%)  |               |       |
|              | 6/691 (6.6%)  |       |                | 2/91 (2.2%)   |       |                     | 2/91 (2.2%)   |       | 10/91 (11%)   |               |       |
| Male         | 1/2 (50%)     | 1/2   | (50%)          |               |       |                     |               |       |               | 2/2 (100%)    |       |
|              | 1/60 (1.7%)   | 1/60  | (1.7%)         |               |       |                     | 2/60 (3.3%)   |       |               |               |       |
| Total        | 7/12 (58.3%)  | 1/12  | (8.3%)         | 2/12 (16.7%)  |       |                     | 2/12 (16.7%)  |       | 12/12 (100%)  |               |       |
|              | 7/151 (4.6%)  | 1/151 | (0.7%)         | 2/12 (16.7%)  |       |                     | 2/151 (1.3%)  |       | 12/151 (7.9%) |               |       |
| Age ≤3 years | 7/70 (57.1%)  |       |                | 1/1 (0.1%)    |       |                     | 1/1 (0.1%)    |       | 10/10 (100%)  |               |       |
|              | 7/121 (5.8%)  |       |                |               |       |                     | 7/121 (5.8%)  |       |               |               |       |
| Age ≥3 years | 7/12 (58.3%)  | 1/2   | (50%)          |               |       |                     |               |       | 2/2 (100%)    |               |       |
|              | 1/30 (3.3%)   |       |                | 1/30 (3.3%)   |       |                     | 1/30 (3.3%)   |       | 2/30 (6.7%)   |               |       |
| Total        | 7/12 (58.3%)  | 1/12  | (8.3%)         | 2/12 (16.7%)  |       |                     | 2/12 (16.7%)  |       | 12/12 (100%)  |               |       |
|              | 7/151 (4.6%)  | 1/151 | (0.7%)         | 2/151 (1.3%)  |       |                     | 2/151 (1.3%)  |       | 12/151 (7.9%) |               |       |
Discussion

Clostridium difficile has been isolated from almost all mammals (Dabard et al., 1979; Frazier et al., 1993; Hasanzade et al., 2013).

In Iran several studies have been performed for detection of C. difficile (Jalali et al., 2012; Fooladi et al., 2014; Rahimi et al., 2014) but this study is the first investigation on C. difficile isolated from dog population in Iran. Many researches on animals concentrate on the presence of the bacterium in healthy animals. Investigation on the role of household pets as a possible reservoir of C. difficile revealed that both healthy and diseased dogs and cats can shed spores of C. difficile (Riley et al., 1991).

Colonization of humans by C. difficile can produce enteric symptoms termed CDI, ranging from asymptomatic intestinal colonization to diarrhea. The most common transmission routes of C. difficile include: direct transmission from human to human, direct contact with the animals and environment, aerosol transmission and consuming contaminated food and water (Ghose, 2013).

There have been several studies worldwide aimed at isolating and molecular typing C. difficile in dogs (Marks et al., 2002; Kevin et al., 2007; Clooten et al., 2008; Koene et al., 2011; Ossiprandi et al., 2012; Silva et al., 2013).

In this study, 12 isolates of C. difficile were isolated from 151 dogs (7.9%) in which the number of dogs being C. difficile positive was lower than other recent reports (O’Neill et al., 1993; Marks et al., 2002; Kevin et al., 2007), however two other studies have reported a lower rate of C. difficile in comparison with the current study (Weese et al., 2001; Wetterwik et al., 2013) 2%, 5.7% positive samples, respectively.

In this study, 8 of the 12 isolates (66.6%) were toxigenic (tcdA⁺, tcdB⁺). Our research was similar to that of Ossiprandi et al. (2012). The last research signifies that 60% of the isolates were toxigenic (tcdA⁺, tcdB⁺). Clooten et al. (2008) found that 69% of the isolates were toxigenic (tcdA⁺, tcdB⁺).

In the current study, one isolate possessed binary toxin gene which was A⁺B⁻ and was derived from non-diarrheic dog. Silva et al. (2013) found one strain with this characteristic.

In this study, we observed the fact that 4 isolates showed tcdA⁺, tcdB⁺ and tcdC⁻ patterns, while the subjects have shown clinical signs of diarrhea which might be due to the variability of tcdC alleles among toxigenic isolates. There have been reports indicating C. difficile isolates being characterized by a non-specific in-frame 18 bp deletion and a specific point deletion at position 117, which results in a frame-shift mutation introducing a stop codon at position 196. This phenomenon leads to a truncated, inactive TcdC protein, the severe truncation of this protein, therefore, seems responsible for the increased toxin production in these pathogenic C. difficile isolates which would usually negatively regulate toxin production (Deneve et al., 2009).

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Conflict of interest

The authors declare no potential conflicts of interest.

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