

# Prevalence of *Clostridium difficile* toxin genes in Pretoria

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The hypervirulent polymerase chain reaction (PCR) ribotype 027 strain of *Clostridium difficile* produces toxins A, B and a binary toxin. Toxin detection kits are commonly used in diagnostic laboratories, but have been unsuccessful in detecting all of the relevant *C. difficile* strains, and the toxins produced. In this study, conventional PCR was used to detect the presence of the genes of toxin A, toxin B and the binary toxin of *C. difficile*. Eighty-four frozen (collected between 2006-2007) and 13 fresh (collected in 2010) stool specimens, obtained in Pretoria, were analysed. The genes for toxin A, toxin B and the binary toxin were detected in one of the fresh stool specimens. This may have implications for healthcare facilities, and suggests the possible emergence of the highly virulent PCR ribotype 027 strain of *C. difficile* in Pretoria. This emphasises the importance of continuous surveillance and monitoring of *C. difficile* outbreaks.

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## Introduction

*Clostridium difficile* is a Gram-positive, spore-forming, anaerobic bacillus, and has frequently been implicated in nosocomial infections.<sup>1,2</sup> *C. difficile* is a major cause of antibiotic-associated diarrhoea and pseudomembranous colitis.<sup>3,4</sup> The treatment, prevention and control of *C. difficile* infections (CDIs) have become increasingly difficult over the last few decades, due to the widespread use of broad-spectrum antibiotics.<sup>5</sup> Therefore, the rapid and accurate diagnosis of CDIs has become crucial in providing proper treatment to patients, and in implementing infection-control principles.<sup>2,5</sup> A highly pathogenic strain of *C. difficile*, polymerase chain reaction (PCR) ribotype 027 has caused numerous outbreaks in North America, Europe and Japan over the last five years,<sup>6</sup> but the prevalence of this strain in South Africa remains unknown.<sup>7</sup> The hypervirulent PCR ribotype 027 strain of *C. difficile* produces three toxins, namely toxin A, toxin B and a binary toxin.<sup>2</sup> Toxin A (Tcd A) and toxin B (Tcd B) express cytopathic and cytotoxic activities in cultured cells in vitro, while the binary toxin may facilitate entry for toxins A and B, and has been implicated in more severe disease.<sup>7,8</sup> Diagnosis of CDIs depends on the detection of toxins by antibody-based cytotoxicity and molecular assays.<sup>9-11</sup> Since most South African diagnostic laboratories use kits that detect only toxin A of *C. difficile*, it is suspected that some cases of CDIs are misdiagnosed. In this study, a conventional multiplex PCR assay was used to determine the prevalence of toxin A, toxin B, and the binary

toxin genes of *C. difficile*, in fresh and frozen stool specimens collected from patients presenting with diarrhoea. These specimens were collected from an academic hospital and private pathology laboratories in Pretoria.

## Method

### Collection of stool specimens

A total of 97 stool specimens from patients suspected of having CDI, and submitted for the detection of *C. difficile*, were analysed in this study. Informed consent was obtained from the patients or their parents or guardians, and human experimentation guidelines (of the National Department of Health, of the Faculty of Health Sciences of the University of Pretoria, and those of the National Health Laboratory Service) were followed when conducting the clinical research. The stool specimens consisted of 84 frozen specimens collected from the Steve Biko Academic Hospital in Pretoria between 2006-2007, and 13 fresh specimens collected from private pathology laboratories in Pretoria in 2010. Specimens were analysed after routine diagnostic tests had been performed by the respective diagnostic and private pathology laboratories.

### DNA from fresh and frozen stool specimens

The deoxyribonucleic acid (DNA) from the fresh stool specimens (n = 13) was extracted using the Zymo Research

Faecal DNA Extraction Kit (Zymo Research, USA) according to the manufacturer's instructions. The QIAamp DNA stool Mini Kit (QIAGEN, USA) was used to extract the DNA of *C. difficile* from the frozen stool specimens ( $n = 84$ ) according to the manufacturer's instructions. The DNA extraction protocol for larger volumes (QIAamp DNA stool Mini Kit, QIAGEN, USA) was modified to use 500 mg of stool specimen, instead of 1 g, since there was not a sufficient volume of each of the 84 frozen stool specimens. Different kits were used for the fresh and frozen stool specimens, due to the unavailability of the QIAamp DNA stool Mini Kit. The two kits were found to be comparable in their ease of use and efficiency. The DNA was stored at  $-20^{\circ}\text{C}$  until required.

#### Detection of toxin A, toxin B and the binary toxin genes using a conventional multiplex PCR assay

Amplification of fragments of the toxin A (*tcdA*), toxin B (*tcdB*) and the binary toxin genes (*cdtA* and *cdtB*) was performed using the DNA extracted from the stool specimens. A multiplex conventional PCR assay, based on the assay described by Stubbs et al,<sup>12</sup> using each primer pair for the detection of the toxin A (*tcdA*), toxin B (*tcdB*) and the binary toxin (which required the presence of both *cdtA* and *cdtB*) genes was performed. The PCR reaction was performed in an Eppendorf thermal cycler, under the conditions described by Stubbs et al,<sup>12</sup> without modifications.

#### Detection of PCR amplification products

The amplicons of the PCR reactions were visualised using an ultraviolet light box following 1.5 hours of electrophoresis on a 1.5% (m/v) agarose gel, which contained 5  $\mu\text{l}$  of a 10 mg/ml ethidium bromide stock solution. A molecular weight marker of a 100 bp GeneRuler DNA ladder was included as a reference for each gel. Expected band sizes were 158 bp for *tcdA*, 101 bp for *tcdB*, 327 bp for *cdtA* and 451 bp for *cdtB*.

#### Activation of *C. difficile* spores

Two methods for spore activation, namely an alcohol shock method and a heat treatment method, were evaluated in this study. In the alcohol shock method, 0.5 ml of each stool specimen was treated with 0.5 ml of 96% ethanol.<sup>13</sup> The solutions were incubated at  $25^{\circ}\text{C}$  for one hour.<sup>13</sup> In the heat treatment method, 200  $\mu\text{l}$  of sterile deionised water was added to 200  $\mu\text{l}$  of stool, and the solution was incubated in a heating block at  $80^{\circ}\text{C}$  for 15 minutes.<sup>14</sup> Twenty microlitres of each solution, from both the alcohol shock and the heat treatment method, was plated onto Brazier's cefoxitin-cycloserine egg yolk (CCEY) agar bases.<sup>13</sup> These agar bases were supplemented with 2% lysed horse blood and 5 mg/ml lysozyme, from a stock solution of 50 mg/ml.<sup>13</sup> Plates were inverted and incubated in an airtight container containing an anaerocult, at  $37^{\circ}\text{C}$  for 48 hours.<sup>13</sup> Gram staining was performed, according to the method of Ryan and Ray.<sup>15</sup>

#### Extraction of DNA from culture-positive isolates of *C. difficile* using the phenol-chloroform method

Agar plates that showed colonies were selected for DNA extraction. DNA was extracted using a standard phenol-chloroform DNA extraction method, as described by Sambrook and Russel.<sup>16</sup>

#### Confirmation of the presence of *tcdA*, *tcdB*, *cdtA* and *cdtB* genes in culture-positive specimens using a conventional multiplex PCR assay

The same conventional multiplex PCR assay (as mentioned previously) was used to amplify the fragments of the suspected *tcdA*, *tcdB*, *cdtA* and *cdtB* genes from the resuscitated *C. difficile* isolates. PCR amplicons were detected by gel electrophoresis.

## Results

Routine diagnostic tests, using the Oxoid toxin A detection kit for *C. difficile*, showed the presence of toxin A in 29.09% (29/97) of the stool specimens (Table I). This detection kit could not detect toxin B or the binary toxins in any of the stool specimens. As shown in Table I, during the first multiplex PCR, a total of 34.02% (33/97) of the isolates tested positive for the *tcdA* gene, and 12.37% (12/97) of the isolates tested positive for the *tcdB* gene. A total of 10.3% (10/97) of the isolates tested positive for both the *tcdA* and *tcdB* genes. The *cdtA* and *cdtB* genes were detected in 2.06% (2/97) of the isolates. These isolates were both fresh stool specimens. One of the fresh stool specimens (7.69%; 1/13); tested positive for the toxin A, toxin B, and the binary toxin genes. The binary toxin genes could not be detected in any of the frozen stool specimens (Table I).

An example of the obtained results in the multiplex PCR assay can be seen in Figure 1. On the agarose gel, the presence of a 158 bp band was indicative of the *tcdA* gene, while the *tcdB* gene gave a 101 bp band. The presence of a 327 bp band was indicative of the *cdtA* gene, while the *cdtB* gene gave a 451 bp band (Figure 1).

A total of 40 (41.2%) of the stool specimens were selected for spore activation and culture. Twenty-seven of these stool specimens were frozen, and 13 were fresh. After spore activation and culture, two of the cultures (original frozen stool specimens) showed mixed colonies, and were discarded. Both of these specimens tested positive for the *tcdA* gene during the first PCR analysis.

The results for the remaining 38 stool specimens were as follows. After the alcohol shock pre-treatment method, growth was observed for 44.7% (17/38) of the stool specimens, and consisted of nine fresh and eight frozen specimens. After the heat treatment method, growth was observed in 23.6% (9/38) of the specimens, all fresh specimens. The alcohol shock pre-treatment method showed the growth of 32% (8/25) of the

Table I: Presence of the *tcdA*, *tcdB*, *cdtA* and *cdtB* genes in fresh and frozen stool specimens, before and after spore activation

	Total positive stool specimens using the Oxoid toxin A detection kit (n = 97)	Total positive stool specimens using multiplex PCR (n = 97)	Positive frozen stool specimens (n = 25*) before spore activation	Positive frozen stool specimens (n = 8**) after spore activation	Positive fresh stool specimens (n = 13) before spore activation	Positive fresh stool specimens (n = 9***) after spore activation
<i>tcdA</i>	29.09% (29/97)	34.02% (33/97)	72% (18/25)	37.5% (3/8)	100% (13/13)	100% (9/9)
<i>tcdB</i>	0	12.37% (12/97)	4% (1/25)	12.5% (1/8)	0	0
<i>tcdA</i> and <i>tcdB</i>	0	10.30% (10/97)	8% (2/25)	25% (2/8)	23.07 (3/13)	22.2% (2/9)
<i>cdtA</i> and <i>cdtB</i>	0	2.06% (2/97)	0	0	15.38% (2/13)	22.2% (2/9)
<i>tcdA</i> , <i>tcdB</i> , <i>cdtA</i> and <i>cdtB</i>	0	1.03% (1/97)	0	0	7.69% (1/13)	11.1% (1/9)

\* Fifty-seven of the frozen stool specimens were not subjected to spore activation, and two of the frozen stool specimen cultures were discarded due to contamination

\*\* Spore activation and culture was only successful in 8/25 frozen stool specimens

\*\*\* Spore activation and culture was only successful in 9/13 fresh stool specimens



Lane 1: 50 bp marker  
 Lane 2: Negative control  
 Lane 3: Blank  
 Lane 4: *tcdA* of specimen 80  
 Lane 5: *tcdB* of specimen 62  
 Lane 6: *tcdB* of specimen 81  
 Lane 7: *tcdA* and *tcdB* of specimen 46  
 Lane 8: *tcdA* and *tcdB* of specimen 96  
 Lane 9 and 10: *cdtA*, *cdtB*, *tcdA* and *tcdB* of specimen 79  
 Lane 11 and 12: *cdtA*, *cdtB* and *tcdA* of specimen 90  
 Lane 13: 50 bp marker

Figure 1: Gel indicating the presence of the *tcdA* (158 bp), *tcdB* (101 bp), *cdtA* (327 bp) and *cdtB* (451 bp) genes of *C. difficile* in stool specimens after multiplex PCR

frozen stool specimens. In 30.7% (4/13) of the fresh, and in 68% (17/25) of the frozen, specimens, spore activation was not successful using any of the two methods. Three of the four fresh stool specimens that did not grow after pre-treatment with both methods, were positive for *tcdA*, and the fourth specimen was positive for both *tcdA* and *tcdB*.

After performing a standard phenol-chloroform DNA extraction protocol<sup>16</sup> and PCR analysis, the remaining nine fresh stool specimens all tested positive for the same toxins that had been detected before during the second round of multiplex PCR. The frozen stool specimens also tested positive for the same toxins. However, the *tcdA* gene was only detected in 37.5% (3/8) of the frozen stool specimens after activation (Table I).

## Discussion

*C. difficile* is a major cause of antibiotic-associated diarrhoea

and pseudomembranous colitis.<sup>17</sup> The correct treatment of CDI is crucial in preventing the progression of the disease, and the rapid and accurate detection of *C. difficile* is of the utmost importance in helping clinicians make decisions regarding the prescription of suitable antibiotics.<sup>18</sup> Although a number of diagnostic tests are available for the detection of *C. difficile*, most are time-consuming, lack sensitivity and do not detect all of the toxins associated with *C. difficile* and CDI.<sup>19</sup> The most important advantage of using PCR as a diagnostic tool is that these assays are rapid, with a high sensitivity and specificity. The aim of this study was to determine the prevalence of toxin A (*tcdA*), toxin B (*tcdB*) and the binary toxin genes (*cdtA* and *cdtB*), of *C. difficile*, in stool specimens collected from patients suspected of having CDI, using conventional multiplex PCR.

At the time of the specimen collection, the diagnostic and pathology laboratories in Pretoria used the Oxoid toxin A detection kit for CDI diagnosis. This kit detected toxin A in 29.09% of the stool specimens, which is lower than the 34.02% detected by the multiplex PCR in this study. The Oxoid toxin A detection kit was also not able to detect the toxin B and binary toxin genes of *C. difficile*, which is a cause for concern, since this suggests that cases of CDI, in which toxin A is not present, are misdiagnosed. Misdiagnosis of CDI may lead to problems such as incorrect treatment, ineffective outbreak control, and infection mismanagement. Furthermore, recently occurring outbreaks of CDIs in the United States, the United Kingdom, Europe, and Japan, have been associated with *C. difficile* PCR ribotype 027 strains, which are antibiotic-resistant, hypervirulent, and do not always produce toxin A.<sup>6</sup>

A study conducted by Samie et al in the Vhembe district of South Africa found the toxin A gene in 40% of the stool specimens, which is higher than the 34.02% found for toxin A in this study. The study conducted in the Vhembe district detected the toxin B gene in 46.7% of the specimens, and both the toxin A and B genes in 30.8% of stool specimens.<sup>7</sup> The four genes for the toxin A, toxin B, and the binary toxin were detected in 20% of the specimens.<sup>7</sup> These findings were also higher than the results obtained in our study,

which was 12.37% for the toxin B gene, 10.3% for the genes for toxins A and B, and 1.03% for the genes of all three of the toxins. The difference in toxin percentages between the study conducted by Samie et al,<sup>7</sup> and the current study, may be due to the difference in the samples investigated (97 *C. difficile* suspected stool specimens in our study vs. 45 confirmed *C. difficile* stools in the study by Samie et al). Additionally, the Vhembe district is mostly rural, with people living in close proximity to animals, including cattle, horses and pigs.<sup>7</sup> This may imply that people in this area, who are suffering from CDI, may have acquired zoonotic strains of *C. difficile*, which may have different toxinotypes compared to those in our study. Further studies on this matter would be beneficial.

The results for the prevalence of binary toxin genes in our study (15.4%) are comparable to those reported in a 2003 study conducted by Geric et al in the United States, where the prevalence was 15.5%.<sup>20</sup> Since the binary toxin was not detected in any of the frozen stool specimens, this might suggest that binary toxin-producing strains of *C. difficile* were not yet present in Pretoria at the time when the frozen stool specimens were collected (2006-2007). The highest prevalence of binary toxin genes recorded to date was in Hungary in 2004, in which occurrence of these genes in *C. difficile* isolates was 23.5%.<sup>21</sup>

To our knowledge, this is the first report on the prevalence of binary toxin-producing *C. difficile* strains in Pretoria. The presence of the binary toxin genes, together with the toxin A and toxin B genes, in one of the fresh stool specimens obtained in 2010 is alarming, since this indicates that these highly virulent *C. difficile* strains might be emerging and circulating in the Pretoria region. Further confirmation of the *C. difficile* PCR ribotype 027 strain using PCR ribotyping is required, but does not form part of the current study.

In this study, two spore activation methods were compared to determine which method provided the best results. These were an alcohol shock method and a heat treatment method. The alcohol shock pre-treatment method seemed to be superior to the heat treatment method for spore activation, since the heat treatment method was not able to activate the spores in any of the frozen stool specimens. After spore activation, and a second round of multiplex PCR, similar results were found for all fresh stool specimens as those recorded for the first multiplex PCR (Table I). Results differed slightly for frozen stool specimens, since spore activation was not successful in the majority of these specimens. The frozen stool specimens which were successfully reactivated, tested positive for the same toxin genes as before, indicating that the multiplex conventional PCR is reliable and reproducible, and abolishes the need for spore activation and culture. Furthermore, the multiplex PCR assay was able to detect all of the genes associated with *C. difficile* toxins. Therefore, it eliminates the problem of misdiagnosis which occurs when using conventional toxin detection kits, such as the Oxoid toxin A detection kit.

## Conclusion

The conventional PCR assay was successfully performed without any optimisation. Results showed that the *tcdA*, *tcdB*, *cdtA* and *cdtB* genes are prevalent in the *C. difficile* strains that are currently circulating in the Pretoria region. Based on the results, molecular-based methods can be used to provide rapid and accurate results that can be used for diagnostic purposes. Conventional PCR is a valuable tool in detecting all of the toxin genes of *C. difficile* in stool specimens, and shows promise in overcoming the issue of CDI misdiagnosis, which may occur when using toxin detection kits. Toxin A-producing *C. difficile* strains were the most prevalent in this study, but may have the potential to acquire binary toxins, which may lead to severe disease.

The role of the binary toxin in CDI pathogenesis is unclear, and further research on this topic is necessary. One limitation of this study was that specimens that were positive for the binary toxin were not subjected to PCR ribotyping, in order to confirm the relatedness of these strains to the highly pathogenic *C. difficile* PCR ribotype 027 strain. Since it is possible that the *C. difficile* strains detected in this study might be *C. difficile* PCR ribotype 027 strains, clinicians and healthcare personnel should take care to follow proper infection control measures, to prevent the spread of these virulent strains in the nosocomial environment.

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## Declaration

None of the authors have a commercial, or other, association which may pose a conflict of interest with this study.

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