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# Isolation and Toxin Gene Profiling of *Clostridium difficile* from Environmental Samples in Andhra Pradesh, India

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## ABSTRACT

This study investigates the presence of *Clostridium difficile* (*C. difficile*) in environmental samples, specifically farm soil (25) and farm water samples (25). A total of 50 samples were collected from nearby poultry (2), sheep farm regions (2) and pig rearing areas (2) and analyzed with cultural tests, identifying that eight isolates (16.0%) were positive for *C. difficile*. PCR testing revealed a lower detection rate, with 2 isolates (4.0%) positive for the tpi (species-specific) gene. Further analysis of isolates for the presence of virulent genes revealed the presence of toxin A and toxin B and binary toxin (cdtA and cdtB). These findings underscore the potential environmental reservoirs of *C. difficile*, highlighting the need for further research to delve into their ecological impact and implications for public health. The presence of *C. difficile* in farm soil and water samples suggests that this pathogen may be disseminated through these environmental sources. However, data on *C. difficile* in environmental samples is limited. Therefore, comprehensive research is necessary to investigate the prevalence of *C. difficile* in food animals and on various environmental surfaces.

**Keywords**: *C. difficile*, CDI, Toxin gene, Profiling

## INTRODUCTION

*Clostridium difficile* (*C. difficile*) is commonly found in the environment, and it persists even on non-living surfaces for a period of several months (Aseeri *et al*., 2008), encompassing the bodies of farm animals and pets, surfaces within hospitals, and foods, besides water and soil, as free-living bacteria. It is primarily linked to infections such as diarrhoea and pseudomembranous colitis. There is a significant increase in community-associated *C. difficile* infections (CDI), though the specific sources of them are not well understood (Wilcox *et al.*, 2008 and Bauer *et al.*, 2014). The characteristic symptoms of CDI are largely due to toxins produced by the bacteria. Most pathogenic strains generate two primary toxins: toxin A, which acts as an enterotoxin, and toxin B, a cytotoxin. These toxins are encoded by the tcdA and tcdB genes, respectively (Voth *et al*., 2005). Additionally, some strains produce a binary toxin known as *C. difficile* transferase (CDT) (Stubbs *et al*., 2000), which is encoded by the cdtA and cdtB genes. While the exact impact of CDT on disease severity is still not fully understood, strains that produce this toxin have been linked to severity in humans and are identified in various animals too (Rupnik *et al*., 2007).

Although there are reports from hospitals in India detailing cases of CDI, there is a significant gap in knowledge on the molecular characteristics of *C. difficile* strains. There is no proper study about the presence of *C. difficile* in environmental samples linked to animal surfaces. More extensive research is needed to understand the transmission patterns, isolation, and possible reservoirs of this pathogen, which could greatly improve our approach in managing CDI (Songer *et al*., 2006 and Hammit *et al*., 2008).

## MATERIALS AND METHODS

The reference strain of *C. difficile* (ATCC 9689) was procured from the American Type Culture Collection, US. A total of 50 environmental samples were collected,

including farm water (50) and soil (Manure) samples (50) near livestock areas such as poultry farms (2), pig farms (2) and sheep farms (2) were collected in sterile vials from two districts, Krishna and Prakasam, in Andhra Pradesh.

The procedure described by Hussain *et al*., (2016) was adopted for isolation and identification of *C. difficile*. Selective enrichment of samples was done in thioglycolate broth at 37$^0$C for 7–10 days under anaerobic conditions. Then the broth culture was treated with absolute alcohol for 45 minutes and centrifuged at 8000 rpm for 15 minutes. The supernatant was removed, and the sediment was inoculated into Cycloserine and Cefoxitin Fructose Agar (CCFA) plates (*C. difficile* agar with CC supplement) [HiMedia Laboratories (Mumbai)] supplemented with 5% sheep blood and incubated at 37°C for 48–72 hours. Colonies showed typical colour (off white coloured colonies) were confirmed by Gram staining, and the results revealed gram-positive rods. Additionally, the motility test indicated that the motility of the bacterium and the non-haemolytic activity of the organism on blood agar, while both the oxidase and catalase tests yielded negative results.

The identification of *C. difficile* in enrichment broth samples was accomplished through species-specific PCR by Lemee *et al*. (2004) and Zheng *et al*. (2007). The primers utilized for targeting the *C. difficile* species–specific gene were tpi-F(5'AAAGAAGCTACTAAGGGTACAAA-3') and tpi-R(5’-CATAATATTGGGTCTATTCCTAC-3'). The targeted gene's expected amplicon size was 230bp. To optimize PCR amplification, a 25µl reaction mixture was prepared. This mixture included 2.0µl of DNA template, 2.5µl of 10 × Taq buffer, 1.0µl of 10 mM dNTP mix, 0.5µl of 25 mM MgCl2, 1.5µl each of forward and reverse primers (10 pmol/µl), 1.0µl of Taq DNA polymerase (1 U/µl), and 15.0 µl of nuclease-free water. The PCR reaction was conducted under standardized cycling conditions.

The PCR assays for the tcdA and tcdB genes were conducted in a 25µl reaction volume, which included 2µl of DNA template, 2.5µl of 10× Taq buffer, 1µl of 10 mM dNTP mix, 0.75µl of 25 mM MgCl2, 1µl each of forward and reverse primers (10 pmol/µl), 1µl of Taq DNA polymerase (1 U/µl), and 8.5µl of nuclease-free water. These components were used under standardized cycling conditions. The PCR assays for the tcdA and tcdB genes were conducted in a 25µl reaction volume, which included 2µl of DNA template, 2.5 µl of 10× Taq buffer, 1µl of 10 mM dNTP mix, 0.75µl of 25 mM MgCl2, 1µl each of forward and reverse primers (10 pmol/µl), 1µl of Taq DNA polymerase (1 U/µl), and 8.5 µl of nuclease-free water. These components were used under standardized cycling conditions.

## RESULTS AND DISCUSSION

### Molecular detection of *C. difficile* by species-specific PCR

PCR testing revealed (Table below) that 2 isolates (4.0%) positive for the tpi gene, confirming *C. difficile*. Further isolates were examined for the presence of virulent genes such as toxin A, toxin B, and Binary toxin by PCR testing with genes (tcdA and tcdB) and binary toxin (cdtA and cdtB) revealed two isolates positive for both tcdA, tcdB and cdtA and cdtB.

| Source | No. of samples analysed | No .of isolates positive for *C. difficile* by cultural tests (%) | No. of isolates positive for *C. difficile* by PCRtpi gene (%) |
|---|---|---|---|
| **Environmental samples** | | | |
| Soil samples | 25 | 4 (16.0%) | 1(4.0%) |
| Farm and Water samples | 25 | 4 (16.0%) | 1(4.0%) |
| Total | 50 | 8 (16.0%) | 2 (4.0%) |

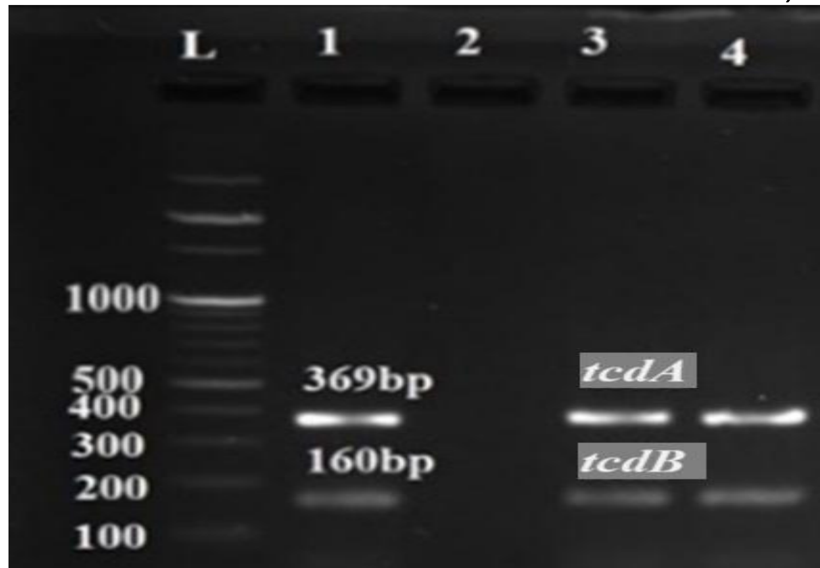


***Figure 1: Gel photograph of C. difficile species-specific PCR (tpi gene)***

*Lane L: DNA ladder (100bp); Lane 1: Positive control of C. difficile (C. difficile ATCC 9689) showing presence of tpi gene (230bp); Lane 2: Negative control (E. coli0157:H7 ATCC 43888); Lane 3: C. difficile isolate carrying tpi gene from farm water sample; Lane 4: C. difficile isolate carrying tpi gene from farm soil sample*

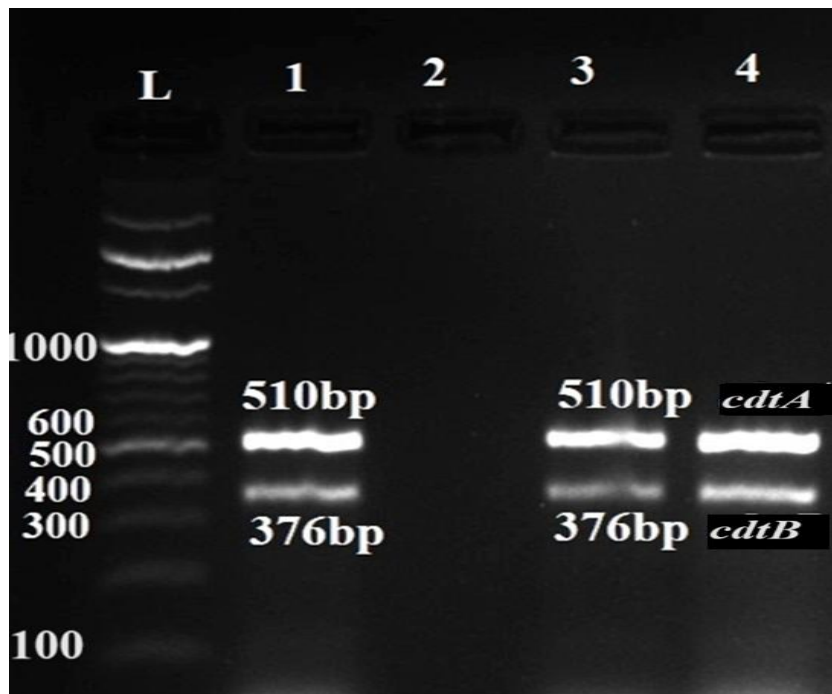


**Figure 2: Gel photograph of the putative virulence genes toxin A and toxin B of C. difficile using multiplex-PCR**

*Lane 1: Positive control of C. difficile (ATCC 9689) carrying toxin A (369bp) and toxin B (160bp) gene; Lane 2: Negative control (E. coli O157:H7 ATCC 43888); Lane 3: C. difficile isolate carrying toxin A and toxin B gene from farm water sample; Lane 4: C. difficile isolate carrying toxin A and toxin B gene from farm soil sample*

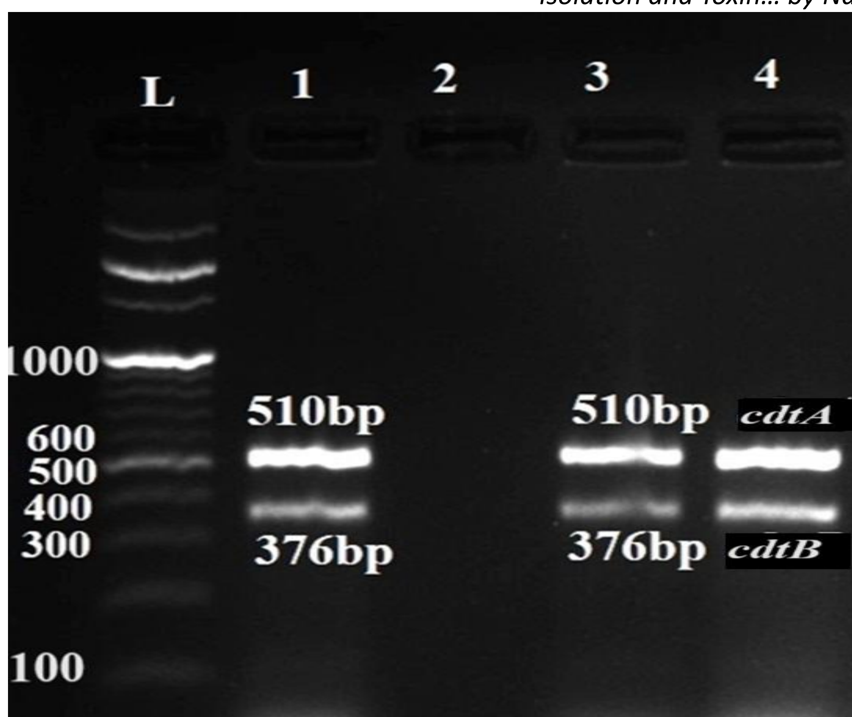


**Figure 3: Gel photograph showing the binary toxin of *C. difficile* using multiplex-PCR**

Lane L: DNA ladder (100bp), Lane 1: Positive control of C. difficile (ATCC 9689) carrying cdtA (510 bp) and cdtB (376 bp) genes, Lane 2: Negative control (*E. coli* 0157:H7 ATCC 43888); Lane 3: *C. difficile* isolate carrying cdtA and cdtB gene from farm water sample and Lane 4: C. difficile isolate carrying cdtA and cdtB gene from farm soil sample

The observed prevalence of *C. difficile* in farm water aligns with previous research by Esfandiari *et al*. (2014), which reported a similar isolation rate of 3.5% from environmental swabs near food processing facilities. However, it contrasts with the findings of Janezic *et al*. (2016), who documented a higher isolation rate of 14.4% from water samples. This discrepancy highlights the variability in *C. difficile* prevalence based on geographical locations, sampling techniques, and environmental conditions. Research on *C. difficile* primarily focuses on hospital environmental surfaces (Vaishnavi *et al*., 2012; Dhawan *et al*., 1999). However, there are limited studies about the prevalence of *C. difficile* in animal-linked environmental surfaces in India to date. This gap highlights the need for further investigation into *C. difficile* on diverse surfaces.

## CONCLUSION

Zoonotic pathogens transmitted through food represent a notable hazard to consumer well-being. The presence of *C. difficile* in farm environments poses a potential risk to both animal and human health. Given that water sources are critical for irrigation and livestock consumption, the contamination of these resources could lead to broader outbreaks of infection. The low but significant prevalence in farm water highlights the need for ongoing surveillance and environmental monitoring to detect and address potential contamination sources early.

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