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Isolation and molecular characterization of *Clostridium difficile* in animal faeces, animal-derived foods, and human samples from Andhra Pradesh

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ABSTRACT

A total of 350 samples including 175 faecal swabs from livestock, 125 samples from animal-derived foods, and 50 human stool samples, were examined. Both bacterial culture methods and PCR techniques were employed to detect *Clostridium difficile* species. The analysis indicated that 2.8% of the tested samples were positive for *C. difficile* through species-specific PCR. Among the faecal samples from livestock, pigs and calves showed a higher prevalence (4%). In the category of animal-derived foods, chicken samples had a notable prevalence (8%), while mutton samples had a lower prevalence (2%). Additionally, 8% of the human stool samples tested positive for *C. difficile*. Using bacterial culture methods, 52 isolates (14.8%) of *C. difficile* were identified. The application of species-specific PCR, targeting gene (*tpi)* further validated the presence of *C. difficile* in faecal samples from animals, animal-derived foods, and humans in Andhra Pradesh, India.

Keywords: Antibiotic Associated Diarrhoea, CDI, *Clostridium difficile*, Food animals, Foods of animal origin,

Clostridium difficile is a gram-positive, anaerobic bacterium known for its ability to produce toxins and form spores. (Vaishnavi 2011, Snbsp 2022). *C. difficile* is ubiquitous and common everywhere in the environment including in the pet bodies, water, soil, farm animals, on different surfaces in hospitals. It can also exist in foods such as vegetables and meat, as a free-living bacteria. (Rogers *et al*. 2012).

C. difficile is the causative agent of *C. difficile*-associated diarrheoa (CDAD) or *C. difficile* infection (CDI), which can lead to conditions ranging from pseudomembranous colitis (PMC) to severe complications such as toxic megacolon and colon perforation in humans. In animals, *C. difficile* is linked to several types of enteric diseases, including PMC (Farooq *et al*. 2015).

Risk factors for CDI include advanced age, extended use of antibiotics, hospitalization, and the presence of either symptomatic or asymptomatic carriers. Prolonged antibiotic therapy is another critical risk factor; antibiotics disrupt the normal gut microbiota, allowing *C. difficile* to thrive and cause infection (Gupta and Khanna 2014).

The Centers for Disease Control and Prevention (CDC) has recently identified *C. difficile* as one of the three urgent threats in its report addressing emerging zoonotic pathogens associated with antibiotic resistance. This classification highlights the severity of the issue and emphasizes the need for ongoing surveillance and effective strategies to combat CDI (CDC 2022). Furthermore, *C. difficile* has emerged as a notable foodborne zoonotic pathogen, particularly in light of recent outbreaks linked to contaminated food sources (CDC 2022).

Several DNA-based fingerprinting techniques are employed for the characterization of *C. difficile*, including pulsed-field gel electrophoresis (PFGE), PCR, MALDI-TOF, Restriction Endonuclease Analysis (REA), Multilocus Variable-Number Tandem-Repeat Analysis, PCR ribotyping, Toxinotyping based on Restriction Fragment Length Polymorphism (RFLP), Surface layer protein A gene sequence typing, Multilocus Sequence Typing (MLST), and Amplified Fragment Length Polymorphism (AFLP).

The true prevalence of (CDI) in India is still unclear, despite the extensive use of antimicrobial drugs. With the recent economic growth in several Asian nations, coupled with aging populations, improved healthcare access, and the widespread misuse of antibiotics, *C. difficile* is likely to be highly prevalent and causing significant disease burden (Monaghan *et al*. 2022, Gupta and Khanna 2014).

There is a scarcity of reports regarding the occurrence and prevalence of *C. difficile* in India, particularly in Andhra Pradesh. The main aim of this research was to investigate the presence of *C. difficile* in various food animals, animal-derived foods, and humans within the region of Andhra Pradesh, India.

MATERIALS AND METHODS

Reference strain: The reference strain of *C. difficile*

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(ATCC 9689) was procured from the American Type Culture Collection (ATCC), United States of America (USA).

Sample Collection: A total of 350 samples including faecal swabs from healthy food animals; consisting of poultry (50), sheep (50), pigs (50) and cattle (25) were collected. Raw food samples of animal origin were also incorporated, such as chicken (50), mutton (50) and pork (25). Additionally, stool samples were also collected from veterinary students and individuals with history of diarrhoea from microbiological labs (50) near Gannavaram and Vijayawada districts. The samples were also collected from Krishna and Prakasam districts in Andhra Pradesh.

Cultural isolation and identification of C. difficile: The procedure described by Hussain *et al*. (2016) was adopted for isolation and identification of *C. difficile*. Selective enrichment of samples was carried out in thioglycolate broth at 37°C for 7–10 days under anaerobic conditions. Then broth culture was treated with absolute alcohol for 45 min and centrifuged at 8,000 rpm for 15 min. The supernatant is removed, and sediment is inoculated into Cycloserine and Cefoxitin Fructose Agar (CCFA) plates (*C. difficile* agar with CC supplement) supplemented with 5% sheep blood and incubated at 37°C for 48–72 h. The typical bacterial colonies (off white coloured colonies) were confirmed by Gram staining and motility test.

C. difficile species-specific PCR: The identification of *C. difficile* spp. in enrichment broth samples was accomplished through species-specific PCR as per the method described by Lemee *et al*. (2004) and Zheng *et al*. (2004). 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. For PCR amplification, a 25 µL reaction mixture was prepared. This mixture included 2.0 µL of DNA template, 2.5 µL of Taq buffer (10x), 1.0 µL of dNTP mix (10mM), 0.5 µL of $MgCl_2$ (25mM), 1.5 µL of forward primer (10 pmol/µL), 1.5 µL of reverse primer (10 pmol/µL), 1.0 µL of Taq DNA polymerase (1 U/µL), and 15.0 µL of nuclease-free water. The PCR reaction was conducted under standard cycling conditions: Initial denaturation step at 95ºC for 1min, succeeded by 35 cycles. Each cycle involved denaturation at 95ºC for 30s, annealing at 60ºC for 30s, and elongation at 72ºC for 30s. The procedure concluded with an extension step at 72ºC for 10 min. Positive controls utilize DNA from the reference strain of *C. difficile* (ATCC 9689), whereas the negative control employs nuclease-free water.

RESULTS AND DISCUSSION

Gram staining of cultures revealed gram-positive rods. Additionally, the motility test indicated motility and non-haemolytic activity of the organism on blood agar and while both the oxidase and catalase tests yielded negative results. The results of study are given in Table 1.

Molecular detection of C. difficile by species-specific PCR: Following species-specific PCR screening of all samples (n=350) after enrichment, 2.85% (10/350) were found to be positive for *C. difficile* species with amplicon size of 230 bp (Fig. 1.) (Table 1.). These results are in line with previous studies that reported a prevalence of 2.0% (Rahimi *et al*. 2014). Documented occurrences of *C. difficile* have been observed in various studies across different regions, emphasizing the bacterium's widespread nature and the importance of monitoring its prevalence in both human and animals. Amongst faecal samples from food animals, a higher prevalence was observed in pigs (4.0%, 2/50), followed closely by cattle (4.0%, 1/25). These findings corresponded with earlier research by Indra *et al*. (2009), which reported detection of 3.3% *C. difficile* in pig and 4.5% in cattle faecal samples. Similar findings have been reported in other studies, indicating that livestock, particularly pigs and cattle, are significant reservoirs of

Table 1. Detection of *C. difficile* isolates by cultural method and PCR assay

Source	Number of samples analysed	Number of isolates positive for *C. difficile* by cultural tests. (%)	Number of isolates positive for *C. difficile* by PCR. *tpi* gene (%)
		Faecal/cloacal samples of food animals	
Poultry	50	04 (8.0%)	-
Sheep	50	04 (8.0%)	-
Pig	50	08 (16.0%)	2 (4.0%)
Cattle	25	04 (8.0%)	1 (4.0%)
Total	175	20 (11.4%)	3 (1.7%)
		Foods of animal origin	
Chicken	50	15 (30.0%)	4 (8.0%)
Mutton	50	4 (8.0%)	1 (2.0%)
Pork	25	5 (20.0%)	-
Total	125	24 (19.2%)	5 (4.0%)
		Human diarrhoeic stool samples	
Human lab diarrhoeic stool samples	50	8 (16.0%)	2 (4.0%)
Total	50	8 (16.0%)	2 (4.0%)
GRAND TOTAL	350	52 (14.8%)	10 (2.85%)

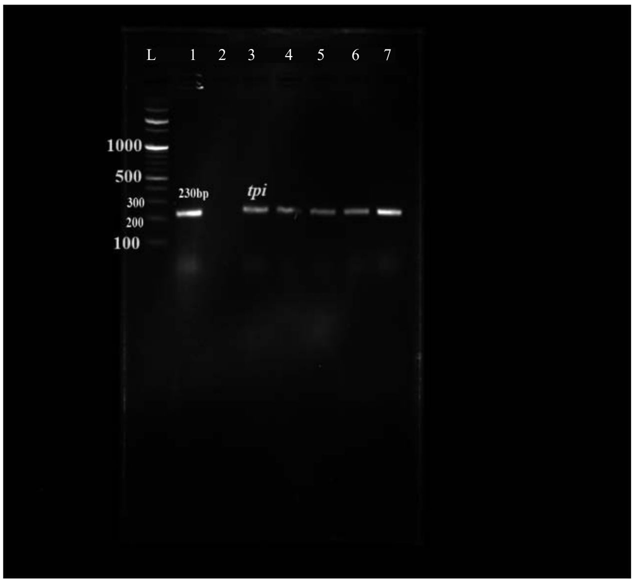

Fig. 1. Gel photograph of *C. difficile* species-specific PCR (*tpi* gene)

C. difficile (Rodriguez *et al*. 2017, Lim *et al*. 2020).

The variations in results can be attributed to differences in sampling criteria, where the present study included faecal swabs from both diarrheic and healthy pigs and cattle along with good feed management practices.

Analysis of animal-derived foods revealed a higher prevalence of *C. difficile* in chicken, recorded at 8.0% (4 out of 50 samples), compared to mutton, which had a detection rate of 2% (1 out of 50 samples). These results are similar to those found in previous research, which indicated an 8% detection rate in chicken (Varshney *et al*. 2011) and 6% in mutton (De boer *et al*. 2011).

The high prevalence of *C. difficile* in poultry may be attributed to various factors, including farming practices, antibiotic use, and contamination during processing. Earlier findings indicate that the use of antibiotics in livestock can disrupt normal gut flora, allowing pathogenic bacteria like *C. difficile* to proliferate. Furthermore, the processing and handling of chicken can lead to cross-contamination, underscoring the importance of stringent hygiene practices in the meat industry (Simango and Mwakurudza 2008). These findings raise important public health considerations. The consumption of contaminated meat can lead to gastrointestinal infections, and there is increasing evidence suggesting a link between foodborne pathogens and human cases of C. difficile-associated diseases (Weese *et al*. 2010).

The presence of *C. difficile* in human stool samples is particularly concerning due to its association with antibiotic use and the subsequent risk of developing infections. Recent research had demonstrated that the use of antibiotics can disrupt normal gut flora, facilitating the overgrowth of *C. difficile* (Mullish *et al*. 2018). This disruption is often seen in hospitalized patients who are frequently prescribed antibiotics, which may lead to increased susceptibility to C. difficile-associated diseases (CDAD) (Gupta and Khanna 2014).

In human stool samples, *C. difficile* was found to be 4.0% (2 out of 50 samples). These findings are consistent with previous research, such as the study by Hussain *et al*. (2016), which reported a prevalence of 9.0% for *C. difficile* in humans, and Segar *et al*. (2017), who identified a rate of 4.5%. This similarity in detection rates underscores the ongoing concern regarding the presence of *C. difficile* in humans and highlights the need for continued surveillance and research into its epidemiology.

However, various factors influence the recovery rates of *C. difficile*, including culturing conditions such as the inability to sustain an anaerobic environment, methods employed for detection and isolation, duration of incubation and potential overgrowth of concurrent microflora that hinders the isolation of pure *C. difficile* cultures. The inclusion of antibiotics in the selective medium, while reducing the growth of other bacteria, can also inhibit the retrieval of the target microorganism, consequently reducing the incidence of the pathogen in different samples.

[Lane L: DNA ladder (100bp), Lane 1: Positive control of *C. difficile* (*C. difficile* ATCC 9689) showing presence of *tpi* gene (230bp), Negative control (*E. coli* 0157:H7 ATCC 43888) (Lane 2), *C. difficile* isolate carrying *tpi* gene from chicken sample (Lane 3), cattle faecal sample (Lane 4), human stool sample (Lane 5), pig faecal sample (Lane 6) and mutton sample (Lane 7)]

In conclusion, zoonotic pathogens transmitted food borne through food pose a significant risk to consumer health. This investigation has unequivocally detected the presence of *C. difficile* in the faeces of ostensibly healthy food animals, emphasizing the potential for contamination within the human food supply chain. Furthermore, the research has confirmed the existence of *C. difficile* in a range of animal-derived foods such as raw chicken, pork and mutton. The correlation between these food sources and the presence of *C. difficile* raises significant concerns regarding potential risks to public health. The misuse of antimicrobials, coupled with the lack of comprehensive culture and toxin testing facilities as well as an inadequate surveillance system, has contributed to the rising prevalence of *C. difficile* in India. It is crucial to undertake further studies to explore and implement effective intervention measures aimed at safeguarding human health.

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