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Molecular detection and epidemiological insights of *Clostridioides difficile* in Egyptian fruit bats

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ABSTRACT

Clostridioides difficile is a major global cause of antibiotic-associated diarrhea, increasingly recognized as a community- and zoonosis-linked pathogen with widespread environmental and animal reservoirs. Given the ecological overlap between bats, humans, and livestock and the limited data on bats, this study investigated the presence of *C. difficile* and its toxin genes in Egyptian bats to evaluate their potential role in the transmission cycle. Fifty fruit bats (Rousettus spp.) were captured and identified using standard morphological keys. Intestinal content samples were collected and cultured anaerobically in Cooked Meat Medium. DNA was extracted from isolates, and PCR was performed to detect *C. difficile* (*tpi*) and its toxin genes (*tcd*A, *tcd*B). *C. difficile* was detected in 64% of intestinal content samples (32/50), with 16 isolates (32%) identified as toxigenic. Among these, 21.8% were *tcd*A$^+$/*tcd*B$^-$, 15.6% *tcd*A$^-$/*tcd*B$^+$, and 12.5% carried both genes, while 32% of isolates were non-toxigenic. This study provides the first molecular evidence of toxigenic and non-toxigenic *C. difficile* in Egyptian fruit bats, prominence their potential role as environmental reservoirs. The findings highlight the need for bats investigation to clarify the possible transmission ways within humans, animals and environment.

Introduction

Clostridioides difficile (*C. difficile*) is an anaerobic, Gram-positive, endospore-forming bacillus that has emerged as a leading cause of antibiotic-associated diarrhea (AAD) and healthcare-associated infections worldwide (Zhu *et al*., 2018; Gawey and Khanna, 2023). Over recent years, the epidemiology of *C. difficile* infection (CDI) has shifted, with increasing recognition of its role as a community-acquired pathogen potentially linked to zoonotic and foodborne transmission (Gould and Limbago, 2010; Gupta and Khanna, 2014; Balsells *et al*., 2018).

Infection by *C. difficile* continues to pose a significant global threat, resulting in hundreds of thousands of cases each year and leading to marked morbidity, increased mortality, and persistently high recurrence rates (Kwon *et al*., 2015; Ilic *et al*., 2024; Akorful *et al*., 2025). The associated economic impact is also significant, as infections lead to prolonged hospitalizations and increased healthcare costs. The emergence of hypervirulent types has further intensified the challenge, emphasizing the need for continuous monitoring and effective control strategies (Fatima and Aziz, 2019; Schley *et al*., 2025).

The detection of *C. difficile* has increasingly been reported in clinical and environmental samples across Egypt, with several hospital-based studies finding surprisingly high isolation rates among patients with diarrhea (Kotb *et al*., 2015; AbdEl-Mongy *et al*., 2018; Elgendy *et al*., 2020). Additionally, recent studies have detected *C. difficile* in various animal species (Abdel-Glil *et al*., 2018; Elshaimaa *et al*., 2019; Fathy *et al*., 2023), highlighting its environmental persistence and potential as an animal reservoir that contributes to its spread beyond clinical settings.

A variety of animal species such as food-producing, companion, and wild animals (Jhung *et al*., 2008; Andrés-Lasheras *et al*., 2017; 2018) play a critical role in the maintenance and dissemination of this pathogen in addition to environmental reservoirs such as soil (Janezic *et al*., 2016; Lim *et al*., 2020a), water (Romano *et al*., 2012), and food (De Boer *et al*., 2011; Hoover and Rodriguez-Palacios, 2013), where its ability to form resilient spores enables survival under harsh environmental conditions, facilitating long-term persistence and transmission (Shen, 2020).

Bats, belonging to the order Chiroptera, comprise over one-fifth of mammalian diversity and inhabit nearly all regions of the world except the polar zones (Bazzoni *et al*., 2024). Their unique ability to fly, migrate over long distances, adapt to diverse environments, live in colonies, and maintain relatively long lifespans contributes to their ecological success (Gonzalez and Banerjee, 2022). However, increasing human–bat interactions, largely driven by anthropogenic habitat disruption, have heightened concerns over zoonotic spillover risks (Jackson *et al*., 2024), underscoring their potential role not only as incidental carriers but also as silent disseminators within the broader epidemiological cycle of *C. difficile*.

Given the close ecological overlap between bats, livestock, and human settlements, the possibility of *C. difficile* transmission through the One Health interface warrants attention (Lim *et al*., 2020b; Sippola *et al*., 2025). Bat guano is often deposited in areas used for agriculture, food storage, or water sources, creating opportunities for indirect human and animal exposure to resilient spores (Sakoui *et al*., 2020; Dimkić *et al*., 2021).

Bats are known to carry numerous bacterial pathogens. Their feces and intestinal contents contain a diverse range of potentially harmful microorganisms and may contribute to the dissemination of these bacteria into the environment, posing significant risks to both human and animal health (Adesiyun *et al*., 2009; Federici *et al*., 2022; Huang *et al*., 2022). Although *C. difficile* has been extensively studied in humans and other animals, information on its occurrence and genetic characteristics in bats remains limited. Nevertheless, *C. difficile* has been detected in various wild animals (Silva *et al*., 2014; Krijger *et al*., 2019; Lima *et al*., 2024), including in bat guano (Bandelj *et al*., 2019), suggesting that bats may represent an overlooked environmental source of *C. difficile*.

The symptoms of CDI range from mild, self-limiting diarrhea to severe and life-threatening conditions such as pseudomembranous colitis and toxic megacolon (Alexiou *et al*., 2025). Its pathogenicity is primarily driven by two major exotoxins, Toxin A and Toxin B (Di Bella *et al*., 2016). Toxin A, encoded by the *tcd*A gene, is an enterotoxin that disrupts the intestinal epithelial barrier, promoting fluid secretion and inflammation. Toxin B, encoded by the *tcd*B gene, is a cytotoxin that damages host cells, triggering apoptosis and causing extensive damage to the colonic mucosa (Chandrasekaran and Lacy, 2017; Alam and Madan, 2024)

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Consequently, molecular findings of these toxin genes are critical to distinguish toxigenic from non-toxigenic strains and to assess their role on both human and animal health (Vedantam *et al*., 2012; Fathy *et al*., 2023). Moreover, studying their distribution among isolates also provides valuable epidemiological insights, helping to identify potential zoonotic reservoirs and track the spread of clinically relevant strains (Knight and Riley, 2019; Redding *et al*., 2022).

Since bats are well-known reservoirs of diverse microbial pathogens and play an important ecological role as highly mobile mammals (Dhivahar *et al*., 2023), the paucity of data on *C. difficile* in this host represents a critical gap in current knowledge. Therefore, the present study was conducted to investigate the occurrence of *C. difficile* in bat populations in Egypt and to characterize the presence of their toxin genes, with the aim of evaluating their possible role as carriers of toxigenic strains and their significance in the epidemiology of CDI.

Materials and methods

Ethics approval and consent to participate

Bats were humanely euthanized in agreement with institutional and international animal welfare guidelines. Euthanasia was made by exposure to isoflurane inhalation till total loss of reflexes and respirational arrest and to confirm death by cervical dislocation. The study was presented in agreement with the ethical guidelines set by the Institutional Animal Care and Use Committee (IACUC) of the Faculty of Veterinary Medicine, Cairo University, Egypt (Approval No. Vet CU110520251177). All experimental protocols were approved by the Institutional Animal Care and Use Committee (IACUC) of the Faculty of Veterinary Medicine, Cairo University, Egypt and are reported in accordance with the ARRIVE guidelines.

Bat Capture and Identification

A total of fifty fruit bats were captured using mist nets that were strategically deployed at both foraging and roosting sites. Species identification was carried out based on external morphological characteristics in accordance with the taxonomic keys of Dietz (2005) and Monadjem *et al*. (2020). Diagnostic features included large eyes and simple ears lacking a tragus or antitragus, which are typical traits of the family Pteropodidae. The presence of claws on the first and second digits further supported this classification. Morphometric parameters, including wingspan, forearm length, and tail length, were measured using a mechanical caliper. Collectively, these morphological features confirmed that all captured specimens belonged to the genus Rousettus.

Sample collection and culturing

Intestinal content samples were aseptically collected from each bat included in this study and stored at -20°C for further analyses. The collected samples were aseptically cultured on sterile, freshly prepared Cooked Meat Medium (HiMedia, India) and after that incubated anaerobically at 37°C for 24 to 48 hours using an anaerobic jar with a gas-generating kit (HiMedia, India).

Molecular investigation of C. difficile and toxin encoding Genes

Extraction of the genomic DNA

DNA was extracted from each isolate using the boiling method (Holland *et al*., 2000; Peng *et al*., 2013). DNA concentration was determined at 260 nm using a spectrophotometer, and purity was evaluated by calculating the A260/A280 ratio. The extracted DNA was stored at −20°C until further molecular analysis.

Direct detection of *C. difficile* and toxin encoding Genes:

The extracted DNA was screened for the presence of *C. difficile* by PCR targeting the *tpi* gene, as described by Lemee *et al*. (2004). For all *tpi*-positive isolates, the presence of the toxin-encoding genes *tcd*A and *tcd*B was determined by PCR according to Titov *et al*. (2000). PCR reactions were performed in a total volume of 25 µl, containing 3 µl of template DNA, 12.5 µl of 2X amaR OnePCR™ Master Mix (GeneDireX, Inc., USA), 0.5 µl of each primer (10 pmol/µl; Metabion, Germany), and PCR-grade water. Amplicons were separated on a 1.5% agarose gel and visualized under UV illumination. Primer sequences and amplification conditions are listed in Table 1.

Results

Occurrence of C. difficile and distribution of toxin genes among isolates

As shown in Table 2. *C. difficile* was detected in 32 of the 50 examined intestinal content samples, yielding an overall prevalence of 64%. Among these isolates, 16 (32% of the total samples) were identified as toxigenic. Specifically, 7/32 isolates (21.8%) carried *tcd*A$^+$/*tcd*B$^-$, 5/32 isolates (15.6%) carried *tcd*A$^-$/*tcd*B$^+$, and 4/32 isolates (12.5%) carried both *tcd*A and *tcd*B. Overall, *tcd*A was detected in 34.4% (11/32) of isolates, while *tcd*B was detected in 28.1% (9/32). The remaining 16 isolates (32%) were non-toxigenic.

Discussion

Over the past decades, *C. difficile* has shifted from being considered a predominantly hospital-acquired pathogen to a widely recognized community-associated and multi-host organism (Xaplanteri *et al*., 2025). Increasing whole-genome sequencing evidence demonstrates that many hospital CDI cases are genetically unrelated, indicating that reservoirs outside healthcare settings may play a major role in transmission (Khanna *et al*., 2012; Lim *et al*., 2020).

In the present study, a relatively high occurrence of *C. difficile* (64%) was detected among fruit bats in Egypt, with 32% of samples harboring toxin-encoding genes (*tcd*A and/or *tcd*B). These findings reinforce

Table 1. Primer sequences and PCR conditions used for amplification of *C. difficile* housekeeping (*tpi*) and toxin (*tcd*A, *tcd*B) genes.

Gene (bp)	Primer sequence	Cycling condition	Reference
tpi (230)	F: AAAGAAGCTACTAAGGGTACAAA R: CATAATATTGGGTCTATTCCTAC	95ºC, 3 min; 40 cycles (95ºC, 30 s; 55ºC, 30 s; 72ºC, 30 s), 72ºC, 5 min	(Lemee *et al.*, 2004).
*tcd*A (602)	F: GCATGATAAGGCAACTTCAGTGG R: GAGTAAGTTCCTCCTGCTCCATCAA	95ºC, 3 min; 40 cycles (95ºC, 20 s; 53ºC, 25 s; 72 ºC, 1 min),72ºC, 7 min	(Titov *et al*., 2000).
*tcd*B (399)	F: GGTGGAGCTGCTTCATTGGAGAG R: GTGTAACCTACTTTCATAACACCA	95ºC, 3 min; 40 cycles (95ºC, 20 s; 49 ºC, 25 s; 72ºC, 1 min),72ºC, 7 min	(Titov *et al*., 2000).

Table 2. Occurrence of *C. difficile* and distribution of toxin genes among isolates.

No. of examined samples	No. of positive					
	C. difficile (%)	Toxigenic *C. difficile* (%)			Total toxigenic isolates (%)	Total non-toxigenic isolates (%)
		*tcd*A$^+$/*tcd*B$^-$	*tcd*A$^-$/*tcd*B$^+$.	*tcd*A$^+$/*tcd*B$^+$		
50	32 (64)	7/32 (21.8)	5/32(15.6)	4/32(12.5)	16/50 (32)	16/50(32)

the growing recognition of wildlife as an important component of the broader ecological cycle of *C. difficile*, highlighting that bats may serve as potential reservoirs or environmental disseminators of toxigenic strains (Alexiou *et al*., 2025).

Several studies have demonstrated that *C. difficile* is widely distributed across the community, having been isolated from animals, the environment, and food sources (Jhung *et al*., 2008; Andrés-Lasheras *et al*., 2018). The pathogen has been recovered from livestock, pets, horses, wildlife, and from soil, water, sewage, and multiple environmental surfaces (Romano *et al*., 2012; Hoover and Rodriguez-Palacios, 2013; Janezic *et al*., 2016). The detection of identical strains in humans, animals, food, and environmental samples supports the likelihood of zoonotic transmission, where food and environmental contamination act as key connecting routes (Gould and Limbago, 2010; Salvarani *et al*., 2025). Collectively, these observations emphasize the expanding importance of community reservoirs beyond hospitals (Lim *et al*., 2020).

The isolation of *C. difficile* from a broad range of wild animal species, underscoring its ecological adaptability and environmental persistence (Krijger *et al*., 2019; Weese *et al*., 2019; Lima *et al*., 2024). Bats, in particular, are recognized reservoirs for numerous pathogenic bacterial pathogens (Huang *et al*., 2022), including several *Clostridium* species such as *C. perfringens* (Allam *et al*., 2025). The detection of *C. difficile* in bat guano further supports the hypothesis that bats may harbor strains of epidemiological relevance (Bandelj *et al*., 2019).

The extensive geographic distribution, migratory behavior, and frequent contact of bats with human-inhabited environments may facilitate the dissemination of *C. difficile* across diverse ecological niches (Devnath *et al*., 2022). Although current data remain limited, evidence from other wildlife species suggests that wildlife can play a significant role in the environmental transmission cycle of this pathogen (Weese, 2020). As the first molecular investigation of *C. difficile* in bats in Egypt, the present study offers new insights into their potential contribution to the epidemiology of CDI.

Fruit bats commonly inhabit agricultural areas where they overlap with human and animal activities, underscoring the need to incorporate wildlife surveillance into national CDI monitoring programs to better characterize cross-species transmission. The origin of *C. difficile* in bats remains unclear; potential sources include contaminated food, water, soil, or indirect exposure to livestock. Conversely, bats may act as environmental amplifiers, shedding spores into ecosystems shared with humans and animals (Ramanantsalama *et al*., 2022; Jackson *et al*., 2024).

Toxigenic strains are the primary cause of *C. difficile*–associated diarrhea in humans (Voth and Ballard, 2005). In this study, 16 isolates (32%) were identified as toxigenic, with the *tcd*A gene detected in 34.4% of isolates, while *tcd*B was found in 28.1%. Pathogenicity is mainly driven by the production of *Tcd*A and *Tcd*B toxins, which damage the colonic epithelium, induce inflammation, increase fluid secretion, and cause progressive tissue injury (Di Bella *et al*., 2016; Chandrasekaran and Lacy, 2017; Alam and Madan, 2024). Notably, the cellular toxicity of *Tcd*A and *Tcd*B is not limited to intestinal tissue; systemic complications involving organs such as the heart, kidneys, lungs, and brain have been increasingly documented (Alam and Madan, 2024).

In our study, four isolates (12.5%) carried both toxin genes (*tcd*A$^+$/*tcd*B$^+$). This toxin profile is typical of most CDI cases (Kuehne *et al*., 2011), and has been identified in various animal and human samples (Fry *et al*., 2012; Ghavidel *et al*., 2016; Zhang *et al*., 2024), including patients with antibiotic-associated diarrhea in hospitals (AbdEl-Mongy *et al*., 2018) and in bird samples collected in Egypt (Abdel-Glil *et al*., 2018). The detection of *tcd*A$^+$/*tcd*B$^+$ isolates in bats suggests that they may serve as potential reservoirs or vectors of clinically significant toxigenic strains.

Five isolates (15.6%) carried only the *tcd*B gene (*tcd*A$^-$/*tcd*B$^+$). This aligns with reports showing that *tcd*A$^-$/*tcd*B$^+$ strains can cause the full spectrum of CDI, from mild diarrhea to pseudomembranous colitis and death (Drudy *et al*., 2007a). *Tcd*B is considered the primary virulence factor and can independently drive CDI pathology (Lyras *et al*., 2009); it is approximately ten times more cytotoxic to the human colon than *Tcd*A (Di Bella *et al*., 2016). Similar *tcd*A$^-$/*tcd*B$^+$ isolates have been reported in several animal species (Rodriguez-Palacios *et al*., 2006; Knight and Riley, 2013) and have been implicated in several nosocomial CDI outbreaks (Drudy *et al*., 2007b; Kim *et al*., 2010; Imwattana *et al*., 2019) raising concerns about potential interspecies transmission.

Additionally, seven isolates (21.8%) carried only the *tcd*A gene (*tcd*A$^+$/*tcd*B$^-$). Although less frequently reported, strains producing only toxin A can still be virulent (Kuehne *et al*., 2010), as demonstrated by Kuehne *et al*. (2014) that toxin A–only strains could cause disease in hamsters. Comparable isolates were previously detected in multiple animal species at lower prevalence (Thanissery *et al*., 2020), in calves (Koene *et al*., 2012), and in pet animals in Egypt (Samir *et al*., 2021). They have also been identified in diarrheic patients in intensive care units (Zarandi *et al*., 2017).

In this study, 16 isolates were non-toxigenic. However, non-toxigenic *C. difficile* can acquire toxin genes from toxigenic strains through horizontal gene transfer, potentially becoming toxin producers (Brouwer *et al*., 2013). This genetic plasticity poses a public health concern, as environmental or animal-associated non-toxigenic strains may evolve into virulent forms, contributing to community- and hospital-acquired infections.

Overall, the present study provides the first evidence of *C. difficile* occurrence and toxin-gene carriage in bats in Egypt, highlighting their potential role as environmental reservoirs within the transmission cycle. These molecular findings underscore the need for further genetic typing and to determine the relatedness of bat-derived strains to human clinical isolates. Future work should incorporate genomic characterization to clarify evolutionary relationships and transmission pathways to provide a more comprehensive understanding of the organism's distribution and its One Health implications.

Conclusion

This study provides the first molecular detection of toxigenic and non-toxigenic *C. difficile* strains in fruit bats in Egypt which highlights the potential role of bats as environmental reservoirs within the transmission cycle of *C. difficile*. The relatively high detection emphasizes the need to consider wildlife, specifically bats, in national surveillance and risk-assessment frameworks. More investigations are required to determine the genetic relatedness of bat-derived isolates. This study is vital for identifying transmission modes and informing approaches to decrease *C. difficile* spread within the human–animal–environment interface.

Conflict of interest

The authors have no conflict of interest to declare.

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