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ORIGINAL RESEARCH

# Some Factors Influencing the Number of *Clostridioides difficile* Spores Detected in Hospital Wastewater

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**Objective:** *Clostridioides difficile* is the most common cause of antibiotic-associated diarrhea. Wastewater from hospitals may be an important source of *C. difficile* transmission between hospitals and communities. The objective of this study is to quantify *C. difficile* spores and to elucidate their potential transmission risk via hospital wastewater.

**Methods:** A prospective study of wastewater from a teaching hospital was conducted weekly, from July 2023 to June 2024. The number of *C. difficile* spores detected in wastewater from hospital settings fluctuated weekly during the study period.

**Results:** There was a borderline association between the number of *C. difficile* spores detected in wastewater at room temperature in hospitals ($p = 0.02$) and the consumption of antimicrobial agents ($p$ *value* = 0.04), particularly cephalosporins ($p = 0.001$). Specifically, the number of *C. difficile* spores detected in the wastewater was highly correlated with first-generation cephalosporin consumption ($p = 0.002$), particularly the consumption of first-generation intravenous cephalosporin (cefazolin) ($p < 0.001$).

**Conclusion:** The number of *C. difficile* spores detected in wastewater from hospital settings is strongly associated with the consumption of antimicrobial agents, particularly cephalosporins. Further evaluation is needed to assess whether antibiotic stewardship programs can reduce the burden of *C. difficile* spores in wastewater.

**Plain Language Summary:** Consumption of antimicrobial agents influences *Clostridioides difficile* spores in hospital wastewater.

**Keywords:** *Clostridioides difficile*, spore, wastewater, cephalosporins, cefazolin, hospital setting

## Introduction

*Clostridioides difficile* remains the most common etiology of healthcare-associated infections in recent years, and significant complications have been observed in severe cases.[1–4] In a review of 59 studies encompassing data from 24 countries across North America, Europe, the Asia-Pacific region, Latin America, and the Middle East, in year 2016–2024, the highest incidence of *C. difficile* infection (CDI) was observed in hospital-onset health-care facility settings, with 5.31 cases per 1,000 admissions (95% CI: 3.76–7.12) and 5.00 cases per 10,000 patient-days (95% CI: 3.96–6.15). Recurrence rates were highest among community-acquired CDI cases at 16.22%. The 30-day all-cause mortality and the overall mortality (unspecified duration) were reported at 8.32% and 16.05%, respectively.[5] Despite important advances in the development of new therapeutic agents and preventative methods, the worldwide prevalence of

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CDI remains high.[1] In addition to unfavorable outcomes, it poses a global concern owing to its resistance to common antibiotics and ease of transmission.[6,7]

*C. difficile* is commonly transmitted via the fecal-oral route. Transmission can even occur due to spore ingestion, because spores can endure extremely acidic conditions within the stomach, germinate into vegetative forms in the intestine, and colonize the host colon.[8] The outermost layer of *C. difficile* spores has an extremely low permeability to small compounds and defends its core against DNA-damaging chemicals or antimicrobials.[9] Owing to its ability to withstand harsh environments, the infectious and transmissible potential of *C. difficile* depends largely on dormant spores, which are characterized by resistance to heat, oxygen, and common disinfectants, such as ethanol-based hand sanitizers.[9–11]

When spores are ingested, *C. difficile* initiates a sporulation pathway that yields dormant spores, resulting in prolonged colonization and further dissemination among patients.[9,12] Germination of *C. difficile* spores and growth of vegetative forms may occur in the intestine, where some compounds such as bile acids and taurocholate can induce germination of spores into dynamically duplicating vegetative bacteria.[13,14] Another primary bile acid, chenodeoxycholate, is a competitive taurocholate inhibitor that inhibits the germination of *C. difficile* spores.[14,15] The sporulation and germination abilities of different clinical strains of *C. difficile* differ.[13,16]

According to the updated international guidelines issued by the Infectious Diseases Society of America (IDSA) and Society for Healthcare Epidemiology of America (SHEA) in 2017, vancomycin and fidaxomicin are recommended therapeutic agents for CDIs.[17] In clinical settings, the high recurrence rate of CDI is mainly not due to the development of antibiotic resistance, but rather due to the formation of spores. Treating CDIs with vancomycin or fidaxomicin is associated with a high recurrence rate because neither antibiotic is effective against spores.[17] The residual spores germinated into vegetative bacteria after the cessation of antibiotics.[18]

Previously, CDIs were often regarded as important healthcare-associated infections. Currently, the majority of CDI cases are acquired within the community as determined by whole-genome sequencing.[19] The origin of community-acquired CDIs is still a topic of debate and is possibly attributed to foodborne transmission since they are usually found in a wide range of foods, including meat, seafood, and fresh products.[20] However, the role of these foods as potential transmission vehicles remains clear.[20] Environmental contamination and the persistence of *C. difficile* in hospital wastewater systems have been reported in German hospital.[21] A genomic survey of *C. difficile* reservoirs in eastern England suggested that environmental contamination of wastewater treatment plants from clinical sources was possible.[22] Therefore, wastewater from health-care facilities may be an important source of CDI transmission. This study aimed to monitor the number of *C. difficile* spores in hospital wastewater and determine the factors associated with the alteration of spore amounts in wastewater.

## Materials and Methods

The amount and characteristics of *C. difficile* spores in hospital wastewater were monitored. *C. difficile* spores from the wastewater of a teaching hospital were collected at 5 p.m. on Wednesdays every week from July 2023 to June 2024. *C. difficile* spores from wastewater were monitored using a culture method.[23]

### Wastewater Sample Collection

Wastewater sampling was conducted at the hospital basement wastewater treatment facility. The source of the wastewater was untreated hospital effluent prior to chlorination. Samples were collected using a water scoop (purchased from Techni Trade[@], D8QA-108000), which was used to retrieve wastewater from the collection tank. The wastewater was transferred into sterile sampling bags (purchased from Techni Trade[@], DP-SB1930W), with each bag containing 1 liter. A total of three bags were collected. One bag was used for subsequent experiments, while the remaining two were stored at 4°C for backup purposes. We first conducted a preliminary test by collecting wastewater samples on several different days within the same week to quantify spore counts. After identifying the day with the highest spore load, we subsequently performed weekly wastewater sampling on that specific day.

### Counting *C. difficile* Spores in Wastewater[23]

Hospital wastewater was collected and concentrated using a 0.45 μm pore-size cellulose acetate membrane filter (Tak Kee Instruments, ADVANTEC[@]). After the entire volume passed through the filter, the membrane was removed and placed into

a 50 mL centrifuge tube containing Brain Heart Infusion-supplemented (BHIS) medium, 0.1% taurocholic acid (TA), and an antibiotic cocktail. The tube was then incubated anaerobically at 37°C for ten days. After ten days, 2 mL of bacterial sediment from the bottom of the centrifuge tube was transferred using a micropipette into a 15 mL centrifuge tube. A heat shock treatment (65°C for 20 minutes) was performed, followed by an ethanol shock treatment (2 mL of 99% ethanol, incubated at room temperature for one hour). The sample was then centrifuged at room temperature (4000 rpm for 10 minutes), and the supernatant was discarded. The spore pellet was resuspended in 1 mL of taurocholic acid (TA) solution. From each tube, 20 µL was plated onto Cycloserine-Cefoxitin Fructose Agar (CCFA) in duplicate. Additionally, 200 µL was plated per tube onto CCFA to prevent false negatives due to low spore counts in the solution. The plates were incubated anaerobically at 37°C for two days. After incubation, colonies were subcultured onto blood agar under anaerobic conditions for confirmation. Colonies identified as *C. difficile* according to the morphology: The colonies are usually circular, slightly raised, and have an irregular edge, and often exhibit a matte or ground-glass appearance and are grayish to yellowish-white in color. These colonies were scraped into BHIS cryovials and stored at –80°C. Statistical analysis was performed using the Prism statistical software (version 10.0). The linear-by-linear associations between the risk factors and spore amounts in wastewater were analyzed using Spearman correlation. Statistical P was set less than 0.05.

### The Amount of Antibiotics Consumed

Hospital-wide antibiotic consumption was recorded using electronic medical records. The most often prescribed antibiotics were grouped into the following classes: cephalosporins, fluoroquinolones, penicillins, and carbapenems. Cephalosporins include first-generation cephalosporins (eg, intravenous cefazolin and oral cephalexin), second-generation cephalosporins (eg, cefuroxime), third-generation cephalosporins (eg, ceftriaxone, cefotaxime, and ceftazidime), and fourth-generation cephalosporins (eg, cefepime). The antibiotic consumption was calculated using the defined daily dose (DDD), which is the assumed average maintenance dose per day for a drug used for its main indication in adults.[24]

### The Number of Patients with CDI, Temperature and Humidity in the Hospital

The number of patients with CDI in the hospital was obtained from the electronic medical records. The Institutional Review Board of the National Cheng Kung University Hospital, Taiwan approved the collection of data from patients with CDI (approval number: B-ER-103-098). Because of the retrospective review of the medical records of hospitalized patients, inability to obtain informed consent, and no specific ethical concerns and minimal safety risks, the study was allowed to waive informed consent in compliance with the Declaration of Helsinki from legally authorized representatives. The weekly temperature and humidity in the study hospital were recorded using a hospital-installed temperature and humidity monitoring device on the first floor, which provides institution-wide data. Since the hospital uses a centralized air conditioning system, the temperature and humidity do not vary significantly across different areas.

### Statistical Analysis

Statistical analysis was performed using the Prism statistical software (version 10.0). The linear-by-linear associations between the risk factors and spore amounts in wastewater were analyzed using Spearman correlation. Statistical *P* was set less than 0.05.

## Results

### Weekly Amount of *C. difficile* Spores in Wastewater

We collected wastewater samples on the following three days within the same week, and the corresponding spore concentrations were as follows:

March 27, 2023 (Monday), 09:00—21,660 CFU/mL

April 12, 2023 (Wednesday), 17:00—113,807 CFU/mL

April 14, 2023 (Friday), 13:00—16,942 CFU/mL

Among these, the sample collected on Wednesday showed the highest spore concentration. Therefore, all subsequent weekly wastewater sampling was performed around 17:00 on Wednesdays.

The number of *C. difficile* spores detected in wastewater from July 2023 to June 2024 fluctuated every week from 95 CFU/mL to 10500 CFU/mL (Figure 1). Many factors may contribute to variations in the detected *C. difficile* spores in wastewater, such as patients with CDI in hospitals, temperature and humidity in hospital settings, and antimicrobial agent consumption.

## Analysis of the Factors Contributing to the Variations in the Detected Spores in Wastewater

There was no association between room temperature and the number of detected *C. difficile* spores in wastewater ($p$ value = 0.02, $\rho = -0.685$) (Figure 2A), but there was no correlation between room humidity and the number of detected *C. difficile* spores in wastewater ($p$ value = 0.22, $\rho = -0.253$) (Figure 2B). There was no correlation between patients with CDI and the number of *C. difficile* spores detected in wastewater ($p$ value = 0.32, $\rho = 0.155$) (Figure 3A). Finally, there was association between the consumption of antimicrobial agents and the number of *C. difficile* spores detected in wastewater ($p = 0.04$, $\rho = 0.627$) (Figure 3B).

## Association Between the Consumption of Antimicrobial Agents and Spores in Wastewater

*C. difficile* spores in wastewater were associated with cephalosporin consumption ($p$ value = 0.001, $\rho = 0.8636$) (Figure 4A) but not with penicillin ($p$ value = 0.69, $\rho = -0.1364$) (Figure 4B), carbapenem ($p$ value = 0.22, $\rho = 0.4$) (Figure 4C), or fluoroquinolone ($p$ value = 0.56, $\rho = 0.2$) (Figure 4D).

A detailed analysis of the consumption of cephalosporins revealed that *C. difficile* spores detected in wastewater were associated with first-generation cephalosporin consumption ($p$ value = 0.002, $\rho = 0.8455$) (Figure 5A) but not with second-generation cephalosporin consumption ($p$ value = 0.95, $\rho = 0.0273$) (Figure 5B), third-generation cephalosporin consumption ($p$ value = 0.08, $\rho = 0.5636$) (Figure 5C), or fourth-generation cephalosporin consumption ($p$ value = 0.56, $\rho = 0.2$) (Figure 5D).

In terms of the association between the number of detected *C. difficile* spores in wastewater and first-generation cephalosporin consumption, there was a correlation between the number of detected *C. difficile* spores in wastewater and the consumption of intravenous first-generation cephalosporins (cefazolin) ($p$ value $\leq 0.001$, $\rho = 0.8909$) (Figure 6A) but not with the consumption of first-generation cephalosporins (cephalexin) in the oral form ($p$ value = 0.15, $\rho = 0.4636$) (Figure 6B).

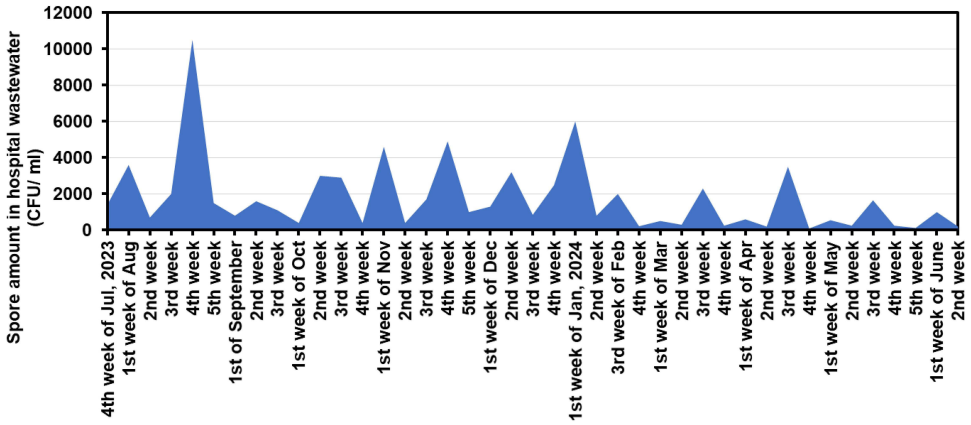


**Figure 1** *C. difficile* spores were detected in wastewater every week from July 2023 to June 2024.

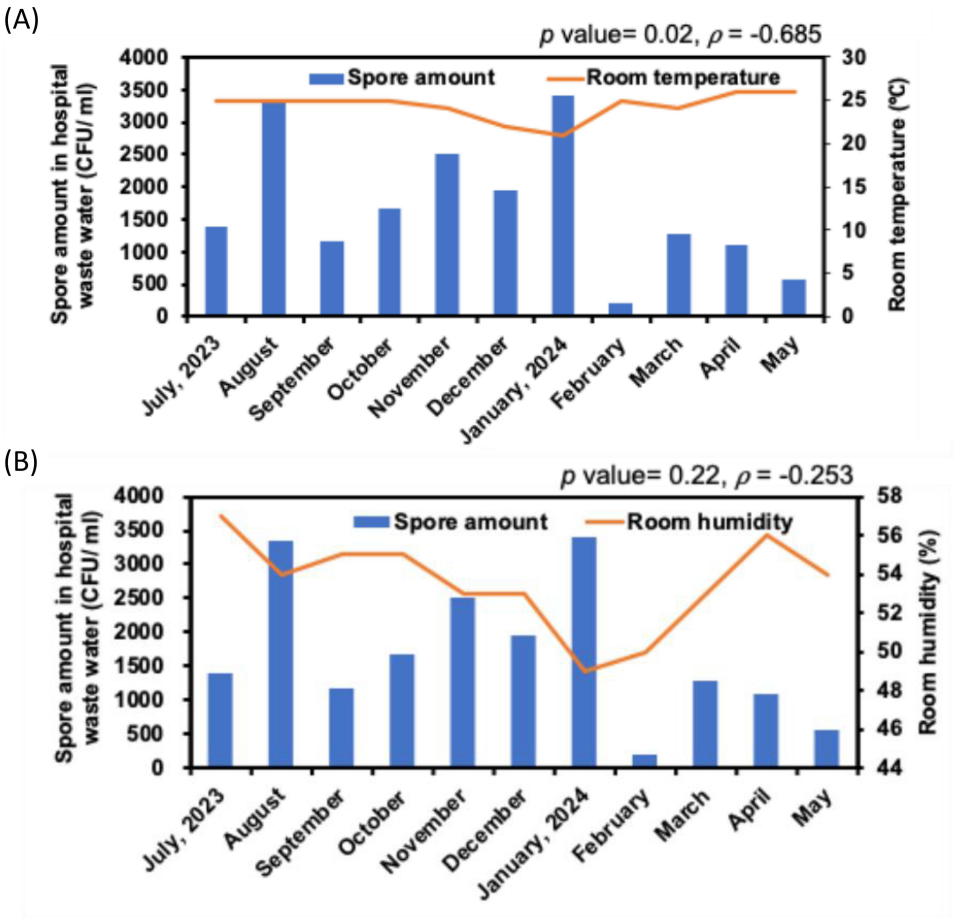


**Figure 2** Factors contributing to the detection of *C. difficile* spores in wastewater. Correlations between *C. difficile* spores detected in wastewater and room temperature (A), and room humidity (B). The linear-by-linear associations between the contributing factors and the spore amounts in wastewater were analyzed via *Pearson* correlation. A two-tailed *P* value of less than 0.05 was considered statistically significant.

## Discussion

The number of *C. difficile* spores detected in the wastewater fluctuated every week during the study period, which was primarily related to differences in antimicrobial agent consumption (Figure 7). Owing to their ability to overcome harsh environments, spores of *C. difficile* can persist in the environment even after exposure to heat, oxygen, and common disinfectants, such as ethanol-based hand sanitizers.[9–11] Therefore, *C. difficile* spores are frequently found in wastewater from wastewater treatment plants.[23,25–27] In a study of 12 wastewater treatment plants in western Australia, spores of *C. difficile* were found in 90.5% of raw sewage influent.[25] *C. difficile* spores in wastewater treatment plants might originate from different sources, including the environment, food, animals, or humans; however, the most important source is the hospital setting.[22,28] A genomic survey of *C. difficile* reservoirs in East England suggested that a major source of environmental contamination in wastewater treatment plants is the clinical settings.[22] Therefore, it is important to monitor the number of *C. difficile* spores in wastewater from hospital settings. Data concerning the weekly amount of *C. difficile* spores in wastewater from a hospital setting and the possible influencing factors analyzed in our study could help manage *C. difficile* spores in hospital wastewater.

In our study, the consumption of antimicrobial agents, especially first-generation cephalosporins, was associated with the number of spores in wastewater from the hospital setting. Almost all classes of antibiotics, including cephalosporins, penicillins, clindamycin, and quinolones, have been associated with CDI.[29–32] Cefazolin, the most popular first-generation cephalosporin, is widely used as perioperative antibiotic prophylaxis in many surgeries.[33,34] The use of cefazolin as a prophylactic antimicrobial agent has been associated with the development of fulminant CDI after


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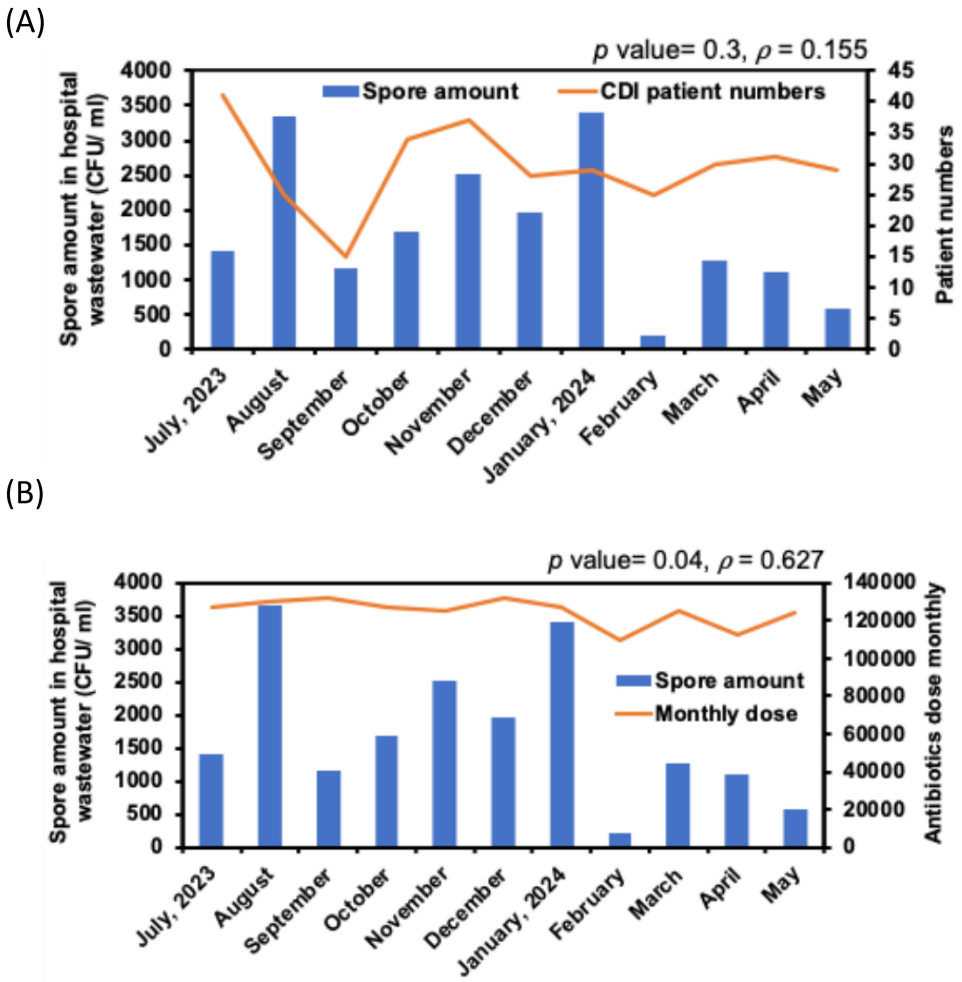


**Figure 3** Factors contributing to the detection of *C. difficile* spores in wastewater. Correlations between *C. difficile* spores detected in wastewater and patients with CDI (**A**), and antimicrobial agent consumption (**B**). The linear-by-linear associations between the contributing factors and the spore amounts in wastewater were analyzed via *Pearson* correlation. A two-tailed *P* value of less than 0.05 was considered statistically significant.

surgery.[34] Therefore, we hypothesize that the widespread use of cefazolin as a prophylactic antibiotic for surgical procedures in our hospital may contribute to an increase in *C. difficile* colonization. However, we do not have direct evidence to support this hypothesis. Although antibiotic therapy is indispensable for treating bacterial infections, it profoundly disrupts the gut microbiota—characterized by reduced microbial diversity and alterations in community composition, particularly affecting beneficial genera such as *Bifidobacterium* and *Eubacterium*.[35] These changes promote the emergence of antibiotic-resistant strains and facilitate the horizontal transfer of resistance genes.[35] Consequently, the disruption impairs colonization resistance, increases intestinal permeability, and heightens susceptibility to opportunistic pathogens such as *C. difficile*. So the reason for the association between antibiotic consumption and the development of CDI is that antibiotic use changes the indigenous intestinal microbiota and creates an environment where *C. difficile* easily colonizes the host colon and influences the bile acid composition in the colon of the host, thereby promoting the growth of *C. difficile*.[36] The correlation between antibiotic consumption and the number of *C. difficile* spores in wastewater indicates an opportunity to decrease the burden of *C. difficile* spores in wastewater through antibiotic stewardship, as up to 30% of the antibiotics prescribed in the United States are unnecessary or inappropriate.[37]

There was no association between room temperature and the number of *C. difficile* spores detected in wastewater in our study. In a population-based spatiotemporal study of CDI in Queensland, Australia, over a 10-year period, peaks in CDI were found in summer.[38] In a study of *C. difficile* spore viability in stored meat products, the change in viability was not significant at 4°C but increased significantly at 23°C.[39] In another in vitro study, the germination rate of *C. difficile*

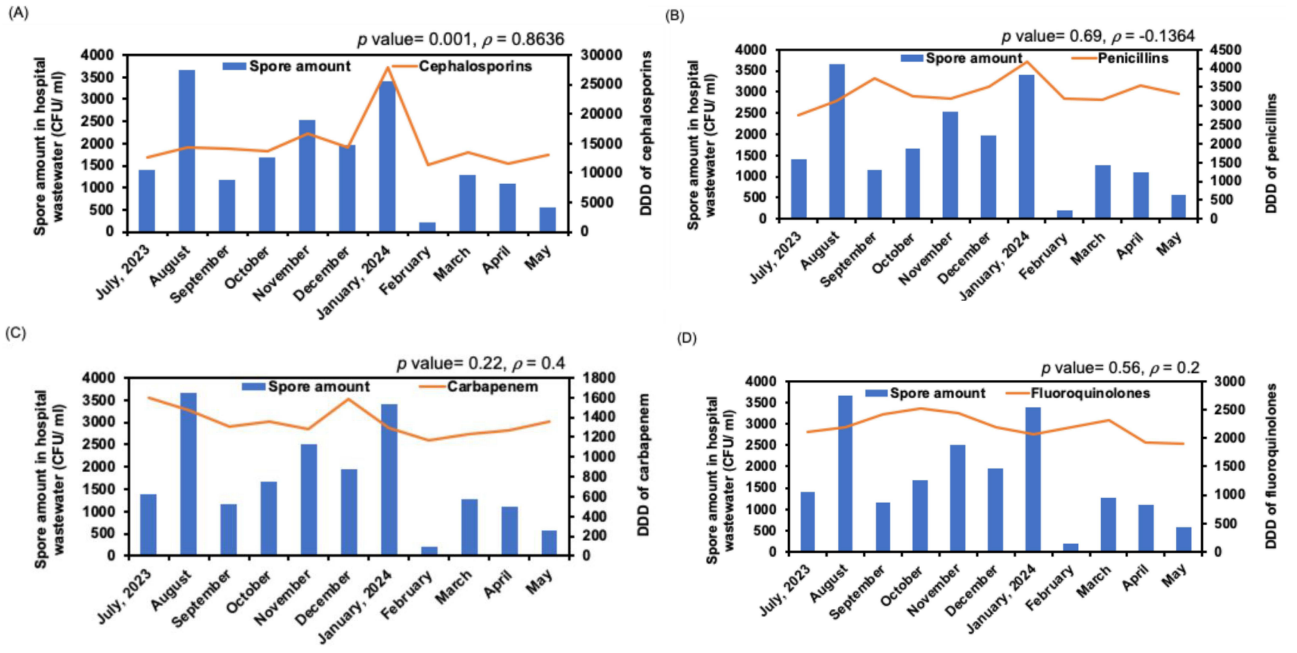


**Figure 4** Associations between the consumption of different classes of antimicrobial agents, including cephalosporins (**A**), penicillins (**B**), carbapenems (**C**), or fluoroquinolones (**D**), and the number of detected *C. difficile* spores in wastewater. The linear-by-linear associations between the contributing factors and the spore amounts in wastewater were analyzed via *Pearson* correlation. A two-tailed *P* value of less than 0.05 was considered statistically significant.

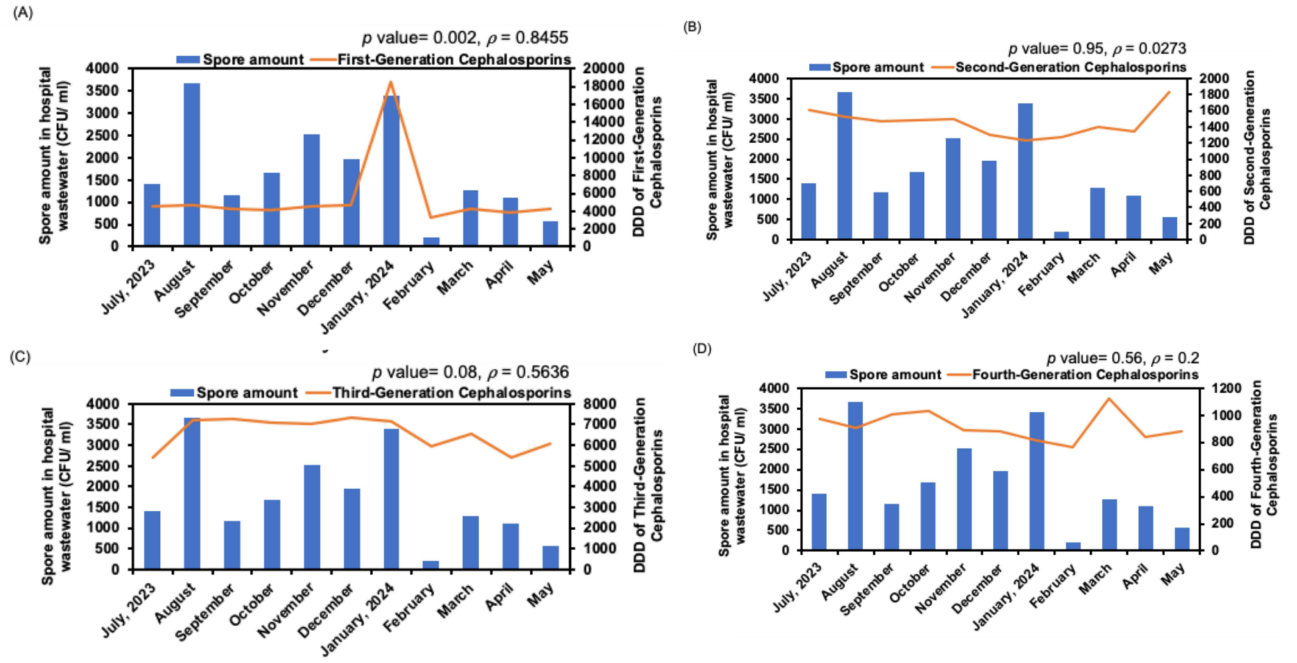


**Figure 5** The correlation between the consumption of different generations of cephalosporins and the number of detected *C. difficile* spores in wastewater, including first-generation cephalosporins (**A**), second-generation cephalosporins (**B**), third-generation cephalosporins (**C**), or fourth-generation cephalosporins (**D**). The linear-by-linear associations between the contributing factors and the spore amounts in wastewater were analyzed via Pearson correlation. A two-tailed P value of less than 0.05 was considered statistically significant.

spores was found to be significantly higher at 37°C than at 20°C.[40] Therefore, the potential impact of lowering room temperature on reducing the incidence of CDI and the concentration of *C. difficile* spores in hospital wastewater requires further evaluation.


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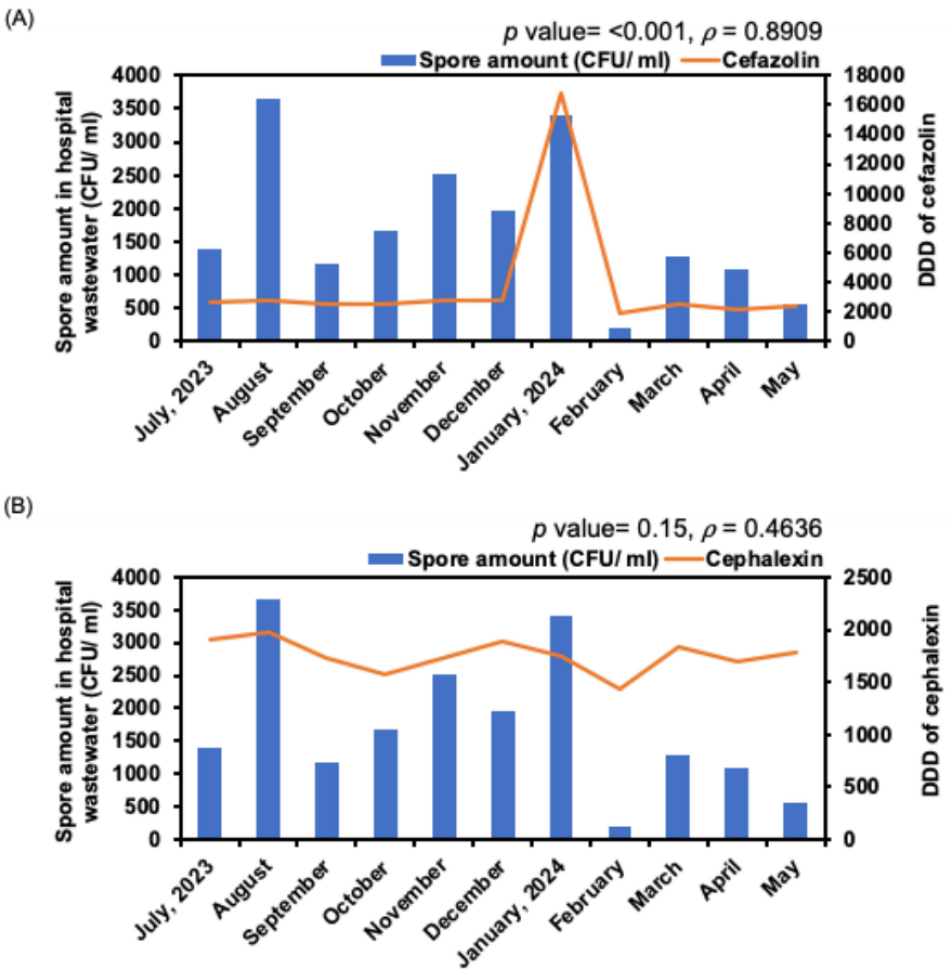


**Figure 6** Associations between *C. difficile* spores detected in wastewater and the consumption of different first-generation cephalosporins, including intravenous first-generation cephalosporins (cefazolin) (**A**) and oral first-generation cephalosporins (cephalexin) (**B**).

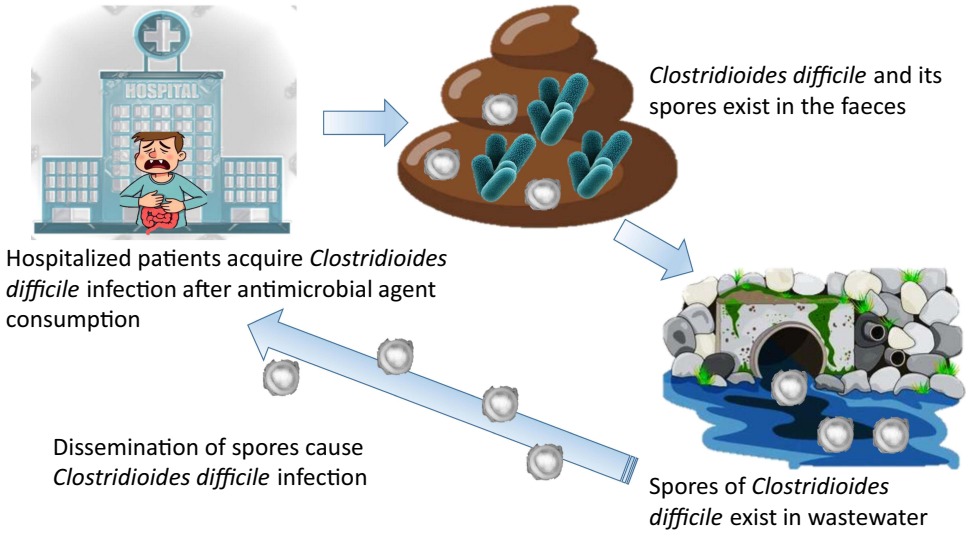


**Figure 7** The number of detected *C. difficile* spores in wastewater fluctuated every week during the study period, which was primarily related to differences in antimicrobial agent consumption.

Although *C. difficile* spore concentration in the wastewater was relatively high in our study, the data did not represent the actual number of spores emitted from hospitals into the environment. First, the spore number was calculated after adding sodium taurocholate to the wastewater to improve the spore germination. Second, the number of spores increased during the culture procedure. Third, *C. difficile* spore concentrations were analyzed in raw sewage wastewater in our study. The number of spores decreased to a small number after treatment before emission from hospitals into the environment. In a study of wastewater treatment plants in western Australia, spores of *C. difficile* were found in 90.5% of the raw sewage influent but decreased to 48.1% in the treated effluent.[25] The actual number of spores expelled from the hospital setting is expected to be very small.

This study had several limitations. First, wastewater was sampled only once per week. As there were fluctuations in the number of spores present in the wastewater at every time point, our data did not represent the number of spores present throughout the week. Continuous monitoring of all spores in wastewater is needed to determine the exact number of spores present throughout the week. Second, CDI spores can exist in the feces of colonized patients. Prospective stool cultures of all patients without diarrhea in the hospital may be needed to determine the exact number of patients with *C. difficile* colonization. Combining patients with CDI and those with *C. difficile* colonization will provide the real number of patients with *C. difficile* spores in feces. Third, the toxin genes and ribotypes of the *C. difficile* isolates from wastewater were not analyzed in our study because the sporulation ability was diverse among different *C. difficile* ribotypes. Fourth, the impact of potential confounding factors, such as differences in patient population, infection control measures, or seasonal variations in prescribing patterns, which might influence the prevalence of CDI, was not analyzed in our study. Finally, since all samples were subjected to sodium taurocholate stimulation, our focus was on the relative changes in values and their potential association with antibiotic use. However, as the reviewer rightly pointed out, these values do not represent the absolute number of spores being shed.

In conclusion, the number of *C. difficile* spores detected in wastewater from hospital settings fluctuated weekly during the study period, which was primarily related to the consumption of different antimicrobial agents, particularly cephalosporins. The effect of reducing unnecessary antibiotic use through antibiotic stewardship on decreasing the burden of *C. difficile* spores in wastewater requires further evaluation.

## Data Sharing Statement

Available from the corresponding author upon reasonable request.

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## Disclosure

The authors have no conflicts of interest to declare for this work.

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