Diagnosis of *Clostridium difficile* infection by toxigenic culture and PCR assay

Elnaze Zare Mirzaei1,2, Mahdi Rajabnia2, Farzin Sadeghi2, Elaheh Ferdosi-Shahandashti2, Mahmoud Sadeghi-Haddad-Zavareh1, Soraya Khafri4, Abolfazl Davoodabadi1,2*

1Infectious Diseases & Tropical Medicine Research Center, Babol University of Medical Sciences, Babol, Iran
2Department of Microbiology, Faculty of Medicine, Babol University of Medical Sciences, Babol, Iran
3Cellular and Molecular Biology Research Center, Health Research Institute, Babol University of Medical Sciences, Babol, Iran
4Infertility and Reproductive Health Research Center, Babol University of Medical Sciences, Babol, Iran

Received: April 2018, Accepted: August 2018

ABSTRACT

Background and Objectives: *Clostridium difficile* is responsible for 15-25% of nosocomial antibiotic associated diarrhea (AAD) cases and all cases of pseudomembranous colitis. *C. difficile* has two major virulence factors, toxin A (enterotoxin) and toxin B (cytotoxin). The aim of this study was to determine the frequency of *C. difficile* strains in patients with diarrhea in Babol’ hospitals with toxigenic culture and PCR assay.

Materials and Methods: One hundred stool specimens were taken from diarrheal patients in hospitals of the city of Babol. All patients had a history of antibiotic use. The samples were cultured on CCFA medium. In the next stage, toxigenic culture was performed for isolated *C. difficile* strains. Then, PCR assay was used to identify *gdh*, *tcdA* and *tcdB* genes among isolated *C. difficile* strains.

Results: From the 100 stool samples, eight (8%) samples were positive in *C. difficile* culture. In toxigenic culture, two (2%) of these strains had cytopathic effects on Vero cells. All eight strains had the *gdh* gene. This gene is specific for *C. difficile*. Two strains that had cytopathic effects on toxigenic culture were positive for toxin genes.

Conclusion: The frequency of toxigenic strains in different parts of the world is variable, and needs to be continually investigated. In the present study, the PCR method had a good correlation with toxigenic culture. Thus, it can replace the laborious and costly cell culture method.

Keywords: Antibiotic associated diarrhea, *Clostridium difficile*, Polymerase chain reaction, Toxigenic culture

*Corresponding author: Abolfazl Davoodabadi, Ph.D, Infectious Diseases & Tropical Medicine Research Center, Babol University of Medical Sciences, Babol, Iran; Department of Microbiology, Faculty of Medicine, Babol University of Medical Sciences, Babol, Iran. Tel: +9811-32199592 Fax: +9811-32190181 Email: davooodabadi89@gmail.com
INTRODUCTION

*Clostridium difficile* is an important cause of nosocomial infections. Symptoms of *C. difficile* infection (CDI) range from asymptomatic carriage to mild diarrhea, colitis, severe life threatening pseudomembranous colitis and to fulminant colitis (1, 2). This microorganism is responsible for 15-25% of nosocomial antibiotic associated diarrhea (AAD) cases and all cases of pseudomembranous colitis (3, 4). The most predisposing factors for CDI include prior antibiotic therapy, age older than 65 years, and recent long-term hospitalization (5). *C. difficile* expresses two major virulence factors, which are toxin A (enterotoxin) and toxin B (cytotoxin) encoded via tcdA and tcdB genes respectively (6, 7).

There are various tests for diagnosis of CDI in laboratories. Some of these tests are enzyme immunoassay (EIA), glutamate dehydrogenase (GDH), cytotoxicity assay (CA), toxigenic culture and PCR. Enzyme immunoassay is a rapid method and is done directly on stool samples. Although this test is very fast, it has very low sensitivity (8). The GDH test detects glutamate dehydrogenase enzyme in the cell wall of *C. difficile*. GDH is considered a screening method because it is expressed by both the toxigenic and nontoxigenic strains. GDH is a very rapid, inexpensive and easy method. Unlike toxin A and B tests, this test has high sensitivity but low specificity. However, to confirm positive GDH test results, complementary tests are needed (9).

The cell culture cytotoxicity assay (CA), based on toxin B detection is the gold standard for diagnosis of CDI. However, this test is not routinely used by clinical microbiology laboratories, because it requires cell culture facilities and a reliable antitoxin for neutralization (10, 11). The PCR is used to detect toxin A or toxin B genes in strains or directly in fecal samples and in terms of sensitivity is similar to the cytotoxicity assay. In comparison to the cytotoxicity assay, PCR is a very fast method for the diagnosis of CDI (12). To the best of our knowledge, no study to date has examined *C. difficile* infection in patients with diarrhea in the hospitals of Babol. The aim of the present study was to determine the frequency of *C. difficile* infection in patients with diarrhea in the hospitals of Babol with toxigenic culture and PCR.

One hundred stool specimens were taken from diarrheal patients in hospitals of Babol. All patients had a history of antibiotic use, and demographics data of patients were collected via a questionnaire. The specimens were immediately transferred to the microbiology laboratory at Babol University of Medical Sciences. About one gram of stool specimen was suspended in a tube containing one mL of BHI broth (Merck, Germany) and one mL of ethanol 96% (ethanol shock) for 45 min. Then this suspension was cultured on CCFA (cycloserine-cefoxitin fructose agar; Merck, Germany) under anaerobic condition at 37°C for 72 h. The colonies which contained Gram-positive bacilli with 1-3 mm diameter, white to gray color, and horse odour were regarded as *C. difficile*. The isolated strains were stocked in BHI broth containing 15% glycerol and stored at -20°C.

Toxigenic culture. In the next stage, toxigenic culture was performed for isolated *C. difficile* strains. Vero cells were grown in a flask containing Dulbecco's modified Eagle's medium (DMEM; Gibco), 100 U/ml penicillin-streptomycin and 10% fetal bovine serum (FBS Gibco), and incubated at 37°C and 5% CO₂ for 3-5 days. The cells were trypsinized and counted. About 10,000 cells were added to the wells of microtiterplate and were incubated at 37°C and 5% CO₂ for 24 h to reach about 80% confluency.

*C. difficile* strains were cultured in BHI broth for 5-7 days at 37°C, then the culture medium was centrifuged (10 min at 1500 g). The obtaining supernatants were filtered (0.22 μm pore size), and 200 μl of filtrate was added to Vero cell culture (96-well microtiter plate; Biofil, China). The microtiter plate was incubated for 24-48 h at 37°C and 5% CO₂. *C. difficile* strains which produce toxin (positive result), cause cytopathic effects in more than 50% of the cell monolayer. Supernatant obtained from a toxigenic *C. difficile* strain, which was previously isolated from a diarrheal patient, was used as a positive control in toxigenic culture test (13).

Identification of gdh, tcdA and tcdB genes by PCR assay. DNA extraction was performed by boiling methods (14). A single colony from every isolate was suspended in 50 mL of TES buffer (containing 50 mM Tris hydrochloride [pH 8.0], 5 mM EDTA, 50 mM NaCl), and the suspension was heated in a boiling water bath at 95°C for 10 min and centrifuged at 15,000 × g for 3 min. The resultant supernatant was
used as DNA template. Extracted DNA was stored at -20°C. *C. difficile* and its toxins were identified by PCR method targeting the *gdh* (glutamate dehydrogenase), *tcdA* and *tcdB* genes. The primers used in this study are listed in Table 1.

For each gene, the PCR was run in 20 μL reaction mixture containing 10 μL master mix PCR, 2 μL DNA template, 20 pmol of each primer and 6.4 μL PCR grade water. PCR was performed in a thermocycler (A & E, England) using the following conditions: 5 min at 95°C, followed by 30 cycles of 1 min s at 94°C, 1 min at 54°C for *gdh* gene, 1 min at 56°C for toxin genes, 1 min at 72°C, and a final extension of 10 min at 72°C. The presence of each gene was determined by electrophoresis on a 1.5% agarose gel. In each PCR run, DNA template from a toxigenic *C. difficile* and water were used as positive and negative controls, respectively.

RESULTS

From the 100 patients with diarrhea, 45 (45%) cases were males and 55 (55%) cases were females. The stool samples were obtained from ICU ward (62%), infectious ward (15%) and other wards including respiratory, hematology, neurology, gastroenterology and internal medicine (23%). From the 100 stool samples, eight (8%) samples were positive in *C. difficile* culture. In toxigenic culture, two (2%) of these strains, had cytopathic effects (CPE) on Vero cells (Fig. 1). Cytopathic effects of *C. difficile* toxins on spindle form Vero cells characterized by rounding up these cells. Demographic data of eight patients with positive *C. difficile* culture is shown in Table 2. A toxigenic *C. difficile* strain was isolated from a woman in infectious ward, and another toxigenic strain isolated from a man in ICU ward.

All eight strains had the *gdh* gene (Fig. 2). This gene is specific for *C. difficile* (2). Among these eight strains, two strains that previously had cytopathic effects on toxigenic culture were positive for toxin genes. The strain isolated from the feces of the 49-year-old man was positive for *tcdA* and *tcdB* genes, and the strain isolated from the feces of a 23-year-old female patient was positive for the *tcdB* gene only (Fig. 3, Table 2).

DISCUSSION

Infections with *C. difficile* have significantly increased over the past two decades (17). Infections with this organism have been reported from Australia (5), European countries (18) and the United States (19). *C. difficile* infections usually occur after treatment with antibiotics in hospitalized patients. Antibiotics such as β-lactams and clindamycin decrease the normal flora of the intestine and ultimately cre-

![Fig. 1. Cytopathic effects (CPE) of *C. difficile* supernatant on Vero cells. A; Toxin negative *C. difficile* B; Toxin positive *C. difficile*. CPE: Toxins deform Vero cells from spindle form to round form in more than 50% of the cells.](image-url)

Table 1. Primers used in this study.

| Genes | Primer name | Sequence (5′–3′) | Amplicon size (bp) | References |
|-------|-------------|-----------------|-------------------|------------|
| *gdh* | *gdh R*     | CTGATTTACACCATTCCAGCCCATAGC | 736 | (15) |
|       | *gdh F*     | GGAAAGATGTAAATGTCTTCGAGATG | 629 | (16) |
| *tcdA*| *tcdA-F3345*| GCAATAGGACAACCTTAGTTGA | 629 | (16) |
|       | *tcdA-R3969*| AGTTCCCTGCTCCATCAATTGA | 629 | (16) |
| *tcdB*| *tcdB-R6079A*| GCATTCTCCATTCTCAGCAAAGTA | 410 | (16) |
|       | *tcdB-F5670*| CCAAARTGGAGTGTTAACAAACAGGTG | 410 | (16) |

http://ijm.tums.ac.ir

IRAN. J. MICROBIOL. Volume 10 Number 5 (October 2018) 287-293 289
iate the condition for further growth of *C. difficile* in the intestine as well as the development of clinical symptoms associated with this infection (20, 21). The pathogenicity of this bacterium is related to two major virulence factors of enterotoxin A and cytotoxin B (22, 23). Strains that have the ability to only produce toxin B are clinically important (24, 25).

This study for the first time examined the frequency of toxigenic *C. difficile* strains in hospitalized patients in Babol hospitals. The frequency of toxin positive *C. difficile* among diarrheal patients by toxigenic culture and PCR was 2%. In 2016, Lotfian et al. studied 171 samples of suspected cases of diarrhea associated with *C. difficile* in Tehran. Their results showed that 10 (5.8%) samples were positive with both PCR and toxigenic culture. Of these 10 strains, 8 strains were *tcdA + B* + and 2 strains were *tcdA-B* +. They found very good agreement between toxigenic culture and PCR. Our study, similar to Lotfian et al., showed that PCR has good agreement with the toxigenic culture method (26).

Another study by Sadeghifard et al. in 2004 was carried out on stool specimens from patients with diarrhea in Tehran hospitals. In their report, the prevalence of toxin producing *C. difficile* by the cytotoxicity method was 6.1% (27). Azizi et al., in 2011, studied 98 diarrheal patients in Tehran and reported 39.8% (39) samples as *C. difficile* culture-positive.

**Table 2.** Demographic data of eight patients with positive *C. difficile* culture.

| Male / Female | Age | Hospital ward | Length of admission (days) | Antibiotic used | Toxigenic culture | Gene |
|---------------|-----|---------------|----------------------------|-----------------|-------------------|------|
| F             | 23  | Infectious    | 10                         | Clindamycin Ceftriaxone | +                 | gdh, tcdB   |
| M             | 49  | ICU           | 16                         | Levofloxacin     | +                 | gdh, tcdA, tcdB |
| F             | 72  | ICU           | 8                          | Ceftriaxone      | -                 | gdh          |
| F             | 80  | ICU           | 44                         | Ciprofloxacin-Cefepime | -              | gdh          |
| F             | 77  | ICU           | 20                         | Meropenem-Ciprofloxacin | -              | gdh          |
| F             | 79  | ICU           | 10                         | Meropenem-Vancomycin-Ciprofloxacin | -       | gdh          |
| F             | 35  | ICU           | 12                         | Meropenem-Levofloxacin-Nitromycin-Ampipotericin-Fluconazole-Cotrimoxazole | -       | gdh          |
| M             | 80  | ICU           | 47                         | Cefepime-Colistin-Levofloxacin-Erythromycin-Fluconazole     | -       | gdh          |

Fig. 2. PCR products of the *gdh* gene. M: DNA size marker, 100 base pair, Lanes 1, 2, and 3: PCR products of the *gdh* gene (736 bp) for three *C. difficile* strains. P: Positive control sample, N: Negative control sample.

Fig. 3. PCR products of *tcdB* and *tcdA* genes. M: DNA size marker, 100 base pair, Lane 1: PCR product of *tcdB* gene (410 bp), 2: PCR product of *tcdA* gene (629 bp), Pa: positive control for *tcdB* gene, Pb: positive control for *tcdA* gene. N: Negative control sample.
Among 39 *C. difficile* strains, 15 (15.3%) strains were positive for toxin genes, 12 (12.2%) strains had toxin B and A, two (2%) strains had only toxin A (A+B-) and one (1%) strains had only toxin B (A-B+) (28).

In another study in Iran, conducted by Goodarzi et al. in 2012, 108 patients with diarrhea were studied at Taleghani Hospital in Tehran. From 108 patients, 17 (15.7%) toxigenic strains were isolated. Among the 17 strains, four (23.9%) strains had only toxin B (A-B+), one (5.9%) strain had only toxin A (A+B-), and 12 (70%) strains had both toxins (A+B+) (29).

In the present study, frequency of toxigenic strains of *C. difficile* isolated from the patients with diarrhea in Babol was low (2%). In the study by Sadeghifard et al. and Lotfian et al., similar to the present study, the prevalence of toxigenic strains of *C. difficile* was reported low (6.1% and 5.8%, respectively) (30).

Prevalence of diarrhea in a Turkish hospital was reported 7% of all hospital infections and *C. difficile* was isolated from 18.2% of hospitalized patients with diarrhea (31). In a study by Garcia et al., which was conducted in Brazil, prevalence of toxigenic strains of *C. difficile* in diarrheal patients was reported at 13.8% (32). Sachu et al., in 2018, studied 660 patients with AAD in India, and they identified *C. difficile* infection in 9.7% patients by NAAT (33).

The prevalence of toxigenic strains of *C. difficile* in other countries like India (34), Indonesia (35), and Germany (36) were reported as 4%, 5.6%, and 11.1%, respectively.

Due to geographical changes, diarrhea accounts for 1-14% of all hospital infections worldwide (30). Prevalence of *C. difficile* diarrhea differ according to the population of different hospitals and is affected by predisposing factors such as age, type and duration of antibiotic use, severity of underlying diseases and duration of admission (37, 38). In this study, for the first time *C. difficile* infection was identified in patients with diarrhea in Babol hospitals. PCR assay had good correlation with toxigenic culture, therefore, it can replace the laborious and costly cell culture method.

ACKNOWLEDGEMENTS

This study was supported by the Babol University of Medical Science (BUMS), Babol, Iran (Grant no. 9541916). We would like to thank Dr Masoumeh Douraghi from Tehran University of Medical Sciences, for gifting us a toxigenic *C. difficile* strain.

REFERENCES

1. De Boer E, Zwartkruis-Nahuis A, Heuvelink AE, Harmanus C, Kuijper EJ. Prevalence of *Clostridium difficile* in retailed meat in the Netherlands. *Int J Food Microbiol* 2011;144:561-564.

2. Dubberke ER, Wertheimer AI. Review of current literature on the economic burden of *Clostridium difficile* infection. *Infect Control Hosp Epidemiol* 2009;30:57-66.

3. Landelle C, Verachten M, Legrand P, Girou E, Barbut F, Buisson CB. Contamination of healthcare workers’ hands with *Clostridium difficile* spores after caring for patients with *C. difficile* infection. *Infect Control Hosp Epidemiol* 2014;35:10-15.

4. Bowman KA, Broussard EK, Surawicz CM. Fecal microbiota transplantation: current clinical efficacy and future prospects. *Clin Exp Gastroenterol* 2015;8:285-291.

5. Davies KA, Longshaw CM, Davis GL, Bouza E, Barbut F, Barna Z, et al. Underdiagnosis of *Clostridium difficile* across Europe: the European, multicentre, prospective, biannual, point-prevalence study of *Clostridium difficile* infection in hospitalised patients with diarrhoea (EUCLID). *Lancet Infect Dis* 2014;14:1208-1219.

6. Deneve C, Janoir C, Poilane I, Fantinato C, Collignon A. New trends in *Clostridium difficile* virulence and pathogenesis. *Int J Antimicrob Agents* 2009;33:S24-28.

7. Bauer MP, van Dessel JT. Alternative strategies for *Clostridium difficile* infection. *Int J Antimicrob Agents* 2009;33:S51-56.

8. Russello G, Russo A, Sisto F, Scaltrito MM, Farina C. Laboratory diagnosis of *Clostridium difficile* associated diarrhoea and molecular characterization of clinical isolates. *New Microbiol* 2012;35:307-316.

9. Hawkins MR, Drew RH, Lewis SS, Anderson DJ, Sexton DJ, Moehring RW, editors. 219 Characteristics of Antimicrobial Stewardship (AS) Activities in Community Hospitals Upon Enrollment in the Duke Antimicrobial Stewardship Outreach Network (DASON). *Open Forum Infect Dis*; 2014: Oxford University Press.

10. Balamurugan R, Balaji V, Ramakrishna BS. Estimation of faecal carriage of *Clostridium difficile* in patients with ulcerative colitis using real time polymerase chain reaction. *Indian J Med Res* 2008;127:472-477.

11. Lemée L, Dhalluin A, Testelin S, Mattrat M-A, Mail-
lard K, Lemeland J-F, et al. Multiplex PCR targeting tpi (triose phosphate isomerase), tcdA (Toxin A), and tcdB (Toxin B) genes for toxigenic culture of Clostridium difficile. J Clin Microbiol 2004;42:5710-5714.

12. Liu Y-L, Lu X-H, Tang YJ, Lam K, Silva J, Leung JW. Polymerase chain reaction for identification and typing of Clostridium difficile isolated from Chinese patients. Int J Infect Dis 1997;2:85-87.

13. Pancholi P, Kelly C, Raczkowski M, Balada-Llasat J. Detection of toxigenic Clostridium difficile: comparison of the cell culture neutralization, Xpert C. difficile, Xpert C. difficile/Epi, and Illumigene C. difficile assays. J Clin Microbiol 2012;50:1331-1335.

14. Kato H, Kato N, Watanabe K, Iwai N, Nakamura H, Yamamoto T, et al. Identification of toxin A-negative, toxin B-positive Clostridium difficile by PCR. J Clin Microbiol 1998;36:2178-2182.

15. Zheng L, Keller S, Lyerly D, Carman R, Genheimer C, Gleave C, et al. Multicenter evaluation of a new screening test that detects Clostridium difficile in fecal specimens. J Clin Microbiol 2004;42:3837-3840.

16. Persson S, Torp Dahl M, Olsen K. New multiplex PCR method for the detection of Clostridium difficile toxin A (tcdA) and toxin B (tcdB) and the binary toxin (cdtA/cdtB) genes applied to a Danish strain collection. Clin Microbiol Infect 2008;14:1057-1064.

17. Khanna S, Pardi DS. The growing incidence and severity of Clostridium difficile infection in inpatient and outpatient settings. Expert Rev Gastroenterol Hepatol 2010;4:409-416.

18. Worth L, Spelman T, Bull A, Brett J, Richards M. Epidemiology of Clostridium difficile infections in Australia: enhanced surveillance to evaluate time trends and severity of illness in Victoria, 2010-2014. J Hosp Infect 2016;93:280-285.

19. Davis BM, Yin J, Blomberg D, Fung IC-H. Impact of a prevention bundle on Clostridium difficile infection rates in a hospital in the Southeastern United States. Am J Infect Control 2016;44:1729-1731.

20. Geric B, Rupnik M, Gerding DN, Grabnar M, Johnson S. Distribution of Clostridium difficile variant toxino-types and strains with binary toxin genes among clinical isolates in an American hospital. J Med Microbiol 2004;53:887-894.

21. Bourgault A-M, Lamothe F, Luo VG, Poirier L, Group C-Cs. In vitro susceptibility of Clostridium difficile clinical isolates from a multi-institutional outbreak in Southern Quebec, Canada. Antimicrob Agents Chemother 2006;50:3473-3475.

22. Wozniak G, Trontelj P, Rupnik M. Genomic relatedness of Clostridium difficile strains from different toxino-types and serogroups. Anaerobe 2000;6:261-267.

23. Sambol SP, Merrigan MM, Lyerly D, Gerding DN, Johnson S. Toxin gene analysis of a variant strain of Clostridium difficile that causes human clinical disease. Infect Immun 2000;68:5480-5487.

24. Rupnik M, Grabnar M, Geric B. Binary toxin producing Clostridium difficile strains. Anaerobe 2003;9:289-294.

25. Van den Berg RJ, Claas EC, Obyh DH, Klaassen CH, Dijkstra L, Brazier JS, et al. Characterization of toxin A-negative, toxin B-positive Clostridium difficile isolates from outbreaks in different countries by amplified fragment length polymorphism and PCR ribotyping. J Clin Microbiol 2004;42:1035-1041.

26. Lotfian S, Douraghi M, Aliramezani A, Ghourchian S, Sarrafnejad A, Zeraati H. Detection of Clostridium difficile in Fecal Specimens: a comparative evaluation of nucleic acid amplification test and toxigenic culture. Clin Lab 2016;62:1887-1892.

27. Sadeghfirad N, Salari M, Ghassemi M, Shirazi M, Feizabadi M, Kazemi B, et al. Prevalence of Clostridium difficile-associated diarrhea in hospitalized patients with nosocomial diarrhea. Iran J Public Health 2005;34:67-72.

28. Azizi O, Aslani MM, Azimi Rad M, Albouyeh M, Mousavi SF, Zali MR. The frequency of toxigenic strains of Clostridium difficile in hospitalized patients with diarrhea in Tehran/Iran by PCR method, 2010. J Kerman Univ Med Sci 2013;20:129-137.

29. Goudarzi M, Goudarzi H, Albouyeh M, Azimi Rad M, Zali MR, Aslan MM. Molecular typing of Clostridium difficile isolated from hospitalized patients by PCR ribotyping. Research in Medicine 2012;36:68-75.

30. Zaidi M, de León SP, Ortiz RM, de León SP, Calva JJ, Ruiz-Palacios G, et al. Hospital-acquired diarrhea in adults: a prospective case-controlled study in Mexico. Infect Control Hosp Epidemiol 1991;12:349-355.

31. Goren I, Dizer U, Besirbilliong B, Eyigun C, Hacibektasoglu A, Van Thiel D. The diagnosis and treatment of Clostridium difficile in antibiotic-associated diarrhea. Hepatogastroenterology 1999;46:343-348.

32. Garcia LB, Uzeda Md. Occurrence of Clostridium difficile in fecal samples of children in Rio de Janeiro, RJ. Rev Inst Med Trop Sao Paulo 1988;30:419-423.

33. Sachu A, Dinesh K, Siyad I, Kumar A, Vasudevan A, Karim S. A prospective cross sectional study of detection of Clostridium difficile toxin in patients with antibiotic associated diarrhea. Iran J Microbiol 2018;10:1-6.

34. Segar L, Easow JM, Srirangaraj S, Hanifi M, Joseph NM, Seetha K. Prevalence of Clostridium difficile infection among the patients attending a tertiary care teaching hospital. Indian J Pathol Microbiol 2017;60:221-225.

35. Collins DA, Gasem MH, Habibie TH, Arinton I, Hendriyanto P, Hartana AP, et al. Prevalence and molecular epidemiology of Clostridium difficile infection in Indo-
36. Arvand M, Rascher C, Bettge-Weller G, Goltz M, Pfeifer Y. Prevalence and risk factors for colonization by *Clostridium difficile* and extended-spectrum β-lactamase-producing Enterobacteriaceae in rehabilitation clinics in Germany. *J Hosp Infect* 2018;98:14-20.

37. Kuijper E, Coignard B, Tüll P. Emergence of *Clostridium difficile*-associated disease in North America and Europe. *Clin Microbiol Infect* 2006;12 Suppl 6:2-18.

38. Goudarzi M, Seyedjavadi SS, Goudarzi H, Mehdizadeh Aghdam E, Nazeri S. *Clostridium difficile* infection: epidemiology, pathogenesis, risk factors, and therapeutic options. *Scientifica (Cairo)* 2014;2014:916826.