

Association of *tcdA*+/*tcdB*+ *Clostridium difficile* Genotype with Emergence of Multidrug-Resistant Strains Conferring Metronidazole Resistant Phenotype

Farahnaz-Sadat Shayganmehr^{1,2}, Masoud Alebouyeh^{*1,3}, Masoumeh Azimirad^{1,3},
Mohammad Mehdi Aslani^{1,4} and Mohammad Reza Zali^{1,3}

¹Foodborne and Waterborne Diseases Research Center, Research Institute for Gastroenterology and Liver Diseases, Shahid Beheshti University of Medical Sciences, Tehran, Iran; ²Dept. of Microbiology, Science and Research Branch, Islamic Azad University, Fars, Iran; ³Gastroenterology and Liver Diseases Research Center, Research Institute for Gastroenterology and Liver Diseases, Shahid Beheshti University of Medical Sciences, Tehran, Iran; ⁴Dept. of Microbiology, Pasteur Institute of Iran, Tehran, Iran

Received 3 April 2014; revised 19 November 2014; accepted 23 November 2014

ABSTRACT

Background: Reduced susceptibility of *Clostridium difficile* to antibiotics is problematic in clinical settings. There is new evidence indicating the cotransfer of toxin-encoding genes and conjugative transposons encoding resistance to antibiotics among different *C. difficile* strains. To analyze this association, in the current study, we evaluated the frequency of toxigenic *C. difficile* among the strains with different multidrug-resistant (MDR) profiles in Iran. **Methods:** Antimicrobial susceptibility patterns and minimal inhibitory concentrations (MIC) of the isolates were determined against metronidazole, imipenem, ceftazidime, amikacin, and ciprofloxacin by agar dilution method. The association of the resistance profiles and toxigenicity of the strains were studied by PCR targeting *tcdA* and *tcdB* genes. **Results:** Among 86 characterized strains, the highest and lowest resistance rates were related to ciprofloxacin (97%) and metronidazole (5%), respectively. The frequency of resistance to other antibiotics was as follow: imipenem (48%), ceftazidime (76%), and amikacin (76.5%). Among the resistant strains, double drug resistance and MDR phenotypes were detected in the frequencies of 10.4% and 66.2%, respectively. All of the metronidazole-resistant strains belonged to *tcdA*⁺/*tcdB*⁺ genotype with triple or quintuple drug resistance phenotypes. MIC₅₀ and MIC₉₀ for this antibiotic was equally ≤ 8 μ g/ml. **Conclusion:** These results proposed the association of *tcdA*⁺/*tcdB*⁺ genotype of *C. difficile* and the emergence of resistance strains to broad-spectrum antibiotics and metronidazole. *Iran. Biomed. J.* 19 (3): 143-148, 2015

Keywords: Multidrug resistance, *Clostridium difficile*, Metronidazole

INTRODUCTION

Clostridium difficile is an anaerobic, spore-forming, Gram-positive bacterium that is able to colonize the human intestinal tract [1]. Infection with this bacterium can be induced through the consumption of contaminated foods or during hospitalization. The infection shows both colonic and extracolonic symptoms. The colonic infestations vary from asymptomatic state to diarrhea, simple colitis, pseudomembranous colitis, fulminant colitis with perforation, prolonged ileus, megacolon, and death [2, 3]. The extracolonic features include small bowel *C. difficile*-associated diseases (CDAD), bacteremia, and reactive arthritis [4]. The main

virulence factors that usually initiate the disease symptoms are two potent toxins, toxin A (enterotoxin) and toxin B (cytotoxin) [5].

In most healthy individuals, the growth of *C. difficile* is controlled by the normal microbiota of the intestine, but in disease conditions, the use of antibiotics and medications, such as proton pump inhibitors, possibly cause the bacterium to proliferate [6]. The emergence of resistant strains of *C. difficile* to different antibiotics is now a reason of great concern worldwide [7]. Despite of the evidence on reduced sensitivity of *C. difficile* strains to common therapeutic regimens, the administration of metronidazole is still considered as the best medicine for treatment of the infections caused by this bacterium [8-10]. The effectiveness of this

*Corresponding Author; Tel.: (+98-21) 2243 2518; Fax: (+98-21) 2243 2517; E-mail: masoud.alebouyeh@gmail.com

Table 1. The ranges of MIC values and MIC₅₀/MIC₉₀ results for 86 *C. difficile* isolates

Antibiotic	MIC Ranges (µg/ml)* n (%)					MIC ₅₀ (µg/ml)	MIC ₉₀ (µg/ml)
Metronidazole	≤8 82 (95%)	16 0 (0%)	32 4 (5%)			≤8	≤8
Amikacin	≤16 17 (20%)	32 3 (3.5%)	64 20 (23%)	≥128 46 (53.5%)		≥128	≥128
Ceftazidime	≤16 21 (24%)	32 10 (12%)	64 20 (23%)	≥128 35 (41%)		64	≥128
Imipenem	≤4 10 (12%)	8 35 (40%)	16 24 (28%)	≥32 17 (20%)		8	≥32
Ciprofloxacin	<4 3 (3%)	4 22 (26%)	8 29 (34%)	16 11 (13%)	≥32 21 (24%)	8	≥32

*The MIC values were determined by agar dilution method using no. 0.5 McFarland standard suspension for each isolate on Brucella agar medium containing 7% defibrinated sheep blood and defined serial two-fold concentrations of each drug.

treatment is considerably challenged with the emergence of new epidemic multidrug resistance (MDR) strains in some countries [11]. The MDR strains are matters of serious concern in hospitals that creates an extensive problem in the management of infected patients [11].

There is new evidence suggesting the cotransfer of toxin-encoding genes and conjugative transposons encoding resistance to antibiotics among different *C. difficile* strains [12]. This study has shown that three transfer-proficient conjugative transposons in the *C. difficile* genome are close to its pathogenicity locus, which encodes toxins TcdA and TcdB. The epidemiology of *C. difficile*-associated infections, their virulence properties, and antimicrobial resistance will provide new insights to design the best treatment strategies against infections with these strains in different geographic regions. Therefore, in the current study, we aimed to analyze the association between the MDR phenotypes and toxin genotypes of *C. difficile* strains which may infect hospitalized patients under the administering prophylactic antibiotics for hospital acquired infections.

MATERIALS AND METHODS

Patients and bacterial strains. A total of 86 suspicious isolates of *C. difficile* collected from fecal samples of hospitalized patients with intestinal disorders were studied in a referral laboratory at Taleghani Hospital in Tehran, Iran. Bacterial cultivation was carried out on proper culture media (*C. difficile* medium, Mast, United Kingdom) supplemented with 7% horse blood and selective

components. The cultured plates were incubated at 37°C for at least 48-72 h under anaerobic conditions (Anoxomat, MART Microbiology, the Netherlands). The grown colonies were initially characterized based on their colony and cell morphologies and common biochemical test reactions [1]. Further identification of the strains was performed by PCR using specific primers [13].

DNA extraction and molecular identification. DNA extraction from the bacterial strains was carried out using boiling method [13]. For the identification of the suspected colonies, PCR to detect the *cdd3* gene fragment was amplified by PCR and specific primer pairs Tim6 and Struppi6, as described by Spigaglia *et al.* [14]. To analyze any relationship between the frequency of resistance phenotypes and genotypes of the strains for toxin A and toxin B, *tcdA* and *tcdB* genes were amplified by PCR as described previously [14].

Determination of antibiotic susceptibility patterns and minimal inhibitory concentrations (MIC). To analyze the susceptibility of the isolates to metronidazole and common CDAD-associated antibiotics (amikacin, imipenem, ceftazidime, and ciprofloxacin), the standard agar dilution method was used according to the clinical and laboratory standard institute guideline [15]. Fresh colonies of each isolate were suspended in a sterile saline buffer (No. 0.5 McFarland standard), and 20 µl of bacterial suspensions were inoculated onto Brucella agar medium plates (Merck Co, Germany) supplemented with 7% defibrinated sheep blood and defined serial two-fold concentrations of each drug (Table 1). The

plates were incubated in an anaerobic jar at 37°C for 24-48 h. MIC of each antibiotic was determined after 48 h of incubation [16]. Cut-off concentrations of ≥ 32 $\mu\text{g/ml}$ for metronidazole, ≥ 4 $\mu\text{g/ml}$ for ciprofloxacin, ≥ 16 $\mu\text{g/ml}$ for imipenem, ≥ 64 $\mu\text{g/ml}$ for amikacin, and ≥ 32 $\mu\text{g/ml}$ for ceftazidime were considered as definitive criteria for the detection of the resistant strains (Table 1) [15].

Statistical analysis. Chi-square and Fisher's exact tests were used to analyze the data. A *P* value less than 0.05 was considered statistically significant.

RESULTS

The current study provides a comprehensive analysis of the antibiotic resistance in 86 *C. difficile* clinical strains collected from different hospitals in Tehran (Iran) during a prospective study in 2011. All the suspected isolates were confirmed as *C. difficile* either by conventional or molecular methods. According to the defined MIC break points, resistance rates among the 86 strains were 97% for ciprofloxacin, 48% for imipenem, 76.5% for amikacin, 76% for ceftazidime, and 5% for metronidazole. The MIC values for each antibiotic are shown in Table 1. The metronidazole

MIC at which 50% (MIC₅₀) and 90% (MIC₉₀) of the tested isolates were inhibited was equally ≤ 8 $\mu\text{g/ml}$. Higher MIC₉₀ values were found for ciprofloxacin (> 32 $\mu\text{g/ml}$), imipenem (32 $\mu\text{g/ml}$), ceftazidime (≥ 128 $\mu\text{g/ml}$), and amikacin (≥ 128 $\mu\text{g/ml}$). Four isolates (5%) presented elevated MIC for metronidazole (32 $\mu\text{g/ml}$) whereas MIC of ciprofloxacin was ≥ 4 $\mu\text{g/ml}$ in 97% of the strains, most of them were inhibited by a concentration of ≤ 8 $\mu\text{g/ml}$ of metronidazole. In the case of amikacin and ceftazidime, the prevalence of strains with higher levels of resistance was considerable (53.5% and 41%, respectively). Double resistance to the studied agents was uncommon and was detected in 10.4% of the strains. However, the results showed a higher percentage of MDR phenotype among the *C. difficile* isolates (66.3%). The overall level of multidrug resistance was 36% for the isolates with resistance to at least three drugs (triple drug resistance), 29% for the isolates with resistance to at least four drugs (quadruple drug resistance), and 1.16% for the isolates with resistance to at least five drugs (quintuple drug resistance). Toxinotyping of the MDR *C. difficile* strains for *tcdA* and *tcdB* showed four strains as *tcdA*⁺/*tcdB*⁻ (7%), one strain as *tcdA*⁻/*tcdB*⁺ (53%), forty seven strains as *tcdA*⁺/*tcdB*⁺ (84.2%), and four strains as *tcdA*⁻/*tcdB*⁻ (7%) (Table 2). Concurrent resistance to the tested antibiotics was significantly

Table 2. Frequency of multidrug-resistant (MDR) phenotype among 86 *C. difficile* isolates.

MDR phenotype*	Frequency n** (%)	MDR%***	<i>tcdA</i> ⁻ / <i>B</i> ⁺	<i>tcdA</i> ⁺ / <i>B</i> ⁻	<i>tcdA</i> ⁺ / <i>B</i> ⁺	<i>tcdA</i> ⁻ / <i>B</i> ⁻
Quintuple Drug Resistance	1/86 (1.16%)	1.7%	0	0	1	0
Metronidazole, Ceftazidime, Amikacin, Imipenem, Ciprofloxacin	1/86 (1.16%)	1/57 (1.7%)	0	0	1	0
Quadruple Drug Resistance	25/86 (29%)	44%	0	3	20	2
Ceftazidime, Amikacin, Imipenem, Ciprofloxacin	25/86 (29%)	25/57 (44%)	0	3	20	2
Triple Drug Resistance	31/86 (36%)	54.2%	1	1	27	2
Ceftazidime, Imipenem, Ciprofloxacin	14/86 (16.2%)	14/57 (24.5%)	1	0	12	1
Ceftazidime, Amikacin, Ciprofloxacin	10/86 (11.6%)	10/57 (17.5%)	0	1	8	1
Metronidazole, Ceftazidime, Ciprofloxacin	2/86 (2.32%)	2/57 (3.5%)	0	0	2	0
Amikacin, Imipenem, Ciprofloxacin	4/86 (4.6%)	4/57 (7%)	0	0	4	0
Metronidazole, Amikacin, Ciprofloxacin	1/86 (1.1%)	1/57 (1.7%)	0	0	1	0
Double Drug Resistance	9/86 (10.4%)		2	0	6	1
Amikacin, Ciprofloxacin	4/86 (4.65%)		1	0	2	1
Ceftazidime, Ciprofloxacin	4/86 (4.6%)		0	0	4	0
Ceftazidime, Amikacin	1/86 (1.16%)		1	0	0	0

*MDR, strains with triple, quadruple, and quintuple drug-resistant phenotypes were defined as strains with multidrug resistant phenotype to different classes of antimicrobial. ** Frequency of resistance isolates among the total isolated bacteria; *** Frequency of each resistance group pattern among the isolates with MDR phenotype.

observed among the strains with *tcdA*⁺/*tcdB*⁺ genotype ($P = 0.015$). All the metronidazole-resistant strains belonged to this genotype group.

DISCUSSION

Effective treatment of CDAD is usually based on common sensitivity reports for the strains in each country. There are a few reports about the prevalence of different MDR phenotypes among the clinical isolates in some countries [17, 18]. We report reduced susceptibility of our strains to ciprofloxacin (97%), amikacin (76.5%), and ceftazidime (76%), which were higher than other resistance phenotypes among the studied isolates. Detection of high level fluoroquinolone-resistant phenotype in *C. difficile* strains was previously reported by Nore'n *et al.* [19] who studied resistance frequency of their isolates to moxifloxacin (23%), levofloxacin (100%), and ciprofloxacin (100%). MIC levels to these antibiotics varied between 0.5 and > 32 mg/L with MIC₅₀ of > 32 mg/L in some studies [19, 20]. The estimated MIC levels for ciprofloxacin among the isolates of this study (MIC_{50/90} of 8 and ≥32 μg/ml, respectively) proposed lower levels of MIC₅₀ among them. The level of resistance to metronidazole varies in different countries. In European countries, MIC₅₀ and MIC₉₀ for metronidazole varied from 0.25 to 1 μg/ml and 0.5 to 2 μg/ml, respectively [19-22]. The highest reported MIC value for metronidazole is 64 μg/ml that was found in one strain in Hong Kong [23].

Data from the present study showed that 95% of our strains were inhibited by metronidazole at a concentration of ≤8 μg/ml; however, 5% of the isolates showed elevated MIC (≥32 μg/ml) that was similar to the overall reported rate of resistance in Spain (6.3%) [24], but higher than results from other studies [19-22]. This resistance level probably was caused by indiscriminate use of metronidazole in CDAD and also in other common cases of protozoal infections in Iran. In the case of ceftazidime, approximately 64% of the isolates showed *in vitro* resistance. In a study conducted in the United States, MIC₉₀ of *C. difficile* isolates for ceftazidime were >128 μg/ml [25]. The results of this study showed MIC₅₀ and MIC₉₀ of 64 and ≥128 μg/ml, respectively. These isolates showed lower resistance rate and MIC value to imipenem (48%, MIC_{50/90} of 8 and 32 μg/ml, respectively) compared with that was determined in Kuwait (86%, with MIC_{50/90} of 32 and > 32 μg/ml, respectively) [26].

In this study, the analysis of the drug resistance phenotypes among the isolates showed 17 strains with single drug resistance (19.8%), 9 strains with double drug resistance (10.4%), and 57 isolates with MDR

phenotypes (66.2%) (Table 2). Triple antibacterial resistance was found as main MDR phenotype among these strains (36%). All the strains with resistance phenotypes to metronidazole belonged to the triple or quintuple drug resistance groups. In a study in Italy, out of 316 *C. difficile* clinical isolates, 12 (3.7%) were resistant to only one antibiotic, 54 (17%) to two antibiotics, and 82 (26%) to at least three antibiotics (MDR) (18), however reduced susceptibility to metronidazole was not found among the MDR strains.

In a similar study in Kuwait, while no resistance was detected to metronidazole, MDR phenotype was reported in 55 isolates (75.3%) and double, triple, and quadruple resistance phenotypes were observed in 11%, 38.3%, and 37% of the strains, respectively [26]. Most of the MDR strains in our study were toxigenic (94.2%). Concurrent resistance to the tested antibiotics was significant in the *tcdA*⁺/*B*⁺ toxigenic group. These results cast new light into the relationship between toxigenic strains and resistance phenotype in *C. difficile*. This association was previously reported by others [18, 27, 28]. It has been shown that toxigenic strains of *C. difficile* (e.g. NAP1/O27) are resistant to broad spectrum antibiotics, such as beta-lactams, clindamycin, and fluoroquinolones [29]. It has been also indicated that mean consumption of several β-lactams, amikacin, imipenem, and fluoroquinolones was higher in affected hospitals with the toxigenic-resistant strains of *C. difficile*, which suggests the involvement of widespread antibiotic prescription in selection of toxigenic strains in these hospitals [30]. The relationship between toxigenicity and resistance phenotype of the *C. difficile* strains was also supported by a recent finding about cotransfer of *C. difficile* pathogenicity locus, encoding the two noted toxins, with conjugative transposons encoding resistance to several antibiotics [12]. *In vitro* transfer of genetic determinants among different strains of *C. difficile* was established by Jorg Wust *et al.* [31] in 1983. They concluded that this transmission cannot occur with plasmid DNA, and mechanism of the transfer seems to be a conjugation-like phenomenon. Pituch *et al.* [32] showed an association between antibiotic resistance strains and toxin B production in Warsaw. Correlation between fluoroquinolone resistance and resistance to macrolide-lincosamide-streptogramin antimicrobials was shown by Ackermann *et al.* [33]. Consistent with these data, our results showed a similar association between the coexistence of *tcdA*⁺/*tcdB*⁺ genes and MDR phenotypes among the clinical isolates of *C. difficile*. This finding emphasizes the need for continuous monitoring of antimicrobial susceptibility patterns among the pathogenic strains for prevention of the occurrence of eradication failure in the infected patients.

ACKNOWLEDGMENTS

This study was part of a PhD thesis that supported by a grant from Gastroenterology and Liver Disease Research Center (no. 560), Shahid Beheshti University of Medical Sciences, Tehran, Iran.

REFERENCES

- Murray PR, Rosenthal K.S, Pfaller A.M. Medical Microbiology. 5th ed. Philadelphia, PA: Elsevier Mosby. 2005.
- Pelleschi ME. *Clostridium difficile*-associated disease: diagnosis, prevention, treatment, and nursing care. *Crit Care Nurse*. 2008 Feb; 28(1):27-35.
- Saidel-Odesa L, Borer A, Odesa S. *Clostridium difficile* infection in patients with inflammatory bowel disease. *Ann Gastroenterol*. 2011; 24(4):263-270.
- Vaishnavi C. Clinical spectrum & pathogenesis of *Clostridium difficile* associated diseases. *Indian J Med Res*. 2010 Apr; 131:487-499.
- Voth DE, Ballard. *Clostridium difficile* toxins: mechanism of action and role in disease. *Clin Microbiol Rev*. 2005 Apr; 247-263.
- Lakhi N, Ahmad F, Woothipoom W. *Clostridium difficile* associated diarrhea and the relationship to antibiotic prescription practices and proton pump inhibitor use in elderly wards. *Iran Red Crescent Med J*. 2010; 12:12-16.
- Pépin J, Saheb N, Coulombe MA, Alary ME, Corriveau MP, Authier S, Leblanc M, et al. Emergence of fluoroquinolones as the predominant risk factor for *Clostridium difficile*-associated diarrhea: a cohort study during an epidemic in Quebec. *Clin Infect Dis*. 2005 Nov; 41(9):1254-1260.
- Yoo J, Lightner AL. *Clostridium difficile* infections: what every clinician should know. *Perm J*. 2010 Summer; 14(2):35-40.
- Kelly CP, LaMont JT. *Clostridium difficile*—more difficult than ever. *N Engl J Med*. 2008 Oct; 359(18):1932-40.
- Issa M, Ananthakrishn A, Binion DG. *Clostridium difficile* and inflammatory bowel disease. *Inflamm Bowel Dis*. 2008 Oct; 14(10):1432-42.
- Bartlett JG. New antimicrobial agents for patients with *Clostridium difficile* infections. *Curr Infect Dis Rep*. 2009 Jan; 11(1):21-8.
- Brouwer MSM, Roberts AP, Hussein H, Williams RJ, Allan E, Mullany P. Horizontal gene transfer converts non-toxicogenic *Clostridium difficile* strains into toxin producers. *Nat Commun*. 2013 Oct; 4:2601.
- Rupnik M, Brazier JS, Duerden BI, Grabnar M, Stubbs SL. Comparison of toxinotyping and PCR ribotyping of *Clostridium difficile* strains and description of novel toxinotypes. *Microbiology*. 2001 Feb; 147(Pt 2):439-47.
- Spigaglia P, Mastrantonio P. Molecular analysis of the pathogenicity locus and polymorphism in the putative negative regulator of toxin production (TcdC) among *Clostridium difficile* clinical isolates. *J Clin Microbiol*. 2002 Sep; 40(9):3470-5.
- Clinical and laboratory standard institute. Performance standards for antimicrobial susceptibility testing; CLSI document. 2012 Jan; M100-S22.
- Rea MC, Clayton E, O'Connor PM, Shanahan F, Kiely B, Ross RP, et al. Antimicrobial activity of lactacin 3147 against clinical *Clostridium difficile* strains. *J Med Microbiol*. 2007 Jul; 56(Pt 7):940-6.
- Wüst J, Sullivan NM, Hardegger U, Wilkins TD. Investigation of an outbreak of antibiotic-associated colitis by various typing methods. *J Clin Microbiol*. 1982 Dec 16(6):1096-101.
- Spigaglia P, Barbanti F, Mastrantonio P. Multidrug resistance in European *Clostridium difficile* clinical isolates. *J Antimicrob Chemother*. 2011 Oct; 66(10):2227-34.
- Nore'n T, Alriksson I, Kerlund TA, Burman LG, Unemo M. *In vitro* susceptibility to 17 antimicrobials of clinical *Clostridium difficile* isolates collected in 1993-2007 in Sweden. *Clin Microbiol Infect*. 2010 Aug; 16(8):1104-10.
- Wultanska D, Banaszekiewicz A, Radzikowski A, Obuch-Woszczatynski P, Mlynarczyk G, Brazier JS, Pituch H, et al. *Clostridium difficile* infection in Polish pediatric outpatients with inflammatory bowel disease. *Eur J Clin Microbiol Infect Dis*. 2010 Oct; 29(10):1265-70.
- Brazier JS, Raybould R, Patel B, Duckworth G, Pearson A, Charlett A, et al. Distribution and antimicrobial susceptibility patterns of *Clostridium difficile* PCR ribotypes in English Hospital, 2007-08. *Euro Surveill*. 2008 Oct; 13(41). pii:19000.
- Mutlu E, Wroe AJ, Sanchez-Hurtado K, Brazier JS, Poxton IR. Molecular characterization and antimicrobial susceptibility patterns of *Clostridium difficile* strains isolated from hospitals in south-east Scotland. *J Med Microbiol*. 2007 Jul; 56(Pt 7):921-9.
- Wong SS, Woo PCY, Luk WK, Yuen KY. Susceptibility testing of *Clostridium difficile* against metronidazole and vancomycin by disk diffusion and Etest. *Diagn Microbiol Infect Dis*. 1999 May; 34:1-6.
- Pelaez T, Alcalá L, Alonso R, Rodríguez-Creixems M, García-Lechuz JM, Bouza E. Reassessment of *Clostridium difficile* susceptibility to metronidazole and vancomycin. *Antimicrob Agents Chemother*. 2002 Jun; 46(6):1647-50.
- Nerandzic MM, Donskey CJ. Effect of ceftibiprole treatment on growth of and toxin production by *Clostridium difficile* in cecal contents of mice. *Antimicrob Agents Chemother*. 2011 May; 55: 2174-7.
- Jamal WY, Mokaddas EM, Verghese TL, Rotimi VO. *In vitro* activity of 15 antimicrobial agents against clinical isolates of *Clostridium difficile* in Kuwait. *Int J Antimicrob Agents*. 2002 Oct; 20(4):270-4.
- Karlowsky JA, Zhanel GG, Hammond GW, Rubinstein E, Wylie J, Du T, et al. Multidrug-resistant North American pulsotype 2 *Clostridium difficile* was the predominant toxigenic hospital-acquired strain in the province of Manitoba, Canada, in 2006-2007. *J Med Microbiol*. 2012 May; 61(Pt 5):693-700.

28. Johnson S, Samore MH, Farrow KA, Killgore GE, Tenover FC, Lyras D, et al. Epidemics of diarrhea caused by a clindamycin-resistant strain of *Clostridium difficile* in four hospitals. *N Engl J Med.* 1999 Nov; 341(22):1645-51.
29. Hedge DD, Strain JD, Heins JR, Farver DK. New advances in the treatment of *Clostridium difficile* infection (CDI). *Ther Clin Risk Manag.* 2008 Oct; 4(5):949-64.
30. Birgand G, Miliani K, Carbonne A, Astagneau P. Is high consumption of antibiotics associated with *Clostridium difficile* polymerase Chain Reaction-ribotype 027 infections in France? *Infect Control Hosp Epidemiol.* 2010 Mar; 31(3):302-5.
31. Wust J., Hardegger U. Transferable resistance to clindamycin, erythromycin, and tetracycline in *Clostridium difficile*. *Antimicrob Agents Chemother.* 1983 May; 23(5):784-6.
32. Pituch H, Brazier JS, Obuch-Woszczatynski P, Wultanska D, Meisel-Mikolajczyk F, Luczak M. Prevalence and association of PCR ribotypes of *Clostridium difficile* isolated from symptomatic patients from Warsaw with macrolide-lincosamide-streptogramin B (MLSB) type resistance. *J Med Microbiol.* 2006 Feb; 55(Pt 2):207-13.
33. Ackermann G, Degner A, Cohen SH, Silva J Jr, Rodloff AC. Prevalence and association of macrolide-lincosamide-streptogramin B (MLS(B)) resistance with resistance to moxifloxacin in *Clostridium difficile*. *J Antimicrob Chemother.* 2003 Mar; 51(3):599-603.