Journal of Global Antimicrobial Resistance 16 (2019) 210–214

Contents lists available at ScienceDirect

Journal of Global Antimicrobial Resistance

journal homepage: www.elsevier.com/locate/jgar

Short Communication

# Emerging metronidazole resistance in *Bacteroides* spp. and its association with the *nim* gene: a study from North India

Shveta Sethi, Ratnakar Shukla, Kiran Bala, Vikas Gautam, Archana Angrup*, Pallab Ray

*Department of Medical Microbiology, Post Graduate Institute of Medical Education and Research, Chandigarh, India*

ARTICLE INFO

*Article history:*
Received 22 May 2018
Received in revised form 9 October 2018
Accepted 10 October 2018
Available online 18 October 2018

*Keywords:*
Metronidazole
*Bacteroides* spp.
*nim* gene

ABSTRACT

*Objectives: Bacteroides* spp. are anaerobic resident intestinal flora but are also known to cause severe morbidity. They are resistant to a wide variety of antimicrobial agents, including metronidazole, which has been shown to be associated with specific nitroimidazole (*nim*) resistance genes. Metronidazole resistance is emerging worldwide, although presently it remains at ca. 5%. This study aimed to determine the metronidazole susceptibility and distribution of *nim* genes in *Bacteroides* spp. clinical isolates in India. The relationship among strains harbouring *nim* genes and their susceptibility to metronidazole was also analysed.

*Methods:* A total of 42 *Bacteroides* spp. clinical isolates were identified using an advanced MALDI-TOF system. Minimum inhibitory concentrations (MICs) for metronidazole were determined by the agar dilution method. Bacterial DNA was extracted and was subjected to *nim* gene PCR and the amplified PCR products were sequenced to determine the prevalent *nim* types.

*Results: Bacteroides fragilis* was the most common isolate (64%) among all *Bacteroides* spp. isolates. Among the total 42 clinical *Bacteroides* spp. isolates, 29 (69%) were susceptible and 13 (31%) were resistant to metronidazole by the agar dilution method. *nim* gene PCR performed on 38 isolates showed positivity in 20 isolates (53%), of which 12 had high metronidazole MICs ($\chi^2$ test, $P < 0.005$). On sequencing, these *nim* genes were most closely related to *nimE* type.

*Conclusion:* Resistance to metronidazole is consistently emerging worldwide. There is a significant association of the *nim* gene with metronidazole resistance. Periodic surveillance is needed to detect geographic and temporal trends in *nim* gene prevalence.



## 1. Introduction

*Bacteroides fragilis* and related members of the genus *Bacteroides* are important opportunistic anaerobic pathogens. They are known to cause intra-abdominal infections, skin and soft-tissue infections, septicaemia and postoperative wound infections. They are easily missed in clinical settings owing to the polymicrobial nature of such infections and difficulty in isolating them in routine laboratories [1]. Metronidazole, a 5-nitroimidazole drug, following its introduction into clinical practice in the 1960s, has been the mainstay both for prophylaxis and treatment of anaerobic infections [2]. *Bacteroides* spp. tend to be resistant to many classes of antimicrobial agents, including β-lactams, clindamycin, tetracyclines and imipenem, making metronidazole resistance a great therapeutic challenge.

The first metronidazole-resistant *B. fragilis* strain (NCTC 11295) was isolated from a patient with Crohn's disease in 1978 in Newcastle, UK [3]. Resistance to metronidazole in *Bacteroides* spp. is known to be associated with nitroimidazole (*nim*) resistance genes, of which there are 10 isoforms (*nimA–J*) that may be plasmid- or chromosomally-encoded [4]. The *nim* genes encode a 5-nitroimidazole reductase that converts 4- or 5-nitroimidazole to 4- or 5-aminoimidazole thus preventing the generation of toxic nitroso residues, which is the main mechanism of bactericidal activity of metronidazole. *nim* genes are also known to be associated with various promoter regions, i.e. insertion sequence (IS) elements, that carry regulatory signals for *nim* expression [5]. Although *nim*-associated metronidazole resistance is not highly prevalent globally, there are reports of isolation of such strains from Europe and Africa [6,7]. In the USA, metronidazole resistance has been reported in <1% of *B. fragilis* isolates, and ca. 3% of *Bacteroides* isolates are believed to carry *nim* genes [7]. Reported metronidazole resistance in *Bacteroides* spp. is ca. 0.5–7.8% in many surveys from European countries, Canada, the Middle East, Asia,

* Corresponding author.
*E-mail address:* archanaangrup@yahoo.com (A. Angrup).

https://doi.org/10.1016/j.jgar.2018.10.015

the USA and Africa [8]. Many other mechanisms of resistance including overexpression of multidrug efflux pumps, overexpression of RecA protein, and deficiency of FeoAB (ferrous-iron transporter) have been suggested, however their understanding remains limited [9].

The exact incidence of metronidazole resistance in India is not known because antimicrobial susceptibility testing for anaerobes is not routinely performed at most centres. This study aimed to determine the metronidazole susceptibility and distribution of *nim* genes in *Bacteroides* spp. clinical isolates in India. The relationship among strains harbouring *nim* genes and their susceptibility to metronidazole was also analysed.

## 2. Materials and methods

### 2.1. Sample collection

*Bacteroides* spp. isolates from various clinical samples, such as pus and wound discharge, in the period July 2016 to December 2016 at the Bacteriology Division, Department of Medical Microbiology, Postgraduate Institute of Medical Education and Research (Chandigarh, India) were included in this study. As this was a retrospective study, we were not able to retrieve clinical details of the patients. Species identification was done by matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF/MS) (Bruker Daltonik GmbH, Bremen, Germany) according to the manufacturer's instructions. MALDI scores were interpreted as follows: score = 2.0, accurate identification to species level; 1.7 = score < 2, accurate identification to genus level; and score < 1.7, no reliable identification [10].

### 2.2. Antimicrobial susceptibility testing

Minimum inhibitory concentrations (MICs) to metronidazole were determined by the agar dilution method according to Clinical and Laboratory Standards Institute (CLSI) guidelines [11]. *Brucella* agar supplemented with 5% laked sheep blood, vitamin K (1 μg/mL) and hemin (5 μg/mL) was inoculated with $10^5$ CFU/spot of a *Bacteroides* strain (0.5 McFarland standard). Serial two-fold dilutions of metronidazole from 0.25–128 μg/mL (0.25, 0.5, 1, 2, 4, 8, 16, 32, 64, and 128 μg/mL) were incorporated into the medium for susceptibility testing. *Bacteroides fragilis* ATCC 25285 was used as a positive control strain (metronidazole MIC = 0.25–2 μg/mL) and *Pseudomonas aeruginosa* ATCC 27853 was used for an anaerobiosis control. Plates were incubated at 37 °C for 48 h in anaerobic conditions using an Anoxomat® system (Mart Microbiology BV, Lichtenvoorde, The Netherlands). The lowest concentration of antibiotic that inhibited bacterial growth was considered the MIC. MIC cut-offs were as follows: ≥32 μg/mL for resistant; 16 μg/mL for intermediate-susceptible; and ≤8 μg/mL for susceptible [11].

### 2.3. DNA extraction

DNA was extracted using the protocol described by Queipo-Ortuño et al. and Akhi et al. [12,13]. One loopful of cultured *Bacteroides* spp. was suspended in 500 μL of phosphate-buffered saline and was centrifuged at 10 000 rpm for 3 min. The supernatant was discarded and the pellet was re-suspended in 100 μL of nuclease-free water. This mixture was vortexed, was kept at 95 °C in a water-bath for 10 min and was then centrifuged at 10 000 rpm for 3 min. The supernatant containing DNA was collected in a fresh Eppendorf tube. The purity of DNA was determined using a Nanodrop spectrophotometer (Thermo Fisher Scientific, Wilmington, DE) and the DNA was stored at −80 °C.

### 2.4. PCR

The 458-bp region of the *nim* gene was amplified in a thermal cycler (Applied Biosystems, Foster City, CA) as described by Akhi et al. [13] using the following primer sequences: NIM-3′ (5′-ATGTTCAGAGAAATGCGGCGTAAGCG-3′); and NIM-5′ (5′-GCTTCCTTGCCTGTCATGTGCTC-3′). Each PCR assay was performed in a final volume of 25 μL containing 12.5 μL of Dream*Taq* Green Master Mix (Thermo Fisher Scientific), 0.5 μL of 10 pmol of forward and reverse primers (Sigma-Aldrich, St Louis, MO) and 100 ng of DNA, with the residual volume made up with nuclease-free water. The thermal cycling parameters included initial denaturation at 94 °C for 5 min, followed by 35 cycles of amplification consisting of denaturation at 94 °C for 30 s, annealing at 63 °C for 55 s and extension at 72 °C for 45 s. The PCR products were subjected to 1.5% agarose gel electrophoresis in TAE (Tris–acetic acid–ethylene diamine tetra-acetic acid) buffer with a 100-bp ladder. PCR products positive for the *nim* gene were sequenced.

### 2.5. Sequencing and phylogenetic analysis

Amplified PCR products were sequenced by commercially available sequencing services (First BASE Laboratories, Seri Kembangan, Malaysia). Sequences were aligned using CLUSTAL_W and were corrected by manual inspection. The nucleotide sequences obtained were compared with the sequence in the National Center for Biotechnology Information (NCBI) database using the BLAST program. A phylogenetic tree was generated by the neighbour-joining method using the MEGA7 program. Bootstrap analysis was performed by repeating the analysis 500 times for the *nim* gene. The GenBank accession no. for the sequence of the *nimE* gene of *B. fragilis* is **MH341532**.

## 3. Results

A total of 42 *Bacteroides* spp. isolates were identified by MALDI-TOF/MS as follows: *B. fragilis* (27; 64%); *Bacteroides thetaiotaomicron* (7; 17%); *Bacteroides ovatus* (4; 10%); *Bacteroides vulgatus* (2; 5%); and *Bacteroides uniformis* (2; 5%).

Of the total 42 clinical isolates, 29 (69%) were susceptible (including intermediate-susceptible) and 13 (31%) were resistant to metronidazole by the agar dilution method (Table 1). PCR was performed on 38 isolates, as 4 isolates could not be revived. A total of 20 isolates (53%) were positive for the *nim* gene. Fig. 1 shows the 458-bp amplified product of the *nim* gene. Of 20 *nim*-positive isolates, 12 showed high metronidazole MICs (32–64 μg/mL) ($\chi^2$ test, $P < 0.005$) and 8 showed low MICs (1–16 μg/mL). Of the 18 *nim*-negative isolates, 1 isolate had a high metronidazole MIC (64 μg/mL) and the other 17 strains had low MICs (1–16 μg/mL) in the susceptible range.

PCR amplicons for the *nim* gene were further sequenced for *nim* typing. Phylogenetic analysis (Fig. 2) showed that the **MH341532** *nimE* nucleotide sequence is most closely related to *nimE* type from *B. fragilis*. The predicted nucleotide and amino acid sequences exhibited 99% and 100% similarities with *nimE* type, respectively.

## 4. Discussion

*Bacteroides* spp. are important members of the resident intestinal flora that, under certain conditions, may act as significant pathogens. *Bacteroides fragilis* has been reported to be the most common isolate in various studies, similar to the findings of the current study [2,5].

Resistance to antimicrobial agents, including metronidazole, has become of great concern among anaerobes over the past few

**Table 1**
Metronidazole minimum inhibitory concentrations (MICs) and types of *nim* gene present in *Bacteroides* spp. clinical isolates.

| Isolate identification[a] | MIC (μg/mL) | Interpretation[b] | *nim* PCR | Sequencing |
|---|---|---|---|---|
| *Bacteroides fragilis* | 64 | R | Positive | *nimE* |
| *Bacteroides fragilis* | 64 | R | Positive | *nimE* |
| *Bacteroides ovatus* | 2 | S | Positive | *nimE* |
| *Bacteroides ovatus* | 2 | S | Positive | *nimE* |
| *Bacteroides fragilis* | 64 | R | Positive | *nimE* |
| *Bacteroides fragilis* | 64 | R | Positive | *nimE* |
| *Bacteroides thetaiotaomicron* | 4 | S | Negative | – |
| *Bacteroides fragilis* | 2 | S | Negative | – |
| *Bacteroides fragilis* | 4 | S | Negative | – |
| *Bacteroides thetaiotaomicron* | 4 | S | Negative | – |
| *Bacteroides fragilis* | 64 | R | Negative | – |
| *Bacteroides fragilis* | 32 | R | Positive | *nimE* |
| *Bacteroides fragilis* | 1 | S | Negative | – |
| *Bacteroides fragilis* | 16 | S | N/R | – |
| *Bacteroides fragilis* | 1 | S | Negative | – |
| *Bacteroides fragilis* | 16 | S | Positive | *nimE* |
| *Bacteroides fragilis* | 64 | R | Positive | *nimE* |
| *Bacteroides fragilis* | 2 | S | Negative | – |
| *Bacteroides fragilis* | 8 | S | Negative | – |
| *Bacteroides fragilis* | 4 | S | Positive | *nimE* |
| *Bacteroides thetaiotaomicron* | 1 | S | Positive | *nimE* |
| *Bacteroides fragilis* | 2 | S | Positive | *nimE* |
| *Bacteroides thetaiotaomicron* | 32 | R | Positive | *nimE* |
| *Bacteroides fragilis* | 32 | R | Positive | *nimE* |
| *Bacteroides uniformis* | 2 | S | Negative | – |
| *Bacteroides fragilis* | 1 | S | N/R | – |
| *Bacteroides fragilis* | 2 | S | N/R | – |
| *Bacteroides fragilis* | 16 | S | Negative | – |
| *Bacteroides ovatus* | 0.5 | S | N/R | – |
| *Bacteroides uniformis* | 2 | S | Negative | – |
| *Bacteroides ovatus* | 2 | S | Negative | – |
| *Bacteroides thetaiotaomicron* | 2 | S | Negative | – |
| *Bacteroides fragilis* | 32 | R | Positive | *nimE* |
| *Bacteroides fragilis* | 16 | S | Positive | *nimE* |
| *Bacteroides fragilis* | 16 | S | Negative | – |
| *Bacteroides fragilis* | 32 | R | Positive | *nimE* |
| *Bacteroides fragilis* | 16 | S | Positive | *nimE* |
| *Bacteroides thetaiotaomicron* | 2 | S | Negative | – |
| *Bacteroides vulgatus* | 1 | S | Negative | – |
| *Bacteroides vulgatus* | 1 | S | Negative | – |
| *Bacteroides fragilis* | 32 | R | Positive | *nimE* |
| *Bacteroides thetaiotaomicron* | 32 | R | Positive | *nimE* |

R, resistant; S, susceptible; N/R, not revived.
[a] By matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF/MS).
[b] Intermediate-susceptible isolates (MIC = 16 μg/mL) were grouped as susceptible (S).

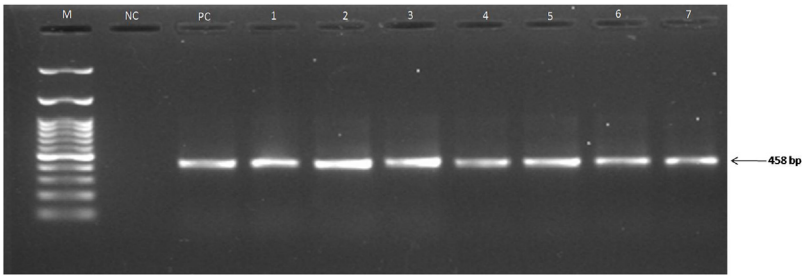


**Fig. 1.** Agarose gel picture for PCR-amplified product (458 bp) of the *nim* gene. Lane M, 100-bp ladder; lane NC, negative control (sterile distilled water); lane PC, positive control; lanes 1–7, samples positive for the *nim* gene.

decades, limiting the therapeutic options available for treating *Bacteroides* infections. In the present study, 31% of isolates were resistant to metronidazole. This is comparable with a study by Akhi et al. where 30% of the total *B. fragilis* isolates were found to be metronidazole-resistant [13]. In another study of Indian isolates, the rate of metronidazole resistance was reported to be 55.5% [14]. Evidence of metronidazole resistance from the Western literature, on the contrary, remains low and varied, with <1% in European isolates [7], 0.3% in Canadian isolates [15] and >15% in UK isolates in various studies [2]. In a study by Goldstein and Snydman metronidazole resistance in community isolates of *B. fragilis* was very rare [1], however most studies have reported metronidazole resistance in nosocomial isolates of *Bacteroides*. In the current study, we could not recover the full history related to the origin of the isolates (nosocomial or community). Although metronidazole resistance among *Bacteroides* spp. varies among diverse

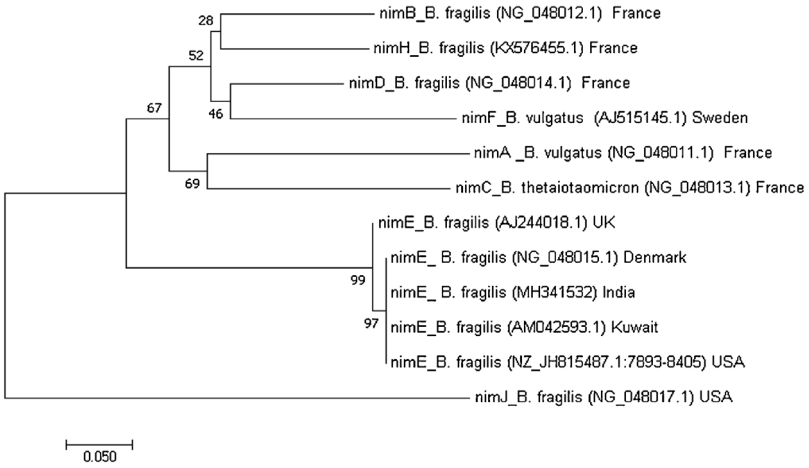


**Fig. 2.** Phylogenetic relationship of **MH341532** *nimE* relative to other *nim* types (*nimA–J* type). The phylogenetic tree was prepared using the neighbour-joining method in MEGA7.0 software. Numbers at the nodes are percent bootstrapping value from 500 replicates.

geographical locations, reliance upon the unrecommended disk diffusion test for metronidazole susceptibility testing might have contributed to an under-reporting of resistant strains owing to lower cut-off values for disk diffusion [7]. Although the prevalence of metronidazole resistance in India is significantly higher compared with the West, the true incidence possibly remains underestimated owing to lack of facilities for performing antimicrobial susceptibility testing of anaerobes at most centres.

The present study reported a high rate of positivity (53%) for the *nim* gene among clinical isolates of *Bacteroides* spp. This rate is slightly higher than that reported in other studies reporting a *nim* positivity of 24% in the UK, 2% in Europe and 38.9% in India [2,7,14]. In contrast, a study by Akhi et al. did not detect the *nim* gene in any metronidazole-resistant *B. fragilis* isolates from patients with surgical site infections [13]. As mentioned above, *nim* gene-associated metronidazole resistance varies among diverse geographical regions [15]. In India, studies on metronidazole resistance and *nim* prevalence are very limited [14,16]. Periodic surveillance is thus needed to detect geographic or temporal trends in *nim* gene prevalence in different geographical locations.

We further looked for a relationship of *nim* gene positivity with metronidazole resistance. Of the total 20 *nim*-positive isolates, 12 had high MICs ($\geq$32 μg/mL). This shows a significant correlation of the *nim* gene with metronidazole resistance ($\chi^2$ test, $P < 0.005$). Among all of the isolates, one isolate was negative for the *nim* gene despite a high metronidazole MIC (64 μg/mL). This suggests that an alternative mechanism of resistance such as overexpression of multidrug efflux pumps or deficiency of the ferrous-iron transporter FeoAB might have played a role [9,13]. Isolates that were positive for the *nim* gene but with MICs within the susceptible range ($n = 8$) point to the role of some other independent factors that might govern the expression of *nim* genes. As corroborated in some studies, the role of certain IS elements (situated upstream of the *nim* genes) has been mentioned [6,7]. The exact mechanism by which these IS elements influence silent *nim* gene expression is still not clear. The present study did not look for IS element in these isolates, which we plan to do in the future.

Phylogenetic tree analysis illustrated that the sequence type of the *nim* gene was most closely related to the *nimE* gene from all *Bacteroides* isolates. The nucleotide sequence of **MH341532** *nimE* in the present study shows similarity with *nimE* type reported from Denmark, Kuwait, the UK and the USA [17–20]. Therefore, this study explains that *nimE* is circulating in our geographical region, in contrast to other studies where *nimA* was found to be the most common type, followed by *nimB* and *nimD* [2,7]. However, in the literature no correlation has been found between the degree of resistance and the type of *nim* gene.

Limitations of this study include the small number of isolates and that PCR for IS elements was not performed to check *nim* expression levels in isolates with a low MIC.

In conclusion, the rate of metronidazole resistance is quite high among *Bacteroides* spp. isolates in India and there is a good association of the genes with metronidazole resistance. Further genetic analysis is required to look for the molecular basis of the *nim* genes. There is a need to perform regular surveillance of resistance among anaerobes in order to determine the prevalence of resistance genes so that appropriate infection control measures can be instituted to prevent the emergence of a multidrug-resistant pathogen.

## Funding

None.

## Competing interests

None declared.

## Ethical approval

Not required.

## References

[1] Goldstein EJC, Snydman DR. Intra-abdominal infections: review of the bacteriology, antimicrobial susceptibility and the role of ertapenem in their therapy. J Antimicrob Chemother 2004;53(Suppl. 2):ii29–36.
[2] Gal M, Brazier JS. Metronidazole resistance in *Bacteroides* spp. carrying *nim* genes and the selection of slow-growing metronidazole-resistant mutants. J Antimicrob Chemother 2004;54:109–16.
[3] Ingham HR, Eaton S, Venables CW, Adams PC. *Bacteroides fragilis* resistant to metronidazole after long-term therapy. Lancet 1978;1:214.
[4] Leitsch D, Soki J, Kolarich D, Urbán E, Nagy E. A study on Nim expression in *Bacteroides fragilis*. Microbiology 2014;160:616–22.
[5] Nakano V, Silva ADN, Merino VRC, Wexler HM, Campos MJA. Antimicrobial resistance and prevalence of resistance genes in intestinal Bacteroidales strains. Clinics (Sao Paulo) 2011;66:543–7.
[6] Schapiro JM, Gupta R, Stefansson E, Fang FC, Limaye AP. Isolation of metronidazole-resistant *Bacteroides fragilis* carrying the *nimA* nitroreductase gene from a patient in Washington State. J Clin Microbiol 2004;42:4127–9.

[7] Lofmark S, Fang H, Hedberg M, Edlund C. Inducible metronidazole resistance and *nim* genes in clinical *Bacteroides fragilis* group isolates. Antimicrob Agents Chemother 2005;49:1253–6.
[8] Ghotasloua R, Baghib HB, Alizadehc N, Yekani M, Arbabia S, Memar MY. Mechanisms of *Bacteroides fragilis* resistance to metronidazole. Infect Genet Evol 2018;64:156–63.
[9] Veeranagouda Y, Husain F, Boente R, Moore J, Smith CJ, Rocha ER, et al. Deficiency of the ferrous iron transporter FeoAB is linked with metronidazole resistance in *Bacteroides fragilis*. J Antimicrob Chemother 2014;69:2634–43.
[10] Bizzini A, Durussel C, Bille J, Greub G, Prodhom G. Performance of matrix-assisted laser desorption ionization–time of flight mass spectrometry for identification of bacterial strains routinely isolated in a clinical microbiology laboratory. J Clin Microbiol 2010;48:1549–54.
[11] Clinical and Laboratory Standards Institute. Methods for antimicrobial susceptibility testing of anaerobic bacteria; approved standard—eighth edition. CLSI document M11-A8. Wayne, PA: CLSI; 2012.
[12] Queipo-Ortuno MI, De Dios Colmenero J, Macias M, Bravo MJ, Morata P. Preparation of bacterial DNA template by boiling and effect of immunoglobulin G as an inhibitor in real-time PCR for serum samples from patients with brucellosis. Clin Vaccine Immunol 2008;15:293–6.
[13] Akhi MT, Ghotaslou R, Alizadeh N, Yekani M, Beheshtirouy S, Asgharzadeh M, et al. *nim* gene-independent metronidazole-resistant *Bacteroides fragilis* in surgical site infections. GMS Hyg Infect Control 2017;12:1–6.
[14] Chaudhary M, Payasi A. Emerging metronidazole resistance in anaerobes and mapping their susceptibility behaviour. Am J Infect Dis 2014;10:56–63.
[15] Karlowsky JA, Walkty AJ, Adam HJ, Baxter MR, Hoban DJ, Zhanel GG. Prevalence of antimicrobial resistance among clinical isolates of *Bacteroides fragilis* group in Canada in 2010-2011: CANWARD Surveillance Study. Antimicrob Agents Chemother 2012;56:1247–52.
[16] Chaudhry R, Mathur P, Dhawan B, Kumar L. Emergence of metronidazole-resistant *Bacteroides fragilis*, India. Emerg Infect Dis 2001;7:485–6.
[17] Sydenham TV, Soki J, Hasman H, Wang M, Justesen US. Identification of antimicrobial resistance genes in multidrug-resistant clinical *Bacteroides fragilis* isolates by whole genome shotgun sequencing. Anaerobe 2015;31:59–64.
[18] Soki J, Gal M, Brazier JS, Rotimi VO, Urban E, Nagy E, et al. Molecular investigation of genetic elements contributing to metronidazole resistance in *Bacteroides* strains. J Antimicrob Chemother 2006;57:212–20.
[19] Stubbs SLJ, Brazier JS, Talbot PR, Duerden BI. PCR-restriction fragment length polymorphism analysis for identification of Bacteroides spp. and characterization of nitroimidazole resistance genes. J Clin Microbiol 2000;38:3209–13.
[20] Sherwood JE, Fraser S, Citron DM, Wexler H, Blakely G, Jobling K, et al. Multidrug resistant *Bacteroides fragilis* recovered from blood and severe leg wounds caused by an improvised explosive device (IED) in Afghanistan. Anaerobe 2011;17:152–5.