Evaluation of the genetic relatedness of *Bacteroides fragilis* isolates by TRs analysis

Niloofar Khodaei 1,2, Behrooz Sadeghi Kalani 3,4, Maryam Zamani 1,2, Rokhsareh Mohammadzadeh 1,2, Malihe Talebi 1,2, Tahmine Nariman1 5, Negar Narimisa 1,2, Faramarz Masjedian Jazi 1,2*

1 Microbial Biotechnology Research Center, Iran University of Medical Science, Tehran, Iran
2 Department of Microbiology, School of Medicine, Iran University of Medical Sciences, Tehran, Iran
3 Clinical Microbiology Research Center, Ilam University of Medical Sciences, Ilam, Iran
4 Department of Microbiology, Faculty of Medicine, Ilam University of Medical Sciences, Ilam, Iran
5 Department of Microbiology, School of Medicine, Isfahan University of Medical Sciences, Tehran, Iran

**Introduction**

*Bacteroides fragilis* is a Gram-negative, anaerobic bacillus residing in the human gut microflora (1). This anaerobic microorganism is regularly isolated from human infections causing serious complications due to the lack of proper antimicrobial therapy. Two molecular subtypes have been attributed to this microorganism: non-toxigenic *B. fragilis* (NTBF) and enterotoxigenic *B. fragilis* (ETBF). The latter subtype is considered an intestinal microorganism contributing to inflammatory bowel disease and colorectal cancer. Reportedly, antimicrobial resistance in this bacterium is increasing worldwide (2-4).

Gut microflora can play vital roles in human health. The overgrowth of anaerobic gut microbiota, especially Bacteroides, can lead to several health outcomes including colon cancer, diarrhea, intrathecal and uterine abscesses, septicaemia, and pelvic inflammation. In this study, multiple locus variable number of tandem repeats analysis (MLVA) was performed to genetically differentiate 50 *B. fragilis* isolates.

**Materials and Methods:** Eight suitable tandem repeats (TRs) were selected by bioinformatics tools and were then subjected to PCR amplification using specific primers. Finally, MLVA profiles were clustered using BioNumerics 7.6 software package.

**Results:** All VNTR loci were detected in all isolates using the PCR method. Overall, *B. fragilis* isolates were differentiated into 27 distinct MLVA types. The highest diversity index was allocated to TR1, TR2, TR3, TR6, and TR8; with this taken into account, strain type 14 was the most prevalent with 12 strains belonging to this type. Clustering revealed three major clusters of A, B, and C. With regards to the pathogenicity of *B. fragilis* and the outcomes of infections related to this microorganism, it is imperative to study this microorganism isolated from both patients and healthy individuals.

**Conclusion:** This study aimed at evaluating the efficiency of MLVA for the genetic differentiation of *B. fragilis*. The results of this study indicate the promising efficiency of MLVA typing for cluster detection of this bacterium.

---

Please cite this article as: Khodaei N, Sadeghi Kalani B, Zamani M, Mohammadzadeh R, Talebi M, Narimani T, Narimisa N, Masjedian Jazi F. Evaluation of the genetic relatedness of *Bacteroides fragilis* isolates by TRs analysis. Iran J Basic Med Sci 2020; 23:1323-1327. doi: 10.22038/ijbms.2020.35816.8532

---

**ARTICLE INFO**

**Article type:** Original article

**Article history:**
Received: Oct 25, 2018
Accepted: Jun 30, 2020

**Keywords:**
*Bacteroides fragilis*
MLVA
PCR amplification
Tandem repeats
Typing

**ABSTRACT**

**Objectives:** Human gastrointestinal tract harbors a variety of bacteria with vital roles in human health. *Bacteroides fragilis* is considered one of the dominant constituents of gut microflora which can act as an opportunistic pathogen leading to various diseases, including colon cancer, diarrhea, uterine and intrathecal abscesses, septicaemia, and pelvic inflammation. In this study, multiple locus variable number of tandem repeats analysis (MLVA) was performed to genetically differentiate 50 *B. fragilis* isolates.

**Materials and Methods:** Eight suitable tandem repeats (TRs) were selected by bioinformatics tools and were then subjected to PCR amplification using specific primers. Finally, MLVA profiles were clustered using BioNumerics 7.6 software package.

**Results:** All VNTR loci were detected in all isolates using the PCR method. Overall, *B. fragilis* isolates were differentiated into 27 distinct MLVA types. The highest diversity index was allocated to TR1, TR2, TR3, TR6, and TR8; with this taken into account, strain type 14 was the most prevalent with 12 strains belonging to this type. Clustering revealed three major clusters of A, B, and C. With regards to the pathogenicity of *B. fragilis* and the outcomes of infections related to this microorganism, it is imperative to study this microorganism isolated from both patients and healthy individuals.

**Conclusion:** This study aimed at evaluating the efficiency of MLVA for the genetic differentiation of *B. fragilis*. The results of this study indicate the promising efficiency of MLVA typing for cluster detection of this bacterium.

---

**Introduction**

*Bacteroides fragilis* is a Gram-negative, anaerobic bacillus residing in the human gut microflora (1). This anaerobic microorganism is regularly isolated from human infections causing serious complications due to the lack of proper antimicrobial therapy. Two molecular subtypes have been attributed to this microorganism: non-toxigenic *B. fragilis* (NTBF) and enterotoxigenic *B. fragilis* (ETBF). The latter subtype is considered an intestinal microorganism contributing to inflammatory bowel disease and colorectal cancer. Reportedly, antimicrobial resistance in this bacterium is increasing worldwide (2-4).

Gut microflora can play vital roles in human health. The overgrowth of anaerobic gut microbiota, especially Bacteroides, can lead to several health outcomes including colon cancer, diarrhea, intrathecal and uterine abscesses, and pelvic inflammation. About 99% of bacterial gut microflora are anaerobic, 20-30% of which are in the Bacteroides group. Moreover, recent reports indicate increased antibiotic resistance levels to methicillin, cephalosporins, tetracycline, and clindamycin (5-7).

Bacteria are often in a commensal relationship with humans; however, pathogenic bacteria can be observed infrequently. Colonization of commensal bacteria is advantageous for human health as it can lead to mucosal and systemic immunity (8, 9).

Gut microbiota can induce maturity of the host immunity and subsequently provide protection against various infections. In spite of the fact that *B. fragilis* constitutes less than 1% of intestinal flora, studies on animal models with colitis have demonstrated that owing to the possession of polysaccharide capsules, this microorganism can rectify the inadequacy of immune system caused by the absence of bacterial colonization (10, 11).

There are two types of polysaccharide capsules, including polysaccharide A (PSA) and polysaccharide B (PSB), both belonging to zwitterion polysaccharides (ZPSs) with both positive and negative charges on each residue. These capsular polysaccharides have been shown to induce TCD4+ immune responses, hence restricting the colonization of other pathogens and the spread of infections (12-14).

In certain conditions, however, *B. fragilis* can cause inflammatory bowel disease, intestinal abscesses, peritonitis, genital infections, deep ulcers, bone marrow infections, pediatric cellulitis and pneumonia, colon cancer, bacteremia, brain abscess, meningitis, and septic
Table 1. Characteristics of variable-number tandem repeat loci used for typing of Bacteroides fragilis YCH46 isolate

| TRs | Repeat motif | TR Size | Genomic Location | Function | Unit | Pi |
|-----|--------------|---------|------------------|----------|------|----|
| TR1 | AAGATCGGAGATTTTTATAT | 23 (bp) | 349742–349830 | non-coding region | 4 | 0 |
| TR2 | GAAAAACATCAAGAAAGATCTTTGGATACCTCTGATTGA | 60 (bp) | 1664955–1665296 | hypothetical protein | 6 | 0 |
| TR3 | AGATAAGCGCTGA | 14 (bp) | 1762135–1762195 | non-coding region | 4 | 0 |
| TR4 | GAAGTGTCAGACAGAAGAGACATTGAAAGAAG | 86 (bp) | 3424467–3425269 | hypothetical protein | 7 | 1 |
| TR5 | AGGCGAGTTAGGCTGGTCGGT | 22 (bp) | 3745326–3745553 | non-coding region | 10 | 0 |
| TR6 | ATGACAGTAAA | 12 (bp) | 3978553–3978616 | hypothetical protein | 5 | 0 |
| TR7 | TCCTGACCTTTACTACCGTACCG | 27 (bp) | 427863–4278232 | hypothetical protein | 6 | 0 |
| TR8 | CGGACGTGGAGGAGCTGCACTCTGAAATGTTAAGAAG | 102 (bp) | 4586247–4586699 | ribosomal large subunit pseudouridine synthase B | 4 | 0 |

*The role of TR-harboring gene, † Percent Indels

Table 2. Primers and their characteristics in the current study

| TRs | Primer sequence 5’→3’ | PF * (bp) | Flanking (bp) | Reference |
|-----|------------------------|----------|---------------|-----------|
| TR1 | TGGACATGCTCCTTGTTGCCCT | 201 | 119 | in this study |
| TR2 | GCCGGATACAGGGAGGTTCG | 498 | 156 | in this study |
| TR3 | GCCGGATACAGGGAGGTTCG | 161 | 141 | in this study |
| TR4 | GGCTCCGTATACAGGGAGGTTCG | 694 | 71 | in this study |
| TR5 | GGCTCCGTATACAGGGAGGTTCG | 285 | 60 | in this study |
| TR6 | GGCTCCGTATACAGGGAGGTTCG | 150 | 86 | in this study |
| TR7 | GGCTCCGTATACAGGGAGGTTCG | 245 | 56 | in this study |
| TR8 | GGCTCCGTATACAGGGAGGTTCG | 550 | 97 | in this study |

* PCR product length in Bacteroides fragilis isolate

Materials and Methods

Bacterial collection and identification

In total, 50 non-toxicogenic B. fragilis isolates were attained from the previous study (22) and were stored in the microbial bank in the Department of Microbiology, Iran University of Medical Sciences, Tehran, Iran.

Identification of VNTR loci

The sequence of the genome of B. fragilis YCH46 was obtained using GenBank NCBI and tandem repeat sequences were analyzed using Tandem Repeats Finder (TRF version 4.09) which have been illustrated in Table 1 (23).

Primer design

Suitable specific primers were designed using Oligo 7, AlleleID, Primer3, and Oligo Analyser software packages. Table 2 shows the oligonucleotide primers and flanking regions of every VNTR locus. Annealing temperatures of the primers were determined in silico using the Primer-BLAST tool in the NCBI website.

Data analysis

MLVA profiles were clustered using the BioNumerics 7.6 software package, with UPGMA (Unweighted Pair
Results

MLVA typing was performed on 50 B. fragilis isolates originating from human stool. Eight regions were chosen for analysis based on the location of the genome, repeat length, percent of matches, and percent of indels between adjacent copies and copy numbers as indicated in Table 1 and Figure 1. The size of the repeat unit of the five VNTRs was in the range of 12 bp and 80 bp. TR1, TR3, and TR5 are located in a non-coding region of the bacterial genome. TR2, TR4, TR6, and TR7 are located in a genomic region with a hypothetical protein function. Finally, TR8 is located in a region of the bacterial genome which is a ribosomal large subunit acting as pseudouridine synthase B.

All isolates presented a PCR product for all VNTR loci. Diversity in the number of repeats between various VNTR loci was observed based on the analysis of all 8 VNTR loci of B. fragilis. Overall, B. fragilis isolates were distinguished into 27 different MLVA types (MT) as indicated in Figure 2. Types 1, 2, 3, 4, 5, 6, 8, 9, 10, 11, 12, 15, 16, 18, 19, 20, 21, 22, 23, 24, 25, and 27 only included one strain each. Type 13 included two strains. Type 17 contained three strains. Type 14 had 12 strains, making it the most prevalent type. Three main clusters called A, B, and C were observed by the dendrogram. Cluster A is categorized into two sub-clusters A1 and A2, sub-cluster A1 with four types and sub-cluster A2 with eight types. In addition, cluster B is divided into two sub-clusters B1 and B2, sub-cluster B1 with two types, and sub-cluster B2 with two types. Cluster C is divided into two sub-clusters C1 and C2, sub-cluster C1 with four types, and sub-cluster C2 with seven types. This study aimed to determine MLVA profiles of 50 strains from eight different origins to determine the relatedness of these isolates. All strains were isolated from the stools of healthy individuals. According to the cluster analysis of the MLVA profiles by a minimum spanning tree algorithm, MLVA was efficient in discriminating B. fragilis isolates from different sources as shown in Figure 3. The Simpson and Hunter index analysis showed the highest diversity in tandem repeats 5, 1, 8, 2, 6, 4, 3, and 7, respectively (Table 3).

Discussion

The current study evaluated the genetic association between 50 B. fragilis isolates from the stools of different
Table 3. Simpson’s Diversity and Hunter-Gaston Diversity

| Locus | Diversity Index | Confidence Interval | K | Max (PI) |
|-------|----------------|---------------------|---|----------|
| TR5   | 0.737          | 0.723 - 0.752       | 4 | 0.333    |
| TR1   | 0.661          | 0.651 - 0.672       | 3 | 0.373    |
| TR8   | 0.654          | 0.638 - 0.671       | 3 | 0.412    |
| TR2   | 0.650          | 0.610 - 0.690       | 4 | 0.490    |
| TR6   | 0.617          | 0.587 - 0.648       | 3 | 0.490    |
| TR4   | 0.498          | 0.491 - 0.506       | 2 | 0.529    |
| TR3   | 0.498          | 0.491 - 0.506       | 2 | 0.529    |
| TR7   | 0.457          | 0.420 - 0.494       | 2 | 0.647    |

Diversity Index (for VNTR data) indicates any variation in the number of repeats at every locus. Within the of 0.0 (indicative of no diversity) to 1.0 (indicative of complete diversity).

Confidence Interval: The level of precision in the diversity Index, indicated as 95% upper & lower cases.

K = Number of different repeats at this locus in sample set.

max(p) = Fraction of samples with the highest repeat numbers in this locus (within the range of 0.0 to 1.0).

TR: Tandem repeats

Table 3. Simpson’s Diversity and Hunter-Gaston Diversity

| Locus | Diversity Index | Confidence Interval | K | Max (PI) |
|-------|----------------|---------------------|---|----------|
| TR5   | 0.752          | 0.738 - 0.767       | 4 | 0.333    |
| TR1   | 0.675          | 0.664 - 0.685       | 3 | 0.373    |
| TR8   | 0.667          | 0.651 - 0.684       | 3 | 0.412    |
| TR2   | 0.663          | 0.623 - 0.703       | 4 | 0.490    |
| TR6   | 0.630          | 0.599 - 0.661       | 3 | 0.490    |
| TR4   | 0.508          | 0.501 - 0.516       | 2 | 0.529    |
| TR3   | 0.508          | 0.501 - 0.516       | 2 | 0.529    |
| TR7   | 0.466          | 0.429 - 0.503       | 2 | 0.647    |

individuals in Tehran, Iran. For a better understanding of the features and varieties of the isolates, B. fragilis isolates were analyzed by MLVA typing, which is considered a valuable method for determining the diversity of bacterial populations in clinical isolates. MLVA is an inexpensive, uncomplicated, and efficient method whose results can be attained within a short period, usually faster than MLST and PFGE techniques (25).

Furthermore, over the past few years, typing has been inclined towards molecular techniques resulting in the development of new techniques such as MLVA. Thus, this new technique can be a promising substitute for previous techniques, including ribotyping, serotyping, and RFLP. B. fragilis is an anaerobic pathogen commonly isolated from clinical specimens with different virulence factors. Among the diseases caused by Bacteroides are cerebrospinal angiomias, meningitis, septic arthritis, inflammatory bowel, intestinal diseases, and soft tissue infections (26, 27).

Owing to the evolution and adaptation of B. fragilis, like other human bacterial flora, quantitative diversity for this bacterium was not observed in this study. A significant relationship was found between MLVA types and strains isolated from stools of different individuals. The limited number of isolates makes it difficult to interpret epidemiological data. Further studies are required to assess the efficacy of MLVA assay in B. fragilis especially in the toxigenic strains.

Conclusion

This technique can provide quick and valuable information for researchers to evaluate pathogenicity, evolution, and epidemiological studies of this microorganism.

Acknowledgment

This study was financially supported by a research grant (No. 96-01-30-30509) for an M.Sc thesis at Iran University of Medical Sciences (Tehran, Iran), for which we are very grateful.

Conflicts of Interest

The authors declare that they have no competing interests.

References

1. Kuwahara T, Yamashita A, Hirakawa H, Nakayama H, Toh H, Okada N, et al. Genomic analysis of Bacteroides fragilis reveals extensive DNA inversions regulating cell surface adaptation. Proc Natl Acad Sci U S A 2004; 101:14919-14924.
2. Bahador A, Kalani BS, Valian F, Irajian G, Lotfollahi L. Phenotypic and genotypic characteristics of Listeria monocytogenes isolated from dairy and meat products. Avicenna J Clin Microbiol Infect 2015; 2:26905.
3. Schwensen SA, Henrisken DP, Justesen US, Sydhem TV. Antimicrobial resistance in the Bacteroides fragilis group in faecal samples from patients receiving broad-spectrum antibiotics. Anaerobe 2017; 47:79-85.
4. Zakharzhevskaya NB, Tsvetkov VB, Vanyushkina AA, Varizhuk AM, Bakitina DV, Podgorsky VV, et al. Interaction of Bacteroides fragilis toxin with outer membrane vesicles reveals new mechanism of its secretion and delivery. Front Cell Infect Microbiol 2017; 7:2.
5. Khodaei F, Kalani BS, Alizadeh N, Hassani A, Najafi M, Kalantar E, et al. Genotyping and phylogenetic analysis of group B streptococcus by multiple locus variable number tandem repeat analysis in iran. Gelen Med J 2018;7:e1121.
6. Joet T, Lacroix C, Braegger CP, Chassard C. New insights in gut microbiota establishment in healthy breast fed neonates. PloSone 2012; 7:e44595.
7. Roi IY, Klimenko N, Zdorovenko G, Goncharuk V. Species identification of water microorganisms resistant to chlorine compounds. J Water Chem Technol 2015; 37:145-150.
8. Ley RE, Peterson DA, Gordon JI. Ecological and evolutionary forces shaping microbial diversity in the human intestine. Cell J 2006; 124:837-848.
9. Pamer EG. Immune responses to commensal and environmental microbes. Nat Immunol 2007; 8:1173.
10. Mazmanian SK, Liu CH, Tzianabos AO, Kasper DL. An immunomodulatory molecule of symbiotic bacteria directs maturation of the host immune system. Cell 2005;122:107-118.
11. Mazmanian SK, Round JL, Kasper DL. A microbial symbiosis factor prevents intestinal inflammatory disease. Nature 2008; 453:620.
12. Tzianabos AO, Onderdonk AB, Rosner B, Cisneros RL, Kasper DL. Structural features of polysaccharides that induce intra-abdominal abscesses. Science 1993; 262:416-419.
13. Tzianabos AO, Wang JY, Lee JC. Structural rationale for the toxicity of Staphylococcus aureus capsular polysaccharides. Proc Natl Acad Sci USA 2001; 98:9365-9370.
14. Tzianabos AO, Finberg RW, Wang Y, Chan M, Onderdonk AB, Jennings HJ, et al. T cells activated by zwitterionic molecules prevent abscesses induced by pathogenic bacteria. J Biol Chem
15. Stranden A, Frei R, Widmer A. Molecular typing of methicillin-resistant *Staphylococcus aureus*: Can PCR replace pulsed-field gel electrophoresis? J Clin Microbiol 2003; 41:3181-3186.

16. Widerström M, Wiström J, Sjöstedt A, Monsen T. Coagulase-negative staphylococci: update on the molecular epidemiology and clinical presentation, with a focus on *Staphylococcus epidermidis* and *Staphylococcus saprophyticus*. Eur J Clin Microbiol 2012; 31:7-20.

17. Wang X-M, Noble L, Kreiswirth BN, Eisner W, McClements W, Jansen KU, et al. Evaluation of a multilocus sequence typing system for *Staphylococcus epidermidis*. J Med Microbiol 2003; 52:989-998.

18. Larsen MV, Cosentino S, Rasmussen S, Friis C, Hasman H, Marvig RL, et al. Multilocus sequence typing of total genome sequenced bacteria. J Clin Microbiol 2012; 50:1355-1361.

19. Lindstedt BA, Heir E, Gjernes E, Vardund T, Kapperud G. DNA fingerprinting of Shiga-toxin producing *Escherichia coli* O157 based on multiple-locus variable-number tandem-repeats analysis (MLVA). Ann Clin Microbiol Antimicrob 2003; 2:12.

20. Kalani BS, Pourajaf A, Sedighi M, Bahador A, Irajian G, Valian F. Genotypic characterization, invasion index and antimicrobial resistance pattern in *Listeria monocytogenes* strains isolated from clinical samples. J Acute Dis 2015; 4:141-146.

21. Dass SC, Abu-Ghannam N, Antony-Babu S, Cummins EJ. Ecology and molecular typing of *Listeria monocytogenes* in a processing plant for cold-smoked salmon in the Republic of Ireland. Food Res Int 2010; 43:1529-1536.

22. Narimani T, Douraghi M, Owlia P, Rastegar A, Esghaei M, Nasr B, et al. Heterogeneity in resistant fecal *Bacteroides fragilis* group collected from healthy people. Microbial Pathogenesis 2016; 95:1-6.

23. Benson G. Tandem repeats finder: a program to analyze DNA sequences. Nucleic Acids Res 1999; 27:573-580.

24. Nunes AP, Silva AC, Paiva AC. Detection of masses in mammographic images using geometry, Simpson's Diversity Index and SVM. IJSISE 2010; 3:40-51.

25. Lindstedt BA, Torpdahl M, Vergnaud G, Le Hello S, Weill E, Tietze E, et al. Use of multilocus variable-number tandem repeat analysis (MLVA) in eight European countries. 2012. Euro Surveill 2012; 18:20385.

26. Doré J, Sghir A, Hannequart-Gramet G, Cortiher G, Pochart P. Design and evaluation of a 16S rRNA-targeted oligonucleotide probe for specific detection and quantitation of human faecal *Bacteroides* populations. Syst Appl Microbiol 1998; 21:65-71.

27. Murphy M, Corcoran D, Buckley JF, O'Mahony M, Whyte P, Fanning S. Development and application of multiple-locus variable number of tandem repeat analysis (MLVA) to subtype a collection of *Listeria monocytogenes*. Int J Food Microbiol 2007; 115:187-194.