Characterization of a Multidrug-Resistant, Novel Bacteroides Genomospecies

Stephen J. Salipante, Aley Kalapila, Paul S. Pottinger, Daniel R. Hoogestraat, Lisa Cummings, Jeffrey S. Duchin, Dhruba J. Sengupta, Steven A. Pergam, Brad T. Cookson, and Susan M. Butler-Wu

Metronidazole- and carbapenem-resistant Bacteroides fragilis are rare in the United States. We isolated a multidrug-resistant anaerobe from the bloodstream and intraabdominal abscesses of a patient who had traveled to India. Whole-genome sequencing identified the organism as a novel Bacteroides genomospecies. Physicians should be aware of the possibility for concomitant carbapenem- and metronidazole-resistant Bacteroides infections.

We previously reported a 2013 case of intraabdominal abscesses and bacteremia caused by a multidrug-resistant anaerobe identified as Bacteroides fragilis (1). In brief, unremitting abdominal pain developed in a 71-year-old man who had been traveling in India for 1 month. The man was hospitalized locally and subsequently received a diagnosis of metastatic colon adenocarcinoma. He returned to Seattle, Washington, USA, for treatment consisting of 5 cycles of chemotherapy, followed by right hemicolectomy and right hepatectomy. On postoperative day 4, the patient showed marked leukocytosis, and abdominal abscesses were noted on computed tomographic scan images. Cultured percutaneous drainage fluid grew Escherichia coli that was resistant to ampicillin, trimethoprim/sulfamethoxazole, and fluoroquinolones. Therapy was then limited to that was resistant to ampicillin, trimethoprim/sulfamethoxazole, and the azole, and fluoroquinolones. Therapy was then limited to that was resistant to ampicillin, trimethoprim/sulfamethoxazole, and the azole, and fluoroquinolones. Therapy was then limited to.

The Study

To better characterize the patient’s clinical isolate, we subjected the organism to whole-genome sequencing by using the MiSeq platform (Illumina, San Diego, CA, USA). In brief, DNA was digested by using NEBNext dsDNA Fragmentase and then end-repaired and A-tailed by using E. coli DNA Polymerase I, T4 PNK, and Taq DNA Polymerase (all from New England Biolabs, Ipswich, MA, USA). Annealed Y adapters (5′-(PO4-) GATCGGAAGAGCGGTTCAGCAG-GAATGCGAGATCTACACTCTTTCCCTACACGACGC-3′) and primer 2 (5′-CGCAGAGCTCTTCCGATCT-3′) were ligated by using T4 DNA Ligase in Rapid Ligation Buffer (Enzymatics, Beverly, MA, USA). The library was PCR-amplified with KAPA HiFi HotStart ReadyMix (Kapa Biosystems, Wilmington, MA, USA) by using primer 1 (5′-AATGATACGGCGACCA-CACCGAGATCTACACTCTTTCCCTACACGACGC-3′) and primer 2 (5′-CAAAGCGAGGACCGCATACGA-TCAAAGGTACCGTGTCGATTTGCTGACGCAA-CG-3′). For sequencing, 250-bp paired-end reads were used with a custom index primer (5′-AGATCGGAAGACGC- GTTACGAGAAATGCGAGACCG-3′); sequencing was performed to an estimated coverage of 61× per base. Oligonucleotides were synthesized by Integrated DNA Technologies (Coralville, IA, USA). De novo genome assembly was performed by using the ABYSS v1.3.5 assembler (2); gene prediction and annotation, using the RAST server v4.0 (3); and comparative genomic analyses, using jSpecies v1.2.1 (4). The assembly was visualized by using BRIG 0.95 (5).

Initial comparison of the clinical isolate with the 3 completed B. fragilis reference genomes (638R, YCH46, NCTC 9343) showed a high degree of sequence divergence (Figure). We expanded our analysis to other sequenced Bacteroides species and observed similar results (Figure). An average nucleotide identity by BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) (AN Ib) analysis (4) was performed (results are shown in online Technical Appendix Table 1, http://wwwnc.cdc.gov/EID/article/21/1/14-0662-Techapp1.pdf). Of note, pairwise AN Ib values of <95% have been used as the cutoff for circumscribing species (4). The clinical isolate demonstrated pairwise AN Ib values of 86.28%–86.54% against B. fragilis reference strains and of antimicrobial drugs, including metronidazole and imipenem (Table). The abscesses ultimately resolved after treatment for 60 days with linezolid and empiric ertapenem.

Author Affiliations: University of Washington, Seattle, Washington, USA (S.J. Salipante, A. Kalapila, P.S. Pottinger, D.R. Hoogestraat, L. Cummings, J.S. Duchin, D.H. Sengupta, S.A. Pergam, B.T. Cookson, and S.M. Butler-Wu); and Fred Hutchinson Cancer Research Center, Seattle (A. Kalapila, S.A. Pergam)

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These first authors contributed equally to this article.
Table. Antimicrobial susceptibility results for a novel Bacteroides genomospecies isolated from the bloodstream and intra-abdominal abscesses of a patient with colon cancer, 2013

| Antimicrobial drug          | MIC, µg/mL* |
|-----------------------------|-------------|
| Ampicillin/sulbactam        | >256/128    |
| Cefotetan                   | 64          |
| Clindamycin                 | >256        |
| Imipenem                    | >32†        |
| Linezolid                   | 2           |
| Metronidazole               | >256†       |
| Minocycline                 | 4           |
| Moxifloxacin                | >32         |
| Piperacillin/tazobactam     | >256        |
| Synercid                    | >32         |
| Tetracycline                | 16          |
| Ticarcillin/clavulanic acid | >256/2      |
| Tigecycline                 | 1           |

*Antimicrobial susceptibility testing performed by using E-test (see online Technical Appendix, http://wwwnc.cdc.gov/EID/article/21/1/14-0662-Techapp1.pdf).†MICs confirmed as ≥64 µg/mL by macrobroth dilution.

Conclusions

Members of the Bacteroides genus constitute a large fraction of the human gut microbiome and are important opportunistic pathogens that can cause a variety of serious infections. Metronidazole is thought to be almost universally effective against the species: only 1 of 1,957 B. fragilis clinical isolates collected across the United States during 2006–2009 was resistant to metronidazole (12). Similarly, previous studies have shown a prevalence of only ≈1% of carbapenem-resistant B. fragilis (13), making this a favored second-line treatment (7). Nevertheless, there are limited but increasing reports of concomitant metronidazole and carbapenem resistance (14), and such resistance poses a threat to current treatment algorithms.

Genomic sequencing revealed the isolate in this study to be a genomospecies related to, but distinct from, B. fragilis. Techniques widely accepted as highly accurate for bacterial identification (16S rRNA sequencing and MALDI-TOF mass spectrometry) incorrectly identified this organism as B. fragilis (1). To determine whether previous multidrug-resistant isolates identified as B. fragilis might instead represent this novel genomospecies, we compared ANIb values for other isolates with those of the clinical isolate in our study. ANIb values indicate that 2 of the multidrug-resistant B. fragilis isolates sequenced in an earlier report (10) actually appear to be members of the genomospecies reported here (online Technical Appendix Table 3). Furthermore, these data highlight the potential for whole-genome sequencing to improve diagnostic accuracy in microbial identification and isolate characterization. Although we detected several antimicrobial drug–resistance factors in this isolate, alternative mechanisms of resistance to carbapenems and metronidazole, including non-cfiA–mediated carbapenem resistance and overexpression of efflux genes, respectively, have been also described and may contribute to the high levels of resistance observed with this isolate.

The increasing prevalence of carbapenem-resistant Enterobacteriaceae worldwide poses a major public health concern, often requiring use of more toxic “last-line agents,” such as colistin and polymyxin B. Although rare thus far, multidrug-resistant Bacteroides infections present a similar challenge to the treating physician and have

Discharges

Although we did not detect homologs of canonical genes nimsA–J, 2 putative nitroimidazole resistance genes were identifiable on the basis of functional annotation (10). We also detected ermF (macrolide resistance) and tetQ genes (tetracycline resistance). The isolate had a substitution in gyrA (Ser82 to Phe) known to confer resistance to fluoroquinolones (11). The draft genome (GenBank accession no. JANI00000000) and sequence reads (Sequence Read Archive accession no. SRP045260) are publicly accessible.

The clinical isolate contained an estimated 5.50 Mbp of DNA, ≈20% more than sequenced B. fragilis reference strains, and 43.81% GC content. The shotgun sequence encodes 5,053 predicted genes, including 1,479 hypothetical proteins without inferred function. This number is significantly (1-tailed z-score = 1) larger than the average predicted gene content of B. fragilis reference genomes (average of 4,760 predicted genes), although we cannot rule out minor contributions from plasmid DNA. Of 1,696 B. fragilis core genes (those present in all B. fragilis reference strains), 1,508 (88.9%) were present in the clinical isolate.

We explored the basis of the isolate’s antimicrobial resistance by performing a BLAST search of the assembly against the nonredundant NCBI (National Center for Biotechnology Information) sequence database that contains published bacterial sequences; confirmation of the relationship indicated that the organism most closely resembles B. fragilis 638R and is not better classified as an alternative species already present in GenBank.

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the potential to become increasingly common in the future. Thus, providers should be aware of the possibility for concomitant carbapenem and metronidazole resistance in these organisms. Future studies will be required to determine what proportion of multidrug-resistant isolates can be ascribed to the novel genomospecies of \textit{Bacteroides} reported here and whether this particular genomospecies offers any opportunities for targeted therapies.

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Dr. Salipante is a laboratory medicine practitioner at the University of Washington, Seattle. His research interests are in the application of genomic technologies to clinical practice. Dr. Kalapila is an Infectious Diseases fellow at the University of Washington. Her research interests are in the management of HIV–hepatitis C virus co-infection.

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\textbf{Figure.} Characterization of circular plot of genome diversity between the clinical isolate of a multidrug-resistant, novel \textit{Bacteroides} genomospecies and other \textit{Bacteroides} spp. isolates. Reading from the center outwards, the map, GC content, and GC skew of the \textit{B. fragilis} reference strain 638R are depicted. The white and colored regions of the following outer rings indicate regions absent and present, respectively, in genomes of the indicated organism compared with the genome of \textit{B. fragilis} reference strain 638R. Intensity of coloration is proportional to the degree of sequence identity relative to the reference genome. The innermost 3 rings indicate the 3 \textit{B. fragilis} reference genomes. The genome of the clinical isolate, separated from other rings by white space, follows. Non-\textit{fragilis} \textit{Bacteroides} species and a \textit{Parabacteroides} species comprise the outermost rings. ATCC, American Type Culture Collection; DSM, Deutsche Sammlung von Mikroorganismen; NCTC, National Collection of Type Cultures.
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Address for correspondence: Susan M. Butler-Wu, University of Washington, Laboratory Medicine, 1959 NE Pacific St, Box 357110, Seattle, WA 98195, USA; email: butlerwu@uw.edu

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Characterization of a Multidrug-Resistant, Novel *Bacteroides* Genomospecies

Technical Appendix

Technical Appendix Table 1. ANIb values for the clinical isolate and other *Bacteroides* species*

| Species                  | B. fragilis strain | B. helcogenes P 36–108 | B. thetaiotaomicron VPI-5482 | B. xylanisolvens XB1A | B. vulgatus ATCC 8482 | B. salanitronis DSM 18170 | Parabacteroides distasonis ATCC 8503 |
|--------------------------|--------------------|------------------------|-----------------------------|----------------------|----------------------|---------------------------|--------------------------------------|
| B. fragilis 638R         | 100                | 72.26                  | 72.65                       | 72.65                | 72.65                | 72.65                     | 72.65                               |
| B. fragilis YCH46        | 98.76†             | 72.26                  | 72.65                       | 72.65                | 72.65                | 72.65                     | 72.65                               |
| B. fragilis NCTC 9343    | 98.77              | 72.26                  | 72.65                       | 72.65                | 72.65                | 72.65                     | 72.65                               |
| Clinical Isolate         | 86.44              | 72.26                  | 72.65                       | 72.65                | 72.65                | 72.65                     | 72.65                               |
| B. helcogenes P 36–108   | 98.58†             | 72.26                  | 72.65                       | 72.65                | 72.65                | 72.65                     | 72.65                               |
| B. thetaiotaomicron VPI  | 74.9               | 72.65                  | 72.26                       | 72.65                | 72.65                | 72.65                     | 72.65                               |
| B. xylanisolvens XB1A    | 74.9               | 72.65                  | 72.26                       | 72.65                | 72.65                | 72.65                     | 72.65                               |
| B. vulgatus ATCC 8482    | 71.01              | 72.65                  | 72.26                       | 72.65                | 72.65                | 72.65                     | 72.65                               |
| B. salanitronis DSM 18170| 69.75              | 72.65                  | 72.26                       | 72.65                | 72.65                | 72.65                     | 72.65                               |
| Parabacteroides distasonis ATCC 8503 | 67.72 | 67.72 | 67.72 | 67.72 | 67.72 | 67.72 | 67.72 |

*Bold font indicates ANIb values >95%, ANIb, average nucleotide identity by BLAST.
†Pairwise ANIb values demonstrate minor differences due to inherent stochasticity in the calculation (1).
| Resistance_type | penta A | Feature_id | start | step | strand | Variantation/Function | Single/Double | iso_sequence |
|----------------|--------|------------|-------|------|--------|------------------------|--------------|-------------|
| Penicillin | 627 | Cg194404666:52319 | 184 | 47597 | 48090 | Pneuovarius S chain (28) | Single | FG0000451-N8 |
| Penicillin | 500 | Cg194404666:52310 | 3805 | 45205 | 61438 | Pneuovarius S chain (28) | Single | FG0000451-N8 |
| βlactamase | 628 | Cg194404666:52312 | 820 | 72520 | 32187 | Eka-lactamase | Single | FG0001229-N6 |
| βlactamase | 629 | Cg194404666:52313 | 2860 | 25829 | 13999 | Eka-lactamase | Single | FG0001586-N3 |
| βlactamase | 687 | Cg194404666:52313 | 3136 | 79679 | 80446 | βlactamase (E 2 3 2.6) | Single | FG0000009-N5 |
| MDR resistance | 442 | Cg194404666:52313 | 378 | 28235 | 27625 | Dimethyladibacatinic transerase | Single | FG0000000-N15 |
|                          | Bacteroides fragilis 638R | Bacteroides fragilis YCH46 | Bacteroides fragilis NCTC 9343 | Bacteroides fragilis HMW 615 | Clinical Isolate | Bacteroides fragilis HMW 610 | Bacteroides fragilis HMW 616 |
|--------------------------|--------------------------|---------------------------|------------------------------|----------------------------|------------------|-----------------------------|----------------------------|
| Bacteroides fragilis 638R | 100*                     | 98.56                     | 98.58                        | 98.3                       | 86.63            | 86.58                       | 87.23                       |
| Bacteroides fragilis YCH46 | 100                     | 100                       | 98.59                        | 98.61                      | 86.64            | 86.59                       | 87.25                       |
| Bacteroides fragilis NCTC 9343 | 100                   | 100                       | 98.18                        | 86.64                      | 86.79            | 86.93                       |                            |
| Bacteroides fragilis HMW 615 | 100                    | 100                       | 86.17                        | 86.55                      | 87.2             |                             |                            |
| Clinical Isolate         |                          |                           |                              |                            |                  | 98                          | 96.66                       |
| Bacteroides fragilis HMW 610 |                        |                           |                              |                            | 100              | 98                          | 97.36                       |
| Bacteroides fragilis HMW 616 |                        |                           |                              |                            |                  | 100                         |                             |

*bold indicates ANIb value above 95%
Susceptibility Testing

A 0.5 McFarland suspension of organism was made in sterile saline and immediately inoculated to Brucella agar with 5% sheep blood, hemin and vitamin K (Remel, Lenexa, KS, USA). The *B. fragilis* ATCC 25285 strain was used for quality control purposes. E-tests corresponding to the antimicrobial drugs shown in the text Table were used in accordance with the manufacturer’s instructions (bioMérieux, Marcy l’Etoile, France). All plates were incubated under anaerobic conditions for 42–48 hours before reading. Where available, antimicrobial susceptibility test results were interpreted according to the Clinical Laboratory Standards Institute (CLSI) MIC interpretive standards for anaerobes. Resistance to metronidazole and imipenem was confirmed by macrobroth dilution using Brucella broth supplemented with hemin (5 µg/mL), vitamin K1 (1 µg/mL) and lyse horse blood (5% v/v). Doubling dilutions of metronidazole and imipenem over a final concentration range of 0.05–64 µg/mL were tested and the tubes incubated anaerobically for 48 hours before reading.

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