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ORIGINAL RESEARCH

Comparison of Phenotypic and Genotypic Patterns of Antimicrobial-Resistant *Bacteroides fragilis* Group Isolated from Healthy Individuals in Vietnam and Japan

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Purpose: Normal non-pathogenic flora can harm the host by acting as a reservoir of resistance determinants that are potentially transferable to human pathogens. This study aimed to assess the phenotypic and genotypic antimicrobial susceptibility patterns of the *Bacteroides fragilis* group (BFG) isolated from healthy individuals in Vietnam and Japan in order to elucidate the prevalence of antimicrobial resistance in human flora in the two economically and geographically different countries.

Materials and Methods: BFG was isolated from fecal samples of 80 healthy individuals in Vietnam (n=51) and Japan (n=29). Isolated strains were identified using MALDI-TOF MS, and the minimum inhibitory concentration (MIC) of 18 antibiotics was determined using the agar dilution method. Additionally, 20 antimicrobial resistance genes were detected using standard PCR.

Results: A total of 139 BFG strains belonging to 11 BFG species were isolated from the two countries, with diversity in the prevalence of each species. *B. fragilis* was not the predominant species. Isolations from Vietnam and Japan showed some similarities in terms of MIC_{50} values, MIC_{90} values, and the percentage of resistant strains. However, isolations from Vietnam showed significantly higher resistance to piperacillin, cefmetazole, clindamycin, tetracycline, and minocycline. *Erm*B, *tet*36, *tet*M, *nim, cat*A, and *qnr*A were not found in either country. *Cep*A was more common in *B. fragilis* than in non-fragilis *Bacteroides*. In contrast, *cfi*A, *erm*G, *mef*A, *msr*SA, *tet*X, *tet*X1, *bex*A, *qnr*B, and *qnr*S were found only in non-fragilis *Bacteroides*. There were differences in the prevalence of *erm*G, *lin*A, *mef*A, *msr*SA, and *qnr*S between isolates from Vietnam and Japan.

Conclusion: This study is the first report on the antimicrobial susceptibility patterns in the BFG isolated from healthy individuals in Vietnam and Japan. Compared to isolations from Japan, isolations from Vietnam showed significantly higher resistance to antimicrobial agents. The distribution of various antibiotic resistance genes also differed between the two countries.

Keywords: antimicrobial resistance patterns, resistance genes, human flora

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Introduction

Anaerobic bacteria, including the most frequently isolated *Bacteroides fragilis* group (BFG) strains, are normal resident members of the gastrointestinal microbiota. BFG members are human opportunistic pathogens that cause severe intra-abdominal, post-operative wound, skin, and soft tissue infections along with aerobic bacteria. The

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number of antimicrobials that are effective against BFG bacteria is relatively limited. Increasing antibiotic resistance in BFG strains has been reported worldwide in recent years, including resistance to β-lactams, tetracyclines, macrolides, clindamycin and fluoroquinolones.[1–6]

The wide distribution of antibiotic resistance genes in BFG harbored by the human gastrointestinal tract could contribute to the maintenance of a balanced microbiota that protects against adverse effects of antibiotic treatments. On the other hand, BFG can potentially harm the host by acting as a reservoir of resistance determinants that may be transferable to human pathogens.[7–9] Moreover, their products may protect commensal bacteria and enteric pathogens against antibiotics, such as membrane vesicles carrying surface-associated β-lactamases that protect *Salmonella typhimurium* against β-lactam antibiotics.[10,11]

Recent studies in Vietnam and Japan showed that various antibiotic resistance genes, including *mcr* and bla_{CTX-M}, found in *E. coli* isolated from healthy individuals could be horizontally transferred among bacterial cells.[12–14] These findings raised questions about the antibiotic susceptibility status and resistance genes carried by other human fecal microbiota species, especially BFG. Unfortunately, little information is available on the antibiotic resistance status and antibiotic resistance genes in BFG in healthy individuals, not only in Vietnam but also in Japan.

This study aimed to investigate the phenotypic and genotypic patterns of antimicrobial-resistant BFG isolated from healthy people in Vietnam and Japan. A wide range of antibiotics (18 antibiotics) and resistance genes (20 genes) were assessed. This research is the first of its kind for these countries and presents a model for similar research to be undertaken elsewhere.

Materials and Methods

Sample Collection, Isolation, and Identification

Fecal samples were collected from 51 and 29 healthy individuals from Thai Binh City, Vietnam and Gifu City, Japan, respectively. All volunteers had no signs of colon or bowel inflammation or disease, and those with a history of antibiotic use during the last three months prior to the study were excluded.

Fecal samples from each person were collected in Puritan® Fecal Opti-Swab CB-206 (Puritan, USA), transported to the laboratory, and inoculated on anaerobic agar plates [Bacteroides Bile Esculin (BBE) agar; Kyokuto Pharmaceutical Industrial, Tokyo, Japan] and BBE-CAZ (BBE with 30 mg/L ceftazidime - CAZ). The plates were then incubated in an anaerobic atmosphere for 48 h at 37 °C. Six well-grown colonies were selected from each plate. Each colony was cultured on modified Gifu Anaerobic Medium (GAM) agar (Nissui Pharmaceutical Co. Ltd, Tokyo, Japan) and incubated in an anaerobic atmosphere (AnaeroPack – Anaero, Mitsubishi Gas Chemical Co. Inc., Tokyo, Japan) for 48 h at 37 °C. Isolates that grew under aerobic conditions were excluded from the study. Isolated strains were identified by matrix-assisted laser desorption ionization - time of flight mass spectrometry (MALDI-TOF) (MALDI Biotyper® MBT, Bruker Japan, Yokohama, Japan). The isolates were stored in 15% skim milk at −80 °C.

Antibiotic Susceptibility Test

Minimum inhibitory concentration (MIC) values were determined using an agar dilution method based primarily on the recommendations of the Clinical and Laboratory Standards Institute (CLSI).[15] The isolates were evaluated for susceptibility to 18 antibiotics: ampicillin, piperacillin, ampicillin/sulbactam, piperacillin/tazobactam, cefmetazole, ceftriaxone, ceftazidime, cefazolin, meropenem, tetracycline, minocycline, erythromycin, clarithromycin, clindamycin, levofloxacin, ciprofloxacin, sulfamethoxazole, and metronidazole. *B. fragilis* ATCC 25285 and *B. thetaiotaomicron* ATCC 29741 were used as quality control strains. Brucella HK agar medium (Kyokuto Pharmaceutical Industrial Co., Ltd., Tokyo, Japan) supplemented with 5% laked sheep blood was used as the test medium. The test strains (10^5 CFU/spot) were inoculated and incubated in an anaerobic chamber (82% N_2, 10% CO_2, and 8% H_2) (Anaerobox, Hirasawa Ltd, Tokyo, Japan) at 35 °C for 48 h.

Detection of β-Lactamase

Nitrocefin discs (BBL Cefinase; Becton, Dickinson, Baltimore, MD, USA) were used to detect β-lactamase production according to the manufacturer's instructions. Isolates with β-lactamase activity will show induce a yellow to red color change on the area where the isolate was smeared. Isolates without β-lactamase activity were scored as negative if there was no change in the color of the nitrocefin disc after 30 minutes.

PCR-Based Detection of Resistance Genes

The following 20 antimicrobial genes were investigated: *cepA, cfxA, cfiA* (β-lactamase), *ermB, ermF, ermG, linA, mefA, msrSA* [macrolide–lincomycin–streptogramin (MLS) resistant], *tetM, tetQ, tetX, tetX1, tet36* (tetracycline resistant), *bexA, qnrA, qnrB, qnrS* (quinolone resistant), *nim* (metronidazole resistant), and *catA* (chloramphenicol resistant). For those strains found to harbor *cfi*A, further investigation of three insertion sequences (IS1186, IS1187, and IS942) was carried out.

Bacterial cells from the colonies on the surface of anaerobic agar plates were suspended in 0.5 mL water in 1.5 mL Eppendorf tubes and incubated at 95 °C for 8 min. The supernatants of the centrifuged suspensions (5 min, 16,000 x g) were used as template DNA and stored at −20 °C. The template DNA (5 µL) was amplified in a 45 µL-reaction mixture consisting of 25 µL of 2x Quick Taq® HS DyeMix (Toyobo Co. Ltd, Osaka, Japan), 2 µL of each primer, and 16 µL of distilled water. Amplification was performed using a Takara PCR Thermal Cycler Dice TP 600 (Takara Bio Inc., Shiga, Japan). The PCR conditions used to detect all 20 genes and 3 insert sequences, primer sequences, and PCR parameters are listed in Table S1. The PCR products were examined by electrophoresis on a 2% agarose gel.

Statistical Evaluation

Comparison of the antimicrobial-resistant percentage and prevalence of different genes between *B. fragilis* and non-fragilis *Bacteroides*, and between the two countries were evaluated using a chi-squared test. The statistical significance was set at $p < 0.05$.

Results

Overview of the Isolates

All the isolates (6 isolates per sample per individual, except isolates that grew under aerobic conditions) were tested for susceptibility to 18 antimicrobial agents and for the presence of 20 antibiotic-resistance genes to build their antimicrobial-resistant profiles. If many isolates in the same person belonged to the same species, only isolates with different profiles were chosen for analysis. Finally, 76 BFG strains from Vietnam and 63 strains from Japan were selected. The identified isolates are listed in Table 1. Eleven BFG species were identified in this study. The first, second, and third most common species were *Parabacteroides distasonis* (35.5%), *B. thetaiotaomicron* (19.7%), and *Phocaeicola* (formerly *Bacteroides*) *vulgatus* (17.1%) in Vietnam, and *B. ovatus* (23.8%), *P. vulgatus* (22.2%), and *P. distasonis* (15.9%) in Japan. The difference in the prevalence of *B. fragilis* between Vietnam and Japan was significant ($p < 0.05$, Table S2). All the isolated strains (139) tested positive for β-lactamase production.

Table 1 Identification of Isolations from Form Healthy Individuals in Vietnam and Japan

Species	Number of Isolations	
	Vietnam	Japan
Bacteroides caccae	1	0
Bacteroides cellulosilyticus	0	1
Bacteroides fragilis	3	9
Bacteroides ovatus	5	15
Bacteroides stercoris	9	5
Bacteroides thetaiotaomicron	15	6
Bacteroides uniformis	1	1
Bacteroides massiliensis	2	0
Parabacteroides distasonis	27	10
Parabacteroides merdae	0	2
Phocaeicola vulgatus	13	14
Total	**76**	**63**

Antimicrobial Susceptibility Profiles

The susceptibility test results are presented in Table 2. Isolations from Vietnam and Japan showed some similarities in MIC_{50} values, MIC_{90} values, and percentages of resistant strains. All isolates were resistant to ampicillin, and none of the isolates were resistant to metronidazole. The rates of resistance to piperacillin, cefmetazole, clindamycin, and tetracycline were high in both countries. However, the isolates from Vietnam showed a significantly higher rate of resistance to these four agents ($p < 0.05$). The MIC_{50} values of three cephalosporins (cefazolin, ceftriaxone, and ceftazidime) and two macrolides (clarithromycin and erythromycin), which have no breakpoint settings, were higher than 128 µg/mL in both countries. The distribution pattern of minocycline was almost the same as that of tetracycline and tended towards lower MIC values. The MIC_{50} and MIC_{90} values of minocycline were 4 µg/mL and 16 µg/mL in Vietnam and 4 µg/mL and 8 µg/mL in Japan. If the breakpoint of tetracycline was applied to minocycline, the resistance rate to minocycline was still much lower than that of tetracycline. The levofloxacin susceptibility status of the two countries

Table 2 Minimum Inhibitory Concentration (MIC) of 18 Antimicrobial Agents Against BFG Isolations from Healthy Individuals in Vietnam and Japan

Antimicrobial Agents	Resistant Breakpoint (μg/mL)[a]	Vietnam (n=76)				Japan (n=63)				*P* value
		MIC Range (μg/mL)	MIC_{50} (μg/mL)	MIC_{90} (μg/mL)	%R[c]	MIC Range (μg /mL)	MIC_{50} (μg/mL)	MIC_{90} (μg/mL)	%R[d]	([c] and [d])
Ampicillin	≥ 2	16 to >128	>128	>128	100	8 to >128	>128	>128	100	_
Ampicillin/sulbactam	≥ 32/16	0.5 to 64	8	32	17.1	1 to 32	8	32	7.9	0.109
Piperacillin	≥ 128	2 to >128	>128	>128	75	4 to >128	128	>128	50.8	0.003
Piperacillin/tazobactam	≥ 128/4	≤0.015 to 128	4	16	1.3	≤0.015 to 32	4	16	0	0.361
Cefazolin	_	16 to >128	>128	>128	_	8 to >128	128	>128	_	_
Cefmetazole	≥ 64	8 to >128	64	>128	61.8	2 to >128	32	128	41.3	0.016
Ceftriaxone	_	16 to >128	>128	>128	_	4 to >128	>128	>128	_	_
Ceftazidime	_	≥128	>128	>128	_	32 to >128	>128	>128	_	_
Meropenem	≥ 16	0.25 to 16	0.5	2	1.3	0.125 to 16	0.25	2	1.6	0.894
Metronidazole	≥ 16	0.25 to 4	1	2	0	0.25 to 2	1	1	0	_
Clindamycin	≥ 8	0.03 to >128	>128	>128	78.9	≤ 0.015 to >128	>128	>128	57.1	0.006
Clarithromycin	_	0.5 to >128	>128	>128	_	0.25 to >128	>128	>128	_	_
Erythromycin	_	2 to >128	>128	>128	_	1 to >128	>128	>128	_	_
Tetracycline	≥ 16	0.25 to 128	32	64	90.8	0.125 to 128	16	64	65.1	< 0.001
Minocycline	≥ 16[b]	≤0.015 to 32	4	16	17.1	≤0.015 to 32	4	8	4.8	0.023
Levofloxacin	_	0.5 to 64	4	32	_	0.5 to 64	4	32	_	_
Ciprofloxacin	_	4 to 128	16	64	_	4 to >128	16	128	_	_
Sulfamethoxazole	_	64 to >128	>128	>128	_	32 to >128	>128	>128	_	_

Notes: [a]Resistant breakpoints from Clinical and Laboratory Standards Institute (CLSI). Performance Standards for Antimicrobial Susceptibility testing. 30th ed. CLSI supplement M100. [b]The breakpoint of tetracycline was used to calculate the resistance percentage of minocycline. [c,d]Percentage of resistant isolations to antibiotics based on CLSI in Vietnam and Japan, respectively. [c,d]Column values were used to calculate the *P* value at the last column.

was similar. However, Japan showed a higher number of strains with high MIC values of ciprofloxacin; only one strain from Vietnam had an MIC value of 128 µg/mL, but 10 (13.9%) strains from Japan had MIC values ≥128 µg/mL (Table 2, Figure S1). Thus, the ciprofloxacin MIC_{90} of isolations from Japan was 128 µg/mL, which was markedly higher than that of isolations from Vietnam (64 µg/mL) (Table 2). All isolates were highly resistant to sulfamethoxazole.

Notably, two meropenem-resistant isolates were found in this study (one from Vietnam and one from Japan). Both isolates were non-fragilis *Bacteroides* (*P. distasonis* from Vietnam and *B. ovatus* from Japan) and had MIC values of 16 µg/mL.

Distribution of Resistance Genes

The distribution of antibiotic resistance genes is presented in Table 3. All the resistance genes present in *B. fragilis* were also found in non-fragilis *Bacteroides*. In contrast, *cfi*A, *erm*G, *mef*A, *msr*SA, *tet*X, *tet*X1, *bex*A, *qnr*B, and *qnr*S were found only in non-fragilis *Bacteroides*. There was a significant difference in the distribution of *ermG, linA, mefA, msrSA*, and *qnrS* between the two countries. The prevalence of *erm*G and *linA* was significantly higher in Japan ($p < 0.001$) and in Vietnam (p=0.003), respectively, than in the other country. *TetQ* was the most prevalent antibiotic resistance gene in both countries. All *bex*A genes were found in *B. thetaiotaomicron*.

β-Lactam Resistance Genes

Of the 12 *B. fragilis* strains isolated from both countries, 11 (91.7%) harbored *cep*A (Table 3). Only one *B. fragilis* strain from Japan was found to be negative for *cep*A, and its MIC value for ampicillin was the lowest (16 µg/mL). The difference in the distribution of *cep*A in *B. fragilis* and non-fragilis *Bacteroides* was significant ($p < 0.001$, Table 3) in both countries. As shown in Figure 1, *cep*A, *cfx*A, and *cfi*A were only found in strains with ampicillin MIC values ≥ 64 µg/mL. Nevertheless, 41% and 44% of isolates from Vietnam and Japan, respectively, were resistant to ampicillin with high MIC values and did not carry any of these genes (Figure 1). All *cep*A-positive isolates (15 strains, Table 3) were susceptible to β-lactamase inhibitor combination agents. Four strains were resistant to cefmetazole with MIC >128 µg/mL (two *B. thetaiotaomicron* and one *B. ovatus* from Vietnam, and one *B. fragilis* from Japan). Among these four strains,

Table 3 Distribution of Antibiotic Resistance Genes in *B. fragilis* and Other *Bacteroides* in Vietnam and Japan

		n	Number of Resistance Genes (%)												
			cepA	*cfxA*	*cfiA*	*ermF*	*ermG*	*linA*	*mefA*	*msrSA*	*tetQ*	*tetX*	*tetX1*	*bexA*	*qnrB*	*qnrS*
Vietnam	*B. fragilis*	3	3 (100)	0 (0)	0 (0)	3 (100)	0 (0)	3 (100)	0 (0)	–	3 (100)	0 (0)	–	0 (0)	0 (0)	0 (0)
	Non-fragilis *Bacteroides*	73	3 (4.1)	37 (50.7)	4 (5.5)	29 (39.7)	5 (6.8)	25 (34.2)	2 (2.7)	–	59 (80.8)	11 (15.1)	–	13 (17.8)	1 (1.4)	5 (6.8)
Total positive strains (%)[a]			6 (7.9)	37 (48.7)	4 (5.26)	32 (42.1)	5 (6.6)	28 (36.8)	2 (2.6)	0 (0)	62 (81.6)	11 (14.5)	0 (0)	13 (17.1)	1 (1.3)	5 (6.6)
Japan	*B. fragilis*	9	8 (88.9)	2 (22.2)	0 (0)	5 (55.5)	0 (0)	2 (22.2)	0 (0)	0 (0)	9 (100)	0 (0)	0 (0)	0 (0)	–	–
	Non-fragilis *Bacteroides*	54	1 (1.8)	23 (42.6)	3 (5.56)	23 (42.6)	19 (36.1)	8 (14.8)	9 (16.7)	4 (7.4)	38 (70.4)	8 (14.8)	1 (1.9)	6 (11.1)	–	–
Total positive strains (%)[b]			9 (14.3)	25 (39.7)	3 (4.8)	28 (44.4)	19 (35.2)	10 (15.9)	9 (14.3)	4 (6.3)	47 (74.6)	8 (12.7)	1 (1.6)	6 (9.5)	0 (0)	0 (0)
***P value* ([a] and [b])**			0.227	0.288	0.892	0.781	<0.001	0.006	0.012	0.026	0.319	0.761	0.270	0.195	0.361	0.038

Notes: *cepA, cfxA, cfiA*: β-lactamase resistance genes. *ermB, ermF, ermG, linA, mefA, msrSA*: macrolide–lincomycin–streptogramin (MLS) resistance genes. *tetM, tetQ, tetX, tetX1, tet36*: tetracycline resistance genes. *bexA, qnrA, qnrB, qnrS* are quinolone resistance genes. *nim*: metronidazole resistance genes. *catA*:chloramphenicol resistance gene. *ErmB, tet36, tetM, nim, catA*, and *qnrA* were not found in either country. [a,b]Are percentage of resistant gene positive strains in Vietnam and Japan, respectively. [a,b]Are used for calculating the *P* value in the last row.

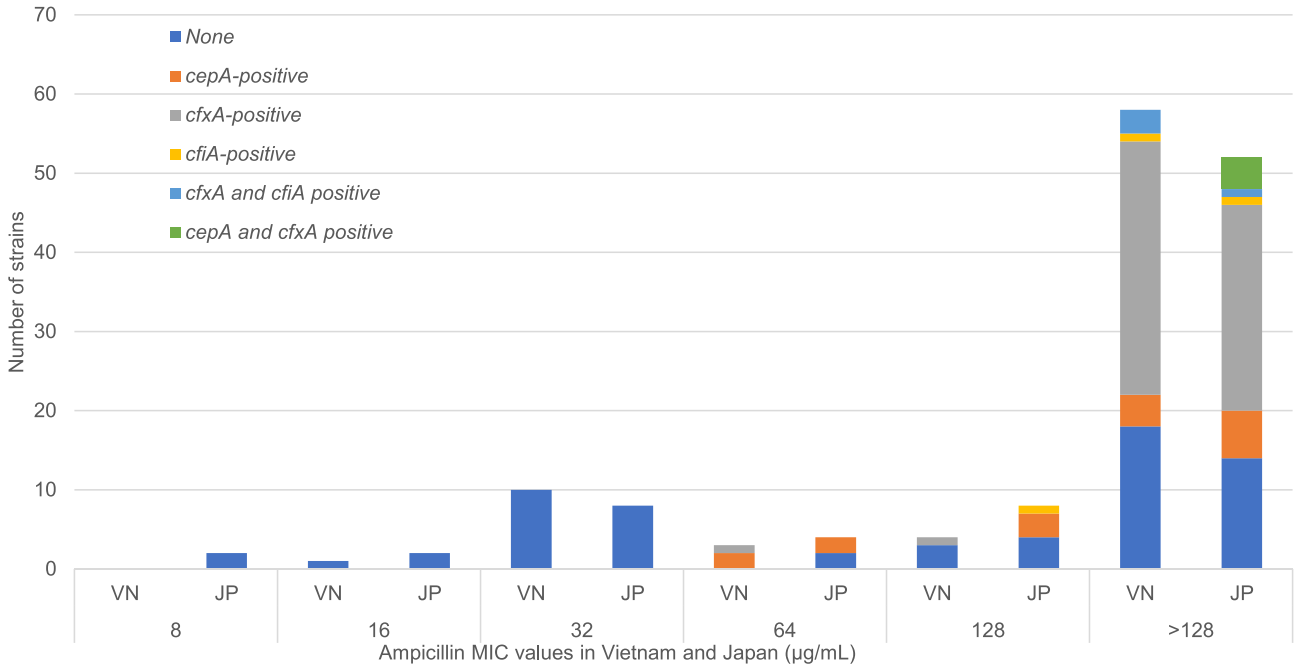

Figure 1 The distribution of *cep*A, *cfx*A, and *cfi*A in BFG strains in Vietnam and Japan based on MIC values for ampicillin.
Abbreviations: BFG, *Bacteroides fragilis* group; MIC, minimum inhibitory concentration; VN, Vietnam; JP, Japan.

B. thetaiotaomicron from Vietnam and *B. fragilis* from Japan did not harbor *cfx*A or *cfi*A.

Notably, two meropenem-resistant isolates were found in this study (one from Vietnam and one from Japan). Both isolates were non-fragilis *Bacteroides* (*P. distasonis* from Vietnam and *B. ovatus* from Japan) and had MIC values of 16 μg/mL. *P. distasonis* did not carry *cfi*A, but *B. ovatus* did. In contrast, four strains from Vietnam and two from Japan harbored *cfi*A but had MIC values ≤1 μg/mL. These six strains were also susceptible to cephalosporins and β-lactamase inhibitor combination agents. Since the insertion of IS elements can increase the expression of the *cfi*A genes, we checked three insertion sequences (IS1186, IS1187, and IS942), but all were negative (data not shown).

Macrolide–Lincomycin–Streptogramin Resistance Genes

In total, clindamycin-resistant strains comprised 83.3% (50/60), 70.8% (17/24), 68.4% (26/38), 81.8% (9/11), and 75.0% (3/4) of the total *erm*F-, *erm*G-, *lin*A-, *mef*A-, and *msr*SA-positive strains, respectively (Table S3B). There was no strict correlation between the presence of these genes and MIC values for clindamycin (Table S3A).

Tetracycline Resistance Genes

Only one strain from Japan (GAI20143) was found to carry *tet*X1 (Table 3). This strain also harbored *tet*Q and *tet*X. MIC values of GAI20143 for tetracycline and minocycline were 32 μg/mL and 4 μg/mL, respectively. Five other strains (two from Vietnam and three from Japan) harbored either *tet*Q or *tet*X and showed phenotypic susceptibility to tetracycline (Figure 2A). In contrast, eight strains did not carry any of the tested resistance genes but showed MIC values for tetracycline >16 μg/mL and for minocycline >2 μg/mL. Interestingly, these strains were isolated from Vietnam (Figure 2A and B).

Fluoroquinolone Resistance Genes

This study investigated the presence of four genes related to fluoroquinolones resistance in *Bacteroides* (*bexA, qnrA, qnrB, qnrS*). The prevalence of those genes is shown in Table 3. The *qnrB* and *qnrS* genes were found in Vietnamese strains only. No strain was found to carry more than one of those genes. The distribution of *bex*A, *qnr*A, *qnr*B, *qnr*S in all strains based on country and levofloxacin and ciprofloxacin MIC values is shown in Figure S1. There was no relationship between those genes and MIC values of both levofloxacin and ciprofloxacin.

Discussion

Many studies on antibiotic resistance in *Bacteroides* spp. have been conducted worldwide, but these have mainly focused on clinical isolates (Table S2). *B. fragilis*, the

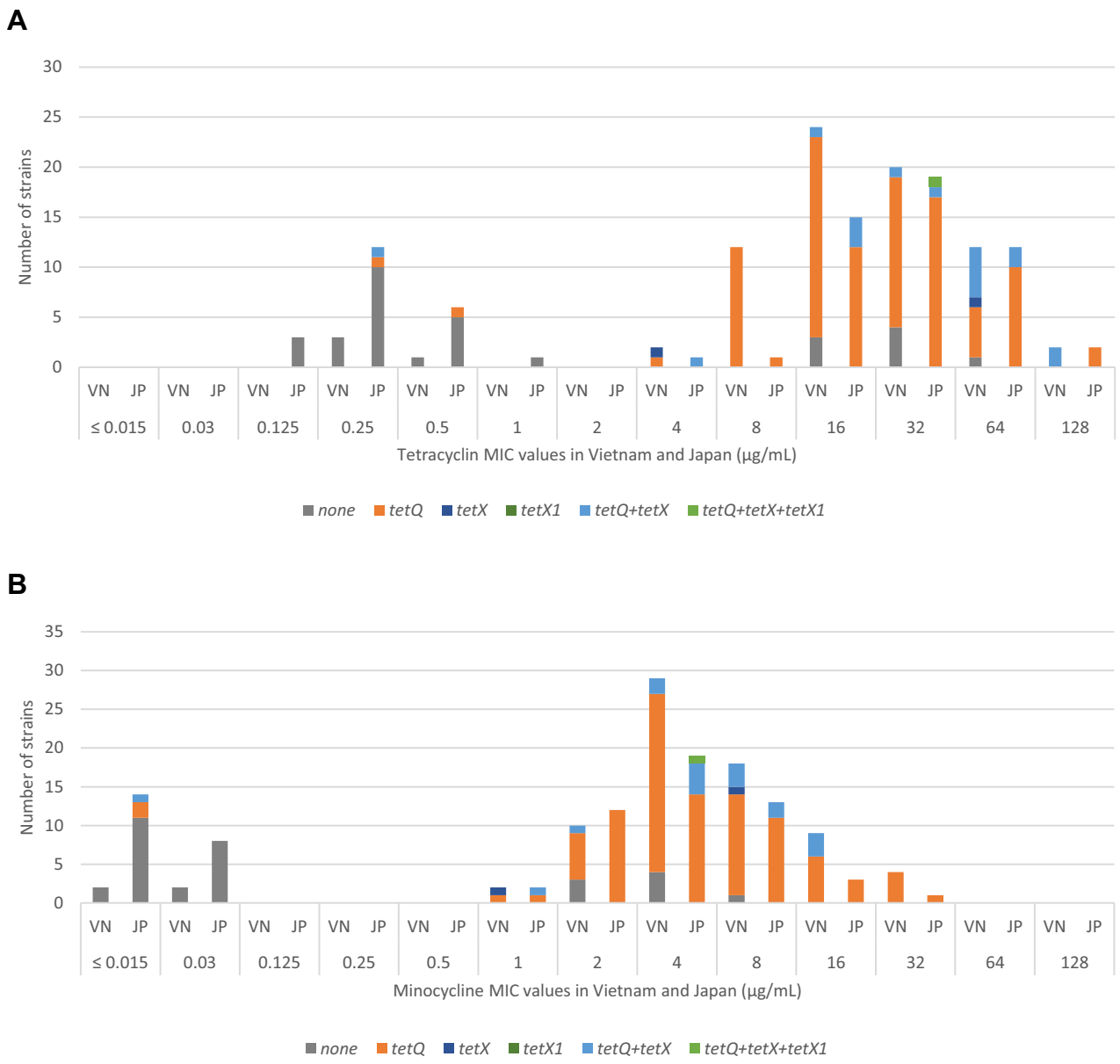

Figure 2 The distribution of resistance genes responsible for tetracycline resistance (*tet*Q, *tet*X, *tet*X1) found in *Bacteroides* strains in Vietnam and Japan based on MIC values for tetracycline and minocycline. (**A**) MIC values for tetracycline in Vietnam and Japan (μg/mL). (**B**) MIC values for minocycline in Vietnam and Japan (μg/mL).
Abbreviations: VN, Vietnam; JP, Japan.

most virulent *Bacteroides* species,[7] is the most commonly detected isolate found in clinical samples, but it was not the dominant species identified in the present study. Among studies on fecal samples from healthy individuals, the percentages of *B. fragilis* are inconsistent. Indeed, studies from Brazil, Iran, and Poland reported a relatively high prevalence of *B. fragilis* in healthy individuals compared to our findings (Table S2).[4,16–18] The observed discrepancies might be attributed to different techniques (sampling, culturing technique, number of pick-ups), variations in the recovery rates of bacterial strains, and the biodiversity of human bacterial flora in different people in different countries. In general, *B. fragilis* is not predominant in BFG.[7,19] Our study supports this finding.

Thirty-one isolates from Vietnam and 29 from Japan were negative for *cepA, cfxA*, and *cfiA*, but all the isolated strains (139) tested positive for production of β-lactamase. This finding suggests that other types of β-lactamase production can be found in these isolates. Notably, *cep*A was reportedly found only in *B. fragilis* as a species-specific β-lactamase-encoding gene.[20–22] The *cepA*-positive non-

fragilis *Bacteroides* were recently reported in Europe[23] and in Iran, with a notably higher prevalence.[24] Our study is the first to document this phenomenon in Vietnam and Japan. In this study, four strains that were highly resistant to cefmetazoles (MIC of 128 μg/mL) were observed, three of which (two *B. thetaiotaomicron* from Vietnam and one *B. fragilis* from Japan) harbored neither *cfx*A nor *cfi*A. This finding raises concerns regarding cefmetazole resistance in *Bacteroides* with inconsistent mechanisms, as recently noted in the literature.[21,25] Of the two non-fragilis *Bacteroides* (*P. distasonis* from Vietnam and *B. ovatus* from Japan) that were moderately resistant to meropenem (MIC values of 16 μg/mL), *P. distasonis* did not carry *cfi*A, but *B. ovatus* did. Notably, six non-fragilis *Bacteroides* with silent *cfiA* were found in both Vietnam and Japan. The observation of silent *cfi*A has been previously described.[21,23] The present study reports two phenomena: *cfi*A-negative *P. distasonis* resistant to meropenem and occurrence of silent *cfi*A in non-fragilis *Bacteroides* (Table 3). BFG carbapenem resistance is still rare but has recently been reported worldwide, including Japan.[23,26–29] The most common mechanism of acquiring resistance to carbapenems in *Bacteroides* is by producing carbapenemase, a metallo-β-lactamase enzyme encoded by *cfi*A. This mechanism mainly contributes to carbapenem resistance in *B. fragilis* and is quite rare in non-fragilis *Bacteroides*. Therefore, the wide prevalence of the silent *cfiA* in non-fragilis *Bacteroides*, which is more resistant than *B. fragilis*, is a serious concern. The *cfi*A-negative meropenem-resistant *P. distasonis* was resistant to other β-lactam antibiotics with high MIC values and harbored neither *cep*A nor *cfx*A. We intend to conduct further investigation to better understand these findings.

In this study, metronidazole was highly effective against all isolates, and *nim* was not found in any of them. Previous studies have shown that the *nim* gene has not yet circulated among anaerobic bacteria in Japan.[20,21] In recent reports from India, the *nim* gene was detected at a notably high prevalence in BFG isolated from clinical samples.[29,30] This study is the first surveillance on the presence of *nim*-carrying anaerobes among isolates from healthy individuals in Vietnam. In Vietnam, metronidazole is widely used to treat anaerobic and parasitic infections; however, metronidazole resistance or circulation of the *nim* gene has not yet been investigated in Vietnam.

Clindamycin-resistant strains carried *erm*F, *erm*G, *lin*A, *mef*A, and *msr*SA, but some clindamycin-non-resistant strains also carried these genes. There was no strict correlation between the presence of these genes and MIC values for clindamycin (Table S3A). This observation indicates that the presence of only some of these genes might not be sufficient to lead to phenotypic clindamycin resistance, although their presence in combination might in some cases (Table S3B). Further studies on the expression of these genes will be necessary to define their contribution to antibiotic resistance. Significant differences in the distribution of *erm*F and *lin*A between clindamycin-resistant and non-resistant groups were observed in Vietnam but not in Japan (Table S3A). This finding partially supports the hypothesis that *erm*F and *lin*A, alone or in combination with other MLS resistance genes, play an important role in the clindamycin-resistance mechanism in BFG.[21,23,28,31,32] A five-year-analysis from Poland showed a very close correlation between the presence of the *erm*F gene and MIC values for clindamycin in BFG.[32] Considering that *erm*F and *lin*A are two of a variety of transmissible elements involved in disseminating antibiotic resistance determinants, this finding addresses a serious concern about the future of clindamycin-based antibiotic therapy, not only for BFG but also other residents of the human flora.[6,8,33]

Compared to recent studies from European countries on clinical samples,[23,34] our results, in Vietnam and Japan separately, showed a considerably higher presence of resistance genes (*erm*F, *erm*G, *lin*A, *mef*A, and *msr*SA) in both clindamycin-resistant and non-resistant groups (Table S3). Our findings were in agreement with a report from Poland in 2019[4] that BFG bacteria isolated from healthy people carry high frequencies of genes encoding resistance to MLS antibiotics. Although our study detected the five most common MLS resistance genes, many clindamycin-resistant strains with higher MIC values did not carry any of those genes, indicating the presence of unknown resistance mechanisms (Table S3B).

Only one strain, *B. thetaiotaomicron*, carried a set of three genes, *erm*G-*mef*A-*msr*SA. This combination was previously reported in a conjugative transposon, CTnGERM1.[35] Our isolate was found to be highly resistant to clindamycin, clarithromycin, and erythromycin. Recent findings from European countries showed a notable prevalence of the *erm*G-*mef*A-*msr*SA combination.[23] However, despite a higher prevalence of resistance genes in the present study, the *erm*G-*mef*A-*msr*SA combination was not common.

Susceptibility tests were performed for all strains with both clarithromycin and erythromycin. The MIC values of clarithromycin and erythromycin showed similarities, but with some exceptions, as listed in Table S4. These strains were found in both the countries. There were no notable

differences in the resistance genes among the strains. Therefore, using MIC values for erythromycin to interpret clarithromycin susceptibility status may cause some misleading results. With such diversity in susceptibility distribution, a holistic approach is needed in the future to test the susceptibility of BFG to macrolides.

Five tetracycline-susceptible strains harbored either *tet*Q or *tet*X. In contrast, eight tetracycline-resistant strains from Vietnam did not carry any of the tested resistance genes (Figure 2A). This raises a question about the possible existence of silent *tet*Q and *tet*X. Another issue that can be considered is that Vietnam, with its over-the-counter antibiotic use, might be nurturing a variety of mechanisms for tetracycline-resistance among BFG, such as drug efflux (*tet*A-E, *tet*K-L) or ribosomal protection (*tet*M, *tet*W), such as those that have been found in recent publications.[4,36] Even though tetracycline is not advised to treat BFG infections, the mechanisms by which a strain can become resistant pose a serious concern in anaerobic research because of the potential for transfer to other human flora via plasmids and transposons, especially under conditions in which there is exposure to antibiotics.[8,33,36,37]

The use of quinolones as monotherapy for mixed infections has been limited by their lack of activity against anaerobic pathogens, especially in the case of ciprofloxacin.[33,38] However, newly developed fluoroquinolones, such as the fourth- and fifth-generation fluoroquinolones, showed promising effects in treating BFG infection.[38–40] Moreover, recent studies of fluoroquinolone-resistance in which *qnr*A, *qnr*B, *qnr*S, and *bex*A were commonly detected also concluded that an effective method for determining fluoroquinolone-resistant mechanisms remains to be developed.[4,23] Further analysis of mutations in *gyr*A needs to be carried out on these strains to better understand this issue.

Differences in antibiotic-resistant patterns and prevalence of resistance genes between countries might be caused by differences in how antibiotics are used in those countries. Some studies on aerobic bacteria in healthy people in Vietnam and Japan also showed a high prevalence of resistance genes.[13,14,41] Further investigations on the relationship between antibiotic usage and the prevalence of resistance genes in the human flora, both aerobic and anaerobic, will need to be carried out.

Conclusion

This study is the first to genetically and phenotypically characterize antimicrobial resistance of *Bacteroides* isolated from healthy individuals in Vietnam and Japan. Data collected revealed the need to broaden the focus of BFG research to include not only the most virulent member *B. fragilis*, but also other members owing to their overwhelming numbers and their role as a source of diverse antibiotic-resistant determinants.

Isolates from Vietnam and Japan showed similarities in antimicrobial-resistant BFG. However, isolates from Vietnam showed significantly higher resistance to piperacillin, cefmetazole, clindamycin, tetracycline, and minocycline.

A comparison of the predominant species in the healthy population of these two Asian countries showed several differences but further research will be required to investigate and extend the suggestive findings of this work.

Abbreviations

BFG, *Bacteroides fragilis* group; MALDI-TOF MS, matrix-assisted laser desorption ionization - time of flight mass spectrometry; MIC, minimum inhibitory concentration; MLS, macrolide–lincomycin–streptogramin; PCR, polymerase chain reaction.

Ethics Approval and Informed Consent

The study was approved by the Ethics Committees of Gifu University (Gifu, Japan; Approval Number 2019-164) and Thai Binh University of Medicine and Pharmacy (Thai Binh, Vietnam; Approval Number 773.1/HĐĐĐ). All study participants provided informed written consent prior to study enrollment. This study was conducted in accordance with the Declaration of Helsinki.

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Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

The authors report no conflicts of interest.

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