Detection of Vancomycin Resistant Genes in Intrinsically Antibiotic Resistant Bacteria from the Gut Microbiota of Indonesian Individuals

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What's Known

- Gut microbiota serves as a reservoir for antibiotic resistant genes.
- Vancomycin-resistant genes are found in Gram-positive bacteria, such as Enterococcus, and Staphylococcus.

What's New

- Indonesian gut microbiota harbors resistant genes for multiple antibiotics with high frequency and abundance.
- Gram-negative bacteria, which are intrinsically vancomycin-resistant bacteria, have vancomycin-resistant genes and can act as a reservoir for vancomycin-resistant genes in the gut.

Abstract

Background: Antibiotic resistance is a global public health concern that has been exacerbated by the overuse and misuse of antibiotics, leading to the emergence of resistant bacteria. The gut microbiota, often influenced by antibiotic usage, plays a crucial role in overall health. Therefore, this study aimed to investigate the prevalence of antibiotic resistant genes in the gut microbiota of Indonesian coastal and highland populations, as well as to identify vancomycin-resistant bacteria and their resistant genes.

Methods: Stool samples were collected from 22 individuals residing in Pacet, Mojokerto, and Kenjeran, Surabaya Indonesia in 2022. The read count of antibiotic resistant genes was analyzed in the collected samples, and the bacterium concentration was counted by plating on the antibiotic-containing agar plate. Vancomycin-resistant strains were further isolated, and the presence of vancomycin-resistant genes was detected using a multiplex polymerase chain reaction (PCR).

Results: The antibiotic resistant genes for tetracycline, aminoglycosides, macrolides, beta-lactams, and vancomycin were found in high frequency in all stool samples (100%) of the gut microbiota. Meanwhile, those meant for chloramphenicol and sulfonamides were found in 86% and 16% of the samples, respectively. Notably, vancomycin-resistant genes were found in 16 intrinsically resistant Gram-negative bacterial strains. Among the detected vancomycin-resistant genes, *vanG* was the most prevalent (27.3%), while *vanA* was the least prevalent (4.5%).

Conclusion: The presence of multiple vancomycin resistance genes in intrinsically resistant Gram-negative bacterial strains demonstrated the importance of the gut microbiota as a reservoir and hub for the horizontal transfer of antibiotic resistant genes.

Introduction

Antibiotic resistance poses a significant and growing threat to public health, including in Indonesia, where it is projected to cause up to 10 million deaths worldwide annually by 2050,[1] primarily due to antibiotic overuse and misuse, which is often caused by lenient regulation and monitoring practices.[2] Antibiotic resistance

was shown to be alarmingly high in individuals with acute respiratory tract infections (ARTIs) in Indonesia. Multiple antibiotics were shown to be less effective, with high resistance values observed for amoxicillin (70.25%), levofloxacin (50.0%), ciprofloxacin (43.03%), cefixime (38.0%), and tetracycline (92.86%).[3-6] Furthermore, a surveillance study across five hospitals detected increased resistance levels against commonly used antibiotics, such as ampicillin, cotrimoxazole, and ciprofloxacin, in *Escherichia coli* and *Klebsiella pneumoniae* isolated from patients with urinary tract infections (UTIs).[7]

The use of antibiotics is associated with significant changes in the composition of the gut microbiota, leading to the emergence of antibiotic resistant strains.[8] As the gut microbiota plays a crucial role in digestive health and disease prevention, disruptions to its composition and function can have a significant impact on overall health.[9] Studies indicated a high frequency of antibiotic resistance in the gut microbiota of healthy individuals, with some estimates claiming that up to 80% of human gut bacteria were resistant to at least one antibiotic.[10] Therefore, this study focused on investigating the presence of genes exhibiting antibiotic resistance, particularly vancomycin resistance, in the gut microbiota of Indonesian populations. Vancomycin is a last-resort antibiotic used to treat Gram-positive bacterial infections such as methicillin-resistant *Staphylococcus aureus* (MRSA) and *Clostridioides difficile*. However, its widespread use has led to the emergence and spread of vancomycin-resistant enterococci (VRE) and other resistant strains, posing a serious threat to public health.[11] Gram-negative bacteria, which are intrinsically resistant to vancomycin due to their cell wall structure, may contain vancomycin-resistant genes that can be horizontally transferred to other bacterial species.[12]

In this study, we aimed to investigate the occurrence of antibiotic resistant genes, namely vancomycin-resistant genes and bacteria, in the gut microbiome of Indonesian populations. The frequency of antibiotic resistant genes in the gut microbiota of selected individuals was determined using metagenomic analysis. Vancomycin-resistant bacteria were isolated and identified, then their resistant genes were determined to gain insights into the prevalence and spread of vancomycin resistance in the human gut microbiota. The findings of this study could have significant implications for public health, as the emergence and spread of antibiotic resistant bacteria can increase morbidity and mortality rates while complicating the treatment of infections. By understanding the prevalence and

mechanisms of antibiotic resistance, strategies can be developed to mitigate resistance spread and preserve the effectiveness of antibiotics as crucial life-saving treatments.

Materials and Methods

The study protocol was approved by the University of Surabaya Health Research Ethics Committee (No. 005-OL/KE/III/2021). All the collected human stool samples were anonymized, and written informed consent was obtained from all the participants.

Study Participants, Stool Sample Collection, and DNA Extraction

Stool samples collected in previous studies[13, 14] were used for this study. They were obtained from the coastal population of Kenjeran, Surabaya (comprising nine males and two females) and the highland population of Pacet, Mojokerto (consisting of five males and six females). The participants were selected based on the following criteria: healthy condition, aged between 20-50 years old, and no recent antibiotic consumption within the previous two months. The DNA was extracted from the collected stool samples using the Zymbiomic DNA Miniprep Kit (Zymo Research, Germany) according to the manufacturer's instructions.

Normalized Read Counts

A process of normalizing read counts was used to assess gene expression levels, as previously described.[15, 16] Metagenomic data from the DNA Data Bank of Japan (DDJP) with submission number SSUB023028 were utilized.[15] The reference or target gene sequences were downloaded from the NCBI database (table 1). Subsequently, all DNA fragments or reads from the samples were mapped against the sequences using Burrows-Wheeler Aligner (BWA). SAMtools were used to extract the corresponding counts from the generated Sam files. To enable meaningful comparisons between samples and genes, the counts were then normalized based on the number of sequenced reads and the length of the respective genes.

Enumeration of Culturable Aerobic Antibiotic Resistant Bacteria

The collected stool samples were diluted in a sterile 0.9% NaCl solution, and serial dilutions were prepared. Enumeration of the bacteria was performed using the total plate count (TPC) method on a tryptic soy agar (TSA) supplemented with specific concentrations of antibiotics as listed in table 2.[17]

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Table 1: Accession numbers of the reference genes used for read count analyses

No.	Gene	Reference species	Resistance against	Accession number	Locus tag
1	*rpoB*	*Bacteroides intestinalis*	Housekeeping gene	NZ_QRKQ01000009.1	DW169_RS10850
2	*tetA*	*Salmonella enterica*	Tetracycline	NC_022372.1	pYT3_0150
3	*tetB*	*Bacillus subtilis*	Tetracycline	NC_020507.1	BSU6051_40770
4	*tetC*	*Escherichia coli*	Tetracycline	NC_024960.1	HXG72_RS00125
5	*tetD*	*Salmonella enterica*	Tetracycline	NC_019114.1	pSH111_227_106
6	*tetE*	*Aeromonas hydrophila*	Tetracycline	NC_016852.1	PAAH01_p10
7	*tetG*	*Pasteurella multocida*	Tetracycline	NC_004771.1	pJR1_p2
8	*tetH*	*Actinobacillus pleuropneumoniae*	Tetracycline	NC_010889.1	p12494_p03
9	*otrB*	*Streptomyces rimosus*	Tetracycline	NZ_CP023688.1	CP984_RS02355
10	*tetM*	*Staphylococcus aureus*	Tetracycline	NC_022604.1	SAZ172_RS02060
11	*tetO*	*Campylobacter jejuni*	Tetracycline	NC_022354.1	N755_01771
12	*tetP*	*Clostridium saccharobutylicum*	Tetracycline	NC_022571.1	CLSA_c15510
13	*tetQ*	*Lactobacillus brevis*	Tetracycline	NC_020819.1	LVISKB_2312
14	*tetS*	*Lactococcus lactis*	Tetracycline	NC_024965.1	D688_p1012
15	*tetW*	*Bifidobacterium animalis*	Tetracycline	NC_017866.1	W7Y_0968
16	*otrA*	*Streptomyces davawensis*	Tetracycline	NC_020504.1	BN159_1010
17	*aacA*	*Escherichia coli*	Aminoglycoside	NC_014615.1	ETN48_p0094
18	*aacC*	*Escherichia coli*	Aminoglycoside	NC_019066.1	pAPEC1990_61_126
19	*aadA*	*Escherichia coli*	Aminoglycoside	NC_019082.1	HS908_RS00060
20	*aadB*	*Salmonella enterica*	Aminoglycoside	NC_022522.2	p164310_0595
21	*aphA*	*Escherichia coli*	Aminoglycoside	NC_008460.1	HXB98_RS00685
22	*aphD*	*Staphylococcus aureus*	Aminoglycoside	NC_005024.1	pSK41_p44
23	*satA*	*Bacillus cereus*	Aminoglycoside	NC_011725.1	BCB4264_RS15655
24	*strA*	*Salmonella enterica*	Aminoglycoside	NC_022522.2	p164310_0580
25	*strB*	*Salmonella enterica*	Aminoglycoside	NC_022522.2	p164310_0575
26	*ermA*	*Staphylococcus aureus*	Macrolide	NC_017341.1	SAA6008_00830
27	*ermB*	*Enterococcus faecium*	Macrolide	NC_021170.1	D687_p2034
28	*ermC*	*Staphylococcus aureus*	Macrolide	NC_007792.1	SAUSA300_pUSA030007
29	*ermE*	*Saccharopolyspora erythraea*	Macrolide	NC_009142.1	SACE_0733
30	*ermF*	*Bacteroides sp.*	Macrolide	NG_034698.1	
31	*ermT*	*Streptococcus pyogenes*	Macrolide	NC_010423.2	D684_p1003, pRW35_1
32	*mphA*	*Escherichia coli*	Macrolide	NC_024955.2	orf00017
33	*cmlA*	*Escherichia coli*	Chloramphenicol	NC_019043.1	ND11Incl1_14
34	*catB*	*Clostridium saccharoperbutylacetonicum*	Chloramphenicol	NC_020291.1	Cspa_c34740
35	*cat*	*Bacteroides fragilis*	Chloramphenicol	NC_003228.3	BF9343_RS21275
36	*floR*	*Salmonella enterica*	Chloramphenicol	NC_012693.1	pAM04528_0040
37	*vanA*	*Enterococcus faecium*	Vancomycin	NC_013317.1	SAP083A_022
38	*vanB*	*Enterococcus faecium*	Vancomycin	NC_021994.1	EFAU085_00735
39	*vanC*	*Enterococcus casseliflavus EC20*	Vancomycin	NC_020995.1	ECBG_RS11575, ECBG_02849
40	*vanD*	*Longicatena caecimuris*	Vancomycin		KJS44_RS09715, L3BBH23_19240
41	*vanF*	*Paenibacillus larvae subsp. larvae*	Vancomycin		ERICIV_RS18855, ERICIV_03885
42	*vanG*	*Clostridioides difficile*	Vancomycin		KNZ77_RS07990, KNZ77_07990
43	*vanI*	*Clostridium tagluense*	Vancomycin		LGL05_RS06360, LGL05_06360
44	*vanM*	*Enterococcus faecium*	Vancomycin		E6A31_RS13925, E6A31_14770
45	*ddlA*	*Bacteroides fragilis NCTC 9343*	Vancomycin	NC_003228.3	BF9343_0418
46	*dfrA*	*Clostridium saccharoperbutylacetonicum*	Sulfonamide	NC_020291.1	Cspa_c56250
47	*sul*	*Bacillus amyloliquefaciens*	Sulfonamide	NC_009725.1	RBAM_000880
48	*ampC*	*Escherichia coli*	Beta lactam	NC_000913.3	b4150, ECK414
49	*bla*	*Salmonella enterica*	Beta lactam	NC_010119.1	pOU7519_76
50	*mecA*	*Staphylococcus aureus*	Beta lactam	NC_002952.2	SAR0039
51	*penA*	*Burkholderia ambifaria*	Beta lactam	NC_008391.1	BAMB_RS21825

Table 2: Antibiotic concentrations used for the enumeration of antibiotic resistant bacteria

Antibiotic	Class	Concentration (µg/mL)
Chloramphenicol	Chloramphenicol	32
Ampicillin	Beta-lactam	16
Tetracycline	Tetracycline	16
Kanamycin	Aminoglycoside	64
Trimethoprim-Sulfamethoxazole	Sulfonamide	4/76
Erythromycin	Macrolide	8
Vancomycin	Vancomycin	32

The diluted samples were plated on agar media and incubated for 24 hours at 37 °C. The formed colonies were then quantified, and the process was repeated three times for each sample.

Isolation and Identification of Vancomycin Resistant Bacteria

Colonies grown on TPC plates were selected based on distinctive features and further purified to obtain monoculture isolates. The purified isolates were then inoculated into TSA agar supplemented with 32 µg/mL of vancomycin and incubated at 37 °C for 24 hours. The colonies were picked and resuspended in 100 µL of phosphate-buffered saline solution. The DNA of the resuspended bacterial cells was extracted using the Wizard®Genomic DNA Purification Kit (Promega, Germany). Using Q5 polymerase (New England Biolabs, Germany), the isolated DNA was subjected to 16s rRNA polymerase chain reaction (PCR) amplification with 27F and 1492R primers.[18-20] The PCR products were purified using Illustra GFX DNA and Gel Band Purification Kit (GE Healthcare, USA). Using the obtained sequences from amplified 16S rRNA genes, the purified products were sequenced and bacterial species were identified by conducting BLASTN against the NCBI 16S rRNA database.

Detection of Vancomycin Resistant Genes in Vancomycin-resistant Isolates

The extracted DNA from the isolates obtained in the previous step was subjected to vancomycin-resistant gene detection. A multiplex PCR was performed to determine the presence of vancomycin-resistant genes, as described by Bhatt and others.[21] This process employed a mixture containing a DNA template, primers (0.5 µM each; table 3), dNTP (50 µM), Taq DNA polymerase (2 U/µL) with an appropriate buffer, and water. The PCR was performed under the following conditions: initial denaturation for 3 min at 94 °C, followed by 35 cycles of amplification consisting of 1 min at 94 °C, 1 min at 45 °C, and 1 min at 72 °C, with a final extension at 72 °C for 7 min. The generated products were then qualitatively analyzed using gel electrophoresis.

Results

Widespread Presence of Antibiotic Resistant Genes in Gut Microbiota

The relative gene abundance was analyzed based on reading counts from the previously collected stool samples of coastal and highland populations in Indonesia.[13-15] Mapping the reads against antibiotic resistant genes presented in table 1 revealed the widespread presence of antibiotic resistant genes from various antibiotic groups in stool samples of both population (figure 1A). The antibiotic resistant genes against tetracycline, aminoglycosides, macrolides, beta-lactams, and vancomycin were found in all participants (100%), while those for chloramphenicol and

Table 3: Primers used in this study

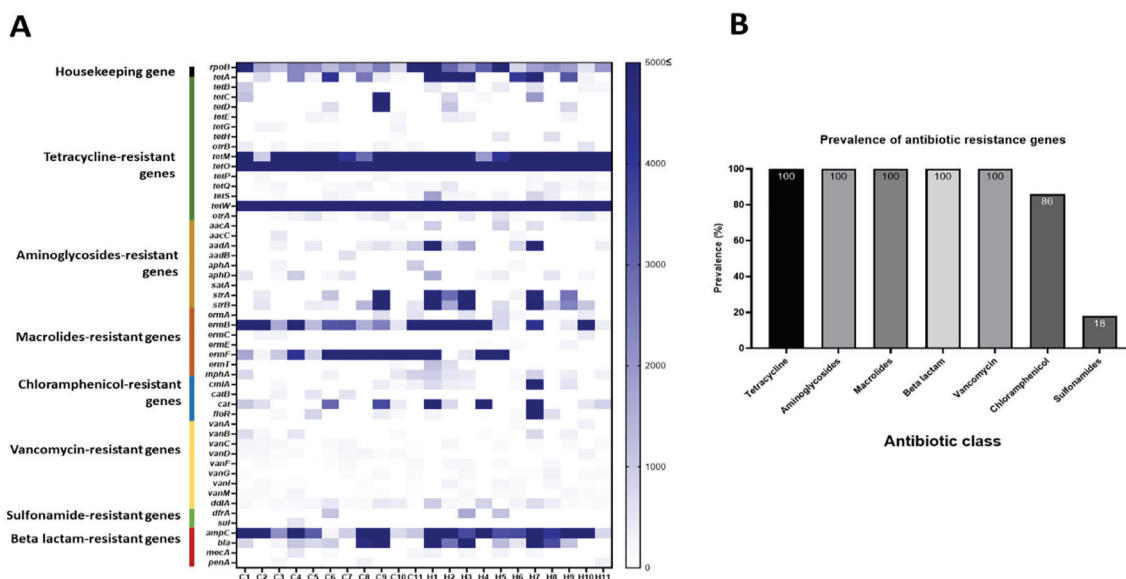| Primer	Sequence (5′→3′)	Gene
vanA (F)	GGGAAAACGACAATTGC	vanA
vanA (R)	GTACAATGCGGCCGTTA	
vanB (F)	ACGGAATGGGAAGCCGA	vanB
vanB (R)	TGCACCCGATTTCGTTC	
vanC (F)	ATGGATTGGTAYTKGTAT	vanC1/2
vanC (R)	TAGCGGGAGTGMCYMGTAA	
vanD (F)	TGTGGGATGCGATATTCAA	vanD
vanD (R)	TGCAGCCAAGTATCCGGTAA	
vanE (F)	TGTGGTATCGGAGCTGCAG	vanE
vanE (R)	ATAGTTTAGCTGGTAAC	
vanG (F)	CGGCATCCGCTGTTTTTGA	vanG
vanG (R)	GAACGATAGACCAATGCCTT	

Figure 1: A high frequency of antibiotic resistant genes was detected in the Indonesian gut microbiota. (A) Heatmap displaying the relative reads of the antibiotic resistance-encoding genes, with *rpoB* (housekeeping gene) as a comparison showed some of the genes, particularly those conferring resistance to tetracycline, macrolides, and beta-lactams, were present in relatively high abundance. (B) The resistance genes for tetracycline, aminoglycosides, macrolides, beta-lactams, and vancomycin were found in all stool samples. C: Coastal; H: Highland.

sulfonamides were found in 86% and 18% of the individuals, respectively (figure 1B). Among the detected antibiotic resistant genes, *tetM, tetO, tetW* (encoded resistance to tetracycline), and *ampC* (encoded resistance to Beta-lactam) were found in all participants, whereas *ermB* (encoded resistance to macrolide) was found in 20 participants. They were abundant in the stool samples, indicating a significant and widespread resistance to tetracycline, beta-lactams, and macrolides in the Indonesian gut microbiota. In contrast, genes encoding resistance to vancomycin were found in very low frequency and

abundance as shown in figure 1A.

High Frequency of Culturable Antibiotic Resistant Bacteria in the Collected Stool Samples

To validate the bioinformatic data obtained from the metagenomic analysis, the collected stool samples were plated on antibiotic-containing enrichment agar under aerobic conditions for 24 hours (table 2). The bioinformatic analysis indicated the ability of gut bacteria to grow on almost all antibiotic-containing agar plates with a frequency exceeding 80%, except for chloramphenicol (63.64%). As shown in figure 2,
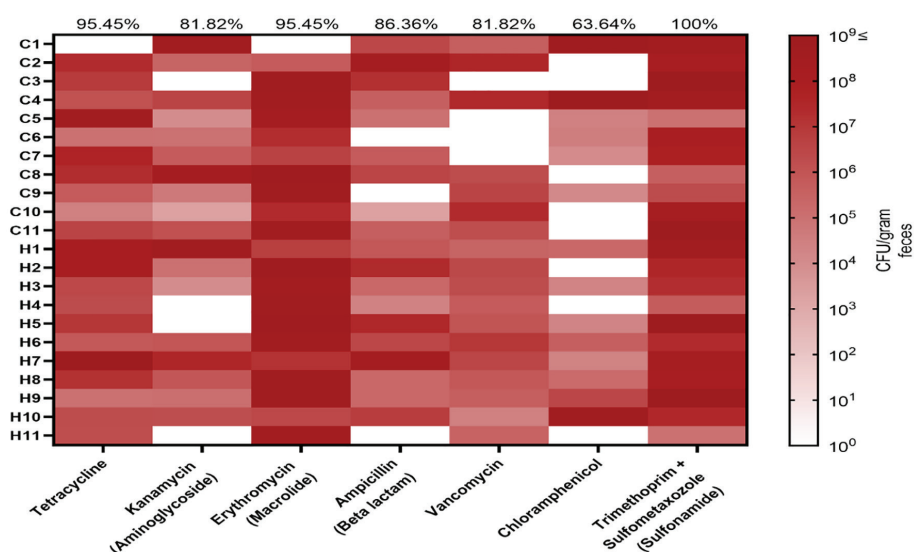

Figure 2: Culturable aerobic antibiotic resistant bacteria in Indonesian stool samples were found to be highly abundant and frequent. The enumeration of culturable aerobic bacteria from stool samples using antibiotic-containing media revealed a high abundance of antibiotic resistant bacteria, with a frequency exceeding 60% for all tested antibiotics. C: Coastal; H: Highland; high CFU: high colony-forming unit

the highest frequency was recorded in media supplemented with trimethoprim and sulfamethoxazole (100%). Enumeration data showed that erythromycin and sulfonamides had the highest number of resistant bacteria per gram of stool samples. These findings confirmed the high frequency of antibiotic resistant bacteria in the gut microbiota.

Isolated Vancomycin-resistant Bacteria are Intrinsically Resistant

This study focused on vancomycin-resistant bacteria in Indonesian gut microbiota, as their genes exhibited diverse but relatively low abundance according to the metagenomic analyses. The colonies grown from the enumeration experiment were isolated and identified based on their 16s rRNA sequence,

leading to the isolation and identification of 65 strains. As expected, vancomycin resistance was found to be an intrinsic mechanism in the majority of these strains. While the isolated vancomycin-resistant bacteria were mostly Gram-negative, certain Gram-positive bacteria, such as *Weisella confusa* and *Lactiplantibacillus plantarum,* were identified in the highland population samples (table 4).

Vancomycin Resistant Genes Detected in Intrinsically Resistant Bacteria

The presence of the vancomycin-resistant genes (*vanA, vanB, vanC, vanD, vanE, vanG*) was further assessed in the isolated strains using multiplex PCR. The results showed that 16 out of the 65 isolated strains harbored vancomycin-resistant genes. Among the six

Table 4: Aerobic vancomycin-resistant bacteria identified from Indonesian stool samples

Participants from the coastal population	Identified vancomycin-resistant bacteria	Participants from the highland population	Identified vancomycin-resistant bacteria
C1	*Escherichia fergusonii*	H1	*Weisella confusa, S. stutzeri*
C2	*E. fergusonii*	H2	*Stutzerimonas stutzeri, S. limneticum, Shigella boydii*
C3	-	H3	*S. stutzeri*
C4	*E. fergusonii*	H4	*E. fergusonii, Lactiplantibacillus plantarum*
C5	-	H5	*E. fergusonii, S. stutzeri*
C6	-	H6	*E. fergusonii, S. stutzeri, Acinetobacter baumannii, P. aeruginosa*
C7	-	H7	*Klebsiella pneumoniae*
C8	*Sphingobium limneticum, Stutzerimonas stutzeri*	H8	*S. limneticum, W. confusa*
C9	*S. stutzeri, Enterobacter mori, E. fergusonii*	H9	*P. aeruginosa, S. flexneri*
C10	*E. fergusonii, Pseudomonas aeruginosa*	H10	*E. fergusonii, S. limneticum, P. aeruginosa*
C11	*Shigella flexneri, S. stutzeri*	H11	*E. fergusonii, S. stutzeri*

C: Coastal; H: Highland

Table 5: Detection of vancomycin-resistant genes in bacteria isolated from stool samples

Source	Isolate	Species	Gene						Number of detected genes
			vanA	vanB	vanC	vanD	vanE	vanG	
Participants from the coastal population	C9.1	*S. stutzeri*			+				1
	C9.2	*S. limneticum*			+				1
	C9.3	*S. stutzeri*				+		+	2
	C11.4	*E. fergusonii*					+	+	2
	C11.6	*S. stutzeri*			+				1
	C12.1	*E. fergusonii*					+		1
	C12.2	*P. aeruginosa*	+			+			2
Participants from the highland population	H3.1	*S. stutzeri*		+			+		2
	H3.21	*S. stutzeri*		+					1
	H3.22	*S. limneticum*		+	+			+	3
	H3.3	*S. boydii*				+		+	2
	H6.11	*S. stutzeri*		+					1
	H6.12	*S. stutzeri*		+					1
	H6.2	*S. stutzeri*			+			+	2
	H1.21	*P. aeruginosa*		+	+	+		+	4
	H1.32	*E. fergusonii*						+	1

The isolate code represents the participant and the isolate number from a certain participant.

Table 6: The frequency of vancomycin-resistant genes in the Indonesian populations

Location	Participants	Vancomycin-resistant genes					
		vanA	*vanB*	*vanC*	*vanD*	*vanE*	*vanG*
Coastal	C1	-	-	-	-	-	-
	C2	-	-	-	-	-	-
	C3	-	-	-	-	-	-
	C4	-	-	-	-	-	-
	C5	-	-	-	-	-	-
	C6	-	-	-	-	-	-
	C7	-	-	-	-	-	-
	C8	-	-	+	+	-	+
	C9	-	+	-	-	+	+
	C10	+	-	-	+	+	-
	C11	-	-	-	-	-	-
Highland	H1	-	-	-	-	-	-
	H2	-	+	+	+	+	+
	H3	-	+	+	-	-	+
	H4	-	-	-	-	-	-
	H5	-	-	-	-	-	-
	H6	-	-	-	-	-	-
	H7	-	-	-	-	-	-
	H8	-	-	-	-	-	-
	H9	-	+	+	+	-	+
	H10	-	-	-	-	-	+
	H11	-	-	-	-	-	-
Prevalence		4.5%	18.2%	18.2%	18.2%	13.6%	27.3%

C: Coastal; H: Highland

genes examined, *vanG* was the most frequent (found in seven strains from six out of 22 participants), while *vanA* was the least frequent (found in a single strain within one out of 22 participants), as indicated in tables 5 and 6. Furthermore, eight bacterial strains were found to have more than one vancomycin-resistant gene, and *Pseudomonas aeruginosa* isolated from the highland population possessed four different genes, including *vanB, vanC, vanD,* and *vanG* (table 5).

Discussion

The findings of this study showed a significant frequency and abundance of antibiotic resistant genes in the gut microbiota of the studied individuals. Vancomycin resistance was found to be highly frequent in the gut microbiota, with intrinsically resistant Gram-negative bacteria harboring the encoding genes. These observations pointed to an alarming prevalence of antibiotic resistance in the community, which was attributed to several factors, including the lenient regulation and monitoring of antibiotic distribution. In Indonesia, antibiotics can be accessed without a prescription due to a lack of awareness regarding their proper usage.[22] Furthermore, the unregulated usage of antibiotics in animals contributes to difficulties.[23]

Given the poor surveillance of antibiotic consumption in Indonesia, obtaining data related to antibiotic consumption in the populations from which stool samples were collected was difficult. However, a previous study reported that tetracycline, beta-lactams, and macrolides were among the most commonly used antibiotics in Indonesia, both for human consumption and in the agriculture sector.[24] The present results were consistent with previous findings, as the metagenomic analysis revealed a relatively high abundance of tetracycline, beta-lactam, and macrolide resistant genes in the stool samples. These findings supported the hypothesis that excessive antibiotic administration was associated with the development of antibiotic resistance.[25, 26]

This study focused on investigating vancomycin resistance, as it is the last-resort antibiotic used to treat severe Gram-positive bacterial infections. The metagenomic analysis suggested the occurrence of widespread but diverse vancomycin resistance genes in the participant's gut microbiota. Currently, there is no recorded data on vancomycin usage in Indonesia, implying that the consumption was relatively low in the studied population. Despite the low level of vancomycin consumption, vancomycin-resistant genes were detected in all of the analyzed stool samples, along with relatively low reads. The antibiotic resistance assays revealed a high colony-forming unit (CFU) count

of vancomycin-resistant bacteria, most of which were Gram-negative. Moreover, Gram-positive *W. confusa* and *L. plantarum*, with intrinsic resistance to vancomycin were isolated.[27-30]

Gram-negative bacteria are intrinsically resistant to vancomycin due to their cell wall structure. Vancomycin is designed to target the peptidoglycan layer of the bacterial cell wall, which is thicker in Gram-positive bacteria and susceptible to antibiotic disruption. However, Gram-negative bacteria have an outer membrane that acts as a barrier, preventing vancomycin from reaching its target. They have efflux pumps, which are frequently used to actively pump out vancomycin molecules, which contributes to their resistance.[31, 32]

It is interesting to note that the isolated Gram-negative bacteria were discovered to be harboring vancomycin-resistant genes. This study demonstrated the presence of the genes in Gram-negative bacteria. However, the majority of the previous studies on vancomycin-resistant genes were on Gram-positive bacteria. The existence of these gut bacteria indicated that they could act as a reservoir for the spread of antibiotic resistance, particularly vancomycin-resistant genes.[33, 34] The collected data suggested that these bacteria could act as a hub for the transmission of the genes through horizontal transfer, as some of the isolated strains possessed more than one vancomycin-resistant gene. This observation highlighted the necessity of understanding the role of Gram-negative bacteria in the spread of antibiotic resistance, as well as the need for further investigations.

P. aeruginosa isolates were found to have multiple *van* genes, which could be related to their type 6 secretion system (T6SS). The T6SS enabled efficient horizontal gene transfer by recruiting outer membrane vesicles through lipopolysaccharide-binding effectors. This mechanism rendered *P. aeruginosa* susceptible to horizontal gene transfer components, which explains why isolate H121 possessed four different *van* genes (*vanBCDG*).[35, 36]

The most frequent vancomycin-resistant genes detected in this study were *vanG*, which was found in seven isolates from six different participants. Additionally, *vanB* and *vanC* were detected in five different isolates. These findings were consistent with a report by Domingo and colleagues, who reported a high frequency of *vanB*, *vanD*, and *vanG* in human fecal flora.[37] Notably, *vanG* conferred low-level resistance to vancomycin, while *vanA* and *vanB* were highly resistant.[38] One possible explanation was that the low resistance-encoding genes might spread more easily than those with high resistance.

Overall, the collected data suggested a high frequency of antibiotic resistant genes in the human gut, implying that the gut microbiota could serve as a reservoir and hub for the spread of resistance genes. This poses a potential risk of multidrug-resistant bacteria emergence, making antibiotic treatment more challenging. Therefore, it is critical to use antibiotics judiciously to avoid the development of resistant bacteria in the human digestive system.

Despite the valuable insights gained from investigating the presence of vancomycin-resistance genes in intrinsically resistant bacteria from Indonesian gut microbiota, the limitations imposed by the study methodology should be highlighted. Due to the small sample size, the obtained results might not be generalizable to a larger population. Further investigation is necessary to determine the role of Gram-negative bacteria as a reservoir for vancomycin-resistant genes and their contribution to the spread of resistance through horizontal transfer.

Conclusion

The Indonesian population revealed a significant prevalence of antibiotic resistance, as evidenced by the abundant detection of tetracycline, beta-lactam, and macrolide resistant genes in stool samples. The presence of vancomycin-resistant genes in all collected stool samples and several bacterial isolates, including Gram-negative bacteria, suggested their potential role in facilitating the spread of antibiotic resistance through horizontal gene transfer. Therefore, further studies in this area are required. Antibiotics should be administered rationally to prevent the development of resistant bacteria in the human digestive system.

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Authors' Contribution

A.L: Conceptualization, methodology, validation, formal analysis, investigation, resources, writing original draft, writing review & editing, visualization, supervision, project administration, funding acquisition; J.S: Conceptualization, methodology, validation, resources, supervision; writing; Y.A.P: Conceptualization, methodology,

validation, resources, writing; A.V.A: Methodology, formal analysis, investigation, writing; A.: Methodology, formal analysis, investigation, writing; S.N.A: Methodology, formal analysis, investigation, writing; E.Z: Methodology, resources, supervision, project administration, funding acquisition, writing; N.D.K: Methodology, resources, supervision, project administration, funding acquisition, writing; F.G: Methodology, resources, supervision, project administration, funding acquisition, writing; A.T.W: Conceptualization, methodology, validation, resources, writing original draft, writing review & editing, visualization, supervision.

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