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Plasmid metagenomics reveals multiple antibiotic resistance gene classes among the gut microbiomes of hospitalised patients

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ABSTRACT

Antibiotic resistance genes are rapidly spread between pathogens and the normal flora, with plasmids playing an important role in their circulation. This study aimed to investigate antibiotic resistance plasmids in the gut microbiome of hospitalised patients. Stool samples were collected from seven inpatients at Siriraj Hospital (Bangkok, Thailand) and were compared with a sample from a healthy volunteer. Plasmids from the gut microbiomes extracted from the stool samples were subjected to high-throughput DNA sequencing (GS Junior). Newbler-assembled DNA reads were categorised into known and unknown sequences (using >80% alignment length as the cut-off), and ResFinder was used to classify the antibiotic resistance gene pools. Plasmid replicon modules were used for plasmid typing. Forty-six genes conferring resistance to several classes of antibiotics were identified in the stool samples. Several antibiotic resistance genes were shared by the patients; interestingly, most were reported previously in food animals and healthy humans. Four antibiotic resistance genes were found in the healthy subject. One gene (*aph3-III*) was identified in the patients and the healthy subject and was related to that in cattle. Uncommon genes of hospital origin such as $bla_{\text{TEM-124-like}}$ and *fosA*, which confer resistance to extended-spectrum β-lactams and fosfomycin, respectively, were identified. The resistance genes did not match the patients' drug treatments. In conclusion, several plasmid types were identified in the gut microbiome; however, it was difficult to link these to the antibiotic resistance genes identified. That the antibiotic resistance genes came from hospital and community environments is worrying.

1. Introduction

Infections with drug-resistant pathogens have become a worldwide health problem following the emergence and spread of antibiotic-resistant organisms. Antibiotics have clear roles in selecting for bacterial resistance genes and creating gene pools and reservoirs of antibiotic-resistant bacteria [1]. The gut microbiome is the largest gene reservoir inside the human body. In humans it is composed of 10^{11}–10^{12} microbial cells per gram of gut contents [2,3]. Some studies have revealed the existence of a gene pool of antibiotic resistance in human microbiomes using culture-based and metagenomic approaches [4,5]. Studies on human microbiomes have revealed that some antibiotic resistance genes, such as *ermG*, *ermB*, *ermF*, *tetM*, *tetQ* and *vanB*, found both in pathogens and in the gut microbiome have high nucleotide sequence similarities [4,5]. However, the culture-based experiments used in these studies are not sufficient to conclude the horizontal transfer of antibiotic resistance genes among anaerobic bacteria in the gut.

Nevertheless, metagenomics can still be used to study the genomes of the bacterial community both under aerobic and anaerobic conditions. Sommer et al. showed that the gut

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microbiome of healthy people contains more than 77 genes conferring resistance to several antimicrobial drugs [6]. Therefore, metagenomics is a potentially suitable approach for identifying the presence and spread of antibiotic resistance genes in the gut microbiome.

Horizontal gene transfer (HGT) rapidly expands the repertoire of antibiotic resistance genes both within and between bacterial species [7–11]. In particular, conjugation is commonly used by bacteria for plasmid-related gene transmission. A plasmid can carry more than one resistance gene by using mobile genetic elements, such as integron-carrying transposons [12]. If newly acquired bacteria carrying plasmids for multidrug resistance encounter a new microbiome, such resistance genes can be easily transferred to the new and possibly antibiotic-sensitive bacterial community. To understand the spread of resistance plasmids, plasmid typing can be used to trace the epidemiological pathways of resistance gene spread [13,14]. Bielak et al. found that an IncI1 plasmid was responsible for spreading the β-lactamase gene $bla_{\text{TEM-52C}}$, whereas plasmid IncX1A spread $bla_{\text{TEM-52B}}$ among *Escherichia coli* and *Salmonella* spp. [7]. Recently, a study showed that plasmids transferred the bla_{KPC} carbapenem resistance gene among Enterobacteriaceae within the gut of the same patient and that spread of this gene occurred in different patients [15]. However, detailed information on antibiotic resistance gene-carrying plasmids in the gut microbiomes of hospitalised patients is lacking.

Antibacterial agents are known to be involved in antibiotic resistance gene selection in bacteria. Resistance to antibiotics not only develops during antibiotic treatment but also continues after the use of such agents ceases. For example, *ermG* emerged following clindamycin use and persisted in *Bacteroides thetaiotaomicron* 18 months after the drug was stopped [16]. Similarly, some resistance genes were found in healthy people who had not been treated with antibiotics for at least 1 year [6,16]. This implies that antibiotics should be used with caution because they can lead to the selection of antibiotic resistance genes in the microbiome as a long-term reservoir. Accordingly, antimicrobial drugs and plasmids both play an important role in antibiotic resistance gene acquisition in the microbiome. Currently, there have been no published studies looking at plasmid-derived drug resistance in the gene pool of a hospital inpatient's gut microbiome. Hence, this study investigated resistance gene acquisition in bacterial plasmids of the gut microbiomes of seven hospital inpatients from a large tertiary university hospital in whom antibiotics had been used intensively.

2. Materials and methods

2.1. Stool preparation, plasmid extraction and high-throughput DNA sequencing

The study investigated stool samples collected from seven patients (designated P1–P7) who were hospitalised in the general internal medicine ward and the internal medicine intensive care unit (ICU) of Siriraj Hospital (Bangkok, Thailand) (Table 1). All of the patients signed informed consent forms, and the study was approved by the Human Research Protection Unit of Siriraj Hospital. The inclusion criteria included the following: (i) hospital admission for >7 days; and (ii) treatment with more than two groups of antimicrobial drugs (Table 1). A stool sample from one healthy subject who had not received antibiotics in the previous 3 years was used as a control. The eight samples from the seven patients (P1–P7) as well as the healthy donor (H) were the maximum samples using the budget allowable for this study. Approximately 100–200 g of each stool sample was collected and was kept frozen at −20 °C until use. Then, 15 g of a divided stool

Table 1
Patient information.

ID	Ward	Antibiotic treatment
P1	Gen. Med.	Colistin, meropenem, vancomycin
P2	Gen. Med.	Meropenem, amikacin, piperacillin/tazobactam
P3	Gen. Med.	Piperacillin/tazobactam, imipenem, colistin
P4	Gen. Med.	Metronidazole, ceftriaxone, cefotaxime, fosfomycin
P5	ICU Med.	Vancomycin, meropenem, ceftriaxone
P6	ICU Med.	Meropenem, colistin
P7	ICU Med.	Meropenem, vancomycin, levofloxacin

Gen. Med., general internal medicine ward; ICU Med., internal medicine intensive care unit.

was used for plasmid extraction. Human cells in each sample were lysed with H_2O, and the food sediment was eliminated by centrifugation at 100 × *g* at 4 °C for 3 min. The supernatant was collected, the centrifugation step was repeated three times and the sample was then transferred to a new 50 mL centrifuge tube. To eliminate the stool pigments, which might inhibit the PCRs, the bacterial cells were washed with phosphate-buffered saline (pH 7.4) and were then centrifuged at 7000 × *g* at 4 °C for 10 min; this step was repeated until the supernatant was clear. The 5–6 g bacterial cell precipitate was subjected to plasmid extraction using a Geneaid™ Maxi Plasmid Kit (Geneaid Biotech Ltd., Taipei City, Taiwan). Plasmid DNA was sequenced using a 454 pyrosequencing platform (GS Junior System; Roche Diagnostics, Bangkok, Thailand). Briefly, plasmid DNA was sheared and a library was constructed using a Rapid Library Preparation Kit (Roche Diagnostics). The clonal DNA templates produced using a Lib-L emPCR Preparation Kit (Roche Diagnostics) were subject to shotgun sequencing.

2.2. Resistance gene analysis

All of the reads from the shotgun sequencing were assembled with Newbler software (Roche Diagnostics). All of the sequences used in the homology search were based on the nucleotide data sets in the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/) and on blastn (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The aligned hits were generated with an e-value of 10^{-7}, and the top hits with the best bit scores were retrieved. Next, the alignment lengths of the selected hits were plotted on a histogram and were arranged with a cumulative curve to observe the frequencies of the alignment lengths. An 80% alignment length was used as a cut-off for characterisation of known (>80%) and unknown (<80%) sequences (Figs. 1 and 2). For known sequences, their DNA types were categorised into the following six groups: genomic DNA; plasmid; mobile genetic elements; phages; human genes; and other genes, such as those of mouse and virus origin. ResFinder (Center for Genomic Epidemiology, Technical University of Denmark, Kgs. Lyngby, Denmark) provided the drug resistance nucleotide data set, and resistance hits with sequence identity ≥30% were further analysed [17]. Resistance hits were confirmed with NCBI blastx [18]. Fisher's exact test was used to evaluate the association between drug treatment and resistance genes. The antibiotic resistance genes identified by ResFinder were investigated further in the context of resistance gene transfer among the hospitalised study group of patients and the healthy subject control. BioEdit v.7.0.9.0 (http://www.mbio.ncsu.edu/Bioedit/bioedit.html) was used to generate multiple alignments of the antibiotic resistance genes and their flanking regions and these were compared with the sequences of the predicted antibiotic resistance genes. Neighbour-joining phylogenetic tree (NJplot) was constructed with a bootstrap value of 1000 (CMG BioTools; Technical University of Denmark) for comparing each resistance gene of patients and the healthy subject

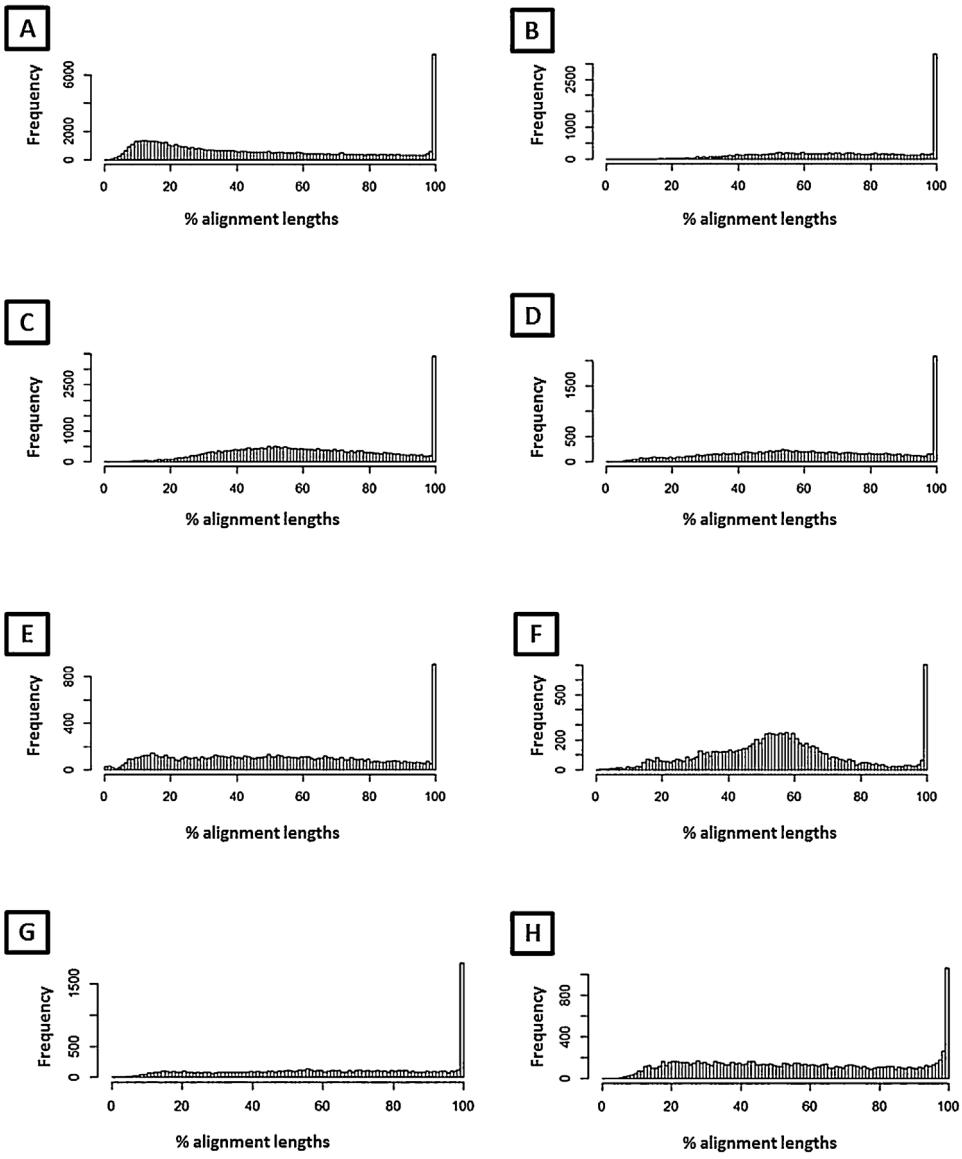

Fig. 1. Frequency of percent (%) alignment length of sequence reads from each sample: (A) healthy subject; and (B–H) patients P1–P7, respectively.

with that of other reservoirs (GenBank accession nos. X92945, AF516335, AF330699, NC_021170 and NC_016973).

2.3. Sequence-based plasmid typing

Replication modules (rep) were used for plasmid typing [13,14]. The replicons were generated as an in-house replicon database. All query sequences were aligned against the replicon data set to find plasmid types among the gut microbiomes. Both antibiotic resistance genes and replicon sequences were concatenated to form complete resistance plasmids. Indirectly, previously reported antibiotic resistance genes were matched to the currently identified plasmid types and resistance genes [11,19–21].

3. Results

3.1. Analysis of the DNA sequences recovered from high-throughput sequencing

Following blastn analysis of the reads and after all the reads from each sample were categorised, most samples contained >50% of unknown reads, except for the sample from P5 (Table 2; Figs. 1 and 2). In P5, the unknown reads comprised 47.17% of the total reads. More than 99% of known reads belonged to bacteria, and mammalian sequences were discarded from further analyses (data not shown).

3.2. Antibiotic resistance genes in the gut microbiomes of the hospitalised patients

All reads were assembled as long DNA sequences using Newbler software and were then aligned against the acquired resistance gene database in ResFinder. The results showed that the sequences obtained comprised genes that confer resistance to most antimicrobial drugs (Fig. 3). The DNA sequences of the antibiotic resistance genes [i.e. *ant(6)-Ia*, *ermB*, *lnuB*, *tetL* and *tetU*] that were shared among P1 and P3 (from the general medicine ward) and P5 (from the ICU) were similar. It should be noted that *ant(6)-Ia*, *ermB*, *lnuB*, *tetL* and *tetU* confer resistance to aminoglycosides, macrolides, lincosamides, streptogramin B and tetracycline. Furthermore, *tetO*, *tet40*, *aph(3′)-III* and *catS*, which are antibiotic resistance genes conferring resistance to tetracycline, aminoglycosides and chloramphenicol, respectively, were also found in the healthy subject. Interestingly, the *aph(3′)-III*

Table 2
DNA read categories.

Sample	Total DNA bases (Mbp)	Total reads (*n*)	% known reads (*n*)	% unknown reads (*n*)
Control	81.5	170,680	17.84 (30,457)	82.16 (140,223)
P1	8.8	37,612	45.54 (17,130)	54.46 (20,482)
P2	16.4	56,667	31.43 (17,812)	68.57 (38,855)
P3	25.8	72,862	34.36 (25,038)	65.64 (47,824)
P4	51.3	126,552	10.66 (13,495)	89.34 (113,057)
P5	76.4	200,673	52.83 (106,024)	47.17 (94,649)
P6	11.0	37,241	36.11 (13,449)	63.89 (23,792)
P7	9.7	36,954	12.75 (4,713)	87.25 (32,241)

sequences were identical among P1, P3, P5 and the healthy subject (H).

In addition, several of the antibiotic resistance genes were uniquely found in particular subjects. For example, *catS*, *tetO* and *tet40*, which confer chloramphenicol and tetracycline resistance, were only identified in the healthy volunteer, whereas the *msrC* gene, which is responsible for macrolide, lincosamide and streptogramin B resistance, was only found in P1. There were several resistance genes in P2, including $bla_{\text{OXA-1-like}}$, $bla_{\text{TEM-124-like}}$, *fosA*, *aac(6′)-Ib*-cr, *arr-3/6*, *catB3*, *dfrA5* and *sul1*, which confer resistance to β-lactams, extended-spectrum β-lactams, fosfomycin, fluoroquinolones, rifampicin, chloramphenicol, trimethoprim and sulfonamide, respectively. *catA2* and *dfrA12*, which confer resistance to chloramphenicol and trimethoprim, respectively, were only found in the P4 sample. The P5 sample contained *aac(6′)–aph(2″)* and *dfrG*, which are responsible for aminoglycoside and trimethoprim resistance. *tetW*, which confers resistance to tetracycline, was only found in the P7 sample.

NJplot was used to evaluate resistance gene sharing between patients, the healthy subject and other reservoirs of previously reported genes (Fig. 4). As a result, *aph(3′)-III* from P1 and P5 were in the same branch of the tree, and that from P3 and the healthy subject (H) were similar. However, the gene from the P1–P5 cluster was far from the P3–H cluster. Interestingly, *aph(3′)-III* from the P3–H cluster was identical to that from *Pasteurella multocida* isolated from cattle. Also, the gene from the P1–P5 cluster was more close to that from *Enterococcus faecium* from different origins.

3.3. Linkage of resistance genes with antibiotic treatment and plasmid type

When the antibiotic resistance genes identified in this study were compared with the antibiotics administered to the patients,

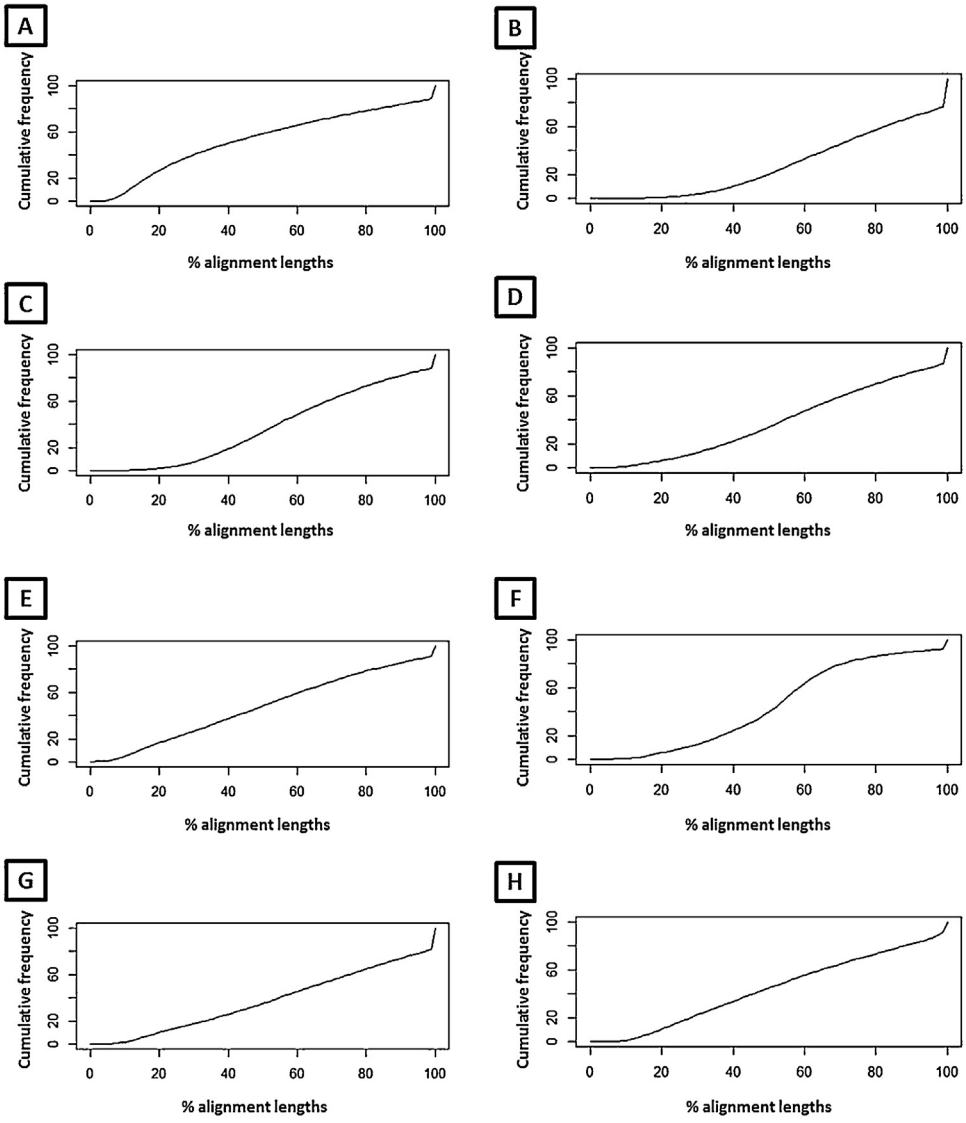

Fig. 2. Cumulative frequency of percent (%) alignment length of sequence reads from each sample: (A) healthy subject; and (B–H) patients P1–P7, respectively.

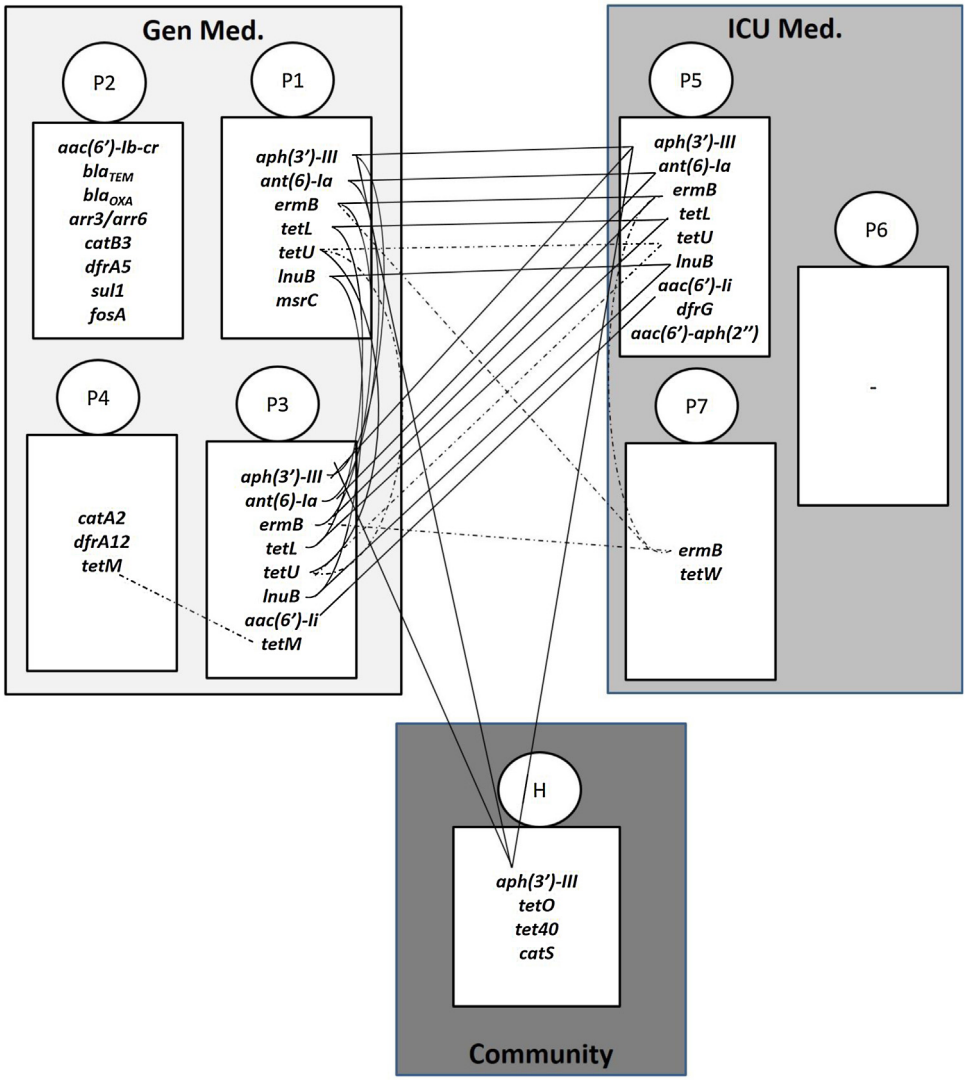

Fig. 3. Resistance gene sharing between hospital patients (P1–P7) and a healthy subject (H), falling into three categories. First, the solid line represents the spread of similar resistance genes with similar flanking regions. Second, the dashed line represents the spread of similar resistance genes with different flanking regions. Finally, the genes without any connected line are individual resistance genes. Gen. Med., general internal medicine ward; ICU Med., internal medicine intensive care unit.

Fisher's exact test (95% confidence intervals) was used to evaluate whether resistance genes depended on their antimicrobial drugs. The results showed that a strong relationship between them was not obvious ($P \geq 0.05$) (Table 3). To further identify the plasmids responsible for the spread of the antibiotic resistance genes, the sequences containing the resistance genes were aligned against the in-house plasmid rep database. However, we did not find any sequences that contained both a rep module and a resistance gene in the same sequence. The assembled sequences were not long enough to contain both resistance genes and rep modules. This might have been caused by the highly repetitive regions on the plasmids. Therefore, it appears that the assembly program was unable to concatenate the plasmid DNA reads from the entire plasmid. Hence, the alignment was repeated using all of the sequences from the gut microbiome plasmids. As a result, 12 different basic replicons (HI1, HI2, I1, FIA, FIB, FIC, FIIA, FIIS, FrepB, N, U and W) were identified (Table 4). We investigated the antibiotic resistance genes reported previously for their association with each plasmid type to try and obtain clues about the spread of resistance genes in the study participants. The results showed that most of the resistance genes found in the patients had never been reported in the various plasmid types, except for *aac(6′)-Ib*-cr from the P2 sample, which was identified in rep FIA, FIB, FIIA, HI2 and N plasmids.

4. Discussion

Theoretically, antibiotic resistance genes can be commonly exchanged among pathogens, environmental bacteria and normal flora. The wide range of bacterial species in the human gut microflora comprises the largest reservoir of antibiotic resistance genes in humans. The consequences of gut microbiomes receiving multiple resistance genes from pathogens and becoming the biggest antibiotic resistance gene pool are an obvious public health concern. Increased use of powerful antibiotics in hospital settings may facilitate the selection of antibiotic resistance genes. In addition, plasmids are the most common mobile genetic elements responsible for the spread of antibiotic resistance genes among bacteria. It is possible that such resistance genes could be transferred to the gut microbiome on plasmids by HGT. Studying the gut microbiome by metagenomics is more suitable than traditional culturing methods because this technique is unbiased from non-culturable aerobic and anaerobic bacteria growth. Sommer et al. found 13 β-lactamase-like genes with the

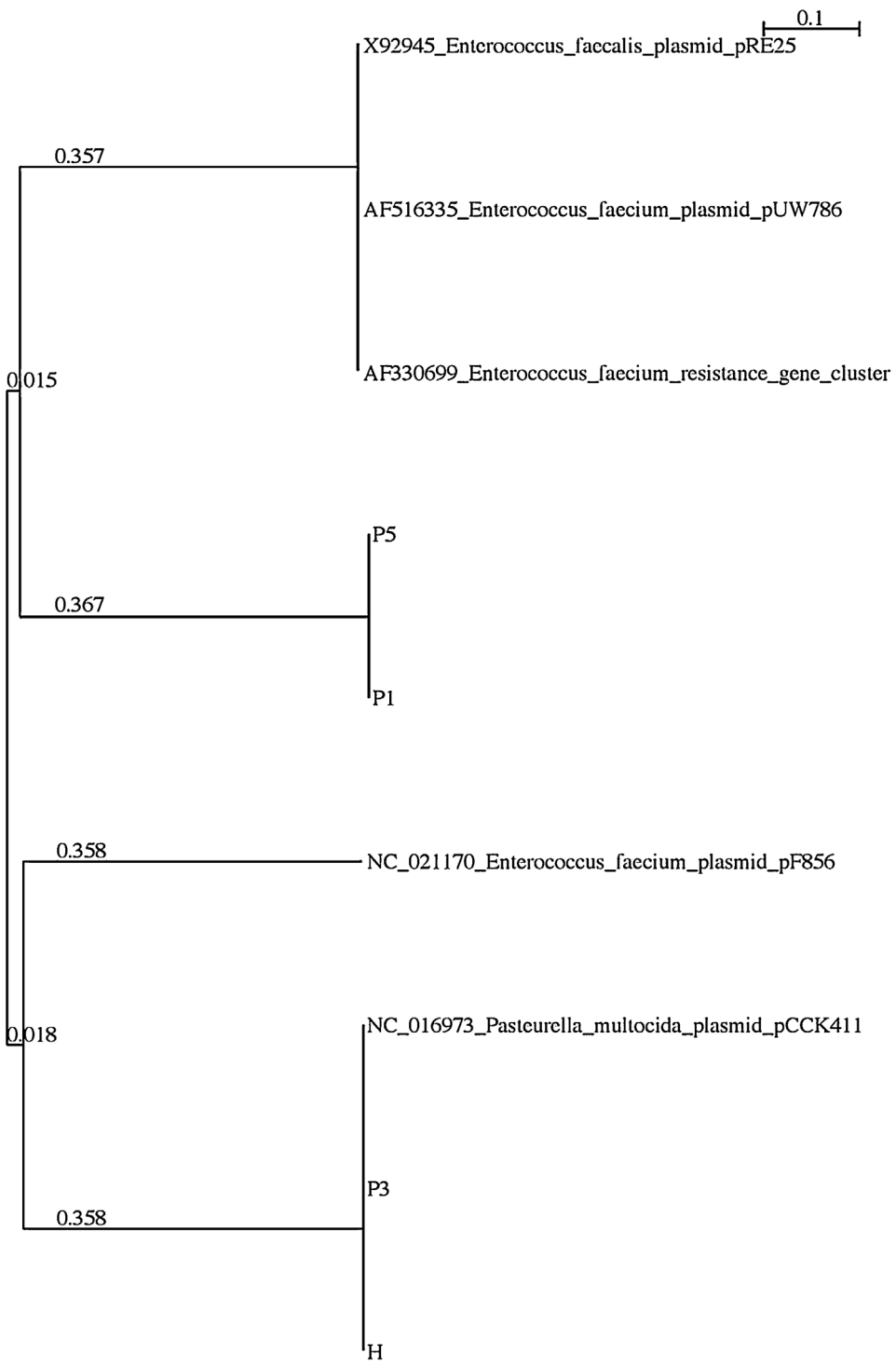

Fig. 4. Phylogenetic tree of *aph(3′)-III* from the gut microbiome of patients (P1, P3, P5) and a healthy subject (H) as well as from other bacteria. X92945 was an *Enterococcus faecalis* isolated from raw fermented sausage; AF516335 was an *Enterococcus faecium* clinical isolate; AF330699 is the identical gene cluster among *E. faecium* in poultry, pig, sewage and patients; NC_021170 was an *E. faecium* isolated from an admitted patient; and NC_016973 was a *Pasteurella multocida* collected from cattle.

metagenomic approach that had never been reported and that could not be identified by traditional culturing methodology [6]. Therefore, the metagenomic approach was used in the present study to identify antibiotic resistance genes on plasmids from the gut microbiomes of hospitalised patients.

From the seven patients investigated here, 46 antibiotic resistance genes were classified into 25 resistance gene types. These genes confer resistance to most antimicrobial drugs and/or drug classes including β-lactams, extended-spectrum β-lactams, aminoglycosides, fosfomycin, tetracycline, chloramphenicol, erythromycin, lincosamides, streptogramins and fluoroquinolones. The current findings for the healthy subject were similar to those of a report by Sommer et al. [6]. We showed that the antibiotic resistance genes in the healthy subject's gut microbiome were genes conferring resistance to aminoglycosides and tetracycline. Whilst Sommer et al. did not inspect the resistance genes in the patient samples, we found that our samples contained many genes that potentially confer resistance to nearly all classes of antibiotics.

Table 3
Acquired resistance genes found among patients (P1–P7) and a healthy human subject (H).

Sample	Antibiotic treatment	Resistance gene	% identity	DNA type[a]	Resistance	Best hits[b]	Country[c]	Accession no.
H	No	*tetO*	99.76	gDNA	Tetracycline	*Streptococcus mutans*	USA	M20925
		tet40	100	gDNA	Tetracycline	Uncultured bacterium	UK	FJ158002
		aph(3′)-III	100	Plasmid	Aminoglycoside	*Campylobacter coli*	France	M26832
		catS	83.16	Unk.	Phenicol	*Streptococcus pyogenes*	France	X74948
P1	Colistin, meropenem, vancomycin	*aph(3′)-III*	99.87	Unk.	Aminoglycoside	*C. coli*	France	M26832
		ermB	99.86	Unk.	MLS_B	*Enterococcus* sp.	Switzerland	X82819
		ant(6)-Ia	100	Unk.	Aminoglycoside	*Enterococcus faecium*	Germany	AF330699
		tetL	100	Unk.	Tetracycline	*Streptococcus agalactiae*	N/A	M29725
		msrC	99.1	Unk.	MLS_B	*E. faecium*	N/A	AY004350
		tetU	99.37	Unk.	Tetracycline	*E. faecium*	USA	U01917
		lnuB	99.72	Unk.	Lincosamide	*E. faecium*	France	AJ238249
P2	Meropenem, TZP, amikacin	$bla_{TEM-124}$	99.03	Unk.	β-Lactam	*Morganella morganii*	Italy	AY327540
		sul1	100	Plasmid	Sulphonamide	*Escherichia coli*	France	AY224185
		catB3	99.75	Plasmid	Phenicol	*Salmonella* Typhimurium	Italy	AJ009818
		bla_{OXA-1}	99.82	Unk.	β-Lactam	*E. coli*	Canada	J02967
		aac(6′)-Ib-cr	100	MGE	Fluoroquinolone and aminoglycoside	*Klebsiella pneumoniae*	Argentina	EF636461
		arr-3/arr-6	100/100	Plasmid	Rifampicin	*E. coli/Pseudomonas putida*	China/Brazil	JF806499/JF922883
		dfrA5	100	Unk.	Trimethoprim	Enterobacteriaceae	Sweden	X12868
		fosA	99.73	Unk.	Fosfomycin	*Klebsiella* sp.	USA	NZ_ACWO01000079
		fosA	100	gDNA	Fosfomycin	*Klebsiella* sp.	USA	NZ_ACWO01000079
		fosA	96.13	gDNA	Fosfomycin	*Klebsiella* sp.	USA	NZ_ACZD01000244
		fosA	98.25	Unk.	Fosfomycin	*Klebsiella* sp.	USA	NZ_ACWO01000079
P3	TZP, imipenem/cilastatin, colistin	*ant(6)-Ia*	100	Unk.	Aminoglycoside resistance	*E. faecium*	Germany	AF330699
		aph(3′)-III	100	Plasmid	Aminoglycoside resistance	*C. coli*	France	M26832
		ermB	99.86	Plasmid	MLS_B	*Enterococcus* sp.	Switzerland	X82819
		tetL	100	Unk.	Tetracycline	*S. agalactiae*	N/A	M29725
		tetM	99.7	Unk.	Tetracycline	*Enterococcus faecalis*	Switzerland	X92947
		tetU	99.37	Unk.	Tetracycline	*E. faecium*	USA	U01917
		lnuB	99.86	Plasmid	Lincosamide	*E. faecium*	France	AJ238249
		aac(6′)-Ii	99.64	gDNA	Aminoglycoside	*E. faecium*	France	L12710
		tetU	97.92	Unk.	Tetracycline	*E. faecium*	USA	U01917
		tetU	97.55	Unk.	Tetracycline	*E. faecium*	USA	U01917
		tetU	98.99	Unk.	Tetracycline	*E. faecium*	USA	U01917
		tetU	98.94	Unk.	Tetracycline	*E. faecium*	USA	U01917
P4	Metronidazole, ceftriaxone, cefotaxime, fosfomycin	*tetM*	99.53	Unk.	Tetracycline	*Staphylococcus aureus*	Netherlands	AM990992
		catA2	95.86	Unk.	Phenicol	*E. coli*	UK	X53796
		dfrA12	98.71	Unk.	Trimethoprim	*Salmonella* Typhimurium	Japan	AB571791
		dfrA12	100	Plasmid	Trimethoprim	*Salmonella* Typhimurium	Japan	AB571791
P5	Vancomycin, meropenem, ceftriaxone	*aac(6′)-Ii*	99.64	gDNA	Aminoglycoside	*E. faecium*	France	L12710
		dfrG	100	Unk.	Trimethoprim	N/A	Japan	AB205645
		tetL	99.93	Plasmid	Tetracycline	*E. faecium*	Germany	AY081910
		aph(3′)-III	100	gDNA	Aminoglycoside	*C. coli*	France	M26832
		aac(6′)–aph(2″)	100	MGE	Aminoglycoside	*E. faecalis*	France	M13771
		ant(6)-Ia	100	gDNA	Aminoglycoside	*E. faecium*	Germany	AF330699
		ermB	99.86	gDNA	MLS_B	*Enterococcus* sp.	Switzerland	X82819
		lnuB	99.86	Plasmid	Lincosamide	*E. faecium*	France	AJ238249
		tetU	80.63	Unk.	Tetracycline	*E. faecium*	USA	U01917
P6	Meropenem, colistin	–	–	–	–	–	–	–
P7	Meropenem, vancomycin, levofloxacin	*tetW*	99.31	gDNA	Tetracycline	*Streptococcus suis*	Italy	FN396364
		ermB	99.78	gDNA	MLS_B	*Clostridium perfringens*	Australia	U18931
		ermB	98.78	Unk.	MLS_B	*C. perfringens*	Australia	U18931

MLS_B, macrolide, lincosamide and streptogramin B; N/A, not available; TZP, piperacillin/tazobactam.
[a] DNA types: six types of DNA, including bacterial genomic DNA (gDNA), bacterial plasmids, mobile genetic elements (MGEs), viruses, other and unknown sequences (Unk.).
[b] Reference microbes with the highest shared homology between their antibiotic resistance genes and the corresponding antibiotic resistance genes from this study.
[c] Countries where the microbes were isolated.

Most were similar among patients from the same ward and from different wards. Interestingly, these genes have never been reported in our hospital. These bacterial genes, however, have been reported in patients, food animals and healthy subjects in several countries including India [22,23], China [24], Vietnam [25], Senegal [26], Portugal [27], the UK [28], the USA and Canada [29], and Brazil [26] (Table 3). This implies that antibiotic resistance genes have spread across every part of the food chain and have been transferred around the globe.

These data revealed that four antibiotic resistance genes [*aph(3′)-III*, *tetO*, *tet40* and *catS*] were identified in the healthy subject who had not received any antibiotics in the 3 years preceding this study. Remarkably, *aph(3′)-III* was also found in patients. This gene has been reported in streptococci that have been isolated from healthy subjects, in *Enterococcus faecalis* from various ecological sources, and in *Campylobacter jejuni* and *Campylobacter coli* from food and patients [28–30]. Surprisingly, the *aph(3′)-III* gene was highly homologous among the healthy subject, patient P3 and *P. multocida* isolated from cattle (Fig. 4). It was possible that *aph(3′)-III* was spread to humans through the food chain. However, *aph(3′)-III* from P1 and P5 that were admitted to different wards were similar to each other, but were far from P3.

Table 4
Resistance genes and plasmid type with rep typing.

ID	rep type	Antibiotic resistance genes found in this study	Resistance gene association reported previously
Healthy control (H)	Q	*aph(3′)-III*, *catS*, *tetO*, *tet40*	*bla*$_{CTX-M-53}$, *qnrS2*, *aac(6′)-Ib*, *bla*$_{OXA}$
	HI2		*bla*$_{CTX-M-3}$, *bla*$_{CTX-M-2}$, *bla*$_{CTX-M-9}$, *bla*$_{CTX-M-14}$, *armA*, *qnrA1* [*bla*$_{SHV-12}$, *bla*$_{CTX-M-14}$, *bla*$_{CTX-M-9}$, *aac(6′)-Ib*-cr], *bla*$_{CMY-8}$, *cat*, *tetD*, *tetC*, *tetA*, *aphA*
	FIIS		N/A
P1	HI1	*ant(6)-Ia*, *aph(3′)-III*, *ermB*, *lnuB*, *msrC*, *tetL*, *tetU*	*bla*$_{TEM-1}$, *tetA*, *tetC*, *tetD*
P2	HI1	*aac(6′)-Ib*-cr, *arr-3* or *arr-6*, *bla*$_{OXA}$, *bla*$_{TEM}$, *catB*, *dfrA*, *fosA*, *sul1*	*bla*$_{TEM-1}$, *tetA*, *tetC*, *tetD*
	HI2		*bla*$_{CTX-M-3}$, *bla*$_{CTX-M-2}$, *bla*$_{CTX-M-9}$, *bla*$_{CTX-M-14}$, *armA*, *qnrA1* [*bla*$_{SHV-12}$, *bla*$_{CTX-M-14}$, *bla*$_{CTX-M-9}$, *aac(6′)-Ib*-cr], *bla*$_{CMY-8}$, *cat*, *tetD*, *tetC*, *tetA*, *aphA*
	FIIA		*armA*, *qnrB4* [*armA*, *bla*$_{CTX-M-14}$, *bla*$_{DHA-1}$, *bla*$_{SHV-12}$, *aac(6′)-Ib*-cr]
	N		*bla*$_{CTX-M-1}$, *armA*, *bla*$_{CTX-M-15}$ [*bla*$_{TEM-1}$, *aac(6′)-Ib*-cr], *bla*$_{CTX-M-32}$, *bla*$_{CTX-M-40}$, *bla*$_{KPC-2}$, *bla*$_{VIM-1}$, *armA*, *qnrA3*, *qnrB2* [*bla*$_{CTX-M-3}$, *aac(6′)-Ib*-cr], *qnrB19* [*bla*$_{SHV-12}$], *qnrS1* [*lap-2*, *aac(6′)-Ib*-cr], *bla*$_{OXA}$, *aadA1*, *sul1*, *tetA*
	FIB		*bla*$_{CTX-M-9}$, *bla*$_{CTX-M-14}$, *bla*$_{CTX-M-15}$ [*bla*$_{TEM-1}$, *aac(6′)-Ib*-cr], *bla*$_{SHV-2}$, *bla*$_{SHV-12}$, *bla*$_{TEM-1}$
	FIC		*aac(3′)-IV*
	FIA		*bla*$_{CTX-M-15}$ [*bla*$_{TEM-1}$, *aac(6′)-Ib*-cr], *bla*$_{DHA-1}$, *bla*$_{TEM-1}$, *qnrB2* [*bla*$_{CTX-M-3}$, *aac(6′)-Ib*-cr], *qnrB4* [*armA*, *bla*$_{CTX-M-14}$, *bla*$_{DHA-1}$, *bla*$_{SHV-12}$, *aac(6′)-Ib*-cr]
	U		*qnrS2*, *sul1*, *aadA2*, *tetA*
	W		*bla*$_{VIM-1}$, *sul1*, *dfrB2*
	FrepB		*bla*$_{CTX-M-15}$, *bla*$_{CTX-M-14}$, *bla*$_{CTX-M-3}$, *cat*, *aad*, *sul*, *tet*
	FIIS		N/A
P3	HI1	*aac(6′)-Ii*, *ant(6)-Ia*, *aph(3′)-III*, *ermB*, *lnuB*, *tetL*, *tetM*, *tetU*	*bla*$_{TEM-1}$, *tetA*, *tetC*, *tetD*
	HI2		*bla*$_{CTX-M-3}$, *bla*$_{CTX-M-2}$, *bla*$_{CTX-M-9}$, *bla*$_{CTX-M-14}$, *armA*, *qnrA1* [*bla*$_{SHV-12}$, *bla*$_{CTX-M-14}$, *bla*$_{CTX-M-9}$, *aac(6′)-Ib*-cr], *bla*$_{CMY-8}$, *cat*, *tetD*, *tetC*, *tetA*, *aphA*
	N		*bla*$_{CTX-M-1}$, *armA*, *bla*$_{CTX-M-15}$ [*bla*$_{TEM-1}$, *aac(6′)-Ib*-cr], *bla*$_{CTX-M-32}$, *bla*$_{CTX-M-40}$, *bla*$_{KPC-2}$, *bla*$_{VIM-1}$, *armA*, *qnrA3*, *qnrB2* [*bla*$_{CTX-M-3}$, *aac(6′)-Ib*-cr], *qnrB19* [*bla*$_{SHV-12}$], *qnrS1* [*lap-2*, *aac(6′)-Ib*-cr], *bla*$_{OXA}$, *aadA1*, *sul1*, *tetA*
	U		*qnrS2*, *sul1*, *aadA2*, *tetA*
	W		*bla*$_{VIM-1}$, *sul1*, *dfrB2*
	FrepB		*bla*$_{CTX-M-15}$, *bla*$_{CTX-M-14}$, *bla*$_{CTX-M-3}$, *cat*, *aad*, *sul*, *tet*
	FIIS		N/A
P4	HI1	*catA2*, *dfrA12*, *tetM*	*bla*$_{TEM-1}$, *tetA*, *tetC*, *tetD*
	HI2		*bla*$_{CTX-M-3}$, *bla*$_{CTX-M-2}$, *bla*$_{CTX-M-9}$, *bla*$_{CTX-M-14}$, *armA*, *qnrA1* [*bla*$_{SHV-12}$, *bla*$_{CTX-M-14}$, *bla*$_{CTX-M-9}$, *aac(6′)-Ib*-cr], *bla*$_{CMY-8}$, *cat*, *tetD*, *tetC*, *tetA*, *aphA*
	I1		*dfrA1*, *aadA1*, *aadB*, *aadA2*, *bla*$_{CMY-2}$, *bla*$_{CMY-7}$, *bla*$_{CTX-M-1}$, *bla*$_{CTX-M-2}$, bla$_{CTX-M-9}$, *bla*$_{CTX-M-14}$, *bla*$_{CTX-M-15}$ [*bla*$_{TEM-1}$, *aac(6′)-Ib*-cr], *bla*$_{CTX-M-24}$, *bla*$_{SHV-12}$, *bla*$_{TEM-1}$, *bla*$_{TEM-52}$, *bla*$_{VIM-1}$, *mphA*, *qnrA1* [*bla*$_{SHV-12}$, *bla*$_{CTX-M-14}$, *bla*$_{CTX-M-9}$, *aac(6′)-Ib*-cr], *aph*
	N		*bla*$_{CTX-M-1}$, *armA*, *bla*$_{CTX-M-15}$ [*bla*$_{TEM-1}$, *aac(6′)-Ib*-cr], *bla*$_{CTX-M-32}$, *bla*$_{CTX-M-40}$, *bla*$_{KPC-2}$, *bla*$_{VIM-1}$, *armA*, *qnrA3*, *qnrB2* [*bla*$_{CTX-M-3}$, *aac(6′)-Ib*-cr], *qnrB19* [*bla*$_{SHV-12}$], *qnrS1* [*lap-2*, *aac(6′)-Ib*-cr], *bla*$_{OXA}$, *aadA1*, *sul1*, *tetA*
	FIB		*bla*$_{CTX-M-9}$, *bla*$_{CTX-M-14}$, *bla*$_{CTX-M-15}$ [*bla*$_{TEM-1}$, *aac(6′)-Ib*-cr], *bla*$_{SHV-2}$, *bla*$_{SHV-12}$, *bla*$_{TEM-1}$
	FIC		*aac(3′)-IV*
	FIA		*bla*$_{CTX-M-15}$ [*bla*$_{TEM-1}$, *aac(6′)-Ib*-cr], *bla*$_{DHA-1}$, *bla*$_{TEM-1}$, *qnrB2* [*bla*$_{CTX-M-3}$, *aac(6′)-Ib*-cr], *qnrB4* [*armA*, *bla*$_{CTX-M-14}$, *bla*$_{DHA-1}$, *bla*$_{SHV-12}$, *aac(6′)-Ib*-cr]
	U		*qnrS2*, *sul1*, *aadA2*, *tetA*
	W		*bla*$_{VIM-1}$, *sul1*, *dfrB2*
	FrepB		*bla*$_{CTX-M-15}$, *bla*$_{CTX-M-14}$, *bla*$_{CTX-M-3}$, *cat*, *aad*, *sul*, *tet*
	FIIS		N/A
P5	N	*aac(6′)–aph(2″)*, *aac(6′)-Ii*, *ant(6)-Ia*, *aph(3′)-III*, *dfrG*, *ermB*, *lnuB*, *tetL*, *tetU*	*bla*$_{CTX-M-1}$, *armA*, *bla*$_{CTX-M-15}$ [*bla*$_{TEM-1}$, *aac(6′)-Ib*-cr], *bla*$_{CTX-M-32}$, *bla*$_{CTX-M-40}$, *bla*$_{KPC-2}$, *bla*$_{VIM-1}$, *armA*, *qnrA3*, *qnrB2* [*bla*$_{CTX-M-3}$, *aac(6′)-Ib*-cr], *qnrB19* [*bla*$_{SHV-12}$], *qnrS1* [*lap-2*, *aac(6′)-Ib*-cr], *bla*$_{OXA}$, *aadA1*, *sul1*, *tetA*
P6	N	–	*bla*$_{CTX-M-1}$, *armA*, *bla*$_{CTX-M-15}$ [*bla*$_{TEM-1}$, *aac(6′)-Ib*-cr], *bla*$_{CTX-M-32}$, *bla*$_{CTX-M-40}$, *bla*$_{KPC-2}$, *bla*$_{VIM-1}$, *armA*, *qnrA3*, *qnrB2* [*bla*$_{CTX-M-3}$, *aac(6′)-Ib*-cr], *qnrB19* [*bla*$_{SHV-12}$], *qnrS1* [*lap-2*, *aac(6′)-Ib*-cr], *bla*$_{OXA}$, *aadA1*, *sul1*, *tetA*
P7	N/A	*ermB*, *tetW*	N/A

rep type, replication modules; N/A, not applicable.

It was not certain whether it was spread between patients in the hospital or in the community before hospitalisation, since the number of samples was not enough to conclude the reason.

Among the antibiotic resistance genes found in the gut microbiome of the patients participating in this study, we were particularly interested in two genes that encode resistance to extended-spectrum β-lactams and fosfomycin. In Thailand, these two antibiotics are only prescribed in hospitals. This is the first report in Thailand of an extended-spectrum β-lactamase gene with 99.03% identity to *bla*$_{TEM-124}$ across its 84% alignment length. The *bla*$_{TEM-124}$ gene was first reported in Italy in *Morganella morganii*, which are normal bacterial flora of humans and other mammals; however, the source of this *bla*$_{TEM-124}$-carrying bacterium was not identified (GenBank AY327540). In Siriraj Hospital, where the present study was conducted, *bla*$_{TEM-1}$ and *bla*$_{TEM-135}$, which are merely restricted-spectrum β-lactamases, were found in patients from 2005 to 2007 [31]. Similarly, *fosA*, a fosfomycin-modifying enzyme, was found in one patient sample (P2). Several studies have previously shown the presence of *fosA* on plasmids in staphylococci and *E. coli* from patients in Japan as well as in other enteric bacteria from patients in Italy [32–34]. It is therefore likely that the *fosA* gene might have been selected in our hospital by fosfomycin use and HGT involving plasmids.

However, the antibiotic resistance genes identified here did not reflect the type of antibiotics used in the study participants. There are several reasons that might account for this. First, it is possible that the next-generation sequencing (NGS) did not cover entire DNA sequences in the gut microbiome genome (which is very

large) and that some antibiotic resistance genes were missed. NGS has a DNA output of not more than 100 mega base pairs (Mbp). In general, 1 g of stool sample contains 10^{12}–10^{13} bacterial cells [2,3], and *E. coli* DNA has 4.6 Mbp; thus, the total DNA would be 4×10^{12}–10^{13} Mbp (or 4×10^{9}–10^{10} giga base pairs). Currently, there are no sequencers that can cope with this large amount of DNA. Second, linkage disequilibrium in resistance genes results in co-transfer of genes in close proximity to each other through recombination [35]. Therefore, genes not involved in antibiotic resistance can be transferred along with a nearby drug resistance gene within the gut microbiome genome and then become selected during antibiotic use. For example, several resistance genes found in the P2 sample were previously found together in the same gene cassette (*intI*–*aacA4*cr–bla_{OXA-1}–*catB3*–*arr-3*) [36]. Lastly, some resistance gene sequences might not be included in the database, although resistance gene databases nowadays are quite large.

Because we were not able to identify sequences that carry resistance genes and a plasmid replicon, we aligned all of the DNA sequences against an in-house plasmid replicon database. When compared with previous studies, several resistance genes were identified (e.g. *aph(3′)-III* and repHI2; *aac(6′)-Ib*-cr and repHI2, FIIA, N, FIB and FIA; bla_{OXA-1} and repN; and *sul1* and repN and U) [11,13,21]. However, no firm conclusions can be made that these plasmid types are the causative agents of antibiotic resistance. Improvements in the efficiency of NGS to increase the length of DNA reads would be helpful, as would redesigning the assembly algorithm to cope with long DNA fragments.

In this study, extracted plasmid portions were used in a metagenomic study to identify antibiotic resistance genes in gut microbiomes of hospitalised patients. Various antibiotic resistance genes in gut microbiome plasmids were shared among the patients and some were also found in the healthy control subject. Although these genes were not linked with antibiotic usage or plasmid type, these findings confirm that the mechanism whereby antibiotic resistance genes are spread is complex. The spread of such genes takes place not only in hospitals but also in the community, through antibiotic misuse, animal farming and self-medication. Therefore, antibiotic usage around the world requires better regulation.

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Competing interests

None declared.

Ethical approval

All of the patients signed informed consent forms, and the study was approved by the Human Research Protection Unit of Siriraj Hospital.

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