

Table 1: Characteristics of 16S RMTase-positive *P. aeruginosa* isolates including region and year of isolation, ST and antibiotic resistance genes.

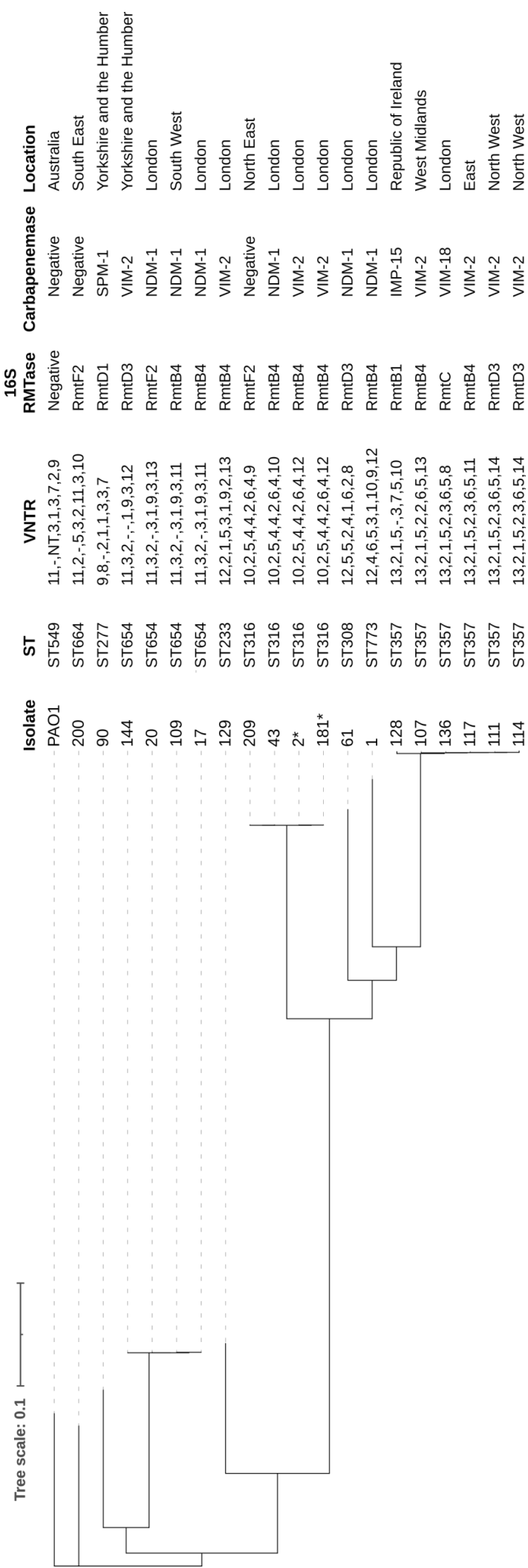
Isolate	Region	Year of isolation	ST	16S RMTase gene	Carbapenemase gene	Other antibiotic resistance genes
128	Republic of Ireland	2009	357	<i>rmtB1</i>	<i>bla_{IMP-15}</i>	<i>aph(3')-XV</i> , <i>aph(6)-Id</i> , <i>bla_{CARB-2}</i> , <i>qnrVC1</i> , <i>floR2</i> , <i>sul1</i> , <i>dfra27</i> and <i>tet(G)</i>
129	London	2009	233	<i>rmtB4</i>	<i>bla_{VIM-2}</i>	<i>aac(3)-Id</i> , <i>sul1</i> , <i>dfdB5</i> and <i>tet(G)</i>
43	London	2014	316	<i>rmtB4</i>	<i>bla_{NDM-1}</i>	<i>aph(6)-Id</i> , <i>bla_{PME-1}</i> , <i>sul1</i> and <i>tet(G)</i>
181 ^a	London	2015	316	<i>rmtB4</i>	<i>bla_{VIM-2}</i>	<i>aac(6')-Ib-cr</i> , <i>aph(6)-Id</i> , <i>bla_{OXA-1}</i> , <i>bla_{OXA-10}</i> , <i>sul1</i> and <i>tet(G)</i>
2 ^a	London	2015	316	<i>rmtB4</i>	<i>bla_{VIM-2}</i>	<i>aac(6')-Ib-cr</i> , <i>aph(6)-Id</i> , <i>bla_{OXA-1}</i> , <i>bla_{OXA-10}</i> , <i>sul1</i> and <i>tet(G)</i>
117	East	2008	357	<i>rmtB4</i>	<i>bla_{VIM-2}</i>	<i>aac(3)-Id</i> , <i>aadA1</i> , <i>ant(2'')-Ia</i> , <i>bla_{OXA-10}</i> , <i>bla_{VEB-1}</i> , <i>sul1</i> , <i>dfdB5</i> and <i>tet(A)</i>
107	West Midlands	2012	357	<i>rmtB4</i>	<i>bla_{VIM-2}</i>	<i>aac(3)-Id</i> , <i>aadA1</i> , <i>aph(3')-Ia</i> , <i>aph(3'')-Ib</i> , <i>aph(6)-Id</i> , <i>bla_{VEB-1}</i> , <i>qnrVC1</i> , <i>ere(A)</i> , <i>mph(A)</i> , <i>sul1</i> , <i>dfdB5</i> , <i>tet(A)</i> and <i>tet(G)</i>
109	South West	2012	654	<i>rmtB4</i>	<i>bla_{NDM-1}</i>	<i>ant(2'')-Ia</i> , <i>aph(3'')-Ib</i> , <i>aph(6)-Id</i> , <i>sul1</i> and <i>tet(G)</i>
17	London	2015	654	<i>rmtB4</i>	<i>bla_{NDM-1}</i>	<i>ant(2'')-Ia</i> , <i>aph(3')-Ib</i> , <i>aph(3'')-Ib</i> , <i>aph(6)-Id</i> , <i>sul1</i> and <i>tet(G)</i>
1	London	2015	773	<i>rmtB4</i>	<i>bla_{NDM-1}</i>	<i>qnrVC1</i> , <i>sul1</i> and <i>tet(G)</i>
136	London	2010	357	<i>rmtC</i>	<i>bla_{VIM-18}</i>	<i>aac(3)-IId</i> , <i>aac(6')-Ib-cr</i> , <i>aadA1</i> , <i>aph(3'')-Ib</i> , <i>aph(6)-Id</i> , <i>bla_{AER-1}</i> , <i>bla_{OXA-10}</i> , <i>cmIA1</i> , <i>sul1</i> and <i>tet(G)</i>
90	Yorkshire and the Humber ^b	2013	277	<i>rmtD1</i>	<i>bla_{SPM-1}</i>	<i>aac(6')-Ib-cr</i> , <i>aadA7</i> , <i>bla_{OXA-56}</i> , <i>cmx</i> and <i>sul1</i>
61	London	2014	308	<i>rmtD3</i>	<i>bla_{NDM-1}</i>	<i>aac(3)-Id</i> , <i>aph(3')-VIa</i> , <i>aph(3'')-Ib</i> , <i>aph(6)-Id</i> , <i>bla_{PME-1}</i> , <i>floR2</i> , <i>msr(E)</i> , <i>qnrVC1</i> , <i>sul1</i> , <i>sul2</i> , <i>dfdB5</i> and <i>tet(G)</i>

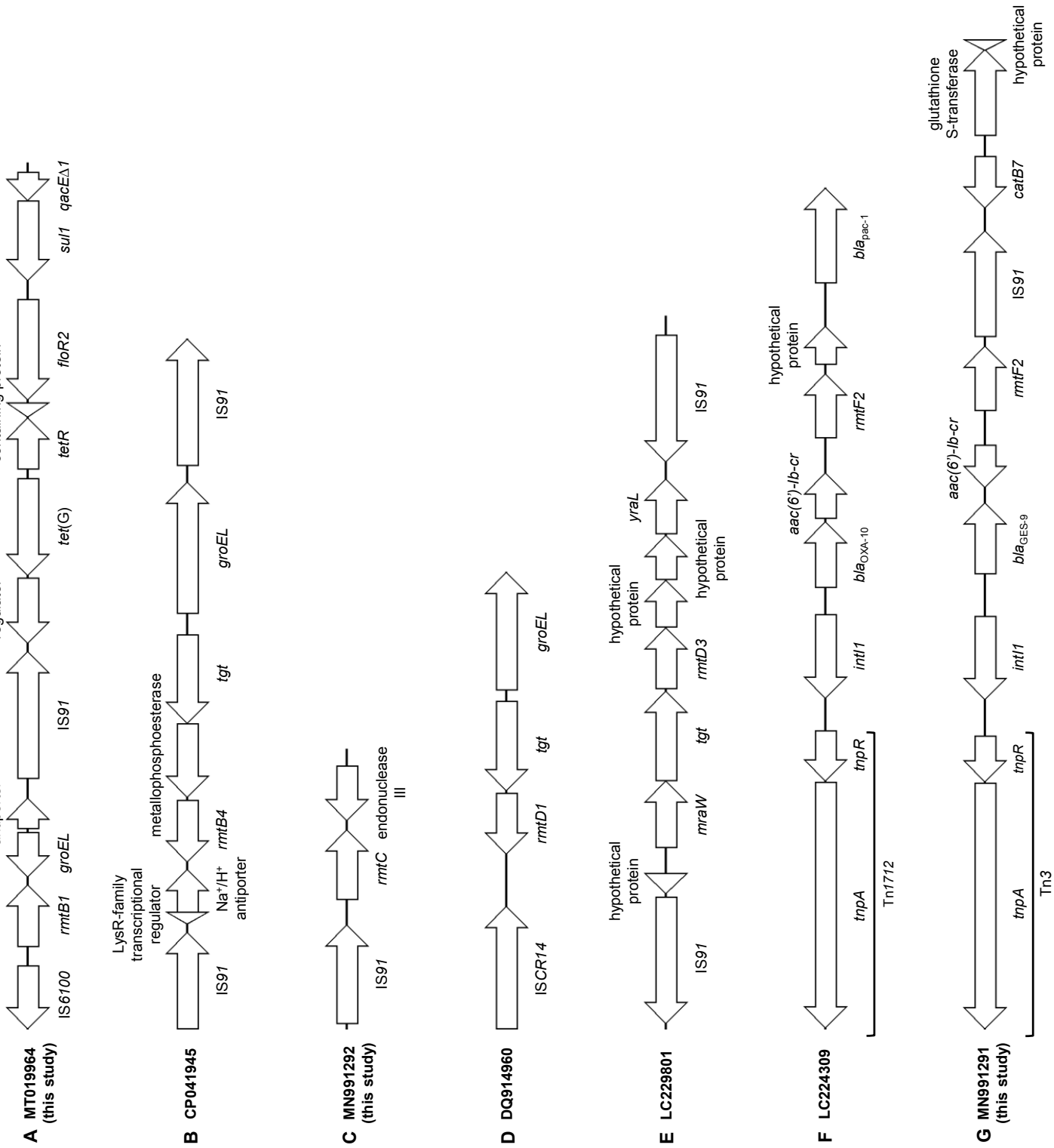
111	North West ^c	2003	357	<i>rmtD3</i>	<i>bla_{VIM-2}</i>	<i>aadA1</i> , <i>ant(2'')-Ia</i> , <i>bla_{OXA-10}</i> , <i>bla_{VEB-1}</i> , <i>sul1</i> , <i>dfrrB2</i> , <i>tet(A)</i> and <i>tet(G)</i>
114	North West ^c	2006	357	<i>rmtD3</i>	<i>bla_{VIM-2}</i>	<i>aadA1</i> , <i>ant(2'')-Ia</i> , <i>bla_{OXA-10}</i> , <i>bla_{VEB-1}</i> , <i>sul1</i> , <i>dfrrB2</i> , <i>tet(A)</i> and <i>tet(G)</i>
144	Yorkshire and the Humber ^b	2010	654	<i>rmtD3</i>	<i>bla_{VIM-2}</i>	<i>ant(2'')-Ia</i> , <i>aph(3'')-Ib</i> , <i>aph(6)-Id</i> , <i>sul1</i> , <i>tet(A)</i> and <i>tet(G)</i>
209	North East	2015	316	<i>rmtF2</i>	Negative	<i>aac(6')-Ib-cr</i> , <i>bla_{OXA-10}</i> , <i>bla_{PAC-1}</i> and <i>sul1</i>
20	London	2015	654	<i>rmtF2</i>	<i>bla_{NDM-1}</i>	<i>aac(6')-Ib-cr</i> , <i>aph(3'')-Ib</i> , <i>aph(6)-Id</i> , <i>bla_{GES-9}</i> and <i>sul1</i>
200	South East	2015	664	<i>rmtF2</i>	Negative	<i>aac(6')-Ib-cr</i> , <i>bla_{OXA-1}</i> , <i>bla_{OXA-10}</i> , <i>bla_{PAC-1}</i> , <i>sul1</i> and <i>tet(G)</i>

^a = same patient; ^b and ^c = same hospital

Figure 1 revised

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Detection and characterisation of 16S rRNA methyltransferase-producing *Pseudomonas aeruginosa* from the UK and Republic of Ireland from 2003-2015

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Abstract

16S rRNA methyltransferase (16S RMTase) genes confer high-level aminoglycoside resistance, reducing treatment options for multi-drug resistant Gram-negative bacteria. *Pseudomonas aeruginosa* isolates (n=221) exhibiting high-level pan-aminoglycoside resistance (amikacin, gentamicin and tobramycin MICs ≥ 64 , ≥ 32 and ≥ 32 mg/L, respectively) were screened for 16S RMTase genes to determine their occurrence amongst isolates submitted to the national reference laboratory from December 2003 to December 2015. 16S RMTase genes were identified using two multiplex PCRs and whole genome sequencing (WGS) was used to identify other antibiotic resistance genes, sequence types (STs) and the genetic environment of 16S RMTase genes. 16S RMTase genes were found in 8.6% (19/221) of isolates, with *rmtB4* (47.4%, 9/19) the most common, followed by *rmtD3* (21.1%, 4/19), *rmtF2* (15.8%, 3/19) and single isolates harboured *rmtB1*, *rmtC* and *rmtD1*. Carbapenemase genes were found in 89.5% (17/19) of 16S RMTase-positive isolates, where *bla_{VIM}* (52.9%, 9/17) was the most common. 16S RMTase genes were found in 'high-risk' clones known to harbour carbapenemase genes: ST233, ST277, ST357, ST654 and ST773. Analysis of the genetic environment of 16S RMTase genes identified that IS6100 was genetically linked to *rmtB1*; IS91 to *rmtB4*, *rmtC* or *rmtD3*; ISCR14 to *rmtD1* and *rmtF2* was linked to Tn3, IS91 or Tn1721.

Although 16S RMTase genes explained only 8.6% of pan-aminoglycoside resistance in the *P. aeruginosa* isolates studied, the association of 16S RMTase genes with carbapenemase-producers and 'high-risk' clones highlights that continued surveillance is required to monitor spread and the importance of suppressing the emergence of dually-resistant clones in hospital settings.

Keywords: 16S RMTase, aminoglycoside resistance, carbapenemases, high-risk clones, genetic environment, *Pseudomonas aeruginosa*

1. Introduction

Pseudomonas aeruginosa is an opportunistic nosocomial pathogen associated with serious disease such as pneumonia, urinary tract infections, meningitis and septicaemia [1]. It has the potential to become highly antibiotic resistant due to an arsenal of antibiotic-modifying enzymes (e.g. β -lactamases and aminoglycoside-modifying enzymes), chromosomal mutations (e.g. within the DNA gyrase and topoisomerase IV genes causing quinolone resistance), upregulation of antibiotic efflux pumps (e.g. MexXY-OprM) and low membrane impermeability [1].

16S rRNA methyltransferases (16S RMTases) confer high-level resistance (MIC >256 mg/L) to all clinically-relevant aminoglycosides with only a single gene, which differs from other aminoglycoside resistance mechanisms that need several genes to achieve the same level of resistance [2-7]. To date, ten 16S RMTase genes (*armA*, *rmtA-rmtH* and *npmA*) have been reported around the world [8]. Their emergence is a cause of public health concern due to the association of 16S RMTase genes with other antibiotic resistance genes (such as carbapenemases and extended-spectrum β -lactamases [ESBLs]) in multidrug-resistant bacteria as well as mobile genetic elements, transposons and plasmids, which allow 16S RMTase genes to spread by horizontal gene transfer [8].

The first 16S RMTase gene to be identified in *P. aeruginosa* was *rmtA*, which was isolated from a sputum sample in Japan in 1997 [7]. Currently, only nine 16S RMTase genes (*armA*, *rmtA-G* and *npmA*) have been reported in *P. aeruginosa* within the clinical setting in Asia [2,4-7,9], Europe [10], and South America [3,11] but the range of 16S RMTase genes present in *P. aeruginosa* in the UK is unknown.

This study sought to determine the occurrence of 16S RMTase genes in pan-aminoglycoside resistant *P. aeruginosa* submitted to Public Health England's Antimicrobial Resistance and

Healthcare Associated Infections (AMRHA) Reference Unit for the investigation of unusual antibiotic resistance (primarily carbapenem resistance).

On 1st October 2021, the UK Health Security Agency (UKHSA) became fully operational, bringing together the health protection functions of Public Health England and the National Health Service Test and Trace, including the Joint Biosecurity Centre. As this study was completed prior to this transition we have retained the former Public Health England designations in the text.

2. Methods

2.1 Bacterial Isolates

P. aeruginosa isolates (n=221) from December 2003 to December 2015 exhibiting high-level aminoglycoside resistance (amikacin, gentamicin and tobramycin MICs ≥ 64 , ≥ 32 and ≥ 32 mg/L, respectively), as determined by agar dilution, were recovered from the AMRHA Reference Unit culture collection. Isolates had been submitted for reference service investigation of unusual (primarily carbapenem) resistance. All isolates exhibited high-level aminoglycoside resistance (amikacin, gentamicin and tobramycin MICs ≥ 64 , ≥ 32 and ≥ 32 mg/L, respectively) as determined by agar dilution. Following an internal review at PHE this study was classified as surveillance and as no ethical issues were identified, hence, there was no requirement for a full review by an ethics committee.

2.2 Bacterial identification and strain typing

Bacterial identification was confirmed by MALDI-TOF (Bruker, Bremen, Germany) and isolates were typed using variable-number tandem-repeat (VNTR) analysis, as previously described [12]. Additionally, multilocus sequence typing (MLST) profiles were derived from whole genome sequencing data (see Section 2.4).

2.3 MIC determination and identification of 16S RMTase genes

Antibiotic susceptibility testing was performed by agar dilution [13] against AMRHA's standard Gram-negative antibiotic panel and the data interpreted using EUCAST clinical breakpoints (version 8.1) [14]. Crude DNA extracts were screened with two multiplex PCRs to detect the 16S RMTase genes *armA*, *rmtA-rmtH* and *npmA* as previously described [15]. Amplicons were analysed using gel electrophoresis on 2% agarose gels.

2.4 Whole genome sequencing (WGS) and analysis of genomic data

For WGS, genomic DNA of the 16S-RMTase producing *P. aeruginosa* isolates were extracted using the automated QIAAsymphony extraction robot (Qiagen, Crawley, UK) and the QIAAsymphony DSP DNA mini kit (Qiagen) according to manufacturer's instructions. WGS was conducted by the Wellcome Trust Sanger Institute using an Illumina HiSeq X Ten (Illumina, San Diego, CA, USA) with 150-bp paired-end reads. Genomes were *de novo* assembled using Velvet with the pipeline and improvements found at <https://github.com/sanger-pathogens/vr-codebase> and https://github.com/sanger-pathogens/assembly_improvement [16]. Reads were uploaded to the European Nucleotide Archive (ENA) under the project number PRJEB23879 and accession numbers ERR3181683; ERR3181601-ERR3181609; ERR3181612-ERR3181614; ERR3181684-ERR3181686; ERR3181688 and ERR3181689. WGS for the *rmtD1*-positive *P. aeruginosa* isolate was conducted at PHE as previously described [10].

STs were derived from WGS data of 16S RMTase-producing isolates using MLST 2.0 [17] and were confirmed using the online *P. aeruginosa* MLST database hosted by PubMLST (<http://pubmlst.org>). To determine the phylogenetic relationship between the strains collected as part of the study, the SNP-calling analysis was carried out by mapping raw reads to the complete reference sequence PAO1 (NC_002516) using SMALT (Wellcome Trust Sanger Institute). Concatenated SNPs in the core genome were used to construct a maximum likelihood tree using RAxML [18]. Phylogenetic trees were visualised and annotated using Interactive Tree Of Life (iTOL) [19].

Antibiotic resistance genes were identified using ResFinder 3.0 (<https://cge.cbs.dtu.dk/services/ResFinder/>) [20] as well as the ARG-ANNOT databases [21] via the read mapping-based tool SRST2 (<https://github.com/katholt/srst2>) [22]. 16S RMTase gene-containing contigs were analysed with BLAST to confirm the genetic environment and identify related DNA sequences in GenBank. Mauve (<http://darlinglab.org/mauve/mauve.html>) was used to visually confirm the alignment of 16S RMTase gene-containing contigs to the published DNA sequences from GenBank [23]. Genetic environments of 16S RMTase genes were visualised using EasyFig version 2.2.2 (<http://mjsull.github.io/Easyfig/>) [24].

3. Results and Discussion

3.1 Identification of 16S RMTase genes

Nineteen (8.6%) isolates were found to harbour 16S RMTase genes including *rmtB4* (47.4%, 9/19), *rmtD3* (21.1%, 4/19), *rmtF2* (15.8%, 3/19), *rmtB1* (5.3%, 1/19), *rmtC* (5.3%, 1/19) as well as a single *rmtD1*-positive isolate, which was previously reported by Hopkins *et al.* [10]. Genes *armA*, *rmtA*, *rmtE*, *rmtG*, *rmtH* and *npmA* were not identified. The occurrence of 16S RMTase genes in this study was lower compared to incidences reported from other countries; studies investigating high-level aminoglycoside-resistant *P. aeruginosa* in China [25] and India [4] identified rates of 88.6% (31/35) and 20.6% (19/92), respectively. However, these rates most likely were higher as the studies only collected isolates from one and two hospitals, respectively, unlike our study, which collected isolates nationally over twelve years (2003-2015).

In this study, the rarely reported 16S RMTase gene variants *rmtB4*, *rmtD3* and *rmtF2* were identified. These were initially isolated in Nepal between 2012 and 2013 [26], Myanmar in 2014 [27] and Nepal between 2012 and 2013 [26], respectively. However, we found that *rmtB4* and *rmtD3* were identified in strains isolated in 2008 and 2003, respectively, meaning that these variants have been circulating in *P. aeruginosa* longer than previously recognised.

3.2 Comparison to our previous study on Enterobacterales

In our previous study, 94.5% (762/806) pan-aminoglycoside resistant Enterobacterales harboured 16S RMTase genes [15]. The low prevalence (8.6%, 19/221) found in this study is likely due to high-level pan-aminoglycoside resistance in *P. aeruginosa* being attributable to alternative resistance mechanisms such as impermeability, MexXY-OprM efflux pumps or a combination of aminoglycoside modifying enzymes (AMEs) [1] rather than 16S RMTases. Additionally, 16S RMTase genes were commonly found on the chromosome in *P. aeruginosa* [5,11,26,27], which was supported by this study as 52.6% (10/19) of 16S RMTase genes were chromosomal. However, it has been reported that 16S RMTases were mostly associated with plasmids in Enterobacterales [8], suggesting that 16S RMTase genes are more readily spread within Enterobacterales through horizontal gene transfer, thereby increasing their prevalence.

3.3 MLST and VNTR analysis

P. aeruginosa isolates belonged to eight STs including the 'high risk' clones ST357 (31.6%, 6/19), ST654 (21.1%, 4/19), **ST233** (5.3%, 1/19), ST277 (5.3%, 1/19) and ST773 (5.3%, 1/19) (Table 1 and Figure 1). These clones are deemed to be 'high-risk' due to being multidrug or extremely-drug resistant, which has allowed them to spread globally [28]. Other STs identified in this study included ST316 (21.1%, 4/19), ST308 (5.3%, 1/19) and ST664 (5.3%, 1/19), which are not currently deemed to be 'high-risk' (Table 1). VNTR analysis identified that there was genomic variation within the STs as five VNTR profiles (13,2,1,5,2,3,6,5,14 [n=2], 13,2,1,5,2,3,6,5,8 [n=1], 13,2,1,5,2,3,6,5,11 [n=1], 13,2,1,5,2,2,6,5,13 [n=1] and 13,2,1,5,-,3,7,5,10 [n=1]) were identified amongst ST357 (n=6) isolates; three VNTR profiles (10,2,5,4,4,2,6,4,12 [n=2], 10,2,5,4,4,2,6,4,9 [n=1] and 10,2,5,4,4,2,6,4,10 [n=1]) were identified in ST316 (n=4) isolates and three VNTR profiles (11,3,2,-,3,1,9,3,11 [n=2], 11,3,2,-,3,1,9,3,13 [n=1] and 11,3,2,-,1,9,3,12 [n=1]) were identified in ST654 (n=4) isolates. The association of 16S RMTase genes with 'high-risk' clones is of concern as 'high-risk' clones have the potential to spread globally due to being multidrug resistant and/or virulent [28].

Different clones, including those deemed 'high-risk', were found to carry *rmtB4*, *rmtD3* and *rmtF2* than previously reported. *rmtB4* has been previously reported in the 'high-risk' clones

ST773 and ST235 in the USA [29] and Nepal [26], respectively, as well as in the emerging 'high-risk' clone ST1047 in Myanmar [9]. However, in this study, *rmtB4* was identified in ST316 as well as the 'high-risk' clones ST233, ST357, ST654 and ST773. Similarly, we identified *rmtD3*-positive isolates belonging to the 'high-risk' clones ST357, ST654 and ST308, whereas *rmtD3* has previously reported associations with ST316 in Myanmar [9] and ST234 in Poland [30], which has not deemed to be 'high-risk'. In this study, *rmtF2* was identified in ST316, ST664 and the 'high-risk' clone ST654. The *rmtF2* gene has also been reported in ST664 in Nepal [26], as well as ST1047 in Nepal [26] and ST983 in Myanmar [9], which has not been deemed to be 'high-risk'. Taken together, these studies highlight the involvement of 'high-risk' clones and gene mobilisation between STs in the spread of 16S RMTase genes.

3.4 Antibiotic susceptibility

In addition to exhibiting pan-aminoglycoside resistance, all 16S RMTase gene-positive isolates were resistant to ceftazidime, ciprofloxacin and piperacillin-tazobactam, and 94.7% (18/19), 89.5% (17/19) and 73.7% (14/19) isolates were resistant to imipenem, meropenem and aztreonam, respectively. All isolates were susceptible to colistin, a last-line antibiotic used to treat multidrug-resistant infections [28], indicating that colistin may be useful in treating infections caused by 16S RMTase-producing *P. aeruginosa*. However, due to the toxicity of colistin and the risk of its overuse driving colistin resistance, newer agents with alternative targets are needed, such as cefiderocol [31].

3.5 Identification of antibiotic resistance genes

As found in our previous study, where 93.4% (712/762) 16S RMTase gene-positive Enterobacterales from AMRHA's isolate collection were carbapenemase producers [15], most (89.5%, 17/19) 16S RMTase gene-positive *P. aeruginosa* isolates also harboured carbapenemase genes. *bla*_{VIM-2} (42.1%, 8/19) was the most common, followed by *bla*_{NDM-1} (31.6%, 6/19), and one each (5.3%) of *bla*_{IMP-15}, *bla*_{SPM-1} and *bla*_{VIM-18} (Table 1). Two *rmtF2*-positive isolates were negative for carbapenemase genes. However, the association between

16S RMTase and carbapenemase genes was not an exclusive association as 68.3% (138/202) 16S RMTase gene-negative *P. aeruginosa* isolates were also carbapenemase producers (data not shown). The high level of association between 16S RMTase and carbapenemase genes in this isolate collection is likely due to carriage of 16S RMTase genes by 'high-risk' clones also reported to harbour carbapenemase genes, with ST233, ST277, ST357 and ST654 reported to harbour *bla*_{IMP}; ST233, ST357, ST654 and ST773 harbour *bla*_{VIM}; ST233, ST654 and ST773 harbour *bla*_{NDM} and ST277 harbour *bla*_{SPM-1} [10,29,32]. Other acquired antibiotic resistance genes that were commonly identified in the 16S-RMTase positive isolates were *sul1* (100%, 19/19) and *tet*(G) (78.9%, 15/19) (Table 1).

3.6 Analysis of the genetic environment of 16S RMTase genes

3.6.1. *rmtB1*

Analysis of the assembled WGS data of the single *rmtB1* gene identified it on a 334,872 bp contig, where IS6100 was found upstream of *rmtB1*. This differs from the literature as *rmtB* is frequently reported to be associated with Tn2 [8,33] and, to our knowledge, this is the first report of IS6100 being associated with *rmtB1*. *tet*(G), *flor2* and *sul1*, encoding trimethoprim, chloramphenicol and sulphonamide resistance, respectively, were found downstream of *rmtB1* (Figure 2A, deposited under GenBank accession number MT019964). This genetic environment appeared to be novel as there were no published DNA sequences in GenBank that shared 100% identity with the entire length of the contig. *rmtB1* appeared to be located on a plasmid as 1-320,547 bp of the contig shared 99.9% sequence identity to regions between 1-467,586 bp of *Pseudomonas koreensis* strain P19E3 plasmid p1 (accession number: CP027478.1); 320,548-324,957 bp of the contig shared 99.9% (3862/3863) identity to 253,261-257,123 bp of *P. aeruginosa* strain AR441 plasmid unnamed3 (accession number: CP029094.1) and 324,460-334,763 bp of the contig contained the genetic environment surrounding *rmtB1*.

3.6.2. *rmtB4*

The genetic environment of *rmtB4* (n=9) was the same as previously described in the *P. aeruginosa* strain ST773 chromosome (accession number: CP041945.1) from 2,438,442-2,446,741 bp of the chromosomal DNA, where *rmtB4* was flanked by two IS91 elements (Figure 2B). This genetic environment has also been reported for *rmtB4* in *P. aeruginosa* strain IOMTU 304 (accession number: LC052325.1) by Tada *et al.* in Nepal [26], but the latter is missing IS91 upstream of the *rmtB4* gene. However, single isolates belonging to ST233, ST316 and ST357 had truncated IS91 genes downstream of *rmtB4* and one ST654 isolate had both IS91 genes truncated and placed on separate contigs, this could be due to short read sequencing not being able to capture the repetitive regions in the IS elements in full. In three isolates, the *rmtB4*-encoding regions were flanked by regions sharing ~99% identity with published *P. aeruginosa* chromosomal DNA sequences, where flanking regions of one *P. aeruginosa* ST357 shared 99.5% identity to 1,284,586-1,288,962 bp of the chromosome of *P. aeruginosa* strain 1334/14 (accession number: CP035739.1); the ST233 isolate shared 100% identity to *P. aeruginosa* strain PASGNM699 (accession number: CP020704.1) chromosome from 3,422,807-3,424,930 bp and one ST773 isolate shared 99.9% identity to the chromosome of *P. aeruginosa* strain ST773 (accession number: CP041945.1) from 2,436,342-2,439,926 bp. This suggests integration of the *rmtB4*-encoding regions into the chromosome. The genetic location of the remaining six *rmtB4* genes could not be determined due to the lack of chromosome- or plasmid-specific genes on the contigs.

3.6.3 *rmtC*

rmtC was identified on a 3,373 bp contig, where the genetic environment of *rmtC* was flanked by IS91 and a gene encoding endonuclease III (Figure 2C, deposited under GenBank accession number MN991292). This genetic environment appeared to be novel as there were no published DNA sequences in GenBank that shared 100% identity to the DNA sequence of the contig. Due to the short length of the contig it was not possible to determine the genetic location of *rmtC*. *rmtC* has previously been described as commonly associated with *ISEcp1* [8,34-35] but the association between IS91 and *rmtC* has also been reported in

Enterobacterales in Australia [35], Canada [35], India [35], New Zealand [35], Romania [36], Taiwan [37] and the USA [38]. But the association of IS91 with *rmtC* therefore appears to be widespread though, to our knowledge, this is the first report of this association in *P. aeruginosa*.

3.6.4 *rmtD1*

rmtD1 was identified on a 5,826 bp contig, which shared 100% identity with *P. aeruginosa* strain PA0905 (accession number: DQ914960.2) from 6,698-11,099 bp (Figure 2D). ISCR14 was found upstream of *rmtD1*, which has previously been reported to be associated with *rmtD1* in *K. pneumoniae* and *P. aeruginosa* isolates from Brazil [39]. Due to the lack of chromosome- or plasmid-specific genes on the contig, the genetic location of *rmtD1* could not be determined. As *rmtD1* was found associated with ISCR14 in the 'high-risk' clone ST277, *rmtD1* may disseminate to other bacteria due to transposition by ISCR14 and through the spread of *P. aeruginosa* ST277.

3.6.5 *rmtD3*

Analysis of the single contigs harbouring *rmtD3* (n=4), which ranged from 6877-14,250 bp in length, identified that the genetic environment was as previously described in *P. aeruginosa* strain MyNCGM481 from Myanmar (accession number: LC229801.1) [27] from 1-8,347 bp *rmtD3* was flanked by two IS91 elements (Figure 2E). The *rmtD3*-encoding region was found on 14,214 and 14,250 bp contigs in two *P. aeruginosa* ST357 isolates, where it was flanked by chromosomal DNA that shared ~99.0% identity to the *P. aeruginosa* strain PA83 chromosome (accession number: CP017293.1) from ~2,801,000-2,808,100 bp and ~6,223,500-6,227,700 bp, respectively. Truncated IS91 genes were found upstream and downstream of *rmtD3* in single ST654 and ST308 isolates, respectively, but due to IS91 genes split between multiple contigs, and the lack of chromosome- or plasmid-specific genes on the contigs, we were not able to determine genetic location of *rmtD3* being identified in these isolates.

3.6.6 *rmtF2*

Two of the three *rmtF2*-positive isolates, belonging to ST316 and ST664, shared the same genetic environment as *P. aeruginosa* strain IOMTU487 from Nepal (accession no: LC224309) [26], where *Tn1721*, *bla*_{OXA-10} and *aac(6')-Ib* were found upstream, and *bla*_{PAC-1} was found downstream of *rmtF2* (Figure 2F). However, *tnpA*, which was part of *Tn1712*, was missing in the *P. aeruginosa* ST316 isolate. In *P. aeruginosa* ST664, 1-26,890 bp of the 38,561 bp contig shared 99.2% identity with 5,979,815-6,006,711 bp of the *P. aeruginosa* strain S04 90 chromosome (accession number: CP011369.1) and in the ST316 isolate 11,940-97,569 bp of the 109,206 bp contig shared 99.9% identity to 331,493-417,122 bp of the *P. aeruginosa* strain Ocean-1155 chromosome (accession number: CP022526.1).

A novel genetic environment was identified for the *rmtF2* gene identified in the *P. aeruginosa* ST654 isolate, which was found on a 62,396 bp contig (Figure 2G, deposited under GenBank accession number MN991291). In this isolate, *Tn3*, *bla*_{GES-9} and *aac(6')-Ib-cr* were found upstream of *rmtF2*, whilst *IS91* and *catB7* were found downstream. *rmtF2* was present on the chromosome as 12,365-60,409 bp of the contig shared 99.5% identity to 3,391,991-3,440,027 bp of the chromosome of *P. aeruginosa* strain H26027 (accession number: CP033684.1) and 60,409-62,396 bp of the contig shared 100% identity to 6,438,383-6,440,370 bp of the chromosome *P. aeruginosa* PA99 (accession number: CP042967.1). Analysis of the genetic environment of the three *rmtF2* genes showed that *rmtF2* was located on the same contigs as *aac(6')-Ib-cr* in all three isolates, therefore use of fluoroquinolones, such as ciprofloxacin, may contribute to selection for *rmtF2*.

3.7 Patient demographics

The 16S RMTase-positive isolates were submitted by 15 hospitals across eight regions in the UK and were obtained from 17 patients who were distributed across London (47.1%, 8/17), the North West of England (11.8%, 2/17), Yorkshire and the Humber (11.8%, 2/17), and single patients (5.9%) in the East, North East, South East and the South West of England and the

West Midlands. One isolate was submitted by a hospital in the Republic of Ireland. Seventeen (94.4%) isolates originated from single patients and one (5.6%) patient was the source of two isolates, which were isolated over a 3-month period from the same site of infection.

Thirteen (72.2%) patients were hospital inpatients, four (22.2%) patients were from the community setting and one (5.6%) patient was from an unknown setting. The most common specimen type was urine (42.1%, 8/19), followed by ear swab (21.1%, 4/19), wound swab (10.5%, 2/19), and single (5.3%) isolates were isolated from a peripheral venous catheter, a throat swab, a lung sample, and a rectal swab. One (5.3%) isolate was from an unknown source.

Information regarding travel history was known for only six patients, including three patients with *rmtB4*-positive *P. aeruginosa* and one patient with an *rmtD3*-positive *P. aeruginosa* isolate, who had all travelled to India. However, one patient with an *rmtD3*-positive *P. aeruginosa* isolate had no history of recent travel. This suggests that *rmtB4*- and *rmtD3*-positive *P. aeruginosa* are circulating outside of Nepal and Myanmar (where these genes were initially reported [26,27]), respectively, as well as Poland (where *rmtD3* has been subsequently identified [30]). The *rmtD1*-producing *P. aeruginosa* ST277 strain originated from a patient who received medical treatment in Brazil, where similar strains are endemic [10,40].

3.8 Limitations of the study

As Public Health England's AMRHAI Reference Unit is the national reference laboratory for investigating unusual antibiotic resistance in healthcare associated infections (particularly carbapenem resistance), this isolate collection is biased and is not representative of the true occurrence of 16S RMTase genes in *P. aeruginosa* in the UK and Republic of Ireland. No denominator data were available so the national prevalence of 16S RMTase genes in *P. aeruginosa* could not be identified; therefore, additional studies looking at an unbiased isolate collection need to be conducted to identify a prevalence rate that is more representative of the UK.

Another limitation to this study was the use of short-read sequencing to identify the genetic environment of 16S RMTase genes and large number of contigs (average 88, range 48-467) meant that the genomic location or the complete genetic environment of certain 16S RMTase genes could not be identified with high confidence. To resolve this issue long-read sequencing technologies such as single-molecule, real-time (SMRT) sequencing (Pacific BioSciences) or the MinION (Oxford Nanopore Technologies) could be used. However, the high cost of long-read sequencing meant it was not feasible for this study.

3.9 Concluding remarks

In conclusion, the occurrence of 16S RMTase genes in high-level pan-aminoglycoside resistant *P. aeruginosa* was low (8.6%, 19/221), meaning 16S RMTases are not the main resistance mechanism causing high-level aminoglycoside resistance in *P. aeruginosa* in the UK and Republic of Ireland. The rarely reported 16S RMTase gene variants *rmtB4*, *rmtD3* and *rmtF2* were found to be circulating in the UK earlier than previously reported in other countries. These variants, along with other 16S RMTase genes, were found to be carried by 'high-risk' clones known to also carry carbapenemase genes. Therefore, the occurrence of 16S RMTases in *P. aeruginosa* in the UK and Republic of Ireland is likely to increase in the future through clonal expansion, as well as through horizontal gene transfer of 16S RMTases facilitated by mobile genetic elements such as IS91. Therefore, continued surveillance should be conducted to limit spread of such high-level aminoglycoside-resistant organisms.

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Competing interests

None.

Ethical approval

Not required.

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Figure 1: Maximum-likelihood phylogenetic tree of 16S RMTase-positive *P. aeruginosa* isolates. *P. aeruginosa* PAO1 (accession number: NC_002516.2) was used as a reference strain. The VNTR profile for *P. aeruginosa* strain PAO1 was previously obtained by PHE when locus 213 was not tested, therefore this has been signified by 'NT'. Isolates from the same patient are indicated by *.

Figure 2: Genetic environments of 16S RMTase genes *rmtB1* (n=1), *rmtB4* (n=9), *rmtC* (n=1), *rmtD1* (n=1), *rmtD3* (n=4) and *rmtF2* (n=3) in *P. aeruginosa* isolates. Three *rmtB4*-positive isolates had truncated IS91 genes downstream of *rmtB4* and one isolate had both upstream and downstream IS91 genes truncated and placed on separate contigs. Two *rmtD3*-positive isolates had truncated IS91 genes upstream and downstream of *rmtD3*.