High prevalence of 16S rRNA methyltransferases among carbapenemase-producing Enterobacteriaceae in the UK & Ireland.

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ABSTRACT

The emergence of 16S rRNA methyltransferases (16S RMTases) worldwide is a growing concern due to their ability to confer high-level resistance (MICs >256 mg/L) to all clinically-relevant aminoglycosides. As the occurrence of 16S RMTases in the United Kingdom has not been investigated to date, we screened 806 Enterobacteriaceae isolates displaying high-level aminoglycoside resistance (amikacin, gentamicin and tobramycin MICs ≥64, ≥32 and ≥32 mg/L, respectively) for 16S RMTases either by analysing whole-genome sequence (WGS) data (which were available for 449 isolates) or by PCR. A total of 94.5% (762/806) pan-aminoglycoside resistant Enterobacteriaceae were positive for one or more 16S RMTase genes; armA was the most common (340, 44.6%) followed by rmtC (146, 19.2%), rmtF (137, 18.0%), rmtB (87, 11.4%) and various two gene combinations (52, 6.8%). Most (93.4%; 712/762) 16S RMTase producers also carried acquired carbapenemase genes, with blaNDM the most common (592/712; 83.1%). Additionally, high-risk bacterial clones associated with blaNDM were identified in the subset of isolates with WGS data. These included E. coli sequence types (STs) 405 [21.8%, 19/87], 167 [20.7%, 18/87] 410 [12.6%, 11/87] and K. pneumoniae STs 14 [35.6%, 112/315], 231 [15.6%, 49/315] and 147 [10.5%, 33/315]. These accounted for 4.2% [15/358], 5.0% [18/358], 3.1% [11/358], 28.2% [101/358], 3.1% [11/358] and 7.0% [25/358] blaNDM-producing isolates, respectively. This study shows that 16S RMTases occur in the UK & Ireland and carbapenemases are particularly prevalent in 16S RMTase-producing Enterobacteriaceae. This association poses a risk to the treatment of multidrug-resistant Gram-negative infections in the clinical setting.

1. Introduction

16S rRNA methyltransferases (16S RMTases) are emerging aminoglycoside resistance mechanisms, first discovered in various Enterobacteriaceae and Pseudomonas aeruginosa in the late 1990s, but which have since been identified globally in Enterobacteriaceae, Acinetobacter baumannii and P. aeruginosa. Ten 16S RMTase-encoding genes are known
These enzymes confer high-level resistance to all clinically-relevant aminoglycosides (MIC >256 mg/L) [2] and the genes encoding them are typically located on plasmids along with those for extended-spectrum beta lactamases (ESBLs), carbapenemases and fluoroquinolone resistance determinants. This potentially renders ineffective multiple classes of antimicrobials used to treat multidrug-resistant Gram-negative infections [1].

Rates of 16S RMTases around the world appear to differ between the East and the West, with rates of 0.12% (19/15,386) in Belgium [3] and 3.9% (38/985) in China [4] in Enterobacteriaceae. However, the occurrence of 16S RMTases in the UK & Ireland is currently unknown. We sought the presence of 16S RMTase genes in a collection of Enterobacteriaceae isolates displaying high-level pan-aminoglycoside resistance that had been submitted to Public Health England’s Antimicrobial Resistance and Healthcare Associated Infections (AMRHAI) Reference Unit for the investigation of unusual antibiotic resistance (primarily carbapenem resistance).

2. Materials and Methods

2.1 Bacterial Isolates

A panel of 806 Enterobacteriaceae referred between June 2004 and December 2015 was recovered from the AMRHAI Reference Unit’s culture collection. Isolates had been submitted for reference service investigation of unusual (primarily carbapenem) resistance. Approval for this study was sought from PHE’s R&D Office, who deemed the project enhanced surveillance, therefore ethical review was not required. All demonstrated high-level pan-aminoglycoside resistance (amikacin, gentamicin and tobramycin MICs ≥64, ≥32 and ≥32 mg/L, respectively) as determined by agar dilution and 729 (90.4%) had been confirmed to be carbapenemase-producing Enterobacteriaceae by in-house PCR.

2.2 DNA extraction and multiplex PCR assay
Crude DNA extracts were subjected to two multiplex PCRs where Multiplex 1, designed by Arakawa et al. [5], detected *armA*, *rmtA*-*rmtD* and Multiplex 2, designed in this study, detected *rmtE*-*rmtH* and *npmA*, including subvariants *rmtD2*, *rmtE2* and *rmtF2* (see Table 1 for primer sequences). The cycling conditions were based on those proposed by Arakawa et al. [5] and were: initial denaturation at 96 °C for 5 min; followed by 30 cycles of 96 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min; followed by a final elongation step at 72 °C for 5 min.

Following PCR, the DNA fragments were analysed using gel electrophoresis on 2% agarose gels.

### 2.3 Bacterial typing

*Escherichia coli* and *Klebsiella pneumoniae* isolates sent to the AMRHAI Reference Unit for bacterial typing were tested by pulsed-field gel-electrophoresis (PFGE) [6] or variable-number tandem-repeat (VNTR) analysis [7], respectively.

### 2.4. Whole-genome sequencing (WGS)

WGS data were available for 449 isolates, and had been derived from a Hiseq sequencing system (Illumina, San Diego, CA, USA) and analysed using AMRHAI’s in-house ‘GeneFinder’ bioinformatics pipeline to infer multilocus sequence typing (MLST) profiles and determine total antibiotic resistance gene contents [8].

### 3. Results

Seven hundred and sixty-two (94.5%) of 806 isolates displaying high-level pan-aminoglycoside resistance were positive for one or more 16S RMTase genes (Table 2). The 16S RMTase genes identified included *armA* (340/762, 44.6%), *rmtC* (146, 19.2%), *rmtF* (137, 18.0%), *rmtB* (87, 11.4%), *armA* + *rmtC* (23, 3.0%), *armA* + *rmtF* (17, 2.2%), *rmtB* + *rmtF* (5, 0.7%), *rmtC* + *rmtF* (3, 0.4%), *armA* + *rmtB* (2, 0.3%) and *rmtB* + *rmtC* (2, 0.3%). No *rmtA*, *rmtE*, *rmtG*, *rmtH* or *npmA* genes were detected in this study.
The 16S RMTase genes were found in the following species: Klebsiella pneumoniae (n=502), Escherichia coli (n=152), Enterobacter cloacae complex (n=33), Klebsiella spp. (n=25), Citrobacter freundii (n=11), Providencia spp. (n=14), Enterobacter spp. (n=10), Proteus mirabilis (n=6), Morganella morganii (n=2), Serratia marcescens (n=2), Citrobacter spp. (n=4) and Escherichia sp. (n=1).

Collectively, K. pneumoniae was the most common host species for rmtF, armA and rmtC genes, accounting for 95.7% (155/162), 74.3% (284/382) and 51.7% (90/174) of rmtF, armA and rmtC isolates, respectively. Conversely, E. coli was the most common host species for rmtB, accounting for 82.3% (79/96) of the rmtB-positive isolates.

16S RMTase-producing isolates were submitted by 109 hospital laboratories across 13 regions in the UK & Ireland and were obtained from 627 patients and seven environmental swabs. Six hundred and six patients were from England where the national distribution of the patients was as follows: London (n=328), West Midlands (n=79), North West (n=48), Yorkshire and the Humber (n=40), East Midlands (n=31), East (n=26), South East (n=25), South West (n=17), and North East (n=12). The 21 remaining patients were from Scotland (n=11), Northern Ireland (n=3), Isle of Man (n=2) and Republic of Ireland (n=5). This includes one patient that was treated in both London and the South East as well as another patient treated in both the East of England and East Midlands. Five of the environmental swabs were from London, one was from the North West and one was from the West Midlands.

Five hundred and forty-one (70.0%) isolates were from patients in the hospital setting, 53 (7.0%) were from GP clinics and 168 (22.0%) came from an unknown setting. The most common specimen type was ‘screening swab’ (32.8%, 250/762), followed by urine (31.1%, 237), tissue and fluid (16.8%, 128), blood and line tip (8.1%, 62), respiratory (6.4%, 49), faecal (1.6%, 12), environmental (0.9%, 7), and 17 (2.2%) were from an unknown source.

Information on travel history was unavailable for 475/627 (75.8%) patients. However, 98 (15.6%) patients had reported recent travel abroad, with travel to India (n=61), Pakistan...
(n=13), Egypt (n=4), Bangladesh (n=3), Greece (n=2), Middle East (n=2), and single patients had travelled to the Asian continent, Democratic Republic of the Congo, Equatorial Guinea, India + Pakistan, Kuwait, Nigeria, Oman, Saudi Arabia, Singapore, Sri Lanka, Syrian Arab Republic, Thailand and Vietnam.

Most (93.4%; 712/762) of the 16S RMTase-positive isolates produced an acquired carbapenemase (Table 3), with clear associations found with NDM (83.1%, 592/712) and OXA-48-like (23.7%, 169/712) carbapenemase families, either alone or in combination with other carbapenemases. *bla*<sub>KPC</sub>, *bla*<sub>VIM</sub> and *bla*<sub>GES-5</sub> were also identified amongst 16S RMTase-positive isolates, but in very low numbers (Table 3).

MLST data were derived from WGS data of 87 *E. coli* and 315 *K. pneumoniae* isolates, where 25 *E. coli* and 30 *K. pneumoniae* STs were identified. The top three *E. coli* STs (accounting for 55.2%, 48/87 isolates) were ST405 (n=19), ST167 (n=18) and ST410 (n=11), which were found in 14 hospitals spanning five regions, 14 hospitals spanning seven regions and eight hospitals spanning four regions, respectively. PFGE was performed on 52.6% (10/19), 55.6% (10/18) and 72.7% (8/11) isolates, respectively, where all isolates had different PFGE profiles indicating that these STs encompass different strains. The top three *K. pneumoniae* STs (accounting for 61.6%, 194/315 isolates) were ST14 (n=112), ST231 (n=49) and ST147 (n=33), which were found in 25 hospitals spanning seven regions, 24 hospitals spanning seven regions and 21 hospitals spanning eight regions, respectively. VNTR was performed on 73.2% (82/112), 69.4% (34/49) and 84.8% (28/33) isolates, respectively. Different strains were identified in STs 14 and 167 with isolates belonging to three and two different VNTR profiles, respectively (data not shown). However, all ST231 isolates shared an identical VNTR profile, which was found in 20 hospitals spanning seven regions.

Analysis of co-resident antibiotic resistance genes in the 449 16S RMTase-positive isolates with WGS data confirmed that *bla*<sub>NDM</sub> (71.7%, 358/499) was the most common
carbapenemase (Table 3) with bla<sub>NDM-1</sub> (82.1%, 294/358) the most common variant identified. This carbapenemase allele was found associated with all 16S RMTases (armA [85.7%, 180/210], rmtC [91.5%, 65/71], rmtF [40.2%, 35/87], rmtB [10.9%, 7/64], armA + rmtC [100%, 2/2], armA + rmtF [25.0%, 2/8], armA + rmtB [100%, 1/1], rmtB + rmtC [100%, 1/1], rmtC + rmtF [100%, 1/1]) but was not found in isolates with rmtB + rmtF (n=4). bla<sub>NDM-5</sub> had a clear association with rmtB-positive isolates as it was found in 62.5% (40/64) of rmtB-positive isolates compared to bla<sub>NDM-4</sub> (14.1%, 9/64) and bla<sub>NDM-1</sub>, (10.9%, 7/64). rmtF had a slightly higher association with bla<sub>OXA-232</sub> (51.7%, 45/87) compared with bla<sub>NDM-1</sub> (40.2%, 35/87), which may be due to 31/45 (68.9%) bla<sub>OXA-232</sub> genes being found in K. pneumoniae isolates belonging to ST231. Other antibiotic genes that were found to be commonly associated with 16S RMTase producers were the quinolone resistance gene aac(6')-Ib-cr and the extended-spectrum beta-lactamase bla<subCTX-M-15</sub>, which were found in 341/499 (75.9%) and 302/499 (67.3%) isolates, respectively. Both were associated with all 16S RMTase genes but were found to be most common in rmtF-positive isolates as they were identified in 86/87 (98.9%) and 79/87 (90.8%) isolates, respectively.

4. Discussion

This study has shown that the vast majority (94.5%; 762/806) of Enterobacteriaceae isolates displaying high-level pan-aminoglycoside resistance (amikacin, gentamicin and tobramycin MICs ≥64, ≥32 and ≥32 mg/L, respectively) from AMRHAI's isolate collection were positive for 16S RMTase genes. Other studies investigating the presence of 16S RMTase genes in pan-aminoglycoside-resistant Enterobacteriaceae have also found high rates of 100% (20/20), 93.6% (204/218) and 86.4% (19/22) in Poland [9], South Korea [10] and Belgium [11], respectively. Absence of a 16S RMTase gene in isolates expressing pan-aminoglycoside resistance may be due to combinations of other aminoglycoside resistance mechanisms such as aminoglycoside-modifying enzymes and upregulation of efflux pumps. Alternatively, it could be due to novel 16S RMTase genes or variants of existing genes that
could not be detected with existing PCR assays. These isolates will be investigated further using WGS to attempt to identify underlying resistance mechanisms.

/armA was the most frequently identified 16S RMTase gene in this study and was mainly found in K. pneumoniae. It is the one of the most widely distributed 16S RMTase genes [1]. In contrast to reports from other countries, /rmtC and /rmtF were more frequent in our isolate collection than /rmtB, which is one of the most predominant global 16S RMTase genes alongside /armA [1]. This may be due to their higher association with /bla\textsubscript{NDM-1} when compared to /rmtB or perhaps plasmids or bacterial clones with /rmtC and /rmtF are more prominent in the UK & Ireland than those with /rmtB.

Most (93.4%; 712/762) of the 16S RMTase-producing bacteria in our collection were carbapenemase producers, indicating a strong association between these resistance mechanisms and providing a possible explanation for the emergence of 16S RMTases in the UK & Ireland. Carbapenemases belonging to the NDM family were the most common, which may be due to co-location of 16S RMTase genes and /bla\textsubscript{NDM} on the same plasmids [12, 13]. Other antibiotic resistance genes were also highly associated with 16S RMTases, e.g. /aac(6')\textsubscript{Ib}-cr, /fosA3 and /bla\textsubscript{CTX-M-15} were particularly associated with /rmtF, as has been reported in Egypt and the USA [14, 15]. This suggests that these genes may be linked on the same plasmids, which has implications for the treatment of bacterial infections as co-selection of genes causing resistance to aminoglycosides, beta-lactams and fluoroquinolones removes the use of the three key antibiotics used to treat Gram-negative infections.

High-risk clones may also play a role in 16S RMTase emergence in the UK & Ireland as the most common STs identified in this study (E. coli ST405, ST167 and ST410 as well as K. pneumoniae ST14, ST231 and ST147) have all been reported to carry /bla\textsubscript{NDM} [16, 17, 18, 19]. However, the reason for the predominance of these STs is unknown as there is insufficient evidence to determine if this is due to carriage of particular virulence genes or
from the presence of 16S RMTase genes and/or carbapenemase genes. Additionally, the lack of clonality in Enterobacteriaceae isolates that had undergone PFGE and VNTR, apart from *K. pneumoniae* ST231 isolates, suggests that different bacterial strains across the UK harbour 16S RMTase genes.

India and Pakistan are known reservoirs of *bla*<sub>NDM</sub> carbapenemases [20] and it appears that they may also act as reservoirs of 16S RMTase genes as 62.2% (61/98) and 13.3% (13/98) of patients with a known travel history had visited these countries, respectively. This is also supported by London and the West Midlands having the highest prevalence of 16S RMTase genes as these regions are known to have multicultural populations with links to the Indian Subcontinent.

PHE’s AMRHAI Reference Unit is the UK & Ireland’s national reference laboratory for the investigation of unusual antibiotic resistance in healthcare-associated infections. It receives multidrug-resistant bacteria from across the country (particularly those demonstrating carbapenem resistance) and so the occurrence of antibiotic resistance genes in AMRHAI’s isolate collection will be much higher than they actually are in the clinical setting. As there are no denominator data, the national prevalence of 16S RMTases cannot be calculated. Further studies need to be done in order to estimate the true prevalence of these resistance genes in the UK & Ireland using unbiased isolate collections.

In conclusion, this is the first report to describe the occurrence of 16S RMTases in pan-aminoglycoside resistant Enterobacteriaceae in the UK & Ireland, albeit in otherwise highly multidrug-resistant isolates. There was a clear association with acquired carbapenemase genes, which is very concerning as these 16S RMTase-positive isolates display resistance to many antibiotic classes used to treat Gram-negative infections, including the new aminoglycoside plazomicin, which is in phase III clinical trials. This highlights that novel agents currently in development with Gram-negative activity will not offer solutions for all multidrug-resistant organisms encountered in the clinical setting. Diagnostic laboratories
should be aware of 16S RMTases and consider their presence in isolates displaying high-level pan-aminoglycoside resistance.

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