were similar to those described from other CC398 strains.\(^1\)\(^,\)\(^2\) In addition, both PVL-positive spa t034 isolates contained genes associated with hibl-converting phages, namely sak, chp and scn.

Although we did not have detailed epidemiological information on all nine patients, it is notable that three of the patients in this study had recent exposure to pigs. In other settings, isolation of CC398 MRSA has been strongly associated with exposure to livestock, including pigs, poultry and calves.\(^2\) This association with livestock may be of particular relevance in our geographic setting, given the large rural and agricultural sector in New Zealand.

To our knowledge, these cases represent the first known human isolations of CC398 MRSA in New Zealand. We found two clusters of CC398 MRSA, each with distinct characteristics. One cluster was due to a PVL-positive spa t034 ST1232 MRSA strain, which was associated with travel to South-East Asia, and the other cluster was due to a PVL-negative spa t011 or spa t571 ST398 strain, similar to the European LA-MRSA lineage. Ongoing clinical and molecular surveillance is essential to monitor the spread of these MRSA strains in our country.

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We thank Mona Schousboe and Antje van der Linden for referral of the isolates and initial patient follow-up.

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**Transparency declarations**

None to declare.

**References**

with ceftazidime and clindamycin. The MRY13-0052 strain was identified as *P. mendocina* by the VITEK2 system (bioMérieux; 96% probability), but subsequently as *P. alcaligenes* based on 16S rRNA gene sequence analysis. MRY13-0052 was resistant to penicillins, cephalosporins, aztreonam and fosfomycin, but susceptible to imipenem, meropenem, amikacin, fluoroquinolones, minocycline and trimethoprim/sulfamethoxazole, according to MICs determined using the VITEK2 system and the Etest (bioMérieux), applying the recommended breakpoints described by CLSI (2013). The production of MBL was screened for using a disc containing sodium mercaptoacetic acid (SMA) (Eiken). Apparent expansion of the growth inhibitory zone around the ceftazidime and meropenem discs was observed around the SMA disc following overnight incubation at 37°C, strongly suggesting that MRY13-0052 produces MBL. PCR tests to detect the *blaNDM*, *blaIMP*, *blaVIM* and *blaTMB* genes in MRY13-0052 were all negative; therefore, we analysed the whole-genome shotgun (WGS) sequence of MRY13-0052, obtained using the GS Junior system (Roche), to identify the responsible MBL gene (DDBJ/EMBL/GenBank accession number of the WGS project: BATO01000000). BLAST-based similarity searches revealed that MRY13-0052 carries three class C β-lactamase genes and a novel subclass B3 MBL gene [which we named *blaPAM-1* (DDBJ/EMBL/GenBank accession number of the gene: AB858498)] that might confer resistance to β-lactams.

The PCR product of the *blaPAM-1* gene was ligated into pUCP19 (ATCC), a *Pseudomonas–Escherichia* shuttle vector, resulting in the PAM-1 expression vector pUCP19–blaPAM-1. *P. aeruginosa* strain PAO1 and *Escherichia coli* strain MC1061 were transformed with this vector, and transformants were selected on agar plates containing 20 mg/L piperacillin. Expression of the *blaPAM-1* gene was driven by the tac promoter regardless of IPTG induction and confirmed by SMA disc-mediated expansion of the growth inhibitory zone around ceftazidime and meropenem discs. As shown in Table 1, *blaPAM-1*-producing *P. aeruginosa* bacteria were more resistant to ceftazidime, imipenem, meropenem and doripenem than control bacteria carrying the empty vector (MICs increased 32-fold, 2-fold, 6-fold and 21-fold, respectively), but were still as susceptible as control bacteria to aztreonam. Although *blaPAM-1*-producing *E. coli* bacteria were slightly more resistant to ceftazidime and meropenem than control bacteria (MICs increased 4-fold and 1.4-fold), there was no apparent change in the susceptibility to aztreonam and other carbapenems. The differences in the contribution of the PAM-1 enzyme to cephalosporin and carbapenem resistance among *P. aeruginosa* and *E. coli* could reflect differences in expression levels, outer-membrane permeability and/or efflux systems in these hosts. The *blaPAM-1* gene in *P. alcaligenes* strain MRY13-0052 is encoded in contig 73, which is part of the chromosome, and there is no transposable element, such as a transposon or integrase, around the gene, suggesting that *blaPAM-1* is an intrinsic species-specific MBL gene of *P. alcaligenes*. *Pseudomonas otitidis*, a *Pseudomonas* species that is associated with otic infections in humans, also produces a resident MBL named POM-1 ([*P. otitidis* MBL-1]), which is active against carbapenems. The PAM-1 protein exhibits close similarity to POM-1 (72.4% amino acid identity), suggesting that these enzymes have a common ancestor (Figure 1). PAM-1 and POM-1 are homologous with the L1 MBL

<table>
<thead>
<tr>
<th>Strain</th>
<th>Antimicrobial agent MIC, mg/L</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>aztreonam</td>
</tr>
<tr>
<td><em>P. alcaligenes</em> MRY13-0052</td>
<td>32</td>
</tr>
<tr>
<td><em>P. aeruginosa</em> PAO1/pUCP19</td>
<td>2</td>
</tr>
<tr>
<td><em>P. aeruginosa</em> PAO1/pUCP19–blaPAM-1</td>
<td>2</td>
</tr>
<tr>
<td><em>E. coli</em> MC1061/pUCP19</td>
<td>0.125</td>
</tr>
<tr>
<td><em>E. coli</em> MC1061/pUCP19–blaPAM-1</td>
<td>0.125</td>
</tr>
</tbody>
</table>

Figure 1. A rooted phylogenetic tree of subclass B3 MBL proteins, generated by ClustalW.
of Stenotrophomonas maltophilia (63.3% and 62.1% identity, respectively, relative to the S. maltophilia strain IAM 1566 protein) (Figure 1). S. maltophilia is a Gram-negative bacterium found in a variety of environments, including soil, water and plants, and is therefore a potential reservoir of the MBL gene. Similar to variety of environments, including soil, water and plants, and is therefore a potential reservoir of the MBL gene. Similar to


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Colistin susceptibility testing: time for a review

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Keywords: polysorbate 80, microtitre plates, MICs

Sir,

Colistin has re-emerged as an important antimicrobial in recent times owing to limited therapeutic options against carbapenem-resistant Gram-negative bacteria. Current guidelines (BSAC, CLSI and EUCAST) recommend routine colistin susceptibility testing by estimation of MIC because the disc diffusion test does not reliably detect low-level resistance. Broth microdilution (BMD) is widely used as a method of MIC estimation in Europe and the USA. Colistin exhibits a varying degree of adherence to organic and inorganic materials due to its polycationic nature, resulting in loss during experimental conditions. Also, polysorbate 80 (P-80), a surfactant widely used as a dispersing agent in BMD panels, may influence the free drug concentration of colistin and hence MIC results.

We evaluated the impact of the use of different BMD panels and the presence of P-80 on colistin MIC estimation. A total of 146 clinical isolates collected from a variety of sources and stored at –70°C were evaluated in this study. The isolates included 56 Pseudomonas aeruginosa, 29 Acinetobacter spp. and 61 Enterobacteriaceae. The MIC testing was carried out on two different types of polystyrene microtitre trays (MTTs), namely non-coated V-bottom MTTs (NMTTs; costar 3896; Corning, NY, USA) and tissue-culture-coated round-bottom MTTs (TCMTTs; costar 3799; Corning). The MICs of colistin for the isolates were determined using the CLSI broth dilution method using colistin sulphate. MIC determination was carried out by using an initial bacterial inoculum of 5 × 10⁶ cfu/mL in Mueller–Hinton broth with or without P-80 (final P-80 concentration of 0.002%) on both types of MTT. The experiments were done in triplicate, and quality control was assured by concurrent testing of P. aeruginosa ATCC 27853 as a control, with all results within the range published by the CLSI.

MICs for the isolates in both types of MTT with or without P-80 are shown in Table 1. The NMTT MICs (mean 0.54 ± 0.58) were

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Transparency declarations

None to declare.

References


6. Shibata N, Doi Y, Yamane K et al. PCR typing of genetic determinants for carbapenemase production by PAM-1-positive MRY13-0052 strain was not categorized as carbapenem resistant. However, the combination of PAM-1-mediated β-lactam hydrolysis with genetic mutations that decrease outer-membrane permeability could confer high-level carbapenem resistance, leading to major concern for the treatment of P. alcaligenes infection.

of Stenotrophomonas maltophilia (63.3% and 62.1% identity, respectively, relative to the S. maltophilia strain IAM 1566 protein) (Figure 1). S. maltophilia is a Gram-negative bacterium found in a variety of environments, including soil, water and plants, and is therefore a potential reservoir of the MBL gene. Similar to POM-1 and L1 MBLs, the ability of the PAM-1 enzyme to hydrolyse β-lactam-lactam hydrolysis with genetic mutations that decrease outer-membrane permeability could confer high-level carbapenem resistance, leading to major concern for the treatment of P. alcaligenes infection.