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Diversity of *bla*$_{POM}$ in carbapenem-resistant opportunistic pathogenic *Pseudomonas otitidis* in municipal wastewater

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ABSTRACT

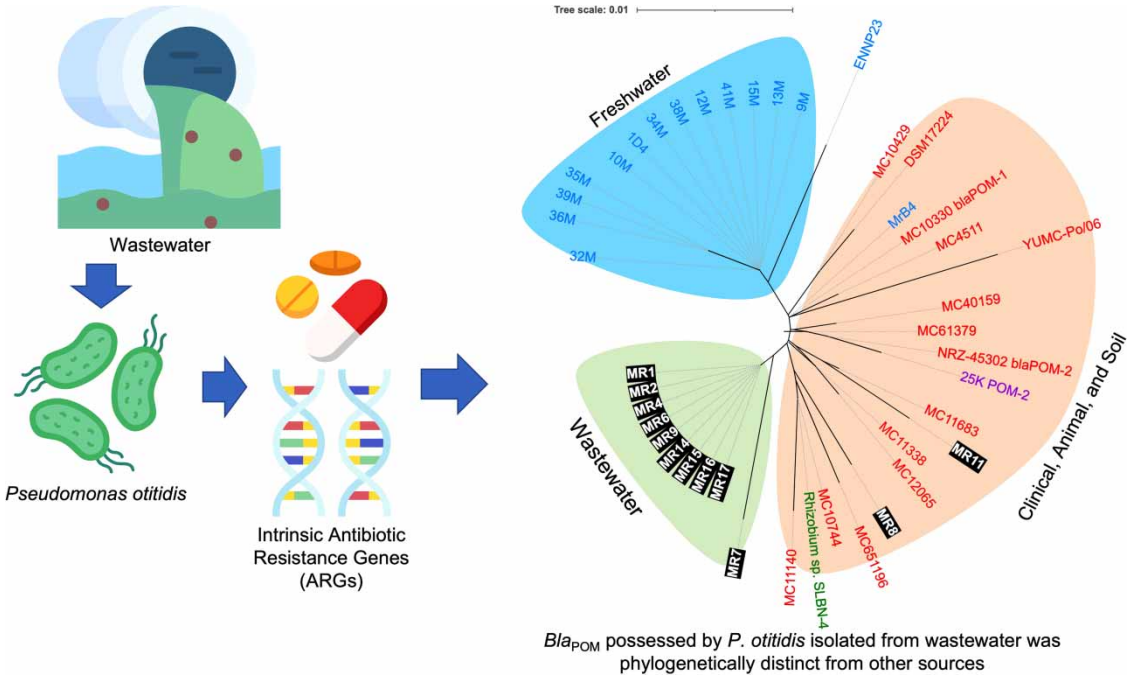
Metallo-β-lactamases (MBLs) encoding carbapenem resistance in wastewater are a well-known serious threat to human health. Twelve *Pseudomonas otitidis* isolates obtained from a municipal wastewater treatment plant (WWTP) in Hawaii were found to possess a subclass B3 MBL – POM (*P. otitidis* MBL), with a minimum inhibition concentration (MIC) range of 8–16 mg/L. The unrooted neighbor-joining phylogenetic tree showed that these *bla*$_{POM}$ genes isolated in wastewater samples ($n = 12$) were distinctly different from other reference genes isolated from clinical, freshwater, animal, and soil samples except for isolates MR7, MR8, and MR11. MR7, MR8, and MR11 were found to have 4, 3, and 3 amino acid substitutions when compared to the type strain MC10330^T and were closely clustered to the clinical reference genes. The meropenem hydrolysis experiment showed that isolates with multiple amino acid substitutions completely hydrolyzed 64 mg/L of meropenem in 7 h. The emergence of the opportunistic pathogen *P. otitidis* chromosomally encoding *bla*$_{POM}$ in the treated municipal wastewater is an alarming call for the spread of this MBL in the environment. Further studies are required to understand the mechanism and regulation of this carbapenem-resistant β-lactamase in order to fill in the knowledge gap.

Key words: *bla*$_{POM}$, carbapenem resistant, intrinsic resistance, metallo-β-lactamase, *Pseudomonas otitidis*, wastewater

HIGHLIGHTS

- Wastewater surveillance detected numerous carbapenem-resistant *Pseudomonas otitidis* isolates that showed minimum inhibition concentrations up to 16 mg/L of meropenem.
- The *bla*$_{POM}$ genes in the wastewater *P. otitidis* isolates possess mutations not observed previously in clinical and environmental sources.
- Some wastewater *P. otitidis* isolates with multiple amino acid substitutions in the *bla*$_{POM}$ gene catalyzed fast hydrolysis of carbapenem.

GRAPHICAL ABSTRACT



Bla_{POM} possessed by *P. otitidis* isolated from wastewater was phylogenetically distinct from other sources

INTRODUCTION

Since the first isolation of carbapenemase and metallo-β-lactamase (MBL) from *Pseudomonas aeruginosa* more than 20 years ago (Watanabe *et al.* 1991), the detection of carbapenem-resistance bacteria has been increasing at an alarming rate globally (Cornaglia *et al.* 2011). MBLs are recognized to be clinically important as resistance determinants because of their potent carbapenemase activities, including the broad substrate specificity covering most β-lactam families (Cornaglia *et al.* 2011). MBLs belong to class B β-lactamases with three major structural subclasses (Bush & Jacoby 2010), encoded either by genes in the chromosomal framework (resident MBL) or by genes acquired through horizontal gene transfer (acquired MBL) (Cornaglia *et al.* 2011). Intrinsic resistance to carbapenems is less common (Meletis 2016), which has been detected in species of clinical significance including *Bacillus* spp., *Stenotrophomonas maltophilia*, *Aeromonas* spp., *Bacteroides fragilis*, various flavobacteria, and *Pseudomonas otitidis* (Walsh *et al.* 2005; Thaller *et al.* 2011).

P. otitidis was first found to intrinsically possess an MBL names POM (*P. otitidis* MBL) isolated from humans infected with acute otitis externa (Clark *et al.* 2006). POM-1 is a subclass B3 MBL, which demonstrates an overall broad substrate specificity, especially for penicillins and carbapenems (Borgianni *et al.* 2015). While POM is highly conserved in *P. otitidis* (Thaller *et al.* 2011), *P. otitidis* is genetically closely related to *P. aeruginosa* (Clark *et al.* 2006) and phenotypically unable to differentiate the two species (Kim *et al.* 2016). *P. otitidis* has been isolated not only from clinical settings (Clark *et al.* 2006; Lee *et al.* 2012; Kim *et al.* 2016; Caixinha *et al.* 2021) but also from food (Wong *et al.* 2015), animal carcass (Vieira *et al.* 2020), and freshwater (Rodríguez-Verdugo *et al.* 2012; Tacao *et al.* 2015; Miyazaki *et al.* 2020).

Wastewater treatment plants (WWTPs) are considered a major source of bacterial antibiotic resistance to the environment (Michael *et al.* 2013). This is not surprising as sewage contains the microbiomes of human populations (Newton *et al.* 2015), and the discharge of treated wastewater degrades water quality and cannot be used for potable water and industrial applications directly (Panagopoulos 2022). Most studies (Picão *et al.* 2013; Galler *et al.* 2014; Hrenovic *et al.* 2016; Naquin *et al.* 2017; Basode *et al.* 2018; Cooper *et al.* 2021) on carbapenem-resistant bacteria (CRB) in the WWTP were focusing on carbapenem-resistant Enterobacteriaceae (CRE) as they are considered urgent threats to public health (CDC 2019). On the other hand, carbapenem resistance in non-Enterobacteriaceae bacteria in wastewater has not been extensively studied, even though some of these non-Enterobacteriaceae can also be human pathogens (Thaller *et al.* 2011; Woodford *et al.* 2014). While the study of antibiotic-resistant *P. otitidis* in wastewater is very limited, other researchers studied the use of

P. otitidis isolated from wastewater for bioelectricity generation (Thulasinathan *et al.* 2019), triphenylmethane dyes decolorization (Jing *et al.* 2009), and biodegradation of sodium dodecyl sulfate (SDS) (Ibrahim & Abd Elsalam 2018).

In this study, we aim to identify opportunistic pathogenic carbapenem-resistant non-Enterobacteriaceae possessing MBL in the wastewater and characterize the MBLs phenotypically (minimum inhibition concentration (MIC) and sensitivity of hydrolyzation of meropenem) and genetically (16S rRNA, pulse-field gel electrophoresis, PFGE; POM-1 amino acid alignments).

MATERIALS AND METHODS

Enumeration of bacteria and CRB in wastewater

Raw municipal wastewater samples (500 mL, $n = 3$) and activated sludge samples (500 g, $n = 3$) were collected from the East Honolulu WWTP (Honolulu, Hawaii) in April 2016. The abundance of total heterotrophic bacterial biomass, *Escherichia coli*, and CRB were enumerated by the direct plating method on Luria-Bertani (LB) and mTEC agar with/without meropenem (2 mg/L; TCI America, Portland, OR, USA). Well-mixed wastewater samples were serially diluted (10^0 to 10^{-7}) in sterilized distilled water, and 100 μL of the serial dilutions were plated in triplicate on LB, LB + meropenem, mTEC, and mTEC + meropenem agars. LB and LB + meropenem agar plates were incubated at 30 °C for 24 h, while mTEC and mTEC + meropenem agar plates were incubated at 44.5 °C for 24 h. Colony-forming units (CFU/mL and CFU/g) were counted.

Isolation of CRB from wastewater

Seventeen individual bacterial colonies that grew on the mTEC agar with 2 mg/L of meropenem were randomly selected and further purified by streaking on mTEC + meropenem (2 mg/L) agar and incubated at 37 °C for 24 h. These colonies did not show the characteristic blue color of *E. coli* on the mTEC agar. The meropenem-resistant bacteria isolates were collected and stored in Tryptic Soy Broth (TSB) (Fluka Biochemika, Buchs, Switzerland) with 15% (v/v) glycerol at −80 °C for further analysis.

Minimum inhibitory concentration

MICs of meropenem of the meropenem-resistant bacteria isolates were determined by using the microbroth dilution assay described by the CLSI (CLSI 2015). Single colonies were inoculated in 10 mL of LB broth + meropenem (2 mg/L) and incubated overnight at 37 °C at 180 rpm. Then, 100 μL of the bacteria-inoculated broth was transferred to a new tube containing 10 mL of LB broth + meropenem (2 mg/L) and incubated at 37 °C until the 0.5 McFarland standards were achieved. Approximately 200 μL of cation-adjusted Mueller Hinton broth (CAMHB) (BD; MD, USA) containing 2–48 mg/L of meropenem were distributed in a 48-well cell culture plates and inoculated with the 0.5 McFarland standards grown isolates. The 48-well plates were incubated at 37 °C for 24 h.

PFGE typing

The whole-cell PFGE method on the 17 meropenem-resistant bacterial isolates was conducted using the standard operating procedure from PulseNet PFGE (CDC 2013). Briefly, the CRB isolates were inoculated in LB broth + meropenem (4 mg/L) at 37 °C overnight, and the cells were collected and resuspended in cell suspension buffer (100 mM Tris–HCl/100 mM EDTA, pH 8.0) to achieve a concentration of OD_{610} 0.8–1.0. Two hundred μL of these cell suspensions was embedded in 0.5% Seakem Gold agarose (Lonza, Allendale, NJ, USA). These PFGE plugs were then incubated in cell lysis buffer (50 mM Tris–HCl/50 mM EDTA, pH 8.0, and 1% Sarcosyl) with proteinase K (1 mg/mL) at 55 °C for at least 1.5 h. Restriction digestion of genomic DNA within the agarose plugs was conducted using *Xba* I (50 U/sample). Electrophoresis was conducted on a CHEF Mapper (Bio-Rad, Richmond, CA, USA) for 18 h at a 10–700 kb molecular weight auto algorithm. Analysis was performed using BioNumerics version 5.10 (Applied Maths, Austin, TX, USA).

16S rRNA and *bla*$_{POM}$ gene identification

Twelve out of the 17 meropenem-resistant bacterial isolates (MR1, MR2, MR4, MR6, MR7, MR8, MR9, MR11, MR14, MR15, MR16, and MR17) that exhibited distinct PFGE patterns were selected for subsequent 16S rRNA gene and POM gene amplification and sequencing. The isolates exhibited the same PFGE patterns and were considered clonal. Briefly, DNA extractions were performed by using GenElute™ Bacterial Genomic DNA Kits (MilliporeSigma, St. Louis, MO, USA) according to the manufacturer's protocol. In short, the CRB strains were inoculated in LB broth + meropenem (4 mg/L) and incubated at 37 °C overnight at 180 rpm. The solutions were centrifuged at 3,000 rpm and the cells were collected and proceeded to DNA extractions. The DNAs extracted were kept at −20 °C for further PCR analysis. 16S rRNA was amplified using the

universal oligonucleotide primers 27F (5′-AGA GTT TGA TCC TGG CTC AG-3′) and 518R (5′-ATT ACC GCG GCT GCT GG-3′), and POM-1 genes were amplified using primers designed in this study: lactaF (5′-GCA TTG ACC TGC GCG ACC AGG CAG T-3′) and lactaR (5′- CTT GTC GGC GTA GGC CTT GCA GCT-3′) in a GeneAmp® PCR System 9700 (Applied Biosystem, Beverly, MA, USA). The PCR mixture with a total reaction volume of 25 μL was comprised of 2.5 μL of 10× PCR buffer, 1.5 mM $MgCl_2$, 0.04 U/μL of Taq Polymerase, 0.1 mM dNTP, 0.4 mg/mL of BSA, 0.15 μM of each forward/reverse primer pairs, 1 μL of DNA template, and nuclease-free water. The thermocycling program included DNA polymerase activation and initial denaturing at 95 °C for 5 min, thermo cycles (16S rRNA = 45 cycles; POM-1 = 35 cycles) of denaturation at 95 °C for 30 s, annealing (16S rRNA = 56 °C for 30 s; POM-1 = 63 °C for 30 s), and extension (16S rRNA = 72 °C for 45 s; POM-1 = 72 °C for 50 s), following by a final extension at 72 °C for 8 min. The PCR amplicon was separated on 1.5% agarose gel through gel electrophoresis and then visualized by a UVP GelStudio (Analytik Jena, Upland, CA, USA). PCR amplicons were gel purified using the QIAquick® Gel Extraction Kit (Qiagen, Valencia, CA, USA) and proceeded with sequencing the reaction using the ABI 3730XL sequencer at the Advanced Studies in Genomics, Proteomics and Bioinformatics (ASGPB), University of Hawaii at Manoa. The sequence reads were quality trimmed and checked manually against the chromatograms by using the Sequence Scanner (version 2.0; Thermo Fisher, Waltham, MA, USA), and the species and antibiotic resistance gene identifications were confirmed by using BLASTn. The 16S rRNA sequences ($n = 12$) and bla_{POM} sequences ($n = 12$) were deposited in the GenBank database, accession numbers ON944110–ON944121 and ON959567–ON959578, respectively.

Phylogenetic tree and amino acid alignments

Partial and full amino acid sequences ($n = 31$) of bla_{POM} from the GenBank database were downloaded to compare with the bla_{POM} sequences detected from wastewater isolates in this study ($n = 12$). The list of reference genes was summarized in Supplementary Table S1. An unrooted neighbor-joining phylogenetic tree of bla_{POM} amino acid sequences was created using iTOL, Interactive Tree of Life (https://itol.embl.de/). The amino acid sequences of 12 bla_{POM} isolated from wastewater (this study) and 9 bla_{POM} reference genes were randomly selected and performed multiple sequence alignments using Clustal Omega by EMBL-EBI (https://www.ebi.ac.uk/Tools/msa/clustalo/).

Hydrolysis of meropenem

The sensitivity and speed of hydrolysis of meropenem were measured by using a modified Kirby-Bauer disk diffusion susceptibility test protocol (Hudzicki 2009). Details of the production of the standard curve are summarized in the Supplementary Material. Five *P. otitidis* isolates (MR1, MR8, MR11, MR16, and MR17) were inoculated in 5 mL of LB broth + meropenem (2 mg/L) and incubated at 37 °C in a shaking incubator overnight. A total of 2 mL of the overnight cultures were transferred to a 10 mL fresh LB broth + meropenem (2 mg/L) and incubated at 37 °C in a shaking incubator overnight. The solutions were centrifuged at 3,000 rpm to collect the bacteria pellets. Approximately 5 mL of the OD_{600} calibrated bacteria suspension (0.2) were transferred to a new tube and added meropenem solution to a final concentration of 64 mg/L. The tubes were incubated at 22 °C in a shaking incubator overnight at 350 rpm. About 100 μL of the samples were extracted every 1 h (up to 12 h), added to a microcentrifuge tube, and centrifuged at 13,000 rpm for 3 min to pellet the bacteria particles. Approximately 5 μL of the supernatant were pipetted and dropped on new sterile antibiotic disks plated on the *E. coli* K12 agar plates and incubated at 37 °C overnight. Each antibiotic disk was duplicated and each *P. otitidis* isolate meropenem hydrolysis test was performed at triplication. A negative control was included in the test where the tubes were incubated with meropenem (64 mg/L) without bacteria. The inhibition zones were measured and the concentrations of meropenem at each hour were measured according to the standard curve.

RESULTS

Characterization of carbapenem-resistant *P. otitidis*

The enumeration of bacteria and CRB in wastewater and sludge samples is shown in Supplementary Table S2. The colony-forming unit (CFU) of general heterotrophic bacteria, *E. coli*, and non-*E. coli* other bacteria enumerated in wastewater and sludge samples ranged from $1.4 \times 10^5 \pm 3.2 \times 10^4$ to $1.9 \times 10^6 \pm 3.3 \times 10^5$ CFU/mL and $3.3 \times 10^4 \pm 1.1 \times 10^4$ to $1.4 \times 10^6 \pm 1.9 \times 10^5$ CFU/g, respectively. On the other hand, carbapenem-resistant heterotrophic bacteria detected in wastewater and sludge samples were $1.0 \times 10^4 \pm 3.8 \times 10^3$ CFU/mL and $3.7 \times 10^4 \pm 1.1 \times 10^4$ CFU/g, respectively. On mTEC agar, carbapenem-resistant other bacteria detected were less than 100 colonies in both wastewater and sludge samples. Since there were no

carbapenem-resistant *E. coli* colonies (Supplementary Table S2), 17 carbapenem-resistant colonies that did not show the characteristic *E. coli* blue color, but grew on the mTEC agar, were isolated. These bacterial isolates were subsequently identified as *P. otitidis* (MR1–MR11, MR13–MR18) by 16S rRNA gene sequencing (Table 1). PFGE fingerprinting patterns showed that among the 17 *P. otitidis*, five isolates were identical to one of the isolates, that is MR3 to MR1, MR5, MR10 to MR4, MR13 to MR11, MR18 to MR16 (Supplementary Figure S1). These identical isolates of *P. otitidis* were not included in the following bla_{POM} analysis. MICs of these 17 *P. otitidis* isolates showed that isolates MR6, MR7, and MR8 showed resistance to meropenem at 8 mg/L. The remaining *P. otitidis* isolates have MIC of 16 mg/L. PCR and sequencing of bla_{POM} showed that 12 *P. otitidis* (MR1, MR2, MR4, MR6, MR7, MR8, MR9, MR11, MR14, MR15, MR16, and MR17) were found to possess a subclass B3 MBL named POM (*P. otitidis* MBL).

Phylogenetic relationship of the wastewater bla_{POM}

In Figure 1, the unrooted neighbor-joining phylogenetic tree showed three main clusters separating the bla_{POM} amino acids from wastewater ($n = 12$), freshwater ($n = 15$), and clinical ($n = 12$). Two bla_{POM} amino acids from *P. otitidis*-type strain and one bla_{POM} amino acid each from soil and animal samples were also included in the phylogenetic analysis, and they were closely clustered in the clinical branch (Figure 1). The wastewater clusters comprised of nine POM amino acids (MR1, MR2, MR4, MR6, MR9, MR14, MR15, MR16, and MR17) were grouped closely together but distantly separated from the clinical and freshwater clusters. Interestingly, MR7 was closely related to the clinical cluster, while MR8 and MR11 were grouped in the clinical cluster, which contained a mixture of bla_{POM} isolated from clinical, type strains, animal, soil, and freshwater. These three isolates were found to have three or four amino acid substitutions (Figure 2).

POM amino acid alignments

All 12 bla_{POM} amino acids isolated from wastewater have one amino acid substitution at position 1, methionine (M) to valine (V), as compared to the bla_{POM} amino acid isolated from other sources (Figure 2). Interestingly, *P. otitidis* MR8 and MR11 have additional two amino acid substitutions, whereas *P. otitidis* MR7 has additional three amino acid substitutions. In *P. otitidis* MR8, valine (V) was substituted by methionine (M) at positions 18 and 154, respectively. Meanwhile, in *P. otitidis* MR11, arginine (R) was substituted by histidine (H) at position 44, and threonine (T) was substituted by asparagine (N) at

Table 1 | Characterization of carbapenem-resistant non-Enterobacteriaceae ($n = 17$) isolated from wastewater

Isolation	Species identified (16S rDNA)	*Xba*I-PFGE	MICs for meropenem (mg/L)	Plasmids	Carbapenem-resistant genes
MR1	*Pseudomonas otitidis*	–	16	97 kb	bla_{POM}
MR2	*Pseudomonas otitidis*	–	16	–	bla_{POM}
MR3	*Pseudomonas otitidis*	Same as MR1	16	–	NT
MR4	*Pseudomonas otitidis*	–	16	–	bla_{POM}
MR5	*Pseudomonas otitidis*	Same as MR4	16	–	NT
MR6	*Pseudomonas otitidis*	–	8	–	bla_{POM}
MR7	*Pseudomonas otitidis*	–	8	–	bla_{POM}
MR8	*Pseudomonas otitidis*	–	8	–	bla_{POM}
MR9	*Pseudomonas otitidis*	–	16	–	bla_{POM}
MR10	*Pseudomonas otitidis*	Same as MR4	16	–	NT
MR11	*Pseudomonas otitidis*	–	16	–	bla_{POM}
MR13	*Pseudomonas otitidis*	Same as MR11	16	–	NT
MR14	*Pseudomonas otitidis*	–	16	–	bla_{POM}
MR15	*Pseudomonas otitidis*	–	16	–	bla_{POM}
MR16	*Pseudomonas otitidis*	–	16	–	bla_{POM}
MR17	*Pseudomonas otitidis*	–	16	–	bla_{POM}
MR18	*Pseudomonas otitidis*	Same as MR16	16	–	NT

NT, not tested.

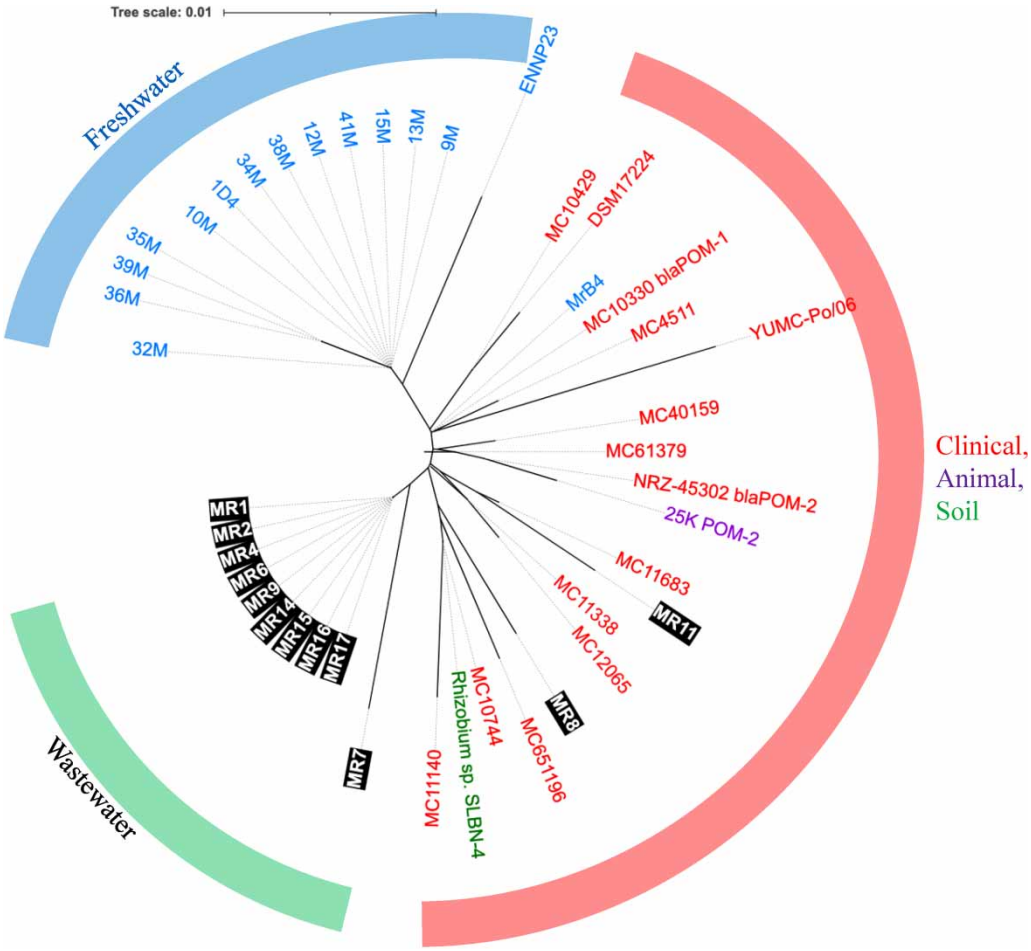


Figure 1 | Unrooted neighbor-joining phylogenetic tree of amino acid sequences isolated in this study (wastewater = 12) and 31 POM amino acids deposited to the GenBank from different sources and countries (freshwater = 15, clinical = 12, type strains = 2, animal = 1, and soil = 1). *P. otitidis* strains MR7, MR8, and MR11 were closely related to clinical references. The blue font was bla_{POM} genes isolated from freshwater, the red font was bla_{POM} genes isolated from clinical, the black font was bla_{POM} genes isolated from type strains, and the purple font was bla_{POM} genes isolated from the animal.

position 189. *P. otitidis* MR7 has amino acid substitutions at position 48 (asparagine, N to aspartic acid, D), position 152 (valine, V to methionine, M), and position 232 (valine, V to an undetermined amino acid, X). The amino acid substitution at position 18 (V to M) of MR8 was matching with MC11140 isolated from clinical and SLBN-4 isolated from soil. These amino acid substitutions in isolates MR7, MR8, and MR11 supported the phylogenetic cluster analysis where they were closely or grouped in the clinical cluster. The key residues involved in metal binding in subclass B3 enzymes (His/Gln116, His118, His196, Asp120, His121, and His263) (Palzkill 2013) were conserved in all bla_{POM} isolates (shown in black arrow).

Hydrolysis of meropenem by *P. otitidis*

In Figure 3, a standard curve of the meropenem inhibition zone (10.5–18 mm) of *E. coli* K12 in the range of concentration of meropenem (2–64 mg/L) was plotted prior to the test (Figure 3(a)). The standard curve has an R^2 value of 0.98 (slope = 0.141; *y*-intercept = 9.2). All five *P. otitidis* isolates showed a faster rate of meropenem hydrolyzation over time (Figure 3(b)). MR11 has the highest rate of hydrolysis, which it completely hydrolyzed 64 mg/L of meropenem in 7 h, while MR8, MR16, MR1, and MR17 hydrolyzed meropenem from 64 to averaged 1.0, 5.3, 6.7, and 10.7 mg/L, respectively, at 7 h. The negative control showed that the concentration of meropenem was reduced to 32 mg/L at 7 h. The isolates MR8 and MR11, which were found to have three amino acid substitutions than original bla_{POM}, showed a higher rate of meropenem hydrolyzation than bla_{POM} isolates, which has only one amino acid substitution than original bla_{POM} (MR1, MR16, and MR17).

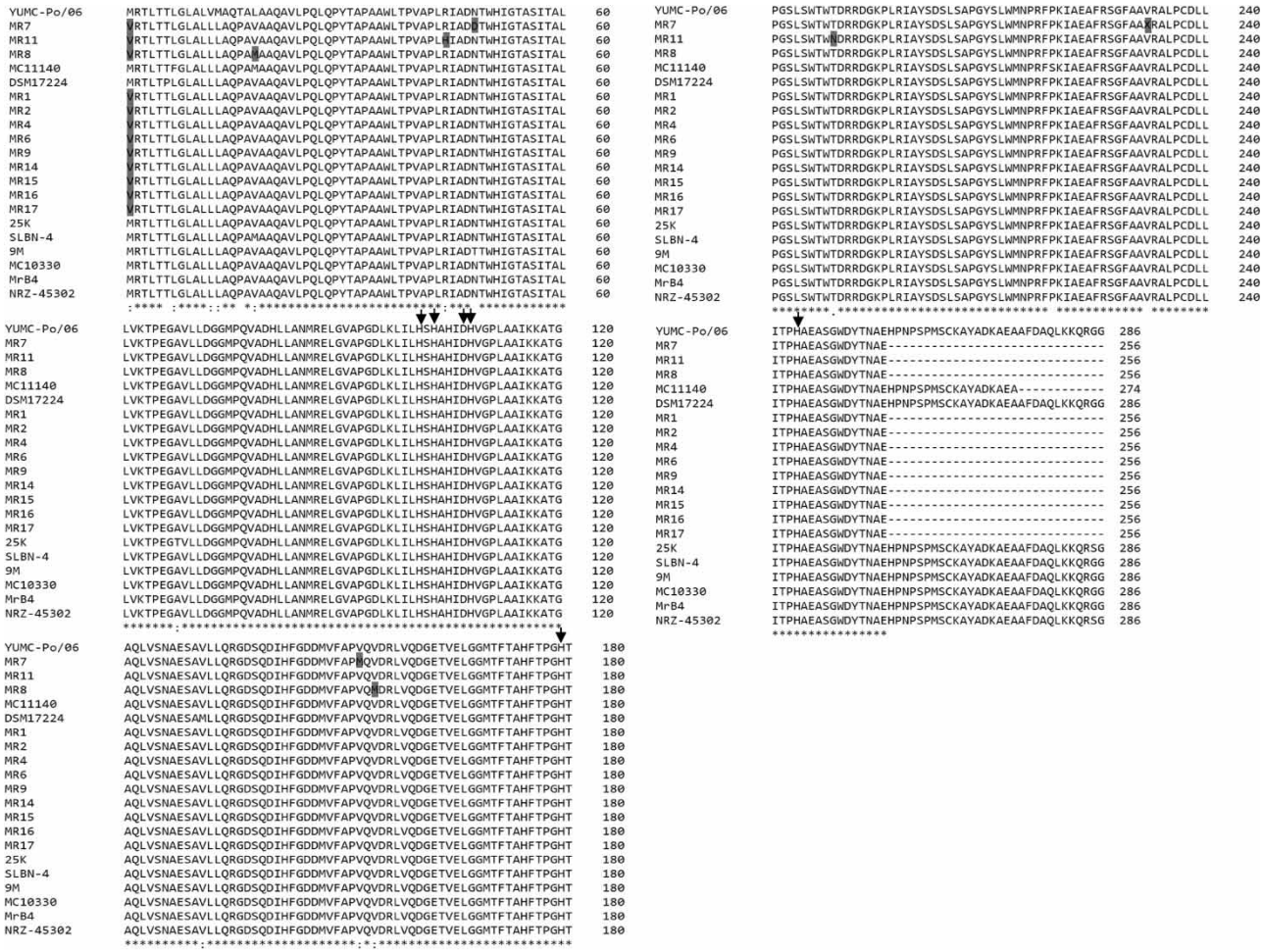


Figure 2 | Amino acid alignment among bla_{POM} genes of *P. otitidis* in this study ($n = 12$) and reference amino acids ($n = 9$). All 12 bla_{POM} obtained from *P. otitidis* strains have at least one amino acid substitution (methione, M to valine, V) at position 1. MR7 has a total of four amino acid substitutions at positions 1, 48, 152, and 232. MR8 has a total of three amino acid substitutions at positions 1, 18, and 154. MR11 has a total of three amino acid substitutions at positions 1, 44, and 189. The amino acid substitutions among the isolates in this study are shown in gray boxes. All the bla_{POM} amino acids have the conserved key residues involved in metal binding in subclass B3 enzymes (His/Gln116, His118, His196, Asp120, His121, and His263), shown in black arrows. The symbols '*' indicate perfect alignment; ':' indicates a site belonging to a group exhibiting strong similarity; '.' indicates a site belonging to a group exhibiting weak similarity.

DISCUSSION

A study by Borgianni *et al.* (2015) showed that POM is a novel broad-spectrum subclass B3 MBL, which manifests high catalytic activity on carbapenems, including ertapenem and doripenem. This has also been proven by Thaller *et al.* (2011) where the expression of bla_{POM-1} in *E. coli* and *P. aeruginosa* increased the MICs of the bacteria hosts to penicillins, cephalosporins, and carbapenems, except aztreonam. Bla_{POM-1} was further proven to show notably high catalytic efficiencies, 2- to 14,000-fold higher k_{cat}/K_m values to imipenem and meropenem than by other subclass B3 enzymes (except bla_{AIM-1} for imipenem) (Borgianni *et al.* 2015). They also found that some of the readings were higher than the globally spread-acquired MBL VIM-2 (Docquier *et al.* 2003). It was also suggested that bla_{POM} could be the earliest member within some *Pseudomonas* species to possess the chromosomal B3 MBLs, considering a similar enzyme bla_{PAM-1} (72% similar at the sequence level) has been reported in *Pseudomonas alcaligenes* (Suzuki *et al.* 2014).

Thaller *et al.* (2011) also showed that the POM-1 enzyme has up to 64% amino acid similarity to several known variants of the L1 enzyme of *S. maltophilia*, which is higher than other subclass B3 enzyme, suggesting a closer ancestry to the L1 enzyme. They also found that bla_{POM-1} has an overall high degree of sequence conservation, with an amino acid homology

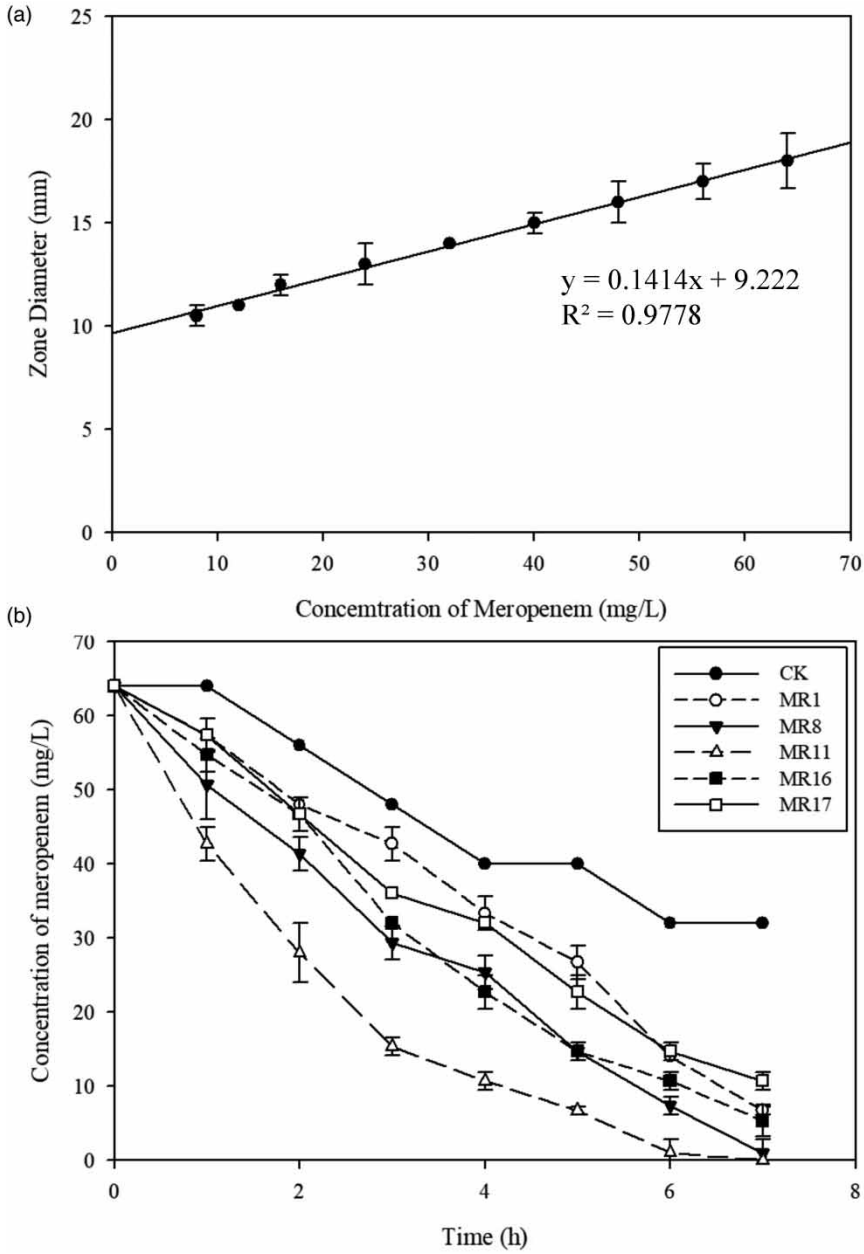


Figure 3 | (a) Standard curve of the meropenem inhibition zones (10.5–18 mm) of *E. coli* K12 on LB agar with concentrations of meropenem ranging from 2 to 64 mg/L. (b) Hydrolysis of meropenem over time by selected strains of *P. otitidis* MR1, MR8, MR11, MR16, and MR17. CK was the negative control without bacteria.

ranging from 97.0 to 100% among the aligned regions. In this study, the bla_{POMs} isolated from wastewater have an amino acid homology ranging from 98.4 to 99.6% compared with the type strain MC10330$^{\mathrm{T}}$. Key residues involved in metal binding in subclass B3 enzymes observed by a previous study (Thaller *et al.* 2011) determined by the BBL numbering scheme (Garau *et al.* 2004) were found to be conserved in all the bla_{POM} isolates in this study (Figure 2). Furthermore, the number of amino acid substitutions in the POM enzyme does not determine the MICs to meropenem. According to Thaller *et al.* (2011) the POM enzymes isolated which have at least 1–3 amino acid substitutions have a wide range of MICs to meropenem (0.25 to >32 mg/L). For example, for an isolate MCC04511 that has one amino acid substitution, the MIC for meropenem was >32 mg/L; in contrary to MCC11140 that has three amino acid substitutions, the MIC for meropenem was 1 mg/L. In addition, MCC51196 has two amino acid substitutions, and three amino acid deletions have the lowest MIC for

meropenem (0.25 mg/L). Wong *et al.* (2015) found that bla_{POM} isolated with one or two amino acid substitutions was resistant to meropenem with MICs >8 mg/L. Similar MICs were observed in this study where bla_{POM} from MR7 that has four and bla_{POM} from MR8 that has three amino acid substitutions have a MIC of 8 mg/L to meropenem, and bla_{POM} from MR11 that has three amino acid substitutions has a MIC of 16 mg/L to meropenem (Table 1).

Since the first publications of the presence of intrinsic β-lactamases in non-fermenting Gram-negative species (Richmond & Curtis 1974; Suginaka *et al.* 1975), the studies of intrinsic β-lactamases in non-Enterobacteriaceae have been increasing, including *Aeromonas* spp. (Bakken *et al.* 1988), *Burkholderia cepacia complex* (BCC) (Poirel *et al.* 2009), *P. aeruginosa* (Fajardo *et al.* 2014), *Shewanella oneidensis* (Poirel *et al.* 2004), and *S. maltophilia* (Saino *et al.* 1982). However, the study of this mechanism in *P. otitidis* was very limited. Carbapenem resistance could also be caused by other mechanisms including the overproduction of AmpC cephalosporinase, changes in the permeability of the outer membrane, and overexpression of efflux pumps (Dantas *et al.* 2017). Wong *et al.* (2015) found that while carbapenem resistance in *P. otitidis* was mainly caused by bla_{POM}, the overexpression of MexAB-OprM efflux pump and the absence of OprD porin were the reason for carbapenem resistance in *P. aeruginosa*; while the overexpression of the TtgABC efflux system in *P. putida* was responsible for carbapenem resistance. The permeability of β-lactams on the outer membrane barrier was also an important role in the hydrolysis of β-lactams by Gram-negative bacteria (Matsumura *et al.* 1999). A study done by Minami (1993) showed that in the addition of β-lactams (ceftazidime and cefpirome), *P. aeruginosa* changed from intermediate to highly resistant to imipenem. Although it is not scientifically proven, these carbapenem mechanisms found in *P. aeruginosa* could have evolved in *P. otitidis* in the future because these two species are genetically closely related as they are in the same genus *Pseudomonas* sensu strict (Peix *et al.* 2009). Other studies also found that originally chromosomally encoded β-lactamases were later found in plasmids, such as IMP-1 in *P. aeruginosa* (Watanabe *et al.* 1991) and IMI-2 in *Enterobacter asburiae* (Aubron *et al.* 2005) and *Enterobacter cloacae* (Yu *et al.* 2006).

This study focused on characterizing the bla_{POM} amplified from *P. otitidis* isolated from wastewater, which was found to be phylogenetically different from bla_{POM} isolated from other sources (e.g. clinics and freshwater) (Figure 1). While most of the studies of *P. otitidis* possessing bla_{POM} were focused on the clinical isolates (Clark *et al.* 2006; Thaller *et al.* 2011; Lee *et al.* 2012; Borgianni *et al.* 2015; Kim *et al.* 2016), the study of bla_{POM} in wastewater is very limited. The bla_{POM} isolated in wastewater was found to have more mutations (i.e. amino acid substitutions) than bla_{POM} isolated from patients in a previous study (Thaller *et al.* 2011) (Figure 2). Some *P. otitidis* isolates MR1, MR8, MR11, MR16, and MR17, which have more than two amino acid substitutions compared to the wild-type strain, were able to hydrolyze meropenem in a shorter time. These were not tested in previous clinical studies.

CONCLUSIONS

The prevalence of opportunistic pathogen *P. otitidis* encoding MBL POM isolated in the wastewater emerged as a substantial threat to public health because wastewater is known as the source of antibiotic-resistant bacteria and genes in the environment. This study isolated bla_{POM} encoding *P. otitidis* in wastewater, which was phylogenetically different from other sources (including freshwater, clinics, soil, and animal). Several isolates with multiple amino acid substitutions in the POM region compared to the wild-type strain showed a higher meropenem hydrolysis rate and were closely clustered to the clinical reference isolates in the phylogenetic tree. This phenomenon not only increases the risk for the development of acquired resistance, but it might also limit and complicate drug selections for treatments because *P. otitidis* is a clinically recognized pathogen.

Due to the lack of the study of bla_{POM} encoding *P. otitidis* in wastewater, our findings have shown the difference in its amino acid sequences compared to other isolation sources and its potential to hydrolyze carbapenems in a shorter time. This information is important for health authorities to formulate plans to prevent the spread in the healthcare system and the environment. More in-depth studies of *P. otitidis* and bla_{POM} should be done to fill in the knowledge gap of this opportunistic pathogen and MBL in wastewater and the environment.

AUTHOR CONTRIBUTION STATEMENTS

D.Y.W.D. analyzed the data and wrote the manuscript. G.X.C. and C.Z. designed and performed the experiments. D.Y.W.D. and G.X.C. corrected and revised the manuscript. All authors read and approved the final manuscript. D.Y.W.D. and G.X.C. contributed equally to the manuscript. T.Y. conceived the idea, supervised the research, and reviewed the manuscript.

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DATA AVAILABILITY STATEMENT

All relevant data are included in the paper or its Supplementary Information.

CONFLICT OF INTEREST

The authors declare there is no conflict.

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