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Antibiotic resistance profiles and mutations that might affect drug susceptibility in metagenome-assembled genomes of *Legionella pneumophila* and *Aeromonas* species from municipal wastewater

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Abstract

Antibiotic resistance (AR) has emerged as a significant global health issue. Wastewater treatment plants (WWTPs) contain diverse bacterial communities, including pathogens, and have been identified as crucial reservoirs for the emergence and dissemination of AR. The present study aimed to identify antibiotic resistance genes (ARGs) and screen for the presence of mutations associated with AR in *Legionella pneumophila* and *Aeromonas* spp. from municipal wastewater. Metagenome-assembled genomes (MAGs) of *L. pneumophila* and *Aeromonas* spp. were reconstructed to investigate the molecular mechanisms of AR in these organisms. A total of 138 nonsynonymous single nucleotide variants (SNVs) in seven genes associated with AR and one deletion mutation in the *lpeB* gene were identified in *L. pneumophila*. In *Aeromonas* spp., two (*aph(6)-Id* and *aph(3")-Ib*) and five (bla_{MOX-4}, $bla_{OXA-1143}$, $bla_{OXA-724}$, *cepH*, and *imiH*) ARGs conferring resistance to aminoglycosides and β-lactams were identified, respectively. Moreover, this study presents β-lactam resistance genes, $bla_{OXA-1143}$ and $bla_{OXA-724}$, for the first time in *Aeromonas* spp. from a municipal WWTP. In conclusion, these findings shed light on the molecular mechanisms through which clinically relevant pathogenic bacteria such as *L. pneumophila* and *Aeromonas* spp. found in natural environments like municipal wastewater acquire AR.

Clinical trial number

Not applicable.

Keywords Antibiotic resistance, Metagenome-assembled genome, Mutations, Molecular mechanisms, Municipal wastewater

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Introduction

Antibiotic resistance (AR) has increased significantly, primarily due to the excessive and inappropriate use of antibiotics in clinical and veterinary settings, increased infection rates, and inadequate hygiene and disinfection practices [1]. Historically, clinical settings were believed to be the primary reservoir of antibiotic-resistant bacteria (ARB) and antibiotic resistance genes (ARGs) [2]. However, ARB and ARGs have recently become ubiquitous contaminants in other environments including wastewater treatment plants (WWTPs) [2, 3]. Numerous studies have implicated WWTPs as key reservoirs and sources of AR in the environment [2, 4]. As such, different AR types and subtypes of almost all ARGs are consistently detected in different types of wastewaters [5, 6].

Legionella spp. are gram-negative fastidious aerobic bacteria found ubiquitously in natural and artificial water systems, including WWTPs. *Legionella pneumophila* is the causative agent of severe atypical pneumonia also called Legionnaires' disease (LD), as well as an acute febrile illness known as Pontiac fever [7]. This organism is responsible for up to 90% of the reported LD cases worldwide, with most of these cases attributed to *L. pneumophila* serogroup 1 [7]. Macrolide and fluoroquinolone antibiotics (usually azithromycin and levofloxacin, respectively) are currently recommended for moderate and severe LD [8]. Despite AR historically not being a primary concern in *Legionella* infections, recent reports have identified resistance to first-line drugs in clinical strains and documented instances of treatment failure in patients [9, 10]. These findings highlight the possibility of reduced efficacy and the emergence of resistance to traditional therapies. Jia et al. (2019) reported that an efflux pump component (*lpeAB*) primarily confers azithromycin resistance in *L. pneumophila* [10]. Through sequencing approaches, several resistance mediating mechanisms have been revealed in *L. pneumophila*, including enzymatic modifications and mutations in genes encoding 23S rRNA, DNA gyrase and topoisomerase IV, as well as ribosomal accessory proteins [11, 12].

The genus *Aeromonas* comprises gram-negative rod-shaped bacteria commonly found in diverse aquatic habitats such as rivers, drinking water, wastewater, soil, and animals. Members of this genus have been implicated in a wide range of human diseases, including gastroenteritis, wound infections, and bacteremia [13]. Notably, *Aeromonas* spp. serve as reservoirs of ARGs in aquatic settings [13]. Moreover, members of the genus *Aeromonas* harbour a plethora of AR determinants located on mobile genetic elements, including plasmids, transposons, integrons, and genomic islands, facilitating the horizontal transfer of ARGs even to phylogenetically distant bacterial species [14]. Notwithstanding the significance of *Aeromonas* in the dissemination of AR, few studies have characterised the genotypic profiles of AR determinants in *Aeromonas* species within WWTP environments [15, 16]. Understanding these genotypic profiles is crucial, as it provides valuable insights into the mechanisms by which ARGs are disseminated in WWTPs and implications for WWTP workers' health.

In 2019, it was approximated that antimicrobial resistant infections led to nearly 1.27 million deaths globally [17]. Moreover, forecasts indicate that by 2050, AR could result in 10 million deaths annually and a 3.8% decline in the global gross domestic product (GDP) [18]. Therefore, efforts to identify the emergence and spread of AR require a broader and non-invasive approach, especially in countries with limited resources and poor surveillance strategies [19]. Antibiotic-resistant bacterial species that are commonly studied in WWTPs predominantly include culturable enteric bacteria such as *Klebsiella pneumoniae*, *Enterobacteriaceae*, *Escherichia coli*, *Citrobacter* spp., and *Serratia* spp [3, 20]. However, culture-dependent methods have drawbacks, as only 1% of total environmental bacteria are culturable. Additionally, some of these bacteria are fastidious or slow growing and are thus difficult to characterise using culture methods [21]. While *L. pneumophila* is an established pathogen, *Aeromonas* is emerging as a potential public health concern because of its presence in water systems and resistance to multiple antibiotics [13, 14]. Therefore, the aim of this study was to characterise metagenome-assembled genomes (MAGs) of *L. pneumophila*, and *Aeromonas* spp. from municipal wastewater and identify their AR genotypes. Additionally, this study investigated the presence of spontaneous mutations in select ARGs known to confer resistance to commonly used antimicrobial drugs in *L. pneumophila*. To the best of our knowledge, this is the first study to perform genome analysis of wild-type *L. pneumophila* detected from municipal wastewater to better understand the potential health risks of environmental pathogenic organisms with no link to outbreaks or clinical cases.

Materials and methods

Wastewater sample collection and culture

Wastewater samples were collected from municipal WWTPs in Tshwane, South Africa. The characteristics of the sampling sites were previously described by Poopedi et al. (2023), see Supplementary Table S1 [22]. Sampling sites were selected based on treatment capacity, representing small (WWTP1: 35 mega litres per day (ML/day)), medium (WWTP1: 60 ML/day), and large (WWTP3: 93 ML/day) WWTPs. Additional selection criteria included the use of surface aeration technology, proximity to the laboratory, and willingness of site management to participate in the study. The Legiolert method (IDEXX Laboratories, Inc., Westbrook, U.S.) was used

Table 1 Sources and type of wastewater used in this study

Sample ID	Source	Sampling point	Collection date
WP1INF4	WWTP1	Bar screen	30 November 2021
WP1INF9	WWTP1	Bar screen	30 November 2021
WP2AS8	WWTP2	Aeration tank	11 January 2022
WP2SST1	WWTP2	Secondary settling tank effluent	25 January 2022
WP3INF4	WWTP3	Bar screen	16 November 2021
WP3INF2	WWTP3	Bar screen	16 November 2021
WP3AS1	WWTP3	Aeration tank	08 February 2022

to analyse 1 mL of wastewater in accordance with the manufacturer's instructions specified for non-portable water. The details of the wastewater samples are outlined in Table 1.

DNA extraction

The positive Legiolert trays were decontaminated with 70% ethanol, and the contents of the wells were extracted with a sterile disposable syringe (Merck, Darmstadt, Germany). The contents were placed into a 50 mL centrifuge tube and concentrated for 45 min at 5000 rpm (Eppendorf, Hamburg, German). Genomic DNA was then extracted from the pellet biomass using a DNeasy Powersoil Kit (Qiagen, Hilden, Germany) as per the manufacturer's instructions.

Sequencing, metagenome-assembled genomes (MAGs) assembly and taxonomic annotation

Sequencing was conducted at the Biotechnology Platform of the Agricultural Research Council in Onderstepoort, South Africa, using the MGI DNBSEQ-G400 sequencing platform according to the manufacturer's instructions, with 150 bp paired-end reads specified. Raw data were subjected to quality control, including adapter removal using BBDuk v.38.91 (https://jgi.doe.gov/data-and-tools/software-tools/bbtools/bb-tools-user-guide/bbduk-guide/;sourceforge.net/projects/bbmap/). De novo assembly of individual samples was performed with metaSPAdes v.3.15.3 [23], and only assembled contigs exceeding 1,500 bp were retained for subsequent analysis. The resulting contigs were then grouped into MAGs using Metabat v.2.15 [24]. Evaluation of MAG completeness and contamination was performed using CheckM v.1.1.3 [25], and only MAGs with completeness ≥ 50% and contamination ≤ 10% (classified as medium to high quality MAGs) were retained for further analyses [26]. Taxonomic classification of the MAGs using the Genome Taxonomy Database-Tk v.1.7.0 [27] identified *Legionella pneumophila* and *Aeromonas* species among the assembled genomes.

Antibiotic resistance genes and point mutations

Genome assemblies were assessed using the National Center for Biotechnology Information (NCBI) AMRFinderPlus (https://github.com/tseemann/abricate) for the in silico prediction of ARGs. This analysis identified ARGs in both *Legionella pneumophila* and *Aeromonas* species MAGs. The presence of the AR-associated genes *lpeA*, *lpeB*, *rplD*, *rplV*, *rrl/23 rRNA*, *gyrA*, *gyrB*, *parC*, *parE* and *rpoB* in the assembled *L. pneumophila* MAGs was screened using BLASTn querying the *L. pneumophila* strain Paris (NC_006368). The obtained gene sequences were subsequently aligned via multiple sequence alignment with the reference strain using Clustal Omega (https://www.ebi.ac.uk/jdispatcher/msa/clustalo). BioEdit [28] was used to perform and visualise the alignments. Amino acid changes were determined by translating the nucleotide sequences using the bacterial genetic code table (*E. coli* numbering system).

Results

General features of the recovered metagenome-assembled genomes (MAGs)

Table 2 summarises the general characteristics of the MAGs classified as *L. pneumophila* and *Aeromonas* spp. In this study, high quality MAGs were recovered, defined by a completeness exceeding 50% and contamination

Table 2 Features of metagenome-assembled genomes (MAGs) classified as *L. pneumophila* and *Aeromonas* spp

Features	*L. pneumophila*				*A. caviae*		*A. hydrophila*
Sampling point	Bar screen	Aeration tank	SST effluent	Bar screen	Bar screen	Bar screen	Aeration tank
Sampling site	WWTP1	WWTP2	WWTP2	WWTP3	WWTP1	WWTP3	WWTP3
Sample ID	WP1INF4	WP2AS8	WP2SST1	WP3INF4	WP1INF9	WP3INF2	WP3AS1
Average coverage	548X	333X	1704X	710X	12X	64X	360X
Completeness (%)	95	99	99	99	95	69	59
Contamination (%)	0,19	0,19	0,19	0,19	0,00	0,00	0,00
Size (bp)	3,177,878	3,435,984	3,335,074	3,295,813	4,245,992	3,782,452	3,046,587
No. of Contigs	60	35	18	19	87	125	315
N50	101,252	160,164	341,210	278,643	85,178	50,913	14,488
GC (%)	38,2	38,2	38,2	38,2	61,9	62,3	62,2
No. of predicted genes	2,838	3,069	3,005	2,961	3,895	2,962	3,512

Note: ID: identification, bp: base pair, GC: guanine cytosine, %: percentage, SST: secondary settling tank

Table 3 Antibiotic resistance genes within the metagenome-assembled genomes (MAGs) classified as *L. pneumophila* and *Aeromonas* spp. from municipal wastewater

Antibiotic class	*L. pneumophila*				*A. caviae*		*A. hydrophila*
	WP1INF4	WP2AS8	WP2SST1	WP3INF4	WP1INF9	WP3INF2	WP3AS1
	Gene (%)	Gene (%)	Gene (%)	Gene (%)	Gene (%)	Gene (%)	Gene (%)
Aminoglycoside	*aph(9)-Ia* (89.8)	*aph(9)-Ia* (90.4)	*aph(9)-Ia* (90.3)	*aph(9)-Ia* (90.5)	*aph(6)-Id* (100) *aph(3")-Ib* (99.9)	*aph(6)-Id* (100) *aph(3")-Ib* (99.9)	-
β-lactams	-	-	-	-	bla_{MOX-4} (95.7) $bla_{OXA-1143}$ (96.5)	bla_{MOX-4} (95.7) $bla_{OXA-1143}$ (96.5)	$bla_{OXA-724}$ (99.8) *cepH* (99.9) *imiH* (97.2)

Note: %: percentage

below 10%. Specifically, MAGs classified as *L. pneumophila* had completeness ranging from 95 to 99%, with contamination levels of less than 1%. Similarly, MAGs classified as *Aeromonas* spp. had completeness ranging from 59 to 95%, with no contamination (0%). The high level of completeness observed in these MAGs was consistent with high sequencing coverage achieved, highlighting the reliability of the genomic reconstructions.

The genome sizes of the WP1INF4, WP2AS8, WP2SST1, WP3INF4 *L. pneumophila* MAGs were approximately 3.18, 3.44, 3.34, and 3.30 Mbp, respectively, with identical low genomic G + C contents of 38.2% in all sequences. The assembled sequences of *L. pneumophila* WP1INF4, WP2AS8, WP2SST1, and WP3INF4 yielded 60, 35, 18, and 19 contigs with N50 values of 101,252, 160,164, 341,210, and 278,643, respectively. In addition, *L. pneumophila* WP1INF4, WP2AS8, WP2SST1, and WP3INF4 each had 2,838, 3,069, 3,005, and 2,961 predicted genes, respectively.

The sizes of the *Aeromonas caviae* MAGs WP1INF and WP3INF2 genomes were approximately 4.24 and 3.78 Mbp, with G + C contents of 61.9 and 62.3%, respectively. The assembly produced 145 and 87 contigs with N50 values of 85,178 and 50,913 in the *A. caviae* WP1INF and WP3INF2 genomes, respectively. The predicted gene counts for the two *A. caviae* genomes were 3,895 and 2,962. The genome of *A. hydrophila* MAGs had the following features: a genome size of 3.05 Mbp, a G + C content of 62.2%, 315 contigs with N50 values of 14,488, and 3,512 predicted genes.

Potential antibiotic resistance mutations and genotype in *L. pneumophila*

Antibiotic resistance genotypes were searched in the NCBI's AMRFinderPlus tool. The gene encoding the aminoglycoside O-phosphotransferase *aph(9)-Ia*, which confers resistance to spectinomycin, was detected in all four *L. pneumophila* MAGs (Table 3). Additionally, point mutations in genes known to be responsible for resistance to macrolides (*lpeA*, *lpeB*, *rplD*, *rplV*, and *rrl* 23 rRNA), fluoroquinolones (*gyrA*, *gyrB*, *parC*, *parE*), and rifampicin (*rpoB*)) drugs used for the treatment and management of LD were explored (Supplementary Table S2). Several nonsynonymous single nucleotide variants (SNVs) in resistance-associated genes (*rpoB*, *lpeB*, *rplD*, *gyrA*, *gyrB*, *parC*, and *parE*) were detected compared to the reference *L. pneumophila* strain Paris. Detailed information regarding the positions and amino acid changes of these nonsynonymous SNVs that were identified are described in Supplementary Table S3. In total, 138 nonsynonymous SNVs corresponding to seven genes and one deletion mutation in the *lpeB* gene were identified. Nonsynonymous SNVs were highly prevalent in the genes *gyrA* and *gyrB*, which had 85 and 17 variants, respectively. No mutations were detected in the *lpeA* efflux pump gene, 23 rRNA gene *rrl* or ribosomal accessory protein gene *rplV*.

Antibiotic resistance patterns of *Aeromonas* species

The ARGs identified from *Aeromonas caviae* and *Aeromonas hydrophila* MAGs are presented in Table 3. Within these MAGs, resistance to the antimicrobial classes aminoglycoside and β-lactam was conferred by two and five ARGs, respectively. Specifically, the MAGs classified as *A. caviae* WP1INF9 and WP3INF2 were found to carry *aph(6)-Id* and *aph(3")-Ib*, known for their strong affinity towards streptomycin and kanamycin, respectively, contributing to aminoglycoside gene resistance. Additionally, both *A. caviae* MAGs presented the β-lactam resistance genes bla_{MOX-4} and $bla_{OXA-1143}$. In contrast, *A. hydrophila* MAG WP3AS1 harboured distinct β-lactam resistance genes, namely, $bla_{OXA-724}$, *cepH*, and *imiH*. The resistance profiles of the two *A. caviae* MAGs (WP1INF9 and WP3INF2) were similar, whereas *A. hydrophila* had unique ARGs. Furthermore, the identified ARGs had a relatively high percent identity (above 95%), suggesting the potential completeness or functionality of the resistance genes.

Discussion

The present study comprehensively investigated AR profiles and point mutations associated with resistance in the MAGs of *L. pneumophila* and *Aeromonas* spp. from municipal wastewater. The aminoglycoside O-phosphotransferase gene (*aph(9)-Ia*) was detected in all *L. pneumophila* MAGs. This gene confers resistance to spectinomycin in *L. pneumophila.* Svetlicic et al. (2023) characterised the genomes of 39 *Legionella* isolates recovered during the environmental surveillance of man-made water systems, including showers, sinks, cooling towers, fountains, and irrigation tanks, across 13 countries and identified the *aph(9)-Ia* gene in 25 strains of *L. pneumophila* and two strains of *Legionella gormanii* [29]. Although the *aph(9)-Ia* gene is currently not considered a serious risk due to the infrequent use of spectinomycin in LD treatment, spectinomycin-resistant *L. pneumophila* has the potential to horizontally transfer the gene to other clinically relevant pathogens.

This study identified several nonsynonymous SNVs in *L. pneumophila* AR genes associated with resistance to macrolide, fluoroquinolone, and rifampicin drugs. An inherent limitation of the present study is the unavailability of culturable isolates to explore the correlation between phenotypic antimicrobial susceptibility patterns and genomic characteristics due to resource constraints. However, genomic findings presented in this study offer valuable information on the potential resistance mechanisms in *L. pneumophila* and *Aeromonas* spp., providing a foundation for future studies that can link these genotypes to phenotypic resistance patterns. The SNV findings of this study were compared with those of previously reported in vitro target gene induced mutations associated with macrolide, fluoroquinolone, and rifampicin in *L. pneumophila* [11–12, 30], but no similarities were found. It should be noted that unlike other ARB, limited studies have described gene mutations conferring AR in *L. pneumophila* [10, 31], resulting in limited data available for comparison. Among the identified mutations in the *L. pneumophila* MAGs recovered in this study, two-point mutations occurring at nucleotide positions 978 and 979 in the *lpeB* gene had previously been detected by Natås et al. (2019), and the other mutations could not be associated [31]. Natås et al. (2019) reported G978S and L979V variants in the *lpeB* gene in clinical and environmental *L. pneumophila* strains with reduced susceptibility to azithromycin [31]. Interestingly, the clinical *L. pneumophila* strain harboured both mutations, whereas the environmental strain possessed only the G978S variant [31]. Unlike clinical *Legionella* strains, the environmental MAGs of *Legionella* in this study may not have developed AR mutations affecting *L. pneumophila* sensitivity to treatment because of a lack of selective pressure. Notably, this study identified different amino acid changes (G978I and L979P), possibly due to distinct nucleotide substitutions resulting in different amino acid changes [32]. Unlike mutations induced by therapeutic interventions, humans can be exposed to resistant *Legionella* strains present in the environment. Macrolides, which are commonly used to treat respiratory infections in humans, are also naturally produced by *Streptomycetes* species in the environment [33]. These macrolides have been detected in various water systems, including both influent and treated effluent wastewater [34, 35]. In a recent one-year study by Senta et al. (2019) in Croatian WWTPs, macrolide concentrations of up to 25 micrograms per liter were observed [34]. The widespread presence of macrolides in WWTPs, coupled with their poor removal through conventional WWTP processes, suggests that *Legionella* species are exposed to macrolides at relatively high levels in these settings, potentially contributing to the development of AR.

Detecting DNA gyrase mutations in *L. pneumophila* can help predict the presence and level of fluoroquinolone resistance (FQR). In this study, DNA gyrase genes harboured a high number of nonsynonymous SNVs compared to other target genes. While previously considered improbable, recent studies have revealed FQR in *L. pneumophila* from LD infected patients [36]. To date, the most common FQR mutation reported in clinical *L. pneumophila* occurs in *gyrA* codon 83 [9, 37]. Although the clinical significance of our findings on FQR could not be determined at this time, the alarming probability of FQR emergence associated with spontaneous mutations in environmental *L. pneumophila* warrants attention. This trend may complicate LD treatment in the future, particularly considering the efficacy of fluoroquinolones against broad spectrum bacteria.

Genetic mutation is one of the major mechanisms driving the development and acquisition of AR in bacteria. Antibiotic resistance caused by mutations frequently reduces drug binding or loss of function, thus promoting drug efflux activity and diminishing the intracellular drug concentration [38]. Spontaneous mutations, such as base pair substitutions, insertions, and deletions in genes encoding drug targets, are common mechanisms in acquired mutations [38]. Understanding mutational AR, especially in clinically relevant bacterial pathogens from the environment including WWTPs, is crucial, as AR can arise spontaneously even without strong selective pressure or antibiotic exposure [39]. Therefore, a more in-depth analysis to explore the mechanisms underlying the acquisition of nonsynonymous SNVs in environmental *L. pneumophila* is needed. Future studies should include sequencing of susceptible and resistant *L. pneumophila* isolates, which would help in determining the magnitude of the effect of the identified mutations.

The common ARGs detected among *Aeromonas* spp. MAGs in this study were β-lactams. This finding is in agreement with other studies indicating that this genus often exhibits high resistance to β-lactams and may serve as a potential reservoir for the dissemination of β-lactam ARGs in water environments [40, 41]. Class C β-lactamase genes are chromosomal or plasmid-encoded and confer resistance to cephamycins, oxyimino-β-cephalosporins, and aztreonam [13]. The main concern with plasmid mediated ARGs is their potential for horizontal transfer, including across different bacterial species. In this study, two β-lactamase genes from class C were identified: $bla_{\text{MOX-4}}$, and *cepH*. Previously, $bla_{\text{MOX-4}}$ was identified in only an *Aeromonas* strain from an urban WWTP in Poland [40]. On the other hand, the *cepH* gene is specific to *A. hydrophila* and has been commonly identified in various environments including wastewater [40, 42], clinical samples [43], and fish [44].

Oxacillin-hydrolysing (OXA)-type β-lactamases (members of class D) are widely distributed among clinically relevant gram-negative bacteria, including *Aeromonas* spp [45]. Their diversity continues to expand to include new bla_{OXA} variants [45]. This study identified $bla_{\text{OXA-1143}}$ and $bla_{\text{OXA-724}}$ in *A. caviae* and *A. hydrophila*, respectively. To the best of our knowledge, this is the first study to report the $bla_{\text{OXA-1143}}$ subtype in municipal wastewater, although it was previously reported in an unpublished study of a rectal swab sample from a patient in France (GenBank accession no. **NG_081795.1**). Conversely, $bla_{\text{OXA-724}}$ has been identified in different *Aeromonas* species isolated from faeces of gastroenteritis patients [40], poultry [46], chickens [47], and fish [48]. Notably, this study represents the first identification of $_{\text{blaOXA-724}}$ in an *Aeromonas* from a municipal WWTP.

Class B metallo-β-lactamases (MBLs) pose a serious AR threat because of their ability to enzymatically hydrolyse most β-lactams, including carbapenems, which are reserved as a last resort for bacterial infections [49]. This study identified the MBL encoding gene *imiH*, which is specific to *A. hydrophila*. Previously, *imiH* was identified in *A. hydrophila* isolated from diverse sources, including a dairy farm in Texas [50], a recreational estuary in Brazil [51], Indian carp [48], and an activated sludge sample from a Polish urban WWTP [40]. Differences in resistance genotypes between *A. caviae* and *A. hydrophila* are associated with species variation. For instance, chromosomal β-lactamase is species-specific in *Aeromonas* species, with *A. caviae* possessing Class C and D β-lactamases, whereas *A. hydrophila* presents a broader spectrum with Class B, C, and D enzymes [13].

This study identified aminoglycoside resistance encoding genes, *aph(6)-Id* and *aph(3")-Ib*, conferring resistance to streptomycin and kanamycin, in both *A. caviae* MAGs. These findings are consistent with prior research investigating circulating ARGs in wastewater, suggesting the widespread presence of *aph(6)-Id* and *aph(3")-Ib* in such environments [52, 53]. It should be noted that aminoglycoside encoding genes are typically located on plasmids or transposons and have been reported in *Aeromonas* spp [41, 54]. Therefore, it is plausible that *A. caviae* could be an important reservoir for the dissemination of aminoglycoside encoding genes, including *aph(6)-Id* and *aph(3")-Ib*, to other bacterial species, particularly those of clinical importance, thereby posing a significant public health risk.

Previous studies have suggested that WWTP workers are potentially exposed to ARB at work via different exposure routes, such as inhalation and/or accidental ingestion of water droplets [20, 55]. Furthermore, an increased prevalence of respiratory and gastrointestinal diseases has been reported among WWTP workers [56], as well as elevated antibodies against certain bacteria [57], suggesting a link to bacterial exposure. Rodríguez-Molina and co-workers (2021) recently investigated ARB carriage in WWTP workers and noted the presence of extended-spectrum β-lactamase producing *E. coli* in the stool of WWTP workers and nearby residents in Romania, Germany, and the Netherlands, with the Romanian population having the highest carriage rate (28%) [58]. Therefore, AR studies are crucial for obtaining a better understanding of the exposure status of workers in WWTPs as well as developing preventive interventions to reduce potential occupational exposure.

The findings of this study highlight the role of municipal wastewater as a significant reservoir for AR, where environmental, human, and animal health intersect. The identified ARGs and SNVs reveal emerging AR patterns in municipal wastewater, providing early insights into potential threats from waterborne pathogens, specifically *L. pneumophila* and *Aeromonas* spp. Although preliminary, these findings are essential for guiding occupational, public health, and environmental monitoring efforts, highlighting the increasing risks posed by AR. Additionally, this study integrates the One Health approach, underscoring the importance of managing AR in the context of climate change, which is accelerating the spread of these pathogens across sectors.

Conclusion

This study characterised MAGs of *L. pneumophila* and *Aeromonas* spp. from municipal wastewater in South Africa. *Legionella pneumophila* MAGs harboured several nonsynonymous genetic variations in the genes *rpoB*, *lpeB*, *rplD*, *gyrA*, *gyrB*, *parC*, and *parE*, which are implicated in LD antibiotic resistance. Future phenotypic testing studies are needed to determine the clinical implications of these mutations in AR. Additionally, this study demonstrated the role of municipal wastewater as

a reservoir of *Aeromonas* spp. carrying multiple functional ARGs, including plasmid-mediated β-lactamase genes that can be easily disseminated among different species. Therefore, implementing large-scale surveillance programs is crucial to uncover AR strains and markers circulating in municipal wastewater, understand factors driving the spread of AR, and enable early detection and mitigation of the potential transmission of *L. pneumophila* and *Aeromonas* spp. from environmental sources to clinical settings and communities. In summary, our findings pave the way in elucidating how clinically relevant pathogenic bacteria such as *L. pneumophila* and *Aeromonas* spp. occurring in engineered water systems such as municipal wastewater can develop AR.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12866-025-03957-x.

Supplementary Material 1

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Author contributions
EP: Conception and design of study, Acquisition of data, Analysis of data, Writing -original draft, and Writing -review and editing. RP: Analysis of data, and Writing -review and editing. TS: Conception and design of study, Resources, Supervision, and Writing -review and editing. AG: Conception and design of study, Funding acquisition, Methodology, Resources, Supervision, and Writing -review and editing. All authors read and approved the final manuscript.

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Data availability
The datasets generated and analysed during the current study are available in the NCBI BioProject repository (BioProject ID: PRJNA1182933), https://www.ncbi.nlm.nih.gov/ PRJNA1182933.

Declarations

Ethical approval
Not applicable.

Consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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References

1. Gao P, Munir M, Xagoraraki I. Correlation of Tetracycline and sulfonamide antibiotics with corresponding resistance genes and resistant bacteria in a conventional municipal wastewater treatment plant. Sci Total Environ. 2012;421:73–183. https://doi.org/10.1016/j.scitotenv.2012.01.061.
2. Rizzo L, Manaia C, Merlin C, Schwartz T, Dagot C, Ploy MC. Urban wastewater treatment plants as hotspots for antibiotic resistant bacteria and genes spread into the environment: a review. Sci Total Environ. 2013;447:345–60. https://doi.org/10.1016/j.scitotenv.2013.01.032.
3. Schwermer CU, Krzeminski P, Wennberg AC, Vogelsang C, Uhl W. Removal of antibiotic resistant *E. coli* in two Norwegian wastewater treatment plants and by nano-and ultra-filtration processes. Water Sci Technol. 2018;77(4):1115–26. https://doi.org/10.2166/wst.2017.642.
4. Karkman A, Do TT, Walsh F, Virta MP. Antibiotic-resistance genes in wastewater. Trends Microbiol. 2018;26(3):220. https://doi.org/10.1016/j.tim.2017.09.005.
5. Tang M, Dou X, Wang C, Tian Z, Yang M, Zhang Y. Abundance and distribution of antibiotic resistance genes in a full-scale anaerobic–aerobic system alternately treating ribostamycin, spiramycin and paromomycin production wastewater. Environ Geochem Health. 2017;39:1595–605. https://doi.org/10.1007/s10653-017-9987-5.
6. Zhang A, Call DR, Besser TE, Liu J, Jones L, Wang H. β-lactam resistance genes in bacteriophage and bacterial DNA from wastewater, river water, and irrigation water in Washington state. Water Res. 2019;161:335–40. https://doi.org/10.1016/j.watres.2019.06.026.
7. Kozak-Muiznieks NA, Lucas CE, Brown E, Pondo T, Taylor Jr TH, Frace M. Prevalence of sequence types among clinical and environmental isolates of *Legionella pneumophila* serogroup 1 in the united States from 1982 to 2012. J Clin Microbiol. 2014;52(1):201–11. https://doi.org/10.1128/jcm.01973-13.
8. Metlay JP, Waterer GW, Long AC, Anzueto A, Brozek J, Crothers K. Diagnosis and treatment of adults with community-acquired pneumonia. An official clinical practice guideline of the American thoracic society and infectious diseases society of America. Am J Respir Crit Care Med. 2019;200(7):45–67. https://doi.org/10.1164/rccm.201908-1581ST.
9. Shadoud L, Almahmoud I, Jarraud S, Etienne J, Larrat S, Schwebel C. Hidden selection of bacterial resistance to fluoroquinolones in vivo: the case of *Legionella pneumophila* and humans. EBioMedicine. 2015;2(9):1179–85. https://doi.org/10.1016/j.ebiom.2015.07.018.
10. Jia X, Ren H, Nie X, Li Y, Li J, Qin T. Antibiotic resistance and Azithromycin resistance mechanism of *Legionella pneumophila* serogroup 1 in China. Antimicrob Agents Chemother. 2019;63(10):768. https://doi.org/10.1128/aac.00768-19.
11. Almahmoud I, Kay E, Schneider D, Maurin M. Mutational paths towards increased fluoroquinolone resistance in *Legionella pneumophila*. J Antimicrob Chemother. 2009;64(2):284–93. https://doi.org/10.1093/jac/dkp173.
12. Descours G, Ginevra C, Jacotin N, Forey F, Chastang J, Kay E. Ribosomal mutations conferring macrolide resistance in *Legionella pneumophila*. Antimicrob Agents Chemother. 2017;61(3):02188–16. https://doi.org/10.1128/aac.02188-16.
13. Janda JM, Abbott SL. The genus *Aeromonas*: taxonomy, pathogenicity, and infection. Clin Microbiol Rev. 2010;23(1):35–73. https://doi.org/10.1128/cmr.00039-09.
14. Bello-López JM, Cabrero-Martínez OA, Ibáñez-Cervantes G, Hernández-Cortez C, Pelcastre-Rodríguez LI, Gonzalez-Avila LU. Horizontal gene transfer and its association with antibiotic resistance in the genus *Aeromonas* spp. Microorganisms. 2019;7(9):363. https://doi.org/10.3390/microorganisms7090363.
15. Varela AR, Nunes OC, Manaia CM. Quinolone resistant *Aeromonas* spp. As carriers and potential tracers of acquired antibiotic resistance in hospital and municipal wastewater. Sci Total Environ. 2016;542:665–71. https://doi.org/10.1016/j.scitotenv.2015.10.124.
16. Drk S, Puljko A, Dželalija M, Udiković-Kolić N. Characterization of third generation cephalosporin-and carbapenem-resistant *Aeromonas* isolates from municipal and hospital wastewater. Antibiotics. 2023;12(3):513. https://doi.org/10.3390/antibiotics12030513.
17. Murray CJ, Ikuta KS, Sharara F, Swetschinski L, Aguilar GR, Gray A. Global burden of bacterial antimicrobial resistance in 2019: a systematic analysis. Lancet. 2022;399(10325):629–55. https://doi.org/10.1016/S0140-6736(21)02724-0.
18. World Health Organization. Global antimicrobial resistance and use surveillance system (GLASS) report: 2021. ISBN 978-92-4-002733-6.

19. CDC, COVID-19:. U.S. Impact on antimicrobial resistance, special report 2022. Atlanta, GA: U.S. department of health and human services, CDC; 2022. https://www.cdc.gov/drugresistance/covid19.html
20. Amador PP, Fernandes RM, Prudêncio MC, Barreto MP, Duarte IM. Antibiotic resistance in wastewater: occurrence and fate of *Enterobacteriaceae* producers of class A and class C β-lactamases. J Environ Sci Health. 2015;50(1):26–39. https://doi.org/10.1080/10934529.2015.964602.
21. Zhang S, Li X, Wu J, Coin L, O'brien J, Hai F, Jiang G. Molecular methods for pathogenic bacteria detection and recent advances in wastewater analysis. Water. 2021;13(24):3551. https://doi.org/10.3390/w13243551.
22. Poopedi E, Singh T, Gomba A. Potential exposure to respiratory and enteric bacterial pathogens among wastewater treatment plant workers, South Africa. Int J Environ Res Public Health. 2023;20(5):4338. https://doi.org/10.3390/ijerph20054338.
23. Nurk S, Meleshko D, Korobeynikov A, Pevzner PA. MetaSPAdes: a new versatile metagenomic assembler. Genome Res. 2017;27(5):824–34. https://doi.org/10.1101/gr.213959.116. http://www.genome.org/cgi/doi/.
24. Kang DD, Li F, Kirton E, Thomas A, Egan R, An H. MetaBAT 2: an adaptive Binning algorithm for robust and efficient genome reconstruction from metagenome assemblies. PeerJ. 2019;7:7359. https://doi.org/10.7717/peerj.7359.
25. Parks DH, Imelfort M, Skennerton CT, Hugenholtz P, Tyson GW. CheckM: assessing the quality of microbial genomes recovered from isolates, single cells, and metagenomes. Genome Res. 2015;25(7):1043–55. https://doi.org/10.1101/gr.186072.114. http://www.genome.org/cgi/doi/.
26. Bowers RM, Kyrpides NC, Stepanauskas R, Harmon-Smith M, Doud D, Reddy TBK. Minimum information about a single amplified genome (MISAG) and a metagenome-assembled genome (MIMAG) of bacteria and archaea. Nat Biotechnol. 2017;35(8):725–31. https://doi.org/10.1038/nbt.3893.
27. Chaumeil PA, Mussig AJ, Hugenholtz P, Parks DH. GTDB-Tk: a toolkit to classify genomes with the genome taxonomy database. 2020. https://doi.org/10.1093/bioinformatics/btz848
28. Hall TA, BioEdit. A user-friendly biological sequence alignment editor and analysis program for windows 95/98/nt. *Nucleic Acids Symposium Series* 1991; *41*:95–98.
29. Svetlicic E, Jaén-Luchoro D, Klobucar RS, Jers C, Kazazic S, Franjevic D. Genomic characterization and assessment of pathogenic potential of *Legionella* spp. Isolates from environmental monitoring. Front Microbiol. 2023;13:1091964. https://doi.org/10.3389/fmicb.2022.1091964.
30. Nielsen K, Hindersson P, Høiby N, Bangsborg JM. Sequencing of the *RpoB* gene in *Legionella pneumophila* and characterization of mutations associated with Rifampin resistance in the *Legionellaceae*. Antimicrob Agents Chemother. 2000;44(10):2679–83. https://doi.org/10.1128/aac.44.10.2679-2683.2000.
31. Natås OB, Brekken AL, Bernhoff E, Hetland MAK, Löhr IH, Lindemann PC. Susceptibility of *Legionella pneumophila* to antimicrobial agents and the presence of the efflux pump *LpeAB*. J Antimicrob Chemother. 2019;74(6):1545–50. https://doi.org/10.1093/jac/dkz081.
32. Griffiths WM, Miller AJF, Suzuki JH, Lewontin DT, Gelbart R. Chapter 1 -Mutation, repair, and recombination. *An Introduction to Genetic Analysis; WH Freemand and Company: New York, NY, USA*. 2000.
33. Dinos GP. The macrolide antibiotic renaissance. Br J Pharmacol. 2017;174(18):2967–83. https://doi.org/10.1111/bph.13936.
34. Senta I, Kostanjevecki P, Krizman-Matasic I, Terzic S, Ahel M. Occurrence and behavior of macrolide antibiotics in municipal wastewater treatment: possible importance of metabolites, synthesis byproducts, and transformation products. Environ Sci Technol. 2019;53(13):7463–72. https://doi.org/10.1021/acs.est.9b01420.
35. Pan M, Yau PC. Fate of macrolide antibiotics with different wastewater treatment technologies. Water Air Soil Pollut. 2021;232:1–13. https://doi.org/10.1007/s11270-021-05053-y.
36. Burdet C, Lepeule R, Duval X, Caseris M, Rioux C, Lucet JC. Quinolones versus macrolides in the treatment of legionellosis: a systematic review and meta-analysis. J Antimicrob Chemother. 2014;69(9):2354–60. https://doi.org/10.1093/jac/dku159.
37. Hennebique A, Bidart M, Jarraud S, Beraud L, Schwebel C, Maurin M. Digital PCR for detection and quantification of fluoroquinolone resistance in *Legionella pneumophila*. Antimicrob Agents Chemother. 2017;61(9):628. https://doi.org/10.1128/aac.00628-17.
38. Woodford N, Ellington MJ. The emergence of antibiotic resistance by mutation. Clin Microbiol Infect. 2007;13(1):5–18. https://doi.org/10.1111/j.1469-0691.2006.01492.x.
39. Merker M, Tueffers L, Vallier M, Groth EE, Sonnenkalb L, Unterweger D, et al. Evolutionary approaches to combat antibiotic resistance: opportunities and challenges for precision medicine. Front Immunol. 2020;11(1938). https://doi.org/10.3389/fimmu.2020.01938.
40. Piotrowska M, Przygodzińska D, Matyjewicz K, Popowska M. Occurrence and variety of β-lactamase genes among *Aeromonas* spp. Isolated from urban wastewater treatment plant. Front Microbiol. 2017;8:863. https://doi.org/10.3389/fmicb.2017.00863.
41. Bertran X, Rubio M, Gómez L, Llovet T, Muñoz C, Navarro F. Taxonomic identification of different species of the genus *Aeromonas* by whole-genome sequencing and use of their species-specific β-lactamases as phylogenetic markers. Antibiotics. 2021;10(4):354. https://doi.org/10.3390/antibiotics10040354.
42. Cooper AL, Carter C, McLeod H, Wright M, Sritharan P, Tamber S. Detection of carbapenem-resistance genes in bacteria isolated from wastewater in Ontario. FACETS. 2021;6(1):569–91. https://doi.org/10.1139/facets-2020-0101sww.
43. Sun Y, Zhao Y, Xu W, Fang R, Wu Q, He H. Taxonomy, virulence determinants and antimicrobial susceptibility of *Aeromonas* spp. Isolated from bacteremia in southeastern China. Antimicrob Resist Infect Control. 2021;10:1–9. https://doi.org/10.1186/s13756-021-00911-0.
44. Bartie KL, Ngô TP, Bekaert M, Hoang Oanh DT, Hoare R, Adams A. *Aeromonas hydrophila* ST251 and *Aeromonas Dhakensis* are major emerging pathogens of striped catfish in Vietnam. Front Microbiol. 2023;13:1067235. https://doi.org/10.3389/fmicb.2022.1067235.
45. Chen Q, Zhou W, Qian C, Shen K, Zhu X, Zhou D. *OXA-830*, a novel chromosomally encoded extended-spectrum class D β-lactamase in *Aeromonas simiae*. Front Microbiol. 2019;10:2732. https://doi.org/10.3389/fmicb.2019.02732.
46. Ramsamy Y, Amoako DG, Abia ALK, Allam M, Ismail A, Mtshali PS. First genome sequence of *Aeromonas hydrophilia* novel sequence type 658 strain isolated from livestock in South Africa. J Global Antimicrob Resist. 2021;24:175–7. https://doi.org/10.1016/j.jgar.2020.12.021.
47. Wang Y, Hou N, Rasooly R, Gu Y, He X. Prevalence and genetic analysis of chromosomal mcr-3/7 in Aeromonas from US animal-derived samples. Front Microbiol. 2021;12:667406. https://doi.org/10.3389/fmicb.2021.667406.
48. Dubey S, Ager-Wick E, Kumar J, Karunasagar I, Peng B, Evensen Ø. *Aeromonas* species isolated from aquatic organisms, insects, chicken, and humans in India show similar antimicrobial resistance profiles. Front Microbiol. 2022;13:1008870. https://doi.org/10.3389/fmicb.2022.1008870.
49. Logan LK, Bonomo RA, April. Metallo-β-lactamase (MBL)-producing *Enterobacteriaceae* in united States children. Open Forum Infect Dis. 2016;3(2):90. https://doi.org/10.1093/ofid/ofw090.
50. Poole TL, Schlosser WD, Crippen TL, Swiger SL, Norman KN, Anderson RC. Whole-genome sequence of *Aeromonas* spp. Isolated from a dairy farm in central Texas. Microbiol Res. 2023;14(1):161–76. https://doi.org/10.3390/microbiolres14010014.
51. Canellas ALB, de Oliveira BFR, Laport MS. Hiding in plain sight: characterization of *Aeromonas* species isolated from a recreational estuary reveals the carriage and putative dissemination of resistance genes. Antibiotics. 2023;12(1):84. https://doi.org/10.3390/antibiotics12010084.
52. Mbanga J, Amoako DG, Abia AL, Allam M, Ismail A, Essack SY. Genomic insights of multidrug-resistant *Escherichia coli* from wastewater sources and their association with clinical pathogens in South Africa. Front Veterinary Sci. 2021;8:636715. https://doi.org/10.3389/fvets.2021.636715.
53. Cabal A, Rab G, Daza-Prieto B, Stöger A, Peischl N, Chakeri A. Characterizing antimicrobial resistance in clinically relevant bacteria isolated at the human/animal/environment interface using whole-genome sequencing in Austria. Int J Mol Sci. 2022;23(19):11276. https://doi.org/10.3390/ijms231911276.
54. Hu X, Zhang H, Liu Y, Liu X, Qiao J, Ge H. Genetic characterization and virulence determinants of multidrug-resistant *NDM-1*-producing *Aeromonas caviae*. Front Microbiol. 2023;13:1055654. https://doi.org/10.3389/fmicb.2022.1055654.
55. Lu R, Frederiksen MW, Uhrbrand K, Li Y, Østergaard C, Madsen AM. Wastewater treatment plant workers' exposure and methods for risk evaluation of their exposure. Ecotoxicol Environ Saf. 2020;205:111365. https://doi.org/10.1016/j.ecoenv.2020.111365.
56. Oza HH, Lee MG, Boisson S, Pega F, Medlicott K, Clasen T. Occupational health outcomes among sanitation workers: A systematic review and meta-analysis. Int J Hyg Environ Health. 2022;240:113907. https://doi.org/10.1016/j.ijheh.2021.113907.

57. Van Hooste W, Charlier AM, Rotsaert P, Bulterys S, Moens G, van Sprundel M. Work-related *Helicobacter pylori* infection among sewage workers in municipal wastewater treatment plants in Belgium. Occup Environ Med. 2010;67(2):91–7. https://doi.org/10.1136/oem.2008.040436.
58. Rodríguez-Molina D, Berglund F, Blaak H, Flach CF, Kemper M, Marutescu L. Carriage of ESBL-producing *Enterobacterales* in wastewater treatment plant workers and surrounding resident -The AWARE study. Eur J Clin Microbiol Infect Dis. 2021;1:1–16. https://doi.org/10.1007/s10096-021-04387-z.

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