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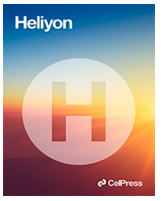

Research article

Comparing antibiotic resistance and virulence profiles of *Enterococcus faecium*, *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa* from environmental and clinical settings

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ABSTRACT

Antibiotic resistance and virulence profiles of *Enterococcus faecium*, *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa*, isolated from water sources collected in informal settlements, were compared to clinical counterparts. Cluster analysis using repetitive extragenic palindromic sequence-based polymerase chain reaction (REP-PCR) indicated that, for each respective species, low genetic relatedness was observed between most of the clinical and environmental isolates, with only one clinical *P. aeruginosa* (PAO1) and one clinical *K. pneumoniae* (P2) exhibiting high genetic similarity to the environmental strains. Based on the antibiograms, the clinical *E. faecium* Ef CD1 was extensively drug resistant (XDR); all *K. pneumoniae* isolates ($n = 12$) (except *K. pneumoniae* ATCC 13883) were multidrug resistant (MDR), while the *P. aeruginosa* ($n = 16$) isolates exhibited higher susceptibility profiles. The *tetM* gene (tetracycline resistance) was identified in 47.4 % ($n = 6$ environmental; $n = 3$ clinical) of the *E. faecium* isolates, while the bla_{KPC} gene (carbapenem resistance) was detected in 52.6 % ($n = 7$ environmental; $n = 3$ clinical) and 15.4 % ($n = 2$ environmental) of the *E. faecium* and *K. pneumoniae* isolates, respectively. The *E. faecium* isolates were predominantly poor biofilm formers, the *K. pneumoniae* isolates were moderate biofilm formers, while the *P. aeruginosa* isolates were strong biofilm formers. All *E. faecium* and *K. pneumoniae* isolates were gamma (γ)-haemolytic, non-gelatinase producing (*E. faecium* only), and non-hypermucoviscous (*K. pneumoniae* only), while the *P. aeruginosa* isolates exhibited beta (β)-haemolysis and produced gelatinase. The *fimH* (type 1 fimbriae adhesion) and *ugE* (uridine diphosphate galacturonate 4-epimerase synthesis) virulence genes were detected in the *K. pneumoniae* isolates, while the *P. aeruginosa* isolates possessed the *phzM* (phenazine production) and *algD* (alginate biosynthesis) genes. Similarities in antibiotic resistance and virulence profiles of environmental and clinical *E. faecium*, *K. pneumoniae*, and *P. aeruginosa*, thus highlights the potential health risks posed by using environmental water sources for daily water needs in low-and-middle-income countries.

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1. Introduction

The indiscriminate and excessive use of antibiotics has escalated the global emergence of antibiotic resistant bacteria (ARB), resulting in a reduced number of therapeutic options available to treat life-threatening disease [1,2]. In fact, it is estimated that 0.7 million people die each year due to infections caused by ARB, and it is expected that this number will increase to 10 million by the year 2050 [3]. Amongst the most clinically relevant opportunistic pathogens, are *Enterococcus faecium* (*E. faecium*), *Klebsiella pneumoniae* (*K. pneumoniae*), and *Pseudomonas aeruginosa* (*P. aeruginosa*). While most studies focus on the prevalence, genetic relatedness, antibiotic susceptibility testing, virulence, and health-associated risks of *E. faecium*, *K. pneumoniae*, and *P. aeruginosa* in clinical settings, multidrug resistant (MDR; bacteria that are resistant to three or more classes of antibiotics), and highly virulent strains of these bacteria, have been isolated from numerous environmental reservoirs including soil, food (e.g., vegetables, fruit, and animals) and various water sources (e.g., surface runoff, drinking water, streams, dams, and rivers, amongst others) [4,5]. The presence of these bacterial species in water sources has primarily been attributed to anthropogenic activities (e.g., bathing and swimming), incorrectly discarded clinical waste, sewage spills, and agricultural waste disposal [6,7]. Furthermore, while these bacterial species significantly decrease the overall water quality; the use of this water for domestic purposes may pose a serious health risk to end-user communities, as it could serve as a vector for the transmission of pathogenic bacteria [8,9].

Community-acquired (CA) infections, linked to the detection of these opportunistic pathogens in the environment, have subsequently been reported in various low-and-middle-income countries (LMIC) [5]. Tang et al. [10] implemented multilocus sequence typing (MLST) and pulse-field gel electrophoresis (PFGE) to highlight a direct correlation between the increasing incidence of vancomycin resistance *E. faecium* infection/colonisation in the community and an increase in hospital-acquired (HA) infections, which was primarily attributed to the admitted patients being carriers of the bacterium. Additionally, Rodrigues et al. [11] linked an invasive CA infection in a human immunodeficiency virus-positive patient with a hypervirulent *K. pneumoniae* strain using whole genome sequencing (WGS) and MLST. The authors identified *K. pneumoniae* ST66-K2 as the causative agent of infection, providing evidence for the persistence of this strain in extra-hospital reservoirs. Similarly, a clonal outbreak of *P. aeruginosa* during a time of high-water stress in Cape Town (South Africa; SA) was observed by Opperman et al. [12]. Based on WGS and MLST, the authors hypothesised that an environmental *P. aeruginosa* strain may have colonised municipal water sources or distribution systems resulting in city-wide dissemination and subsequent infection. Thus, while CA infections are frequently observed for pathogens such as *E. faecium*, *K. pneumoniae*, and *P. aeruginosa*, studies often fail to compare the genetic relatedness of environmental bacteria to well-characterised clinical strains. This differential comparison is crucial for determining the precise origin of the pathogens and the potential existence of an unexplored environmental reservoir.

Enterococci, including *E. faecium*, are natural inhabitants of both the human and animal gut microbiota [13–15], but also commonly inhabit environmental reservoirs such as soil and surface water, amongst others [16]. In clinical settings, *E. faecium* HA outbreaks [e.g., urinary tract infections (UTIs), amongst others] are associated with highly resistant and virulent strains of this species [17–19]. Resistant *E. faecium* isolates exhibit intrinsic resistance to cephalosporins, beta (β)-lactams, clindamycin (lincosamide), quinupristin and dalfopristin (streptogramins), and trimethoprim-sulfamethoxazole or acquired resistance to β-lactams, aminoglycosides, glycopeptides, streptogramins, linezolid, daptomycin, and tigecycline [20,21]. Resistance has been attributed to the acquisition of antimicrobial resistance gene (ARG) variants [e.g., *van* (vancomycin resistance), *tet* (tetracycline resistance)], expression of β-lactamases, point mutations in antibiotic binding regions, and modifications of antibiotic precursors, amongst others [22–24]. Subsequently, *E. faecium* was categorised under the "Priority 2: High" group on the World Health Organisation (WHO) priority pathogens list [25]. Additionally, *E. faecium* produces virulence factors such as cytolysin, aggregation substance, hyaluronidase, gelatinase, and enterococcal surface protein (encoded by *cyl*, *asa*, *hyl*, *gel*, and *esp* genes) contributing to the persistence of this species in both clinical and environmental settings [26–29]. Dos Santos et al. [16] then isolated 40 *E. faecium* strains from 171 soil and water samples, with all isolates characterised as MDR exhibiting resistance to fluoroquinolones, linezolid, and vancomycin. Resistance was further confirmed through the detection of the *vanC1* [enzyme responsible for peptidoglycan precursors terminating in D-alanyl-D-serine (D-Ala-D-Ser)], *ermB, ermC* (erythromycin ribosomal methylases), *mefAE* (energy dependent efflux pump), *tetM*, *tetL* (ribosomal protection), *ant(6′)-Ia*, *ant(4′)-Ia*, *aph(3′)-IIIa* and *aac(6′)-Ie-aph(2″)-Ia* (aminoglycoside-modifying enzymes) genes amongst the environmental *E. faecium* isolates, with these MDR *E. faecium* isolates additionally harbouring the *esp*, *gelE* and *ace* [adhesin to collagen of *Enterococcus faecalis* (*E. faecalis*)] virulence genes [30,31]. While the study offered valuable insights, further analysis comparing the genetic relatedness of the environmental isolates and clinically strains is needed to identify highly virulent and antibiotic resistant strains in environmental reservoirs unique to LMIC.

Klebsiella pneumoniae typically inhabits the nasopharynx and gastrointestinal tract of humans and animals, while also being widely distributed in environments such as soil, and water [32,33]. In clinical environments *K. pneumoniae* is responsible for diseases such as meningitis, pulmonary liver abscess, and pneumonia (in immunocompromised individuals) [34]. *Klebsiella pneumoniae* exhibits intrinsic resistance to penicillins (e.g., ampicillin), due to the presence of the chromosomally encoded SHV-1 (sulfhydryl variant) penicillinase, while *Klebsiella* carbapenemase (KPC) enzymes are highly efficient at hydrolysing cephalosporins, β-lactams, monobactams, and carbapenems [35–39]. Additional resistance mechanisms include efflux pumps (e.g., via *tet* genes), mutations in antibiotic target genes, modification of enzymes and proteins pumps, and the acquisition of ARGs through horizontal gene transfer (HGT; mainly carried on large, conjugative plasmids) [32,40] Classified as a "Priority 1: Critical" group by the WHO, *K. pneumoniae* possesses virulence factors such as siderophores (e.g., aerobactin, enterobactin, salmochelin, and yersiniabactin), capsular polysaccharides (CPS; *magA* and *ugE*), outer membrane porins (OMPs) and lipopolysaccharides (LPS; *wabG*), hypermucoviscous phenotypes (*rmpA*), and fimbriae (*fimH*), which facilitates pathogenicity and survival under unfavourable conditions [41–43].

Although environmental *K. pneumoniae* isolates are not extensively studied and characterised, research has shown that clinical and

environmental strains may be similar with regards to their phenotypic and genotypic features, but may differ in their virulence characteristics [44,45]. For example, Runcharoen et al. [45] isolated 77 extended-spectrum β-lactamase (ESBL) *K. pneumoniae* from hospitalised patients, sewage, farms and canals and compared them to 286 *K. pneumoniae* genomes from a global collection (i.e., globally circulating lineages). Despite close genetic relatedness, clinical isolates exhibited a higher prevalence of β-lactamase resistance and virulence, with 40 % of the analysed virulence genes [i.e., iron transport (*iro*), capsule synthesis (*rmpA*), transcription regulation (*kvgA*), transport and regulation (various *clb* genes), and phospholipid transport (*mce*)] exclusively detected in the clinical isolates. The analyses revealed that while clinical and environmental *K. pneumoniae* isolates may be closely related, clinical settings can amplify resistant and virulent determinants. Additionally, the study emphasises that environmental water is a potential reservoir for antibiotic resistant *K. pneumoniae* strains.

Pseudomonas aeruginosa, categorised alongside *K. pneumoniae* in the "Priority 1: Critical" group on the WHO's priority pathogens list, inhabits wet and moist environments, such as soil, plants, and water [25,46–49]. Despite its environmental presence, *P. aeruginosa* is a notorious opportunistic pathogen, responsible for the onset of various fatal human infections (e.g., UTIs, cystitis, endocarditis, surgical wound infections, and ventilator-associated pneumonia, amongst others) in immunocompromised patients [48,49]. Its intrinsic antibiotic resistance mechanisms include, decreased permeability of the outer membrane, efflux pump expression (e.g., MexXY and MexAB-OprM), and the production of antibiotic inactivating enzymes (e.g., OXA-type oxacillinase, inducible AmpC cephalosporinase) [50,51]. Acquired resistance mechanisms may include mutation-induced resistance (leading to the over-expression of efflux pumps, reduced antibiotic uptake, antibiotic target alteration, or the production of enzymes which inactivate antibiotics), or the acquisition of ARGs [51]. Additionally, virulence-associated traits of *P. aeruginosa* include biofilm formation, LPS and OMPs expression (e.g., OprF, OprH, OprD), swarming or swimming motility, type iv pili, exotoxins, haemolytic activity (PlcHR phospholipase), and various secretion systems [52].

In contrast to *K. pneumoniae*, studies have extensively analysed environmental *P. aeruginosa* stains, offering valuable insights into the genotypic and phenotypic characteristics of both environmental and clinical strains [53–55]. For example, Liew et al. [54] implemented Enterobacterial repetitive intergenic consensus polymerase chain reaction (ERIC-PCR) typing, antimicrobial susceptibility testing and virulence factor gene screening of clinical ($n = 105$) and fresh water environmental ($n = 114$) *P. aeruginosa* isolates. Overall, clinical, and environmental *P. aeruginosa* isolates were genotypically heterogeneous, with some clinical and environmental isolates showing identical ERIC-PCR patterns. Environmental *P. aeruginosa* isolates exhibited resistance to piperacillin/tazobactam, ciprofloxacin, piperacillin and carbapenems, with both clinical and environmental *P. aeruginosa* isolates exhibiting high prevalence (>60 %) of the virulence factor genes, except for *exoT*, *exoU* (type III secretion system effector enzymes), *pvdA* (pyoverdine) and *pilB* (pili).

As research has primarily focused on the antibiotic resistance and virulence profiling of clinical isolates of *E. faecium*, *K. pneumoniae*, and *P. aeruginosa*, the primary aim of the current study was to compare the genotypic [e.g., conventional PCR; REP-PCR and virulence gene PCR] and phenotypic (i.e., biofilm formation, haemolytic activity, hypermucoviscosity and gelatinase production) characteristics of these opportunistic pathogens, isolated from environmental water sources in informal settlements in the Western Cape (SA), to clinical counterparts. This study is significant for LMIC where large portions of the population reside in informal settlements. These informal settlements are densely populated areas where community members have limited access to basic public services such as water supply and water drainage and sanitation infrastructure, and often rely on environmental water sources for non-potable and potable purposes [56]. The potential presence of *E. faecium*, *K. pneumoniae*, and *P. aeruginosa* in surface water and surface runoff is thus a public health concern, particularly in regions with high rates of CA infections recorded amongst immunosuppressed

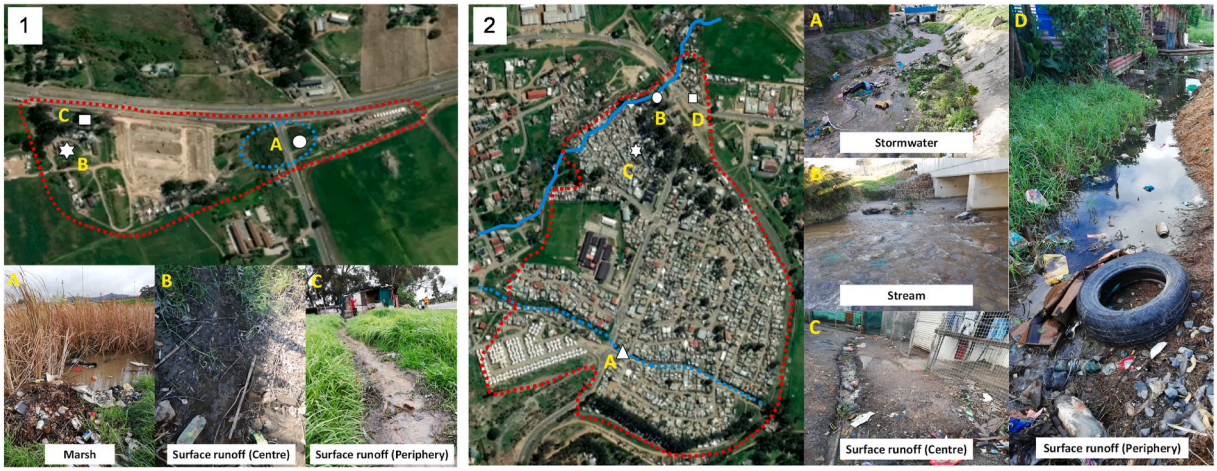

Fig. 1. Sampling sites for the collection of various environmental water samples in (**1**) Vlottenburg informal settlement [marsh (**1A**), surface runoff (centre) (**1B**) and surface runoff (periphery) (**1C**)] (rural settlement in Cape Town, SA; GPS co-ordinates: 33°95′35″ S; 18°79′75″ E) and (**2**) Sir Lowry's Pass Village [stormwater (**2A**), stream (**2B**), surface runoff (centre) (**2C**) and surface runoff (periphery) (**2D**)] (urban settlement in Cape Town, SA; GPS co-ordinates: 34°11′95″ S; 18°90′86″ E).

individuals.

2. Materials and methods

2.1. Reference and clinical bacterial isolates

The reference strains, *K. pneumoniae* ATCC 13883, and *P. aeruginosa* ATCC 27853, as well as one clinical *E. faecium* (Ef CD1), three clinical *K. pneumoniae* (P2, P3, and MCC3) and three clinical *P. aeruginosa* (MCC4, MCC5, and PAO1) were obtained from the Water Resource Laboratory Culture Collection (Department of Microbiology, Stellenbosch University). Additionally, six clinical *E. faecium* isolates (Ef S33a, Ef R174b, Ef R174c, Ef N36b, Ef R217c, and Ef S20d) were obtained from the Water and Health Research Centre Culture Collection (Faculty of Health Sciences, University of Johannesburg). The reference and clinical isolates were re-streaked onto Nutrient Agar (NA; Biolab, Merck, Midrand, SA) after being stored at −80 °C in 40 % (v/v) glycerol solution and were incubated at 37 °C for 24 h.

2.2. Environmental sample collection

Ethical clearance, for the collection and processing of environmental water samples, was obtained through the Research Ethics Committee: Biosafety and Environmental Ethics at Stellenbosch University (BEE-2019-9466). Sampling was conducted over six sampling sessions, from June to September 2021, with samples (1–5 L) collected in sterile bottles from various environmental water sources located in the Vlottenburg informal settlement (rural settlement in SA; GPS co-ordinates: 33°95′35″ S; 18°79′75″ E), and Sir Lowry's Pass Village (urban settlement in SA; GPS co-ordinates: 34°11′95″ S; 18°90′86″ E) (Fig. 1, Fig. S1 and Fig. S2). Samples were collected from three sites in the Vlottenburg informal settlement [i.e., marsh water, surface runoff water (from the periphery and the centre of the settlement); Fig. 1 and Fig. S1], while four sites were identified for the collection of environmental water samples at Sir Lowry's Pass Village [i.e., stream water, stormwater runoff, surface runoff water (from the periphery and centre of the settlement); Fig. 1 and Fig. S2].

Isolation of environmental *E. faecium*, *K. pneumoniae*, and *P. aeruginosa* was conducted using a primary enrichment step. Briefly, 20 mL of each sample was inoculated into 200 mL Luria Bertani (LB) broth (Oxoid, ThermoFisher Scientific, Johannesburg, SA) and incubated for 24 h at 37 °C [57]. Following primary enrichment, serial dilutions of each sample were prepared (10^{-1} to 10^{-6}) in 0.85 % (w/v) saline solution and 100 μL of each sample dilution was spread plated onto Slanetz and Bartley agar (Oxoid), HiCrome *Klebsiella* Selective agar base supplemented with *Klebsiella* Selective Supplement (Sigma-Aldrich, Gauteng, SA), and Cetrimide agar (Biolab) in duplicate, to isolate *E. faecium*, *K. pneumoniae*, and *P. aeruginosa*, respectively [58]. The agar plates were incubated for 24 h at 37 °C. Hereafter, colonies that morphologically resembled *E. faecium* (small, red-maroon colonies), *K. pneumoniae* (purple-magenta, mucoid colonies), and *P. aeruginosa* (fluorescent, yellow-green colonies) [59–61] were selected and purified by streaking onto NA (Biolab) at least once (to potentially prevent the loss of plasmid encoded resistance genes). The NA (Biolab) plates were incubated at 37 °C for 24 h to obtain pure cultures for molecular identification.

2.3. Conventional species-specific PCR and DNA sequencing for the identification of the target pathogens

To confirm the species identity of all the presumptive environmental *E. faecium*, *K. pneumoniae*, and *P. aeruginosa* isolates, and the reference and clinical isolates (Table 1) for each of the target species, a single colony from purified cultures (NA plates; Sections 2.1 and 2.2) was inoculated into LB broth and incubated overnight at 37 °C. Genomic DNA was extracted from the overnight cultures using the

Table 1
Primers, cycling parameters and PCR components used for conventional species-specific PCR analysis of the target pathogens.

Organism	Primers	Primer Sequence (5' – 3')	PCR Cycling Parameters	Gene Size (bp)	PCR Components	References
E. faecium	pstS1-F pstS2-R	TTGAGCCAAGTCGAAGCTGGAG CGTGATCACGTTCTACTTCC	94 °C for 3 min; 35 cycles of 94 °C for 30 s, 50 °C for 30 s, and 72 °C for 30 s; final extension step at 72 °C for 5 min	*pstS* (583)	1X Green GoTaq® Flexi Buffer, 1 mM MgCl2, 0.2 mM dNTP, 0.6 μM of the forward and reverse primers, 1.25 U GoTaq® G2 DNA Polymerase	Homan et al. [62]
K. pneumoniae	Kpn-F Kpn-R	GTGCGATGCGGTCTTTG GGGCGAACTGAACTGATG	95 °C for 5 min; 30 cycles of 95 °C for 15 s, 60 °C for 30 s, and 72 °C for 90 s; final extension step at 72 °C for 5 min	*phoE* (398)	1X Green GoTaq® Flexi Buffer, 2.5 mM MgCl2, 0.2 mM dNTP, 0.3 μM of the forward and reverse primers, 0.65 U GoTaq® G2 DNA Polymerase	Kaushik et al. [64]
P. aeruginosa	kpd1 kpd2	GCCCACGACCAGTTCGAC CATCCCCCTCCCTATGAC	94 °C for 2 min; 30 cycles of 94 °C for 15 s, 54 °C for 15 s, and 72 °C for 15 s; final extension step at 72 °C for 2 min	*rhlB* (226)	1X Green GoTaq® Flexi Buffer, 1 mM MgCl2, 0.2 mM dNTP, 0.5 μM of the forward and reverse primers, 1.25 U GoTaq® G2 DNA Polymerase	Bodour et al. [63]

Quick-DNA Fecal/Soil Microbe Miniprep Kit [Zymo Research, Irvine, CA, United States of America (USA)], as per the manufacturer's instructions. Species-specific conventional PCR analysis was subsequently performed to amplify the *pstS* (583 bp), *phoE* (398 bp), and *rhlB* (226 bp) genes of *E. faecium*, *K. pneumoniae*, and *P. aeruginosa*, respectively [62–64]. The primers, cycling parameters and amplification reactions are outlined in Table 1.

The PCR products were visualised using gel electrophoresis (80 V for 80 min) on a 1 % (w/v) agarose gel (CSL-AG500 LE Multi-Purpose Agarose, Cleaver Scientific, Warwickshire, United Kingdom) containing 0.5 μg/mL ethidium bromide, in 1X tris/acetate/ethylenediaminetetraacetic acid buffer. The amplicon size for each target pathogen was determined using the Generuler™ 1 kb Plus DNA ladder (ThermoFisher Scientific, Waltham, Massachusetts, USA). The amplicons for each of the reference, clinical and environmental target organisms were purified and concentrated using the Wizard® SV Gel and PCR Clean-up System (Promega, Wisconsin, USA) and were sent for DNA sequencing at Inqaba Biotechnical Industries (Pty) Ltd (Pretoria, SA). The FinchTV version 1.4.0 software was used to examine the chromatogram of each sequence; whereafter sequence identification for each isolate was carried out using the National Centre for Biotechnology Information (NCBI), Basic Local Alignment Search Tool (BLAST). Sequences of the representative isolates showing a similarity of ≥97 % to *E. faecium*, *K. pneumoniae*, and *P. aeruginosa* on the NCBI database, were recorded as positive.

2.4. Repetitive extragenic palindromic sequence-based PCR (REP-PCR)

To differentiate between the reference, clinical, and environmental *E. faecium*, *K. pneumoniae*, and *P. aeruginosa* isolates respectively, REP-PCR was used as outlined in Versalovic et al. [65]. Specifically, the REP-PCR analysis was used to generate DNA fingerprints and thereby assess the genetic relatedness (differentiate between strains of the same species) of the target species. The universal REP1R-1 (5′-IIIICGICGICATCIGGC-3′) and REP2-1 (5′-ICICTTATCIGCCTAC-3′) were used. The cycling parameters and amplification reactions are outlined in Table S1 Following amplification, the PCR products were visualised as outlined in Section 2.3. The fragment sizes of each DNA fingerprint were determined using the Generuler™ 1 kb Plus DNA ladder (ThermoFisher Scientific). The REP-PCR profiles were visualised under UV illumination using a MiniBIS Pro (DNR Bio-Imaging System, Neve Yamin, Israel) and the subsequent images were captured using GelCapture Version 4.25 (DNR Bio-Imaging System). The images of the DNA fingerprints of the *E. faecium*, *K. pneumoniae*, and *P. aeruginosa* isolates were imported into the GelJ software version 2.0 and analysed as outlined in De Vos et al. [66], with minor modifications. Briefly, the Generuler™ 1 kb Plus DNA ladder (ThermoFisher Scientific), was used to normalise the DNA fingerprints. The Pearson correlation coefficient was used to evaluate the similarity of the DNA fingerprints, whereafter Cluster Analysis was performed using the Unweighted Pair Group Method with Arithmetic Mean Algorithm (UPGMA). Isolates exhibiting a banding pattern of ≥85 % similarity were considered part of the same REP-PCR Type group [66].

2.5. Kirby-Bauer antibiotic assay

The antibiograms of the *E. faecium*, *K. pneumoniae*, and *P. aeruginosa* isolates were determined using the Kirby-Bauer antibiotic assay. Briefly, each isolate was streaked onto NA (Biolab) and grown for 18–24 h at 37 °C. The optical density (OD) of the bacterial

Table 2
Primers and conventional PCR cycling parameters for the detection of antibiotic resistance and virulence genes.

Gene	Organism (s)[a]	Primers	Primer Sequence (5' – 3')	PCR Cycling Parameters**	Size (bp)	References
bla$_{KPC}$	EF, KP, PA	KPC-F KPC-R	CAGCTCATTCAAGGGCTTTC GGCGGCGTTATCACTGTATT	95 °C for 3 min; 40 cycles of 95 °C for 30 s, 60 °C for 45 s	196	Subirats et al. [72]
tetM	EF, KP, PA	tetM-F tetM-R	GTGGACAAAGGTACAACGAG CGGTAAAGTTCGTCACACAC	94 °C for 5 min; 35 cycles of 94 °C for 30 s, 56 °C for 30 s, 72 °C for 1 min	406	Khalaf et al. [73]
mcr-1	KP, PA	MCR1-F MCR1-R	AGTCCGTTTGTTCTTGTGGC AGATCCTTGGTCTCGGCTTG	94 °C for 15 min; 25 cycles of 94 °C for 30 s, 58 °C for 90 s, 72 °C for 60 s	320	Rebelo et al. [74]
mcr-5	KP, PA	MCR5-F MCR5-R	ATGCGGTTGTCTGCATTTATC TCATTGTGGTTGTCCTTTTCTG	95 °C for 5 min; 30 cycles of 95 °C for 30 s, 50 °C for 30 s, 72 °C for 95 s	1644	Borowiak et al. [75]
esp	EF	esp-F esp-R	TATGAAAGCAACAGCACAAGTT ACGTCGAAAGTTCGATTTCC	95 °C for 5 min; 30 cycles of 94 °C for 30 s, 59 °C for 1 min, 72 °C for 1 min	680	Ahmed et al. [76]
gelE	EF	gelE-F gelE-R	TATGACAATGCTTTTTGGGAT AGATGCACCCGAAATAATATA	95 °C for 3 min; 30 cycles of 95 °C for 30 s, 56 °C for 30 s, 72 °C for 60 s	213	Molale and Bezuidenhout [27]
algD	PA	algD-F algD-R	TTCCCTCGCAGAGAAAACATC CCTGGTTGATCAGGTCGATCT	95 °C for 2 min; 30 cycles of 94 °C for 1 min, 60 °C for 1 min, 72 °C for 1 min	520	Taee et al. [77]
phzM	PA	phzM-F phzM-R	AGACTTCTACAGCTACCTGAAGC GATGGCCTTGGTCAATTCGC	95 °C for 10 min; 50 cycles of 95 °C for 15 s, 61 °C for 20 s, 72 °C for 30 s	166	Hendiani et al. [78]
ugE	KP	ugE-F ugE-R	TCTTCACGCCTTCCTTCACT GATCATCCGGTCTCCCTGTA	95 °C for 5 min; 30 cycles of 95 °C for 1 min, 56 °C for 45 s, 72 °C for 1 min	535	Barati et al. [42]
fimH	KP	fimH-F fimH-R	TGCTGCTGGGCTGGTCGATG GGGAGGGTGACGGTGACATC	95 °C for 5 min; 30 cycles of 95 °C for 1 min, 58 °C for 45 s, 72 °C for 1 min	550	Barati et al. [42]

[a] EF = *E. faecium*, KP = *K. pneumoniae*, PA = *P. aeruginosa*, **A final elongation step at 72 °C for 5 min was included for each PCR assay.

suspensions were measured using a GENESYS™ 20 Visible Spectrophotometer (ThermoFisher Scientific) at 625 nm (OD_{625}) and were adjusted to an OD_{625} of 0.08–0.13 (corresponding to 1.5×10^8 colony forming units/mL) using 0.85 % (w/v) saline solution (European Committee on Antimicrobial Susceptibility Testing [EUCAST, [67]). One hundred microliters (100 μl) of each adjusted bacterial suspension were subsequently spread plated onto Mueller-Hinton agar (Biolab). Commercially prepared, fixed concentration, antibiotic discs (Oxoid) (Table S2) were placed in triplicate onto the surface of the agar media and the plates were incubated at 37 °C for 16–20 h. A quality control (*P. aeruginosa* ATCC 27853) and a negative control (6 mm blank filter paper discs; Oxoid) were included for the disc diffusion assays. Following incubation, the diameter of the zone of inhibition around each antibiotic disc was measured to the closest mm and compared to the EUCAST [67] and Clinical and Laboratory Standards Institute (CLSI, [68]) standards. Based on the resistance profiles, the isolates were classified as MDR or extensively drug resistant (XDR) [69].

2.6. Screening for antibiotic resistance and virulence genes

The reference, environmental and clinical *E. faecium*, *K. pneumoniae*, and *P. aeruginosa* isolates were screened for the bla_{KPC} (carbapenem resistance), and *tetM* (tetracycline resistance) genes (Table 2). Only the *K. pneumoniae* and *P. aeruginosa* isolates were screened for the *mcr-1* and *mcr-5* (colistin resistance) genes (Table 2). The bla_{KPC} and *tetM* genes are amongst the most prevalent ARGs detected in the target species, while colistin is considered a last-resort antibiotic for the treatment of Gram-negative bacterial infections [35,36]. The presence of common virulence genes associated with each of the target species was determined for the respective *E. faecium* [*gelE* (encodes gelatinase production) and *esp* (encodes for the enterococcal surface protein)], *K. pneumoniae* [*fimH* (regulates the length and mediation of type 1 fimbriae adhesion) and *ugE* (encodes the synthesis of a uridine diphosphate galacturonate 4-epimerase)], and *P. aeruginosa* [*algD* (encodes for alginate production) and *phzM* (encodes for phenazine production)] isolates.

Amplification for each of the ARGs and virulence genes was performed using the PCR cycling parameters outlined in Table 2, Table S3 and Table S4. For each PCR assay, sterile milliQ was used as a negative control. To obtain positive controls for the ARGs and virulence genes, genomic DNA was extracted from wastewater (potential hotspot for the selection of AR, virulence, and HGT [79]) using the *Quick*-DNA Fecal/Soil Microbe Miniprep Kit (Zymo Research) according to the manufacturer's instructions. Following amplification, the PCR products were visualised, purified, concentrated sequenced and analysed as outline in Section 2.3.

2.7. Quantitative biofilm formation assay

The biofilm formation capabilities of the reference, clinical, and environmental *E. faecium*, *K. pneumoniae*, and *P. aeruginosa* isolates was determined using the methodology outlined in De Breij et al. [80] and Havenga et al. [81]. Briefly, all the *K. pneumoniae*, and *P. aeruginosa* isolates were inoculated into 5 mL LB broth (Biolab), while all the *E. faecium* isolates were inoculated into 5 mL Tryptic Soy broth (TSB; Biolab). The LB and TSB test tubes were incubated for 18–24 h at 37 °C. The overnight cultures were diluted (1:100) in LB (*K. pneumoniae* and *P. aeruginosa*) or TSB broth (*E. faecium*) (Biolab), and 50 μL of each dilution was aliquoted into a CELLSTAR® 96-well plate (Sigma-Aldrich) containing 150 μL LB (*K. pneumoniae* and *P. aeruginosa*) or TSB broth (*E. faecium*) (Biolab), respectively. Eight repeats were included for all experiments as well as a negative control of sterile LB/TSB broth (Biolab).

The 96-well plate was incubated for 48 h at 37 °C under static conditions to allow for biofilm formation. Following incubation, the planktonic cells were removed from the wells and the wells were washed with distilled water (ddH_2O). Thereafter, 1 % (v/v) crystal violet was used to stain the biofilms for 15 min, whereafter the crystal violet was removed, and the wells were washed with ddH_2O. Stained biofilms were solubilised with 95 % (v/v) ethanol and quantified at 570 nm (OD_{570}) using an iMark™ Microplate Absorbance Reader (Bio-Rad Laboratories).

Biofilm formation was assessed as per the criteria outlined by Stepanović et al. [82]. The OD cut off value (OD_c) was calculated based on the formula in Equation (1):

$$OD_c = \text{average OD of negative control} + (3 \times \text{SD of negative control}) \quad (1)$$

According to the OD readings, the isolates were divided into four categories: (1) strong biofilm producers ($4 \times OD_c < OD$); (2) moderate biofilm producers ($2 \times OD_c < OD \leq 4 \times OD_c$); (3) weak biofilm producers ($OD_c < OD \leq 2 \times OD_c$); and (4) non-biofilm producers ($OD \leq OD_c$) [82].

2.8. Haemolytic assay, hypermucoviscosity test, and gelatinase production

For the haemolytic activity assay, the reference, clinical and environmental *E. faecium*, *K. pneumoniae*, and *P. aeruginosa* isolates were inoculated into 5 mL LB broth (Biolab) and incubated overnight for 18–24 h at 37 °C. Ten microlitres (10 μL) of each overnight culture was spotted in triplicate onto sheep blood agar [5 % (w/v) sheep blood (ThermoFisher Scientific)]. The inoculated agar plates were incubated for 48 h at 37 °C, whereafter the haemolytic activity of the isolates was recorded. Isolates were categorised as alpha (α)-haemolytic (incomplete destruction of red blood cells in the medium), gamma (γ)-haemolytic (non-destruction of red blood cells in the medium), or beta (β)-haemolytic (complete destruction of red blood cells in the medium). A negative control (*K. pneumoniae* ATCC 13883) and a positive control (*Staphylococcus aureus* ATCC 25923) were included for the haemolytic assays [42].

The reference, clinical, and environmental *K. pneumoniae* isolates were assessed for the hypermucoviscous phenotype using a modified string test, as outlined by Mohammed and Flayyih [83]. The hypermucoviscosity test was not performed for *E. faecium* or *P. aeruginosa* as no protocols or literature exist on this phenomenon for these two species. The *K. pneumoniae* isolates were thus streaked

onto MacConkey agar (Biolab) and incubated for 18–24 h at 37 °C. A positive result (i.e., the hypermucoviscous phenotype) was recorded as the production of a viscous string ≥1 cm in length, by the isolates [83].

Gelatinase production was determined for the reference, clinical, and environmental *E. faecium*, and *P. aeruginosa* isolates using a gelatin liquefaction protocol [83]. The gelatinase test was not conducted for the *K. pneumoniae* isolates as this species does not produce gelatinase. Briefly, all the *E. faecium*, and *P. aeruginosa* isolates were streaked onto NA (Biolab) and incubated overnight at 37 °C. Following incubation, the streak plate cultures were stab inoculated into NA (Biolab) slants containing 12 % (w/v) gelatin (Difco Laboratories, Franklin Lakes, USA). Following inoculation, the agar slants were incubated at 26 °C for seven days. Thereafter, the tubes were cooled on ice for 30 min and cultures that remained liquefied after cooling were considered gelatinase positive (i.e., capable of hydrolysing gelatin) [83]. A negative control (*K. pneumoniae* ATCC 13883) and a positive control (*P. aeruginosa* ATCC 27853) were included for the gelatinase production assays.

2.9. Statistical analysis

All statistical analyses were conducted using Microsoft Excel version 2018 (Microsoft, Redmond, Washington, USA) and GraphPad Prism version 8.4.3 (GraphPad Software, San Diego, California, USA) software. Quantitative data were assessed using non-parametric Mann-Whitney *U* Test. Significance was observed at $p < 0.05$.

3. Results

3.1. Identification of the target pathogens

Culture-based analysis onto selective media presumptively isolated 123 *E.* faecium, 130 *K. pneumoniae*, and 54 *P. aeruginosa* from the environmental samples. For *E. faecium*, the highest isolation frequency of presumptive isolates was obtained at the Sir Lowry's Pass stream site [19.5 % (24/123)]. Similarly, the highest number of presumptive *K. pneumoniae* isolates were obtained from the Vlottenburg marsh site and Sir Lowry's Pass stream sites (18.5 % each; 24/130), while the highest number of presumptive *P. aeruginosa* isolates was obtained from the Vlottenburg marsh site (20.4 %; 11/54) (Fig. S3 and Table S5).

The environmental isolates were subsequently assigned code names based on their morphological species identity [*E. faecium* (Ef), *K. pneumoniae* (Kp), and *P. aeruginosa* (Pa)] and number of presumptive isolates obtained for each species isolated (i.e., Ef1, Ef2, Ef3 etc.) (Table S5). The code names of the clinical and reference isolates align to the code names used in the Water Resource Laboratory Culture Collection and the Water and Health Research Centre Culture Collection databases (Table S6).

Conventional species-specific PCR and DNA sequencing was performed on the presumptive environmental *E. faecium*,

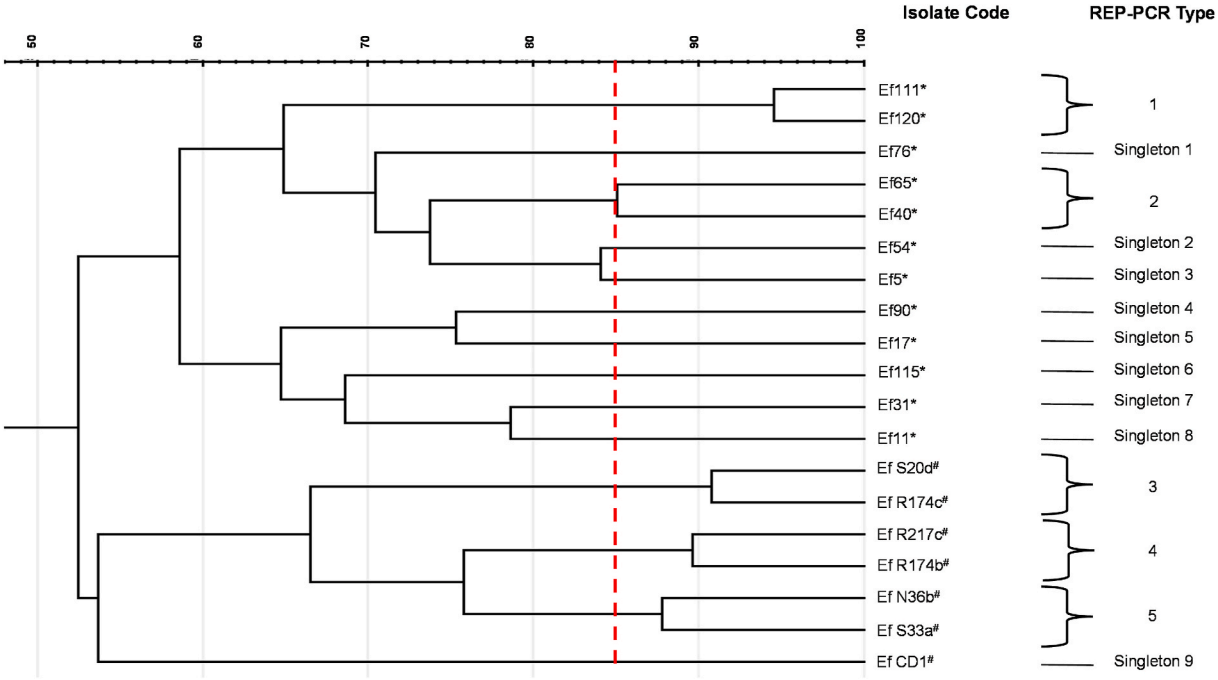

Fig. 2. REP-PCR dendrogram based on UPGMA and Pearson correlation coefficient, representing genetic similarity among the *E. faecium* isolates ($n = 19$). Red dashed line (——) refers to ≥ 85 % similarity [66]. * - Environmental Isolate. # - Clinical Isolate. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

K. pneumoniae, and *P. aeruginosa* isolates to confirm their identity (Table S7). Overall, 42.3 % (52/123) of the presumptive *E. faecium* isolates were positive for the *pstS* gene (583 bp) (Table S7). Additionally, positive species-specific PCR results were recorded for 27.7 % (36/130) of the presumptive *K. pneumoniae* isolates (*phoE* gene – 398 bp) and 66.7 % (36/54) of the presumptive *P. aeruginosa* isolates (*rhlB* gene – 226 bp) (Table S7). As many PCR-positive *E. faecium*, *K. pneumoniae*, and *P. aeruginosa* were obtained over the sampling period, the number of environmental isolates selected for further downstream analysis was reduced (i.e., 12 *E. faecium*, 9 *K. pneumoniae*, and 12 *P. aeruginosa*) based on the sampling session and sampling sites from which they were obtained.

Sequencing analysis further confirmed the identity of the reference (*K. pneumoniae* ATCC 13883 and *P. aeruginosa* ATCC 27853) and all clinical *E. faecium* (Ef CD1, Ef S33a, Ef N36b, Ef R217c, Ef R174b, Ef R174c, Ef S20d), *K. pneumoniae* (P2, P3, MCC3), and *P. aeruginosa* (MCC4, MCC5, PAO1) isolates as the correct species (Table S8). Therefore, in total 19 *E. faecium* (7 clinical and 12 environmental), 13 *K. pneumoniae* (1 reference, 3 clinical, and 9 environmental), and 16 *P. aeruginosa* (1 reference, 3 clinical, and 12 environmental) isolates were included in further analyses.

3.2. Repetitive extragenic palindromic sequence-based PCR (REP-PCR)

Genotyping of the reference, clinical and environmental *E. faecium* ($n = 19$), *K. pneumoniae* ($n = 13$), and *P. aeruginosa* ($n = 16$) isolates was conducted using REP-PCR, whereafter dendrograms were generated for each species respectively, using the GelJ software version 2.0 (Fig. 2 to Fig. 4). Overall, analysis of the DNA fingerprints for the *E. faecium* isolates resulted in the designation of five REP-PCR Types (REP-PCR Type 1 to 5; similarity ≥85 %) and nine singleton isolates (Fig. 2). For the environmental *E. faecium* isolates, cluster analysis designated Ef76, Ef54, Ef5, Ef90, Ef17, Ef115, Ef31, and Ef11 (Singleton 1 to 8, respectively) as singleton isolates (Fig. 2). Two REP-PCR Types were then identified for the remaining environmental isolates, as Ef111 and Ef120 grouped together in REP-PCR Type 1, while Ef65 and Ef40 grouped together in REP-PCR Type 2 (Fig. 2). For the clinical *E. faecium* isolates, cluster analysis designated three REP-PCR Types, with Ef CD1 (Singleton 9) designated as a singleton isolate. The clinical isolates Ef S20d and Ef R174c then grouped together in REP-PCR Type 3, while clinical isolates Ef R217c and Ef R174b grouped together in REP-PCR Type 4. The remaining clinical isolates, i.e., Ef N36b and Ef S33a, grouped together in REP-PCR Type 5 (Fig. 2).

For the environmental *K. pneumoniae* isolates, cluster analysis designated two REP-PCR Types, as Kp102, Kp103, Kp116, Kp105, Kp55, Kp82, and Kp53 grouped together in REP-PCR Type 2, while Kp111 and Kp60 were grouped into REP-PCR Type 3 (Fig. 3). For the clinical *K. pneumoniae* isolates, cluster analysis designated two REP-PCR Types. The clinical isolates MCC3 and P3 grouped together in REP-PCR Type 1, while the remaining clinical isolate, P2, was grouped in REP-PCR Type 3 alongside the environmental isolates Kp111 and Kp60 (Fig. 3). The reference strain, *K. pneumoniae* ATCC 13883, was then designated as a singleton isolate (Singleton 1; Fig. 3).

Cluster analysis of the environmental *P. aeruginosa* isolates then designated Pa48, Pa37, Pa41, and Pa33 (Singleton 1 to 4, respectively) as singleton isolates (Fig. 4). One REP-PCR Type was identified for the remaining environmental isolates, as Pa21, Pa28, Pa13, Pa12, Pa32, Pa29, Pa26, and Pa11, grouped together in REP-PCR Type 1. It should be noted that the clinical isolate PAO1 was grouped in REP-PCR Type 1, alongside the environmental isolates (Fig. 4). For the remaining clinical *P. aeruginosa* isolates, cluster analysis designated isolates MCC4 and MCC5 (Singletons 5 and 6, respectively) as singleton isolates. The reference strain, *P. aeruginosa* ATCC 27853, was also designated as a singleton isolate (Singleton 7; Fig. 4).

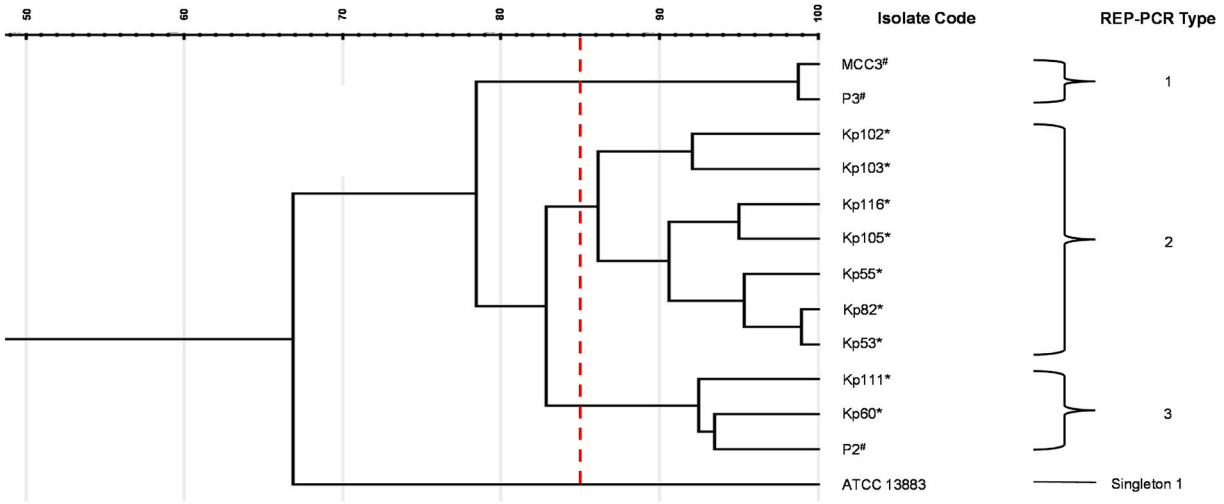

Fig. 3. REP-PCR dendrogram based on UPGMA and Pearson correlation coefficient, representing genetic similarity among the *K. pneumoniae* isolates ($n = 13$). Red dashed line (——) refers to ≥ 85 % similarity [66]. * - Environmental Isolate. # - Clinical Isolate. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

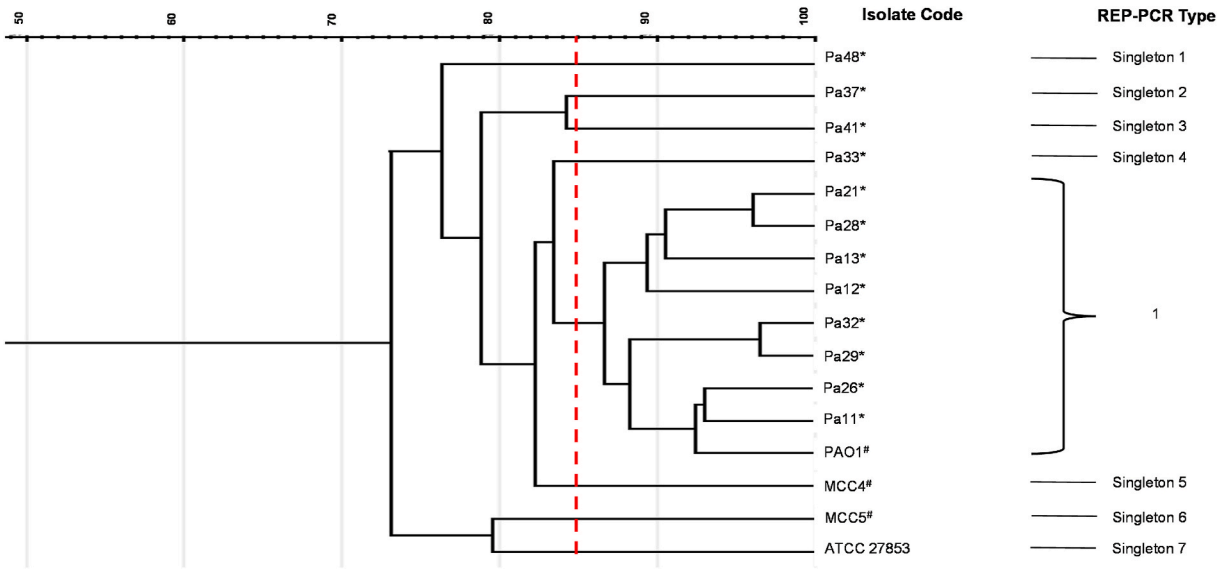

Fig. 4. REP-PCR dendrogram based on UPGMA and Pearson correlation coefficient, representing genetic similarity among the *P. aeruginosa* isolates ($n = 16$). Red dashed line (——) refers to ≥ 85 % similarity [66]. * - Environmental Isolate. # - Clinical Isolate. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

3.3. Kirby-Bauer antibiotic assay

The Kirby-Bauer assay was used to determine the resistance, intermediate susceptibility, or susceptibility (i.e., the antibiogram) of the reference, clinical, and environmental *E. faecium* ($n = 19$), *K. pneumoniae* ($n = 13$), and *P. aeruginosa* ($n = 16$) isolates, to various commercially available antibiotics (Table S2). Based on EUCAST [67] guidelines, in comparison to the clinical isolates, environmental *E. faecium* isolates exhibited increased susceptibility to the tested antibiotics (Fig. 5A). Overall, 63.2 % [12/19; environmental isolates ($n = 10$) and clinical isolates ($n = 2$)] of the *E. faecium* isolates exhibited identical antibiograms and were susceptible to 75 % (3/4) and intermediately susceptible to 25 % (1/4) of the tested antibiotics (Fig. 5A). Additionally, isolates Ef31 (environmental), Ef R217c (clinical), and Ef S33a (clinical) exhibited identical antibiograms, with 25 % (1/4) resistance and 75 % (3/4) susceptibility recorded

A

EUCAST								
Isolate Type	Isolate Code	Antibiotic						
		AMP	CIP	DO	LEV	MH	TE	IPM
Environmental	Ef11	S	R		R			I
	Ef5	S	S		S			I
	Ef40	S	S		S			I
	Ef31	S	S		S			R
	Ef17	S	S		S			I
	Ef65	S	S		S			I
	Ef54	S	S		S			I
	Ef76	S	S		S			I
	Ef90	S	S		S			I
	Ef120	S	S		S			I
	Ef111	S	S		S			I
	Ef115	S	S		S			I
Clinical	Ef N36b	S	R		R			I
	Ef R174b	S	S		S			I
	Ef R174c	S	R		S			I
	Ef R217c	S	S		S			R
	Ef S20d	S	S		S			I
	Ef S33a	S	S		S			R
	Ef CD1	R	R		R			R

B

CLSI								
Isolate Type	Isolate Code	Antibiotic						
		AMP	CIP	DO	LEV	MH	TE	IPM
Environmental	Ef11	S	R	R	I	R	R	
	Ef5	S	I	I	S	R	R	
	Ef40	S	S	S	S	S	S	
	Ef31	S	R	R	I	R	R	
	Ef17	S	I	S	S	S	I	
	Ef65	S	S	S	S	S	S	
	Ef54	S	I	S	S	S	S	
	Ef76	S	I	S	S	S	S	
	Ef90	S	I	R	S	R	R	
	Ef120	S	S	R	S	R	R	
	Ef111	S	I	S	S	S	S	
	Ef115	S	I	R	S	R	R	
Clinical	Ef N36b	S	R	S	I	S	S	
	Ef R174b	S	I	S	I	S	S	
	Ef R174c	S	R	S	I	S	S	
	Ef R217c	R	S	R	S	R	R	
	Ef S20d	S	I	S	S	S	S	
	Ef S33a	S	I	R	S	R	R	
	Ef CD1	R	R	R	R	R	R	

Fig. 5. Antibiotic susceptibility profiles of the *E. faecium* isolates, as determined using the Kirby-Bauer disc diffusion assay and classified according to the (**A**) EUCAST [67] and (**B**) CLSI [68] guidelines. Susceptible (S; Green); Intermediate (I; Orange); Resistant (R; Red); Thin diagonal stripes = Data not available (N/A) for EUCAST [67]/CLSI [68] guidelines, thus antibiotics not tested. Ampicillin (AMP), ciprofloxacin (CIP), doxycycline (DO), levofloxacin (LEV), minocycline (MH), tetracycline (TE), imipenem (IPM). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

against the tested antibiotics.

Similarly, the Ef11 (environmental) and Ef N36b (clinical) isolates exhibited identical antibiograms and were resistant to 50 % (2/4) of the antibiotics, while Ef R174c (clinical) was resistant to 25 % (1/4) of the tested antibiotics [67]. In contrast, the Ef CD1 (clinical) isolate exhibited resistance to 100 % (4/4) of the tested antibiotics (Fig. 5A).

Based on the CLSI [68] breakpoints, the Ef11 and Ef31 (both environmental) isolates exhibited identical antibiograms and were resistant to 66.6 % (4/6) of the antibiotics tested. The Ef5 (environmental) isolated exhibited resistance to 33.3 % (2/6) of the tested antibiotics, while isolate Ef120 (environmental) was resistant to 50 % (3/6) of the tested antibiotics (Fig. 5B).

The environmental isolates Ef40 and Ef65 displayed susceptibility to 100 % ($n = 6$) of the tested antibiotics, while isolates Ef54, Ef76, Ef111 (all environmental), and Ef S20d (clinical) were susceptible to 83.3 % (5/6) and intermediately susceptible to 16.7 % (1/6) of the tested antibiotics (EUCAST, [67]; Fig. 5B). Isolates Ef90, Ef115 (both environmental) and Ef S33a (clinical) exhibited identical antibiograms and were resistant to 50 % (3/6) of the tested antibiotics. The Ef N36b and Ef R174c (both clinical) isolates also exhibited identical antibiograms and were susceptible to 66.6 % (4/6), intermediately susceptible to 16.7 % (1/6) and resistant to 16.7 % (1/6) of the tested antibiotics. Similarly, the Ef R217c (clinical) was resistant to 66.7 % (4/6) of the tested antibiotics, while isolate Ef CD1 (clinical) exhibited 100 % (6/6) resistance to the tested antibiotics. While many of the environmental and clinical isolates were resistant to numerous antibiotics, overall, based on the EUCAST [67] and CLSI [68] guidelines, only isolate Ef CD1 was classified as XDR (Fig. 5A and B) [69].

In accordance with the EUCAST [67] breakpoints, the *K. pneumoniae* ATCC 13883 reference strain was the most susceptible isolate and exhibited resistance to only 11.1 % (2/18; ampicillin, ceftazidime) of the tested antibiotics (EUCAST, [67]; Fig. 6A). The environmental and clinical *K. pneumoniae* isolates then exhibited the highest and most diverse levels of resistance (Fig. 6A and B).

Environmental isolates, Kp55, Kp82, and Kp103 exhibited identical antibiograms and were resistant to 33.3 % (6/18) of the antibiotics, while isolates Kp53 and Kp116 were susceptible to 61.1 % (11/18), intermediately susceptible to 5.6 % (1/18), and resistant to 33.3 % (6/18) of the tested antibiotics. Isolate Kp111 displayed susceptibility to 77.8 % (14/18) and resistance to 22.2 % (4/18) of the tested antibiotics, while isolate Kp60 was susceptible to 44.4 % (8/18), intermediately susceptible to 11.1 % (2/18), and resistant to 44.4 % (8/18) of the antibiotics (EUCAST, [67]; Fig. 6A). Isolate Kp105 was found to be resistant to 27.8 % (5/18) of the tested antibiotics, while isolate Kp102 was the most resistant environmental isolate, with resistance observed towards 55.6 % (10/18) of the antibiotics analysed. The *K. pneumoniae* clinical isolate MCC3 was then susceptible to 27.8 % (5/18), intermediately susceptible to 5.6 % (1/18), and resistant to 66.6 % (12/18) of the tested antibiotics, while the *K. pneumoniae* isolates P2 and P3 were identified as the most resistant isolates, as they only displayed susceptibility to 22.2 % (4/18) of the tested antibiotics and were intermediately susceptible to 5.6 % (1/18) and resistant to 72.2 % (13/18) of the antibiotics analysed (EUCAST, [67]; Fig. 6A).

Comparable results were obtained for the CLSI [68] guidelines, with the reference strain (*K. pneumoniae* ATCC 13883) exhibiting resistance to 10.5 % (2/19) and increased susceptibility (89.5 %; 17/19) to the tested antibiotics (CLSI, [68]; Fig. 6B). In comparison to the CLSI [68] breakpoints, the environmental isolate Kp111 exhibited susceptibility to 78.9 % (15/19), intermediately susceptibility to 5.3 % (1/19), and resistance to 15.8 % (3/19) to the antibiotics analysed. Isolate Kp60 (environmental) was resistant to 26.3 % (5/19) of the antibiotics, while isolate Kp102 was resistant to 36.8 % (7/19) of the tested antibiotics (Fig. 6B). Isolates Kp53, Kp55, Kp82, Kp103, Kp105, and Kp116 displayed identical antibiograms and were susceptible to 63.2 % (12/19), and resistant to 36.8 % (7/19) of the tested antibiotics.

In comparison to the reference and environmental isolates, the clinical *K. pneumoniae* isolates exhibited increased resistance [68]. Isolate MCC3 was susceptible to 42.1 % (8/19), intermediately susceptible to 10.5 % (2/19), and resistant to 47.4 % (9/19) of the antibiotics, while isolates P2 and P3 were identified as the most resistant isolates, with resistance displayed towards 63.2 % (12/19) and 68.4 % (13/19) of the tested antibiotics, respectively (CLSI, [68]; Fig. 6A). Based on the breakpoint values defined in the EUCAST [67] and CLSI [68] guidelines, all the *K. pneumoniae* isolates except *K. pneumoniae* ATCC 13883 (reference strain), were classified as MDR (Fig. 6A and B) [69].

The *P. aeruginosa* environmental and clinical isolates exhibited similar antibiograms, with increased susceptibility to the tested antibiotics recorded according to the EUCAST [67] and CLSI [68] guidelines (Fig. 7A and B). According to EUCAST [67] guidelines, *P. aeruginosa* ATCC 27853 (reference strain) exhibited susceptibility to 41.7 % (5/12) and intermediate susceptibility to 58.3 % (7/12) of the tested antibiotics (Fig. 7A).

All the environmental *P. aeruginosa* ($n = 12$) isolates, as well as the clinical isolate PAO1, exhibited identical antibiograms and were susceptible to 25 % (3/12) and intermediately susceptible to 75 % (9/12) of the tested antibiotics (Fig. 7A). The clinical isolates MCC4 and MCC5 exhibited identical antibiograms and were resistant to only 8.3 % (1/12) of the tested antibiotics (EUCAST, [67]; Fig. 7A). Based on the CLSI [68] guidelines, 83.3 % (10/12) of the environmental *P. aeruginosa* isolates, as well as the clinical isolate MCC5, displayed 92.9 % (13/14) susceptibility and 7.1 % (1/14) intermediate susceptibility to the antibiotics tested (Fig. 7B). Similarly, the environmental isolate Pa13 was susceptible to 85.7 % (12/14) and intermediately susceptible to 14.3 % (2/14) of the antibiotics, while isolate Pa11 (environmental) was resistant to 7.1 % (1/14) of the antibiotics and susceptible (92.9 %; 13/14) to all the other antibiotics analysed. The *P. aeruginosa* ATCC 27853 (reference) and MCC4 and PAO1 (both clinical), displayed susceptibility to all (100 %; $n = 14$) of the tested antibiotics (Fig. 7B). In accordance with EUCAST [67] and CLSI [68] guidelines, none of the *P. aeruginosa* isolates were classified as MDR [69].

3.4. PCR analysis of antibiotic resistance and virulence genes

Based on the PCR analysis of the ARGs, 47.4 % (9/19) of the *E. faecium* [environmental ($n = 6$) and clinical ($n = 3$] isolates were PCR positive for the *tetM* gene(Table S9). However, while several of the environmental and clinical *K. pneumoniae* isolates (Fig. 6A and

A

Isolate Type	Isolate Code	EUCAST Antibiotic																			
		AK	ATM	AMP	CAZ	CIP	CN	CRO	DO	DOR	FEP	IPM	LEV	MEM	PRL	SAM	TE	SXT	TOB	TZP	W
Environmental	Kp53	S	S	R	S	S	S	S		S	I	S	S	S	R	R		R	S	R	R
	Kp55	S	S	R	S	S	S	S		S	S	S	S	S	R	R		R	S	R	R
	Kp60	S	R	R	S	I	S	R		S	R	S	I	S	R	R		R	S	S	R
	Kp82	S	S	R	S	S	S	S		S	S	S	S	S	R	R		R	S	R	R
	Kp103	S	S	R	S	S	S	S		S	S	S	S	S	R	R		R	S	R	R
	Kp105	S	S	R	S	S	S	S		S	S	S	S	S	R	R		R	S	S	R
	Kp102	S	R	R	I	S	R	R		S	R	S	S	S	R	R		R	R	S	R
	Kp116	S	S	R	S	S	S	S		S	I	S	S	S	R	R		R	S	R	R
	Kp111	S	S	R	S	S	S	S		S	S	S	S	S	R	S		R	S	S	R
Clinical	P2	S	R	R	R	R	R	R		S	R	I	R	S	R	R		R	S	R	R
	P3	S	R	R	R	R	R	R		S	R	I	S	S	R	R		R	R	R	R
	MCC3	S	R	R	R	R	R	R		S	R	I	S	S	R	R		R	R	S	R
Reference	ATCC 13883	S	S	R	R	S	S	S		S	S	S	S	S	S	S		S	S	S	S

B

Isolate Type	Isolate Code	CLSI Antibiotic																			
		AK	ATM	AMP	CAZ	CIP	CN	CRO	DO	DOR	FEP	IPM	LEV	MEM	PRL	SAM	TE	SXT	TOB	TZP	W
Environmental	Kp53	S	S	R	S	S	S	S	R	S	S	S	S	S		R	R	R	S	R	R
	Kp55	S	S	R	S	S	S	S	R	S	S	S	S	S		R	R	R	S	R	R
	Kp60	S	I	R	S	I	S	R	I	S	I	S	S	S		I	R	R	S	I	R
	Kp82	S	S	R	S	S	S	S	R	S	S	S	S	S		R	R	R	S	R	R
	Kp103	S	S	R	S	S	S	S	R	S	S	S	S	S		R	R	R	S	R	R
	Kp105	S	S	R	S	S	S	S	R	S	S	S	S	S		R	R	R	S	R	R
	Kp102	S	R	R	I	S	R	R	S	S	I	S	S	S		R	S	R	S	I	R
	Kp116	S	S	R	S	S	S	S	R	S	S	S	S	S		R	R	R	S	R	R
	Kp111	S	S	R	S	S	S	S	S	S	S	S	S	S		S	S	R	S	I	R
Clinical	P2	S	R	R	R	R	R	R	S	S	R	S	R	S		R	S	R	S	R	R
	P3	S	R	R	R	R	R	R	R	S	R	S	S	S		R	R	R	I	R	R
	MCC3	S	R	R	R	R	I	R	S	S	R	S	S	S		R	S	R	S	I	R
Reference	ATCC 13883	S	S	R	R	S	S	S	S	S	S	S	S	S		S	S	S	S	S	S

Fig. 6. Antibiotic susceptibility profiles of the *K. pneumoniae* isolates as determined using the Kirby-Bauer disc diffusion assay and classified according to the (**A**) EUCAST [67] and (**B**) CLSI [68] guidelines. Susceptible (S; Green); Intermediate (I; Orange); Resistant (R; Red); Thin diagonal stripes = Data not available (N/A) for EUCAST [67] and CLSI [68] guidelines, thus antibiotics not tested. Amikacin (AK), aztreonam (ATM), ampicillin (AMP), ceftazidime (CAZ), ciprofloxacin (CIP), doripenem (DOR), gentamicin (CN), ceftriaxone (CRO), doxycycline (DO), cefepime (FEP), imipenem (IPM), levofloxacin (LEV), meropenem (MEM), piperacillin (PRL), piperacillin-tazobactam (TZP), tobramycin (TOB), trimethoprim (W), ampicillin-sulbactam (SAM), tetracycline (TE), trimethoprim-sulfamethoxazole (SXT). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

B) exhibited tetracycline resistance based on the Kirby-Bauer assays, none of the *K. pneumoniae* isolates ($n = 13$) were PCR positive for the *tetM* gene. Similarly, none of the environmental and clinical *P. aeruginosa* isolates ($n = 16$) tested positive for the *tetM* gene (Table S9). In contrast, the bla_{KPC} gene was detected in 52.6 % (10/19) of the *E. faecium* [environmental ($n = 7$) and clinical ($n = 3$)] isolates (Table S9). Furthermore, only 15.4 % (2/13; Kp116 and Kp111) of the *K. pneumoniae* isolates, and none (0/16) of the *P. aeruginosa* isolates possessed the bla_{KPC} gene. The *mcr-1* and *mcr-5* genes were also not detected in any of the environmental and clinical *K. pneumoniae*, or *P. aeruginosa* isolates.

In addition, PCR analysis of the virulence genes in the target pathogens revealed that none (0/19) of the *E. faecium* isolates possessed the *esp* or *gelE* genes. In contrast, the *fimH* and *ugE* genes were present in 100 % ($n = 13$) of the reference, environmental and clinical *K. pneumoniae* isolates. Similarly, for the *P. aeruginosa* reference, clinical and environmental isolates, 100 % ($n = 16$) of the isolates possessed both the *phzM* and *algD* genes (Table S9).

3.5. Quantitative biofilm formation assay

Quantitative biofilm assays were performed to investigate the biofilm formation ability of all the *E. faecium*, *K. pneumoniae*, and *P. aeruginosa* isolates. An OD of 570 nm (OD_{570}) was used to measure biofilm formation, whereafter the OD_c was calculated using Equation (1) (Section 2.7) to classify the isolates as (1) strong biofilm producers ($4 \times OD_c < OD$); (2) moderate biofilm producers ($2 \times OD_c < OD \leq 4 \times OD_c$); (3) weak biofilm producers ($OD_c < OD \leq 2 \times OD_c$); and (4) non-biofilm producers ($OD \leq OD_c$).

Analysis of the biofilm assay results for the *E. faecium* isolates ($n = 19$) indicated that the clinical and environmental isolates analysed in the current study were poor biofilm formers. It was interesting to note that the clinical *E. faecium* isolates exhibited a significantly ($p < 0.05$) lower biofilm formation ability, with the average optical density (OD_{570}) recorded as 0.142 ± 0.041, compared to the environmental isolates, which had an average OD_{570} of 0.237 ± 0.077 (Fig. 8A). Overall, 66.7 % (8/12) of the environmental, and 57.1 % (4/7) of the clinical *E. faecium* isolates were weak biofilm formers ($OD_c < OD \leq 2 \times OD_c$), while 25 % (3/12) and 42.9 % (3/7) of the environmental and clinical *E. faecium* isolates respectively, were not capable of producing biofilms (i.e., non-formers; $OD \leq OD_c$). Only one (8.3 %; $n = 12$) environmental isolate (Ef11), was classified as a moderate biofilm producer ($2 \times OD_c < OD \leq 4 \times OD_c$).

In contrast to the results obtained for the *E. faecium* isolates, the clinical *K. pneumoniae* isolates exhibited a significantly ($p < 0.05$)

A

EUCAST														
Isolate Type	Isolate Code	Antibiotic												
		AK	ATM	CAZ	CIP	CN	DOR	FEP	IPM	LEV	MEM	NET	PRL	TOB	TZP
Environmental	Pa11	S	I	I	I		I	I	I	I	S		I	S	I
	Pa12	S	I	I	I		I	I	I	I	S		I	S	I
	Pa13	S	I	I	I		I	I	I	I	S		I	S	I
	Pa41	S	I	I	I		I	I	I	I	S		I	S	I
	Pa26	S	I	I	I		I	I	I	I	S		I	S	I
	Pa28	S	I	I	I		I	I	I	I	S		I	S	I
	Pa29	S	I	I	I		I	I	I	I	S		I	S	I
	Pa37	S	I	I	I		I	I	I	I	S		I	S	I
	Pa21	S	I	I	I		I	I	I	I	S		I	S	I
	Pa32	S	I	I	I		I	I	I	I	S		I	S	I
	Pa33	S	I	I	I		I	I	I	I	S		I	S	I
	Pa48	S	I	I	I		I	I	I	I	S		I	S	I
Clinical	MCC4	S	I	I	I		I	I	R	I	S		I	S	I
	MCC5	S	I	I	I		I	I	R	I	S		I	S	I
	PAO1	S	I	I	I		I	I	I	I	S		I	S	I
Reference	ATCC 27853	S	S	I	I		I	I	I	S	S		I	S	I

B

CLSI															
Isolate Type	Isolate Code	Antibiotic													
		AK	ATM	CAZ	CIP	CN	DOR	FEP	IPM	LEV	MEM	NET	PRL	TOB	TZP
Environmental	Pa11	S	S	S	S	S	S	S	S	S	S	R	S	S	S
	Pa12	S	S	S	S	S	S	S	S	S	S	I	S	S	S
	Pa13	S	I	S	S	S	S	S	S	S	S	I	S	S	S
	Pa41	S	S	S	S	S	S	S	S	S	S	I	S	S	S
	Pa26	S	S	S	S	S	S	S	S	S	S	I	S	S	S
	Pa28	S	S	S	S	S	S	S	S	S	S	I	S	S	S
	Pa29	S	S	S	S	S	S	S	S	S	S	I	S	S	S
	Pa37	S	S	S	S	S	S	S	S	S	S	I	S	S	S
	Pa21	S	S	S	S	S	S	S	S	S	S	I	S	S	S
	Pa32	S	S	S	S	S	S	S	S	S	S	I	S	S	S
	Pa33	S	S	S	S	S	S	S	S	S	S	I	S	S	S
	Pa48	S	S	S	S	S	S	S	S	S	S	I	S	S	S
Clinical	MCC4	S	S	S	S	S	S	S	S	S	S	S	S	S	S
	MCC5	S	S	S	S	S	S	S	I	S	S	S	S	S	S
	PAO1	S	S	S	S	S	S	S	S	S	S	S	S	S	S
Reference	ATCC 27853	S	S	S	S	S	S	S	S	S	S	S	S	S	S

Fig. 7. Antibiotic susceptibility profiles of the *P. aeruginosa* isolates as determined using the Kirby-Bauer disc diffusion assay and classified according to the (**A**) EUCAST [67] and (**B**) CLSI [68] guidelines. Susceptible (S; Green); Intermediate (I; Orange); Resistant (R; Red); Thin diagonal stripes = Data not available (N/A) for EUCAST [67] guidelines, thus antibiotics not tested. Amikacin (AK), aztreonam (ATM), cefepime (FEP), ceftazidime (CAZ), ciprofloxacin (CIP), doripenem (DOR), gentamicin (CN), imipenem (IPM), levofloxacin (LEV), meropenem (MEM), netilmicin (NET), piperacillin (PRL), piperacillin-tazobactam (TZP), tobramycin (TOB). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

higher capability to form biofilms (average OD_{570} of 0.622 ± 0.204; Fig. 8B) than the environmental isolates (average OD_{570} of 0.268 ± 0.027; Fig. 8B). More specifically, the reference strain (*K. pneumoniae* ATCC 13883) and 33.3 % (3/9) of the environmental isolates were classified as weak biofilm formers ($OD_c < OD \leq 2 \times OD_c$), while 66.7 % (6/9) of the environmental and 33.3 % (1/3) of the clinical isolates, were classified as moderate biofilm formers ($2 \times OD_c < OD \leq 4 \times OD_c$). The clinical isolates MCC3 and P3 (66.7 %; $n = 2$) were classified as strong biofilm formers ($4 \times OD_c < OD$).

The biofilm formation ability of the clinical and environmental *P. aeruginosa* isolates was, however, not significantly different ($p > 0.05$), with the environmental and clinical isolates exhibiting an average OD_{570} of 0.956 ± 0.077 and 0.993 ± 0.071, respectively (Fig. 8C). Overall, the reference strain (*P. aeruginosa* ATCC 27853), 100 % ($n = 3$) of the clinical isolates, and 83.3 % (10/12) of the environmental isolates, were classified as strong biofilm formers ($4 \times OD_c < OD$). The environmental isolates, namely Pa11 and Pa29, were classified as moderate biofilm formers ($2 \times OD_c < OD \leq 4 \times OD_c$).

3.6. Haemolytic assay, hypermucoviscosity test, and gelatinase production

To investigate the virulence and possible mode of persistence of all the *E. faecium*, *K. pneumoniae*, and *P. aeruginosa* isolates in environmental and clinical settings, various phenotypic virulence assays (i.e., the haemolytic assay, hypermucoviscosity test, and gelatinase production) were performed. Thereafter, the isolates were classified as either α-, β-, or γ-haemolytic, hyper-, or non-hypermucoviscous, and gelatinase positive or negative.

Upon analysis of the haemolytic activity assay results, all the environmental and clinical *E. faecium* ($n = 19$) and *K. pneumoniae* ($n =$

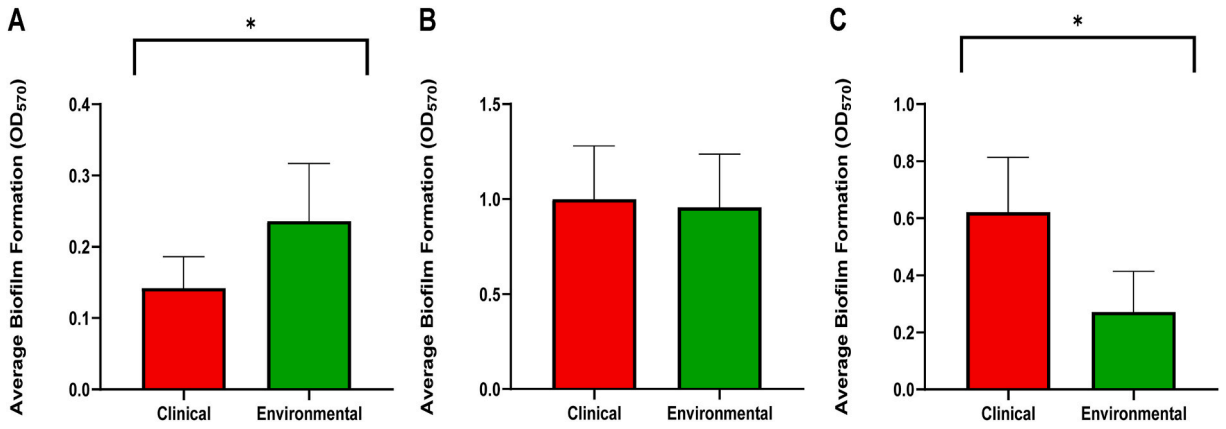

Fig. 8. Average biofilm formation at 37 °C after 48 h of the (**A**) *E. faecium*, (**B**) *P. aeruginosa* and (**C**) *K. pneumoniae* environmental and clinical isolates, measured by staining with 1 % (v/v) crystal violet solution for measurement of the optical density at OD_{570}. Standard deviation is represented by the error bars. Statistical analysis was performed using a non-parametric Mann-Whitney *U* Test.; * - $p < 0.05$. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

13) isolates grew as greyish colonies on blood agar plates, and did not display haemolytic activity (i.e., γ-haemolytic phenotype). In contrast, all ($n = 16$) the reference, environmental and clinical *P. aeruginosa* isolates grew as grey-green colonies and displayed haemolytic activity (i.e., β-haemolytic phenotype) on the blood agar plates (Fig. S5). As indicated in Section 2.8, the hypermucoviscosity test was conducted on all ($n = 13$) the reference, environmental and clinical *K. pneumoniae* isolates however, none (0 %) displayed the hypermucoviscous phenotype (results not shown). The gelatinase test was conducted on all the *E. faecium* ($n = 19$), and *P. aeruginosa* ($n = 16$) isolates, but not for the *K. pneumoniae* isolates ($n = 13$) as this species is considered gelatinase negative [70]. Overall, none (0 %) of the *E. faecium* isolates displayed gelatinase production. In contrast, 100 % ($n = 16$) of the reference, environmental and clinical *P. aeruginosa* isolates produced gelatinase and were capable of hydrolysing gelatin.

4. Discussion

The antibiotic resistance and virulence profiling of *E. faecium*, *K. pneumoniae*, and *P. aeruginosa* isolated from environmental water sources in informal settlements in the Western Cape (SA) were compared to clinical strains. Culture- and molecular-based analyses detected high frequencies of *E. faecium, K. pneumoniae* and *P. aeruginosa* in the environmental water samples obtained from both Vlottenburg informal settlement and Sir Lowry's Pass Village. As enterococci and coliform bacteria (which includes *K. pneumoniae*) are common indicators of faecal contamination, the presence of specifically *E. faecium* and *K. pneumoniae* suggests increased contamination with human and animal faecal matter [13–15,71]. This result is similar to the observation made by Salvador et al. [84], where coliform bacteria were detected in 100.0 % (24/24), faecal coliforms in 75.0 % (18/24), *E. coli* in 70.8 % (17/24), and intestinal enterococci in 66.7 % (16/24) of surface water samples (river and a dam reservoir) collected in Portugal. The authors hypothesised that the faecal contamination may have been due to the close proximity of the sampling point to industrial, agricultural, and animal farming activities, where manure-based fertiliser was used. The faecal contaminants may have permeated the soil or directly drained into the river water [84]. Correspondingly, Lepuschitz et al. [85] observed that all water samples obtained from rivers located on the outskirts of cities in Austria were negative for carbapenemase- and ESBL-producing *K. pneumoniae*, while all samples collected one to 3 km downstream from the primary wastewater treatment plant (WWTP) release points in Austria, were positive for these specific *K. pneumoniae* [85]. The results of this study thus confirmed that the release of wastewater effluents into the environment may impact the bacterial communities present in aquatic environments [86]. In line with these studies, both areas sampled (i.e., Vlottenburg informal settlement and Sir Lowry's Pass Village) in the current study were located close to farming communities, WWTPs and communal ablution facilities (located at specific points in informal settlements).

According to literature, *P. aeruginosa* is commonly recovered from wet and moist sites such as soils and plants, as well as several aquatic environments [87]. It is thus hypothesised that the high isolation frequency of *P. aeruginosa* from the marsh water in the Vlottenburg informal settlement and Sir Lowry's Pass Village, may be due to these sites acting as catchments for rainwater, environmental and human waste. The discarded greywater (which ultimately leads to streams/pools of surface runoff) regularly contains cleaning agents and chemicals, which may affect the survival of microorganisms in surface runoff [88]. However, the increased isolation frequency in the Sir Lowry's Pass surface runoff samples is indicative of this species' survival and persistence in environmental water sources [89]. This is concerning as children were often observed to play alongside the sites where the surface runoff samples were collected and may thus be at higher risk of contracting life-threatening infections or diseases from these water sources.

As genotyping often indicates that pathogenic environmental isolates may originate from clinical environments [90], the genetic relatedness of the reference, clinical and environmental *E. faecium*, *K. pneumoniae*, and *P. aeruginosa* species was determined using REP-PCR analysis. Based on *E. faecium* REP-PCR analysis, five REP-PCR Types (REP-PCR Type 1 to 5; similarity ≥85 %) and nine singleton isolates were identified, with no clustering [low (~52 %) genetic similarity] observed for clinical and environmental isolates.

In contrast, cluster analysis for *K. pneumoniae* identified only one singleton isolate, while the remaining isolates grouped together across three REP-PCR Type groups. Notably, clinical isolates (MCC3 and P3) clustered together whereas clinical isolate (P2) grouped with two of the environmental isolates (Kp111 and Kp60) in REP-PCR Type 3. The environmental isolates clustered together in REP-PCR Type 2 as all isolates were obtained from the same sampling site (Sir Lowry's Pass stormwater runoff). In addition, clustering of clinical and environmental isolates (Kp111; Kp60 and P2) suggests the potential contamination of the sampling site with clinical waste (Sir Lowry's Pass stream). This is further validated as a local healthcare centre is located within ~260 m of the sampling site, which may have served as a reservoir for the *K. pneumoniae* isolates. For *P. aeruginosa*, cluster analysis revealed seven singleton isolates, and only one REP-PCR Type group. Notably, one clinical isolate (PAO1) shared genetic similarity of ~87 % within the REP-PCR Type 1 group with eight environmental isolates (Pa21, Pa28, Pa13, Pa12, Pa32, Pa29, Pa26, Pa11). The inclusion of the environmental and clinic isolates in one group suggest that environmental isolates may have originated from a clinical setting, as the sampling sites are also located in close proximity to medical clinics present in both the Vlottenburg informal settlement and Sir Lowry's Pass Village.

While limited literature is available on the extensive epidemiological survey of *E. faecium*, *K. pneumoniae*, and *P. aeruginosa* in environmental reservoirs, REP-PCR has previously been implemented in the medical, agricultural and food industries for the identification of contamination sources, cross-transmission routes, and the recognition of virulent strains [91,92]. For example, Farkas et al. [92] investigated the extent to which anthropogenic contamination contributes to the spread of antibiotic resistant enterococci in aquatic environments and additionally explored the genetic relationships (REP-PCR with ERIC primers) amongst *Enterococcus* strains (i.e., *E. avium, E. faecalis* and *E. faecium*). In contrast to the current study, Farkas et al. [92] observed that *E. faecium* isolates from aquatic matrices exhibited the same or similar ERIC-PCR fingerprints to that of hospital-derived variants as they clustered within the same clade. This is significant as *E. faecium* has been associated with HA infections, however the bacteria are rarely observed amongst healthy individuals [92]. The results of the current study are however, comparable to observations made by Serrano et al. [93], as the *P. aeruginosa* REP-PCR demonstrated that a wide genetic diversity was observed across the *P. aeruginosa* ($n = 16$) clinical and environmental isolates. Similarly, Serrano et al. [93] used the REP-PCR technique to investigate the genetic relatedness of *P. aeruginosa* isolates ($n = 73$), obtained from veterinary samples (e.g., wild animals as well as zoo and farm animals), to a global population [i.e., environmental, and clinical (animal and human) samples from around the world] of *P. aeruginosa*. Based on the comparative genotyping no animal-specific cluster was detected, with the authors concluding that *P. aeruginosa* isolates obtained from animals form a population with a non-clonal epidemic structure (i.e., there are no explicit clones of *P. aeruginosa* that are related to specific animal species, habitats, or associated with particular diseases) and cannot be distinguished from the globally circulating population of *P. aeruginosa* (in which no "animal clonal lineage" was detected).

Based on the antibiotic susceptibility assays selective environmental *E. faecium* isolates exhibited resistance to one or more of the antibiotics tested, however none were classified as MDR. In contrast, based on the EUCAST [67] and CLSI [68] breakpoints, clinical isolate Ef CD1, was classified as XDR. While *E. faecium* is not inherently resistant to tetracycline antibiotics, a common pattern of resistance was observed towards tetracyclines (i.e., minocycline, doxycycline, tetracycline) in the environmental and clinical isolates, based primarily on the CLSI [68] breakpoints. These resistance profile results, for the environmental *E. faecium* isolates, are comparable to results obtained by Dos Santos et al. [16], where environmental isolates ($n = 40$) exhibited susceptibility to ampicillin while resistance towards several antibiotics including tetracycline ($n = 10$; 25 %), doxycycline ($n = 10$; 25 %), minocycline ($n = 6$; 15 %) was recorded. Tetracyclines are widely used in human and animal medicine due to the broad-spectrum activity and lower cost of application [94]. However, tetracyclines are not easily metabolised in the human and animal digestive system leading to 50–80 % excretion in human and animal excrement [94,95]. Indiscriminate and excessive use of tetracyclines has also resulted in increased bacterial resistance and significant fluctuations in environmental concentrations. The tetracycline resistance observed amongst *E. faecium* environmental and clinical isolates in the current study may thus be due to the presence of tetracyclines at the sample site environments. In addition to tetracycline, penicillins (ampicillin), fluoroquinolones (ciprofloxacin, levofloxacin), and carbapenem (imipenem) resistance was observed amongst specific *E. faecium* isolates. As with tetracycline, ciprofloxacin (fluoroquinolone) is one of the most common globally used antibiotics and is similarly excreted by humans and animals, who fail to metabolise the antibiotic, leading to the increased flow of the antibiotic into environmental waters via hospital wastewater or animal waste from the agricultural sector [96–99]. Furthermore, factors such as chromosomal mutations, acquired resistance genes, and chromosomally encoded efflux pumps may have played a role in promoting the development of resistance amongst the clinical and environmental isolates [100].

For *K. pneumoniae*, clinical and environmental strains displayed high levels of resistance, with all (excluding the reference *K. pneumoniae* ATCC 13883) exhibiting resistance to ampicillin, piperacillin, trimethoprim-sulfamethoxazole and trimethoprim. As a result, clinical and environmental *K. pneumoniae* isolates were resistant to three or more of the tested antibiotics, based on both EUCAST [67] and CLSI [68] breakpoint values and were classified as MDR. The observed antibiotic resistance may have resulted from several mechanisms including chromosomal mutations, however, most antibiotic resistant strains of *K. pneumoniae* develop resistance via the acquisition of ARGs (due to the HGT of large, conjugative plasmids) [32,40,101]. The results obtained in the current study, for the antibiogram of the clinical and environmental *K. pneumoniae* isolates, correspond to results outlined by Giri et al. [102], where the antibiotic resistance profiles of *K. pneumoniae* isolated from clinical [stool samples ($n = 15$)] and environmental [fish market effluent ($n = 38$) and well water; ($n = 11$)] samples were investigated. Overall, *K. pneumoniae* isolates from stool samples exhibited resistance to ampicillin (86.6 %), ceftazidime (80 %), cefepime (80 %), ceftazidime clavulanic acid (66.6 %), whereas isolates from fish market effluent ($n = 38$) exhibited resistance to ampicillin (92.1 %), cefoperazone–sulbactam (92.1 %), and piperacillin–tazobactam (42.1 %), while well water ($n = 11$) isolates exhibited resistance to ampicillin (100 %), cefuroxime (54.5 %), ertapenem (36.3 %), and meropenem (36 %).

In contrast to *E. faecium* and *K. pneumoniae*, *P. aeruginosa* isolates were highly susceptible or intermediately susceptible to the tested antibiotics, with only one environmental isolate (Pa11) exhibiting resistance to netilmicin (aminoglycoside), based on CLSI [68]

guidelines, while two ($n = 3$) clinical isolates (MCC4 and MCC5) exhibited resistance to imipenem (carbapenem), based on EUCAST [67] guidelines. The current results are comparable to Liew et al. [54], where the environmental isolates ($n = 104$) obtained from different fresh water sources exhibited 100 % susceptibility to ceftazidime and amikacin, gentamicin and netilmicin (aminoglycosides), while low resistance to piperacillin-tazobactam (1.8 %) and ciprofloxacin (1.8 %), and relatively higher resistance to piperacillin (4.4 %), doripenem (11.4 %), meropenem (8.8 %) and imipenem (2.6 %) (carbapenems), was recorded. Several articles have also indicated that clinical *P. aeruginosa* isolates exhibit inherent resistance to multiple antibiotics [103–105]. Moreover, Hafiz et al. [104] investigated the epidemiological, microbiological and clinical characteristics of clinical *P. aeruginosa* isolates ($n = 3579$), with results indicating that strains were sensitive to amikacin (92.6 %) and resistant to aztreonam (29.8 %), imipenem (29.5 %), ceftazidime (26.1 %), meropenem (25.6 %), and cefepime (24.3 %). While not investigated by the authors, the increased resistance could be attributed to various resistance mechanism primarily being associated with the low permeability of the cell envelope, the presence of multidrug efflux pumps, enzymes that inactivate antibiotics, and detoxification systems that are chromosomally encoded in this species [103].

As the Kirby-Bauer assay results indicated that many of the *E. faecium* and *K. pneumoniae* isolates exhibited phenotypic resistance to tetracyclines (e.g., minocycline, doxycycline and tetracycline), PCR analysis was conducted to screen for the *tetM* gene (tetracycline resistance) in the *E. faecium*, *K. pneumoniae*, or *P. aeruginosa*[1] isolates. Subsequently, the *tetM* gene was detected in 47.4 % [9/19; environmental ($n = 6$) and clinical ($n = 3$)] of the *E. faecium* isolates, while none of the *K. pneumoniae* or *P. aeruginosa* isolates possessed the gene. Dos Santos et al. [16] also reported that amongst 40 *E. faecium* isolates, the *tetM* gene was the second most detected gene and occurred in 22.5 % ($n = 9$) of the *E. faecium* isolates. The phenotypic tetracycline resistance observed in many of the *K. pneumoniae* isolates may, however, have been induced via other tetracycline resistance genes (e.g., *tetA*, *tetB*, *tetO*, *tetL*) and/or mutations, efflux pumps, and/or mutations in the ribosomal binding site [106,107].

The bla_{KPC} gene (carbapenem resistance) was also screened for in the *E. faecium*, *K. pneumoniae*, and *P. aeruginosa* isolates [108,109] and was detected in 52.6 % (10/19) of the *E. faecium* isolates [environmental ($n = 7$) and clinical ($n = 3$)]. Although carbapenemase genes are mostly found in MDR pathogens such as *K. pneumoniae*, *Acinetobacter* spp., and *P. aeruginosa*, mobile genetic elements allow for the easy transfer (via HGT) of these genes to other species [109], and to the best of our knowledge, this is the first report of the presence of the bla_{KPC} gene in *E. faecium*. Although carbapenemase producing *Klebsiella* spp. have been detected in non-clinical environments such as WWTPs (bla_{GES} variants, bla_{IMP}, bla_{KPC}, and bla_{VIM}), agricultural samples (bla_{KPC}, $bla_{OXA\text{-}48}$, and bla_{NDM}), lakes and rivers ($bla_{NDM\text{-}1}$), only 15.4 % of the *K. pneumoniae* [two environmental isolates (Kp116 and Kp111)] isolates were found to harbour the bla_{KPC} gene [110–113]. Similarly, in the study conducted by Ebomah and Okoh [8], where *Klebsiella* spp. ($n = 234$) were isolated from various environmental niches (i.e., hospitals, farms, rivers, WWTPs) in the Eastern Cape Province (SA), low incidence rates of the bla_{KPC} gene (7.3 %; $n = 17$) were detected. The absence of this gene in most of the isolates analysed in the current study, corresponds to the Kirby-Bauer assay results, where none of the isolates were resistant to any of the tested carbapenems (i.e., imipenem, meropenem, doripenem). Nonetheless, KPC-producing bacteria are becoming an emerging threat due to their increased resistance profiles, and significant association with increased morbidity and mortality [114,115]. In comparison to *E. faecium* and *K. pneumoniae*, none of the environmental or clinical *P. aeruginosa* isolates ($n = 16$), possessed the bla_{KPC} gene. *Klebsiella* carbapenemase (KPC; Ambler class A β-lactamase) has however, been reported in *P. aeruginosa* strains [116]. Thus, while none of the clinical or environmental *P. aeruginosa* isolates possessed the bla_{KPC} gene in this study, it is possible for *P. aeruginosa* to possess KPC gene variations [115].

The *mcr-1* and *mcr-5* genes (colistin resistance) were also screened for in the *K. pneumoniae* and *P. aeruginosa* isolates, with none of the isolates containing these genes. Moreover, while various clinical isolates in SA have been found to possess the *mcr-1* gene [117], limited data is available on the environmental distribution of the *mcr-1* gene in the South African environment [118,119]. It has however, been detected in *E. coli* isolates obtained from pigs, broiler chickens and final effluents of WWTPs in SA and is thus potentially present in environmental waters [120–122]. In contrast, a recent study conducted by Snyman et al. [117] detected *mcr-3* gene variants and the *mcr-5.1* gene in water samples collected from the Berg-, Eerste- and Plankenburg rivers, as well as in stormwater from Muizenburg and Fish Hoek (*mcr-5.1* gene only) (Western Cape, SA).

Several virulence assays (i.e., biofilm formation, haemolytic activity, gelatinase production, and hypermucoviscosity) and virulence gene PCRs were also performed on the *E. faecium*, *K. pneumoniae*, and *P. aeruginosa* isolates to determine their possible mode of persistence in environmental and clinical settings. Overall, the *E. faecium* isolates ($n = 19$) were not effective biofilm formers, with 31.6 % (6/19), 63.3 % (12/19) and 5.3 % (1/19) characterised as non-biofilm formers, weak biofilm formers and moderate biofilm formers, respectively. Biofilm formation of *Enterococcus* spp. has been linked to the presence of virulence genes such as *esp* (enterococcal surface protein), *asa* (aggregation substance), *ebp* (endocarditis and biofilm-associated pili), *gelE* (gelatinase production), amongst others [26]. In the current, study, all the *E. faecium* isolates then displayed γ-haemolytic (i.e., non-haemolytic) activity on blood agar media, and were not capable of hydrolysing gelatin. These results correspond to previous findings as *E. faecium* is known to display low haemolytic activity, while the production of gelatinase is more commonly associated with *E. faecalis* [123,124]. Results of the PCR assays then indicated that none of the *E. faecium* isolates ($n = 19$) possessed the *gelE* or *esp* genes. The absence of the *gelE* gene in the *E. faecium* isolates thus confirms the lack of phenotypic gelatinase production observed. While many studies have detected the *esp* gene in *E. faecium* isolates [16,26,123] the result of the current study correspond to the study conducted by Rehman et al. [29], where the *esp* gene was not detected in *E. faecium* isolates obtained from clinical, sewage and freshwater samples. As the *gelE* and *esp* genes have been linked to biofilm formation [26,28] the lack of these genes in the *E. faecium* isolates in the current study may elucidate

[1] Tetracycline was not tested for using the Kirby-Bauer analysis, due to a lack of EUCAST [67] and CLSI [68] breakpoint values for tetracycline resistance in *P. aeruginosa*.

their low biofilm formation capabilities. Research has additionally shown that the enterococcal surface protein is restricted to HA *E. faecium* isolates and may be associated with the clonal complex 17 (CC17) subpopulation [125]. This was further validated by the study conducted by Ferguson et al. [126] where the *gelE* and *esp* genes were found to have a higher prevalence in human samples (clinical and non-clinical), in comparison to environmental (sewage, plants, water), and animal samples (birds and dogs).

For *K. pneumoniae*, 30.8 % (4/13) of the isolates were classified as weak biofilm formers, 53.8 % (7/13) as moderate biofilm formers, and 15.4 % (2/13) as strong biofilm formers, while none of the isolates displayed the hypermucoviscous phenotype. The non-hypermucoviscous phenotype that was observed in all the isolates is likely because the gene responsible for this phenotype (*rmpA*) is carried on virulence plasmids which are not generally found in "common" or "classic" *K. pneumoniae* strains [127]. All the *K. pneumoniae* isolates exhibited the γ-haemolytic phenotype which is similar to the results reported by Mohammed et al. [128], where 54 *K. pneumoniae* isolates of clinical origin did not exhibit any haemolytic activity. The absence of haemolytic activity in the *K. pneumoniae* isolates may thus be attributed to the lack of activators required for the expression of genes encoding haemolytic enzymes [129,130]. The primary virulence factors enabling *K. pneumoniae* biofilm growth includes the capsule, capsular polysaccharide CPS (*cpsD*, *treC*, *wabG*, *wcaG*, *wzc*, *k2A*, and *wzyK2*), and type 1 and 3 fimbriae (*fimA*, *fimH*, *mrkA* and *mrkD*) [43,131,132]. The presence of the *ugE* (CPS) and *fimH* (fimbriae) genes were therefore screened for in the *K. pneumoniae* isolates, with 100 % ($n = 13$) of the *K. pneumoniae* isolates possessing the *fimH* and *ugE* genes. These genes (*ugE* and *fimH*) are commonly found in both clinical and environmental *K. pneumoniae* isolates [42,133]. For example, Barati et al. [42] detected both the *ugE* and *fimH* virulence genes in 70.9 % ($n = 55$) environmental (estuary water and sediment) *K. pneumoniae* isolates. Similarly, Shen et al. [133] detected the *ugE* and *fimH* virulence genes amongst 89 (94.7 %) and 87 (92.6 %) carbapenem resistant clinical *K. pneumoniae* isolates ($n = 94$). The detection of these virulence genes amongst both environmental and clinical isolates, is noteworthy as *ugE* and *fimH* have been linked to the infectivity of *K. pneumoniae* [134]. This is particularly concerning given that in the current study, many of these isolates were obtained from water sources collected from densely populated informal settlements.

Overall, 87.5 % (14/16) of the *P. aeruginosa* isolates were classified as strong biofilm producers, with two isolates (environmental; Pa11 and Pa29) classified as moderate biofilm formers. Biofilm formation is a key virulence factor of *P. aeruginosa* and is mediated by rhamnolipid production, LPS, elastase, and alginate [135,136]. The β-haemolytic phenotype and gelatin hydrolysis was observed in all the *P. aeruginosa* isolates in the current study. Secretion of toxic haemolysins by *P. aeruginosa* enables the spread of this pathogen throughout the target tissues. In contrast, gelatinase production facilitates gelatin (found in bones, skin, and cartilage) degradation allowing for the release and uptake of nutrients (e.g., amino acids, peptides and polypeptides) [137]. While screening for a gelatinase production gene in *P. aeruginosa* is recommended for confirmation, research predominantly focuses on phenotypic analysis of gelatinase production, with limited literature confirming the gene associated with gelatinase production [138,139]. However, as the *phzM* (pyocyanin production; green-blue secondary metabolite) and *algD* (alginate production) genes also contribute to biofilm formation and protection from oxidative stress (via the production of reactive oxygen species) in *P. aeruginosa*, respectively [140], these two genes were screened for in the *P. aeruginosa* isolates [141]. The results of the PCR analysis indicated that 100 % ($n = 16$) of the *P. aeruginosa* isolates analysed in this study possessed both the *phzM* and *algD* virulence genes. Similarly, Martins et al. [142] observed the presence of the *algD* gene amongst clinical (70 %; $n = 20$), water (75 %; $n = 20$), and soil (30 %; $n = 20$) *P. aeruginosa* isolates, suggesting that although clinical isolates primarily express this gene [141], it may also be found in environmental strains. The results also align to the results reported by Eladawy et al. [143], where the *algD* gene was detected in 100 % ($n = 103$) of the *P. aeruginosa* isolates, while also observing a significant ($p < 0.001$) correlation between the presence of the *pelA* and *phzM* genes and strong biofilm formation. The high detection of the *algD* and *phzM* genes amongst all the *P. aeruginosa* isolates ($n = 16$) analysed in the current study may thus have contributed to the strong biofilm forming capabilities that were observed.

5. Conclusions

With the exception of the clinical *P. aeruginosa* (PAO1) and clinical *K. pneumoniae* (P2), which exhibited high genetic similarity to the environmental isolates, the REP-PCR analysis indicated low genetic similarity or relatedness between clinical and environmental isolates of *E. faecium*, *K. pneumoniae*, and *P. aeruginosa*. The clinical and environmental *E. faecium* isolates also shared similar resistance profiles, with one clinical isolate (Ef CD1) classified as XDR. In contrast, all the *K. pneumoniae* isolates (except *K. pneumoniae* ATCC 13883) were classified as MDR, while *P. aeruginosa* isolates exhibited increased susceptibility to the tested antibiotics. Correspondingly, *E. faecium* isolates possessed the *tetM* and bla_{KPC} genes at higher detection rates in compared to the other species, while *K. pneumoniae* isolates possessed the bla_{KPC} gene. Biofilm formation capabilities also varied amongst the species, with environmental *E. faecium* isolates exhibiting higher biofilm capabilities, while clinical *K. pneumoniae* isolates displayed stronger biofilm formation. In contrast, most of the clinical and environmental *P. aeruginosa* isolates were characterised as strong biofilm formers. Additionally, virulence characterisation revealed species specific differences, with *E. faecium* isolates lacking the presence of the genes associated with gelatinase (*esp*) and enterococcal surface protein (*gelE*) production and exhibited no haemolytic and gelatinase activity. All *K. pneumoniae* isolates possessed the genes associated with type 1 fimbriae adhesion (*fimH*) and CPS (*ugE*) production but did not exhibit haemolytic activity and hypermucoviscosity. In contrast, all the *P. aeruginosa* possessed both the genes associated with alginate (*algD*) and phenazine production (*phzM*) and exhibited haemolytic activity and gelatinase production.

Overall, the study thus highlighted the similarities and differences in the antibiotic resistance and virulence characteristics of environmental isolates obtained from surface water collected from two informal settlements located in SA versus clinical isolates of *E. faecium*, *K. pneumoniae*, and *P. aeruginosa*. While previous studies have investigated the genetic comparison of clinical isolates, limited research has been conducted or published on the comparison of environmental and clinical *E. faecium*, *K. pneumoniae*, and *P. aeruginosa* isolates. This comparison is crucial as the presence of these antibiotic resistant bacterial species in environmental

reservoirs is of significant concern to communities in LMIC countries, who may rely on exposed environmental water sources for their daily water needs.

Several limitations, however, need to be addressed in future research, including the selection of a larger number of sampling sites and sample size, with a focus on incorporating more regions within SA. Constraints in terms of genetic analysis and antibiotic susceptibility testing, and antibiotic resistance and virulence gene detection must also be expanded upon through the inclusion of MLST, WGS and broth microdilutions. Additionally, virulence assays such as cytotoxicity assays, liquid haemolysis assays, adherence and invasion assays, quorum sensing assays and in vivo infections assays, should be included in further studies.

Institutional review board statement

Ethical clearance was granted by the Research Ethics Committee: Biosafety and Environmental Ethics (REC: BEE) at Stellenbosch University (Ref No. BEE-2019-9466).

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Data availability statement

The datasets generated during the current study are available from the corresponding author upon reasonable request.

CRediT authorship contribution statement

Julia Denissen: Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Benjamin Havenga:** Writing – review & editing, Writing – original draft, Supervision. **Brandon Reyneke:** Writing – review & editing, Supervision, Data curation, Conceptualization. **Sehaam Khan:** Writing – review & editing, Resources, Funding acquisition. **Wesaal Khan:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Wesaal Khan reports equipment, drugs, or supplies was provided by Water Research Commission. Wesaal Khan reports equipment, drugs, or supplies was provided by National Research Foundation.

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Appendix A. Supplementary data

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