Antibiotic resistant gene of Pseudomonas aeruginosa from non-clinical environment: public health implications in Mthatha, Eastern Cape Province, South Africa

Mojisola C. Hosu
Walter Sisulu University Faculty of Health Sciences
Sandeep Vasaikar
Walter Sisulu University Faculty of Health Sciences
Grace. E. Okuthe
Walter Sisulu University
Teke Apalata (ruffinapalata@gmail.com)
Walter Sisulu University Faculty of Health Sciences https://orcid.org/0000-0002-1329-3864

Research article

Keywords: Pseudomonas aeruginosa, non-clinical, wastewater, multiple antibiotic resistance, antibiotic resistant gene, extended-spectrum -β-lactamase, metallo-β-lactamase

Posted Date: June 8th, 2020

DOI: https://doi.org/10.21203/rs.3.rs-31330/v1

Abstract

Background

Pseudomonas aeruginosa (*P. aeruginosa*) are Gram-negative bacilli that are ubiquitous in nature particularly plentiful in soils and in aquatic milieu because they thrive well in humid or wet environments. It has extensive metabolic versatility, ability to thrive well and colonize diverse ecological habitats such as soil, rhizosphere, wastewaters that, enhances its potential threat to public health. Abattoirs, aquatic ecosystem including generated wastewaters are latent sources of pathogenic bacteria, serve as reservoirs and contribute to the spread of antibiotic resistant genes. Several studies have focused on clinical environment while scarce data exist from non-clinical environments, which sometimes are hotspots of antibiotic resistance. Thus, the present investigation aimed to identify antibiotic resistant gene of *P. aeruginosa* from non-clinical sources in Mthatha, Eastern Cape and evaluate its public health implications.

Results

Fifty-five (55) *Pseudomonas* species and other organisms recovered from effluent and surface water samples included *P. aeruginosa* (65.4%), *P. fluorescens* (27.3%), *Burkholderia gladioli* (5.5%) and *Burkholderia cepacia* (1.8%). The *P. aeruginosa* isolates showed high resistance to aztreonam (86.1%), ceftazidime (63.9%), piperacillin (58.3%) and cefepime (55.6%); with moderate resistance displayed to imipenem (50%), piperacillin/tazobactam (47.2%), meropenem (41.7%) and levofloxacin (30.6%). Twenty out of thirty-six (36) *P. aeruginosa* displayed multidrug resistance profiles and were classified as multidrug resistant (MDR) (55.6%). Most of the bacterial isolates exhibited a high Multiple Antibiotic Resistance (MAR) Index of > 0.2 ranging from 0.08–0.69 with a mean MAR Index of 0.38. PCR analysis of fifteen (15) *P. aeruginosa* isolates detected 14 (93.3%) harbored bla_{SHV}, 6 (40%) harbored bla_{TEM} with the least occurring ESBL as bla_{CTX-M} at 3 (20%).

Conclusions

Results of the current study indicating resistance of environmental *P. aeruginosa* isolates to front-line clinically relevant antipseudomonal drugs is concerning and poses risk to the environment and receiving water bodies. Given the public health relevance, the results of this study highlight the importance and urgent need of monitoring multidrug-resistant pathogens in effluent environments. These non-clinical environments are potential reservoirs of resistance genes that would further serve as avenues for the dissemination of multidrug resistant bacteria in the community.

Background

Pseudomonas aeruginosa, an environmental bacterium can be found in various terrestrial and aquatic habitats. *P. aeruginosa* is an opportunistic human pathogen competent for a wide array of infections including respiratory tract, blood, and urinary tract and skin infections. It causes nosocomial infections and is responsible for persistent infections in individuals with weakened defense walls and for the chronic lung infections of patients with cystic fibrosis. This competence for infections and ability for antibiotic resistance has made the organism to be recognized as a threat to public health [1-2]. The aquatic environment, terrestrial plants, animals and humans constitute a broad host range of *P. aeruginosa.* It is widely dispersed in nature and particularly abundant in soils and water. This is due to their narrow nutritional requirements, which enhances their distribution, proliferation and survival despite adverse physical and chemical conditions [3]. Its extensive metabolic versatility enhances its ecological success and potential threat to public health [4-5]. It has been designated as an emerging waterborne pathogen of economic, health and ecological relevance. It colonizes moist environment and preferentially lives in aquatic habitats such as rivers, wastewater and recreational waters [1, 4, 6].

Abattoir waste have the ability to contaminate both surface and groundwater with the introduction of pathogens that can affect land and water qualities when appropriate management protocols are not adhered to. Discharge from abattoir effluent contaminates the environment, thus endangering human, animal and aquatic ecosystem's health thereby constituting a menace to human health and environmental safety [7, 8]. The fact that, pathogens from abattoir and animal waste effluents upon reaching receiving water bodies become resistant to antibiotics once they cause infection is of grave concern. This is because they are usually difficult to treat and often cause morbidity and mortality especially to the most vulnerable members of the community [9].

Antimicrobial resistance (AMR) is a public health crisis in both human and veterinary medicine. This has been made worse with the emergence and spread of carbapenemase-producing Enterobacteriaceae (CPE) displaying resistant genes, KPC-2 and NDM-1 [10, 11]. The drivers and burden of AMR are not restricted by national territories, sector or discipline hence there is need for a coordinated and all-inclusive global response to combat AMR [12]. Globally, due to the speedy emergence and spread of resistant bacteria and associated antimicrobial resistant genes amongst human populations, animals and the environment, there is an urgent need to achieve optimum state of health in these biomes as necessitated by the One Health strategy through concerted effort of national, regional and global bodies. The strategy involves a multi-sectoral approach of combating the crisis through the alliance of FAO, OIE and WHO [13-15]. The apprehension with respect to antibiotic use is justified because of the close relation between antibiotics used in both human medicine and animal production, which has resulted in cross-species transmission of ARB and ARG from the environment and animals to humans [16]. Little attention and regulation has been put on antibiotic use in aquatic and abattoir environments. This subsequently affects environmental and human health. The use of antibiotics for prophylaxis, therapy or as growth promoters in western countries has reduced intensively because of the great advances in production of effective vaccines. On the contrary, in developing countries the use of antibiotics in animal production is still on the rise. Recent research findings revealed that occurrence of antimicrobial resistance in food-producing animals has nearly tripled within the last twenty years [6, 17].

Wastewater and wastewater treatment plants (WWTPs) serve to disseminate antibiotic resistant bacteria into surface waters and other water bodies and are thus hotspots for HGT. Consequently, there is a latent risk of colonization with antibiotic-resistant bacteria (ARB) upon contact with drinking water, surface water and wastewater, and upon causing infections may result in treatment failure [18]. It has been proved that the environment is a massive reservoir of resistant organisms and antibiotic resistant genes (ARGs), which are mostly acquired through HGT via transferable genetic materials such as plasmids and transposons and have been introduced to clinical strains. It has also been reported that the antibiotic resistant gene, CTX-M and qnrA (linked to fluoroquinolone resistance) gene actually emanated from environmental reservoir present in the environmental bacterium, Kluyvera and marine bacterium, Shewanella respectively [19, 20]. Antibiotic usage in the agricultural sector have compounded the spread of resistance in the human community due to environmental dissemination of transferable resistance genes [20]. Several studies have investigated the prevalence and detection of ESBL- and MBL-producing *P. aeruginosa* isolated from clinical samples, but there is scarcity of data on the occurrence in non-clinical samples. The need to evaluate antibiotic resistant gene in water bodies wastewater inclusive is necessary to assess their potential risk to human health. This study was designed for the molecular identification of antibiotic resistant gene of *Pseudomonas aeruginosa* from non-clinical sources in Mthatha, Eastern Cape and its public health implications.

Results

Isolation And Antimicrobial Susceptibility Testing

During the study period, identification of the fifty-five organisms isolated revealed that thirty-six (36) were *P. aeruginosa* (65.4%), fifteen (15) were *P. fluorescens* (27.3%), three (3) were *Burkholderia gladioli* (5.5%) and one (1) was *Burkholderia cepacia* (1.8%). *P. fluorescens* and *B. gladioli* isolates did not grow at 42 °C. Result of antibiotic susceptibility testing of *P. aeruginosa* isolates showed a high resistance to aztreonam, ceftazidime, piperacillin and cefepime (86.1%, 63.9%, 58.3% and 55.6% respectively). Moderate resistance was displayed to imipenem, piperacillin/tazobactam, meropenem and levofloxacin at 50%, 47.2%, 41.7% and 30.6% respectively (Table 1). Twenty out of thirty-six (36) *P. aeruginosa* presented multidrug resistance profiles and were classified as MDR (55.6%).

Table 1
Antibiotic resistance pattern of P. aeruginosa isolates

Antibiotic	No (%) resistant	No (%) susceptible
Amikacin	6 (16.7)	30 (83.3)
Aztreonam	31 (86.1)	5 (13.9)
Ceftazidime	20 (55.6)	16 (44.4)
Cefepime	23 (63.9)	13 (36.1)
Ciprofloxacin	8 (22.2)	28 (77.8)
Doripenem	5 (13.9)	31 (86.1)
Gentamicin	6 (16.7)	30 (83.3)
Imipenem	18 (50)	18 (50)
Levofloxacin	11 (30.6)	25 (69.4)
Meropenem	15 (41.7)	21 (58.3)
Piperacillin	21 (58.3)	15 (41.7)
Piperacillin/tazobactam	17 (47.2)	19 (52.8)
Tobramycin	3 (8.3)	33 (91.7)

Multiple Antibiotic Resistance (mar) Index

Most of the bacterial isolates showed a high MAR Index of > 0.2 ranging from 0.08–0.69 with a mean MAR Index of 0.38. The mean MAR index of isolates from abattoir wastewater was 0.42 while that of Mthatha dam equals 0.34 (Fig. 1)

Molecular detection of extended spectrum β-lactamase (ESBL) and metallo-β-lactamase (MBL) encoding genes in P. aeruginosa

PCR screening of genes encoding ESBL and MBL revealed the amplification of bla_{SHV}, bla_{CTX-M} and bla_{TEM} in some of the *P. aeruginosa* isolates, while all but one of the isolates harbored the MBL, bla_{VIM}. ESBL production was detected genotypically in fifteen (15) of them. Results of molecular detection of ESBL genotypes in environmental strains of *P. aeruginosa* by singleplex rPCR are presented in Table 2. Among the 15 *P. aeruginosa* isolates evaluated for the molecular analysis, a total of 14 (93.3%) harbored the bla_{SHV} ESBL, 6 (40%) harbored the bla_{TEM} with the least occurring ESBL as bla_{CTX-M} at 3 (20%). Only one isolate (6.7%) harbored the bla_{VIM} gene while no isolate was detected harboring the MBL, bla_{IMP}.

Table 2
Extended spectrum β-lactamase (ESBL) and MBL genotypes in P. aeruginosa non-clinical isolates

Positive by PCR for ESBL genes	Number amplified (N = 15)	Total (%)
A. Single ESBL gene		
bla_{SHV} only	14	93.3
bla_{TEM} only	6	40.0
bla_{CTX-M} only	3	20.0
bla_{VIM} only	1	6.7
bla_{IMP} only	0	0
B. Two or more ESBL genes		
bla_{TEM} + bla_{SHV}	6	40
bla_{TEM} + bla_{CTX-M}	3	20
bla_{SHV} + bla_{CTX-M}	3	20
bla_{SHV} +bla_{VIM}	1	6.7

Discussion

P. aeruginosa strains isolated from hospital settings have been associated with hospital-acquired infections particularly when the immune defence of the host is compromised such as in neutropenia, severe burns or cystic fibrosis [21, 22]. It has been linked to outbreaks especially in ICU and onco/hematological units with reported overall incidence of carriage varying from 11.7–37.0% [23–25], resulting in significant morbidity and mortality rate close to 80% [26]. The impact of the origin and evolution of antimicrobial resistance of *P. aeruginosa* from non-clinical settings on human health has not received so much attention as has been with the clinical counterparts. The use of antimicrobial drugs in both human medicine and animal production for growth-promoting purposes, metaphylaxis and prophylaxis have escalated the emergence and spread of antimicrobial resistance resulting in aggravated public health and environmental risks [27, 28]. Antibiotics are incompletely biodegradable and therefore, residues are usually washed into aquatic ecosystems through wastewater, presenting an environmental hazard, posing danger to human health [29]. The hazard of AMR posed to human health is particularly concerning in low- to middle- income countries (LMICs) due to higher possibility of community-acquired resistant infections, the high transmissible disease burden in the general populace, poor access to health services and weak guidelines and implementation of antibiotic use in food production and healthcare

[14]. It also leads to increased morbidity, prolonged hospitalization, increased healthcare costs, and exert economic burden on family units and the society [30].

In this study, 65.4% of the isolates recovered were *P. aeruginosa*, 27.3% were *P. fluorescens*, while *Burkholderia gladioli* and *Burkholderia cepacia* constituted 5.5% and 1.8% respectively from abattoir wastewater and surface water (Mthatha dam) during the study period. In agreement with our studies, Igbinosa *et al*., Ejikeugwu *et al*. and Tapela and Rahube, [6, 31, 32] have all reported the occurrence of *P. aeruginosa* and other species from hospital drains, environmental, and wastewater networks from various parts of the world. The occurrence of these microorganisms is a cause of concern given that they are opportunistic human pathogens and can infect people whose immunity are compromised [33]. The discharge of effluents from abattoir directly into water bodies without prior treatment have triggered serious health risks subsequent to its contamination by bacteria [9].

The highest prevalence rate of *P. aeruginosa* (65.4%) seen in the current study is comparable to previous reports from Nigeria on water samples from fish pond sites and cattle waste, where *P. aeruginosa* was found to be the most prevalent with highest occurrence rate of 62.8% and 71.5% respectively, among other species [34, 35]. This possibly could be due to the physiological versatility and limited nutritional requirements that enables it to adapt in adverse conditions [36]. Likewise, in agreement with our findings, a study carried out in Mafikeng in the North West Province of South Africa, isolated *P. aeruginosa* and other *Pseudomonas* species from both drinking and surface waters [37]. However, contrary to our findings, a study carried out in Alice, Eastern Cape, South Africa on wastewater samples found a lower occurrence rate of 11.1% [38]. This disparity is most likely due to different treatment processes used in water purification, or it can be assumed that WWTP does not totally eliminate bacteria especially MDR strains since these organisms are resilient to the treatment processes and eventually play a role in the transmission and spread of antimicrobial resistance.

The pathogenicity of *P. aeruginosa* favours its resistance to potent antimicrobials thereby resulting in therapeutic challenge and elongation of patients' stay in the hospital settings [39]. Table 1 shows the resistance profiles of the isolates and reveals 63.9% and 55.6% resistance to second and third generation cephalosporin respectively (ceftazidime and cefepime) and low resistance to aminoglycosides. The observed resistance pattern in cephalosporins observed in the current study is at par with previous report of Ejikeugwu *et al*. [31], but lower compared to that of Tapela and Rahube, and Elhariri *et al*. [32, 40] with 100% resistance rate. However, Benie *et al*. from Cote d'Ivoire [41] reported lower rates of resistance of 6.9% and 17% to ceftazidime and cefepime respectively. A cause for concern is the high resistance displayed to the cephalosporins, which are frontline antipseudomonal drugs for treating *P. aeruginosa* infections, increased resistance to this class of antibiotics will not be favorable and will result in limited treatment options. The present study revealed that 16.7% isolates were resistant to the aminoglycoside, amikacin and gentamicin. A similar low rate of resistance to amikacin (19%) but slightly higher rate to gentamicin (28.5%) was reported by a study carried out in Egypt [40]. However, an elevated resistance rate of 79% in gentamicin had been reported by a study carried out in Nigeria [31]. Amikacin, gentamicin and tobramycin are first or second line empirical drugs of choice in the access group of antibiotics for treating

common or severe clinical conditions [42]; particularly, inhaled tobramycin is used to eradicate early infections in cystic fibrosis while amikacin and gentamicin are also used in combination therapy with other antibiotics to improve overall efficacy [43].

Resistance to carbapenems including imipenem (50%) and meropenem (41.7%) was also observed in our study. This is quite unexpected, given the fact that carbapenems represent one of the most effective and among the best option for treating Gram-negative infections particularly MDR infections. Multifaceted interactions of several mechanisms mediate resistance to the carbapenems and these may include loss of outer membrane porins, efflux overexpression and production of carbapenemases. Carbapenem-resistant *P. aeruginosa* isolates are frequently associated with a higher mortality rate due to the enzyme carbapenemase mediating the resistance and a higher likelihood of extensive spread of resistance through mobile genetic elements [44]. Antimicrobial resistance affects both human and animal health. The abuse of antimicrobials in human and veterinary medicine often leads to proliferation of ARB and ARGs that can be transferred to human pathogenic bacteria. This transfer eventually nullifies the efficacy of current and upcoming antibiotics, thereby leading to treatment failure for some life-threatening diseases [45, 46]. The impact of AMR leading to antibiotic-resistant infections suggests that close to a million people die annually and further projections of 10 million people could die globally by the year 2050 due to these infections [47].

Occurrence of 55.6% MDR *P. aeruginosa* seen in this study in a non-clinical setting is high and alarming. Notwithstanding, higher percentages of *P. aeruginosa* has been reported in hospital wastewater treatment plants (HWWTP) in other parts of the tropics, with an occurrence of 82% and 93.2% in Brazil and Nigeria respectively [48, 49]. This is expected since hospital wastewater and WWTP are regarded as hotspot of antibiotic resistance due to the discharge of antibiotics, disinfectants and materials containing MDR pathogens [50, 51]. Similarly, studies of Olga *et al*. and Kateete *et al*. [52, 53] reported lower rates of MDRPA of 32% and 38% respectively from aquatic environments and clinical and environmental samples

MAR indexing method is a simplified rapid method of distinguishing organisms from different origins either from high-risk sources of contamination where antibiotics are frequently used or low risk sources [54] and as an indicator of the level of contamination potentially unsafe for humans [55]. A MAR index > 0.2 indicates that isolates originate from high-risk sources of contamination [56] while MAR index > 0.4 is an indication of faecal origin contamination [51]. Antibiotics are increasingly present in wastewater and this contribute to the emergence and selection of AMR in the environment [57]. In the current study, analysis of MAR index of the *P. aeruginosa* strains showed that all of them had a MAR index above 0.2 with Mean MAR index of 0.42 and 0.34 of abattoir wastewater and aquatic isolates respectively (Fig. 1). It therefore validates the theory that these isolates originated from high risk contaminated sources with frequent antibiotic use. This is particularly evident with the mean MAR index of 0.42 from the abattoir wastewater. The findings reflect the overuse of antibiotics in animal production and highlights the sources of these pathogens, which eventually translocate into water bodies and pose health risks to humans. Odjajare *et al*. [58] in Alice, Eastern Cape, South Africa, reported similar result with a mean MAR index of 0.46. A MAR index of isolates with values higher than 0.2 has also been reported in fish samples

sold in informal markets in Zimbabwe [59] and River Nile in Egypt [57]. These isolates with MAR indices greater than 0.2 indicate that they are from potentially health risk niche.

The release of MDR bacteria including ESBL and MBL producers into water bodies is a cause of concern. These organisms could act as opportunist pathogens when they persist in the environment and serve as resistance pool that could fast-track the evolution of antimicrobial resistance in a community [48]. ESBL belonging to the Class A category have been identified in *P. aeruginosa* conferring resistance to the cephalosporins and this is specifically due to the production of β-lactamases. The main ESBL of clinical significance include bla_{CTX-M}, bla_{SHV} and bla_{TEM} [40]. In the current study, bla_{SHV} was the most prevalent ESBL detected by PCR. This was detected in 14 isolates (93.3%). bla_{TEM} was detected in 40% of our isolates while the least detected ESBL was bla_{CTX-M} at 20%. Our study is in agreement with the study of Rezai *et al*. [60], who detected bla_{SHV} as the most prevalent ESBL by PCR. Other authors from Malaysia and India reported bla_{TEM} to be the prevalent ESBL [61, 62], whereas some others from Brazil, Egypt, Nigeria have reported bla_{CTX-M} [40, 63–64]. Together, these statistics suggest successful dissemination of the ESBL-encoding genes universally.

Conclusion

The result of this study indicating resistance of environmental *P. aeruginosa* isolates to front-line clinically relevant antipseudomonal drugs is concerning and poses a risk to the environment and to the receiving water body as these waste effluents gets discharged freely into the environment. Given the public health relevance, the results of this study reveal the importance and necessity of monitoring multidrug-resistant pathogens in effluent environments. This non-clinical environment could harbor *P. aeruginosa* as a potential reservoir of resistance genes and could serve as a path for the dissemination of multidrug resistant bacteria in a community. In addition, the high multiple antibiotic resistance index and the occurrence of ESBL-producing *P. aeruginosa* represent a public health threat since the genetic elements responsible for this resistance are present on mobile genetic elements (MGEs) that can be transferred to other Gram-negative bacteria through horizontal gene transfer. This increases the antimicrobial resistance rate and complicates the treatment if the infection is caused by these pathogens. It also portends great risk to the vulnerable population whose immunity is compromised due to HIV/AIDS when exposed to contaminated water supply.

Furthermore, the swift detection of various beta lactamases is imperative for improving therapy, halting of the emergence and spread of resistant organisms and for epidemiological tracing of resistance determinants. This will also enhance timely execution of strict infection control protocols as well as clinical guidance regarding the potential risks for therapeutic failure. Precise prevalence data will be necessary to appraise the success of control measures.

Methods

Study design and setting

A prospective, cross sectional study conducted between the periods January to June 2019. The study site was Umzikantu Red Meat Abattoir, Zimbane Mthatha, Mthatha River and Mthatha dam. Umzikantu Red Meat Abattoir is a high throughput abattoir located in Zimbane location in Mthatha, Eastern Cape. It is the only operational red meat abattoir serving Mthatha and its environs in the O.R Tambo District Municipality. It is certified and have the capacity to slaughter 50 units of animals on a daily basis. One unit equals to one cow/ox or two calves or six sheep or four pigs. The abattoir is open to the public and offers slaughter and cutting services at an affordable price. It also doubles as a wholesaler supplying meat to butcheries, supermarkets and hospitals.

Mthatha Dam (31°33'2"S 28°44'24"E Coordinates) is an earth-fill type dam on the Mthatha River, located close to Mthatha Town, in the OR Tambo District Municipality of the Eastern Cape. This dam was founded in 1977 to serve municipal and industrial purposes. The Department of Water and Sanitation oversees the affairs of the dam. The catchment area of the dam is 886 km^2 with surface area measuring 25.42 km^2. It has a height of 38 m with the length measuring 620 m. The reservoir capacity of the dam is 253, 674, 000 m^3.

Sample collection

Abattoir environment samples: Using standard methods, one hundred (100) mililitres of abattoir effluents were taken from two sampling points into sterile bottles appropriately labelled. All samples were stored in cooler boxes for transportation to the Medical Microbiology laboratory at Walter Sisulu University Mthatha, for further analyses, within 4 h of sample collection.

Aquatic environment samples: Water samples from the Mthatha dam were collected aseptically in sterile 100mL Duran Schott glass bottles from different sampling points by directly dipping the bottles about 20cm below the surface of the water. After collection, the samples were placed in iced cooler boxes, transported to the laboratory and kept at about 4^oC until analysed.

Bacteriological analysis

Membrane filtration method was used for isolation and enumeration according to standard methods [65]. For all samples, three volumes of 100mL were filtered through 0.45 μm pore sized gridded membrane filter (Whatman Laboratory Division, Maidstone, England) using a water pump (model Sartorius 16824). Filter was removed and aseptically placed on CHROMagar^{TM} *Pseudomonas* (CHROMagar^{TM}; Paris, France) plates ensuring that no air bubbles were trapped. All media were prepared according to the manufacturers' instructions (CHROMagar^{TM}; Paris, France). Each sample was analysed in triplicate. Effluent samples from Abattoir were serially diluted and 1mL of the 5 fold serial dilutions was filtered through the 0.45 μm pore sized gridded membrane filter (Whatman Laboratory Division, Maidstone, England) using a water pump (model Sartorius 16824) before being placed aseptically on to the CHROMagar^{TM} *Pseudomonas* (CHROMagar^{TM}; Paris, France) agar plates. The plates were incubated

aerobically at 37^0C for 24-48 hours. Blue colonies characteristics of *Pseudomonas* spp. were enumerated.

Characterization of Pseudomonas aeruginosa

Blue colonies typical of *Pseudomonas* species on the chromogenic medium were subcultured on both Cetrimide agar and CHROMagar to get pure colonies. Characteristics grape-like odour was a useful marker of identification. Phenotypic tests such as Gram stain, oxidase test and catalase test were performed [66]. Species identification was carried out using Gram-negative ID type 2 panels (Beckman Coulter, Inc. USA) of MicroScan autoScan-4 automated system (Dade Behring Inc., Deerfield, IL). Growth at 42^0C [66] in an aerobic incubator was also used to confirm the identity of the *P. aeruginosa* isolates.

Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was performed by MicroScan autoScan-4 system using Microscan dehydrated broth microdilution negative MIC Panel Type 44 (NM44) (Beckman Coulter, Inc. USA) following the manufacturer's guidelines [67]. The following antibiotics were tested in the panels: amikacin, aztreonam, cefepime, ceftazidime, ciprofloxacin, doripenem, gentamicin, imipenem, levofloxacin, meropenem, piperacillin/tazobactam, piperacillin and tobramycin. MICs were interpreted following CLSI guidelines [68]. ATCC Quality control organisms used were *P. aeruginosa* ATCC 27853 and *Escherichia coli* ATCC 25922. Non-susceptibility included the combination of resistance and intermediate resistance. MDR *P. aeruginosa* was defined as non-susceptibility to at least one agent in three or more antimicrobial categories according to Magiorakos *et al*. [69].

Multiple antibiotic resistance (MAR) index: MAR was calculated and interpreted for the isolates as described by Gufe *et al*. [59]. Briefly, it is described as the ratio of the number of antibiotics to which isolates were resistant (a) to the total number of antibiotics to which the isolates were exposed (b) i.e: MARI = a/b. The MARI of individual *P. aeruginosa* from the two different sources was calculated. Bacteria having MARI (>0.2) originate from high risk sources of contamination where several antibiotics have been used, while, MARI value of $\leq$0.2 indicates strains from sources where antibiotics have seldom or never been used.

Molecular confirmation of strains by rPCR

DNA Extraction: DNA was extracted from overnight colonies of bacterial culture grown on Cetrimide agar. This was re-suspended in Roche Magna Pure Bacteria Lysis Buffer, vortexed briefly, heated at 95°C for 10 minutes and pelleted by centrifugation at 13000 g for 10 minutes. Four hundred microliters were used as a specimen in the MagNa Pure Compact System (Roche Applied Science, Indianapolis), using MagNa Pure Compact Nucleic Acid isolation kit 1 according to manufacturer's instructions. Elution tubes containing 200 µl purified nucleic acids were stored at – 80^0C until further use. The LightCycler 2.0 instrument (Roche Applied Science, Germany) and Fast start LightCycler 480 Hybprobes Master kit (Roche Diagnostics, USA) were used for polymerase reaction. Specific primers targeting the gene, species-

specific *gyrB* were amplified by singleplex *rPCR* using primers and probes designed by TIB Molbiol, Germany. 16S rRNA analysis was also carried out to confirm identity of the isolates.

Molecular ESBL and MBL detection by rPCR

Real-time PCR for *bla*$_{CTX-M}$, *bla*$_{SHV}$, *bla*$_{TEM}$, *bla*$_{IMP}$ and *bla*$_{VIM}$. Isolated *P. aeruginosa* colonies on Cetrimide agar and CHROMagar *Pseudomonas* were selected for genomic DNA extraction. Template DNA was extracted by MagNA Pure Compact (MPC) using MPC Nucleic Acid isolation kit according to manufacturer's instruction. Real time PCR was carried out in the LightCycler 2.0 instrument (Roche Applied Science, Germany) using Fast start LightCycler 480 Hybprobes Master kit (Roche Diagnostics, USA). Specific primers and probes (Table 3) targeting the genes CTX-M, SHV, TEM, IMP and VIM were amplified by singleplex rPCR. Primers were designed by TIB-Molbiol (Berlin, Germany). rPCR assay was performed in a 32 carousels using 20 μL capillaries with each capillary containing a total volume of 20 μL; including 2μL of LightCycler FastStart DNA Master Hybridization Probes (Roche Diagnostics), 2 μL of primers (0.5mM for each forward and reverse), 2 μL of probe, 2.4 μL of MgCl2, 2 μL of extracted DNA, and water to make up the volume of 20 μL. The rPCR protocol are as shown in Table 4.

Table 3: Primer sequences for detection of *bla*$_{CTX-M}$, *bla*$_{SHV}$, *bla*$_{TEM}$, *bla*$_{IMP}$,*bla*$_{VIM}$ genes and gyrB

Target gene	Primers	Primers sequences (5'-3')	Tm in 0^0C	References
bla$_{CTX-M}$	CTX-M forward primer	ATGAGYACCAGTAARGTKATGGC	58.7	70
	CTX-M reverse primer	ATCACKCGGRTCGCCIGGRAT	59.3	
	CTX-M Probe	FAM-CCCGACAGCTGGGAGACGAAACGT-BBQ	70.2	
bla$_{SHV}$	SHV forward primer	TCCCATGATGAGCACCTTTAAA	56.8	71
	SHV reverse primer	TCCTGCTGGCGATAGTGGAT	58.6	
	SHV Probe	FAM-TGCCGGTGACGAACAGCTGGAG-BBQ	68.3	
bla$_{TEM}$	TEM forward primer	GCATCTTACGGATGGCATGA	56.6	71
	TEM reverse primer	GTCCTCCGATCGTTGTCAGAA	57.7	
	TEM Probe	FAM-CAGTGCTGCCATAACCATGAGTGA-BHQ1	62.2	
bla$_{IMP}$	IMP forward primer	GGGCGGAATAGAGTGGCTTA	57.6	72
	IMP reverse primer	GGCTTGAACCTTACCGTCTTTTT	59.3	
	IMP Probe	FAM-CGATCTATCCCCACGTATGCATCTGAATTAACA-BHQ1	67.4	
bla$_{VIM}$	VIM forward primer	TGCGCTTCGGTCCAGTAGA	59.0	72
	VIM reverse primer	TGACGGGACGTATACAACCAGAT	58.5	
	VIM Probe	FAM-CTTCTATCCTGGTGCTGCGCATTCG-BHQ1	67.6	
*gyr*B	*gyr*B forward primer	CCT GAC CAT CCG TCG CCA CAA		73
	*gyr*B reverse primer	CGC AGC AGG ATG CCG ACG CC		
	*gyr*B probe	6-FAM-CCG TGG TGG TAG ACC TGT TCC CAG ACC-BHQ 6-FAM-CCG TGG TGG TAG ACC TGT TCC CAG ACC-BBQ		This study

Table 4 Real-time PCR cycle protocol

Protocol	Temperature	Acquisition mode	Time	Ramp rate	Cycle
Denaturation	95^0C	None	5 minutes	4.4	1
Quantification: annealing	95^0C	None	30 seconds	4.4	45
Extension	60^0C	Single	1 minute	4.4	
	No third step				
Cooling	40^0C	None	30 seconds	4.4	1

Absolute quantification was carried out using the Light Cycler software 4.05. Data were obtained during annealing period. Fluorescence was measured once every cycle immediately after the 60°C incubation (extension step). Fluorescence curves were analyzed with the LightCycler software, version 4.05. Results were expressed by determination of threshold cycle (Ct) value which signified the cycle at which sample fluorescence became remarkably different from baseline signal. Positive control strains used included *Klebsiella pneumoniae* ATCC 51503 (bla_{CTX-M}), *Klebsiella pneumoniae* ATCC 700603 (bla_{SHV}), *Escherichia coli* NCTC 13351 (bla_{TEM}), *P. aeruginosa* NCTC 13437 (bla_{VIM}) and *Escherichia coli* NCTC 13476 (bla_{IMP}). These were obtained from the National Institute of Communicable Diseases (NICD), Johannesburg, South Africa.

Abbreviations

AMR: antimicrobial resistance; ARB:antibiotic-resistant bacteria; ARG:antibiotic-resistant gene; CPE:carbapenemase-producing Enterobacteriaceae; ESBL:extended-spectrum-β-lactamase; FAO:Food and Agriculture Organization; HGT:horizontal gene transfer; HWWTP:hospital wastewater treatment plant; ICU:intensive care unit; MAR:multiple antibiotic resistance; MBL:metallo-β-lactamase; MDR:multidrug resistance; OIE:World Organization for Animal Health; rPCR:real-time polymerase chain reaction; WWTP:wastewater treatment plant.

Declarations

Ethics approval

Human Research Ethics Committee of the Faculty of Health Sciences of Walter Sisulu University approved this study (Reference number: 024/2016).

Consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Competing interests

The authors declare that they have no competing interests.

Funding

MCH received PhD bursary from National Research Foundation (NRF) in this study. The study was conducted within the Thuthuka Rating Track, Ref. No. TTK150625121238; UID: 99307) project, funded by NRF (South African). The funders had no role in study design, data collection, analysis and interpretation of data, writing of manuscript and decision to publish.

Authors' contributions

TA, SV and MCH conceived and designed the study. MCH and GEO were involved in field studies. MCH collected the data, analyzed the data, and drafted the manuscript. TA. SV, MCH, and GEO revised the manuscript critically for intellectual content. All authors read and approved the final manuscript.

Acknowledgements

The authors are grateful to the Nelson Mandela Academic Hospital (NMAH) and the Microbiology Laboratory at the National Health Laboratory Services (NHLS) at the NMAH for support during sample collection. The National Institute of Communicable Diseases (NICD) is appreciated for supply of reference strains.

References

1. Deredjian A, Colinon C, Hien E, Brothier E, Youenou B, Cournoyer B, Dequiedt S, Hartmann A, Jolivet C, Houot S, Ranjard L. Low occurrence of *Pseudomonas aeruginosa* in agricultural soils with and without organic amendment. Front Cell Infect Microbiol. 2014 Apr 29;4:53.
2. Golle A, Janezic S, Rupnik M. Low overlap between carbapenem resistant *Pseudomonas aeruginosa* genotypes isolated from hospitalized patients and wastewater treatment plants. PLoS One. 2017;12(10).
3. Elshafiee EA, Nader SM, Dorgham SM, Hamza DA. Carbapenem-resistant *Pseudomonas aeruginosa* originating from farm animals and people in Egypt. J Vet Res. 2019 Aug 9.

4. Vujović B, Teodorović S, Rudić Ž, Božić M, Raičević V. Phenotypic heterogeneity of *Pseudomonas aeruginosa* isolates in the protected nature park 'Palić'(Serbia). Water Science Technology: Water Supply. 2016 Oct;16(5):1370–7.
5. Kordes A, Preusse M, Willger SD, Braubach P, Jonigk D, Haverich A, Warnecke G, Häussler S. Genetically diverse *Pseudomonas aeruginosa* populations display similar transcriptomic profiles in a cystic fibrosis explanted lung. Nat Commun. 2019 Jul 30;10(1):1–0.
6. Igbinosa IH, Beshiru A, Igbinosa EO. Antibiotic resistance profile of *Pseudomonas aeruginosa* isolated from aquaculture and abattoir environments in urban communities. Asian Pac J Trop Dis. 2017;7(1):47–52.
7. Asemota UK, Odutayo OI, Oyewole I, Abubakar S. Preliminary Bacteriological Evaluation of Abattoir Waste water Treated With Moringa Oleifera Seed Powder. Amer J Phytomed Clin Ther. 2016 Mar 31;4(3):098–104.
8. Atuanya EI, Nwogu NA, Orah CU. Antibiotic Resistance and Plasmid Profiles of Bacteria Isolated from Abattoir Effluents around Ikpoba River in Benin City, Nigeria. J Appl Sci Env Man. 2018;22(11):1749–55.
9. Iroha IR, Eromonsele OB, Moses IB, Afiukwa FN, Nwakaeze AE, Ejikeugwu PC. In vitro antibiogram of multidrug resistant bacteria isolated from Ogbete abattoir effluent in Enugu State, Nigeria. Int Res J Public Env Health. 2016;3(1):1–6.
10. Wall BA, Mateus AL, Marshall L, Pfeiffer DU, Lubroth J, Ormel HJ, Otto P, Patriarchi A. Drivers, dynamics and epidemiology of antimicrobial resistance in animal production. Food and Agriculture Organization of the United Nations;2016.
11. Ahmed ZS, Elshafiee EA, Khalefa HS, Kadry M, Hamza DA. Evidence of colistin resistance genes (mcr-1 and mcr-2) in wild birds and its public health implication in Egypt. Antimicrob Resist Infect Cont. 2019 Dec 1;8(1):197.
12. The PLOS Medicine editor. Antimicrobial resistance: is the world UNprepared?. PLoS Med. 2016 Sep;13(9).
13. Goulas A, Livoreil B, Grall N, Benoit P, Couderc-Obert C, Dagot C, Patureau D, Petit F, Laouénan C, Andremont A. What are the effective solutions to control the dissemination of antibiotic resistance in the environment? A systematic review protocol. Env Evidence. 2018 Dec;7(1):3.
14. Rousham EK, Unicomb L, Islam MA. Human, animal and environmental contributors to antibiotic resistance in low-resource settings: integrating behavioural, epidemiological and One Health approaches. Proc Royal Soc B. 2018 Apr 11;285(1876):20180332.
15. Kraemer SA, Ramachandran A, Perron GG. Antibiotic pollution in the environment: from microbial ecology to public policy. Microorganisms. 2019 Jun;7(6):180.
16. Mouiche MM, Moffo F, Akoachere JF, Okah-Nnane NH, Mapiefou NP, Ndze VN, Wade A, Djuikwo-Teukeng FF, Toghoua DG, Zambou HR, Feussom JM. Antimicrobial resistance from a one health perspective in Cameroon: a systematic review and meta-analysis. BMC Public Health. 2019 Dec 1;19(1):1135.

17. Van Boeckel TP, Pires J, Silvester R, Zhao C, Song J, Criscuolo NG, Gilbert M, Bonhoeffer S, Laxminarayan R. Global trends in antimicrobial resistance in animals in low-and middle-income countries. Science. 2019 Sep 20;365(6459):eaaw1944.
18. Müller H, Sib E, Gajdiss M, Klanke U, Lenz-Plet F, Barabasch V, Albert C, Schallenberg A, Timm C, Zacharias N, Schmithausen RM. Dissemination of multi-resistant Gram-negative bacteria into German wastewater and surface waters. FEMS Microbiol Ecol. 2018 May;94(5):fiy057.
19. Wright GD. Antibiotic resistance in the environment: a link to the clinic?.Curr Opin Microbiol. 2010 Oct 1;13(5):589–94.
20. Heuer H, Schmitt H, Smalla K. Antibiotic resistance gene spread due to manure application on agricultural fields. Curr Opin Microbiol. 2011 Jun 1;14(3):236 – 43.
21. Havenga B, Ndlovu T, Clements T, Reyneke B, Waso M, Khan W. Exploring the antimicrobial resistance profiles of WHO critical priority list bacterial strains. BMC Microbiol. 2019 Dec 1;19(1):303.
22. Magalhães B, Valot B, Abdelbary MM, Prod'hom G, Greub G, Senn L, Blanc DS. Combining standard molecular typing and whole genome sequencing to investigate *Pseudomonas aeruginosa* epidemiology in Intensive Care Units. Front Public Health. 2020 Jan 28;8:3.
23. Gomes MZ, Machado CR, da Conceicao MD, Ortega JA, Neves SM, da Silva Lourenço MC, Asensi MD. Outbreaks, persistence, and high mortality rates of multiresistant *Pseudomonas aeruginosa* infections in a hospital with AIDS-predominant admissions. Braz J Inf Dis. 2011 Jul 1;15(4):312 – 22.
24. Mudau M, Jacobson R, Minenza N, Kuonza L, Morris V, Engelbrecht H, Nicol MP, Bamford C. Outbreak of multi-drug resistant *Pseudomonas aeruginosa* bloodstream infection in the haematology unit of a South African Academic Hospital. PLoS One. 2013;8(3).
25. Buhl M, Kästle C, Geyer A, Autenrieth I, Peter SM, Willmann M. Molecular evolution of extensively drug-resistant (XDR) *Pseudomonas aeruginosa* strains from patients and hospital environment in a prolonged outbreak. Front Microbiol. 2019;10:1742.
26. Kossow A, Kampmeier S, Willems S, Berdel WE, Groll AH, Burckhardt B, Rossig C, Groth C, Idelevich EA, Kipp F, Mellmann A. Control of multidrug-resistant *Pseudomonas aeruginosa* in allogeneic hematopoietic stem cell transplant recipients by a novel bundle including remodeling of sanitary and water supply systems. Clin Infect Dis. 2017 Sep 15;65(6):935 – 42.
27. Hoelzer K, Wong N, Thomas J, Talkington K, Jungman E, Coukell A. Antimicrobial drug use in food-producing animals and associated human health risks: what, and how strong, is the evidence?. BMC Vet Res. 2017 Dec 1;13(1):211.
28. Yaw Anane A, Apalata T, Vasaikar S, Okuthe GE, Songca S. Prevalence and molecular analysis of multidrug-resistant *Acinetobacter baumannii* in the extra-hospital environment in Mthatha, South Africa. Braz J Infect Dis. 2019 Nov 1;23(6):371 – 80.
29. Faleye AC, Adegoke AA, Ramluckan K, Bux F, Stenström TA. Antibiotic residue in the aquatic environment: status in Africa. Open Chem. 2018 Jan 1;16(1):890–903.

30. Carvalho IT, Santos L. Antibiotics in the aquatic environments: a review of the European scenario. Environ Int. 2016 Sep 1;94:736–57.
31. Ejikeugwu C, Esimone C, Iroha I, Eze P, Ugwu M, Adikwu M. Genotypic and Phenotypic Characterization of MBL Genes in *Pseudomonas aeruginosa* Isolates from the Non-hospital Environment. J Pure Appl Microbiol. 2018;12(4):1877–85.
32. Tapela K, Rahube T. Isolation and antibiotic resistance profiles of bacteria from influent, effluent and downstream: A study in Botswana. Afr J Microbiol Res. 2019;13:279–89.
33. Onuoha SC. Distribution and antibiogram of bacterial species in effluents from abattoirs in Nigeria. J Environ Occup Sci. 2018;7(1):1–8.
34. Falodun OI, Ikusika EO. Extended Spectrum Beta-Lactamase and Metallo Beta-Lactamase Producing *Pseudomonas* Species Isolated From Fish Pond Water in Ibadan, Nigeria. Int J Environ Stud. 2019 Dec 18:1–1.
35. Falodun OI, Musa IB. *Pseudomonas* Species from cattle dung producing extended spectrum and metallo-beta lactamases. Eur J Biol Res. 2020: 10(1)1–10.
36. Moradali MF, Ghods S, Rehm BH. *Pseudomonas aeruginosa* lifestyle: a paradigm for adaptation, survival, and persistence. Front Cell Infect Microbiol. 2017 Feb 15;7:39.
37. Mulamattathil SG, Bezuidenhout C, Mbewe M, Ateba CN. Isolation of environmental bacteria from surface and drinking water in Mafikeng, South Africa, and characterization using their antibiotic resistance profiles. J Pathogens. 2014;2014.
38. Igbinosa IH, Nwodo UU, Sosa A, Tom M, Okoh AI. Commensal *Pseudomonas* species isolated from wastewater and freshwater milieus in the Eastern Cape Province, South Africa, as reservoir of antibiotic resistant determinants. Int J Environ Res Public Health. 2012 Jul;9(7):2537–49.
39. Imanah EO, Beshiru A, Igbinosa EO. Antibiogram profile of *Pseudomonas aeruginosa* isolated from some selected hospital environmental drains. Asian Pac J Trop Dis. 2017;7(10):E604-9.
40. Elhariri M, Hamza D, Elhelw R, Dorgham SM. Extended-spectrum beta-lactamase-producing *Pseudomonas aeruginosa* in camel in Egypt: potential human hazard. Ann Clin Microbiol Antimicrob. 2017 Dec 1;16(1):21.
41. Benie CKD, Nathalie G, Adjéhi D, et al. Prevalence and Antibiotic Resistance of *Pseudomonas aeruginosa* Isolated from Bovine Meat, Fresh Fish and Smoked Fish. Arch Clin Microbiol. 2017;8:3.
42. Sharland M, Pulcini C, Harbarth S, Zeng M, Gandra S, Mathur S, Magrini N. Classifying antibiotics in the WHO Essential Medicines List for optimal use—be AWaRe. Lancet Infect Dis. 2018 Jan 1; 18(1):18–20.
43. Ren H, Liu Y, Zhou J, Long Y, Liu C, Xia B, Shi J, Fan Z, Liang Y, Chen S, Xu J. Combination of Azithromycin and Gentamicin for Efficient Treatment of *Pseudomonas aeruginosa* Infections. J Infect Dis. 2019 Oct 8;220(10):1667-78.
44. Juayang AC, Lim JP, Bonifacio AF, Lambot AV, Millan SM, Sevilla VZ, Sy JK, Villanueva PJ, Grajales CP, Gallega CT. Five-year antimicrobial susceptibility of *Pseudomonas aeruginosa* from a local tertiary hospital in Bacolod City, Philippines. Trop Med Infect Dis. 2017 Sep;2(3):28.

45. Fletcher S. Understanding the contribution of environmental factors in the spread of antimicrobial resistance. Environ Health Prev Med. 2015 Jul;20(4):243.
46. Goulas A, Livoreil B, Grall N, Benoit P, Couderc-Obert C, Dagot C, Patureau D, Petit F, Laouénan C, Andremont A. What are the effective solutions to control the dissemination of antibiotic resistance in the environment? A systematic review protocol. Environ Evid. 2018 Dec;7(1):3.
47. O'Neill JI. Antimicrobial resistance: tackling a crisis for the health and wealth of nations. Rev Antimicrob Resist. 2014 Dec;11:20:1–6.
48. Miranda CC, de Filippis I, Pinto LH, Coelho-Souza T, Bianco K, Cacci LC, Picão RC, Clementino MM. Genotypic characteristics of multidrug-resistant *Pseudomonas aeruginosa* from hospital wastewater treatment plant in Rio de Janeiro, Brazil. J Appl Microbiol. 2015 Jun;118(6):1276–86.
49. Falodun OI, Akinbamiro TF, Rabiu AG. Hospital wastewater: reservoir of antibiotic resistant *Pseudomonas* strains in Ibadan, Nigeria. Emergent Life Sci Res. 2019 Jun;5:1–7.
50. Eduardo-Correia B, Morales-Filloy H, Abad JP. Bacteria From the Multi-Contaminated Tinto River Estuary (SW, Spain) Show High Multi-Resistance to Antibiotics and Point to *Paenibacillus spp*. as Antibiotic-Resistance-Dissemination Players. Front Microbiol. 2020 Jan 10;10:3071.
51. Mandal M, Das SN, Mandal S. Principal component analysis exploring the association between antibiotic resistance and heavy metal tolerance of plasmid-bearing sewage wastewater bacteria of clinical relevance. Access Microbiol. 2020 Mar 1;2(3):e000095.
52. Olga P, Apostolos V, Alexis G, George V, Athena M. Antibiotic resistance profiles of *Pseudomonas aeruginosa* isolated from various Greek aquatic environments. FEMS Microbiol Ecol. 2016 May 1;92(5).
53. Kateete DP, Nakanjako R, Okee M, Joloba ML, Najjuka CF. Genotypic diversity among multidrug resistant *Pseudomonas aeruginosa* and *Acinetobacter* species at Mulago Hospital in Kampala, Uganda. BMC Res Notes. 2017 Dec;10(1):1–0.
54. Nain Z, Islam M, Minnatul Karim M. Antibiotic Resistance Profiling and Molecular Phylogeny of Biofilm Forming Bacteria From Clinical and Non-clinical Environment in Southern Part of Bangladesh. Int J Enteric Pathog. 2019;7(2):37–43.
55. Kathleen MM, Samuel L, Felecia C, Reagan EL, Kasing A, Lesley M, Toh SC. Antibiotic resistance of diverse bacteria from aquaculture in Borneo. Int J Microbiol. 2016;2016.
56. Stenström TA, Okoh AI, Adegoke AA. Antibiogram of environmental isolates of *Acinetobacter calcoaceticus* from Nkonkobe Municipality, South Africa. Fresenius Environ Bull. 2016;25:3059–62.
57. Azzam MI, Ezzat SM, Othman BA, El-Dougdoug KA. Antibiotics resistance phenomenon and virulence ability in bacteria from water environment. Water Sci. 2017 Oct 1;31(2):109 – 21.
58. Odjadjare EE, Igbinosa EO, Mordi R, Igere B, Igeleke CL, Okoh AI. Prevalence of multiple antibiotics resistant (MAR) *Pseudomonas* species in the final effluents of three municipal wastewater treatment facilities in South Africa. Int J Environ Res Public health. 2012 Jun;9(6):2092–107.
59. Gufe C, Canaan Hodobo T, Mbonjani B, Majonga O, Marumure J, Musari S, Jongi G, Makaya PV, Machakwa J. Antimicrobial Profiling of Bacteria Isolated from Fish Sold at Informal Market in

Mufakose, Zimbabwe. Int J Microbiol. 2019;2019.

60. Rezai MS, Ahangarkani F, Rafiei A, Hajalibeig A, Bagheri-Nesami M. Extended-Spectrum Beta-Lactamases Producing *Pseudomonas aeruginosa* Isolated From Patients With Ventilator Associated Nosocomial Infection. Arch Clin Infect Dis. 2018 Aug;13(4).
61. Tew LS, She LY, Chew CH. Isolation, antimicrobial susceptibility profile and detection of Sul1, blaTEM, and blaSHV in amoxicillin-clavulanate-resistant bacteria isolated from retail sausages in Kampar, Malaysia. Jundishapur J Microbiol. 2016 Oct;9(10).
62. Bajpai V, Govindaswamy A, Khurana S, Batra P, Aravinda A, Katoch O, Hasan F, Malhotra R, Mathur P. Phenotypic & genotypic profile of antimicrobial resistance in *Pseudomonas* species in hospitalized patients. Indian J Med Res. 2019 Feb;149(2):216.
63. Poloto M, Casella T, Oliveria M, Rubio F, Nogueira M, Almeida M. Detection of *P. aeruginosa* harboring blaCTX-M-2, blaGES-1 and blaGES-5, blaIMP-1 and blaSPM-1 causing infectious in Brazilian tertiary-care hospital. BMC Infect Dis. 2012;12:176.
64. Olowe OA, Adewumi O, Odewale G, Ojurongbe O, Adefioye OJ. Phenotypic and molecular characterisation of extended-spectrum beta-lactamase producing *Escherichia coli* obtained from animal fecal samples in Ado Ekiti, Nigeria. J Environ Public Health. 2015;2015.
65. American Public Health Association (APHA). (2012) Standard methods for the examination of water and wastewater. APHA 22nd Edition, Washington DC.
66. Goudarzi M, Fazeli M, Azad M, Seyedjavadi SS, Mousavi R. Aloe vera gel: effective therapeutic agent against multidrug-resistant *Pseudomonas aeruginosa* isolates recovered from burn wound infections. Chemother Res Pract. 2015;2015.
67. Lutgring JD, Kim A, Campbell D, Karlsson M, Brown AC, Burd EM. Evaluation of the MicroScan colistin well and gradient diffusion strips for colistin susceptibility testing in Enterobacteriaceae. J Clin Microbiol. 2019;57(5):e01866-18.
68. Clinical and Laboratory Standards Institute (CLSI). Performance Standards for Antimicrobial Susceptibility Testing; 20th Informational Supplement. CLSI document M100-S20, 2010. CLSI, Wayne, PA: Clinical and Laboratory Standards Institute.
69. Magiorakos AP, Srinivasan A, Carey RB, Carmeli Y, Falagas ME, Giske CG, Harbarth S, Hindler JF, Kahlmeter G, Olsson-Liljequist B, Paterson DL. Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance. Clin Microbiol Infect. 2012 Mar;18(3):268–81.
70. Angeletti S, Gherardi G, De Florio L, Avola A, Crea F, Riva E, Vitali MA, Galluzzo S, Dicuonzo G. Real-time polymerase chain reaction with melting analysis of positive blood culture specimens in bloodstream infections: diagnostic value and turnaround time. New Microbiol. 2013;36:65–74.
71. Roschanski N, Fischer J, Guerra B, Roesler U. Development of a multiplex real-time PCR for the rapid detection of the predominant beta-lactamase genes *CTX-M, SHV, TEM and CIT*-type AmpCs in Enterobacteriaceae. PLoS One. 2014;9(7).

72. Wendel AF, Brodner AH, Wydra S, Ressina S, Henrich B, Pfeffer K, Toleman MA, MacKenzie CR. Genetic characterization and emergence of the metallo-β-lactamase GIM-1 in *Pseudomonas* spp. and Enterobacteriaceae during a long-term outbreak. Antimicrob Agents Chemother. 2013;57(10):5162–5.
73. Anuj SN, Whiley DM, Kidd TJ, Bell SC, Wainwright CE, Nissen MD, Sloots TP. Identification of *Pseudomonas aeruginosa* by a duplex real-time polymerase chain reaction assay targeting the ecfX and the gyrB genes. Diagn Microbiol Infect Dis. 2009;63(2):127–31.

Figures

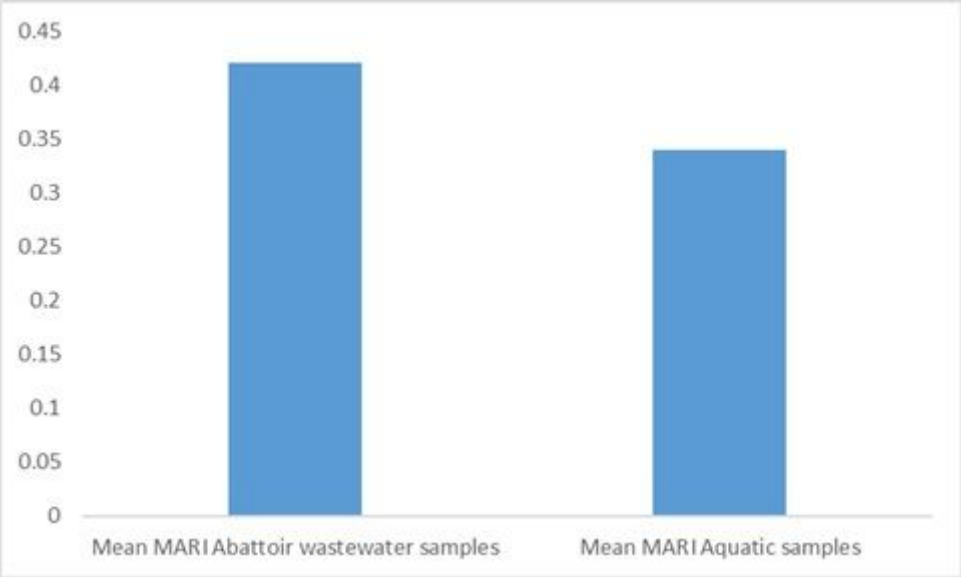

Figure 1

Mean Multiple antibiotic resistance index of non-clinical P. aeruginosa isolates