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Antibiotic resistance and biofilm formation of *Acinetobacter baumannii* isolated from high-risk effluent water in tertiary hospitals in South Africa

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ABSTRACT

Objectives: Discharge of drug-resistant, biofilm-forming pathogens from hospital effluent water into municipal wastewater treatment plants poses a public health concern. This study examined the relationship between antibiotic resistance levels and biofilm formation of *Acinetobacter baumannii* strains isolated from hospital effluents.

Methods: Antibiotic susceptibility of 71 *A. baumannii* isolates was evaluated by the Kirby–Bauer disk diffusion method. Minimum inhibitory concentrations (MICs) were determined by the agar dilution method, while the minimum biofilm eradication concentration (MBEC) was determined by the broth dilution method. Genotyping was performed for plasmid DNA. Biofilm formation was evaluated by the microtitre plate method and was quantified using crystal violet. A *P*-value of <0.05 was regarded as statistically significant in all tests.

Results: Extensively drug-resistant (XDR) strains made up 58% of the isolates, while multidrug-resistant (MDR) and pandrug-resistant (PDR) strains made up 50% of the isolates from final effluent. The MBEC of ciprofloxacin increased by 255-fold, while that of ceftazidime was as high as 63–1310-fold compared with their respective MICs. Isolates were classified into four plasmid pattern groups with no association between biofilm formation and plasmid type ($P = 0.0921$). The degree of biofilm formation was independent of the level of antibiotic resistance, although MDR, XDR and PDR isolates produced significant biofilm biomass ($P = 0.2580$).

Conclusion: These results suggest that hospital effluent is a potential source of MDR biofilm-forming *A. baumannii* strains. Appropriate treatment and disposal of effluents are essential to prevent the presence of drug-resistant pathogens in wastewater.

1. Introduction

Antibiotic resistance is currently one of the greatest challenges facing global health. It is estimated that antimicrobial resistance will lead to more deaths than other major causes of death, including cancer, at approximately 10 million by 2050 [1]. The high rate of abuse, misuse and inappropriate use of antibiotics together with poor awareness of the factors and mechanisms that promote drug resistance contribute to the progression of this global health concern [2]. The release of toxic chemicals, heavy metals and sewage elements especially from industrial and hospital effluent through wastewater treatment plants (WWTPs) has been reported largely as a driver of antibiotic resistance [3,4]. The procedure of indexing bacterial isolates using the multiple antibiotic resistance index (MARI) indicates that hospital effluents constitute an origin of high-risk isolates that could be disseminated to drinking water sources [5]. The extent to which micro-organisms develop drug resistance suggests poor control strategies and/or the continued innovation by microbial cells of new mechanisms to

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evade antimicrobial agents and the acquisition of plasmids harbouring resistance determinants within the microbial environment [6]. One of the most widely reported mechanisms for the selection of antibiotic-resistant bacteria through plasmid acquisition and evasion of drug action is the existence of microbial cells in community-like life forms known as biofilms [7].

Bacterial biofilm formation enhances many mechanisms of drug resistance, including enzymatic degradation, drug exclusion and permeability defeats [8]. For example, in adverse environments, the biofilm phenotype offers bacterial cells a strength-through-unity-like protection and promotes their persistence, unlike individual planktonic bacteria [9]. Several research reports have documented the ability of biofilms to demonstrate increased resistance to environmental pressures and chemical stressors [10]. Although researchers have devoted much effort in investigating antimicrobial resistance in single-celled bacteria, it is indisputable that most bacterial cells scarcely exist alone in the infectious state [7]. The close interaction between microbial cells in biofilms encourages the possible sharing of resistance determinants and other associated virulence genes between participating organisms [11]. Identification of common antibiotic resistance determinants as part of biofilm-specific determinants have revolutionised biofilm research [11].

Biofilm formation in *Acinetobacter baumannii* has been widely reported over the last two decades [12–14]. *Acinetobacter baumannii* was recently listed by the World Health Organization (WHO) as one of the critical priority pathogens of interest for research [15] and has been extensively reported in hospital- and device-associated infections in intensive care units [16]. *Acinetobacter baumannii* is significantly associated with resistance to almost all currently available antibiotics [15,17]. The ability of *A. baumannii* to survive in desiccated and harsh environments through biofilm formation has promoted its success as a nosocomial pathogen [14]. One of the predisposing factors for treatment failure of biofilm-associated infections is a lack of understanding in the selection of appropriate antibiotics for treatment and/or inaccurate dependence on minimum inhibitory concentration (MIC) data for bacteria in the biofilm state [18]. Thus, it is pertinent to investigate the relationship between biofilm formation and antibiotic resistance to inform appropriate treatment regimens [18].

It remains controversial whether biofilm formation incurs a fitness cost in multidrug-resistant (MDR) bacteria.

This study reports on the association between biofilm formation and antibiotic resistance of *A. baumannii* strains isolated from hospital effluent water from two hospitals in KwaZulu-Natal Province, South Africa.

2. Methodology

2.1. Sample collection

Effluent water samples were obtained on two separate occasions from two hospitals in South Africa: Appelsbosch Hospital, a rural district hospital located at Ozwathini with a bed capacity of approximately 159 (designated Hospital A); and Greys Hospital, an urban tertiary hospital in Pietermaritzburg providing services to approximately 3.5 million persons in Pietermaritzburg, KwaZulu-Natal Province (designated Hospital B). The first sample collection was on 8 August 2017 and the second on 22 August 2017. On each occasion, three samples were obtained from both hospitals using a sterile plastic bottle and were immediately transferred on ice to the laboratory and analysed within 3 h. The different sampling points included the main ward (at the South block), final effluent (collection point for all kinds of effluent) and effluent from the pathology laboratory in Greys Hospital. Effluents from the main ward (maternity), paediatrics and the final effluent from Appelsbosch Hospital were also collected.

2.2. Isolation and identification of Acinetobacter baumannii from hospital effluent

A volume of 50 mL of each sample was filtered through a 0.45 μm membrane filter (Millipore, Billerica, MA, USA) and the filter was placed on Leeds *Acinetobacter* Medium (LAM) agar with multidrug-resistant selective supplement (HiMedia™ Laboratories, India) and incubated at 37°C for 24 h. Presumptive *A. baumannii* isolates were selected by screening with antibiotic punch test and incubation at 44°C for 24 h. Mucoid pink colonies were sub-cultured on Mueller–Hinton agar (Thermo Scientific™ Oxoid™, South Africa), incubated at 44°C for 24 h, followed by growth on LAM agar at 37°C for 24 h. Preliminary identification by Gram staining, catalase test and oxidase test was conducted to select oxidase-negative and catalase-positive Gram-negative bacteria. Isolates were screened for the presence of plasmids by the alkaline lysis method to select isolates with diverse plasmid types [19]. Isolates were confirmed by VITEK®2 automated system (bioMérieux, Marcy-l'Étoile, France) as *A. baumannii*. Stock cultures of strains with plasmids ($n = 71$) were stored at −80°C. No isolates were obtained from the paediatrics ward in Appelsbosch Hospital. All isolates were kept on tryptic soy agar (Sigma-Aldrich, South Africa) plates and stored at 4°C for short-term use. The type strain *A. baumannii* ATCC 19606 (American Type Culture Collection, Manassas, VA, USA) was used as a reference strain in all assays [20].

2.3. Determination of antimicrobial susceptibilities and minimum inhibitory concentrations (MICs) of Acinetobacter baumannii isolates

The antimicrobial susceptibility of 71 *A. baumannii* isolates to ampicillin/sulbactam (10/10 μg), piperacillin/tazobactam (100/10 μg), ticarcillin/clavulanic acid (75/10 μg), ceftazidime (30 μg), cefepime (30 μg), cefotaxime (30 μg), ceftriaxone (30 μg), imipenem (10 μg), doripenem (10 μg), meropenem (10 μg), gentamicin (10 μg), tobramycin (10 μg), amikacin (30 μg), doxycycline (30 μg), minocycline (30 μg), tetracycline (30 μg), ciprofloxacin (5 μg), levofloxacin (5 μg), trimethoprim/sulfamethoxazole (1.25/23.75 μg) and piperacillin (100 μg) was determined by the Kirby–Bauer disk diffusion method (Oxoid Ltd., UK) [21]. Inoculated Mueller–Hinton agar plates were incubated at 37°C for 24 h. Isolates were characterised as susceptible, intermediate or resistant to the antibiotics based on their inhibition zones. The results were interpreted according to Clinical and Laboratory Standard Institute (CLSI, 2020) guidelines [22]. Colistin sulfate was tested on all strains by the broth dilution method as described previously and was interpreted according to CLSI guidelines [22]. MICs of imipenem, ciprofloxacin, cefotaxime, ceftazidime and tetracycline were determined using the agar dilution method as reported previously and were interpreted according to the CLSI standard [22]. *Acinetobacter baumannii* ATCC 19606 and *Pseudomonas aeruginosa* ATCC 27853 were used as reference strains [23].

The multiple antibiotic resistance index (MARI) was calculated according to the following equation [5]:

$$\text{MARI} = \frac{\text{No. of antibiotics to which resistance occurred}}{\text{Total no. of antibiotics to which the isolates were tested}}$$

Isolates were classified as multidrug-resistant (MDR) if they were non-susceptible to at least one agent in three or more antimicrobial categories, extensively drug-resistant (XDR) if they were non-susceptible to at least one agent in all but two or fewer categories, and pandrug-resistant (PDR) if they non-susceptible to all agents in all antibiotic categories [24].

2.4. Measurement of biofilm-forming ability

Biofilm formation of the 71 *A. baumannii* isolates was assessed by the modified microtitre plate assay in triplicate and repeated three times as described previously [25,26]. Briefly, an overnight tryptic soy broth culture standardised to a 1.0 McFarland in Anacker and Ordal broth (EAOB) was incubated at 37°C for 24 h without agitation. After washing, the dried plates were stained with 2% Hugo's crystal violet for 10 min. The stain was washed, solubilised in 33% (v/v) glacial acetic acid (ChemLab, South Africa) and the optical density at 570 nm (OD_{570}) was measured using a spectrophotometer (GloMax® Discover spectrophotometer; Promega, Australia), using uninoculated EAOB and *A. baumannii* ATCC 19606 as negative and positive controls, respectively. The results were interpreted as previously described: The optical density cut-off value (OD_C) was established as three standard deviations (SD) above the mean of the OD of the negative control: OD_C = average OD of negative control (uninoculated growth media). Isolates were classified as follows: OD ≤ OD_C = non-adherent; ODc < OD ≤ ($2 \times OD_C$) = weakly adherent; ($2 \times OD_C$) < OD ≤ ($4 \times OD_C$) = moderately adherent; and ($4 \times OD_C$) < OD = strongly adherent [27].

2.5. Plasmid typing

Plasmid DNA typing and interpretation were carried out as previously described with minor modifications [28]. Briefly, frozen stocks of bacterial isolates were revived on tryptic soy agar at 37°C for 24 h. Discrete colonies were subcultured in Luria–Bertani (Sigma-Aldrich) broth with shaking overnight at 37°C. Plasmid DNA was extracted using a GeneJET Plasmid Miniprep Kit (Thermo Scientific, Waltham, MA, USA) as per the manufacturer's instructions, after which 5 μL of the sample was electrophoresed in 1% agarose gel for 2 h at 70 V. Gels were stained with 0.1 μg/mL ethidium bromide, incubated for 15–20 min and visualised under ultraviolet irradiation with a gel documentation system (Mild Steel Rectangular Gel Documentation System IG-618GD; iGene Labserve India). Lambda DNA-*Hind*III (Sigma-Aldrich) was used as a molecular marker. Different plasmid types were defined as any pattern that varied from another in the number and size of the bands. Two strains were considered related if the banding pattern was identical or not more than one band was present or absent in one of them. Plasmid DNA was run in duplicate in different gels [28].

2.6. Biofilm antibiotic susceptibility testing

The minimum biofilm eradication concentrations (MBECs) of ciprofloxacin and ceftazidime against 24 strains randomly selected as representative of the 71 *A. baumannii* strains were determined using the broth microdilution method according to a previously described method [25]. The selected strains included representatives of the strong, medium and weak biofilm-producing strains. The selected antibiotics demonstrated the highest and lowest activity, respectively, as shown by their MICs. Briefly, 10 μL of an overnight Mueller–Hinton broth (MHB) (Sigma-Aldrich) culture of the isolates made up with 90 μL of autoclaved MHB was cultured in sterile 96-well round-bottomed polystyrene microtitre plates and incubated at 37°C for 24 h to allow for biofilm formation. The biofilms formed were then exposed to ciprofloxacin and ceftazidime at concentrations between 256 μg/mL and 262 144 μg/mL overnight at 37°C. The wells were made up to a final volume of 400 μL using sterile distilled water before incubating at 37°C for 24 h. Wells were then washed three times with sterile distilled water and were incubated in MHB for 24 h at 37°C. The MBEC was defined as the concentration at which no viable cell was observed in the biofilm material as demonstrated by an OD_{570} < 0.1 [25]. All tests were performed in triplicate.

2.7. Statistical analyses

Biofilm-forming capacities (OD values) were expressed either as the mean ± SD or as median values and interquartile range. The biofilm-forming capacity among the susceptible, resistant and intermediate strains and the MDR, XDR and PDR strains as well as between MICs and MBECs were compared using Spearman's rank correlation test. The Wilcoxon rank sum test was used to compare biofilm-forming capacity among isolates that were either susceptible or non-susceptible to each antimicrobial category. Data analyses were performed using GraphPad Prism v.5. A *P*-value of <0.05 was considered statistically significant for all comparisons.

3. Results

3.1. Predominance of multidrug-resistant (MDR) and extensively drug-resistant (XDR) strains of Acinetobacter baumannii in hospital effluent samples

During the sampling period, 71 *A. baumannii* isolates, comprising 36 (50.7%) from final effluent, 19 (26.8%) from the pathology laboratory and 16 (22.5%) from the main hospital ward were obtained. No isolate was obtained from the paediatric ward of the rural hospital. The 71 *A. baumannii* isolates were tested against 21 antibiotics in nine antibiotic classes, namely aminoglycosides, carbapenems, cephalosporins, β-lactam/β-lactamase inhibitors, fluoroquinolones, folate pathway inhibitors, penicillins, tetracyclines and polymyxins (Fig. 1). Whilst all of the isolates were resistant to at least 1 antibiotic, none of the isolates were susceptible to all 21 antibiotics tested. Of all nine categories of antimicrobials tested, only one isolate was non-resistant to at least one antibiotic in seven categories, none was susceptible to all antibiotics in all categories, while 17% were resistant to all antibiotics in all the categories (Supplementary Table S1). MDR, XDR and PDR isolates were observed from each source (Fig. 1), with 24.0% classified as MDR, 57.7% as XDR and 16.9% as PDR. The majority of the MDR (47%), XDR (51%) and PDR (50%) strains were obtained from the final effluent of the hospitals, while only 24.4% of XDR strains (10/41) originated from the pathology laboratory. Of the three sampling sites, main waste contained the fewest numbers of MDR and PDR isolates. There was no significant association between the source of isolates and the resistance phenotype ($P = 0.9696$) (Supplementary Fig. S1). Generally, the MARI of all of the isolates was above the threshold of 0.20 [23], ranging from 0.4 to 1.0. The final effluent from the rural tertiary hospital (Hospital A) and the pathology laboratory of an urban healthcare centre (Hospital B) contained isolates with the highest MARI (Supplementary Tables S2 and S3). Based on the criteria, 56.3%, 39.4% and 4.3% were categorised as strong, moderate and weak biofilm-producing strains, respectively, but no significant difference was observed among the strong, moderate and weak biofilm-formers.

3.2. Measurement of biofilm formation

The OD_{570} values of the positive control (reference strain *A. baumannii* ATCC 19606) and the negative control (uninoculated broth) were 0.22 ± 0.02 and 0.06 ± 0.01, respectively. Based on the criteria, 56.3%, 39.4% and 4.2% were categorised as strong, moderate and weak biofilm-producing strains, respectively. No significant difference in the biofilm-forming capacity of the strong, moderate and weak biofilm-formers was observed (Fig. 2A) among the different groups of resistance phenotypes, indicating that differences

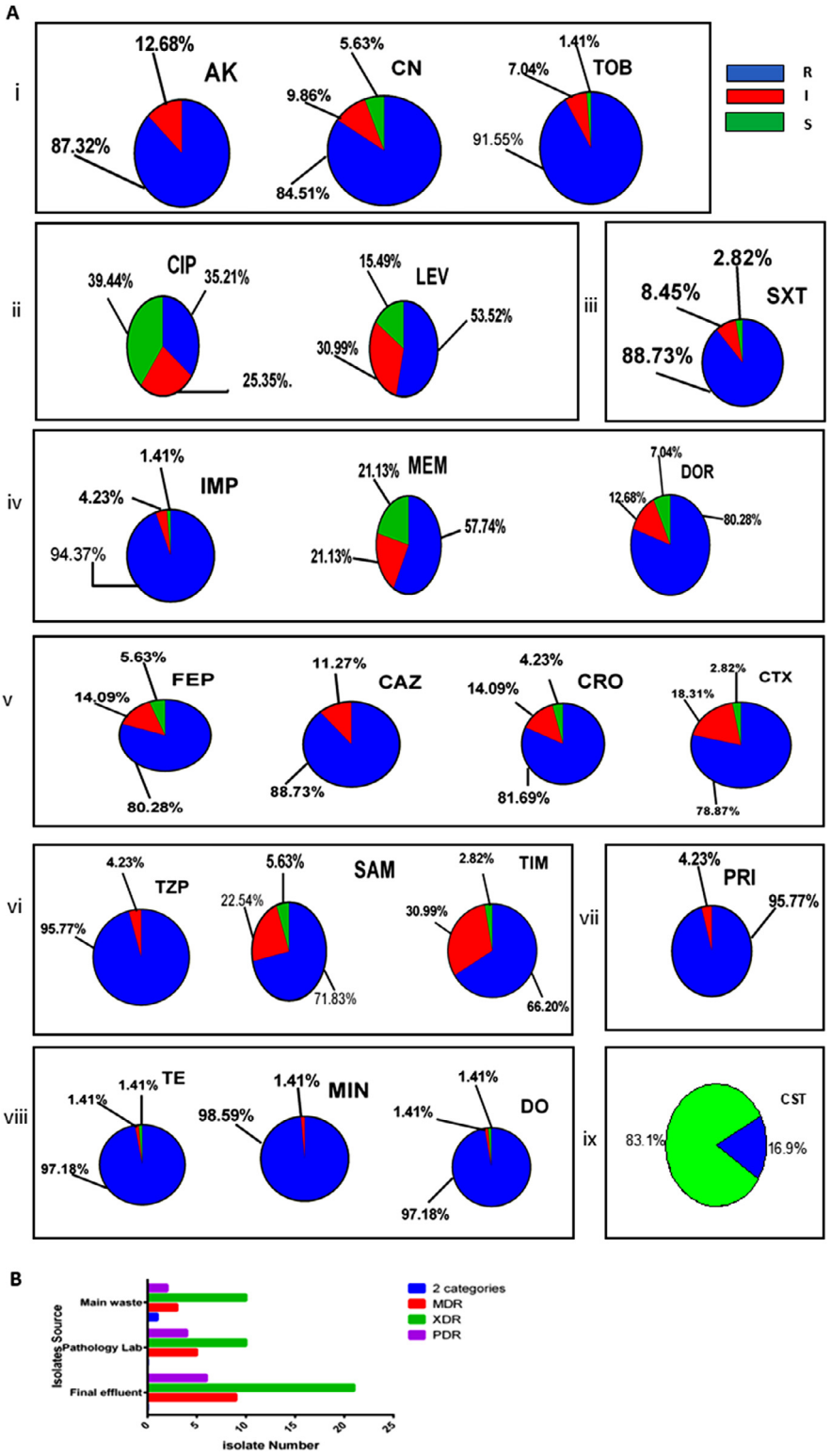

Fig. 1. Antimicrobial resistance phenotype of 71 *Acinetobacter baumannii* isolates to the 21 antibiotics tested in nine classes. The resistance rate showed that tetracyclines were the most resisted, while fluoroquinolones were most intermediate. (i) Aminoglycosides, (ii) fluoroquinolones, (iii) folate pathway inhibitors, (iv) carbapenems, (v) cephalosporins, (vi) β-lactam/β-lactamase inhibitors, (vii) penicillins, (viii) tetracyclines and (ix) polymyxins. Most of the isolates (57.7%) were XDR. AK, amikacin; CN, gentamicin; TOB, tobramycin; CIP, ciprofloxacin; LEV, levofloxacin; SXT, trimethoprim/sulfamethoxazole; IMP, imipenem; MEM, meropenem; DOR, doripenem; FEP, cefepime; CAZ, ceftazidime; CRO, ceftriaxone; CTX, cefotaxime; TZP, piperacillin/tazobactam; SAM, ampicillin/sulbactam; TIM, ticarcillin/clavulanic acid; PRI, piperacillin; TE, tetracycline; MIN, minocycline, DO, doxycycline, CST, colistin. MDR, multidrug-resistant; XDR, extensively drug-resistant; PDR, pandrug-resistant.

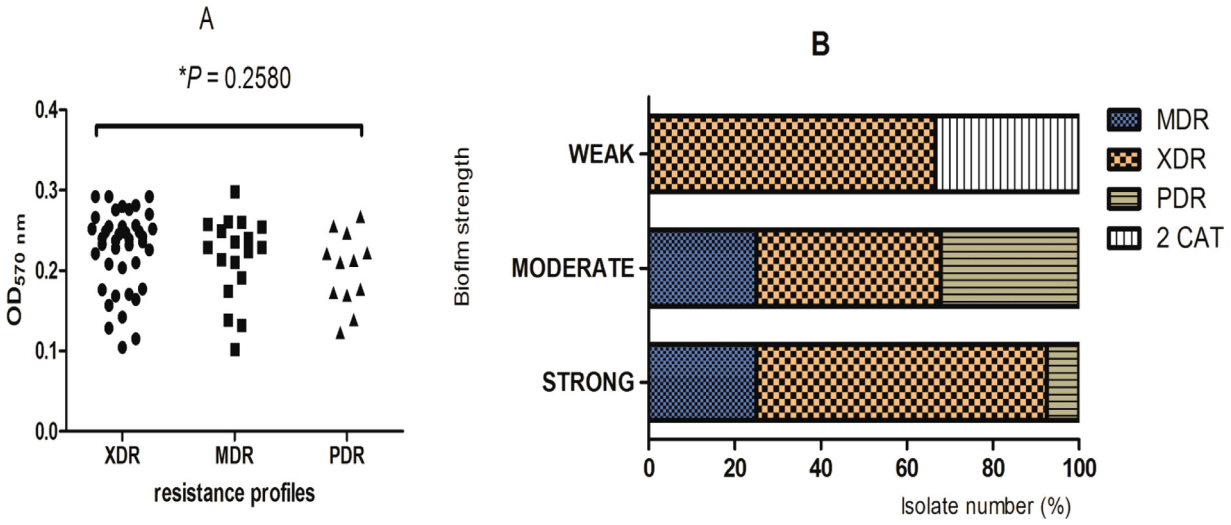

Fig. 2. Association between drug resistance and biofilm-forming capacity of isolates measured at an optical density at 570 nm (OD_{570}). Isolates were divided into three groups according to their antibiotic resistance phenotypes, as multidrug-resistant (MDR), extensively drug-resistant (XDR) or pandrug-resistant (PDR). Two categories (2 CAT) means resistance to only two antimicrobial categories. (A) A larger proportion of XDR phenotypes appear to form strong biofilm (higher OD_{570} values) as indicated in the stacked bar graph. (B) Distribution of biofilm formation of isolates with different resistance phenotypes indicated no statistically significant difference among the phenotypes. A larger proportion of the PDR isolates formed either moderate or weak biofilms.

Table 1
Correlation between level of antibiotic resistance and biofilm formation of 21 antibiotics in 71 *Acinetobacter baumannii* isolates from hospital effluent water

Antimicrobial category	Antimicrobial agent	OD_{570} [median (IQR)]			r_s	*P*-value
		S	I	R		
Aminoglycosides	Gentamicin	0.243 (0.229–0.260)	0.221 (0.138–0.266)	0.217 (0.101–0.298)	0.316	0.0041
	Tobramycin	–	0.220 (0.138–0.298)	0.218 (0.101–0.292)	0.155	0.196
	Amikacin	–	0.240 (0.138–0.298)	0.216 (0.101–0.216)	0.178	0.1373
Carbapenems	Imipenem	–	0.266 (0.248–0.298)	0.217 (0.101–0.292)	0.3025	0.0103
	Meropenem	0.208 (0.115–0.261)	0.200 (0.104–0.298)	0.226 (0.101–0.292)	–0.154	0.1996
	Doripenem	0.235 (0.191–0.256)	0.230 (0.128–0.281)	0.216 (0.101–0.298)	0.3065	0.0093
Fluoroquinolones	Ciprofloxacin	0.216 (0.101–0.292)	0.212 (0.104–0.298)	0.226 (0.123–0.281)	0.0104	0.9322
	Levofloxacin	0.228 (0.170–0.270)	0.229 (0.128–0.298)	0.210 (0.101–0.292)	0.214	0.0732
β-Lactam/β-lactamase inhibitors	Piperacillin/tazobactam	–	0.212 (0.138–0.249)	0.219 (0.101–0.298)	0.162	0.170
	Ticarcillin/clavulanic acid	0.174 (0.138–0.210)	0.228 (0.128–0.298)	0.216 (0.104–0.292)	–0.0826	0.4932
	Ampicillin/sulbactam	0.231 (0.138–0.298)	0.208 (0.101–0.261)	0.221 (0.104–0.292)	–0.086	0.7648
Cephalosporins	Cefotaxime	0.187 (0.138–0.235)	0.246 (0.101–0.281)	0.214 (0.101–0.298)	0.0711	0.5656
	Ceftriaxone	0.202 (0.138–0.235)	0.216 (0.115–0.276)	0.220 (0.101–0.298)	0.042	0.7280
	Ceftazidime	–	0.233 (0.138–0.298)	0.217 (0.101–0.292)	0.1579	0.1884
	Cefepime	0.222 (0.138–0.261)	0.240 (0.191–0.292)	0.215 (0.101–0.298)	0.2357	0.0479
Folate pathway inhibitors	Trimethoprim/sulfamethoxazole	0.240 (0.229–0.251)	0.231 (0.132–0.292)	0.216 (0.101–0.298)	0.0667	0.5801
Penicillins	Piperacillin		0.1945 (0.139–0.250)	0.197 (0.102–0.292)	0.0079	0.9488
Tetracyclines	Tetracycline	–	–	0.217 (0.101–0.292)	0.0680	0.6253
	Doxycycline	–	–	0.217 (0.101–0.292)	0.1539	0.2001
	Minocycline	–	–	0.218 (0.101–0.292)	0.0617	0.6088
Polymyxins	Colistin	0.2035 (0.115–0.292)		0.213 (0.170–0.256)	–0.5294	0.0210

OD_{570}, optical density at 570 nm; IQR, interquartile range; S, susceptible; I, intermediate; R, resistant; r_s, Spearman's correlation coefficient.

in the capacity of the isolates to form biofilm was not impacted by their level of antibiotic resistance.

3.3. Biofilm formation does not depend on the extent of antibiotic resistance of the isolates

Isolates were grouped into different resistance phenotypes to ascertain any association between antibiotic resistance and biofilm formation (Table 1; Fig. 2). Of the 40 strong biofilm-formers, 25%, 67.5% and 7.5% were MDR, XDR and PDR strains, respectively (Fig. 2B). The proportion of moderate biofilm-producing strains that were MDR, XDR and PDR was 25%, 42.9% and 32.1%, respectively. Among the three strains with weak biofilm-forming ability, two were classified as XDR and one was resistant to only two categories of antibiotics. None of the MDR strains formed weak biofilms. A smaller proportion of PDR *A. baumannii* isolates tended to form stronger biofilms than MDR and XDR strains. No significant association was observed between biofilm capacity and the resistance phenotype ($P = 0.2580$) (Fig. 2A) and this was confirmed by χ^2 analysis ($\chi^2 = 0.4092$). The distribution of the biofilm formation capacity among the resistance profiles of the isolates is summarised in Table 1, Fig. 2 and Supplementary Fig. S2.

3.4. Association of biofilm formation and resistance to specific antibiotics

The biofilm-forming abilities of the 71 *A. baumannii* isolates were compared with their respective resistance profiles using 21 specific antibiotics to determine any association between the isolates. With the exception of meropenem, ticarcillin/clavulanic acid, ampicillin/sulbactam and colistin that showed negative correlations, a positive correlation was observed between biofilm biomass and the resistance profile to the remaining 16 antibiotics ($r_s = 0.010–0.316$) (Table 1). Statistically significant correla-

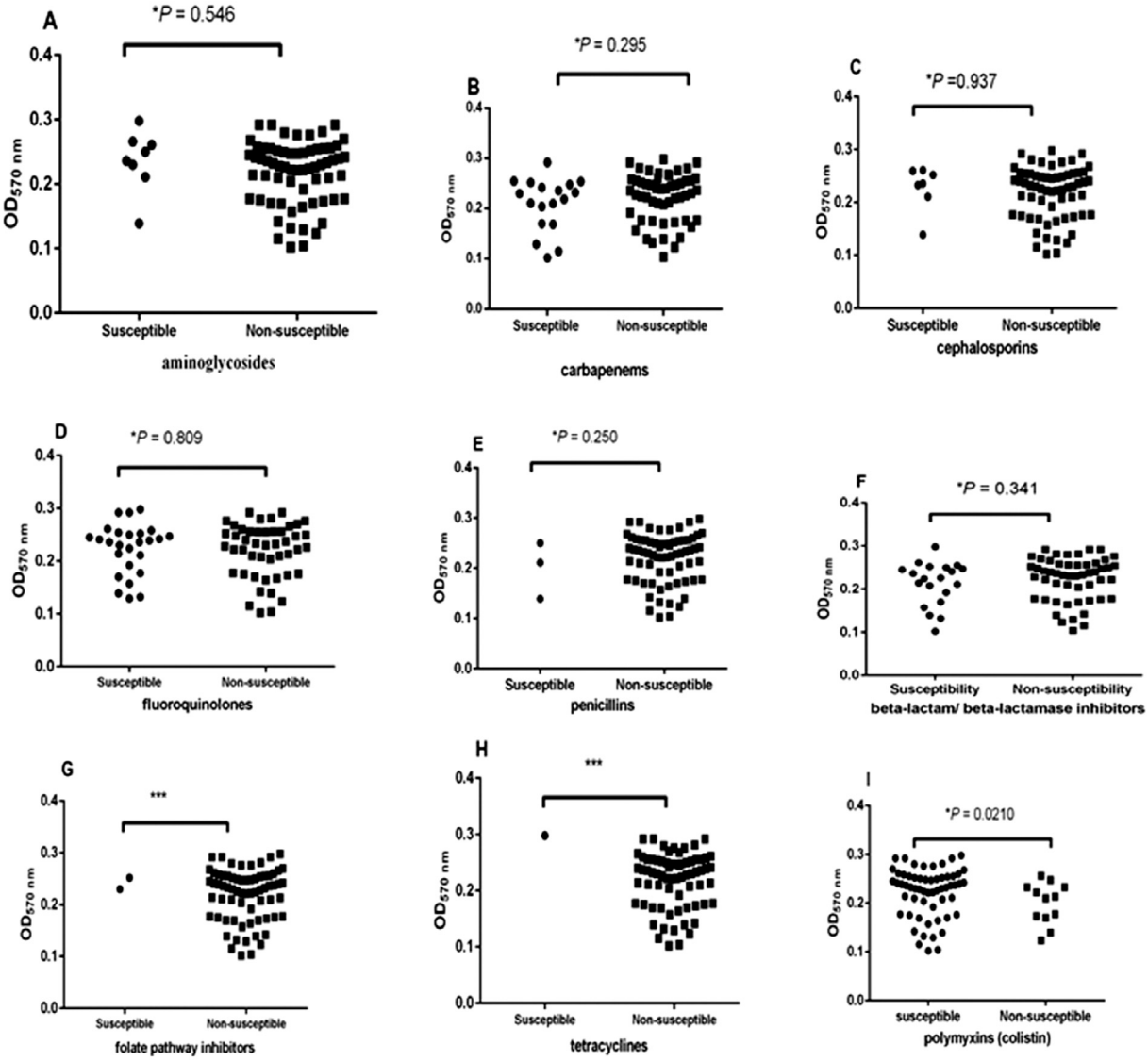

Fig. 3. Relationship between biofilm formation measured at an optical density at 570 nm (OD_{570}) and resistance of *Acinetobacter baumannii* isolates to each of the nine antimicrobial categories. (A–F) For aminoglycosides, carbapenems, cephalosporins, fluoroquinolones, penicillins and β-lactam/β-lactamase inhibitors, there was no statistical difference between susceptible and non-susceptible isolates regarding biofilm formation ($P > 0.05$). (G,H) For folate pathway inhibitors and tetracyclines, almost all biofilm-formers were non-susceptible. *** Insufficient data for correlation. (I) Colistin demonstrated a statistical difference in biofilm formation between susceptible and non-susceptible strains.

tions were observed for gentamicin, imipenem, doripenem, colistin and cefepime ($P < 0.05$) (Table 1). Analysis of the correlation between biofilm and resistance to the nine antimicrobial categories revealed no statistically significant difference for six categories, including aminoglycosides, carbapenems, cephalosporins, fluoroquinolones, penicillins and β-lactam/β-lactamase inhibitors ($P > 0.05$) (Fig. 3).

3.5. The lowest antimicrobial concentration at which isolates are inhibited does not influence their biofilm-forming abilities

The MICs and MBECs of the selected antibiotics were evaluated against 24 randomly selected *A. baumannii* isolates (Table 2). MIC ranges were 64–128 μg/mL for imipenem, 64–200 μg/mL cefotaxime, 128–200 μg/mL for ceftazidime, 16–128 μg/mL for tetracycline and 1–8 μg/mL for ciprofloxacin. The MBEC of ceftazidime was as high as 8192–262 144 μg/mL, which was 63–1310-fold higher than the MICs. Whereas for ciprofloxacin the MBEC ranged from 256–2048 μg/mL, which was 255-fold higher than the MICs. The result indicated that the lower MIC of ciprofloxacin did not result in an increased effect on the MBEC level of the isolates. Except for ciprofloxacin, a negative correlation existed between the MICs of four selected antibiotics and the biofilm-forming capacities of the 24 strains ($r_s = -0.09705$ to -0.197; $P > 0.05$) (Supplementary Table S4), indicative of an inverse relationship between biofilm formation and resistance to each antibiotic (Table 2). In addition, for ciprofloxacin a negative correlation was observed between antibiotic resistance (MICs) and biofilm-specific resistance (MBECs) ($r = -0.2601$; $P > 0.05$). As shown in Table 1, the lowest concentration of the five antibiotics at which each of the selected isolates were inhibited did not have an appreciable influence on the biofilm biomass produced. Also, Spearman's rank correlation analysis showed that for ciprofloxacin, biofilm-specific resistance was independent of the level of biomass produced ($r = -0.07956$; $P > 0.05$) (Supplementary Fig. S2).

Table 2
Comparison of drug susceptibility, MIC and MBEC to selected antibiotics of 24 biofilm-producing *Acinetobacter baumannii* strains

Antimicrobial agent	Biofilm-forming isolates (n = 24)			r_s	P-value
	No. (%) resistant	MIC (μg/mL)	MBEC (μg/mL)		
Imipenem	21 (87.5)	≥64	– [a]	–0.09705	0.6519
Cefotaxime	17 (70.8)	≥64	–	–0.1417	0.5089
Ceftazidime	20 (83.3)	≥128	≥8192	–0.197	0.3562
Tetracycline	22 (91.6)	≥16	–	–0.1338	0.5332
Ciprofloxacin	9 (37.5)	≥1	≥256	0.1663	0.4374

MIC, minimum inhibitory concentration; MBEC, minimum biofilm eradication concentration; r_s, Spearman's correlation coefficient.
[a] –, not tested.

3.6. Biotyping and plasmid profiles

Four plasmid types were observed among the 71 isolates examined. Plasmid types 2–5 were harboured by 47, 8, 11 and 5 isolates, respectively. The final effluent and the main ward contained 27 and 11 isolates with plasmid type 2, respectively, while the majority of isolates with type 3 and 4 plasmids were from the pathology laboratory. The main ward from the urban hospital contained four isolates that harboured a type 5 plasmid. The plasmids were of various sizes ranging from 2.3 kb to 23.3 kb. Isolates resistant to a greater number of antibiotics were most likely to harbour diverse plasmid types ($P < 0.05$). No significant correlation was found between biofilm-forming capacity and the plasmid type harboured ($P > 0.05$) (Supplementary Fig. S3). Strains with the same plasmid profile was observed to have different resistance phenotypes, biofilm-forming capacities and MARI values, indicating that despite the similarity in their band characteristics, isolates may show different resistance to antibiotics.

4. Discussion

Antibiotic-resistant *A. baumannii* has emerged in recent years as a serious threat to public health globally [24] and is regarded as one of the top priorities requiring urgent attention. Discharge of resistant bacterial strains from hospital wastewater feeding into municipal WWTPs is a potential risk to public health [29–31]. In the current study, assessment of antibiotic resistance phenotypes and its risk assessment through measurement of the MARI and the associated biofilm-specific resistance of *A. baumannii* obtained from effluents from three sites each in two local hospitals was determined. A total of 71‘ confirmed *A. baumannii* isolates comprising 50.7% from the final effluent, 26.8% from the pathology laboratory and 22.5% from the main hospital ward were obtained from both hospitals. No isolate was obtained from the paediatric ward in the rural hospital. The prevalence of MDR and XDR isolates in the effluent was 81.7%, while the MARI of more than 90% of the isolates was above the threshold of 0.2 [5]. Fluoroquinolones and penicillins + β-lactamase inhibitors had the highest proportion of isolates that were intermediate-resistant. PDR isolates accounted for 16.9% of all isolates examined. Tetracyclines and cephalosporins appeared to be the most highly resisted antibiotics in this study. Previously, there were concerns regarding the rate of *A. baumannii* resistance to third- and fourth-generation cephalosporins in South Africa [32]. A study reported 100% resistance to cefuroxime, 67% to ceftriaxone and 67% to cefepime [33] in 2015. The current study showed that 78.9% of *A. baumannii* were resistant to cefotaxime, 88.7% to ceftazidime, 81.7% to ceftriaxone and 80.2% to cefepime. A report has attributed the rise on the approval of third-generation cephalosporins as a part of the Integrated Management of Childhood Illness Guidelines [34] and Sexually Transmitted Infection Management Guideline in South Africa [32,34]. However, selection pressure exerted on the isolates owing to the environmental niche cannot be underemphasised.

More than 95% of the isolates produced higher biofilm biomass than the reference strain, prompting the investigation into the relationship between biofilm formation and antibiotic resistance. The results demonstrated that the degree of resistance to antibiotics does not significantly affect biofilm biomass produced by the isolates ($P = 0.2580$). Although individual antibiotics, including imipenem, doripenem, gentamicin and cefepime, showed statistical significance, no significant correlation was observed between biofilm formation and resistance to any of the antibiotic categories tested except for polymyxins (Fig. 3; Table 1). It therefore suggests that for *A. baumannii*, the influence of any particular antibiotic on the biofilm-producing capacity differs even within the same category. Antibiotics such as meropenem, colistin, ticarcillin/clavulanic acid and ampicillin/sulbactam showed a negative correlation, while others showed a positive correlation with biofilm-forming ability. Previous studies reported associations of biofilm formation with MDR phenotypes of *A. baumannii* [35,36], while others reported a significant positive correlation with imipenem alone [32,34]. In contrast, other researchers showed non-dependence of biofilm formation on either multidrug resistance or resistance to antibiotics [25,37,38]. The current study indicated that for *A. baumannii*, the extent of resistance to antibiotics does not necessarily influence the level of biofilm production. It appears that antibiotics may provide a trigger for specific transcriptional regulators for biofilm production, and in a pool of *A. baumannii* where such a regulator is absent or silent, antibiotics have a minimal effect. Thus, the effect of different antibiotics on the transcriptional level of biofilm-specific determinants and the loss of fitness with the acquisition of biofilm-related genes warrants future investigation. All selected *A. baumannii* isolates exhibited up to 255-fold and 63–1310-fold greater biofilm-dependent resistance to ciprofloxacin and ceftazidime, respectively, than those under planktonic growth conditions. The data further demonstrated that *A. baumannii* resistance in the biofilm mode of growth was neither influenced by resistance in planktonic mode of growth nor the level of biofilm biomass produced. While Qi et al. previously reported a positive correlation between the MIC and MBEC values for imipenem, ciprofloxacin and cefotaxime in *A. baumannii* [25], the present findings showed a contrast and a negative correlation between the two quantities for only ceftazidime.

Plasmid analysis showed that all of the isolates harboured one or more plasmids. The final effluent from the rural hospital contained most of the isolates with the type 2 plasmid, while the pathology laboratory contained the highest number of isolates harbouring type 5 plasmids. Our data could not link biofilm formation and the type of plasmid harboured by the isolates ($P > 0.05$) (Supplementary Fig. S3). A previous report also showed that there was no significant correlation between plasmid occurrence and biofilm formation [25]. However, it would be interesting to investigate whether a relationship exists between plasmid map and copy number and biofilm production ability of *A. baumannii* strains. The results presented in this report are limited to the two hospitals studied and therefore cannot be extrapolated to other healthcare

systems in South Africa for policy administration. The specific plasmids associated with the acquisition of biofilm-promoting genetic determinants and specific antibiotics require identification to provide relevant information regarding the pathogenicity of *A. baumannii* from hospital wastewater.

In conclusion, hospital effluent may be a reservoir of MDR *A. baumannii* strains with strong biofilm-forming ability that pose a serious risk to public health. Biofilm formation characteristics are not dependent on the level of resistance of a particular strain to antibiotics. Hence, biofilm phenotype and their associated infections should be managed independent of the data generated through planktonic cell investigations. The hospital effluent disposal system is a crucial aspect of the tertiary health system that could bring about selection of resistant strains of microbial species when improperly applied and requires continuous monitoring and surveillance. The findings of the present study suggested that hospital effluent is a potential source of MDR biofilm-forming *A. baumannii* strains. While MDR, XDR and PDR strains produced biofilms, the degree of biofilm formation was independent of the level of drug resistance. Therefore, appropriate treatment and disposal as well as guidelines for handling such hazardous waste are essential to prevent disasters in WWTPs. Awareness and education among workers at hospital effluent plants are required to avoid occupational exposure to health hazards.

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Competing interests

None declared.

Ethical approval

Ethical approval of the study was granted by the University of KwaZulu-Natal Biomedical Research Ethics Committee (UKZN BREC) [Registration Number of BE063/19].

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jgar.2021.08.004.

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