ORIGINAL ARTICLE

Effects of Antibiotic, Nicotine and Aminoacid starvation stresses on biofilm production in respiratory *Klebsiella pneumoniae*

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Key words:
K.pneumoniae, MAR index, biofilm, antibiotics, amino acid starvation; ERIC-PCR

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ABSTRACT

Background: *Klebsiella pneumoniae is a major cause of hospital-acquired infections in Jamaica.* ***Objective:*** *We aimed to determine their antimicrobial resistance profiles and to assess biofilm formation in the presence of antibiotic, nicotine and amino acid starvation stresses.* ***Methodology:*** *Antimicrobial susceptibility and multiple antimicrobial resistance (MAR) index were determined for 23 K. pneumoniae strains. Biofilm production was evaluated in the presence of 50 μg/ml ceftazidime or gentamicin, 0–4 mg/ml nicotine, or 0.5 mg/ml serine hydroxamate (to induce amino acid starvation). Genetic relatedness, and the presence of type 3 fimbriae (mrkA) and determinants for extended spectrum β-lactamase and carbapenamases (bla-IMP, bla-VIM, bla-GIM and bla-SIM) were assessed by PCR-based amplification.* ***Results:*** *All strains were susceptible to imipenem ($p<0.05$); frequencies of resistance varied from 4% (for amikacin) and 8.7% (for meropenem) to over 30% for the other antimicrobials. About half of strains were resistant to ceftazidime, gentamicin and piperacillin. Mean MAR index was 0.31. The presence of antibiotics and nicotine at 2 and 4 mg/ml negatively affected biofilm formation for most strains. However, with amino acid starvation, almost 60% of strains retained medium or high biofilm production. Most strains harboured determinants for carbapenemase or metallo-β-lactamase, and one-third were PCR-positive for the OXA-1 gene. Strains were clustered into three groups based on ERIC-PCR analysis.* ***Conclusion:*** *These data suggest that certain antibiotics could inhibit biofilm production in K. pneumoniae even as multidrug resistance in this organism is evident. Further, this species has the propensity to harbour several genetic determinants for antimicrobial resistance.*

INTRODUCTION

Klebsiella pneumoniae, the opportunistic encapsulated Gram-negative pathogen, is responsible for many cases of nosocomial pneumonia with increasing implication in community-acquired pneumonia (CAP) and ventilator-associated pneumonia[1,2]. In many of these cases, there have been high morbidity and mortality rates among high risk patients due in part to extended-spectrum β-lactamase (ESBL) production [3,4].

Traditionally, the β-lactam class of antibiotics was the first line treatment for infections caused by *K. pneumoniae*, with carbapenems being the most effective. In fact, the use of carbapenems have long been advised for the treatment of infections caused by ESBL-producing Enterobacteriaceae, especially *E. coli* and *K. pneumoniae.* A common feature of this family of microorganisms is the presence of genes responsible for resistance to the Group I cephalosporinases AmpC β-lactamases[4]. With the association of antimicrobial resistance with plasmids, coupled with antimicrobial overuse and misadministration, there is widespread dissemination via class 1 integrons and other mobile gene cassettes[5]. Class 1 integrons carry the majority of the resistance genes, including those associated with ESBL families [5]. We have previously shown that *E. coli* associates most of its genetic determinants on class 1 integrons [6].

The ability of *K. pneumoniae* to form biofilm is very important for colonization and pathogenesis. This feature has been reported in a large proportion of *K. pneumoniae* from sputum and endotracheal tubes from ventilator-assisted pneumonia patents [7,8]. Because many strains of *K. pneumoniae* have increased adherence and invasive capabilities, these bacteria can persist even

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when appropriate antibiotics are applied [9]. While types 1 and 3 fimbriae are thought to be vital for adherence to surfaces [10], most clonal *K. pneumoniae* strains express type 3 fimbriae for attachment to both biotic and abiotic surfaces[11]. These fimbriae are coded for on chromosomal gene clusters designated *mrkABCDF* [10]. In Jamaica, *K. pneumoniae* is second only to *Pseudomonas aeruginosa* in terms of isolates from respiratory sources [12]. However, there is a lack of information about the genetic characterization of *K. pneumoniae* implicated in these infections, which in many ways mirror *P. aeruginosa* in terms of difficulty in treatment or management of infections, and associated morbidity and mortality. This is particularly true in the case of smokers as nicotine can boost the virulence potential of bacteria, including biofilm production [13,14]. Therefore, the aims of this study were to determine the multiple antimicrobial resistance index of *K. pneumoniae* strains and to assess biofilm formation in the presence of ceftazidime, gentamicin, nicotine and serine hydroxamate (amino acid starvation). We also examined the clonal diversity and the presence of determinants for the fimbriae type 3 gene (*mrkA*), and extended spectrum β-lactamases in these *K. pneumoniae* strains.

METHODOLOGY

Identification of Clinical Strains

Twenty-three non-duplicate *Klebsiella pneumoniae* strains (one strain / person) were previously obtained from the sputum samples of acutely-ill patients with clinically-significant lower respiratory tract infections admitted to Hospitals in Kingston and St. Andrew and Clarendon, Jamaica. Sputum samples were recovered by expectoration and collected in sterile transparent containers. Because it was not possible to identify individuals from the limited informations provided. The study was deemed not to require ethical approval. However, approval for the use of the strains was obtained from the respective laboratory chiefs where isolations were done. *K. pneumoniae* was identified by microscopic morphology, catalase and urease activities, esculin hydrolysis, carbohydrate fermentation, indole and methyl red-Voges-Proskauer (MR-VP) tests, using standard microbial techniques. Any other microbial pathogen (for example, *Pseudomonas aeruginosa*) present was similarly identified [12].

Antimicrobial Susceptibility Test

Following the guidelines outlined in the Clinical and Laboratory Standards Institute [15], susceptibility testing was performed using amikacin (AN, 30 µg), aztreonam (ATM, 30 µg), ceftazidime (CAZ, 30 µg), ciprofloxacin (CIP, 5 µg), gentamicin (GEN, 10 µg), imipenem (IPM, 10 µg), meropenem (MEM, 10 µg), norfloxacin (NOR, 10 µg) and piperacillin (PIP, 10 µg). The multiple antibiotic resistance (MAR) index, used to identify whether strains were from a region of high or low antibiotic use, was calculated as the ratio of the number of antibiotics to which the isolate displayed resistance to the number of antibiotics to which the isolate was evaluated for susceptibility [16]. Obtaining a MAR index value greater than 0.20 would indicate that strains were from a 'high-risk' region. *Pseudomonas aeruginosa* ATCC27853 and *K. pneumoniae* ATCC700603 were used as quality control strains in these analyses.

Detection of antibiotic resistance genes

K. pneumoniae strains were screened for the presence of OXA-1 β-lactamase and the metallo-β-lactamases (MBL) genes (*bla*-GIM-1, *bla*-IMP, *bla*-SIM-1 and *bla*-VIM) using the PCR conditions previously described [17]. Essentially, extracted DNA was amplified using the PCR parameters: initial denaturation at 94°C for 5 min, followed by 25 cycles of 94°C for 30 s, 57°C for 45 s and 72°C for 60 s, followed by a final extension at 72°C for 7 min, and the following primers: GIM-F (5'-TCGACACACCTTGGTCTG-3') and GIM-R (5'-AACTTCCAACTTTGCCAT-3'), IMP-F (5'-GAAGGCGTTTATGTTCATAC-3') and IMP-R (5'- GTATGTTTCAAGAGTGATGC-3'), SIM-F (5'-TACAAGGGATTCGGCATCC-3') and SIM-R (5'-TAATGGCCTGTTCCCATG-3'), VIM-F (5'-GTTTGGTCGCATATCGCAAC-3') and VIM-R (5'-AATGCGCAGCACCAGGACAG-3').

Microtiter Plate Biofilm Susceptibility Assay

Biofilm assessment was done using the Merritt et al.[18] and O'Toole [19] protocols with some modification. Strains were grown in a semi-defined biofilm medium supplemented with 40% glucose as the carbohydrate source without agitation for 24 hr at 37°C. Subsequently, wells were washed with 0.1% crystal violet and the bound dye was extracted from the cells using 33% acetic acid in water. The extent of biofilm production was estimated by absorbance at 570 nm using a Victor microtiter plate reader (Perkin Elmer, USA). Experiments were done in triplicates.

The extent of biofilm production in the presence of various agents was carried out as detailed above, except that strains were incubated in M9 minimal medium with ceftazidime or gentamicin (50 µg/ml), nicotine (0, 0.5, 1, 2 or 4 mg/ml), or serine hydroxamate (0.5 mg/ml). In the latter case, serine hydroxamate was used to induce amino acid starvation. *K. pneumoniae* ATCC 43816 was used as quality control strain. Experiments were done in triplicate.

Enterobacterial intergenic consensus (ERIC) PCR analysis

Strains were grown in tryptic soy broth (TSB) for 18 hrs at 37^{o}C. DNA was extracted using the Wizard Genomic DNA Purification Kit (Promega) and ERIC-PCR analysis was done using the primers, ERIC-2 (5'-AAGTAAGTGACTGGGGTGAGC-3') and ERIC-1R (5'-ATGTAAGCTCCTGGGGATTCA-3') to amplify the intergenic regions of the chromosomal DNA that contained the specific sequence of *K. pneumoniae*. Primers were obtained from Integrated DNA Technologies (Iowa, USA). PCR mixtures consisted of 10 µl 2x Promega Gotaq Green master mix reaction buffer (containing 1 U *Taq* DNA polymerase, 400 µM of each dNTPs, 3 mM $MgCl_2$) and reaction buffers at optimal concentrations, ~0.2 µg *K. pneumoniae* DNA, and 1 µl (10 µM) of each primer. Amplification parameters included an initial denaturation at 94°C for 2 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 1 min and extension at 72°C for 5 min, and a final extension at 72°C for 10 min [20,21]. Amplified PCR products were separated and visualized on ethidium bromide-stained 2.0% agarose using the UVP BioDoc-it system.

A binary data framework was formulated using the electrophoretogram for each isolate in order to create the ERIC banding pattern information, where '1' was denoted as band present and '0' as band absent. The binary data produced were then entered into the DendroUPGMA online programme and then subjected to statistical analysis using Dice's coefficient and unweighted-pair group method analysis (UPGMA) to produce a dendrogram [20,22].

Detection of Type 3 Fimbriae mrkA Biofilm Gene

Strains were grown in Luria-Bertani broth (LB) for 18 hrs at 37^{o}C. DNA was extracted using the Wizard Genomic DNA Purification Kit (Promega) and *mrkA* analysis was done using the primers, mrkA-F: (5'-CCATGCAGCTGATACCAATG-3') and mrkA-R: (5'-GCAGCCTGGCAGTTAGAGAC-3') to amplify the *mrkA* gene from the chromosomal gene cluster that contains the specific sequence. PCR mixtures consisted of 12.5µl 2x Promega Gotaq Green master mix reaction buffer (containing 1 U *Taq* DNA polymerase, 400 µM of each dNTPs, 3 mM $MgCl_2$) and reaction buffers at optimal concentrations, ~0.2 µg *K. pneumoniae* DNA, and 1.5µl (10 µM) of each *mrkA* primers (Integrated DNA Technologies, USA). Amplification parameters included a 5 min hot-start at 94°C, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 62°C for 30 s and extension at 72°C for 2 min, and a final extension at 72°C for 10 min [23]. Amplified PCR products were separated and visualized on ethidium bromide-stained 1.5% agarose, using the UVP BioDoc-it system.

Statistical Analyses

The average biofilm absorbance values corresponding to the various treatments were statistically analyzed using one-way analysis of variance (ANOVA) (post-hoc Bonferonni, Tukey and Duncan's tests) and Box and Whisker plots using SPSS version 14.0. In all cases, $p < 0.05$ was considered statistically significant.

RESULTS

Antimicrobial susceptibility and MAR index

The antibiotic susceptibility profiles of the *K. pneumoniae* strains are summarized in table 1 and resistance frequencies are illustrated in figure 1. The carbapenem, imipenem was the only antibiotic to which all strains were susceptible, while 48% were resistant to ceftazidime, gentamicin and piperacillin. Multi-drug resistance (MDR) was observed in 11 (48 %) strains. Cross resistance to other antibiotics was observed for fluoroquinolone-resistant strains which were also resistant to aztreonam, gentamicin, ceftazidime, and piperacillin. The mean MAR index for these strains was 0.31 (range 0.00 – 0.56).

Table 1: Summary of antibiotic susceptibility patterns, biofilm production and presence of antibiotic resistance genes in *K. pneumoniae*.

Isolate Name	Antimicrobial resistance patterns										Biofilm	Antibiotic Resistance Genes				
	AN	ATM	CAZ	CIP	GM	IPM	MEM	NOR	PIP	TOTAL RESISTANCE	Assay	OXA-1	bla-GIM	bla-IMP	bla-SIM	bla-VIM
KP01	S	R	R	R	R	S	S	R	R	6	Good	+	+	+	+	+
KP02	S	S	S	S	R	S	S	S	ND	1	Poor	-	-	-	-	-
KP03	R	S	R	S	S	S	S	S	S	2	Poor	-	-	+	-	+
KP04	S	S	S	S	S	S	S	S	R	1	Good	-	+	+	+	+
KP05	S	R	R	R	R	S	S	S	R	5	Poor	-	+	+	+	+
KP06	S	S	S	S	S	S	R	S	R	2	Good	-	+	-	+	+
KP07	S	R	S	S	S	S	R	S	R	3	Good	-	+	+	+	+
KP09	S	S	S	S	S	S	S	S	S	0	Poor	+	+	+	+	+
KP10	S	S	S	S	S	S	S	S	S	0	Poor	-	-	+	-	+
KP11	S	S	S	S	S	S	S	S	S	0	Good	-	+	-	+	-
KP12	S	S	S	S	S	S	S	S	S	0	Good	ND	ND	ND	ND	ND
KP13	S	S	S	S	S	S	S	S	S	0	Good	-	+	+	+	+
KP14	S	R	R	R	R	S	S	R	R	6	Good	-	-	+	-	+
KP15	S	R	R	R	R	S	S	R	R	6	Good	+	+	+	+	+
KP16	S	R	R	S	R	S	S	S	R	4	Poor	-	+	+	+	+
KP17	S	S	S	S	S	S	S	ND	S	0	ND	-	+	+	+	+
KP18	S	S	S	S	S	S	S	S	S	0	ND	+	+	+	+	+
KP19	S	R	R	R	R	S	S	R	ND	5	ND	+	+	+	-	-
KP24	S	S	S	S	S	S	S	S	S	0	ND	-	+	-	+	-
KP25	S	R	R	S	R	S	S	R	R	5	Poor	+	+	+	-	+
KP26	S	S	R	R	R	S	S	S	S	3	ND	-	-	-	+	-
KP27	S	S	R	R	R	S	S	R	R	5	Good	ND	ND	ND	ND	ND
KP28	S	S	S	S	S	S	S	S	S	0	Poor	-	+	+	+	+
KP29	S	R	R	R	R	S	S	R	R	6	Good	-	+	+	+	+
Total n (%)	1	9	11	8	11	0	2	7	11	6.2	11	6	17	17	16	17
	4.3	39.1	47.8	34.8	47.8	0.0	8.7	30.4	47.8	5.1	47.8	26.1	73.9	73.9	69.6	73.9

(+), Present; - , Absent; S, Sensitive; I, Intermediate; R, Resistant; ND, Not Determined

S, Sensitive; R, Resistant; ND, not determined; Total, count of R phenotypes for the respective antimicrobial agents assessed, or presence of the respective antimicrobial resistance gene; +, Present; - , Absent.

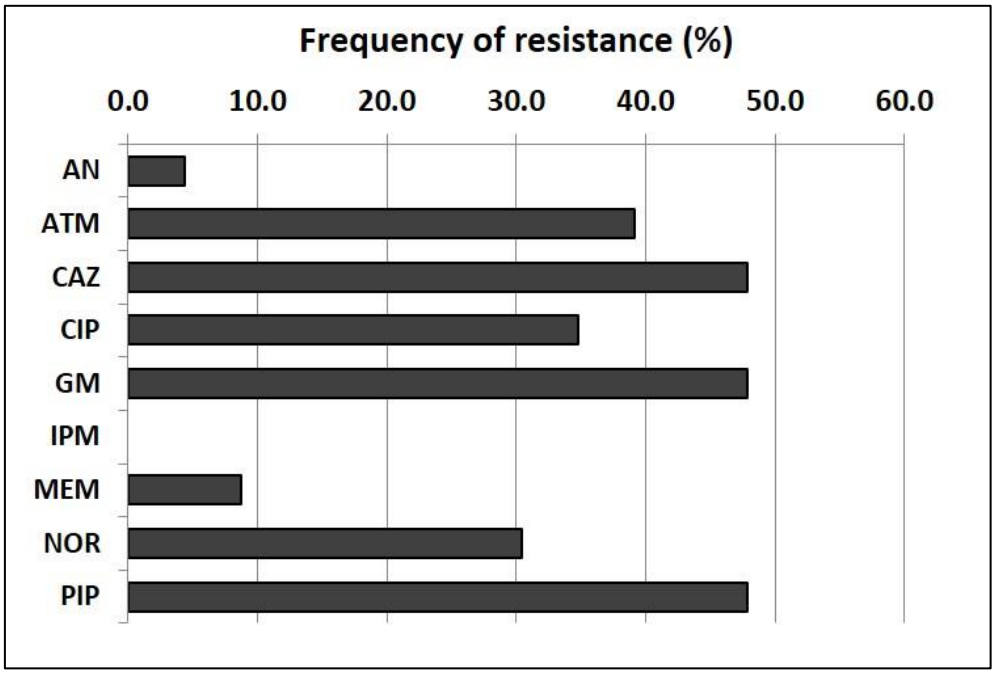

Fig. 1: Frequency of resistance among *K. pneumoniae* strains to antibiotics analyzed in this study. AN, amikacin; ATM, aztreonam; CAZ, ceftazidime; CIP, ciprofloxacin; GM, gentamicin; IPM, imipenem; MEM, meropenem; NOR, norfloxacin; PIP, piperacillin

Microtitre Plate Biofilm Susceptibility Assay

Figure 2 illustrates the extent of biofilm formation in strains supplemented with glucose (control), antibiotics (ceftazidime and gentamicin), and 0.5 mg/ml serine hydroxamate (SHX). Dataset for analysis was based on grouping isolates as follows: low biofilm producers (absorbance, 0.100-0.249), medium producers (0.250-0.399) and high biofilm producers (>0.400). Under control settings, just over 50% of strains were medium biofilm producers, 18% were high producers (i.e., about 70% of strains were medium or high biofilm producers). In the presence of antibiotic exposure, there were 2.7-fold and 1.8-fold decreases in medium and high biofilm producers, respectively ($p<0.05$). Conversely, in the presence of the antibiotics, there were at least 2.0-fold increases in low biofilm producers, respectively ($p<0.05$). Strains KP07, KP20 and KP23 were the only three strains in which biofilm production was most significantly enhanced in the presence of CAZ; similar enhanced biofilm production was observed for strains KP22 and KP23 in the presence of GEN. SHX (aminoacid starvation) treatment resulted in a modest 1.2-fold decrease in medium and high biofilm production among these strains, and a 1.5-fold increase in low biofilm producers.

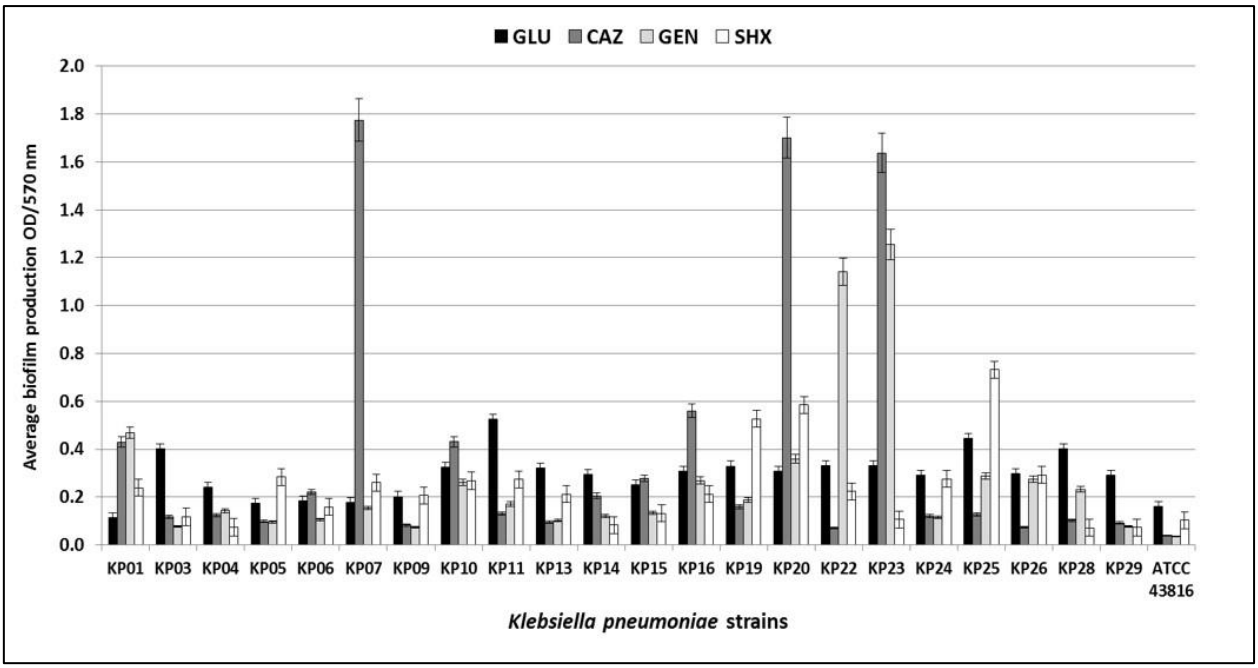

Fig. 2: Composite biofilm formation for *K. pneumoniae* strains in the presence of glucose, ceftazidime (CAZ), gentamicin (GEN) and serine hydroxamate (SHX; amino acid starvation). Values are means (of triplicates) ± SEM. *K. pneumoniae* KPPR1 ATCC 43816 was included as control strain.

Figure 3 represents the extent of biofilm production of the *K. pneumoniae* strains exposed to nicotine and illustrates the distribution of percentage biofilm production relative to the untreated strains. As expected, the median percentage biofilm formation was lowest at 4.0 mg/ml nicotine and highest at 0.5 mg/ml. There was a statistical significant difference ($p< 0.05$) between groups for biofilm production with varying concentrations of nicotine but there was no statistical significant difference between groups of strains KP04, KP05, KP06, KP20, KP28 and ATCC 43816 for biofilm production across the varying nicotine concentrations using one-way ANOVA.

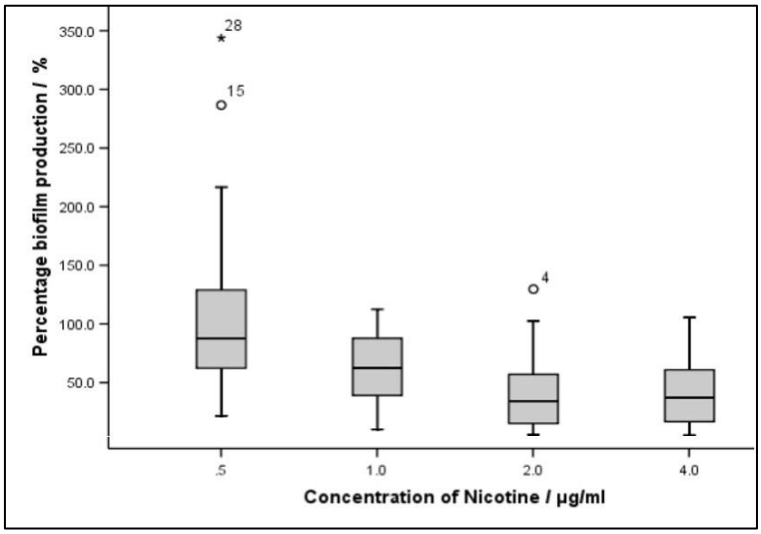

Fig. 3: Box and whisker plots illustrating the distribution of biofilm production at varying concentrations of nicotine for *K. pneumoniae* strains relative to the untreated strain cluster. Boxes range from the 25th to the 75th percentile and are intersected by the median line. Whiskers extending below and above the box range from the 10th to 90th percentile respectively. Outliers are indicated as individual data points of asterisk or circle with strain identifier beside.

ERIC-PCR types

Utilizing a 0.05 cut-off level on the dendrogram produced three clusters (I, II and III) with about 73% similarity (Fig. 4). Cluster I contained 15 strains, while clusters II and III contained two and six strains, respectively. A closer inspection of the dendrogram revealed that cluster I contained more strains that were deemed to be good biofilm formers (indicated by asterisk) even though those that produced limited or no biofilm were also found in this cluster. All except KP25 in Group III were multidrug resistant (indicated by dagger symbol); they had between two and four OXA/MBL genes. In both clusters I and III, three strains that were MDR were also good biofilm producers. Two of the strains that had enhanced biofilm production in the presence of SHX treatment (aminoacid starvation) were found in cluster III; the other was found in cluster I. Up to 74% of the strains harboured MBL genes and eight strains were positive for the OXA gene.

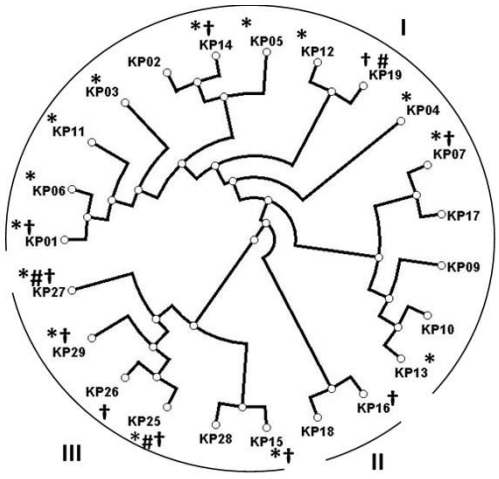

Fig. 4: Radial ERIC-PCR dendrogram for *K. pneumoniae* strains in this study. Percentage similarity of profiles for strains was calculated using Dice's coefficient and UPGMA. Using a 0.05 cut-off, three clusters were observed. Asterisks, hashtags and daggers indicate strains with good biofilm production, enhanced biofilm with aminoacid starvation, and multidrug resistance (MDR), respectively.

DISCUSSION

In this study we examined the antimicrobial resistance and biofilm production in a small set of *Klebsiella pneumoniae* strains obtained from respiratory specimens in Jamaica; this was part of a larger study of respiratory isolates. As is the case in many countries worldwide, *K. pneumoniae* is established as a significant cause of severe infections in hospitalized patients. Part of the difficulty in effectively treating this pathogen is its propensity for multidrug resistance and its innate and acquired ability to form biofilms and adhere and multiply on surfaces.

Gentamicin, amikacin, imipenem and ciprofloxacin are considered potent treatments of infection caused by multidrug resistant *K. pneumoniae*. In this study, we found low frequency of resistance to the aminoglycoside, amikacin and complete susceptibility to the carbapenem, imipenem; close to 50% of the strains expressed resistance to gentamicin. Consequently, it is critical that the use of these antimicrobial agents be monitored and controlled in the hospital setting to prevent the emergence or entrenchment of aminoglycoside- or carbapenem-resistant strains, and restricted when resistant strains are detected, as in the case of meropenem resistance approaching 10%. The finding of close to 50% of the strains resistant to piperacillin and ceftazidime is worrying as piperacillin and other β-lactam antibiotics can induce clonal dissemination of strains producing the extended-spectrum β-lactamases (ESBLs), thus resulting in the spread of resistance in the hospital setting [24].

Resistance to ceftazidime, aztreonam and piperacillin has been attributed to production of ESBLs or AmpC type cephalosporinases that is encoded in the chromosomes of many Enterobacteriaceae and other bacteria including *K. pneumoniae.* It is believed that such a resistance is conferred by an over-expression of *ampC* gene, which is caused by a mutation in AmpD and is hyper-inducible in the presence of β-lactams [25,26]. Due to a relatively high frequency of resistance of *K. pneumoniae* to ceftazidime (36%) and aztreonam (48%), and an overall 44% MDR in this study, ESBL production in this group of organisms is a significant issue, and was borne out in this study when the genetic bases for these phenotypes were assessed. It is well established that among the metallo-β lactamase (MBLs), *bla*-VIM and *bla*-IMP are the more geographical widespread. Consequently, the finding of significant association of MBLs with the *K. pneumoniae* strains in this study is worrying, not only because of the small size of Jamaica, and the less than ideal infrastructure and contribution to healthcare, but also because these bacteria are already aetiological agents of severe respiratory and nosocomial infections.

A good tool for risk assessment is the MAR index, for which a nominal value of 0.20 differentiates low- and high-risk use of antibiotics with a certain area [27]. This kind of analysis allows an estimation of the proportion of resistant bacteria in the particular risk zone. The analysis revealed that *K. pneumoniae* strains from teaching hospitals in Kingston & St. Andrew had significantly higher MAR index values (>0.20) when compared to those from hospitals in St. Thomas and Clarendon.

The ability of *K. pneumoniae* to form biofilms on surfaces is very important in the pathogenesis of infections. In this study, the strains showed varying potential to form biofilm in the presence of several stressors. Pili and fimbriae (in particular, type 3) have been previously reported to be associated with biofilm formation in *K. pneumoniae* [28]. This was supported in this study by the presence of the *mrkA* gene in all strains, which underscore the ability of these strains to colonize both endothelial and epithelial cells of the respiratory tract [29]. Although there was no strong association between antimicrobial resistance or MDR and biofilm production, biofilm production should be taken into consideration in antimicrobial therapy given the significant association of *K. pneumoniae* and biofilms on medically inserted devices like endotracheal tubes [11]. This lack was also seen in a population of respiratory *Pseudomonas aeruginosa* strains from Jamaica [12].

Biofilm production in majority of *K. pneumoniae* strains were negatively affected by gentamicin and ceftazidime. Clearly, these strains have a low fitness in the presence of both antibiotics, with ceftazidime exerting the greater effect. Serine hydroxamate (SHX), a structural analog of L-serine that inhibits transfer RNA ($tRNA^{Ser}$) aminoacylation, which causes an increase in production of uncharged tRNA and subsequent induction of the stringent response [30], impaired biofilm production the least.

Concentrations of nicotine in the saliva of persons who smoke range from 0.07–1.6 mg/ml for light smokers to 0–2.27 mg/ml for persons who smoke heavily [31]. Based on these reported ranges, we used 0, 1, 2, and 4 mg/ml of nicotine to assess the effect of nicotine on the formation of *K. pneumoniae* biofilms. From the current study, it was apparent that nicotine did not promote biofilm formation in *K. pneumoniae*. This contrasts with a previous report [14], which found that increased nicotine concentration promoted biofilm production in *Streptococcus gordonni.* However, this could be a case of difference based on species (i.e., Gram positive vs Gram negative). Nicotine has been shown to upregulate the surface expression of salivary agglutinin antigen I/II, which is used by cariogenic agents such as *S. mutans* and other Gram positive bacteria in conjunction with the cell surface protein P1,

sortase A. Consequently, biofilm formation is enhanced in these species. In the case of Gram negative bacteria, including *Pseudomonas aeruginosa*, biofilm promoting genes *pilF* and *flgK* are unregulated by cigarette smoking while *rhlA* which is a quorum sensing mediator is down-regulated[32], Notwithstanding, biofilm formation was more evident at a lower concentration (0.5 mg/ml) and much reduced at 2.0 and 4.0 mg/ml concentration. It is possible that *K. pneumoniae* is less able to metabolize nicotine, compared to *Pseudomonas aeruginosa* (unpublished data), and hence fitness is compromised.

The ERIC-PCR analyses confirmed that the *K. pneumoniae* strains included in this study were quite diverse genetically, with three groups identified. Notwithstanding the general non-association between multidrug resistance and biofilm production, it was clear that the strains grouped in cluster III were less heterogeneous as they showed good association between these two main phenotypes.

CONCLUSION

Based on the findings of this study, it is important to control the use of aminoglycoside antibiotics in hospital setting and the use of piperacillin and other β-lactam antibiotics should be monitored in the treatment of respiratory infections. Ceftazidime and gentamicin showed enhanced efficacy against biofilms formed by these respiratory strains of *K. pneumoniae*, while serine hydroxamate (through aminoacid starvation induction) was less effective. Further, nicotine did not promote biofilm production in these strains. Finally, being genetically diverse, the presence of respiratory *Klebsiella pneumoniae* in the hospital environment indicates that they are from multiple introductions.

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Author Contributions

Conceived and designed the experiments: RD and PB. Performed the experiments: RD. Analyzed the data: RD and PB. Wrote the paper: RD and PB. Coordinated the work plan, manuscript writing and is the corresponding author: PB

- The authors declare that they have no financial or non financial conflicts of interest related to the work done in the manuscript.
- Each author listed in the manuscript had seen and approved the submission of this version of the manuscript and takes full responsibility for it.
- This article had not been published anywhere and is not currently under consideration by another journal or a publisher.

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