Multiple antibiotic resistance index, fitness and virulence potential in respiratory *Pseudomonas aeruginosa* from Jamaica

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Respiratory infections are common causes of morbidity and mortality worldwide. We sought to assess the multiple antibiotic resistance (MAR) index, fitness and virulence potential in *Pseudomonas aeruginosa* from patients with lower respiratory tract infections. Isolates were assessed for antimicrobial susceptibility, *in vitro* competitive fitness, and pigment, elastase and rhamnolipid production. Oxidative stress tolerance was determined on both planktonic and biofilm cells, and virulence potential was tested in a plant model. Mean MAR index for isolates was 0.34 (range 0.17–0.50). Whilst isolates exhibited good biofilm formation in the presence of ciprofloxacin, there was no significant difference in biofilm production over the concentration range assessed. Several drug-resistant strains were out-competed by a susceptible strain even in the presence of antibiotic. H$_2$O$_2$ exerted a greater oxidative stress than tert-butyl-hydroperoxide and, as expected, biofilms were more resistant than planktonic cells. Whilst most (81%) isolates were pigmented there was no significant difference between pigmented and non-pigmented isolates when elastolytic activity was compared (P > 0.05). More than half of the isolates produced the quorum sensing mediator rhamnolipid and infection of the plant model by bacteria occurred whether elastase or rhamnolipid was present or absent. These data suggest that non-pigmented strains of *P. aeruginosa* might pose an equally significant microbiological threat as pigmented strains even though pigment production appeared to be strongly associated with elastase expression. Whilst dual expression of elastase and rhamnolipid by these bacteria would cause severe tissue damage (as seen in the plant model), non-production of either does not prevent bacteria from causing serious infection.

**INTRODUCTION**

Respiratory infections are common causes of morbidity and mortality in both developed and developing countries (Fine, 1996). Whilst antibiotic resistance is increasingly associated with the most common pathogens (*Streptococcus pneumoniae*, *Haemophilus influenzae* and *Moraxella catarrhalis*), *Pseudomonas aeruginosa* is often a causative agent in fatal infections of the respiratory tract. In a study carried out at the University Hospital of the West Indies Intensive Care Unit, Akpaka et al. (2002) found *P. aeruginosa* to be the second most common isolate amongst patients in the unit, with > 30% of the isolates being resistant to standard antipseudomonal antibiotics. Furthermore, unpublished local data revealed that *P. aeruginosa* was a significant cause of lower respiratory tract infections, accounting for 18% of hospitalizations with lower respiratory tract infections (R. U. Kumar, National Public Health Laboratory, Kingston, Jamaica, unpublished data).

Although *P. aeruginosa* rarely causes community-acquired pneumonia, there have been high morbidity and mortality rates amongst high-risk patients, including those who are > 62 years old or immunocompromised with pre-existing conditions, such as human immunodeficiency virus infection, diabetes mellitus, renal failure, burns, chronic obstructive pulmonary disease, smoker or congestive heart failure (Jorgensen & Ferraro, 2009; Li *et al.*, 2011; Shah *et al.*, 2010). In addition, *P. aeruginosa* has increased resistance to many antibiotics due to several adaptations, which include membrane impermeability, efflux pumps, biofilm formation and enzyme expression (Henwood *et al.*, 2001; Landman *et al.*, 2007).
Biofilm formation by \textit{P. aeruginosa} is critical to the establishment of a persistent lung infection. This process is mediated by a switch from planktonic to sessile forms, and attachment to organic tissues or abiotic surfaces, and involves virulence factors such as alginate, elastase, LPS and rhamnolipids (Bjarnsholt et al., 2010). Colonization as biofilms enables bacterial communication and coordination of virulence factor expression via cell-density-dependent (quorum sensing, QS) regulation, which employs the \textit{las} and \textit{rhl} encoded systems. Together, these factors, which include elastase, several cytotoxins, iron scavengers and mediators of oxidative stress tolerance, amongst others, affect receptor molecules on various respiratory tissues and organs of patients, resulting in significant morbidity (Wagner et al., 2008). Historically, the QS systems of \textit{P. aeruginosa} were thought to be arranged hierarchically, with the \textit{rhl} system being controlled by the \textit{las} system (Pesci et al., 1997). However, these two systems have been shown to be activated independently, with the involvement of a third regulatory pathway (Dekimpe & Dézel, 2009; Diggle et al., 2003).

Even in acute respiratory infections, there is high selective pressure, and \textit{P. aeruginosa} cope by genetic variations mediated by mutation and selection. In this regard, the development of ‘hypermutable’ strains is expected to improve the adaptability of the bacteria to the diverse microenvironments of the respiratory system (Hogardt & Heesemann, 2013). Furthermore, mixed populations of \textit{P. aeruginosa}, including QS WT and \textit{lasR} mutants, have demonstrated cooperative behaviour, where the QS mutants appear to exploit the functional QS systems of other members of the population (Diggle et al., 2007; Sandoz et al., 2007). In this way, bacteria that have reduced fitness (such as \textit{lasR} mutants) might be able to persist and benefit from the activities of the QS-active members of the community. Similarly, antimicrobial resistance associated fitness costs might be due to mutations in cellular structures such as DNA gyrase, RNA polymerase, ribosome and cell wall biosynthetic enzymes, which are amongst the targets of antibiotics (Kugelberg et al., 2005).

There is a paucity of information regarding the characteristics of \textit{P. aeruginosa} implicated in respiratory tract infections in Jamaica, coupled with the fact that these infections result in increased hospitalization and thus pose a major public health challenge for clinicians. Therefore, the aims of this study were to determine the multiple antibiotic resistance (MAR) index of respiratory \textit{P. aeruginosa}, and to assess the fitness cost in mixed populations, oxidative stress resistance and virulence potential using a plant model of pathogenesis.

**METHODS**

**Bacterial strains and growth conditions.** A total of 92 non-duplicate \textit{P. aeruginosa} isolates (one isolate per person) were previously isolated from the sputum samples of acutely ill patients with clinically significant lower respiratory tract infections admitted to hospitals in Kingston & St Andrew and Clarendon, Jamaica. Sputum samples were obtained by expectoration and collected in sterile transparent containers. As it was not possible to identify individuals from the limited information provided, the study was deemed not to require ethical approval. However, approval for the use of the isolates was obtained from the respective laboratory chiefs where isolations were done. \textit{P. aeruginosa} was identified by microscopic morphology, catalase, oxidase and urease activity, casein and starch hydrolysis, citrate and indole utilization, and methyl red–Voges–Proskauer and gelatin liquefication tests, using standard microbial techniques. Growth with or without the production of pigment was determined on various growth media, such as Mueller–Hinton II agar, \textit{Pseudomonas} F medium (King B medium; fluorescein or pyoverdin, a green/yellow pigment) or \textit{Pseudomonas} P medium (King A medium; pyocyanin, a blue/green pigment) at 37 °C. In the absence of visible pigment, colonies were examined using UV illumination (Huston et al., 2004). \textit{P. aeruginosa} was further grown on cetrimide (Difco) isolation agar and tryptic soy agar (TSA), and streaked on Mueller–Hinton agar to assess purity at 37 °C for 18 h. Any other microbial pathogen (e.g. \textit{Klebsiella pneumoniae}) present was similarly identified.

**Antibiotic susceptibility tests.** Susceptibility testing was performed using the Clinical and Laboratory Standards Institute’s disc diffusion method (CLSI, 2011) using amikacin (AN; 30 μg), gentamicin (GM; 10 μg), piperacillin (PIP; 100 μg), ceftazidime (CAZ; 30 μg), imipenem (IPM; 10 μg), meropenem (MEM; 10 μg), aztreonam (ATM; 30 μg), ciprofloxacin (CIP; 5 μg) and norfloxacin (NOR; 10 μg). The MAR index 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 had been evaluated for susceptibility (Krumperman, 1983). The MAR index is a good tool for health risk assessment which identifies if isolates are from a region of high or low antibiotic use. A MAR index > 0.2 indicates a ‘high-risk’ source of contamination.

Biofilm susceptibility assays against CIP were carried out on a subset of 90 isolates and \textit{P. aeruginosa} ATCC 27853 control strain using the biofilm assay protocol of Merritt et al. (2011) and Khan et al. (2010) in 96-well polystyrene microtitre plates. CIP, a broad-spectrum fluoroquinolone used clinically to treat \textit{P. aeruginosa} infections, was chosen because it exerts significant effect on growth rate and the minimal biofilm eradication concentration is of the order of 4 μg ml⁻¹ (Agarwal et al., 2005). Mueller–Hinton broth containing the inoculum was mixed with a final CIP (Oxoid/Remel) concentration of 0.5, 0.3, 0.15, 0.05, 0.0125, 0.00625 and 0.00098 μg ml⁻¹ in a 96-well microtitre plate and incubated at 37 °C for 24 h. Wells were washed twice to remove planktonic water, subsequently stained in 0.1 % crystal violet solution followed by washing again and then de-stained in 200 μl 95 % ethanol. OD₅₇₀ was measured with a Victor microtitre plate reader (Perkin Elmer) and experiments were performed in triplicate.

**Enterobacterial intergenic conserved (ERIC)-PCR conditions and primers.** The specific banding pattern of the amplified PCR products obtained from the ERIC-PCR can be used to decipher the clonal diversity or heterogeneity of the bacteria present. Strains (90 clinical isolates and ATCC 27853) were grown in tryptic soy broth (TSB) and DNA was extracted using a Wizard Genomic DNA Purification kit. PCR analysis was performed using the primers ERIC2 (5’-AAAGTGCAGGTTGACG-3’) and ERIC1R (5’-AGCTTCCTGGGATTCA-3’) (Integrated DNA Technologies). Reactions were carried using the Promega GoTaq Green system with ~0.2 μg \textit{P. aeruginosa} DNA. Amplification conditions included a 2 min hot start at 94 °C, 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 for 10 min at 72 °C (Prabhhu et al., 2010; Moreno et al., 2011). Amplified PCR products were analysed on ethidium bromide-stained agarose gels. Banding pattern information was coded as binary data, where ‘1’ denoted band present and ‘0’ denoted band absent. These binary data were entered into the online DendroUPGMA program, and subjected
to statistical analysis using Dice’s coefficient and unweighted-pair group method analysis (UPGMA) to produce a dendrogram (Prabhu et al., 2010; Meacham et al., 2003).

**In vitro competition and relative fitness.** For mixed growth in vitro competition assays, fluoroquinolone-resistant (n=6 CIP-resistant and n=6 NOR-resistant) and carbapenem-resistant (n=5 IPM-resistant and n=5 MEM-resistant) isolates were tested against the WT *P. aeruginosa* ATCC 27853 strain (1:1 ratio) according to Rozen et al. (2007). These clinical strains were selected because they were drug resistant and produced either no pigment or pyorurbin and could be easily differentiated from the WT ATCC strain, which was drug susceptible and produced pyoverdin. Antibiotics were prepared from a stock to get a final concentration in the media containing 0.6 µg CIP ml⁻¹, 1.5 µg NOR ml⁻¹, 0.6 µg IPM ml⁻¹ or 0.1 µg MEM ml⁻¹ for antibiotic-selective plates. Mixed cultures were plated onto *Pseudomonas* F (King B) agar with or without antibiotic pressure, and observed over 24–48 and 72–96 h. The relative fitness value (RFV; W) under non-selective (absence of antibiotic) and selective (presence of antibiotic) conditions was calculated as the ratio of the Malthusian parameters (m) of the two competing strains as determined by:

\[ m_A = \ln \left( \frac{A(n)}{A(0)} \right) \text{ for strain A} \]
\[ m_B = \ln \left( \frac{B(n)}{B(0)} \right) \text{ for strain B} \]
\[ W = \frac{m_A}{m_B} \]

where A(0) or B(0) and A(n) or B(n) are the population sizes of the strains at time points 0 and n, respectively (Lenski, 1991; Travisano & Lenski, 1996). The RFV assesses how well one strain does in relation to another within a population, i.e. whether its population is increasing or decreasing. RFV>1 indicates increased or higher fitness and RFV<1 indicates reduced or lower fitness. RFV=1 or 0 indicates no fitness loss or gain between the two competitors.

**Oxidative stress analysis.** The ability of biofilms to tolerate oxidative stress was assessed by exposing 72 h stationary-phase cultures at 37 °C in 96-well microtitre plates to 100 mM hydrogen peroxide, or 40 or 80 mM tert-butyl-hydroperoxide (t-BOOH) for 15 min. After addition of 0.2 % (w/v) sodium thiosulfate to stop the reaction, OD₅₅₀ was measured with a Victor microtitre plate reader (Perkin Elmer), using the optical approach proposed by Kwasny & Opperman (2010). Biofilm optical density has been shown to correlate well with biofilm mass and cell mass (Brakke et al., 2010), and is less invasive (as a technique) particularly when working with small volumes. Planktonic cells (exponential-phase cultures) were assessed in a similar manner except there was no pre-incubation prior to addition of oxidative stress.

**Elastase and rhamnolipid production.** Elastase production and the presence of genes encoding elastase (lasA and lasB) and QS mediators (rhlA and rhlB) were assessed according to Finlayson & Brown (2011). For elastase production, overnight broth cultures were centrifuged and 100 µl supernatant was transferred to 900 µl 100 mM Tris/1 mM CaCl₂ at pH 7.5 containing 1 mg elastin-Congo red. This mixture was then incubated at 37 °C for 24 h and was centrifuged again, and 1 ml supernatant was transferred to a clean cuvette and OD₅₅₀ of the supernatant measured using a spectrophotometer.

Rhamnolipid production was confirmed using the mineral salt/cetrimide methylene agar plate method of Gunther et al. (2005). A single isolate was picked from previously prepared TSA plates and placed in 1.5 ml Kay’s medium at 37 °C for 24 h with continuous shaking. A 10 ml glass sterile pipette was used to cut into the surface of cetyltrimethylammonium bromide plates and 10 µl appropriate isolate prepared in Kay’s medium placed into each well. The plates were then incubated at 37 °C for 48 h and then further incubated at 4 °C for 24 h for blue halo development around culture wells.

**Assessment of pathogenicity using the romaine lettuce leaf model.** The virulence potential of *P. aeruginosa* isolates (using 20 isolates grouped into four categories based on the presence or absence of elastase production and rhamnolipid formation) was performed as described by Rahme et al. (1997) and modified by Filiatrault et al. (2006) using romaine lettuce as a model of infection. Romaine lettuce has been shown to be a suitable model system for assessment of virulence, and as noted by Rahme et al. (1997), bacterial pathogenesis in *P. aeruginosa* might use an overlapping set of basic virulence genes for plant and animal hosts. Midribs were inoculated in triplicates with 10 µl of each strain (containing ~10⁶ bacteria). As controls, midribs were inoculated with 10 mM MgSO₄ only (negative control) or control strain *P. aeruginosa* ATCC 27853 (rhamnolipid-positive/elastase-negative). The assessment parameters according to Rahme et al. (1997) were chlorosis, discoloration at site of inoculation and along the midrib, water soaking, necrotic lesions or decomposition of leaf tissues.

**Statistical analysis.** One-way ANOVA, Student’s t-test, box-and-whisker plots and the χ²-test were used to analyse differences observed in mean biofilm absorbance values, frequency of elastin hydrolysis, rhamnolipid production and RFVs. spss version 14.0 was used for analyses. P<0.05 was considered statistically significant.

**RESULTS**

**Antimicrobial susceptibility and MAR index**

Antimicrobial susceptibility profiles of the 92 isolates are illustrated in Fig. 1. The highest frequencies of resistance were recorded for PIP (22.4 %) and ATM (17.8 %), whilst the lowest numbers of resistant isolates were observed for AN (6.7 %) and IPM (7.8 %). Multidrug resistance (defined as resistance to at least three antibiotic classes) was observed in 15 (17 %) isolates, whilst three isolates expressed resistance to all nine antibiotics used. The mean MAR index for these isolates was 0.34 (range 0.17–0.50).

From the biofilm susceptibility assay, there were no significant differences (P=0.838) observed for the isolates at the

![Fig. 1. Frequency of antimicrobial resistance amongst *P. aeruginosa* strains to the antibiotics analysed in this study.](http://jmm.microbiologyresearch.org)
Various concentrations of CIP examined (Fig. 2), and there was tight clustering of isolates between the 25th and 75th percentiles with similar variability for the whiskers (10th and 90th percentiles). Notwithstanding, it was clear that as the concentration of antibiotic increased, there appeared larger variation in the extent of biofilm production as indicated by the presence of several outliers. It was apparent that CIP had reduced efficacy against the biofilm forms of these strains. In fact, those strains considered good biofilm producers showed increased fitness (based on production of biofilm) in the presence of antibiotic. However, whilst the majority of strains were good biofilm producers, there did not appear to be a strong correlation between biofilm production and CIP resistance.

**In vitro competition and relative fitness**

Resistant isolates (six each of CIP- and NOR-resistant isolates and five each of IPM- and MEM-resistant isolates) were tested against the susceptible pyoverdin-producing WT *P. aeruginosa* ATCC 27853 strain and the fitness of isolates was based on RFVs. As expected, antibiotic-resistant strains out-competed the susceptible strain (evidenced by increases in RFVs) in several instances, in the absence and presence of antibiotics (Figs 3 and 4). However, some drug-resistant strains (including in the presence of antibiotics) had decreased fitness against the susceptible strain, which indicated that over time the WT strain out-competed the resistant isolates. There were some cases in which there was selective neutrality (RFV = 0 or 1): there was no fitness effect. It was clear from these studies that competition in the presence of one fluoroquinolone or carbapenem was not a predictor of outcome for competition in the presence of another antibiotic of the same class, even if the same organisms were in question. This was observed for PA06, PA27 and PA76 for CIP/NOR and PA75 and PA83 for IPM/MEM combinations (Figs 3 and 4). There was no statistically significant difference ($P > 0.05$) between mean RFVs of the carbapenem and fluoroquinolone assays over the course of the study.

**Pigment production, oxidative stress tolerance and QS mediators**

Most *P. aeruginosa* isolates (38; 51.3%) produced pyoverdin, whilst 12 (16.2%) and 10 (13.5%) produced pyorubin.
and pyocyanin, respectively (Fig. S1, available in the online Supplementary Material). No isolate produced more than one pigment. The remainder were non-pigmented.

Fig. 5 illustrates an ascending order of oxidative stress tolerance of planktonic cells and biofilms to H$_2$O$_2$ and t-BOOH. As expected, biofilms were more resistant to oxidative stress (except for isolates exposed to 100 mM H$_2$O$_2$) when compared with planktonic cells ($P<0.05$). However, there were several planktonic forms that were quite resilient when exposed to 80 mM t-BOOH and approached the growth density of biofilm forms exposed to similar conditions. Overall, H$_2$O$_2$ was more effective at exerting oxidative stress when compared with t-BOOH.

Fig. 6 illustrates the quantity of solubilized elastin-Congo red produced for each of the $P. aeruginosa$ strains assessed in this study. The isolates were categorized as low producers (elastase production $<0.10$ mg ml$^{-1}$), medium producers (elastase production $0.1$–$0.40$ mg ml$^{-1}$) and high producers (elastase production $>0.40$ mg ml$^{-1}$). Almost half (49 %) of isolates were designated low elastase producers (including at least five elastase-deficient strains), 13 % as medium producers and 38 % as high producers ($\chi^2=38.012; P<0.05$).

Positive rhamnolipid production, evidenced by the formation of a blue halo around inoculated wells (Fig. S2), was observed for 78.9 % of isolates. Most of the strains were PCR-positive for lasA (92.3 %), lasB (91 %), rhlB
(93.2 %) and rhlA (86.6 %) genes. It was apparent that whilst there was congruence with phenotype, there were several cases where the presence of the gene did not result in expression.

**Virulence potential using the romaine lettuce leaf model**

Isolates categorized on the basis of whether they produced none, either or both QS mediators (i.e. rhamnolipid and/or elastase) were used for infection studies with romaine lettuce. We found that the plant model was not only susceptible to infection by strains that were rhamnolipid-positive/elastase-positive, but was also susceptible to rhamnolipid-negative/elastase-positive and rhamnolipid-positive/elastase-negative strains (Table 1). Strains from these latter two groups caused moderate symptoms with rotting at the site of inoculation along with localized water soaking by day 2, followed by rapid progression of symptoms at 3–5 days post-inoculation with systemic soft rotting of the entire stem followed by complete decomposition and collapse of the entire leaf tissue (Fig. S3). Interestingly, strains that were grouped as rhamnolipid-negative/elastase-negative showed only weak, slight yellowing discolouration at the site of inoculation by day 2. However, by days 3 and 4, leaves showed moderate and severe symptoms, respectively, with localized water soaking and chlorosis of tissue circumscribing the inoculation site. By day 5, there was complete decomposition and severe water soaking in the leaves.

![Fig. 4. RFVs of resistant *P. aeruginosa* strains co-inoculated with WT strain ATCC 27853 on non-selective and selective plates containing (a) IPM and (b) MEM. Analyses in triplicates were performed at 24–48 and 72–96 h.](image-url)
Control romaine lettuce leaves inoculated with 10 mM MgSO₄ showed no symptoms during the course of the study, whilst *P. aeruginosa* ATCC 27853 (positive control strain) showed similar symptoms to those elicited by strains that were rhamno-lipid-positive/elastase-positive.

### ERIC types

For the ERIC-PCR analysis, utilizing a 0.05 cut-off on the dendrogram produced two main clusters with ~68% similarity and an unclustered group of 19 isolates (Fig. 7). Cluster A contained 59 isolates, with far greater genetic diversity than cluster B, which contained 13 isolates. On closer inspection, cluster A included the fitter

### Table 1. Virulence of *P. aeruginosa* strains in the romaine lettuce leaf infection model

Symptoms were recorded over 5 days post-inoculation.

<table>
<thead>
<tr>
<th>Strain category</th>
<th>Symptoms elicited*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rhamnolipid-positive/elastase-positive</td>
<td>Days 1–2 Moderate</td>
</tr>
<tr>
<td>Rhamnolipid-positive/elastase-negative</td>
<td>Days 3–5 Severe</td>
</tr>
<tr>
<td>Rhamnolipid-negative/elastase-positive</td>
<td></td>
</tr>
<tr>
<td>Rhamnolipid-negative/elastase-negative</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td></td>
</tr>
<tr>
<td>Moderate to severe</td>
<td></td>
</tr>
<tr>
<td>Moderate</td>
<td></td>
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<tr>
<td>None</td>
<td></td>
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*None, no signs of infection; weak, slight yellow discolouration at the site of inoculation; moderate, localized water soaking and chlorosis of tissue circumscribing the inoculation site; severe, complete decomposition of entire leaves characterized by systemic water soaking, necrotic lesions and chlorosis.
Fig. 7. ERIC-PCR dendrogram for *P. aeruginosa* strains in this study. Percentage similarity of profiles for strains was calculated using Dice's coefficient and UPGMA. Using a 0.05 cut-off, two main clusters were observed. Asterisks indicate strains with at least two of the following: good biofilm, high elastase and rhamnolipid production.
(RFV > 1) isolates, those deemed good biofilm producers, those more tolerant to oxidative stress, those with phenotypic and genotypic presence of QS mediators, the more virulent isolates based on pyoverdin production, and those that caused severe symptoms in the plant model.

**DISCUSSION**

The overuse and frequent misuse of antibiotics in developing populations of micro-organisms amongst bacterial populations (Byarugaba, 2004). In Jamaica, patients with respiratory illness caused by *P. aeruginosa* are usually treated empirically with an aminoglycoside, either alone or in combination with another agent or class (Brown & Izundu, 2004; Finlayson & Brown, 2011). GM, AN, IPM and CIP are considered potent treatments of infections caused by multiresistant *P. aeruginosa*; this was corroborated by the findings of this study. We found that the aminoglycoside AN and the carbapenem IPM were associated with fewer resistance frequencies, followed by NOR and CIP. Therefore, it is important to control the use of these antibiotics in the hospital setting to prevent the emergence of aminoglycoside- and fluoroquinolone-resistant strains, and to restrict the use of antimicrobials when these resistant strains are detected. The MAR index is a good risk assessment tool and the value of the MAR index (nominal 0.200) has been applied to differentiate low- and high-risk regions where antibiotics are overused (Riaz et al., 2011). Such an analysis gives an idea of the number of bacteria showing antibiotic resistance in the risk zone of the susceptibility study. Based on our findings, most isolates had MAR indices of >0.25, confirming that there was high antibiotic use and high selective pressure in these environments. However, the practical significance of such an analysis in a developing country such as Jamaica may be lost because antibiotic use and abuse are widespread (Olayinka et al., 2009). Furthermore, given CIP’s reduced efficacy against the biofilm forms of the strains in this study, cationic antimicrobial peptides might be attractive alternatives as they have been reported to degrade established biofilms and kill planktonic cells (Segev-Zarko et al., 2015).

It was not expected that a susceptible strain would out-compete a resistant strain during competition between resistant and susceptible strains, particularly in the presence of antibiotics. This begs the question how resistant strains could lose fitness over the time period. Given that CIP-resistant strains express resistance through the acquisition of *qnr* determinants or via mutation in topoisomerase IV genes, these determinants normally incur a fitness cost in the strains, but not sufficient to prevent them from out-competing a susceptible strain in the presence of antibiotics. Bhatter et al. (2012) reported that the strain type and the assay system (culture medium) may influence the fitness cost imposed on the micro-organisms exposed to competition. West & Buckling (2003) showed that the production of pyoverdin, favoured on King B medium, was metabolically costly to the micro-organism that produced it. However, Griffin et al. (2004) noted that a population of mutant *P. aeruginosa* strains that did not produce the siderophore declined in numbers when grown in the presence of other strains that produced it. From this study, it can be inferred that by producing pyoverdin the susceptible strain adversely affected the population of the drug-resistant strain and this explains how the susceptible strain could out-compete the resistant strains as observed in this study.

Furthermore, the observation of the susceptible strain growing faster than the drug-resistant strains by 48 h suggests that mixed strain infections including a drug-resistant strain may increase the opportunity for susceptible strains, even without acquiring drug resistance determinants, to persist in areas where they would more likely be killed or out-competed. Consequently, allowing longer persistence of drug-resistance drug-resistant *P. aeruginosa* strains in a co-infection or mixed system could provide these bacteria with adequate opportunities to accumulate additional mutations and thus become problematic in further treating patients with such infections (Hogardt & Heesemann, 2013).

Whilst pigment production by an organism or a group of organisms is a characteristic of taxonomic importance, in *P. aeruginosa* it is a key expression of virulence. We noted that the majority (51.3 %) of the *P. aeruginosa* isolates produced pyoverdin. Pyorubin production, observed in 16.2 % of isolates, is also believed to be involved in the protection of the organism from oxidative stress (Meyer et al., 1997). Furthermore, 13.5 % of the isolates in this study produced pyocyanin, a pigment with oxygen-dependent antimicrobial activity and cytotoxic effects, which could contribute to the pathogenicity of the organisms in respiratory infections (Owen & Ackerley, 2011).

The presence of enzymes such as catalase and dismutase facilitates the ability to withstand oxidative stress, particularly in the respiratory tract. In this study, *H₂O₂* had a greater oxidative stress than *t-BOOH* at both tested concentrations on biofilm and planktonic cells. The lack of efficacy of *t-BOOH* could either be as a result of the utilization of this compound as a carbon source by *P. aeruginosa* (Garnier et al., 1999) or due to the expression of an anti-oxidative resistance mechanism (Somprasong et al., 2012). Consequently, resistant biofilms could conceivably produce more viable cells after treatment with *t-BOOH*.

Elastase has long been considered a virulence-associated factor that aids in the pathogenicity of *P. aeruginosa*. The connective tissues and cell surface receptors of host cells are usually attacked and degraded by this metalloprotease (Olson & Ohman, 1992). Results from this study showed that elastase activity was strongly associated with pigment-producing strains compared with non-pigment producers. Rhamnolipids are biosurfactants and, like elastase, are also considered virulence factors in *P. aeruginosa* (Bazire et al., 2009). These virulence factors work in synergy with the LasRI QS system and are important for
full elastolytic activity. Based on this study, there was a good correlation between elastase production and the presence of rhamnolipid.

Using the romaine lettuce leaf model, those strains grouped as rhamnolipid-positive/elastase-positive and rhamnolipid-negative/elastase-negative showed significant infection potential due to severe maceration of tissues as early as day 1. However, bacteria grouped as rhamnolipid-negative/elastase-negative and rhamnolipid-negative/elastase-positive showed reduced infection potential as the more severe effects were not observed until day 4 or 5. Of significance, then, is that strains that were rhamnolipid-negative/elastase-negative could still cause serious infection even if these virulence factors were not immediately expressed, as was observed in this study. In addition, there was no correlation between the different strains from the four rhamnolipid/elastase categories with biofilm formation. There was, however, some correlation with antibiotic resistance, where once the rhl gene for rhamnolipid expression was present, isolates showed resistance to more than five of the antibiotics tested. The ERIC-PCR analyses confirmed that the strains were genetically heterogeneous, with two main groups identified, with the apparently more virulent strains clustering together (cluster A).

In conclusion, this study is, to the best of our knowledge, the first major study on fitness and oxidative stress tolerance of respiratory pathogens in Jamaica, and suggests that nonpigmented strains of P. aeruginosa might pose an equally significant microbiological threat as pigmented strains even though pigment production appeared to be strongly associated with elastase expression. This study also showed that during competition, a susceptible strain has the potential to gain fitness within a mixed population that includes a resistant strain. Furthermore, respiratory P. aeruginosa in Jamaica appears to be genetically heterogeneous, suggesting multiple introductions of organisms into the hospital environment. In addition, whilst dual expression of rhamnolipid and elastase are important as virulence factors in these bacteria, non-production of either does not preclude the causation of severe symptoms in susceptible hosts.

**ACKNOWLEDGEMENTS**

Special thanks to the staff of the University Hospital of the West Indies and Central Medical Laboratory, Kingston, Jamaica, for assisting with isolate collection. R. D. received a grant from the Office of Graduate Studies and Research, University of the West Indies, Mona. Material assistance provided by the Department of Basic Medical Sciences is greatly appreciated.

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