Comparison of Antibiotic Resistance and Virulence Factors in Pigmented and Non-pigmented *Pseudomonas aeruginosa*

EA Finlayson, PD Brown

ABSTRACT

Objective: Pseudomonas aeruginosa *produces multiple virulence factors that have been implicated in pathogenesis and quorum sensing. The aim of this study was to determine differences in the virulence factors of pigmented and non-pigmented* P aeruginosa *isolates.*

Methods: *Associations were assessed between pigment production (pyocyanin and pyoverdin) and production of DNase, elastase, lipase, protease, siderophore, twitching motility, antibiotic resistance patterns and virulence-associated genes in 57 non-duplicate* P aeruginosa *isolates from wounds, sputum, urine, high vaginal swab (HVS), ear, eye and respiratory tract swabs and aspirates of peritoneum and ulcers.*

Results: *Most (82.5%) of the isolates produced either pigment. Pigmented isolates produced more frequently and significant more (*$p < 0.05$*) DNase, elastase, lipase protease, and siderophore. Imipenem was the only antibiotic to which all isolates were susceptible (*$p < 0.05$*), while 93% and 32% were resistant to tetracycline and norfloxacin, respectively. There was however no significant difference between pigmented and non-pigmented isolates when antibiotic resistance was compared.*

While isolates had multiple virulence-associated genes, exoS (51%), rhlA *(37%) and* rhlB *(46%) were the predominant genes detected. Except for* exoY, *genes were present in pigmented isolates more frequently than in non-pigmented isolates.*

Conclusion: *The results of this study suggest that antibiotic resistance* per se *might not be associated with the pigment production in* P aeruginosa. *However, pigment production appeared to be more significantly associated with multi-drug resistance, presence of virulence-associated genes, and expression of certain virulence factors, most notably elastase, protease, siderophore and DNase activity. Since pigment production is easy to determine, this might to be a good starting point to identify the virulence status of an isolate.*

Keywords: Antiobiotic resistance, *Pseudomonas aeruginosa*, virulence factors

Comparación de la Resistencia Antibiótica y los Factores de Virulencia en las *Pseudomonas aeruginosa* Pigmentadas y no Pigmentadas

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RESUMEN

Objetivo: Pseudomonas aeruginosa *produce múltiples factores de virulencia que han estado implicados en patogénesis y detección de quórum* (quorum sensing). *El objetivo de este estudio fue determinar las diferencias en los factores de virulencia de aislados de* P aeruginosa *pigmentada y no pigmentada.*

Método: *Se evaluaron las asociaciones entre la producción de pigmentos (piocianina y pioverdina) y la producción de Dnasa, elastasa, lipasa, proteasa, sideróforos, motilidad asociada a superficies (twitching), patrones de resistencia antibiótica, y genes asociados con virulencia en 57 aislados de* P aeruginosa *no duplicados, de heridas, esputo, orina, exudado vaginal, exudados de oídos, ojos, y vías respiratorias, y aspirados de peritoneo y úlceras.*

Resultados: *La mayor parte (82.5%) de los aislados produjeron uno de los pigmentos. Los aislados pigmentados produjeron con mayor frecuencia y más significativamente (*$p < 0.05$*). Dnasa, elastasa,*

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*lipasa, proteasa, y siderósforos. Imipenem fue el único antibiótico al que todos los aislados eran susceptibles (*p < *0.05), mientras que el 93% y el 32% fueron resistentes a la tetraciclina y a la norfloxacina, respectivamente. Sin embargo, no hubo diferencia significativa entre los aislados pigmentados y los no pigmentados cuando se comparaba la resistencia antibiótica. Si bien los aislados tenían múltiples genes asociados con la virulencia, exoS (51%),* rhlA *(37%) y* rhlB *(46%) fueron los genes predominantes detectados. Con excepción de exoY, los genes estuvieron presentes en aislados pigmentados con mayor frecuencia que en los aislados no pigmentados.*

Conclusión: *Los resultados de este estudio sugieren que la resistencia antibiótica per se podría no estar asociada con la producción de pigmentos en* P aeruginosa. *Sin embargo, la producción de pigmentos parecía estar asociada más significativamente con la resistencia a las multidrogas, la presencia de genes asociados con la virulencia, y la expresión de ciertos factores de virulencia, en particular la actividad de la elastasa, la proteasa, los sideróforos, y la Dnasa. Puesto que la producción de pigmentos es fácil de determinar, esto podría ser un buen punto de partida para identificar el estado de virulencia de un aislado.*

Palabras claves: Resistencia antibiótica**,** *Pseudomonas aeruginosa*, factores de virulencia

West Indian Med J 2011; 60 (1): 25

INTRODUCTION

Pseudomonas aeruginosa is a gram negative opportunistic pathogen often implicated in nosocomial infections and which causes many severe and often fatal infections particularly affecting immunocompromised patients with severe burns or those with HIV infection (1). The bacterium's intrinsic and acquired resistance to many structurally-unrelated antibiotics is due to several adaptations, including active efflux systems, reduced cell wall permeability, plasmid acquisition, expression of various enzymes or by biofilm formation (2, 3). Pathogenesis involves production of both extracellular and cell associated virulence factors (4). Many virulence factors are expressed through a cell density dependent mechanism known as quorum sensing (4). These additional virulence factors include elastase, lipase, protease and several cytotoxins, encoded by *exo* genes. Elastase and alkaline protease are known to degrade a large variety of tissue components such as proteinaceous elements of connective tissue and cleave the cell surface receptors on neutrophils (5). Elastase production in *P aeruginosa* is regulated by the *las*R, *las*I, *rhl*R and *rhl*I genes (6).

Further, this opportunistic pathogen is found in a diverse range of ecological niches and has evolved a variety of iron acquisition mechanisms mainly through the production of pigments (7). The mechanism of twitching motility is pilus retraction and extension (8). Twitching motility has been shown to be required for initial attachment and development of biofilms (9), which once developed, are hard to eradicate (10) and are up to 1000 times more resistant to the effects of antibiotics than their planktonic counterparts (11).

In this study, the genotype (*exoT*, *exoS*, *exoU*, *exoY*, *lasA, lasB*, *rhlA*, *rhlB*, Bacto 1, Pf1, PAGI-1, -2 and -3) and phenotype (DNase, elastase, lipase, protease, siderophore, twitching motility, antibiotic resistance patterns) of *P aeruginosa* isolates implicated in serious infections from hospitalized patients were determined and compared between pigmented and non-pigmented isolates. The hypothesis tested was that pigmented *P aeruginosa* possess distinguishing virulence characteristics when compared to their non-pigmented counterparts, which might have implications for treatment and control.

MATERIALS AND METHODS

Bacterial strains and growth conditions

A collection of 57 non-duplicate *P aeruginosa* isolates were obtained from wounds, sputum, urine, high vaginal swab (HVS), ear, eye and respiratory tract swabs, and aspirates of peritoneum and ulcers submitted by hospitalized patients. The criteria for identifying an isolate as *Pseudomonas aeruginosa* were oxidase positivity, catalase positivity, growth at 42°C and pigment production (12). *P aeruginosa* PAO1 and PAOR1 (gifts from B Iglewski) and ATCC 27853 (gift from RKhan) were used as the reference strains in all tests completed. *P aeruginosa* isolates were grown on blood agar and MacConkey agar plates to assess purity and on Mueller-Hinton agar plates to assess pigment production. Unless otherwise noted, bacteria were grown in Luria-Bertani medium (Oxoid, Basingstoke, UK) or in Mueller-Hinton broth or agar (Becton Dickinson, Cockeysville, MD) at 37°C for 18 hour. After incubation, centrifugation was carried out, and the supernatant was filter-sterilized and transferred to a new test tube for phenotypic tests and the remaining cell pellet was resuspended in sterile phosphate-buffered saline for Polymerase Chain Reaction (PCR) amplification.

Phenotypic tests

DNase production was determined after spot inoculation of DNase test agar (Difco) plates, incubation at 37°C for 24 to 48 hours, and flooding of the surface of the plates with 1.0 N

HCl. The test agar contained an emulsion of DNA and peptides as a nutrient source. Bacterial colonies that secrete DNase hydrolyze the DNA in the medium into smaller fragments which results in clearing around the bacterial growth. Elastolytic activity of bacterial supernatants was determined by the elastin Congo red assay. Following overnight growth of cultures in liquid LB medium, centrifugation at 13 000 rpm was carried out for 15 minutes in microcentrifuge tubes. A 100 µl volume of supernatant was added to 900 µl of 100 mM Tris-1 mM $CaCl_2$ (pH 7.5) containing 1 mg of elastin Congo red, and the mixture was incubated with shaking at 37°C for 24 hours. Centrifugation was repeated as before and the optical density of the supernatants measured at 495 nm. Lipase activity was measured with Tween 20 (Sigma) as a substrate. The reaction mixture consisted of 0.1 ml of 10% Tween 20 in 50 mM Tris HCl (pH 7.6) [Tris buffer], 0.1 ml of 1 M $CaCl_2$ in Tris buffer, 0.5 ml of culture supernatant and 2.3 ml of Tris buffer. Duplicate samples were prepared for each isolate tested and incubated in a 37°C water bath for two hours. Reagent blanks was prepared with 0.5 ml of deionized water instead of supernatant. The extent of precipitate formation was measured turbidimetrically at 400 nm. The ability of the isolates to produce protease was determined on a medium containing 1% agar, 2% skim milk dissolved in 3 mM $CaCl_2$, and 50 mM Tris-HCl (pH 7.5). Briefly, the medium was inoculated with 100 µl of an overnight culture in holes made on the plates. Following overnight incubation at 37°C, clear zones around the holes indicated protease production.

Siderophore production was detected using the universal siderophore detection medium chrome azurol S (CAS) agar (13). After incubation (1 to 3 days, at 37°C), the relative halo size ([halo diameter – colony diameter]/halo diameter]) was determined. Halo determinations were done in triplicate and repeated at least three times. Twitching motility was assessed after overnight incubation (at 37°C) of stab inoculated 1% LB agar plates (11). All test isolates were assessed for the extent of pigment production on King A (for pyocyanin) and King B (for pyoverdin) agar (14).

Antibiotic susceptibility tests

Susceptibility testing was performed by the standard CLSI (formerly known as NCCLS) disk diffusion method (15) using common anti-pseudomonad antibiotics: tetracycline (30 µg), amikacin (30 µg), tobramycin (10 µg), piperacillin (10 µg), norfloxacin (10 µg), ciprofloxacin (5 µg), ceftazidime (30 µg) and imipenem (10 µg). Inocula were prepared by suspending growth from LB broth to a starting concentration of 5 x 10^5 CFU/ml. Mueller-Hinton plates were incubated for 24 hours after inoculation with organisms and placement of the disks and zones of inhibition were measured. Ceftazidime-resistant isolates were assessed for IMP-1 β-lactamase production by suspending a colony of each in Mueller-Hinton (MH) broth to 10^6 CFU/ml and spreading on an MH agar plate according to the protocol recommended by the NCCLS. Two commercially supplied disks, each containing 30 µg of CAZ (Difco), were placed on the plates about 4 to 5 cm apart. Subsequently, a filter disk was placed near one of the CAZ disks within a centre-to-centre distance of 1.0 to 2.5 cm, and 5 µl of 100 mM EDTA or 2-3 µl 2-mercaptopro-pionic acid added to assess IMP-1 inhibition. Plates were incubated at 37°C overnight (16).

PCR analysis of virulence-associated genes

The presence of several virulence-associated genes encoding exotoxins T (*exoT*), S (*exoS*), U (*exoU*) and Y (*exoY*), elastase (*lasA* and *lasB*), and quorum sensing mediators (*rhlA* and *rhlB*), the presence of prophage (Bacto 1 and Pf1) and genomic islands (PAGI-1, -2 and -3) was assessed by PCR amplification as previously described (17, 18), except for primers for *rhlA* (rhlAF, 5'-GCGCGAAAGTCTGTTG GTAT; rhlAR, 5'-CAGGTGATTGACCTCGAAGC) and *rhlB*, (rhlBF, 5'-GAGCGACGAACTGACCTACC; rhlBR, 5'- GTTGAACTTGGGGTGTACCG) which were designed in this study. A typical 20 µl PCR mixture for every primer set consisted of 1x Green GoTaq Reaction buffer (containing 1.5 mM $MgCl_2$; pH 8.5; Promega), 200 µM of each dNTP (Promega), 1 U GoTaq DNA polymerase (Promega), 0.5 µM of each primer, and 1 µl of DNA template. Amplifications of virulence genes and prophage were carried out in a Techne TC-3000 DNA Thermal Cycler (Techne, Inc, USA) using the following protocol: 94°C for 1 minute, 34 cycles consisting of 94°C for 1 minute, 54°C for 2 minutes, and 72°C for 2 minutes and a final extension step at of 72°C for 8 minutes (18). Each gene was amplified in a separate reaction to obviate possible competition among primers. The presence of genomic islands was investigated using the following parameters: initial denaturation at 96°C for 5 minutes, 30 cycles at 94°C for 30 seconds, 47 to 64°C at 30 seconds, 1 to 4 minutes at 72°C and final extension for 8 minutes (17). PCR products were separated in a 1% agarose gel for one hour at 100 V, stained with ethidium bromide and detected by UV trans-illumination. Amplified genes were identified on the basis of fragment size and reference strain PAO1 was used as the positive control.

Statistical analysis

Statistical analysis was performed using the χ^2 test. Differences were considered significant at $p \leq 0.05$.

RESULTS

Fifty-seven isolates of *P aeruginosa* were obtained from the clinical specimens as indicated in Table 1. These isolates grew on blood, MacConkey and Mueller Hinton plates, and were identified by their morphology, smell and pigmentation, as well as their Gram reaction and specific confirmatory biochemical tests. Most isolates were obtained from wounds (17 or 29.3%), respiratory tract (14 or 24.5%), ear swab (11 or 19.3%) and urinary catheter or urine samples (6 or 10.3%). Forty-eight (82.8%) specimens yielding isolates were from

Table 1: Distribution, by parish and source of specimens positive for *Pseudomonas aeruginosa*

Isolate #	Gender	Specimen/swabs	Origin	Isolate #	Gender	Specimen/swabs	Origin
D061	M	Aspirate	St Andrew	B131	M	Sputum	Kingston
B021	M	Ear	Kingston	A304	F	Ulcer	St Thomas
B043	M	Ear	Kingston	C183	M	Ulcer	Kingston
B044		Ear	Kingston	U068	M	Urinary catheter	Kingston
B076	F	Ear	Kingston	U126	M	Urinary catheter	Kingston
B132	F	Ear	Kingston	P075	M	Catheter urine	Kingston
C055	M	Ear	Manchester	C090	M	Urethral Swab	St Catherine
C078	M	Ear	Kingston	A210	F	Urine	Kingston
C097	A	Ear	Kingston	U342	F	Urine	Kingston
C175	F	Ear	Kingston	C038	F	High Vaginal Swab	Kingston
P089	F	Ear	Kingston	P038	F	Vagina	Kingston
P090	F	Ear	Kingston	A045	M	Wound	Kingston
C058	M	Eye	Kingston	A074	F	Wound	Kingston
D011	F	Peritoneal	Kingston	A087	F	Wound	Kingston
A307	M	Pus	Kingston	A089	F	Wound	Kingston
P011	F	Pus	Kingston	A185	M	Wound	Kingston
D008	F	Respiratory Tract	Kingston	A273	F	Wound	Kingston
B027		Sputum	Clarendon	A278	F	Wound	Kingston
B047	M	Sputum	Kingston	A302	F	Wound	Kingston
B071	F	Sputum	St Andrew	C102	F	Wound	Kingston
B073	M	Sputum	Kingston	C138	M	Wound	St Andrew
B083	M	Sputum	St Catherine	C320	M	Wound	Kingston
B084	M	Sputum	Kingston	P021	M	Wound	Kingston
B102	M	Sputum	Kingston	P046	F	Wound	Kingston
B104	M	Sputum	Kingston	P058	F	Wound	St Catherine
B111	F	Sputum	Clarendon	P077	F	Wound	Kingston
B112	F	Sputum	Clarendon	P079	M	Wound	Kingston
B123	M	Sputum	Kingston	P083	F	Wound	Portland
B128	M	Sputum	Kingston				

the Kingston metropolis region, and the largest number of positive samples were from wound swabs from female patients (n = 11) and sputum samples from male patients (n = 9).

Forty-seven (82.5%) of the isolates produced either pigment, ranging from 64.7% (wound isolates) to 100% [pus/abscess, vaginal, septic ulcer and eye isolates] (Table 2).

The distributions of the virulence factors of pigmented and non-pigmented *P aeruginosa* isolates are given in Table 3. Pigmented isolates produced more frequently and significantly more ($p < 0.05$) DNase, elastase, lipase, protease and siderophore; all pigmented and a non-pigmented isolates produced lipase. All isolates from urine expressed DNase

Table 2: Frequency of isolation and pigment production by isolates of *P aeruginosa* from various sites of infection or specimen.

Site of infection/ specimen	Total isolates (%)	Pyoverdin production (%)	Pyocyanin production (%)	Pigment production (%)
Wound	17 (29.3)	10 (58.8)	8 (47.1)	11 (64.7)
Respiratory	14 (24.5)	12 (85.7)	11 (78.6)	13 (92.9)
Ear	11 (19.3)	9 (81.8)	7 (63.6)	9 (90.9)
Urine/urinary catheter	6 (10.3)	5 (83.3)	3 (50.0)	5 (83.3)
Pus/abscess	4 (7.0)	4 (100.0)	1 (25.0)	4 (100.0)
Vagina	2 (3.5)	2 (100.0)	0 (0.0)	2 (100.0)
Septic ulcer	2 (3.5)	2 (100.0)	2 (100.0)	2 (100.0)
Eye	1 (1.8)	1 (100.0)	1 (100.0)	1 (100.0)
Total	**57**	**45 (78.9)**	**33 (57.9)**	**47 (82.5)**

Overall, more isolates produced pyoverdin (78.9%) than pyocyanin (57.9%; $p < 0.05$). When the source of isolates was considered, except for those from wound, septic ulcers and eye, production of pyoverdin *versus* pyocyanin was significantly different ($p < 0.05$).

activity while only 41%, 54% and 64% of isolates from wound swabs, sputum and ear swabs, respectively, showed DNase activity.

Table 3: The distribution of virulence factors of pigmented and non-pigmented *P aeruginosa* isolates

Virulence Factor		Pigmented		Non-Pigmented		χ^2 Test
		n	%	n	%	*p*-value*
Twitching	Total	17	100.0	0	0.0	< 0.0001
Dnase	Total	25	75.8	8	24.2	< 0.0001
Lipase	(+)	28	49.1	6	10.5	< 0.0001
	(++)	19	33.3	4	7.0	0.0001
	Total	47	82.5	10	17.5	< 0.0001
Elastase	(+)	27	58.7	7	15.2	< 0.0001
	(++)	7	15.2	0	0.0	< 0.0001
	(+++)	5	10.9	0	0.0	< 0.0001
	Total	39	84.8	7	15.2	< 0.0001
Protease	(+)	11	28.2	5	12.8	0.0288
	(++)	11	28.2	3	7.7	0.0015
	(+++)	9	23.1	0	0.0	< 0.0001
	Total	31	79.5	8	20.5	< 0.0001
Siderophore	(+)	12	36.4	3	9.1	0.0001
	(++)	15	45.5	3	9.1	< 0.0001
	Total	27	81.8	6	18.2	< 0.0001

(+), (++), (+++), levels of activity based on diameter of zone around well or spectrophotometric measurement; Total, total number of isolates expressing specific virulence factor.
*$p \leq 0.05$ considered significant

Twenty-nine (51%) isolates were PCR-positive for *exoS* (Table 4). Except for *exoY*, genes were present in pigmented isolates more frequently than in non-pigmented (Table 5). Twelve (21%) isolates were PCR-positive for *lasB* and two (3%) for *lasA*. While the *lasB* gene was disseminated among isolates from various sites of infection, *lasA* was only found in isolates from the ear (Table 5), and all except one *lasB* positive isolate was pigment producing.

The designed *rhlA* and *rhlB* primers resulted in an amplified product of about 550 bp. Twenty-one (37%) and 26 (46%) of the isolates possessed the *rhlA* and *rhlB* gene, respectively, and these were from various sites of infection. Again, more pigmented isolates were positive for *rhlA* and/or *rhlB*.

Table 4: Distribution of virulence-associated genes between pigmented and non-pigmented isolates of *P aeruginosa*.

Virulence genes	Pigment producing	Non-pigment producing
lasA	2	0
lasB	11	1
rhlA	19	2
rhlB	19	7
exoS	21	8
exoT	2	0
exoU	0	0
exoY	6	5
Bacto1	0	0
Pf1	1	0
PAGI-1	2	1
PAGI-2	0	0
PAGI-3	1	1

A single pigmented isolate from ear was positive for pf1. Only one non-pigmented isolate (each) was positive for PAGI-1 and PAGI-3. These isolates were from urine and ear, respectively.

Antibiotic susceptibility of the 57 *P aeruginosa* isolates is illustrated in Fig. 1. For the purpose of this analysis,

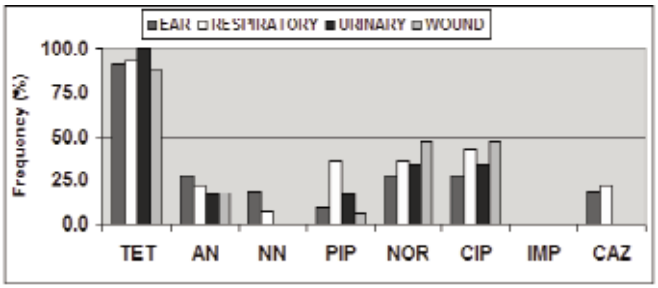

Fig. 1. Frequency of resistance among *P aeruginosa* isolates to common anti-pseudomonal antibiotics. TET, tetracycline; AN, amikacin; NN, tobramycin; PIP, piperacillin; NOR, norfloxacin; CIP, ciprofloxacin; IMP, imipenem; CAZ, ceftazidime.

isolates with resistant phenotypes excluded those that were classified as intermediate. The carbapenem, imipenem, was the only antibiotic to which all isolates were susceptible ($p < 0.05$), while 93%, 30%, 32%, 19% and 11% were resistant to

Table 5: Distribution of virulence-associated genes between sites of infection.

Source	n	Virulence Genes							Prophage/Genomic Islands		
		las A	*las B*	*rhl A*	*rhl B*	*exoS*	*exoT*	*exoY*	Pf1	PAGI-1	PAGI-3
Respiratory	13	0	1	6	7	7	0	1	0	1	0
Wound	17	0	4	8	7	11	0	5	0	1	1
Ear	11	2	3	5	5	8	0	4	1	0	1
Others	15	0	4	2	7	3	2	1	0	1	0
Total	**57**	**2**	**12**	**21**	**26**	**29**	**2**	**11**	**1**	**3**	**2**

tetracycline, amikacin, norfloxacin, ciprofloxacin and ceftazidime, respectively. Isolates from ear swabs were marginally more frequently resistant to amikacin and significantly more frequently resistant ($p < 0.05$) to tobramycin. Respiratory isolates were significantly more frequently resistant to piperacillin ($p < 0.001$) and wound isolates were significantly more frequently resistant to norfloxacin ($p < 0.05$). In addition, all wound isolates were susceptible to ceftazidime. Urinary isolates were all resistant to tetracycline, and 33%, 17%, and 17% were also resistant to amikacin, the fluoroquinolones and piperacillin, respectively. However, all urinary isolates were susceptible to tobramycin and ceftazidime. There was no significant difference ($p > 0.250$) between pigmented and non-pigmented isolates when antibiotic resistance was compared (Fig. 2).

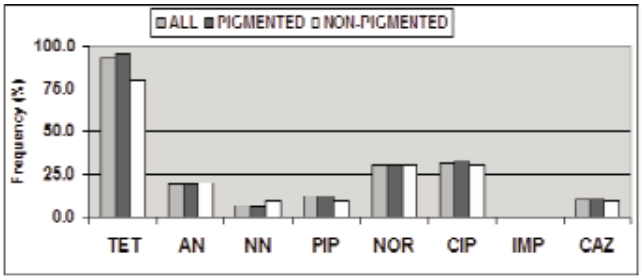

Fig. 2: Comparison of frequency of resistance between pigmented and non-pigmented *P aeruginosa* isolates to common anti-pseudomonal antibiotics. TET, tetracycline; AN, amikacin; NN, tobramycin; PIP, piperacillin; NOR, norfloxacin; CIP, ciprofloxacin; IMP, imipenem; CAZ, ceftazidime.

Multi-drug resistance (MDR – defined as resistance to at least three antibiotics) was observed in six pigmented and one non-pigmented isolate. Four of the MDR pigmented isolates were obtained from respiratory samples; an isolate each from an ulcer and an ear swab were resistant to at least four antibiotics. The lone non-pigmented MDR isolate was from an ear swab.

When cross-resistance to other classes of antibiotics was examined, it was noted that tetracycline resistant isolates were largely resistant to all other (except imipenem) anti-pseudomonal antibiotics assessed. Tobramycin-resistant isolates were also resistant to tetracycline, amikacin and ceftazidime. Fluoroquinolone-resistant isolates were susceptible to tobramycin and ceftazidime. Of isolates resistant to ceftazidime, 83%, 67% and 50% were also resistant to amikacin, tobramycin and piperacillin, respectively. None of the ceftazidime resistant isolates expressed IMP-1 metallo-β-lactamase activity.

DISCUSSION

Because of the widespread use of antibiotics, especially in developing countries, the resistance profile of microorganisms is changing, as evidenced by the increasing occurrence of antibiotic resistance among bacterial populations (19, 20). *Pseudomonas aeruginosa* is naturally resistant to β-lactams, including broad-spectrum cephalosporins, quinolones, chloramphenicol and tetracyclines, mainly due to the very low permeability of their cell wall, as well as the presence of inducible cephalosporinase, active efflux and poor affinity for the target (DNA gyrase). These latter three mechanisms are known to synergize with poor cell wall permeability (21). Consequently, infections with these organisms are often refractory to treatment with many currently available drugs, including the anti-pseudomona antibiotics. This emphasizes the need for the implementation of local surveillance with antibiograms to guide the current use of antibiotics. In this study, the examination of some virulence factors for several clinical isolates of *P aeruginosa* from hospitalized patients mainly in the Kingston metropolitan region, and the relationship between these virulence factors, antibiotic resistance profiles and pigment production, were carried out.

It was found that isolates that produced either pyocyanin or pyoverdin were more likely to produce additional virulence factors, including elastase, protease, siderophores, DNase, and twitching motility ($p < 0.05$). Results from the selective plating on *Pseudomonas* agar *P* identified most of the isolates with the ability to produce pyocyanin, a redox-active secondary metabolite, which is regulated by quorum sensing (22). The pigment has been determined to display cytotoxic properties and therefore contributes to the pathogenesis of *P aeruginosa* as a human pathogen (23). Significantly, the compound has been identified in sputum samples from patients with chronic pulmonary infections, especially cystic fibrosis patients (24), thus is considered to be an infection-associated virulence factor. Eleven of the 13 respiratory isolates produced pyocyanin, which concurs with the literature. However, cystic fibrosis is rarely observed in patients in Jamaica, while other causes of chronic lung infections, such as pneumonia and tuberculosis, occur frequently. Similarly, pyoverdin (fluorescein), produced by 45 of the 57 isolates, is another virulence factor produced by *P aeruginosa* (4). Pyoverdin, encoded by the *pvd* genes (25) acts as a siderophore, involved in a complex iron acquisition system tightly binding and transporting soluble iron (Fe^{III}) from the environment under iron-deficient condition and has been determined to be an essential component in biofilm formation (4).

Furthermore, elastase (metalloprotease) and alkaline protease, two important invasins known to be important virulence factors for *P aeruginosa* can degrade a large variety of tissue components such as proteinaceous elements of connective tissue and cell surface receptors on neutrophils (5). Elastase was the virulence factor most strongly associated with pigment production by isolates, but was more associated with ear, urinary and respiratory isolates. In contrast, twitching motility was the virulence factor that was least expressed and was more associated with catheter and respiratory isolates.

The different antibiotic resistance patterns observed in the isolates indicate that the organism uses several mechanisms of resistance simultaneously, and that all isolates do not necessarily use the same mechanisms for resistance to particular classes of antibiotics. Further, isolates that were resistant to one class of antibiotics were also resistant to at least one other class. Infections with *P aeruginosa* are often treated with aminoglycosides either alone or in combination with other antimicrobial agents. There appears to be a trend to increasing resistance by *P aeruginosa* to these antibiotics, particularly gentamicin. We found that 19% and 7% of the isolates were resistant to the aminoglycosides, amikacin and tobramycin, respectively. This contrasts with the results of a previous multi-parish study in Jamaica, where none and 25% of *P aeruginosa* isolates were resistant to amikacin and tobramycin (26). However, the problem of increasing aminoglycoside resistance is becoming a cause for concern as the results from a study published a year later, conducted at the University Hospital of the West Indies, in Kingston, found that 11% and 23% of *P aeruginosa* isolates were resistant to amikacin and gentamicin (27). In Jamaica, amikacin and other aminoglycosides are among the main antibiotics administered for urinary tract infections (UTIs) and other infections including wounds and sepsis and could be driving the increase observed.

Many of the aminoglycoside-resistant isolates were pigmented and were obtained from ear and respiratory samples, in contrast to the association elsewhere of these with outbreaks of UTIs (28). These isolates also had low levels of twitching motility and lipase, DNase and protease activities, which suggest that they may not be as virulent as their aminoglycoside-susceptible counterparts (29). It has been inferred that increased adherence of aminoglycoside-resistant strains to various cells (when compared to aminoglycoside-susceptible strains) result in these being excellent colonizers or epithelial surfaces.

The production of β-lactamase is the most prevalent mechanism of resistance to β-lactam antibiotics. The metallo -β-lactamase, IMP-1, encoded by a mobile gene (bla_{IMP}) located on an integron like element, was recently found in some clinical isolates including *P aeruginosa*. Due to the ability of the bla_{IMP} gene to be rapidly spread among pathogens, IMP-1 currently represents the most dangerous metallo-β-lactamase as it is hardly blocked by β-lactamase inhibitors such as clavulanate, sulbactam and tazobactam (30). Therefore, the absence of this characteristic in our isolates is a welcomed finding.

The analysis and comparison of virulence genes provide an overall understanding of the disease causing factors and their genetic regulation as well as providing a means of identifying the subsets of parameters in characterizing a pathogen. In this study, 57 isolates of epidemiologically unrelated isolates of *P aeruginosa* were assessed for the presence or absence of 13 genetic regions associated with virulence. Two quorum-sensing systems (*las* and *rhl*) regulate virulence gene expression in *P aeruginosa*. Of the two *Las* quorum sensing genes analysed, significantly more of the isolates were positive for *lasB* than *lasA*. It has been shown previously that more strains are positive for *lasB* when compared to the *lasA* gene (17). The *LasA* protein has been shown to be involved in the final processing of elastase by broadening the substrate specificity of elastase and the *lasB* locus is responsible for the regulation of both as elastase and protease (4). While none of these isolates were obtained from blood, the presence of elastase-producing strains with the ability to cause invasive damage may be a signal for efficient antimicrobial treatment to prevent the spread of a localized infection to one that is systemic.

The *rhl* system regulates production of rhamnolipid which has both haemolytic and biosurfactant properties, and is important for full elastase activity (4). The quorum sensing system is based on a hierarchal system where the *las* system is dominant over the *rhl* system. However, the *rhl* system can activate the *las* system and hence its genes and the synergy that exists between these two systems might prove devastating for a patient if a systemic infection is established. The data show that there was not much variability between frequency of *rhlA* and *rhlB* genes, which probably indicate that there is no association with *rhl* gene and the site of the infection.

Most of the isolates were found to have two of the four cytotoxins normally secreted *via* type 3 secretion (TTS) systems. The *exoS* gene, found in 72%, 65% and 54% of ear, wound, and sputum isolates, respectively, has been correlated with the ability of *P aeruginosa* to spread from epithelial colonization sites to the bloodstream (31). Because ExoS may compete with other more potent cytotoxins for the TTS systems, it is likely to cause effects on multiple cellular processes, including inhibition of DNA synthesis and interference of cell matrix adherence (32). The finding of a majority of the isolates containing *exoS* and none with *exoU*, is supported by previous work (33), which suggests that these genes are mutually exclusive (34). *ExoT*, found in three isolates, has been implicated in increased cellular and toxic effects in experimental models and human infections (35). The presence of pathogenicity islands or prophages is often the only genetic difference between virulent and avirulent strains (17). We identified PAGI-1 and PAGI-2 in 5% and 4% of the isolates, respectively, and one isolate with the prophage *pf1*. PAGI-1 encodes two transcriptional regulators, which not only regulate the expression of PAGI-1, but also virulence genes that are located in the chromosome, analogous to plasmid encoded regulators of chromosome genes. It is well established that pathogenicity islands and prophages harbour virulence genes and/or TTS systems for delivery of the virulence effectors in many Gram negative bacteria (36, 37).

In conclusion, the results of this study suggest that antibiotic resistance *per se* does not appear to be associated with the production of pyocyanin and pyoverdin in

P aeruginosa. However, the ability of the isolates to produce pigment appears to be more significantly associated with MDR and expression of certain virulence factors, most notably elastase, protease, siderophore and DNase activity. There was no evidence of IMP-1 ESBLs detected in these isolates.

This study suggests that empirical therapy for non-complicated infections with *P aeruginosa* should include a carbapenem singly or in combination with another class of anti-pseudomonal antibiotic. This is the first report of genetic analysis of clinical isolates of *P aeruginosa* in Jamaica and the Caribbean region. Based on these results and others in the literature, pigmented strains of *P aeruginosa* pose a far greater threat than those that are non-pigmented. The fact that there appears to be no direct correlation between virulence factors is not surprising since any combination of virulence factors aids infection and horizontal transmission or exchange of genes. However, further studies are needed to more fully understand the actual role of these virulence factors in pathogenesis and the genetic basis for their regulation.

ACKNOWLEDGEMENTS

The authors thank the staff of the National Public Health and Central Medical Laboratories, Kingston, Jamaica, for assistance with isolate collection. Material assistance was received from the Department of Basic Medical Sciences, The University of the West Indies, Mona, Jamaica.

REFERENCES

1. Meyer JM, Stintzi A, De Vos D, Cornelis P, Tappe R, Taraz K et al. Use of siderophores to type pseudomonads: the three *Pseudomonas aeruginosa* pyoverdine systems. Microbiology 1997; **143:** 35–43.
2. Deplano A, Denis O, Poirel L, Hocquet D, Nonhoff C, Byl B et al. Molecular characterization of an epidemic clone of panantibiotic resistant *Pseudomonas aeruginosa*. J Clin Microbiol 2005; **43:** 1198–204.
3. Köhler T, van Delden C, Curty LK, Hamzehpour MM, Pechere J. Overexpression of the MexEF-OprN multidrug efflux system affects cell to cell signalling in *Pseudomonas aeruginosa*. J Bacteriol 2001; **183:** 5213–22.
4. Wagner VE, Filiatrault MJ, Picardo KF, Iglewski BH. *Pseudomonas aeruginosa*: Virulence and pathogenesis issues. In: Cornelis P. Pseudomonas: genomics and molecular biology. Caister Academic Press, Norfolk, UK 2008; 129–58.
5. Lomholt JA, Poulsen K, Kilian M. Epidemic population structure of *Pseudomonas aeruginosa*: Evidence for a clone that is pathogenic to the eye and that has a distinct combination of virulence factors. Infect Immun 2001; **69:** 6284–95.
6. Hamood AN, Griswold J, Colmer J. Characterization of elastase-deficient clinical isolates of *P. aeruginosa*. Infect Immun 1996. **64:** 3154–60.
7. Gensberg K, Hughes K, Smith AW. Siderophore-specific induction of iron uptake in *Pseudomonas aeruginosa*. J Gen Microbiol 1992; **138:** 2381–7.
8. Beatson SA, Whitchurch CB, Semmler ABT, Mattick JS. Quorum sensing is not required for twitching motility in *Pseudomonas aeruginosa*. J Bact 2002; **184:** 3598–604.
9. O'Toole GA, Kolter R. Flagellar and twitching motility are necessary for *Pseudomonas aeruginosa* biofilm development. Mol Microbiol 1998; **30:** 295–304.
10. Mah TF, O'Toole GA. Mechanisms of biofilm resistance to antimicrobial agents. Trends Microbiol 2001; **9:** 34–8.
11. Shan Z, Xu H, Shi X, Yu Y, Yao H, Zhang X et al. Identification of two new genes involved in twitching motility in *Pseudomonas aeruginosa*. Microbiology 2004; **150:** 2653–61.
12. Kiska DL, Gilligan PH. Pseudomonas. In: Manual of clinical microbiology. Volume 47. 8th edition. Edited by: Murray PR. Washington DC, ASM Press. 2003; 719–28.
13. Mercado-Blanco J, Van der Drift KMGM, Olsson PE, Thomas-Oates JE, van Loon LC, Bakker PAHM. Analysis of the *pmsCEAB* gene cluster involved in biosynthesis of salicylic acid and the siderophore Pseudomonine in the biocontrol strain *Pseudomonas fluorescens* WCS374. J Bact 2001; **183:** 1909–20.
14. Huston WM, Potter AJ, Jennings MP, Rello J, Hauser AR, McEwan AG. Survey of ferroxidase expression and siderophore production in clinical isolates of *Pseudomonas aeruginosa*. J Clin Microbiol 2004; **42:** 2806–9.
15. National Committee for Clinical Laboratory Standards: Performance Standards for Antimicrobial Susceptibility Testing; Twelfth Informational Supplement M100-S12. NCCLS, Wayne, PA, USA; 2002.
16. Arakawa Y, Shibata N, Shibayama K, Kurokawa H, Yagi T, Fujiwara H et al. Convenient test for screening metallo-β-lactamase-producing Gram-negative bacteria by using thiol compounds. J Clin Microb 2000; **38:** 40–3.
17. Finnan S, Morrissey JP, O'Gara F, Boyd EF. Genome diversity of *Pseudomonas aeruginosa* isolates from cystic fibrosis patients and the hospital environment. J Clin Microbiol 2004. **42:** 5783–92.
18. Head NE, Yu H. Cross sectional analysis of clinical and environmental isolates of *Pseudomonas aeruginosa*: Biofilm formation, virulence, and genome diversity. Infect Immun 2004; **72:** 133–44.
19. Byarugaba DK. Antimicrobial resistance in developing countries and responsible risk factors. Internat J Antimicrob Agents 2004; **24:** 105–10.
20. Sgambatti de Andrade S, Gales AC, Sader HS. Antimicrobial resistance in Gram-negative bacteria from developing countries. In: Sosa A, Byarugaba DK, Amábile-Cuevas CF, Hsueh PR, Kariuki S, Okeke IN (Eds.) Antimicrobial resistance in developing countries. Springer, NY; 2010: 249–66.
21. Poole K. Multidrug efflux pumps and antimicrobial resistance in *Pseudomonas aeruginosa* and related organisms. J Mol Microbiol Biotechnol 2001; **3:** 255–64.
22. Mavrodi DV, Bonsall RF, Delaney SM, Soule MJ, Phillips G, Thomashow LS. Functional analysis of genes for biosynthesis of pyocyanin and phenazine-1-carboxamide from *Pseudomonas aeruginosa* PAO1. J Bacteriol 2001; **183:** 6454–65.
23. Reszka KJ, O'Malley Y, McCormick ML, Denning GM, Britigan BE. Oxidation of pyocyanin, a cytotoxic product from *Pseudomonas aeruginosa*, by microperoxidase 11 and hydrogen peroxide." Free Radical Biol Med 2004; **36:** 1448.
24. Lee B, Haagensen JAJ, Ciofu O, Andersen JB, Hoiby N, Molin S. Heterogeneity of biofilms formed by nonmucoid *Pseudomonas aeruginosa* isolates from patients with cystic fibrosis." J Clin Microbiol 2005; **43:** 5247–55.
25. Lamont IL, Martin LW. Identification and characterization of novel pyoverdine synthesis genes in *Pseudomonas aeruginosa*. Microbiology 2003; **149:** 833–42.
26. Brown PD, Izundu A. Antibiotic resistance in clinical isolates of *Pseudomonas aeruginosa* in Jamaica. Rev Panam Salud Publica 2004; **16:** 125–30.
27. Tennant I, Harding H, Nelson M, Roye-Green K. Microbial isolates from patients in an intensive care unit, and associated risk factors. West Indian Med J 2005; **54:** 225–31.
28. Poole K. Aminoglycoside resistance in *Pseudomonas aeruginosa*. Antimicrob Agents Chemother 2005; **49:** 479–87.
29. Clarke RB, Janda JM, Bottone EJ. Phenotypic factors correlated with the absence of virulence among gentamicin-resistant *Pseudomonas aeruginosa* strains. J Clin Microbiol 1984; **20:** 235–8.

30. Laraki N, Galleni M, Thamm I, Riccio ML, Amicosante G, Frere JM et al. Structure of In31, a bla_{IMP}-containing *Pseudomonas aeruginosa* integron phyletically related to In5, which carries an unusual array of gene cassettes. Antimicrob Agents Chemother 1999; **43:** 890–901.
31. Fleiszig SMJ, Wiener-Kronisii JP, Miyazaki H, Vallas V, Mostov KE, Kanada D et al. *Pseudomonas aeruginosa*-mediated cytotoxicity and invasion correlate with distinct genotypes at the loci encoding exoenzyme S. Infect. Immun 1997; **65:** 579–86.
32. Wolfgang MC, Kulasekara BR, Liang X, Boyd D, Wu K, Yang Q et al. Conservation of genome content and virulence determinants among clinical and environmental isolates of *Pseudo-monas aeruginosa*. Proc Natl Acad Sci USA 203; **100:** 8484–9.
33. Dacheux D, Toussaint B, Richard M, Brochier G, Croize J, Attree I. *Pseudomonas aeruginosa* cystic fibrosis isolates induce rapid, type III secretion-dependent, but ExoU-independent, oncosis of macrophages and polymorphonuclear neutrophils. J Infect Immun 2000; **68:** 2916–24.
34. Feltman H, Schulert G, Khan S, Jain M, Peterson L, Hauser AR. Prevalence of type III secretion genes in clinical and environmental isolates of *Pseudomonas aeruginosa*. J Microbiol 2001; **147:** 2659–69.
35. Roy-Burman A, Savel RH, Racine S, Swanson BL, Revadigar NS, Fujimoto J et al. Type III protein secretion is associated with death in lower respiratory and systemic *Pseudomonas aeruginosa* infections. J Infect Dis 2001; **183:** 1767–74.
36. Ochman H. The evolution of bacterial pathogens. In: Groisman EA (ed.) Principles of Bacterial Pathogenesis. Academic Press, NY, 2000; 1–41.
37. Marcus SL, Brumell JH, Pfeifer CG, Finlay BB. *Salmonella* pathogenicity islands: big virulence in small packages. Microbes Infect 2000; **2:** 145–56.