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Heterogeneous Virulence Potential and High Antibiotic Resistance of *Pseudomonas aeruginosa* Strains Isolated from Korean Pneumonia Patients

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Pseudomonas aeruginosa **is an opportunistic human pathogen of clinical importance that causes airway infections in immunocompromised patients. Here, we report the virulence-associated characteristics of strains of *P. aeruginosa*, isolated from the sputa of 25 Korean pneumonia patients. A high degree of genomic plasticity was observed by random amplified polymorphic DNA genotype analysis, suggesting that the infections were caused by strains with diverse genomic backgrounds. Biofilm formation of each isolate was heterogeneous in terms of their relative motilities. In addition, 48% of isolates were defective in the production of 3-oxo-C_{12}-HSL (PAI-1), a quorum sensing signal molecule. In these strains, PAI-1-dependent elastase production was correspondingly decreased, suggesting that a large number of strains were presumed to be quorum sensing deficient. Multidrug resistance (MDR) was seen in 56% of the isolates tested, and 44% of the MDR strains were resistant to five or more antibiotics. Taken together, our results provide additional insights into the virulence traits of *P. aeruginosa* clinical isolates, which will aid in treating *P. aeruginosa* infections in pneumonia patients.**

Keywords: *P. aeruginosa*, virulence, pneumonia, biofilm, quorum sensing

Hospital-acquired pneumonia continues to be a serious healthcare problem, despite the availability of effective antibiotics. *Pseudomonas aeruginosa* is a common human opportunistic pathogen that is associated with nosocomial pneumonia (El Solh and Alhajhusain, 2009). Because of its intrinsic resistance to many antimicrobial agents and sophisticated virulence mechanisms, pneumonia patients infected with *P. aeruginosa* develop more severe pathological symptoms than patients with other types of pneumonia (Kurahashi *et al.*, 1999).

P. aeruginosa is a highly adaptable bacterium that can colonize harsh environmental niches, including the human airway (Yoon and Hassett, 2004). Phenotypic changes in *P. aeruginosa* resulting in greater resistance are thought to occur as a result of active responses to host immune attack (Mathee *et al.*, 1999; Leid *et al.*, 2005) and/or antibiotic treatment (Zhanel *et al.*, 1995; Mah *et al.*, 2003). Moreover, phenotypic improvement to a more recalcitrant form can also be achieved by (i) genetic mutations that lead to an alginate overproducing mucoid form (Mathee *et al.*, 1999) or (ii) formation of a sessile community known as biofilm, a process dependent on quorum sensing (Whiteley *et al.*, 2001; Yoon and Hassett, 2004; de Kievit, 2009). Therefore, in-depth knowledge of the selective pressures that direct the adaptation of this clinically important pathogen and precise analysis of the phenotypic adjustments that occur in the human airway are needed to develop a better strategy for treating *P. aeruginosa* infections.

The pathogenesis of *P. aeruginosa* infection depends on multiple cell-associated or extracellular virulence factors (Doring, 1987; Tingpej *et al.*, 2007; Kobayashi *et al.*, 2009). Virulence determinants associated with motility, toxin production, invasion, and secretion are usually necessary for acute infections, while persistence factors such as biofilm growth, anaerobic metabolism (Hassett *et al.*, 2002), and antibiotic resistance are needed to establish chronic infection (Yoon and Hassett, 2004). Among the diverse virulence determinants, biofilm formation, and antibiotic resistance are of particular interest for chronic airway infections, because *P. aeruginosa* biofilm is more resistant to antimicrobial treatment than the free-living form (Mah *et al.*, 2003).

Although *P. aeruginosa* infections pose a large threat to public healthcare in Korea, no detailed, molecular-based epidemiological study has been reported. This study was undertaken to gain a global insight into the virulence-associated genotypic and phenotypic features of clinical *P. aeruginosa* strains, isolated from Korean pneumonia patients. Our results provide a reference point to compare virulence traits of Korean *P. aeruginosa* isolates from other clinical or non-clinical strains, thereby allowing us to develop a better treatment strategy.

Materials and Methods

Bacterial isolates

Sputum isolates of *P. aeruginosa* were collected in 2009 from hospitalized patients diagnosed with pneumonia in the Yonsei University Severance Hospital and Kyungpook National University Hospital in Korea. Each isolate was cultivated on Pseudomonas Isolation agar (PIA) plate and identified as *P. aeruginosa* using the ATB 32 GN system (bioMérieux, France). For further verification,

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16S rRNA gene sequencing was performed using a subset of strains and GenBank nr database search revealed query sequences clearly matched with sequences derived from *P. aeruginosa*. Patient information is summarized in Table 1.

DNA fingerprint analysis

Clinical isolates were genotyped by random amplified polymorphic DNA (RAPD) fingerprinting, as described elsewhere (Mahenthiralingam *et al.*, 1996; Campbell *et al.*, 2000). Genomic DNA was extracted by G spin kit (Invitrogen, Korea) according to the manufacturer's instructions. A dendrogram was generated with XLSTAT software (Addinsoft USA, USA) based on the unweighted pair group method using arithmetic algorithm (UPGMA).

Colony morphology

The colony morphology of each isolate was examined after 24 h on LB plates and classified into six groups following the guidelines described elsewhere (Kirisits *et al.*, 2005). The mucoid phenotype of each isolate was visually inspected, and designated as negative (–) or positive (+).

Motility and biofilm assay

Swimming, swarming, and twitching motilities were determined as described previously (Denervaud *et al.*, 2004; Winstanley and Fothergill, 2009). The ability of *P. aeruginosa* to form *in vitro* biofilms was quantified as described previously (O'Toole and Kolter, 1998).

Assays for secretory virulence factors

Production of pyocyanin and elastase were measured following procedures described elsewhere (Azghani *et al.*, 2000; Denervaud *et al.*, 2004).

Quantification of a QS signal

The production of the QS signal molecule, 3-oxo-C_{12}-HSL (PAI-1) was assessed with monitor strain *Escherichia coli* DH5α harboring pKDT17, which carries *lasR* under the control of the *lac* promoter, and a *lasB-lacZ* reporter (Pearson *et al.*, 1994). Filter-sterilized culture supernatant from each isolate was added to the monitor strain culture in mid-logarithmic phase as an exogenous source of PAI-1 to induce the production of β-galactosidase. β-Galactosidase assays were performed as described previously (Pearson *et al.*, 1994).

Antibiotic susceptibility test

The antibiotic susceptibility of the isolates was determined by the disk diffusion method on Muller-Hinton (MH) agar plates (Difco, USA). The antibiotics tested were tobramycin (10 μg; Oxoid Ltd., UK), ciprofloxacin (5 μg), ticarcillin (75 μg), ceftazidime (30 μg), imipenem

Table 1. Morphological features of *P. aeruginosa* recovered from the septum of pneumonia patients

Isolate[a]	Gender[b]	Age (yr)	Morphology of colony on LB plate	Pyocyanin (U)[c]	Mucoid type[d]
Y06	M	57	Green, rough, opaque, undulate	1.04	-
Y09	M	69	White, smooth, glossy, undulate	0.33	-
Y25	M	2	Green, rough, translucent, entire	0.51	-
Y31	F	58	Green, smooth, glossy, undulate	0.21	-
Y33	M	34	Green, rough, translucent, curled	0.61	-
Y38	M	2	White, smooth, glossy, undulate	ND	-
Y46	M	85	White, smooth, glossy, entire	ND	-
Y47	M	72	White, smooth, glossy, entire	0.26	-
Y59	F	77	Green, smooth, glossy, entire	0.75	-
Y64	F	43	White, smooth, glossy, entire	0.42	-
Y71	M	70	White, smooth, glossy, entire	0.22	-
Y73	M	58	Green, smooth, glossy, undulate	0.43	-
Y82	M	58	Green, smooth, glossy, undulate	0.94	-
Y89	M	69	White, smooth, glossy, undulate	ND	-
Y94	F	80	White, smooth, glossy, undulate	ND	-
K05	M	66	Green, smooth, glossy, undulate	0.57	-
K15	M	69	Green, rough, translucent, undulate	0.25	-
K43	M	41	Green, smooth, opaque, undulate	0.52	-
K46	F	67	White, smooth, opaque, entire	0.21	+
K48	M	55	White, rough, translucent, undulate	ND	-
K57	F	0	Green, smooth, glossy, entire	0.27	+
K59	M	83	Green, smooth, glossy, entire	0.27	-
K60	M	44	Green, smooth, glossy, entire	0.26	+
K62	F	0	White, smooth, glossy, entire	ND	+
K69	F	73	White, rough, translucent, entire	0.30	-
PAO1			Green, smooth, glossy, entire	0.54	-

[a] The isolates were collected from patients diagnosed with pneumonia and hospitalized in the Yonsei University Severance Hospital and the Kyungpook National University Hospital in 2009, and they were named Y and K respectively.
[b] F, female; M, male
[c] Pyocyanin was quantified by absorbance at 520 nm. One U was arbitrarily defined as a OD_{520nm} of 1.0. The detection limit was 0.2. ND, not detectable.
[d] +, mucoid; -, nonmucoid.

(10 μg), polymixin B (300 units), and penicillin/tazobactam (110 μg). The minimal inhibitory concentration (MIC) of selected antibiotics was determined as described previously (Wong *et al.*, 1997). Results were interpreted using Clinical Laboratory Standards Institute (CLSI) guidelines (CLSI, 2009).

Statistics

Statistical analysis was performed using Student *t*-test. Regression analysis was performed using SigmaPlot (Systat Software Inc., USA) and Microsoft Excel software.

Results and Discussion

RAPD genotypes

To compare genome-wide polymorphic profiles of isolates, we performed RAPD genotyping. To better appreciate the genetic distances between isolates, a dendrogram was constructed by the software-aided gel image analysis. As shown in Fig. 1B, a high degree of genetic diversity was revealed in our RAPD analysis. Among 25 strains, four groups were identified and each group contained a pair of strains with identical RAPD genotype (Y64 and Y71; Y09 and Y25; Y73 and Y82; K05 and K43, please note thick solid lines in the y-axis of Fig. 1B). This result demonstrates that (i) although clinical isolates were collected from patients hospitalized in two local clinics, isolates are of divergent RAPD genotypes, and thus (ii) patients were not colonized with clonally expanded strains by patient-to-patient spread. These findings are similar with those of a previous study, in which 131 distinct RAPD types were identified in 200 *P. aeruginosa* isolates (Campbell *et al.*, 2000).

Morphological features of *P. aeruginosa* isolates

Next, we sought to categorize the isolates based on colony morphology (Table 1). Of the tested strains, only four pneumonia isolates were initially mucoid (Table 1), and this is

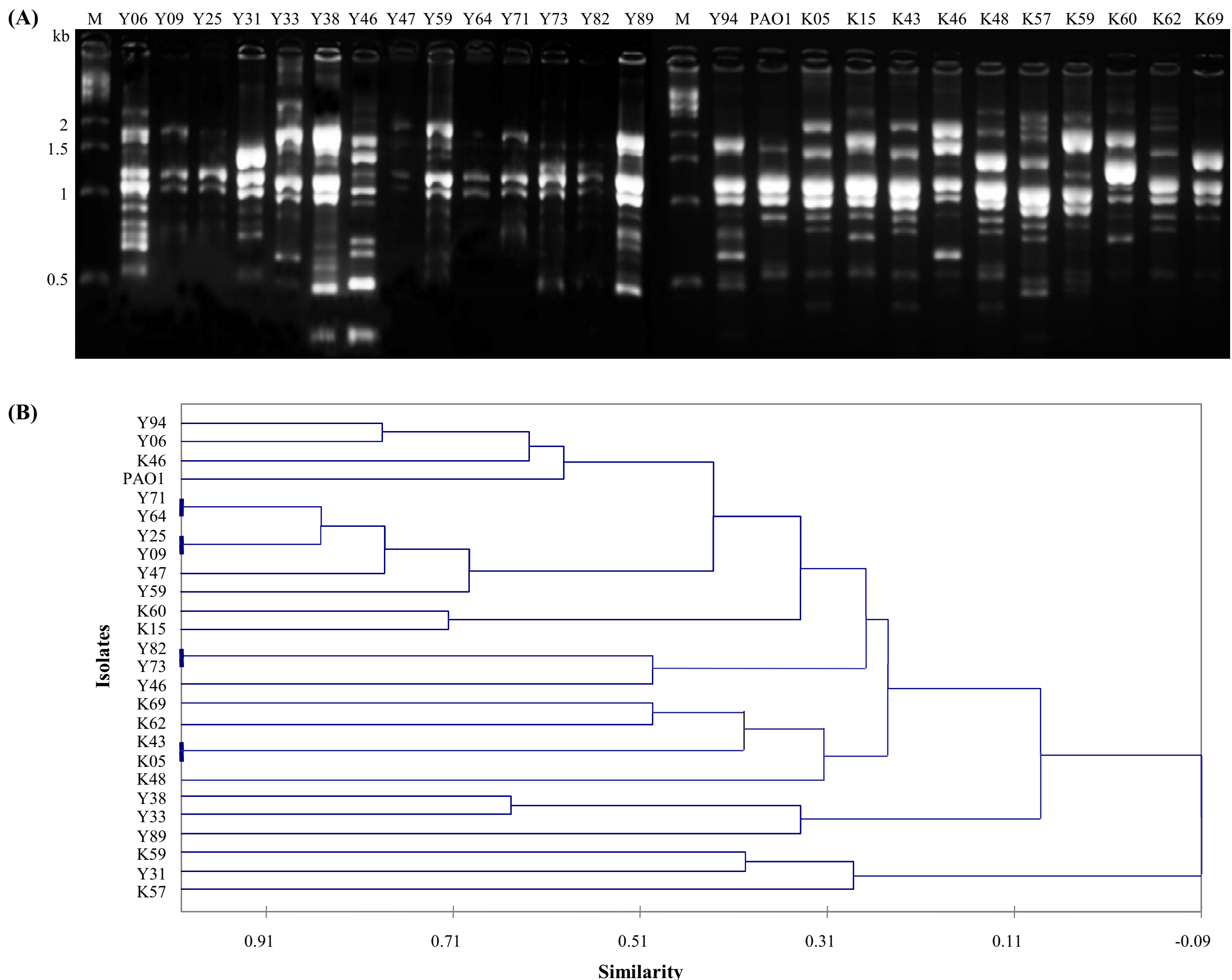


Fig. 1. Random amplified polymorphic DNA (RAPD) typing of *P. aeruginosa* clinical isolates. (A) Agarose gel of PCR products. PCR was performed as described in 'Materials and Methods'. Molecular weight marker (1 kb) was run in lane M. (B) Dendrogram was constructed by the XTSTAT software based on the unweighted pair group method using arithmetic algorithm (UPGMA). Thick solid lines on the y-axis indicate pairs of strains with identical RAPD genotypes.

in contrast to chronic cystic fibrosis (CF) patient isolates, a majority of which were mucoid (Yoon *et al.*, 2006). However, as subculturing proceeded, all reverted to the nonmucoid phenotype, suggesting that the alginate-producing trait of these isolates is not stable. Of our isolates, 36% showed a white, smooth, and glossy morphology, while a comparable number (32%) were green, smooth, and glossy. A clear positive correlation existed between pyocyanin production and green colony formation. A total of twelve isolates that formed white colonies produced either no pyocyanin (6 isolates) or a level of pyocyanin lower than that produced by the reference strain PAO1 (6 isolates).

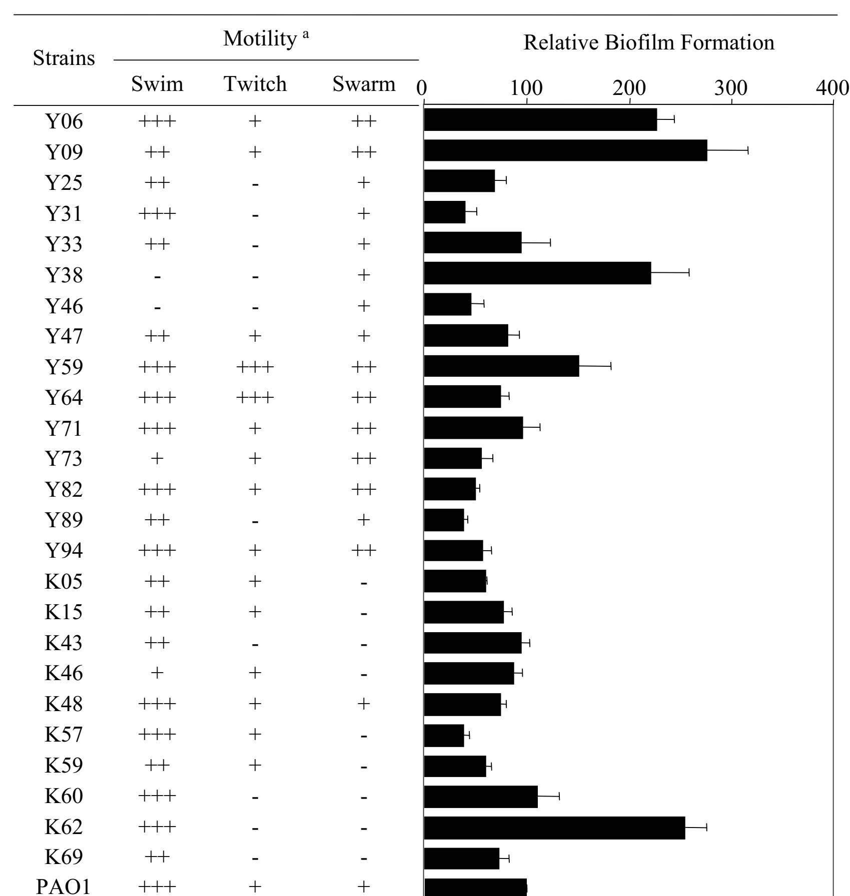
Motility and biofilm formation of the isolates

Then, we assessed motility and biofilm formation of the isolates. These two phenotypic features were co-evaluated because biofilm formation was reported to be dependent on motility (O'Toole and Kolter, 1998). We measured three types of motilities: swimming, swarming, and twitching. As shown in Fig. 2, most isolates exhibited swimming motility comparable to PAO1, with only two strains (Y38 and Y46) showing no swimming motility. The isolates showed varying degrees of twitching and swarming motilities (Fig. 2), however none had lost all three types of motility. Next we examined the biofilm forming capability of the isolates. As shown in the bar graphs (Fig. 2, right), all strains formed biofilms *in vitro*, although with a somewhat reduced capacity than PAO1. Six isolates formed a more robust biofilm than PAO1 under the same growth conditions.

Motility is an important determinant governing biofilm formation in *P. aeruginosa* (O'Toole and Kolter, 1998). The pneumonia patient isolates tested in this study, however, displayed heterogeneous biofilm-forming capability with respect to their motility and thus, no clearly defined correlation was observed between these two virulence-associated phenotypes. Interestingly, the Y38 strain was defective in motility, but formed more robust biofilm than PAO1. Consistent with this finding, a recent study by Rampioni and colleagues demonstrated that a *P. aeruginosa rsaL* mutant formed significantly impaired biofilm while it displayed increased swimming and twitching motility (Rampioni *et al.*, 2009). Further investigation using this particular strain may lead us to uncover a motility-independent mechanism for biofilm formation.

Production of elastase and PAI-1

Elastase, production of which has been reported to be controlled mainly by *lasI-lasR* QS system (Pearson *et al.*, 1997), is one of the most abundant proteins in the culture supernatant



Strains	Motility [a]		
	Swim	Twitch	Swarm
Y06	+++	+	++
Y09	++	+	++
Y25	++	-	+
Y31	+++	-	+
Y33	++	-	+
Y38	-	-	+
Y46	-	-	+
Y47	++	+	+
Y59	+++	+++	++
Y64	+++	+++	++
Y71	+++	+	++
Y73	+	+	++
Y82	+++	+	++
Y89	++	-	+
Y94	+++	+	++
K05	++	+	-
K15	++	+	-
K43	++	-	-
K46	+	+	-
K48	+++	+	+
K57	+++	+	-
K59	++	+	-
K60	+++	-	-
K62	+++	-	-
K69	++	-	-
PAO1	+++	+	+

Fig. 2. Motility and biofilm formation by the *P. aeruginosa* isolates. The three different motilities are indicated qualitatively. [a] +++, motility zone ≥20 mm; ++, motility zone ≥10 mm and ≤20 mm; +, motility zone ≤10 mm; -, motility zone=0 mm. Quantitative biofilm formation of each isolate was normalized against the control PAO1. Mean±standard deviation (SD) is presented for each biofilm.

of *P. aeruginosa* (data not shown). Exhibiting a broad spectrum of substrate specificity, *P. aeruginosa* elastase degrades (i) structural host components, such as elastin (Moneto de Ledesma *et al.*, 1985) and collagen (Heck *et al.*, 1986) and (ii) immune-related host proteins including immunoglobulins (Diebel *et al.*, 2009), airway lysozyme (Jacquot *et al.*, 1985), complement components (Schultz and Miller, 1974), and bronchial mucus protease inhibitor (Johnson *et al.*, 1982). Therefore, elastase not only destroys tissue components but also interferes with host defense mechanisms. Studies in animal models demonstrated that mutants defective in elastase production are less virulent than their parental strains, further supporting the role of elastase as a critical virulence factor (Azghani *et al.*, 2000).

To gain an insight into the QS capability of the clinical isolates, we quantified the level of elastase produced by each (Fig. 3, gray bars). Although most were capable of producing elastase, 21 strains produced a lower level of elastase than PAO1. Only four (Y47, Y59, Y64, and K46) produced higher levels of elastase during overnight growth in LB.

Next, we analyzed the correlation between elastase activity and the level of PAI-1, which regulates its production. As shown in Fig. 3 (black bars), 13 strains produced PAI-1 at either comparable or higher levels than PAO1. The other 12 strains produced very low or undetectable levels of PAI-1. Of these 12 strains, 10 strains (Fig. 3, black asterisks) also produced very low levels of elastase, further demonstrating the positive correlation between these two virulence traits.

As shown in Table 1, 76% of the isolates were positive for pyocyanin production, an event that is known to be dependent on *rhlI-rhlR* system, another arm of *P. aeruginosa* QS (Bratu *et al.*, 2006). Importantly, four (Y38, Y46, K48, and K62) out of six isolates that were negative in the pyocyanin assay (Y38, Y46, Y89, Y94, K48, and K62) also produced considerably decreased levels of both elastase and PAI-1 than PAO1, suggesting that the QS machinery in these strains may be impaired. Consistent with this finding, recent reports demonstrated that a large number of isolates recovered from the airways of chronic CF patients harbored mutations in *lasR* gene (Hoffman *et al.*, 2009, 2010).

The extent to which the lack of elastase secretion affects the overall virulence or survival rate within the host airway is not clearly defined. Interestingly, elastase-positive strains are more frequently isolated from patients with acute *P. aeruginosa* infections than from chronically infected patients (Woods *et al.*, 1986; Lee *et al.*, 2005). Furthermore, the mucoid *P. aeruginosa* isolates that are routinely recovered from chronic CF patients are reported to secrete less elastase than nonmucoid strains (de Kievit, 2009). This suggests that the production of elastase may decrease as chronicity increases and thus, the detection of elastase activity could serve as a biomarker that reflects the degree of chronicity. The strains used in our study produced a varying range of elastase. Y64 produced ~7.5 fold more than PAO1, while the level was minimal in the culture supernatant of strains Y38 and Y46 (Fig. 3). Unfortunately, the duration for which patients have been infected with each of the strains is not known. Further study is needed to assess the effects of the chronicity on the elastase-dependent bacterial virulence traits.

Of note, two hyper-elastase producers, Y64 and K46 produced below-average levels of pyocyanin, suggesting that *lasI-lasR* and *rhlI-rhlR* QS systems may work in a distinct manner. This notion was supported in a recent report by Dênervaud and colleagues (Denervaud *et al.*, 2004), where a chromosomal insertion of *lasR* gene into QS-null patient isolates restored elastase production, but not pyocyanin production. In the same work, pyocyanin production was restored by the insertion of the *rhlR* gene alone.

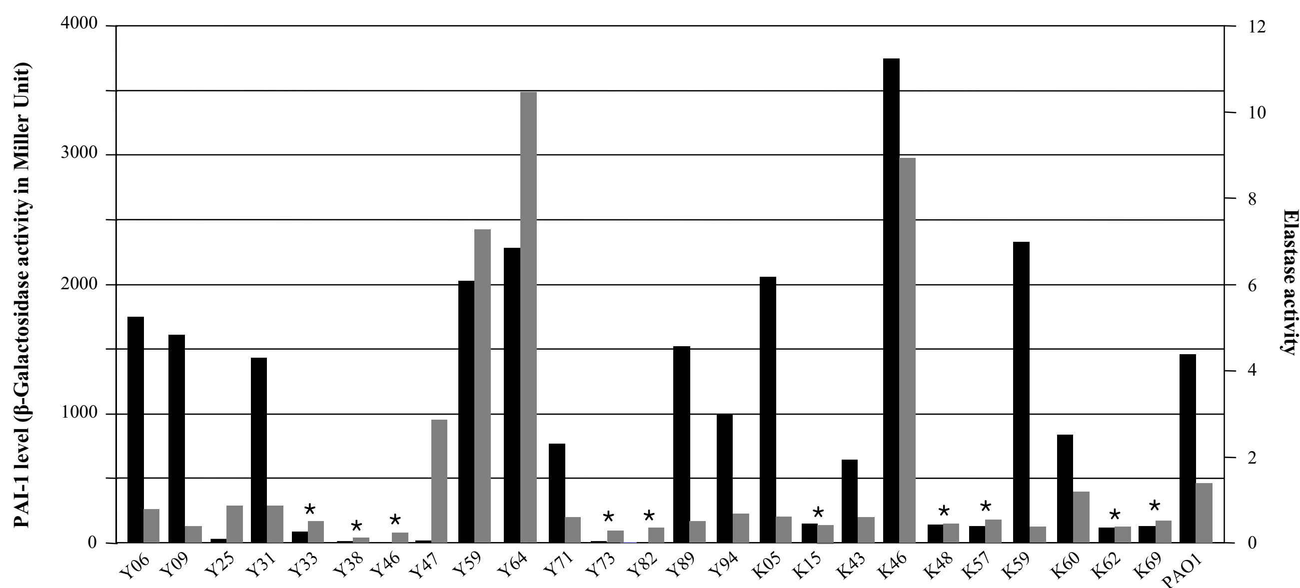


Fig. 3. Elastase and PAI-1 production for each *P. aeruginosa* isolate. Levels of PAI-1 and elastase in the culture supernatant of each isolate are plotted together. PAI-1 level was quantified using a β-gal reporter assay and is presented in Miller units (black bars). Elastase activity shown in gray bars was measured as described in 'Materials and Methods'. One U was arbitrarily defined as OD_{495nm} of 1.0. Results are representative of three independent assays with similar results.

Table 2. Antibiotic susceptibility of *P. aeruginosa* isolates

Strains	Resistance to antibiotics[a]	MIC range (mg/L)[b]	
		Tobramycin	Ciprofloxacin
Y06	-	4	0.5
Y09	TM, CIP, CAZ, IPM, TZP, TIC	256	32
Y25	CAZ, PB	4	0.5
Y31	-	2	0.5
Y33	TM, CIP, IPM, TIC	64	32
Y38	CAZ, IPM, TZP, TIC	2	0.5
Y46	CAZ, TIC	4	0.5
Y47	CIP, TZP, TIC	4	>256
Y59	-	4	0.5
Y64	TZP, PB	4	0.5
Y71	TM, CIP, IPM, TZP, TIC	256	16
Y73	TM, CIP, IPM, TZP, TIC	>512	4
Y82	TM, CIP, IPM, TZP, TIC	256	4
Y89	TM, CIP , IPM, TZP, TIC	>512	16
Y94	CIP	4	4
K05	CAZ	4	2
K15	TM, IPM, TIC	16	2
K43	CIP	4	16
K46	-	4	1
K48	CIP, TZP	4	16
K57	CIP, IPM	4	8
K59	TM, CIP, CAZ, TZP, TIC	128	32
K60	TM, CIP, TZP	128	16
K62	-	4	0.5
K69	-	4	2
PAO1	-	1	0.5

[a] Antibiotic resistance was determined by agar diffusion method according to CLSI guidelines.
[b] MIC was determined by broth micro-dilution method.
Drug abbreviations: TM, tobramycin; CIP, ciprofloxacin; CAZ, ceftazidime; IPM, imipenem; TZP, tazobactam; TIC, ticarcillin; PB, polymixin B.

Antibiotic resistance

Finally, we examined the antibiotic resistance of the isolates. Antibiotic resistance is arguably one of the most important virulence features of clinically isolated *P. aeruginosa*. Over 76% of the isolates showed resistance to more than one antimicrobial agent currently in use to combat *P. aeruginosa* infections (Table 2). Importantly, six isolates (Y09, Y71, Y73, Y82, Y89, and K59) were highly resistant to five different antibiotics, by disk diffusion assay (Table 2). We also determined MIC values for two selected antibiotics, tobramycin, and ciprofloxacin. MIC assay results were in accordance with resistance profiles determined by disk diffusion (Table 2). Interestingly, the six MDR isolates exhibited a high level of resistance, especially to tobramycin. Isolate Y47 showed high level resistance to ciprofloxacin (MIC>256), but was not resistant to tobramycin. A group of only six isolates and the reference strain PAO1 were susceptible to all antimicrobial agents, as tested by both disk-diffusion assay and MIC test.

P. aeruginosa is an important nosocomial pathogen in patients with significant underlying disease, and colonization is frequently selected by broad-spectrum antimicrobial usage. Our results shows that Korean isolates, similar to clinical strains isolated in other regions of the world, have also acquired MDR. Importantly, the *P. aeruginosa* isolates in this study showed a higher level of antibiotic resistance than in previous reports (Wolter *et al.*, 2009). Whether region-specific or race-specific determinants influence this problematic phenotypic change is unknown. Our results also demonstrate that a subset of MDR strains exhibited a high level of resistance to tobramycin (Table 2). Further investigation is necessary to clearly understand whether acquired resistance to tobramycin can render subsequent resistance to other types of antibiotics. It is also of particular importance to examine if (i) there is an integrative pathway leading to the resistance to a wide range of diverse antibiotics or (ii) multi-drug resistance is achieved after acquiring individual genetic change for each antibiotic resistance. Moreover, two isolates Y25 and Y64 were resistant to polymyxin B at the chosen breakpoint of 300 units. Being a cyclic and positively charged peptide antibiotic, polymyxin B is considered to be the last resort for the treatment of MDR *P. aeruginosa* pneumonia (Zaborina *et al.*, 2006).

Correlation between RAPD genotypes with tested phenotypes

Determining if strain genotype reflects phenotypic characteristics is important, because identification of a distinct genotype might be useful for predicting corresponding phenotypes. Our RAPD analysis revealed four pairs of strains with identical genotypes (Fig. 1). As summarized in Table 3, similar, although not identical, patterns of antibiotic resistance, biofilm formation and elastase/PAI-1 production were observed in the strains of each group. Specifically, Y82 and Y73 in group C produced negligible amount of elastase and PAI-I, while strains in group D produced comparable levels of elastase and PAI-1. This suggests that strains in a given genotypic group match well with most phenotypic characteristics.

Conclusion

This report provides the first insight into the virulence-associated characteristics of *P. aeruginosa* sputum isolates recovered from Korean pneumonia patients. Our preliminary

Table 3. Phenotype patterns of the strains with identical RAPD genotypes

Groups	Strains	Antibiotics resistance	Elastase production	PAI-1 level	Biofilm formation
A	Y71	+++++	+	++	++
	Y64	++	++++++	++++	++
B	Y25	+++	+	-	++
	Y09	++++++	+	+++	++++
C	Y82	+++++	-	-	+
	Y73	+++++	-	-	+
D	K43	+	+	++	+++
	K05	+	+	++++	++

Phenotypes of strains are qualitatively displayed.

investigation revealed following findings; (i) 25 strains, isolated from two local hospitals, exhibited a high level of genome plasticity, (ii) strains also exhibited heterogeneous virulence-associated phenotypes, (iii) strains' capability to form biofilm was not critically dependent on their motility, (iv) a relatively large portion of strains were impaired in the production of QS-controlled virulence determinants, and (v) a high level of antibiotic resistance was observed in most isolates. These findings further proved that *P. aeruginosa*, as a versatile organism, can employ a variety of mechanisms to maximize its survival fitness in the patient's airway. A better understanding of fundamental mechanisms by which *P. aeruginosa* acquires phenotypic and genotypic diversity will allow us to further develop evidence-based clinical guidelines for the management of pneumonia caused by *P. aeruginosa*.

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