Pol. J. Environ. Stud. Vol. 23, No. 6 (2014), 2327-2331

Short Communication

Molecular Detection of Metallo-β-Lactamase and Putative Virulence Genes in Environmental Isolates of *Pseudomonas* Species

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Received: 7 October 2013
Accepted: 17 February 2014

Abstract

The aim of this study was to investigate the presence of metallo-β-lactamase (*Imp-1*, *Vim-1*, *Vim-2*) -resistant genes and putative virulence genes (*ExoS*, *Apr*, *LasB*, *PhzM*) in some environmental isolates of *Pseudomonas* species recovered from freshwater, wastewater, cultivated soil, plant root, and plant rhizosphere samples. Polymerase chain reaction (PCR) was employed to evaluate the presence and established metallo-β-lactamase and virulence genes using specific primer sets. About 6.7% of the *Pseudomonas* isolates from wastewater treatment plant were found to harbour the cytotoxin gene, while 20%, 20.6%, and 6.7% of the isolates were positive for alkaline protease, phenazine and *Vim-2* genes, respectively. For the *Pseudomonas* species isolated from plant rhizosphere, 14.3% were positive for phenazine gene, while 10.7% were positive for *Vim-2* gene. Dissemination of the target genes were in the following pattern: all isolates that harboured *ExoS*, *Apr*, and *Vim-2* genes from wastewater sample were found to be *Pseudomonas putida,* while phenazine gene was found in 75% of *Pseudomonas putida* isolates and 25% of other *Pseudomonas* species. All plant rhizosphere isolates harbouring phenazine and *Vim-2* genes were observed to be *Pseudomonas putida* species. The incidence of *Vim-2* and virulence genes in some environmental isolates of *Pseudomonas* species suggest that these isolates are reservoirs of metallo-β-lactamase-resistant genes and are potential pathogens that may be of serious public health significance.

Keywords: wastewater, plant rhizosphere, phenazine, cytotoxin, *Pseudomonas putida*

Introduction

The pseudomonads are Gram-negative, oxidase positive organisms ubiquitous in diverse ecological niches including water, soil, and rhizosphere environment. *Pseudomonas* species cause several infections among which include cystic fibrosis. Carbapenems are broad-spectrum antibiotics that are often used in the treatment of such infections [1]. The emergence of metallo-β-lactamases (MBLs) of the *Imp* or *Vim* type among Gram-negative non-fermenting bacteria is alarming due to its increased frequency, and represents an epidemiological risk as these enzymes confer resistance not only to carbapenems, but to almost all β-lactams [2, 3]. Furthermore, bla_{Imp} and bla_{Vim} are usually carried on integrons in association with aminoglycoside-resistant cassettes. These mobile elements can easily spread horizontally between different bacteria species due to their association with transposons or plasmids [2, 4, 5]. Currently the *Vim*-type enzymes comprise the second most dominant group of β-lactamases, and have been reported in different bacteria species from 23 countries worldwide [3, 6].

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The pathogenesis of *Pseudomonas* is partly due to the production of both extracellular and cell-associated virulence factors [7]. Extracellular factors include hydrogen cyanide, proteases, and elastase. Proteases are considered to play a role in the pathogenesis of some *Pseudomonas aeruginosa* infections. Elastase is a metalloprotease that reduces elastin and collagen and deactivates human immunoglobulin G, serum alpha-1, proteinase inhibitor, and several complementary components [8]. One of the factors that regulates the production of elastase protein in *Pseudomonas* is its growth rate. More elastase is produced when cells are at the late-logarithmic phase of growth or when cell density is high [7, 9]. Cell-associated virulence factors include flagella, lipopolysaccharide, pili, type III system effector proteins, a type III secretion system and alginate. At present, four type III effector proteins have been identified in *P. aeruginosa* and include *ExoS*, *ExoU*, *ExoT*, and *ExoY* [7]. Intoxication with *ExoS*, *ExoY*, and *ExoT* causes cell rounding and detachment and may play a role in causing infection by preventing or inhibiting bacterial uptake and phagocytosis [7, 10, 11]. Secreted products include phenazines. Over 90% of *P. aeruginosa* isolates produce pyocyanin [12], and high concentrations of pyocyanin present in the sputa of cystic fibrosis patients suggests that this compound plays a role in pulmonary tissue damage observed in chronic lung infection [13].

Many studies have focused on characterization of the virulence diversity and cabapenem resistance in clinical isolates of *Pseudomonas* species and with emphasis on *P. aeruginosa*; but there are limited studies on virulence factors distribution and cabapenem resistance in environmental isolates of *Pseudomonas* species. This study was initiated to investigate some virulence gene distribution in environmental isolates of *Pseudomonas* species and also to determine the incidence of metallo-β-lactamase genes in the bacteria.

Table 1. Primer sequence used in the study.

Target gene	Primer	Sequence 5'-3'	Reference
Cytotoxin	*ExoS* F	ATCCTCAGGCGTACATCC	[7]
	ExoS R	ACGACGGCTATCTCTCCAC	
Elastase	*LasB* F	ACAGGTAGAACGCACGGTTG	[7]
	lasB R	GATCGACGTGTCCAAACTCC	
Alkaline protease	*Apr* F	TGTCCAGCAATTCTCTTGC	[7]
	Apr R	CGTTTTCCACGGTGACC	
Phenazine	*PhzM* F	ATGGAGAGCGGGATCGACAG	[7]
	PhzM R	ATGCGGGTTTCCATCGGCAG	
Vim-1	*Vim-1* A	TCTACATGACCGCGTCTGTC	[28]
	Vim-1 B	TGTGCTTTGACAACGTTCGC	
Vim-2	*Vim-2* A	ATGTTCAAACTTTTGAGTAGTAAG	[28]
	Vim-2 B	CTACTCAACGACTGAGCG	
Imp-type gene	*Imp-1* A	CTACCGCAGCAGAGTCTTTGC	[28]
	Imp-1 B	GAACAACCAGTTTTGCCTTACC	

Materials and Methods

Sample Collection and Processing

Water samples were collected from the Kat and Tyume rivers in the Eastern Cape Province, South Africa. Also, samples were collected from two wastewater treatment plants in Alice and Fort Beaufort in the Eastern Cape Province. The water samples were processed as described elsewhere [14]. Soil samples (butternut, spinach, cabbage, and maize-cultivated soil), plant roots and rhizosphere samples (spinach, cabbage, and grass) were also collected from Lovedale farms in Eastern Cape Province. Samples were immediately transported in cooler boxes to the laboratory for processing and analyses as described by Igbinosa et al. [15].

Isolation of Genomic DNA

Isolation of genomic DNA from the bacterial isolates was done following the description of Igbinosa et al. [14, 15]. Briefly, single colonies of the identified *Pseudomonas* species grown overnight at 37ºC for 24 h on Nutrient agar plates were picked, suspended in 500 µl of sterile Milli-Q PCR grade water (Merck, SA), and the cells were lysed using Dri-block DB.2A (Techne, SA) for 10 min at 100ºC. The cell debris was removed by centrifuge at 11,000 × g for 5 min. using a MiniSpin micro centrifuge (Merck, SA) and the supernatant used directly as template DNA for the PCR reaction or stored at -80ºC until ready for use.

Genetic Detection of Metallo-β-Lactamases and Virulence Genes in *Pseudomonas* Species

The primer sets used in this study are as shown in Table 1. The PCR condition for the detection of metallo-β-lactamases was as follows; 25 cycles of denaturation at 94ºC for 50s, annealing at 55ºC for 60s, extension at 72ºC for 90s, and a final extension step of 72ºC for 5 min. The PCR condition for the detection of virulence genes were as follows: *ExoS* gene (an initial denaturation step at 96ºC for 5 min, followed by 30 cycles of denaturation at 94ºC for 30s, primer annealing at 47-63ºC, and 4 min of primer extension at 72ºC); *Las B* gene (96ºC for 5 min, 94ºC; 30s, 57ºC; 30s,

Table 2. Percentage distribution of metallo-β-lactamases and virulence genes among group sources.

Isolate source	Target gene (%)						
	Cytotoxin	Elastase	Alkaline protease	Phenazine	Vim-1	Vim-2	Imp-type gene
Fresh water	0	0	0	0	0	0	0
Wastewater	6.7	0	20	26.7	0	6.7	0
Plant root	0	0	0	0	0	0	0
Plant rhizosphere	0	0	0	14.3	0	10.7	0
Cultivated soil	0	0	0	0	0	0	0

Table 3. Distribution of virulence gene and *Vim-2* gene among *Pseudomonas* species.

Species	Target gene distribution (%)			
	Cytotoxin	Alkaline protease	Phenazine	Vim-2
Wastewater				
P. putida	100%	100%	75%	100%
Other *Pseudomonas* spp	-	-	25%	-
Plant Rhizosphere				
P. putida	-	-	100%	100%

72ºC; 30s for 30 cycles, final extension 72ºC for 5 min); *Apr* gene (95ºC for 5 min, 94°C for 30s, 51ºC for 30s, 72ºC for 3 min, 72ºC for 3 min); and *PhzM* gene (96ºC for 5 min, 30 cycles at 94ºC for 30s, 51ºC for 30s, 72ºC for 3 min, 72ºC for 8 min).

Results

Pseudomonas isolates used in this study were previously isolated, characterized to species level, and the antibiogram study carried in our previous studies [14, 15]. In this study, a total of 46 *Pseudomonas* isolates were investigated of which 15 were from wastewater, 2 from fresh water, 1 from plant root, 5 from cultivated soil, and 28 from plant rhizosphere. Metallo-β-lactamases and virulence genes were detected only in some *Pseudomonas* isolates from wastewater and plant rhizosphere. The proportions of the isolates from the wastewater sources harbouring the target genes are in the following order: cytotoxin gene (6.7%), alkaline protease gene (20%), phenazine gene (26.7%), and *Vim-2* gene (6.7%) (Table 2), while those isolated from plant rhizosphere followed the order: phenazine gene (14.3%) and *Vim-2* gene (10.7%) (Table 2). Elastase, *Vim-1* and *Imp*-type genes were not detected in any of the isolates in this study. The species that harboured these target genes were encapsulated from data of previous studies [14, 15] and were found to be distributed as follows: all isolates that harboured cytotoxin, alkaline protease, and *Vim-2* genes from wastewater sample were found to be *P. putida,* while 75% of the isolates that were positive for phenazine gene belong to *P. putida* and the remaining 25% that were positive for the phenazine gene belonged to other *Pseudomonas* spp. (Table 3). All isolates that harboured phenazine and *Vim-2* gene from plant rhizosphere source were *P. putida* species as shown in Table 3.

Discussion

The presence of putative virulence genes and the metallo-β-lactamase gene in environmental isolates of *Pseudomonas* species were investigated in this study. Among the three metallo-β-lactamase (*Imp*, *Vim-1*, *Vim-2*) genes screened, only the $bla_{\text{Vim-2}}$ gene was detected. Studies have demonstrated the presence of $bla_{\text{VIM-2}}$ in clinical isolates from *Pseudomonas* species. The first description of $bla_{\text{Vim-2}}$ gene was a clinical isolate of *P. aeruginosa* in France [16]. However, $bla_{\text{Vim-2}}$ is becoming dominant in other species including *P. putida* [5]. Our study shows the dominance of the $bla_{\text{Vim-2}}$ gene in *P. putida* isolates from wastewater and plant rhizosphere in the Eastern Cape Province of South Africa. *Vim-6* type genes that differ from the *Vim-2* type gene at nucleotide positions 179 was found with *P. putida* isolated from patients in a tertiary care hospital [17]. In Greece, Siarkou et al. [18] reported the presence of the *Vim-2* gene in *P. aeruginosa* and an outbreak caused by multidrug-resistant *P. aeruginosa* isolates carrying the new variant $bla_{\text{Vim-17}}$ gene in a university hospital. An environmental isolate of *Pseudomonas pseudoalcaligenes* from urban sewage receiving untreated hospital effluents was found to harbour the *Vim-2* gene [19]. Our observation suggests that carbapenems resistance, which is

widely disseminated among clinical strains of *Pseudomonas* species, is increasingly detected among environmental isolates.

Production of cytotoxin in *Pseudomonas aeruginosa* is of interest because it is involved in phagocytosis and lung injury in human host [7]. In this study, *ExoS* gene, one of the genes responsible for cytotoxin production, was detected. The presence of *ExoS* gene was reported in environmental strain of *P. aeruginosa* in Ireland [7]. Choy et al. [20] reported the presence of *ExoS* gene in about 63% of *P. aeruginosa* isolated from contact-lens and non-contact lens related keratitis in Australia with higher dominance in non-contact lens-related keratitis isolates; the reason for this observation was attributed to the association between *ExoS*/invasiveness and ocular trauma. Another study reported that 98% of cystic fibrosis isolates, including both clonal and non-clonal *P. aeruginosa* strains, carried an *ExoS* gene [21]. The above studies reported the presence of *ExoS* gene in *P. aeruginosa* from clinical sources, in contrast to the current study, where *ExoS* gene was detected in *P. putida* from wastewater source.

Phenazine production causes cell death. A number of genes are involved in phenazine production (*phzH*, *phzS*, and *phzM*). *PhzM* gene is one of the genes that encode proteins required for phenazine production [7], and this gene was detected in *Pseudomonas* isolates from wastewater and plant rhizosphere in the study. The dominance of these phenazine genes has been reported in clinical isolates of *P. aeruginosa*, and only *phzH* was present in environmental strain [7]. In contrast, our data shows the presence of *phzM* in *P. putida* and other *Pseudomonas* species, but absent in *P. aeruginosa*.

Alkaline protease is a protein involved in lysis of fibrin. It interferes with fibrin formation, and inactivates important host defence proteins such as antibodies, complement, IFN-γ, and cytokines [22]. Alkaline protease (*Apr* gene) was detected in *P. putida* from wastewater sample (Table 3). The detection of cytotoxin (*ExoS* gene), phenazine production gene (*PhzM*), and alkaline protease (*Apr* gene) in *P. putida* from wastewater samples suggest wastewater source as a reservoir of potential pathogenic *P. putida* strain and may serve as epidemiological risk due to the dissemination of wastewater-treated effluent into the wider environment.

Pseudomonas putida is known as an opportunistic pathogen that rarely cause human infection [23], causing it to be considered as a low-grade pathogen [1, 24]. Recently, multi-drug resistant *P. putida* have been found in connection with difficult-to-treat infections [25-27]. This study demonstrates the presence of some putative virulence factors and $bla_{\text{Vim-2}}$ in environmental strains of *P. putida*. Despite the fact that this bacterium is not common in human infections, they can act as a reservoir for antibiotic resistance and virulence genes determinant in the environment.

Conclusion

Pseudomonas putida is considered an emerging pathogen with great risk to public and environmental health. *P. putida* isolated from wastewater and plant rhizosphere was found to possess some virulence genes and metallo-β-lactamase gene, portraying this microhabitat as a potential reservoir for virulent and antibiotic-resistant determinant genes in the environment.

Acknowledgements

We are grateful to the University of Fort Hare for financial support.

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