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SHORT COMMUNICATION

Clonal Analysis of Clinical and Environmental *Pseudomonas aeruginosa* Isolates from Meknes Region, Morocco

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Abstract

From 123 clinical and environmental *Pseudomonas aeruginosa* isolates, 24 strains were selected for their similar antibioresistance, virulence and biofilm formation profiles, to examine their diversity and occurrence of clones within two hospitals and different natural sites in Meknes (Morocco). Pulsed-field gel electrophoresis, using DraI enzyme, didn't reveal a close relationship between clinical and environmental isolates nor between strains of the two hospitals. 19 genotypes were obtained, including two virulent environmental clones and three clinical clones virulent and resistant to antibiotics. Intra-hospital transmission of high-risk clones detected, in and between wards, constitutes a great public health concern.

Key words: *Pseudomonas aeruginosa*, clonal transmission in hospital, genetic diversity, genotyping of environmental and clinical isolates

Pseudomonas aeruginosa is one of the major opportunistic human pathogens known to cause severe nosocomial infections often associated with high morbidity and mortality, particularly in immunocompromised or vulnerable patients (Armour *et al.*, 2007; Kerr *et al.*, 2009). Typing methods are essential in tracking sources, pathways of spreading infections and studying population structure. Phenotypic methods as biotyping, serotyping, pyocin typing, phage typing and antibiotyping are not discriminatory enough to identify strains belonging to same genotype. Thus, molecular typing methods are required to investigate diversity of *P. aeruginosa* collections (Speert, 2002). Molecular typing is an important tool in epidemiological surveillance and outbreak investigations of human *P. aeruginosa* infections. Although many typing systems have been developed and show a variety of discriminatory powers, analysis of macrorestriction fragment patterns created by pulsed-field gel electrophoresis (PFGE) is one of the most powerful discriminating methods to type *P. aeruginosa* (Romling *et al.*, 1994a; Johnson *et al.*, 2007). PFGE has demonstrated its efficacy, efficiency, and usefulness for *P. aeruginosa* genotyping. It has been used to detect spread of *P. aeruginosa* clones within wards, within hospitals, among hospitals in the same city, and within state borders (Romling *et al.*, 1994b; Landman *et al.*, 2002). The purpose of present study was to assess genetic diversity and eventual relatedness among a collection of phenotypically close *P. aeruginosa* strains isolated from different natural environmental sites, and distinct pathological specimens from different wards of two major public hospitals in Meknes (Morocco).

A total of 123 environmental and clinical *P. aeruginosa* strains were isolated in Meknes city from June 2012 to June 2014. These isolates originated from different environmental sites and various pathological specimens of patients admitted at two major hospitals in Meknes region. Environmental samples included soil (n = 20), aliments (n = 3), amurca olive (n = 6), rivers water (n = 16), wells (n = 5) and public swimming pools (n = 5). Clinical samples included distal bronchial levy protected (n = 6), pus (n = 35), urine (n = 18), Pleural

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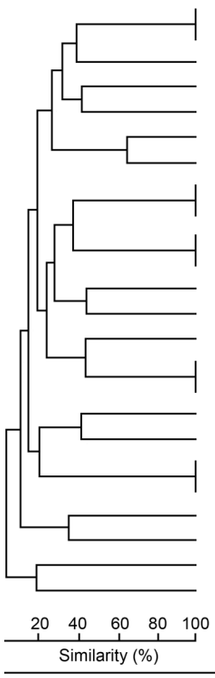
fluid (n = 4), biopsy (n = 2), blood culture (n = 1), bronchial aspirate (n = 1) and vaginal levy (n = 1). These isolates were investigated in previous studies for antimicrobial resistance against 14 antipseudomonal drugs (Maroui *et al.*, 2016a), some secreted and cell-associated virulence factors (production of β-haemolysin, caseinase, lipase, lecithinase, pyocyanin and pyoverdin; ability to swim, swarm, and twitch) and biofilm formation kinetics (Maroui *et al.*, 2016b). Their antibiograms, structural and biochemical profiles were compared to determine whether these isolates were related. Strains showing distinct antibiotypes, phenotypic expression of studied virulence factors and biofilm formation kinetics were first discarded. Remaining strains (24 isolates) which presented closely profiles were selected for further typing by a molecular test.

Genetic relationship among isolates was evaluated using PFGE following a standardized protocol developed by Durmaz *et al.* (2009), and using the restriction enzyme Dra I (Promega, Madison, WI, USA). Dice similarity coefficient was calculated between pairs of lanes, and strains were grouped using the dendrogram construction utility Dendro UPGMA (Biochemistry and Biotechnology Department, Rovirai Virgili University, Tarragona, Spain) (http://genomes.urv.cat/UPGMA/index.php). The Dice similarity coefficient of ≥ 80% was used to define genetically related strains.

Nineteen distinct genetic profiles determined by PFGE were detected among *P. aeruginosa* studied strains (Fig. 1). Clinical strain genotypes were not similar to that of environmental isolates. Furthermore, clinical strains showed 10 patterns indicating none identical genotype identified when pulsotypes obtained for Military hospital and Regional hospital strains were compared. Among Military hospital sample two strains from cardiology ward isolated at distant period (35 days) shared the same clonal profile (clone 12), while remaining isolates presented distinct clonal profiles (clones 2, 13, 16, 17 and 18). For Regional hospital isolates, two strains isolated in burn ward at distant period (4 months) belonged to the same clone (clone 15), one strain isolated from intensive care unit and another from burn ward (after 2 months) shared the same genotype (clone 8), while other strains presented distinct clonal profiles (clones 14 and 19). Pulsotypes analysis of environmental strains showed 9 genotypes. Two strains from amurca olive isolated in the same habitat at distant period (one month) belonged to the same clone (clone 1), one isolate detected in soil and another from well water of sciences faculty of Meknes at distant period (6 months) shared the same clonal profile (clone 7), while other strains presented distinct genotypes (clones 3, 4, 5, 6, 9, 10 and 11).

Analysis of PFGE patterns revealed that examined *P. aeruginosa* strains showed a great genetic variabil-



Dendrogram	PFGE patterns	Strain code	Origin	Isolation site	Isolation date	Ward*	Gender*	PFGE type
		2	Env.	Amurca olive	11/01/2013	–	–	C1
		42	Env.	Amurca olive	15/02/2013	–	–	C1
		BP601	MH	Biopsy	21/02/2014	Intensive care	M	C2
		41–1	Env.	River Boufekrane water	07/02/2013	–	–	C3
		11	Env.	River Bouishak water	02/02/2013	–	–	C4
		47	Env.	Public pool water	15/07/2013	–	–	C5
		38	Env.	River Bouishak soil	25/12/2012	–	–	C6
		22	Env.	Sc. Fac. well water	14/07/2012	–	–	C7
		25–1	Env.	Sc. Fac. soil	31/01/2013	–	–	C7
		Pu241	RH	Pus	28/10/2013	Burn	M	C8
		102–1	RH	Pus	30/08/2013	Intensive care	M	C8
		43	Env.	Public pool water	15/07/2013	–	–	C9
		31–1	Env.	River Ouislane water	15/02/2013	–	–	C10
		30	Env.	River Ouislane water	15/02/2013	–	–	C11
		Pu301''	MH	Pus	28/01/2014	Cardiology	M	C12
		Pu505	MH	Pus	06/03/2014	Cardiology	M	C12
		Pu601	MH	Pus	22/03/2014	Thoracic	M	C13
		115–1	RH	DBP	07/05/2013	Intensive care	M	C14
		Pu141	RH	Pus	03/07/2012	Burn	M	C15
		Pu234	RH	Pus	29/10/2012	Burn	M	C15
		Pu402	MH	Pus	30/04/2014	Oncology	F	C16
		Pu304	MH	Pus	27/05/2014	Oncology	M	C17
		Pu305'	MH	Pus	03/06/2014	Thoracic	M	C18
		Pu247	RH	Pus	04/11/2013	Intensive care	M	C19

Fig. 1. Dendrogram of environmental and clinical *P. aeruginosa* isolates after DNA digestion with Dra I enzyme and PFGE.

*, for clinical isolates; DBP, distal bronchial levy protected; Env., Environmental; F, Female; M, male; MH, Military hospital; RH, Regional hospital; Sc. Fac., Sciences Faculty.

ity, regardless of the strains origin. Most *P. aeruginosa* selected isolates belonged to distinct genotypes (79.2% of polymorphism). Environmental isolates showed more polymorphism (81.8%) than clinical ones (76.9%). PFGE did not reveal a close relatedness between clinical and environmental isolates nor between strains of the two hospitals. High level of heterogeneity recorded in this *P. aeruginosa* strains collection is in agreement with several studies suggesting that this species exhibits a nonclonal population structure (Picard *et al.*, 1994; Wiehlmann *et al.*, 2007; Pirnay *et al.*, 2009; Maatallah *et al.*, 2011).

Higher diversity of environmental isolates can be explained by their origin, since most of these strains were isolated in various habitats geographically scattered in and around Meknes city. Even more, a genetic variability was also observed even among strains isolated from the same natural site at the same time. Only two clones with two strains each were identified, the first concerns strains isolated with one month interval from amurca olive directly discharged in nature by an olive oil factory. These isolates showed the same antibiotype and virulence profile and formed denser biofilm. They would be from the same parent strain that was able to resist and grow on amurca olive. The second clone originated from sciences faculty of Meknes with one strain from well water and other isolated in soil after 6 months. These isolates showed the same virulence profile and biofilm formation kinetics, but distinct antibiotypes. This observation can be explained by the circulation of clones between these geographically close biotopes. These isolates would have arose from the same ancestor strain, and the imipenem and meropenem resistant one could evolved in response to changing environmental conditions and developed this resistance.

Clinical isolates were also highly diverse, the level detected (76.9%) is higher than that reported by Selim *et al.* (2015) signaling 50% of polymorphism in *P. aeruginosa* isolates from Egypt. However it remains slightly lower than that obtained by Freitas and Barth (2004) indicating 71 PFGE types in 81 clinical isolates from 3 Brazilian hospitals. Obtained polymorphism is also lower than that reported by Lim *et al.* (2009) signaling 93.7% of polymorphism among 48 *P. aeruginosa* isolates from 6 Malaysian hospitals. In fact, tested clinical strains were isolated from various samples of different patients at distinct times in two hospitals. Three clones with two strains each were identified, each clone contains strains showing the same virulence profile and biofilm formation kinetics, but distinct antibiograms. These clones contain virulent and carbapenem resistant strains, with a particular attention to a high risk clone (clone 15) from burn ward that includes 2 multi drug resistant isolates harboring $bla_{\text{VIM-2}}$ gene. In fact, metallo-β-lactamases VIM have a potential for horizontal transfer and are among the most clinically threatening carbapenemases (Gupta, 2008).

Findings of isolates showing the same genotype but having distinct antibiotypes and others with same antibiograms but belonging to distinct genotypes were also highlighted by Freitas and Barth (2004) as well as Selim *et al.* (2015). Among various features of *P. aeruginosa* its capability to customize its genome to adapt and fit the needs for thriving in any environmental niche (Mathee *et al.*, 2008). Intra-clonal phenotypic diversity detected in this study may be explained by the remarkable plasticity of *P. aeruginosa* genome. Indeed, genomic dynamism mainly by microevolution events (mutations) or acquisition or discard of genomic segments *via* horizontal gene transfer and recombination modulate *P. aeruginosa* strain's phenotype and differentiate it (Mathee *et al.*, 2008; Bezuidt *et al.*, 2013).

We have detected two transition cases of clones between patients admitted to the same ward and 1 case of clone transmission between 2 distinct wards. Persistence of these resistant and virulent *P. aeruginosa* strains within hospital can be explained by their resistance and high biofilm formation. Indeed, biofilm was identified as a mechanism promoting persistence of clonal groups (Sommerfeld Ross and Fiegel, 2012), and more antimicrobial selection pressure could allow their persistence and silent spread in hospital wards. Because of difficulty in accessing patient histories, the method of transmission between patients or wards could not be determined.

Clinical implications of this study are of concern. Indeed, intra-hospital persistence and dissemination of multi drug resistant *P. aeruginosa* high-risk clones is a serious epidemiological problem. This result causes alarm and should serve as the basis for nationwide strategies to improve infection prevention and control measures in hospitals, aiming to bring down costs and damages caused by this life-threatening microorganism.

To our knowledge, this study is the first genotyping of environmental and clinical *P. aeruginosa* isolates in Morocco. Genetic technique supported by some phenotypic tests has enabled us to conduct a detailed characterization of *P. aeruginosa* strains isolated from distinct samples at particular times. Our findings are in agreement with several studies showing high discriminatory power of PFGE method, and failure to obtain reliable results based only on phenotypic methods (Muller and Gubina, 2000; Freitas and Barth, 2004; Yousefi *et al.*, 2013; Selim *et al.*, 2015).

In conclusion, we detected a great genetic variability and a clear distinction between clinical and environmental isolates. We also described clonal transmission of high-risk *P. aeruginosa* in and between wards in Meknes hospitals which can be deemed a major public health concern.

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