APMIS
ACTA PATHOLOGICA, MICROBIOLOGICA
ET IMMUNOLOGICA SCANDINAVICA

APMIS 122: 92–100

DOI 10.1111/apm.12112

Pathogenic potential and genetic diversity of environmental and clinical isolates of *Pseudomonas aeruginosa*

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Martins VV, Pitondo-Silva A, Manço LM, Falcão JP, Freitas SS, da Silveira WD, Stehling EG. Pathogenic potential and genetic diversity of environmental and clinical isolates of *Pseudomonas aeruginosa*. APMIS 2014; 122: 92–100.

The aim of this study was to investigate the occurrence of virulence genes among clinical and environmental isolates of *Pseudomonas aeruginosa* and to establish their genetic relationships by Enterobacterial Repetitive Intergenic Consensus PCR (ERIC-PCR). A total of 60 *P. aeruginosa* isolates from environmental and clinical sources were studied. Of these, 20 bacterial isolates were from soil, 20 from water, and 20 from patients with cystic fibrosis. Analysis of ERIC-PCR demonstrated that the isolates of *P. aeruginosa* showed a considerable genetic variability, regardless of their habitat. Numerous virulence genes were detected in both clinical and environmental isolates, reinforcing the possible pathogenic potential of soil and water isolates. The results showed that the environmental *P. aeruginosa* has all the apparatus needed to cause disease in humans and animals.

Key words: *Pseudomonas aeruginosa*; virulence genes; environmental and hospital isolates; ERIC-PCR; pathogenic potential.

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Pseudomonas aeruginosa is an aquatic and soil bacterium that can infect several organisms, including plants and mammals (1). In humans, *P. aeruginosa* has become a significant cause of infection, particularly in patients with compromised host defense mechanisms. Of particular further importance, *P. aeruginosa* biofilms develop in the lungs of chronically infected cystic fibrosis (CF) patients, in which they defend the bacteria against the immune response and antibiotics (2).

Received 1 February 2013. Accepted 19 March 2013

Pseudomonas aeruginosa possesses a large number of virulence factors which are cell-associated and extracellular, and most of them seem to be controlled by quorum-sensing, a cell-to-cell signaling system (3–5). In *P. aeruginosa*, two main quorum-sensing systems, *las* and *rhl*, have been described. Some genes, including *aprA, lasA*, *lasB*, *and toxA* are controlled by the las system and *rhlAB* rhamnolipid synthesis genes, and to some extent *lasB* are controlled by the rhl system (6, 7).

The alginate is encoded by (alg) genes, including *algD,* and is involved in the establishment

of mucoid colonies of *P. aeruginosa*. It contributes to chronic pulmonary inflammation because it helps the bacterium to protect from antibiotics and the host's immune system (8–10). The switch of the non-mucoid to a mucoid phenotype is considered an indication that eradication of the infection is extremely difficult and the mechanisms responsible for this switch appear to involve post-translational regulation, a complex arrangement of transcriptional regulation, and the mutation of the genome in hyper-mutable regions (11).

The cause of pulmonary damage is also related to other virulence factors that act by different mechanisms. The inhibition of protein biosynthesis is performed by exotoxin A, a toxin encoded by the gene *toxA* (12) and the elastolytic activity on lung tissue is performed by the zinc metalloprotease LasB elastase, an enzyme encoded by the *lasB* gene (13, 14). Exoenzyme S, an ADP-ribosyltransferase encoded by the gene *exoS*, is secreted inside the host cell (15, 16) and can be related to cytotoxicity caused by *P. aeruginosa* (17). Hydrolysation of the phospholipids included in pulmonary surfactants may be performed by two phospholipases C which are encoded by *plcH* and *plcN* genes (18, 19) and bacterial binding can be significantly enhanced by the change in the epithelial surface by the *P. aeruginosa* exoproducts, particularly by proteases and neuraminidases (20, 21).

In this work, 60 isolates (40 environmental and 20 clinical) were analyzed to verify the presence of virulence genes to compare them, and afterward, to determine if clinical isolates possess a major virulence potential in comparison to those from the environment. In addition, the genetic relationship among the isolates was evaluated by Enterobacterial Repetitive Intergenic Consensus PCR (ERIC-PCR) to establish if clinical isolates were genetically more similar than environmental isolates.

MATERIAL AND METHODS

Isolation and identification of *P. aeruginosa*

Isolation and identification of *P. aeruginosa* was performed as described previously by Mukherjee et al. (22). The isolates from soil were identified as proposed by Zanetti et al. (Zanetti, M. O., Martins, V. V., Pitondo-Silva, A., Stehling, E. G., unpublished data) and are part of a collection of the Environmental Microbiology and Bioremediation Laboratory from School of Pharmaceutical Sciences of Ribeirão Preto, University of São Paulo. The soil samples were collected from plantation areas of different crops (lettuce, sugar cane, soya, orange, chili, chrysanthemums, cabbage) and also from sand. The samples were collected 5 cm below the land surface into the sterile containers. One gram of each soil sample was dispersed in 9 mL of 0.85% NaCl in sterile test tubes and a series of dilutions from 10^{-2} to 10^{-10} were prepared in 0.85% NaCl. A 0.2 mL aliquot of the appropriate dilution was spread aseptically onto Cetrimide Agar plates (Fluka Analytical, Sigma-Aldrich, St. Louis, MO, USA) and was incubated at 37 °C for 48–72 h.

Water samples were collected from wells, and drinking water from residences and schools in different states of the southeastern Brazil into sterile flasks. For the isolation of bacterial colonies of *P. aeruginosa*, 100 mL aliquots were filtered through sterile 0.45-mm pore membrane filters (Millipore), 47 mm in diameter, with the aid of a vacuum pump. Membranes aseptically removed from the filtration equipment were placed on the surface of Cetrimide Agar plates (Fluka) and incubated at 37 °C for 48–72 h.

The clinical isolates were obtained from patients with CF who attended the School Hospital of Campinas State University (UNICAMP), Brazil, between April 1996 and January 1998 (Ethical Process number 045/98 CEP/FCM from 05/27/98).

Identification of the isolates was performed by colony pigmentation, growth at 42 °C, biochemical tests [carbohydrate fermentation (−), citrate assimilation (+), DNAse (−), indol (−), lysine decarboxylase (−), and oxidase (+)] and by PCR using specific primers to amplify the open reading frame of the *oprL* gene (23). The primers were ordered from Invitrogen-Life Technologies (Brazil) and had the following sequences: PAL1, 59-ATGGAAATGCT GAAATTCGGC-39 (a 21-mer corresponding to the beginning of the open reading frame of *oprL*); and PAL2, 59-CTTCTTCAGCTCGACGCGACG-39 (a 21-mer corresponding to the end of the open reading frame of *oprL*). Genomic DNA was extracted with the *QIAamp DNA Mini Kit* (QIAGEN, Germany) following the manufacturer's instructions. Reaction parameters were performed as described by De Vos et al. (24). PCR products were visualized after agarose gel (1.5%) electrophoresis and stained with ethidium bromide. *P. aeruginosa* ATCC 27853 served as positive control and a reaction mixture without a DNA template served as negative control.

ERIC-PCR conditions and primers

Genomic DNA was extracted as previously described and 100 ng was used in each ERIC-PCR reaction,

using the primers ERIC 1 (5′-CACTTAGGGGTC CTCGAATGTA-3′) and ERIC 2 (5′-AAGTAAGT-GACTGGGGTGAGCG-3′) as described by Versalovic et al. (25). The PCR reaction mixture were prepared in a final volume of 50 μL containing: 1.25 mM of each deoxyribonucleotide, 5 mM $MgCl_2$, 1.0 U Taq DNA polymerase (Fermentas Life Sciences, Burlington, Ontário, CA, USA) and 50 pmol of each primer. PCR conditions were performed as follows: an initial denaturation (94 °C, 7 min), followed by 30 cycles of denaturation (94 °C, 30 s), annealing (52 °C, 1 min), and extension (72 °C, 8 min) with a single final extension (72 °C, 16 min). Each ERIC-PCR was performed in triplicate to ensure conformity of each fingerprint. Reactions without the DNA template were used as negative controls and *P. aeruginosa* ATCC 27853 served as positive control to verify the reproducibility of the experiment.

The ERIC-PCR amplified products (amplicons) were resolved by 1.5% agarose gel electrophoresis into bands, which were stained with ethidium bromide and revealed when subjected to UV light. A 1 kb Plus DNA Ladder from Invitrogen (Life Technologies) was included three times on each gel, to normalize images and thus allow valid comparisons of fingerprints on different gels. Only bands between 298 and 4072 bp were included in the analyses. For each fingerprint, individual bands were identified and the data were analyzed with the BioNumerics 5.1 software package (Applied Maths, Sint-Martens-Latem, Belgium). A similarity dendrogram was constructed by the UPGMA method, using the Dice similarity coefficient for cluster analysis.

Detection of virulence genes by PCR

PCR reactions were performed to detect the prevalence of virulence genes encoding alginate (*algD*), alkaline protease (*aprA*), exoenzyme S (*exoS*), elastase (*lasB*), the putative neuraminidase genes (*nan1* and *nan2*), hemolytic phospholipase C (*plcH*), non-hemolytic phospholipase C (*plcN*), rhamnolipids (*rhlAB*), and exotoxin A (*toxA*). The PCR procedure adopted in this study was proposed by Lanotte et al. (26). The PCR reaction conditions used for detection of the *aprA* and *rhlAB* genes were as described by Zhu et al. (27). The primers used and the number of base pairs of the respective products are presented in Table 1. The PCR products were analyzed by agarose gel electrophoresis and were visualized by UV light after staining of the gel with ethidium bromide. A standard molecular weight ladder (1 kb Plus DNA Ladder from Invitrogen, Life Technologies) was included on each gel and the amplified genes were identified on the basis of fragment size. Reaction mixtures without the DNA template were used as negative controls. *P. aeruginosa* ATCC 27853 carrying all the virulence genes examined was used as a positive control.

Statistical methods

The relationship between virulence genes distribution with respect to the source of isolation was compared using chi-square (χ^2) test.

Table 1. Primers used for PCR amplification of virulence factors

Gene	Primers sequences (forward and reverse, 5′–3′)	Product (bp)	Reference
algD	ATGCGAATCAGCATCTTTGGT CTACCAGCAGATGCCCTCGGC	1310	Lanotte et al., 2004
aprA	ACCCTGTCCTATTCGTTCC GATTGCAGCGACAACTTGG	140	Zhu et al., 2004
exoS	CTTGAAGGGACTCGACAAGG TTCAGGTCCGCGTAGTGAAT	504	Lanotte et al., 2004
lasB	GGAATGAACGAAGCGTTCTC GGTCCAGTAGTAGCGGTTGG	300	Lanotte et al., 2004
nan1	AGGATGAATACTTATTTTGAT TCACTAAATCCATCTCTGACCCGATA	1316	Lanotte et al., 2004
nan2	ACAACAACGGGGACGGTAT GTTTTGCTGATGCTGGTTCA	1161	Lanotte et al., 2004
plcH	GAAGCCATGGGCTACTTCAA AGAGTGACGAGGAGCGGTAG	307	Lanotte et al., 2004
plcN	GTTATCGCAACCAGCCCTAC AGGTCGAACACCTGGAACAC	466	Lanotte et al., 2004
rhlAB	TCATGGAATTGTCACAACCGC ATACGGCAAAATCATGGCAAC	151	Zhu et al., 2004
toxA	GGTAACCAGCTCAGCCACAT TGATGTCCAGGTCATGCTTC	352	Lanotte et al., 2004

RESULTS AND DISCUSSION

Pseudomonas aeruginosa is an environmental bacterium, and it is among the top three opportunistic bacteria which can cause human infections. It forms biofilms on wet surfaces such as those of rocks and soil (28). The presence of virulence genes and/or biofilms related to human diseases, such as CF, is well known and well studied (28). However, the occurrence of these virulence features in both clinical and environmental sources are not well established. Therefore, in this study, the genetic relationships among *P. aeruginosa* isolated from environmental and clinical samples and their pathogenic potential were investigated.

A total of 60 *P. aeruginosa* isolates from environmental and clinical sources were studied. Of these, 20 bacterial isolates were from soil plantation areas of different vegetation, 20 were from water, and 20 clinical isolates were from CF patients.

The isolates were initially analyzed by ERIC-PCR to verify the genetic relationship among them, as well as to verify if clinical isolates were genetically more similar than environmental isolates. ERIC-PCR is a rapid, reproducible, and highly discriminatory assay that proved to be a powerful surveillance screening tool for the typing of clinical *P. aeruginosa* isolated from patients with CF (29, 30). Moreover, this technique appears to be a more reliable typing strategy for *P. aeruginosa* than other novel PCR-based typing methodologies (31).

There are few works that used ERIC-PCR to type *P. aeruginosa* isolated from the environment, specifically from soil and water (32). In this study, all *P. aeruginosa* analyzed by ERIC-PCR presented a high genetic diversity. Most of the clinical isolates (16 of the 20) grouped into a single cluster, showing a 42.5% genetic similarity among them. Among the remaining four clinical isolates, three of them (C4, C15, and C13) were grouped together into a smaller cluster, with genetic similarity of 60% and only the strain C14 was grouped with environmental isolates. Except for the isolate S3, soil and water isolates were grouped into smaller clusters with variable genetic similarities, ranging from 28% to 100% (Fig. 1). Regarding the environmental isolates, it was not possible to observe clusters related to the source of isolation, i.e., isolates from soil and water were mixed up. These results indicate that the ERIC-PCR technique can discriminate clinical and environmental isolates of *P. aeruginosa*, and the clinical isolates tend to be genetically closer than the environmental isolates. However, both clinical and environmental isolates showed very low genetic similarity (below 80%), indicating that the isolates are genetically distinct (Fig. 1).

Besides, ERIC-PCR results were compared with the occurrence of the virulence genes. It was observed that only two clinical isolates (C06 and C08), which showed 100% genetic similarity, were also identical regarding the presence of some virulence genes analyzed.

Water isolates W01 and W02, with 100% genetic similarity, were different for the presence of genes *toxA* and *rhlAB*, both present in the isolate W01 and absent in isolate W02. The soil isolates (S01 and S11, S05, S08, and S09) were also analyzed and it was observed that they were different regarding the presence of the genes *exoS* and *plcN*. These results demonstrated that ERIC-PCR was not able to differentiate isolates according to their pathogenic potential.

As expected, the virulence genes were predominant in clinical isolates. However, environmental isolates also showed a high percentage of virulence genes. Our data demonstrated that the tested genes *algD*, *oprA*, exoS, *lasB*, *nan1*, *nan2*, *plcH*, *plcN*, *rhlAB*, and *toxA* were presented in 58%, 75%, 63%, 80%, 7%, 18%, 62%, 67%, 68%, and 32% of all studied *P. aeruginosa*, respectively.

The chi-square analyses indicated that the distribution of virulence genes, according to the source of isolation, was significant ($p \leq 0.01$) for genes *algD*, *exoS*, *nan1*, *nan2*, *plcN*, *rhlAB*, and *toxA* (Table 2).

All virulence genes were found in all sources (clinical, soil, and water), except the putative neuraminidase genes (*nan1* and *nan2*), which were found just in clinical isolates (Fig. 2). In some cases, the virulence genes of the environmental isolates surpassed the frequency of the clinical isolates such as *plcN* and *algD* for water isolates and *exoS* for soil and water isolates (Fig. 2 and Table 2).

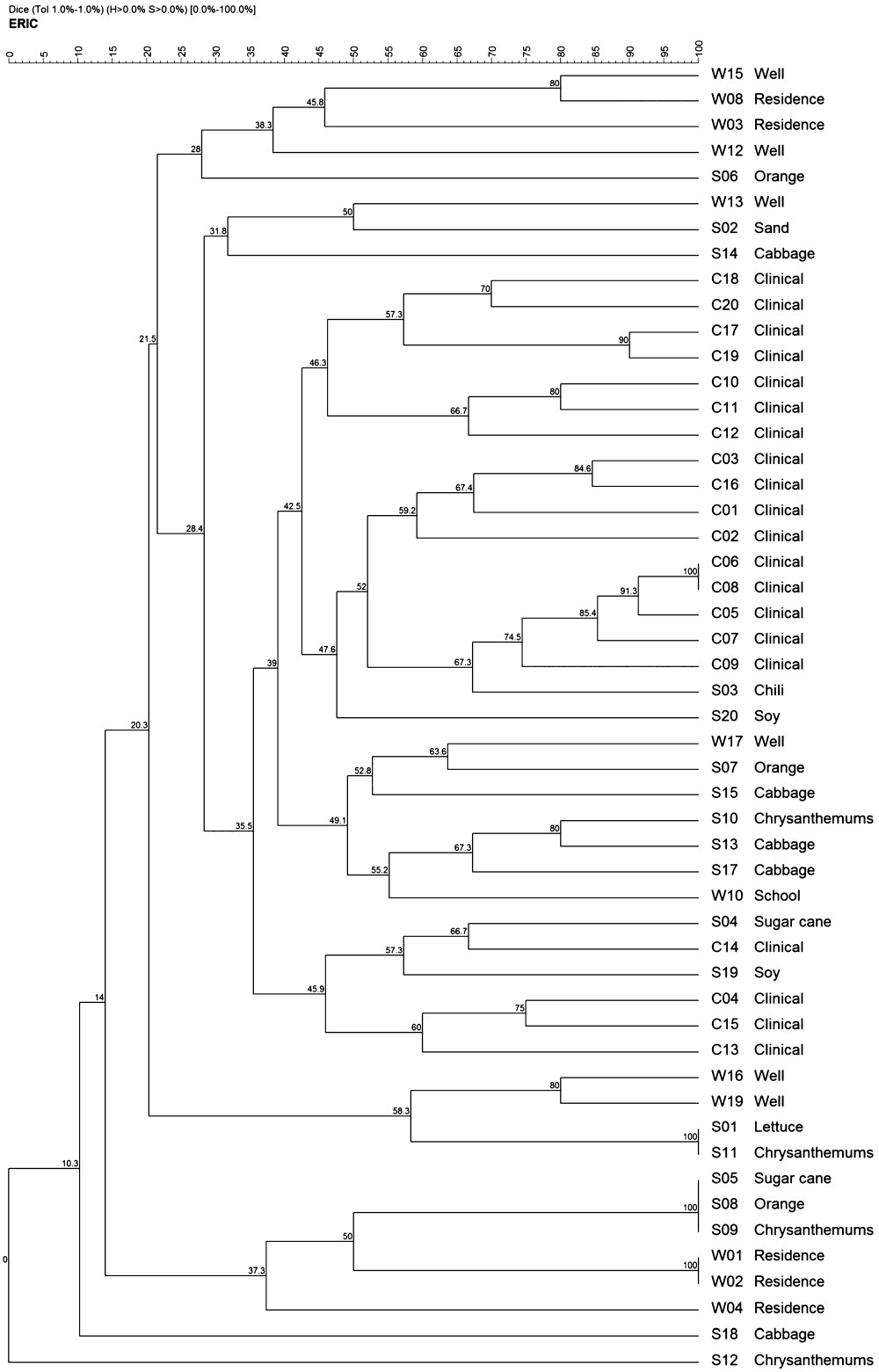

Fig. 1. Dendrogram representing genetic relationships among the 60 *Pseudomonas aeruginosa* studied based on Enterobacterial Repetitive Intergenic Consensus PCR (ERIC-PCR) fingerprints. Isolates from water, clinical, and soil sources are represented by W, C, and S, respectively.

Table 2. Distribution, Frequency (%), and p-value of virulence genes in all *Pseudomonas aeruginosa* studied. (Isolates from water, clinical and soil sources are represented by W, C, and S, respectively)

Isolates	*algD*	*oprA*	*exoS*	*lasB*	*nan1*	*nan2*	*plcH*	*plcN*	*rhlAB*	*toxA*
W01	+	+	+	+	–	–	+	+	+	+
W02	+	+	+	+	–	–	+	+	–	–
W03	+	+	+	–	–	–	+	+	–	–
W04	+	+	–	–	–	–	+	+	–	–
W05	+	+	+	+	–	–	+	+	+	+
W06	+	+	+	+	–	–	+	+	+	+
W07	+	+	+	+	–	–	+	+	+	+
W08	+	+	+	+	–	–	+	+	+	+
W09	+	+	+	+	–	–	+	+	+	+
W10	+	+	+	+	–	–	+	+	+	–
W11	+	+	+	+	–	–	+	+	+	–
W12	+	–	+	–	–	–	–	+	–	–
W13	+	+	+	+	–	–	+	+	+	–
W14	–	–	+	–	–	–	–	+	–	–
W15	–	–	+	–	–	–	–	+	–	–
W16	–	–	+	–	–	–	–	+	–	–
W17	–	–	+	–	–	–	–	+	–	–
W18	+	–	+	–	–	–	–	+	–	–
W19	+	–	+	–	–	–	–	+	–	–
W20	–	–	+	+	–	–	–	+	–	–
C01	+	+	–	+	–	+	+	–	+	+
C02	+	+	–	+	–	+	+	–	+	+
C03	+	+	–	+	–	+	+	+	+	+
C04	+	+	–	+	–	+	+	+	+	+
C05	+	+	–	+	–	+	+	+	+	+
C06	+	+	–	+	–	+	+	–	+	+
C07	+	+	–	+	–	+	–	+	+	–
C08	+	+	–	+	–	+	+	–	+	+
C09	–	+	+	+	–	–	+	–	+	+
C10	–	+	+	+	–	–	+	–	+	+
C11	+	+	+	+	–	+	+	+	+	–
C12	–	+	+	+	–	+	+	+	+	–
C13	–	+	–	+	–	–	+	–	+	–
C14	+	+	–	+	–	–	+	+	+	–
C15	–	+	–	+	–	–	–	+	+	–
C16	+	+	+	+	+	–	–	–	+	–
C17	+	+	+	+	+	–	–	–	+	–
C18	+	+	+	+	+	–	–	–	+	–
C19	–	+	+	+	+	–	–	–	+	+
C20	+	+	–	+	–	–	+	+	+	+
S01	–	+	–	+	–	–	+	–	+	–
S02	–	+	+	+	–	–	–	+	–	–
S03	–	+	–	+	–	–	+	–	+	–
S04	+	–	+	+	–	–	–	+	–	–
S05	–	+	+	+	–	–	+	+	+	–
S06	–	+	–	–	–	–	+	–	–	–
S07	–	+	+	+	–	–	+	+	–	–
S08	–	+	–	+	–	–	+	+	+	–
S09	–	+	+	+	–	–	+	+	+	–
S10	+	+	+	+	–	–	+	+	+	–
S11	–	+	+	+	–	–	+	–	–	–
S12	–	+	–	+	–	–	–	+	+	–
S13	+	+	+	+	–	–	+	+	+	+
S14	+	+	+	+	–	–	–	+	+	–

Table 2. *(continued)*

Isolates	*algD*	*oprA*	*exoS*	*lasB*	*nan1*	*nan2*	*plcH*	*plcN*	*rhlAB*	*toxA*
S15	+	–	–	+	–	–	–	–	+	–
S16	–	–	–	+	–	–	–	–	+	–
S17	–	–	–	–	–	–	–	–	–	–
S18	–	–	–	–	–	–	–	–	–	–
S19	+	–	+	+	–	–	–	+	+	–
S20	–	–	+	+	–	–	+	–	–	+
Frequency (%)	58	75	63	80	7	18	62	67	68	32
p-value	0.0067	0.8027	0.0002	0.0126	<0.0001	<0.0001	0.06105	0.0004	0.0006	0.0091

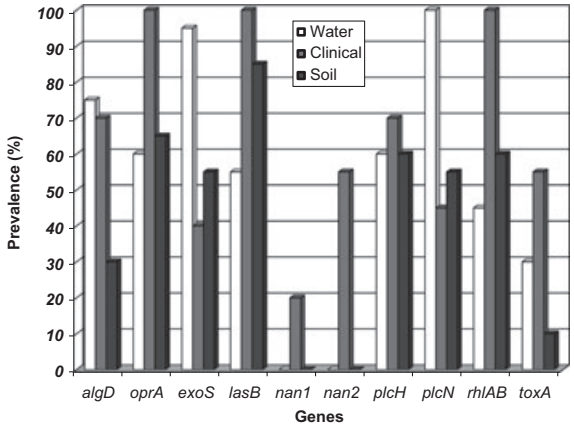

Fig. 2. Prevalence (as percentages) of the virulence genes detected by PCR, among *Pseudomonas aeruginosa* isolated from water, clinical and soil samples.

Lanotte et al. (26) reported that the prevalence of *nan1* is strongly associated with CF isolates. Mitov et al. (33) established a low frequency of spread of *nan1* among non-CF *P. aeruginosa* isolates compared with CF isolates. Regarding the *nan2* gene, Lanote et al. (26) demonstrated that this gene is also highly prevalent in isolates from CF, but more often than *nan1*. Our results correspond to these statistics and also suggest that, same as *nan1*, the *nan2* gene is not common in environmental isolates.

The isolates from water presented a high level of virulence genes and the most frequent were *plcN* (100%), *exoS* (95%), and *algD* (75%) (Fig. 2). Some works have demonstrated that the expression of these genes is directly associated with osmotic stress during the infectious process of *P. aeruginosa* in patients with CF (8, 34). Berry et al. (35) showed that the transcriptional activation of *algD* is directly proportional to the increase in osmolarity. In a study that analyzed the osmotic stress response in *P. aeruginosa*, the authors found that the majority of genes that had some alteration in their expression were associated with virulence factor expression of encoding proteins of the type III secretion system (36). As the products of these genes have the function of osmoprotectants, their importance is evident not only in the pathogenic process but also on the survival of these bacteria in different environments, especially in water and soil helping *P. aeruginosa* to survive in an extreme osmotic stress.

Fergunson et al. (37) demonstrated that exoenzyme S (ExoS) also is essential for the survival of *P. aeruginosa* in soil, but it is not so common in some clinical settings, and suggested that the lower frequency of expression of ExoS in certain clinical settings can be related to the loss of this gene or its expression, and not due to its limited acquisition. This may explain the higher frequency of this gene in relation to soil and water isolates.

In conclusion, analysis of ERIC-PCR technique could discriminate clinical and environmental isolates of *P. aeruginosa* and the clinical isolates tend to be genetically closer than the environmental isolates; however, both clinical and environmental isolates showed a considerable genetic variability, regardless of their habitat. The environmental isolates, both from soil and water, and the clinical isolates proved to harbor several virulence genes. These virulence genes may not be expressed in the environment or are used by these bacteria as a defense and adaptation mechanisms in different environments. However, as these strains are transmitted to other organisms, such as humans and animals, they can cause diseases as a consequence of their great

pathogenic potential. To our knowledge, this is the first work comparing *P. aeruginosa* isolated from CF patients, soil, and water by ERIC-PCR and their pathogenic potential.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

This study was supported by the Fundação de Amparo à Pesquisa do Estado de São. Paulo (FAPESP, grants 2009/16657-3). We also thank John Carpenter for the English revision.

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