Journal of Microbiological Methods 114 (2015) 66–74

Contents lists available at ScienceDirect

Journal of Microbiological Methods

journal homepage: www.elsevier.com/locate/jmicmeth

A cultivation-independent PCR-RFLP assay targeting *oprF* gene for detection and identification of *Pseudomonas* spp. in samples from fibrocystic pediatric patients

Antonio Lagares Jr. [a], Betina Agaras [a], Marisa P. Bettiol [b], Blanca M. Gatti [b], Claudio Valverde [a,*]

[a] Laboratorio de Bioquímica, Microbiología e Interacciones Biológicas en el Suelo, Departamento de Ciencia y Tecnología, Universidad Nacional de Quilmes, Roque Sáenz Peña 352, Bernal B1876BXD, Buenos Aires, Argentina
[b] Sala de Microbiología HIAEP "Sor María Ludovica", Calle 14N° 1631, La Plata B1904CSI, Buenos Aires, Argentina

ARTICLE INFO

Article history:
Received 20 April 2014
Received in revised form 6 May 2015
Accepted 6 May 2015
Available online 8 May 2015

Keywords:
Cystic fibrosis
oprF
PCR-RFLP
Pseudomonas aeruginosa

ABSTRACT

Species-specific genetic markers are crucial to develop faithful and sensitive molecular methods for the detection and identification of *Pseudomonas aeruginosa* (Pa). We have previously set up a PCR-RFLP protocol targeting *oprF*, the gene encoding the genus-specific outer membrane porin F, whose strong conservation and marked sequence diversity allowed detection and differentiation of environmental isolates (Agaras et al., 2012). Here, we evaluated the ability of the PCR-RFLP assay to genotype clinical isolates previously identified as Pa by conventional microbiological methods within a collection of 62 presumptive Pa isolates from different pediatric clinical samples and different sections of the Hospital de Niños "Sor María Ludovica" from La Plata, Argentina. All isolates, but one, gave an *oprF* amplicon consistent with that from reference Pa strains. The sequence of the smaller-sized amplicon revealed that the isolate was in fact a *mendocina Pseudomonas* strain. The *oprF* RFLP pattern generated with *Taq*I or *Hae*III nucleases matched those of reference Pa strains for 59 isolates (96%). The other two Pa isolates (4%) revealed a different RFLP pattern based on *Hae*III digestion, although *oprF* sequencing confirmed that Pa identification was correct. We next tested the effectiveness of the PCR-RFLP to detect pseudomonads on clinical samples of pediatric fibrocystic patients directly without sample cultivation. The expected amplicon and its cognate RFLP profile were obtained for all samples in which Pa was previously detected by cultivation-dependent methods. Altogether, these results provide the basis for the application of the *oprF* PCR-RFLP protocol to directly detect and identify Pa and other non-Pa pseudomonads in fibrocystic clinical samples.

1. Introduction

Pseudomonas aeruginosa (Pa) is an opportunistic human pathogen considered as a major causative agent of nosocomial infections. Pa is commonly isolated from patients with skin and soft tissue infections, hospital-acquired pneumonia, urinary tract and bloodstream infections, organ transplantations, and in particular it is among the most frequent pathogens found in the airways of patients with the genetic disease cystic fibrosis (Lyczak et al., 2000; Obritsch et al., 2005; Veesenmeyer et al., 2009). Because of the severity and unfavorable prognosis of Pa infections of the lower respiratory tract of fibrocystic pediatric patients, an early detection and identification of the microorganism is especially relevant for a successful antimicrobial treatment before the infection becomes chronically established as a multidrug-resistant biofilm (Folkesson et al., 2012; Hoiby, 2011). It is well known that Pa has virulence factors and adaptive mechanisms to evade host defense and to resist antimicrobial treatments, thus allowing its survival over decades in the respiratory tract of the fibrocystic patients (Folkesson et al., 2012; Oliver, 2010; Tummler and Kiewitz, 1999). Although *Pseudomonas* spp. other than *aeruginosa* are of lower clinical relevance than Pa in fibrocystic patients, recently published works have pointed out the role of non-Pa pseudomonads, such as *Pseudomonas putida*, as clinical and environmental reservoirs of antimicrobial resistance genes that can be transferred to Pa clones (Docquier et al., 2003; Gilarranz et al., 2013; Juan et al., 2010; Juan and Oliver, 2010; Viedma et al., 2014). These authors underline the importance of establishing an active parallel surveillance of other pseudomonads than *aeruginosa* and of taking special care to design detection methods aiming to reduce misidentification of non-Pa as Pa.

Molecular methods are sensitive, specific and rapid for the detection and genotyping of microorganisms, thus representing a powerful complementary approach to classical microbiological methods based on sample culturing. In order to develop protocols aiming at the detection and identification of pseudomonads, specific and robust genetic markers are required. We have already tested the utility of the *oprF* gene, encoding a non-specific outer membrane porin, to specifically

Abbreviations: Pa, *Pseudomonas aeruginosa*; PCR-RFLP, polymerase chain reaction-restriction fragment length polymorphism.

* Corresponding author.
E-mail address: cvalver@unq.edu.ar (C. Valverde).

http://dx.doi.org/10.1016/j.mimet.2015.05.008
0167-7012/

monitor *Pseudomonas* populations in soil and plant root samples (Agaras et al., 2012). Although *oprF* is conserved in the genus *Pseudomonas*, its sequence shows enough diversity to be used as a genetic marker to explore its allelic variation among different species (Bodilis and Barray, 2006; Bodilis et al., 2004; Bodilis et al., 2006). Based on this, we have developed a PCR-RFLP protocol targeting the *oprF* gene that allows species level differentiation in environmental pseudomonad isolates (Agaras et al., 2012).

Here, we evaluated the robustness of the *oprF* PCR-RFLP protocol for detection and species identification of pseudomonad isolates from

Table 1
Collection of *Pseudomonas aeruginosa* isolates from the Hospital de Niños of La Plata (Buenos Aires, Argentina) that were used in this study.

Isolate	ID	Section[a]	Sample type[b]	Growth on S1	Colony types	Pigment[c]	UV fluoresc.	Features
Y806	1	CV	BS	+	1	lg	+	–
Y1123	2	CV	TS	+	1	lg	+	–
Y699	3	NEO	NP	+	1	lb	–	Almost transparent colonies
Y1071	4	NEO	TS	+	1	ly	+	–
Y773 (V)	5	NEO	TS	+	1	g	+	Mixed colonies
Y773 (N)	6	NEO	TS	+	1	dg	+	–
Y711	7	NUT	NP	+	1	g	+	–
Y1127	8	CLIN	FA	+	1	g	+	–
P229	9	CLIN	HE	+ (poor)	1	y	+	Small and transparent colonies
Q175	10	BURN	SB	+	1	–	–	–
Y731	11	CLIN	TS	+	1	g	–	–
Y863	12	ICU	NP	+	1	–	–	–
Y800	13	ICU	TS	+	1	gy	+	–
Y851	14	ICU	ES	+	1	b/g	+	–
P812 (V)	15	ICU	HE	+	1	g	+	Mixed colonies
Y787	16	ICU	TS	+	1	g	+	–
U767	17	ICU	UC	+	1	g	+	–
P812 (N)	18	ICU	HE	+	1	g	+	–
Y788	19	CLIN	NP	+	1	–	+	–
Y846	20	CLIN	TS	+	1	bg	+	–
Y1053	21	CLIN	TS	+	1	lg	+	–
Y1125	22	EXT	BS	+	1	gy	+	–
FQ8373.1	23	PNE	BS	+	1	ly	–	–
FQ8374	24	PNE	BS	+ (poor)	1	gy	+	Small colonies
FQ8371 (1)	25	PNE	BS	+	2	lg	+	Mixed colonies (some mucoid)
FQ8371 (2)	26	PNE		+		lg	+	
FQ8373.2	27	PNE	BS	+	1	lg	+	Mucoid colonies
FQ8388	28	PNE	BS	+	1	g	+	–
FQ8396	29	PNE	BS	+	1	g	+	–
FQ8375 (1)	30	PNE	BS	+	2	ly	+	Mixed colonies
FQ8375 (2)	31	PNE		+		ly	+	
FQ8382	32	PNE	BS	+	1	ly	+	Hypermucoid
D170	33	CV	CT	+	1	lg	+	–
D199	34	CV	CT	+	1	lg	–	Transparent colonies
L330	35	NEO	CRF	+	1	lg	+	Rough colonies
P1951	36	NEO	HE	+	1	yg	+	Radiated rough colonies
P2943-1	37	NEO	HE	+	1	lg	+	–
P2943-2	38	NEO	HE	+	1	lg	+	Mucoid colonies
P2968	39	NEO	HE	+	1	lyg	+	–
P1138	40	CLIN	HE	+	1	lg	+	Mucoid colonies
P2984	41	CLIN	HE	+	1	yg	+	–
P2946	42	CLIN	HE	+	1	lg	+	–
P2652	43	HEM	HE	+	1	lyg	+	Mucoid colonies
Y1471	44	TRAU	A	+	1	lg	+	Slightly mucoid colonies
P2942	45	ICU	HE	+	1	lg	+	–
Y1258	46	ICU	TS	+	1	lg	+	–
Y1268	47	ICU	TS	+	1	yg	+	Slightly mucoid colonies
Y1312	48	ICU	BS	+	1	lg	+	Mucoid colonies
U831	49	EXT	UC	+	1	lg	–	Mucoid colonies
FQ8396	50	PNE	BS	+	1	g	–	–
FQ8399	51	PNE	BS	+	1	db	–	Mucoid colonies
FQ8415	52	PNE	BS	+	1	lg	–	–
FQ8417	53	PNE	BS	+	1	lg	+	Rough colonies
FQ8418	54	PNE	BS	+	1	vlg	–	Small, rough and transparent colonies
FQ8418 m	55	PNE	BS	+	1	bg	–	Mucoid colonies
FQ8433	56	PNE	BS	+	1	g	–	Small colonies
FQ8438	57	PNE	BS	+	1	ly	–	Small transparent colonies
FQ8470	58	PNE	BS	+	1	lg	+	Large and rough colonies
FQ8471 m	59	PNE	BS	+	1	vlb	–	Mucoid colonies
FQ8472 m	60	PNE	BS	+	1	vlb	–	–
FQ8472 nm	61	PNE	BS	+	1	bg	–	Mucoid colonies
FQ8514 p.r.	62	PNE	BS	+ (poor)	1	lb	–	Tiny colonies

[a] CV, Cardiovascular; NEO, Neonatology; NUT, Nutrition; CLIN, General Clinics; BURN, Burns Unit; ICU, Intensive Care Unit; EXT, External; PNE, Pneumology; HEM, Hemotherapy; TRAU, Traumatology.

[b] BS, bronchial secretion; TS, tracheal secretion; NP, nasopharyngeal aspirate; FA, foot abscess; HE, hemoculture; SB, skin burn; ES, eschar; UC, uroculture; CT, catheter tip; CRL, cephalorachidian fluid; A, abscess.

[c] lg, light green; lb, light brown; ly, light yellow; g, green; dg, dark green; y, yellow; gy, greenish yellow; b, brown; yg, yellowish green; bg, brownish green; vlb, very light brown; vlg, very light green; db, dark brown; lyg, light yellowish green.

Table 2
Collection of sputa sampled from fibrocystic pediatric patients from the Hospital de Niños of La Plata (Buenos Aires, Argentina).

Sample ID[a]	Microorganism(s) isolated by classical cultivation-based approaches
S1	*Staphylococcus aureus*, *P. aeruginosa*
S2	mucoid *P. aeruginosa*
S3	*S. aureus*
S4	*S. aureus*, *Stenotrophomonas maltophilia*
S5	*S. aureus*, *Aspergillus* sp.
S6	Mixed flora
S7	*S. aureus*, *Serratia* sp.
S8	*Burkholderia cepacia* complex
S9	*S. aureus*, *P.aeruginosa*
S10	mucoid *P. aeruginosa*
S11	*S. aureus*
S12	*S. aureus*, *S. maltophilia*
S13	*S. aureus*
S14	*S. aureus*, mucoid and non-mucoid *P. aeruginosa*
S15	*S. aureus*, *Achromobacter* sp.

[a] Samples from which Pa could be isolated are highlighted in gray.

hospitalized pediatric patients, and we tested the effectiveness of the *oprF* PCR-RFLP when applied directly on clinical samples of the respiratory tract of infected fibrocystic patients.

2. Materials and methods

2.1. Strains and clinical isolates

A collection of sixty two isolates from the Central Laboratory of Hospital de Niños de La Plata "Sor María Ludovica" (La Plata, Buenos Aires, Argentina) was generated (Table 1). All isolates had been identified as Pa by classic microbiological and biochemical tests, namely, not fermenting in TSI, oxidase positive and able to grow at 42 °C (Mac Faddin, 2000), and later confirmed by the Vitek-2 automated system (bioMérieux, France). Until transported to our lab, isolates were conserved at 4 °C as aqueous suspensions. Once in the lab, isolates were first streaked on the *Pseudomonas*-selective medium Gould's S1 (Gould et al., 1985) and incubated at 37 °C for 72 h. Isolated colonies were re-streaked onto nutrient agar plates (NA; tryptone soy agar 4% w/v, yeast extract 0.5% w/v).

2.2. DNA preparation from isolates and PCR assay targeting the oprF gene

Template DNA was obtained from fresh colonies developed on NA plates. One single colony (1–2 mm in diameter) was resuspended in 100 μl of deionized water and treated at 100 °C for 10 min. After centrifugation 1 min at 12,000 ×g, the supernatant containing the template DNA was recovered and conserved at −20 °C. PCR reactions were carried out in a total volume of 20 μl containing 1 μl of template DNA, 200 μM of each dNTP, 20 pmol of each oligonucleotide, 2.5 mM of $MgCl_2$, 1× Taq buffer and 1 U of recombinant Taq DNA polymerase (Taq Pegasus; PB-L). The cycling program consisted in an initial denaturation step of 2 min at 95 °C, followed by 30 cycles of 1 min at 95 °C, 1 min at 57 °C and 1 min at 72 °C, and a final elongation step of 2 min at 72 °C. The oligonucleotides *oprF*f (5′-ATYGGYTACTTCCTKACCGA-3′) and *oprF*r (5′-GACAGYTTYTGGTTGTAAGCGTC-3′) were designed upon alignment of the *oprF* sequences from all the pseudomonad species that were available in GenBank (NCBI) in February 2009. With this primer combination, the expected size of the *oprF* amplicon is 635 bp for *Pseudomonas protegens* strain CHA0 (Crespo and Valverde, 2009; Stutz et al., 1986) and 710 bp for Pa strain PAO1 (Holloway, 1969). Such polymorphism among pseudomonad groups is due to an internal deletion of 15–25 residues within the cysteine-rich motif present in the central linker region of the protein (Bodilis et al., 2006). The Pa isolates PAO1 (Holloway, 1969), Hex1T (Pezza et al., 2002) and MPAO1 (Luján et al., 2007) were included as positive controls. Deionized water was utilized as negative control for PCR reactions.

An aliquot of 4 μl from each PCR reaction was analyzed by agarose gel electrophoresis (1% w/v) for 60 min at constant voltage (5 V/cm). The rest of the positive PCR reactions were kept at −20 °C for further treatment with restriction endonucleases. Gels were stained with ethidium bromide (50 μg/ml), destained by immersion in deionized water and visualized on a UV transilluminator. Images were captured with a Kodak Electrophoresis Documentation and Analysis System-DC 120 (Eastman Kodak Company, Rochester, NY).

2.3. RFLP analysis of oprF amplicons from isolates

Each amplicon was restricted separately with the endonucleases *Taq*I (Promega) and *Hae*III (Fermentas). These enzymes were chosen based on their ability to generate distinguishable RFLP patterns for different pseudomonad strains (Agaras et al., 2012). Each restriction reaction was carried out in a final volume of 20 μl containing 7 μl of PCR product, 1× buffer and 2 U of the corresponding restriction enzyme. Reactions were incubated overnight at 37 °C for *Hae*III or at 62 °C for *Taq*I. The restriction reaction time can be reduced to 3 h without changes in the restriction profile. The digestion products were separated in 2% w/v agarose gels for 120 min at 5 V/cm. Gels were processed as described above for image documentation.

2.4. DNA sequencing of 16S rDNA and oprF PCR products from isolates

The 16S rDNA gene of the chosen isolates was PCR amplified with primers P0 and P6 (Picard et al., 2000). Partial DNA sequencing was carried out by Macrogen Inc. (Seoul, Korea) with primers P0 and P6 for 16S rDNA amplicons, and with primers *oprF*f and *oprF*r for *oprF* amplicons. The set of partial 16S rDNA and *oprF* sequences has been deposited in GenBank under Accession codes KJ721166 to KJ721172. Partial 16S rDNA sequences were used to query the Seqmatch tool of the Ribosomal database project II (Cole et al., 2009). Partial *oprF* sequences were analyzed with the BlastN tool in the NCBI database.

2.5. DNA extraction from clinical samples, PCR amplification and RFLP analysis of oprF amplicons

A set of fifteen sputa from infected and non-infected patients (listed in Table 2) was collected and stored at −20 °C. 100 μl of each of the samples were initially treated for 20 min at 90 °C with 1 volume of the inactivation/digestion solution (SDS 2%, NaOH 0.2 M, NaCl 2 M). Total DNA was prepared from the pre-treated samples by a phenol–chloroform extraction and subsequent nucleic acid precipitation with isopropanol (Sambrook, 1989). The DNA pellets were resuspended in 25 μl of deionized water. PCR reactions were carried out each in a total volume of 20 μl containing 1 μl of template DNA (either undiluted, or as 5- or 50-fold dilutions), 200 μM of each dNTP, 20 pmol of each oligonucleotide, 1.25 mM of $MgCl_2$, 1× Taq buffer and 1 U of recombinant Taq DNA polymerase (Taq Pegasus; PB-L). The cycling program consisted in an initial denaturation step of 2 min at 95 °C, followed by 35 cycles of 1 min at 95 °C, 30 s at 59 °C and 1 min at 72 °C, and a final elongation step of 2 min

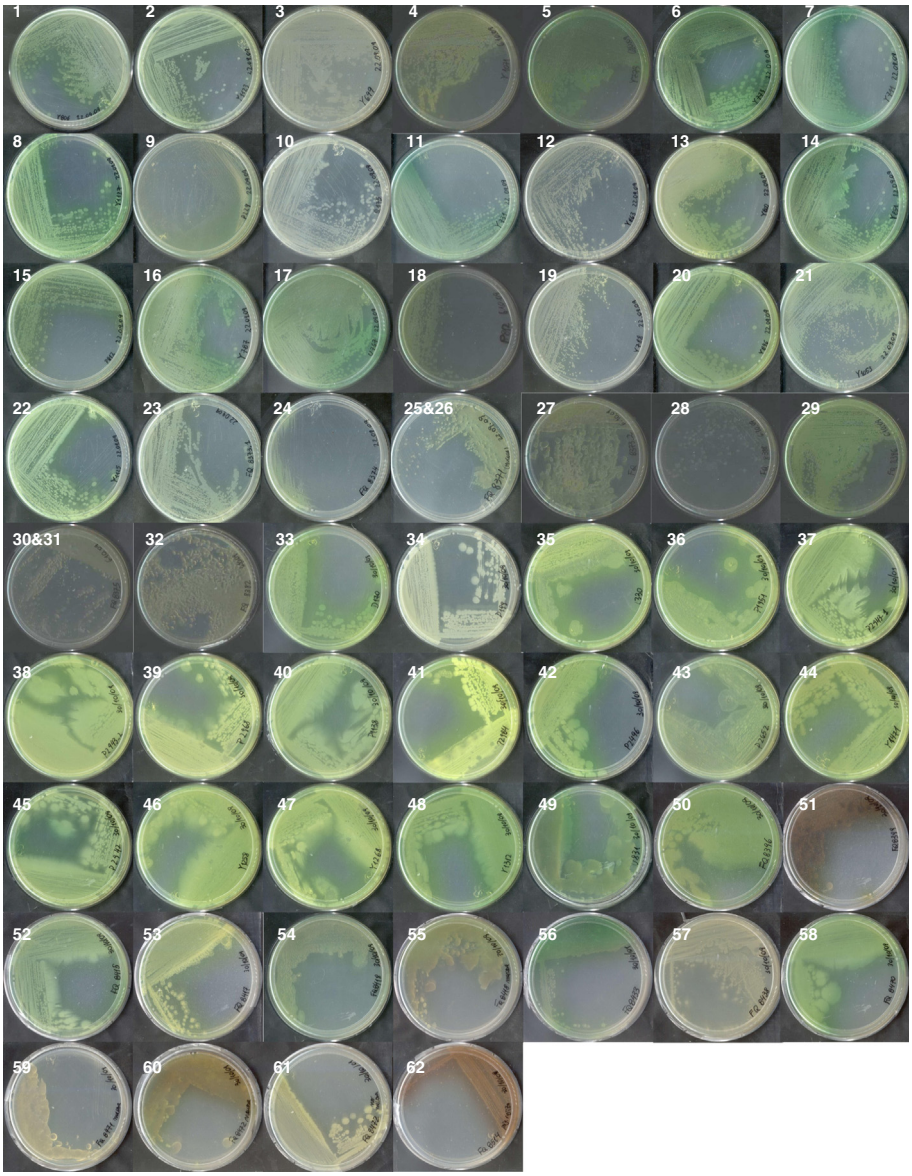

Fig. 1. Overview of the pseudomonad collection from pediatric patients isolated at the Central Laboratory of the Hospital de Niños of La Plata (Buenos Aires, Argentina). The corresponding sample IDs (see Table 1) are indicated at the upper left corner of each plate. Isolates were grown in Gould's S1 selective medium for 72 h at 37 °C. All isolates had been firstly identified as *P. aeruginosa* based on microbiological and biochemical criteria.

at 72 °C. The PCR reaction products were analyzed by agarose gel electrophoresis (1% w/v) for 60 min at constant voltage (5 V/cm). For each sample, the best performing dilution of the template DNA was chosen to repeat the PCR for amplicon restriction analyses with the endonuclease *Taq*I (Promega) or *Hae*III (Fermentas). Each restriction reaction was carried out in a final volume of 20 μl containing 10 μl of PCR product,

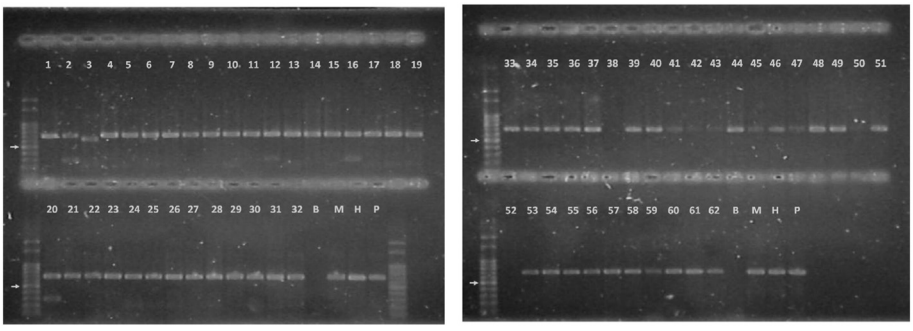

Fig. 2. Agarose gel electrophoresis analysis of the PCR reaction targeting an internal fragment of the *Pseudomonas* marker gene *oprF*, applied to the collection of isolates described in Table 1. Each number refers to the corresponding isolate ID as shown in Table 1. M, *P. aeruginosa* MPAO1; H, *P. aeruginosa* Hex1T; P, *P. aeruginosa* PAO1. The reference bands correspond to a 100 bp-ladder (PB-L, Argentina) covering the range 100–1000 bp, with two additional bands of 1500 and 2080 bp, being the stronger band that of 500 bp (white arrows). Amplicons of the expected size were obtained for isolates 38, 50 and 52 after a second amplification attempt (not shown).

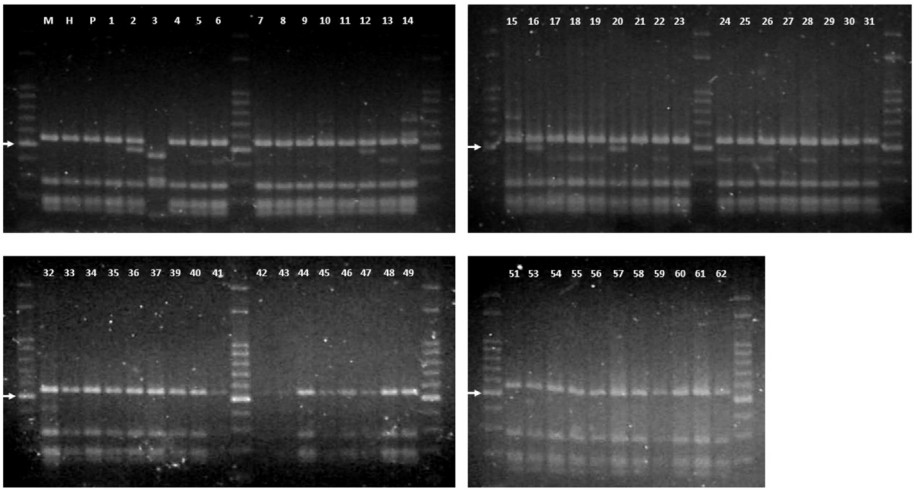

Fig. 3. RFLP pattern of the *oprF* amplicons (Fig. 2) after treatment with *Taq*I endonuclease. Each number refers to the corresponding isolate ID as shown in Table 1. M, *P. aeruginosa* MPAO1; H, *P. aeruginosa* Hex1T; P, *P. aeruginosa* PAO1. The reference bands correspond to a 50 bp-plus ladder (PB-L, Argentina) covering the range 50–500 bp, with two additional bands of 750 and 1000 bp, being the stronger band that of 250 bp (white arrows).

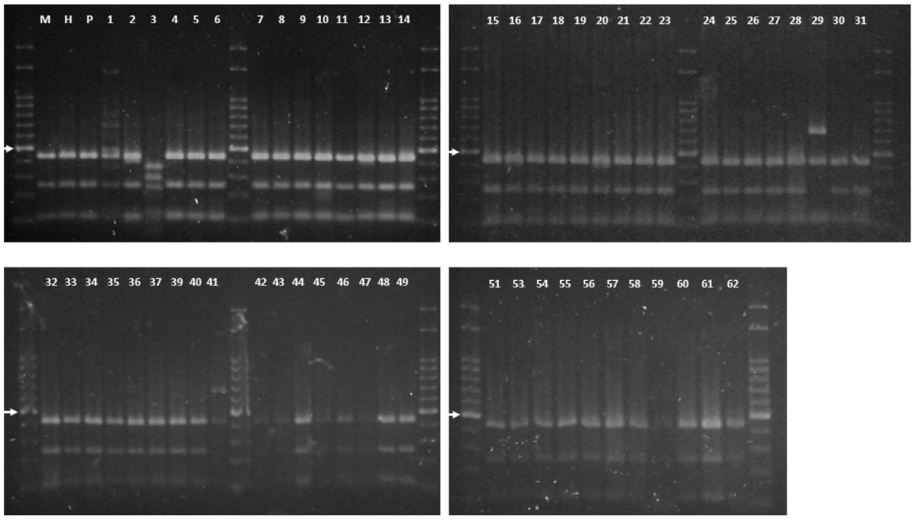

Fig. 4. RFLP pattern of the *oprF* amplicons (Fig. 2) after treatment with *Hae*III endonuclease. Each number refers to the corresponding isolate ID as shown in Table 1. M, *P. aeruginosa* MPAO1; H, *P. aeruginosa* Hex1T; P, *P. aeruginosa* PAO1. The reference bands correspond to a 50 bp-plus ladder (PB-L, Argentina) covering the range 50–500 bp, with two additional bands of 750 and 1000 bp, being the stronger band that of 250 bp (white arrows).

```
  41 TACGAAACCGGCAACAAGAAGGTCCACGGCAACCTGACCTC**T**CTGGACGCCATCTACCAC 106
PAO1 TACGAAACCGGCAACAAGAAGGTCCACGGCAACCTGACCTCCCTGGACGCCATCTACCAC 300
  29 TACGAAACCGGCAACAAGAAGGTCCACGGCAACCTGACCTC**T**CTGGACGCCATCTACCAC 90
     ****************************************▲******************

  41 TCCGACTCCGACAACGACGGCGTTTGCGACAACGTCGACAAGTGCCCGGATACCCCGGC**T** 466
PAO1 TCCGACTCCGACAACGACGGCGTTTGCGACAACGTCGACAAGTGCCCGGATACCCCGGCC 660
  29 TCCGACTCCGACAACGACGGCGTTTGCGACAACGTCGACAAGTGCCCGGATACCCCGGC**T** 450
     ********************************************************▲

  41 GACTCCGTCGGCACCG**T**CGCTTAC---------------------------------- 670
PAO1 GACTCCGTCGGCACCGACGCTTACAACCAGAAGCTGTCCGAGCGTCGTGCCAACGCCGTT 900
  29 GACTCCGTCGGCACCG**T**CGCTTACAACC------------------------------ 658
     ***************▲*******
```

Fig. 5. Single nucleotide polymorphisms detected within the *oprF* PCR products of isolates 29 and 41 (Table 1). The figure shows the alignment of *oprF* sequences from isolates 29 and 41 together with that of reference strain PAO1. Only those sequence segments bearing nucleotide replacements are shown; the rest of the amplified sequences were identical to that of PAO1. The substituted positions are highlighted in bold. Nucleotides shadowed in gray within PAO1 sequence point to a *Hae*III recognition site that is lost in isolates 29 and 41, and that results in a distinct PCR-RFLP profile (Fig. 4). The numbers at the right margin correspond to the nucleotide position of the *oprF* gene of strain PAO1, and to the first nucleotide of the sequenced PCR fragment of isolates 29 and 41.

1× buffer and 2 U of the corresponding restriction enzyme. Reactions were incubated overnight at 37 °C for *Hae*III or at 62 °C for *Taq*I. The digestion products were separated in 2% w/v agarose gels for 120 min at 5 V/cm. Gels were processed as described above for image documentation.

The integrity and amplificability of the DNA samples were verified via PCR targeting a partial sequence of the human β-globin gene, with primers GH20/PC04 (Bauer et al., 1991). PCR reactions were carried out in a total volume of 20 μl containing 1 μl of template DNA, 200 μM of each dNTP, 20 pmol of each oligonucleotide, 2.5 mM of $MgCl_2$, 1× Taq buffer and 1 U of recombinant Taq DNA polymerase (Taq Pegasus; PB-L). An initial denaturation step was performed at 94 °C for 2 min, followed by 40 cycles of 30 s at 94 °C, 30 s at 55 °C and 30 s at 72 °C, and a final elongation step at 72 °C for 5 min. PCR products were run in a 1.5% agarose gel electrophoresis. Gel staining and image acquisition were performed as described before.

3. Results

3.1. Collection of clinical isolates from pediatric patients identified as *P. aeruginosa*

A collection of isolates identified as Pa was compiled aiming to cover a broad range of clinical origins. Sixty two isolates were provided by the Central Laboratory of the Hospital de Niños "Sor María Ludovica" from La Plata (Buenos Aires, Argentina), from both internal and external patients. Isolates were derived from different pediatric sections at the Hospital, i.e. Pneumology (37%), Intensive Care (18%) and Neonatology (14%), among others (Table 1). Most of the isolates were obtained from bronchial secretions (42%), followed by those from nasopharyngeal aspirates (20%) and tracheal secretions (18%) (Table 1). Once at our lab, all isolates were streaked onto pseudomonads-selective medium Gould's S1. The growth on S1 plates served to analyze the diversity of colony features and pigment production (Table 1). Non-mucoid isolates

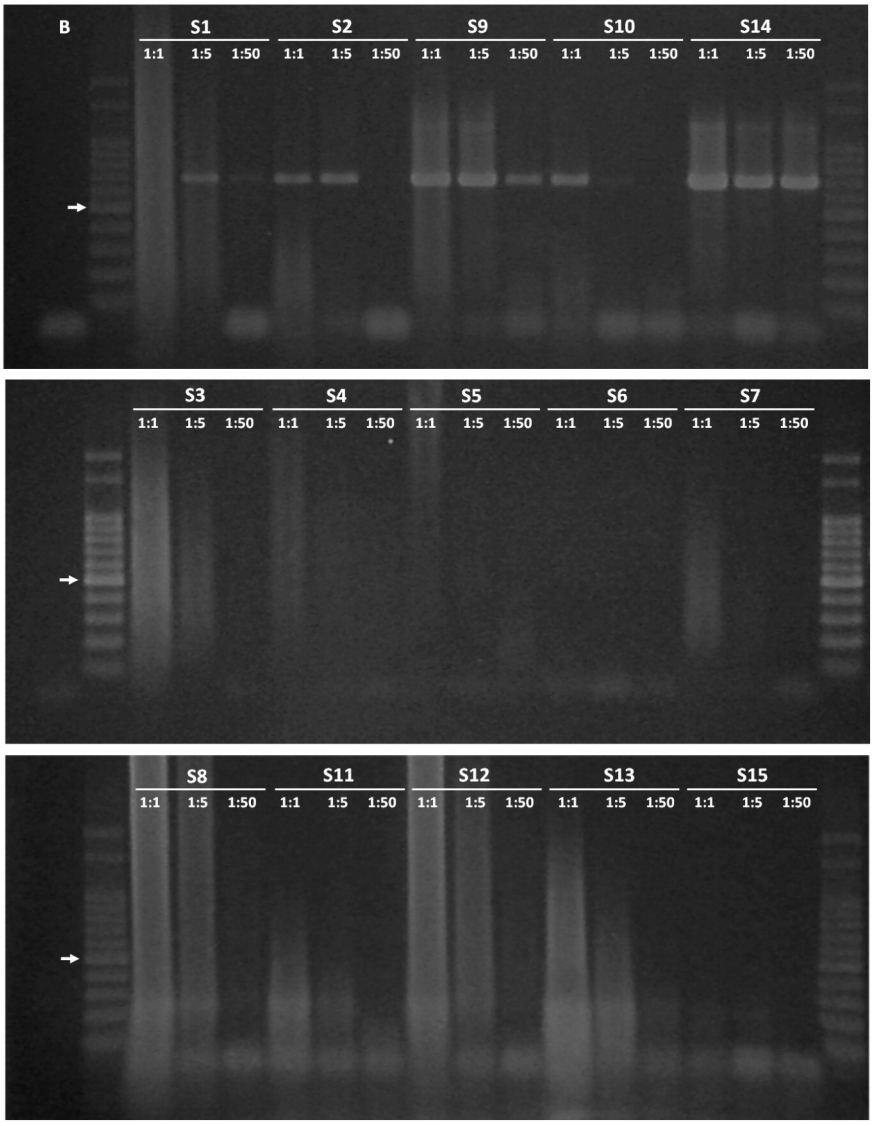

Fig. 6. Agarose gel electrophoresis analysis of the PCR reaction targeting an internal fragment of the pseudomonads marker gene *oprF*, applied to total DNA extracts directly prepared from clinical samples. For each sample, the PCR reaction results using as template the undiluted DNA preparation and their 5-fold and 50-fold dilutions are shown. The numbers refer to the corresponding sample ID as shown in Table 2. B, blank (negative control). The reference bands correspond to a 100 bp-ladder (PB-L, Argentina) covering the range 100–1000 bp, with two additional bands of 1500 and 2080 bp, being the stronger band that of 500 bp (white arrows). All the samples gave an expected amplicon for the PCR targeting the human β-globin gene, proving their integrity and amplificability (data not shown).

were dominant (79%), followed by mucoid (19%) ones; only one isolate was hypermucoid. With regard to pigment production, most isolates produced diffusible pigments (92%); moreover, 71% of the compounds were fluorescent under UV light. The wide phenotypic diversity of the collection is shown in Fig. 1.

3.2. Successful detection of the genus-specific oprF amplicon in all presumptive P. aeruginosa isolates

Fresh colonies suspended in deionized water were lysed by thermal treatment to provide a source of DNA for PCR reactions targeting an internal fragment of the *Pseudomonas*-specific gene *oprF*. Reference Pa strains produced an amplicon with the expected size (710 bp) (Fig. 2). The size of the PCR products from 61 out of 62 isolates was indistinguishable from that of the reference strains (Fig. 2). Notably, isolate 3 produced an amplicon of smaller size (ca. 650 bp) (Fig. 2). DNA sequencing of both *oprF* and 16S rDNA amplicons confirmed that isolate 3 was actually a *Pseudomonas mendocina* strain, bearing a deletion within the OprF central linker domain, a trait described for a selected group of pseudomonad taxa (Bodilis et al., 2004). Although no specific amplicon was obtained for isolates 38, 50 and 52 at the first amplification attempt, the expected PCR product was observed after repeating the reaction (data not shown).

3.3. The oprF PCR-RFLP is useful for species identification of Pseudomonas spp. clinical isolates

In order to explore the diversity contained within the *oprF* amplicon from the isolate collection, *oprF* PCR products were treated with the restriction endonuclease *Taq*I or *Hae*III, which are commonly used in molecular fingerprinting methods. Most of the isolates showed the same RFLP pattern with either nuclease, with 4 bands of approx. 80, a doublet of 100, 140 and 270 bp for *Taq*I (Fig. 3), and 3 bands of approx. 60 (doublet), 130 and 220 bp (doublet) for *Hae*III (Fig. 4). These patterns coincided with those of the reference strains, as expected from the available *oprF* sequence. In contrast, isolate 3 produced different RFLP profiles with both enzymes, which is also consistent with the smaller size detected for the *oprF* amplicon (Fig. 2). Interestingly, in spite of having an *oprF* PCR product of comparable size to those from the rest of the isolates, isolates 29 and 41 generated a differential *Hae*III RFLP profile with three bands of approx. 70, 220 and 350 bp (Fig. 4). This suggested that these two isolates had nucleotide changes within a *Hae*III site that in most isolates allows cleavage of the 350 bp-fragment into the 220 bp- and 130 bp-bands (Fig. 4). This was confirmed by sequencing the *oprF* amplicon of isolates 29 and 41, which revealed a C → T silent transition in the third position of the 220th codon encoding alanine, thus resulting in the loss of a *Hae*III restriction site (Fig. 5). Moreover, sequencing of the *oprF* amplicon allowed detection of two additional single nucleotide polymorphisms (SNPs) (Fig. 5). These observations suggest that there are at least two different *oprF Hae*III-RFLP profiles indicative of the presence of Pa in samples from pediatric patients.

3.4. The oprF-targeted PCR-RFLP allows detection and identification of Pa in clinical samples without cultivation

Upon demonstration of the robustness of the PCR-RFLP assay for the identification of clinical isolates of Pa, we next evaluated the performance of the assay when applied directly to clinical samples without strain isolation. A set of 15 clinical samples from fibrocystic pediatric patients were analyzed (Table 2). The expected *oprF* PCR product was obtained for all samples in which Pa had been previously isolated by culture-based microbiological assays (S1, S2, S9, S10 and S14; shown in Fig. 6). Furthermore, the *Taq*I and *Hae*III-based RFLP analyses performed on the PCR products obtained directly from the clinical samples confirmed the presence of Pa strains (Fig. 7).

4. Discussion

The PCR protocol reported here allowed amplification of an internal fragment of approx. 710 bp corresponding to the *Pseudomonas oprF* gene in 61 out of 62 tested clinical isolates. The application of restriction endonucleases *Taq*I and *Hae*III on the *oprF* amplicons resulted in highly prevalent RFLP patterns (over 94%). Moreover, in a previous work, none of the Pa RFLP profiles coincided with those registered for other pseudomonad species (Agaras et al., 2012). This observation supports the application of this RFLP protocol for identification Pa strains present in clinical samples.

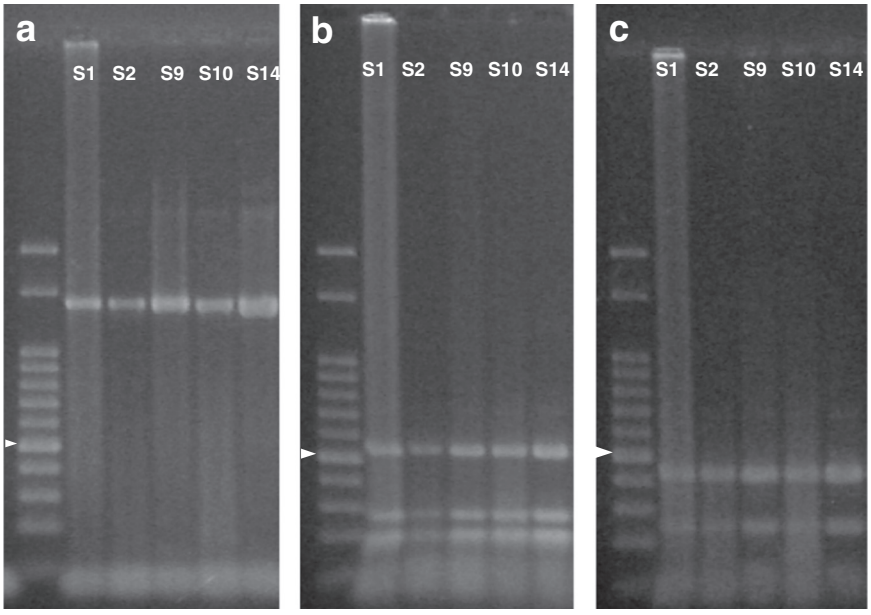

Fig. 7. RFLP pattern of the *oprF* amplicons from DNA extracts of samples without cultivation, (a) after treatment with *Taq*I (b) or *Hae*III (c) endonucleases. Each sample code refers to the corresponding isolate ID as shown in Table 2. The reference bands correspond to a 50 bp-plus ladder (PB-L, Argentina) covering the range 50–500 bp, with two additional bands of 750 and 1000 bp, being the stronger band that of 250 bp (white arrowheads).

Unlike reported PCR assays that target other Pa-specific genetic markers as *algP*, *gyrB* and *groES* (Clarke et al., 2003; da Silva Filho et al., 1999; Deschaght et al., 2011; Motoshima et al., 2007), the *oprF* PCR-RFLP assay reported here is — to our knowledge — the first genus-specific PCR-RFLP assay that allows identification of pseudomonads at the species level, provided both the PCR reaction and the RFLP profiling proved useful to detect and differentiate strains present in environmental (Agaras et al., 2012) and clinical samples (Figs. 2–4). Although there is no clear clinical relevance yet for the presence of infrequent non-Pa pseudomonads in patients with cystic fibrosis, the epidemiological meaning of detecting non-Pa *Pseudomonas* has started to be a focus of study upon recognition of environmental strains as reservoirs of antimicrobial resistance determinants (Docquier et al., 2003; Gilarranz et al., 2013; Juan et al., 2010, Juan and Oliver, 2010; Viedma et al., 2014). In this context, we propose that the application of the PCR-RFLP assay reported here to monitor the presence of non-Pa pseudomonads in clinical samples (even if not originated in the respiratory tract of fibrocystic patients), will be useful for the epidemiologic surveillance of *Pseudomonas* spp. populations in the hospital environment. Classical microbiological practices based on culturing methods and characterization of biochemical properties of the isolates could eventually lead to misidentification of non-Pa *Pseudomonas* strains (Figs. 2–4). The method presented here would help to overcome this issue.

Moreover, we provide evidence that the *oprF*-targeted PCR assay is able to detect pseudomonads in DNA extracts from samples of fibrocystic pediatric patients without sample cultivation (Fig. 6). In addition, the RFLP analysis of the *oprF* amplicons generated from DNA extracts from clinical samples, revealed the characteristic banding profile of Pa in all samples that were also positive for Pa upon microbiological and biochemical characterizations (Fig. 7). Thus, the *oprF* PCR-RFLP is able to detect Pa in clinical pediatric samples without cultivation. We observed that detection of the expected *oprF* amplicon required some samples to be diluted before the PCR reaction (Fig. 6), possibly due to the co-extraction of inhibitory substances present in the clinical sample. For this reason, we suggest assaying different dilutions of the same sample DNA extract. A positive result for the *oprF* PCR-RFLP on sample DNA extracts is particularly relevant because detecting the presence of the pathogen through its DNA without its isolation at first attempt, may prompt the laboratory to focus on isolation of the strain from new collected samples without major delay.

In addition to Pa, there are other pathogens associated to cystic fibrosis, i.e. species of the *Burkholderia cepacia* complex (Martina et al., 2013), *Achromobacter xylosoxidans* (Liu et al., 2002) and *Stenotrophomonas maltophilia* (Gallo et al., 2013; Pinot et al., 2011; Roscetto et al., 2008), for which molecular detection methods are available. It would be worth trying to consider setting up a multiplex PCR-RFLP assay targeting the parallel detection and identification of these microorganisms.

As reported elsewhere (Deschaght et al., 2009; Deschaght et al., 2011), a wide variety of methods have been used so far to extract DNA from respiratory tract samples, from those based on nucleic acid organic extraction to those silica-based commercial extraction kits. Considering the macroscopic heterogeneity of the sputum samples that we have processed, we selected the phenol-chloroform extraction as the gold standard technique to be used for the downstream application of the *oprF* PCR-RFLP procedure. We are currently working to develop a cheaper, easily accessible, non-commercial protocol for DNA template preparation from clinical samples that does not require organic extraction but retains the high effectiveness for the detection.

5. Conclusions

Altogether, our results support the use of the PCR-RFLP protocol targeting *oprF* for the detection and species identification of Pa strains present in clinical samples from fibrocystic pediatric patients with high effectiveness and, more importantly, without cultivation. In addition, the *oprF* PCR-RFLP assay proved useful to detect the presence of other less frequent pseudomonads of increasing epidemiological relevance. Their surveillance will hopefully contribute to the understanding of the dynamics of pseudomonad population diversity in a clinical niche and their contribution to the clinical evolution of fibrocystic patients. For the reasons exposed above, the *oprF* PCR-RFLP assay represents an excellent, simple and cheap complement to the conventional microbiological methods for rapid and early diagnosis of Pa infection and for detection on non-Pa strains in fibrocystic patients.

Acknowledgments

This work was funded by Universidad Nacional de Quilmes (grant PUNQ 1009/11). AL and BA were supported by PhD and postdoc fellowships from CONICET. CV is a member of CONICET.

References

Agaras, B., Wall, L.G., Valverde, C., 2012. Specific enumeration and analysis of the community structure of culturable pseudomonads in agricultural soils under no-till management in Argentina. Appl. Soil Ecol. 61, 305–319.

Bauer, H.M., Ting, Y., Greer, C.E., Chambers, J.C., Tashiro, C.J., Chimera, J., Reingold, A., Manos, M.M., 1991. Genital human papillomavirus infection in female university students as determined by a PCR-based method. J. Am. Med. Assoc. 265, 472–477.

Bodilis, J., Barray, S., 2006. Molecular evolution of the major outer-membrane protein gene (*oprF*) of *Pseudomonas*. Microbiology 152, 1075–1088.

Bodilis, J., Calbrix, R., Guerillon, J., Merieau, A., Pawlak, B., Orange, N., Barray, S., 2004. Phylogenetic relationships between environmental and clinical isolates of *Pseudomonas fluorescens* and related species deduced from 16S rRNA gene and OprF protein sequences. Syst. Appl. Microbiol. 27, 93–108.

Bodilis, J., Hedde, M., Orange, N., Barray, S., 2006. OprF polymorphism as a marker of ecological niche in *Pseudomonas*. Environ. Microbiol. 8, 1544–1551.

Clarke, L., Moore, J.E., Millar, B.C., Garske, L., Xu, J., Heuzenroeder, M.W., Crowe, M., Elborn, J.S., 2003. Development of a diagnostic PCR assay that targets a heat-shock protein gene (*groES*) for detection of *Pseudomonas* spp. in cystic fibrosis patients. J. Med. Microbiol. 52, 759–763.

Cole, J.R., Wang, Q., Cardenas, E., Fish, J., Chai, B., Farris, R.J., Kulam-Syed-Mohideen, A.S., McGarrell, D.M., Marsh, T., Garrity, G.M., Tiedje, J.M., 2009. The Ribosomal Database Project: improved alignments and new tools for rRNA analysis. Nucleic Acids Res. 37, D141–D145.

Crespo, M.C., Valverde, C., 2009. A single mutation in the *oprF* mRNA leader confers strict translational control by the Gac/Rsm system in *Pseudomonas fluorescens* CHA0. Curr. Microbiol. 58, 182–188.

da Silva Filho, L.V., Levi, J.E., Oda Bento, C.N., da Silva Ramos, S.R., Rozov, T., 1999. PCR identification of *Pseudomonas aeruginosa* and direct detection in clinical samples from cystic fibrosis patients. J. Med. Microbiol. 48, 357–361.

Deschaght, P., De Baere, T., Van Simaey, L., Van Daele, S., De Baets, F., De Vos, D., Pirnay, J.P., Vaneechoutte, M., 2009. Comparison of the sensitivity of culture, PCR and quantitative real-time PCR for the detection of *Pseudomonas aeruginosa* in sputum of cystic fibrosis patients. BMC Microbiol. 9, 244.

Deschaght, P., Van Daele, S., De Baets, F., Vaneechoutte, M., 2011. PCR and the detection of *Pseudomonas aeruginosa* in respiratory samples of CF patients. A literature review. J. Cyst. Fibros. 10, 293–297.

Docquier, J.D., Riccio, M.L., Mugnaioli, C., Luzzaro, F., Endimiani, A., Toniolo, A., Amicosante, G., Rossolini, G.M., 2003. IMP-12, a new plasmid-encoded metallo-beta-lactamase from a *Pseudomonas putida* clinical isolate. Antimicrob. Agents Chemother. 47, 1522–1528.

Folkesson, A., Jelsbak, L., Yang, L., Johansen, H.K., Ciofu, O., Hoiby, N., Molin, S., 2012. Adaptation of *Pseudomonas aeruginosa* to the cystic fibrosis airway: an evolutionary perspective. Nat. Rev. Microbiol. 10, 841–851.

Gallo, S.W., Ramos, P.L., Ferreira, C.A., Oliveira, S.D., 2013. A specific polymerase chain reaction method to identify *Stenotrophomonas maltophilia*. Mem. Inst. Oswaldo Cruz 108, 390–391.

Gilarranz, R., Juan, C., Castillo-Vera, J., Chamizo, F.J., Artiles, F., Alamo, I., Oliver, A., 2013. First detection in Europe of the metallo-beta-lactamase IMP-15 in clinical strains of *Pseudomonas putida* and *Pseudomonas aeruginosa*. Clin. Microbiol. Infect. 19, E424–E427.

Gould, W.D., Hagedorn, C., Bardinelli, T.R., Zablotowicz, R.M., 1985. New selective media for enumeration and recovery of fluorescent pseudomonads from various habitats. Appl. Environ. Microbiol. 49, 28–32.

Hoiby, N., 2011. Recent advances in the treatment of *Pseudomonas aeruginosa* infections in cystic fibrosis. BMC Med. 9, 32.

Holloway, B.W., 1969. Genetics of *Pseudomonas*. Bacteriol. Rev. 33, 419–443.

Juan, C., Oliver, A., 2010. Carbapenemases in *Pseudomonas* spp. Enferm. Infecc. Microbiol. Clin. 28 (Suppl. 1), 19–28.

Juan, C., Zamorano, L., Mena, A., Alberti, S., Perez, J.L., Oliver, A., 2010. Metallo-beta-lactamase-producing *Pseudomonas putida* as a reservoir of multidrug resistance elements that can be transferred to successful *Pseudomonas aeruginosa* clones. J. Antimicrob. Chemother. 65, 474–478.

Liu, L., Coenye, T., Burns, J.L., Whitby, P.W., Stull, T.L., LiPuma, J.J., 2002. Ribosomal DNA-directed PCR for identification of *Achromobacter* (*Alcaligenes*) *xylosoxidans* recovered from sputum samples from cystic fibrosis patients. J. Clin. Microbiol. 40, 1210–1213.

Luján, A.M., Moyano, A.J., Segura, I., Argaraña, C.E., Smania, A.M., 2007. Quorum-sensing-deficient (*lasR*) mutants emerge at high frequency from a *Pseudomonas aeruginosa mutS* strain. Microbiology 153, 225–237.

Lyczak, J.B., Cannon, C.L., Pier, G.B., 2000. Establishment of *Pseudomonas aeruginosa* infection: lessons from a versatile opportunist. Microbes Infect. 2, 1051–1060.

Mac Faddin, J.F., 2000. Biochemical Tests for Identification of Medical Bacteria. 3rd ed. Lippincott Williams & Wilkins, Philadelphia.

Martina, P., Bettiol, M., Vescina, C., Montanaro, P., Mannino, M.C., Prieto, C.I., Vay, C., Naumann, D., Schmitt, J., Yantorno, O., Lagares, A., Bosch, A., 2013. Genetic diversity of *Burkholderia contaminans* isolates from cystic fibrosis patients in Argentina. J. Clin. Microbiol. 51, 339–344.

Motoshima, M., Yanagihara, K., Fukushima, K., Matsuda, J., Sugahara, K., Hirakata, Y., Yamada, Y., Kohno, S., Kamihira, S., 2007. Rapid and accurate detection of *Pseudomonas aeruginosa* by real-time polymerase chain reaction with melting curve analysis targeting *gyrB* gene. Diagn. Microbiol. Infect. Dis. 58, 53–58.

Obritsch, M.D., Fish, D.N., MacLaren, R., Jung, R., 2005. Nosocomial infections due to multidrug-resistant *Pseudomonas aeruginosa*: epidemiology and treatment options. Pharmacotherapy 25, 1353–1364.

Oliver, A., 2010. Mutators in cystic fibrosis chronic lung infection: prevalence, mechanisms, and consequences for antimicrobial therapy. Int. J. Med. Microbiol. 300, 563–572.

Pezza, R.J., Smania, A.M., Barra, J.L., Argarana, C.E., 2002. Nucleotides and heteroduplex DNA preserve the active conformation of *Pseudomonas aeruginosa* MutS by preventing protein oligomerization. Biochem. J. 361, 87–95.

Picard, C., Di Cello, F., Ventura, M., Fani, R., Guckert, A., 2000. Frequency and biodiversity of 2,4-diacetylphloroglucinol-producing bacteria isolated from the maize rhizosphere at different stages of plant growth. Appl. Environ. Microbiol. 66, 948–955.

Pinot, C., Deredjian, A., Nazaret, S., Brothier, E., Cournoyer, B., Segonds, C., Favre-Bonte, S., 2011. Identification of *Stenotrophomonas maltophilia* strains isolated from environmental and clinical samples: a rapid and efficient procedure. J. Appl. Microbiol. 111, 1185–1193.

Roscetto, E., Rocco, F., Carlomagno, M.S., Casalino, M., Colonna, B., Zarrilli, R., Di Nocera, P.P., 2008. PCR-based rapid genotyping of *Stenotrophomonas maltophilia* isolates. BMC Microbiol. 8, 202.

Sambrook, J., 1989. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press.

Stutz, E.W., Défago, G., Kern, H., 1986. Naturally occurring fluorescent pseudomonads involved in suppression of black root rot of tobacco. Phytopathology 181–185.

Tummler, B., Kiewitz, C., 1999. Cystic fibrosis: an inherited susceptibility to bacterial respiratory infections. Mol. Med. Today 5, 351–358.

Veesenmeyer, J.L., Hauser, A.R., Lisboa, T., Rello, J., 2009. *Pseudomonas aeruginosa* virulence and therapy: evolving translational strategies. Crit. Care Med. 37, 1777–1786.

Viedma, E., Estepa, V., Juan, C., Castillo-Vera, J., Rojo-Bezares, B., Seral, C., Castillo, F.J., Saenz, Y., Torres, C., Chaves, F., Oliver, A., 2014. Comparison of local features from two spanish hospitals reveals common and specific traits at multiple levels of the molecular epidemiology of metallo-beta-lactamase-producing *Pseudomonas* spp. Antimicrob. Agents Chemother. 58, 2454–2458.