Molecular Characterization Of Pseudomonas Aeruginosa Clinical Isolates Among Patients Of The Hospital Del Niño, Republic Of Panama

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Citation

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Abstract

The molecular characterization of 37 clinical isolates of P. aeruginosa obtained during September 2013 and September 2014 was conducted based on the 16S rDNA and oprL genes. The genetic diversity among isolates was determined with ERIC-pcr. The segments of the opr L and 16s rDNA genes were used to detect all P. aeruginosa isolates. The ERIC-PCR bands generated patterns that allowed the separation of isolates into different groups or clades and classify them into different genotypes.

The susceptibility pattern of the isolated strains against ceftazidime, imipenem, gentamicin and amikin was assessed by a disk diffusion method. The percentage of isolates resistant to ceftazidime, imipenem, gentamicin and amikin was 14%, 16%, 16% and 24% respectively. In general these percentages are relatively low compared to those reported in other countries and only one of the isolates (64113) showed multidrug resistance.

INTRODUCTION

Pseudomonas aeruginosa is a ubiquitous gram-negative rod shaped bacterium with a polar flagellum. Among fluorescent pseudomonads belonging to the rRNA group I of the gamma subclass of Proteobacteria, it is the only recognized human pathogen (De Vos et al., 2001, Palleroni, 2008).

Pseudomonas aeruginosa is a nosocomial human pathogen in immuno-compromised patients, such as those with cystic fibrosis, cancer, burns, and those hospitalized in intensive care units. Infections caused by Pseudomonas species include endocarditis, pneumonia, and infections of the urinary tract, central nervous system, wounds, eyes, ears, skin, and musculoskeletal system. (Pirnay et al., 2000, 2002, Campana et al., 2004).

The use of catheters, medical devices, and chemical disinfectants represent an optimal means for acquiring nosocomial infections and therefore efforts to control the spread of the infections are difficult. In addition the emergence of multidrug resistant isolates of P. aeruginosa may lead to severe infections and life-long treatments. These infections are untreatable because of the higher resistance to antimicrobial agents and lack of new drug development (Aendekerk et al., 2005, Stheling et al., 2010, Luna de Araujo, de et al., 2012, Nikbin et al., 2012, Kan et al., 2014).

The virulence factors of P. aeruginosa such as exotin A, exoenzyme S, pyoverdine, elastase and sialicidase among others are strongly regulated by cell to cell signaling systems that contribute to its pathogenecity (Cornelis et al., 1989, Van Delden & Iglewski, 1998, Khalifa et al., 2011). The outer membrane proteins of P. aeruginosa play an important role in the bacteria-environment interactions and therefore to the pathogenic infections. The inherent resistance of P. aeruginosa to antibiotics is a consequence of the presence of specific proteins in the outer membrane, efflux pumps that affect cellular permeability. The outer membrane lipoproteins OprL and OprI have been implicated in these efflux transport systems (De Vos et al., 1997, Matthijs, Khattab, et al., 2015).

P. aeruginosa identification has been based on phenotypic observations like the growth on selective media such as cetrimide agar, bacto casamino acids, and serotyping and anti-microbial-testing. However other gram-negative bacilli including other Pseudomonas species may interfere with the phenotypic typing of P. aeruginosa and this represents a drawback for the treatment of patients, particularly with regard to antimicrobial therapy and control of infection in hospitals. The genotyping methods solve the variable phenotype problem and are specific for the identification of P. aeruginosa. Since OprL and OprI proteins are found only in this organism they could be a reliable factor for rapid identification of P. aeruginosa in clinical samples. The 16S rDNA gene and the enterobacterial repetitive intergenic consensus sequences (ERIC) have been successfully used in epidemiological studies of several microorganisms, including P. aeruginosa (Stehling et al., 2010).

In this study 37 P. aeruginosa clinical isolates from patients of the Hospital del Niño, Republic of Panama were characterized phenotypically with selective media and their susceptibility to antibiotics. The oprL and 16s rDNA molecular markers were used for rapid identification of the isolates and the ERIC-PCR was used to determine the genetic diversity between the isolates.

MATERIALS AND METHODS

Bacterial Isolates and Culture methods

A total of 37 strains of P. aeruginosa were isolated from various sites of infections (blood, tracheal secretions, wound, eye and ear discharge and ulcer) of patients from the Hospital del Niño, República de Panamá and provided to the microbiology laboratory, between September 2013 and September 2014. First the isolates were grown on selective media such as cetrimide agar (AC) (Scharlau Chemie S.A., España) and bacto casaminoacids (CAA) (Becton, Dickinson Company, Estados Unidos) and incubated for 24 h at 37°C to verify pigment (pyoverdine) production as previously described (De Vos et al.,2001). Samples of all strains were store in 50% glycerol at -80°C.

Antimicrobial susceptibility

The antimicrobial susceptibility was performed using Kirby-Bauer's disc-diffusion method as per CLSI (Clinical Laboratory Standard Institute) guidelines for antimicrobial susceptibility. The antibiotics used were ceftazidime (30 g) (CAZ), amikin (30 g) (AK), imipenem (10 g) (IPM) and gentamicin (10 g). P. aeruginosa ATCC 27853 was used as a control strain for this study.

DNA Extraction

Isolates were grown in CAA liquid medium at 37°C overnight and the DNA was extracted according to methods for gram-negative bacteria previously described (Chen and Kuo, 1993)

PCR amplification of the oprL and 16S rDNA genes

The amplification of these genes was carried out in a final volume of 25 L following a method previously described. (De Vos, et al., 1997, Nikbin et al., 2012, Spilker, et al., 2004).

ERIC PCR

ERIC-PCR typing was carried out using the primers ERIC1 (5' ATG TAA GCTCCTGGGGATTCAC-3') and ERIC2 (5' AAGTAAGTGACTGGGGTGAGCG3') in a final volume of 50 L as previously described (Tosin et al., 2003). The amplification products (10 L) were separated in a 0.8% agarose gel and stained with ethidium bromide for 3 h at 80V. The genomic fingerprints obtained were compared for similarity by visual inspection of band patterns. These patterns were converted to a two-dimensional binary matrix (1 = presence of band; 0 = absence of band)

The genetic distance between isolates was calculated using Nei's genetic standard distance (Nei, 1987) and the allele distance DAS (Chakraborty y Jin, 1993) using the software Populations 1.2.30 beta (Languella, 2005). The cluster analysis of the Isolates was carried out using the software MEGA 6.06 and the UPGMA method (Unweighted Pair Group Method using Arithmetic averages); 1000 bootstrap samplings were used to obtain a confidence tree.

RESULTS

Microbial and antibiotic susceptibility analysis

The 37 clinical isolates of P. aeruginosa were grown on AC agar and CAA agar. Only 3 strains did not produce pyoverdine in CAA agar (Table 1). The antibiotic sensitivity and resistance pattern were as studied in these isolates (Table 2). The highest rate of resistance was amikin (24%) and the lowest rate of resistance the antibiotic ceftazidime (14%). The pattern of susceptibility was 76% to ceftazidime, 78% to imipenem, 73% to gentymicin and 68% to amikin. Three strains showed an intermediate

susceptibility to ceftazidime that represents an 8%. In few isolates the susceptibility pattern was not determined, it represents 2% in ceftazidime, 5% in imipenem, 11 % in gentamycin and 8% in amikin. The strain 20 (641113) showed a multidrug resistance profile to all antibiotics (Table 3).

Molecular Analysis

The rapid identification of P. aeruginosa was performed with fragment amplification of the oprL and 16S rDNA genes. Two PCR products of 504 and 618 bp for the oprL y 16S rDNA respectively were obtained (figure 1, 2).

The ERIC-PCR fingerprints showed a banding pattern with sizes that ranged from approximately 3000 to 1500bp and an average of 6 amplicons (figure 3). By the UPGMA analysis based on the Nei genetic distance and the DAS allele distance, there were 3 major clusters, 2 of which are the negative control strains. The UPGMA based on Nei genetic distance is shown (figure 4). Among the isolates the positive control is included and the dendogram showed identical clusters that include strains 4 and11, strains 23 and 24; strains 3 and 7; and strains 12, 26, 30 are genetically identical (figure 4).

DISCUSSION

Pseudomonas aeruginosa is a nosocomial pathogen responsible for the high morbidity and mortality of immunocompromised patients. The infections caused by this bacterium are detected by standard methods such as growth on specific culture medias. This has been one of the most important techniques for diagnosis in microbiology due to easy quantification and the possibility of obtaining a pure sample for further studies, however other additional phenotypic test are always needed to obtain the exact identification. Biochemical tests have facilitated the identification of P. aeruginosa but are time consuming. The API 20E test for identification of P. aeruginosa is not so useful as it does not clearly distinguish between related species and can only be applied at genus level (Ferguson et al., 2007, Hassan et al., 2012 Workentine et al., 2013).

In this study all P. aeuruginosa strains were isolated and identified at the Microbiology Laboratory of the Hospital del Niño. All isolates showed the characteristic odor and pyoverdine production in the CA and CAA media (Hassan et. al., 2012, 2014). The CA is a differential media for the identification of P. aeruginosa, in which cetrimide acts as a detergent that inhibits the growth of most bacteria and improves the production of pyoverdine and piocianine pigments (Hameed et al., 2014). CAA is an iron-restricted media that is used for siderophore production (Meyer et al., 1996)

The resistant patterns of the 37 clinical isolates were determined. In the present study out of the 37 isolates 14% showed resistance to ceftazidime, 16% to imipenem, 16% to gentymicin and 24% to amikin. The resistance pattern to ceftazidime is low compared to the 90% and 71% values reported for Brazil and Peru respectively (Goncalves et al., 2009; Luján et al., 2008) and similar to the values reported in Cuba and France (Pérez et al., 2006; Minchella et al., 2010). The resistance pattern to imipenem is considerably low compared to those reported in Mexico, Brazil, Venezuela, India and Nigeria (Luján-Roca, 2014). The resistance values reported for gentymicin and amikin are also low compared to those reported in Nigeria, Tunisia and Korea (Luján-Roca, 2014).

The susceptibility patterns of the 37 clinical isolates were determined as well. In this study out of the 37 isolates, 76% were sensitive to ceftazidime, 78% to imipenem, 73% to gentymicin and 68% to amikin. In a previous study the susceptibility values were different, 35% to ceftazidime and 36% to gentymicin; but were similar to imipenem and amikin with 73% and 66 % values respectively (Luján-Roca et al., 2008). In Clinical isolates obtained in Jamaica, the susceptibility values for ceftazidime, 80% and 78% Gentamycin are similar to the values for the same antibiotics in this study and the sensitive values were different to the values exhibited for the same antibiotics in this study, 90% and 100% for imipenem and amikin respectively (Brown e Izundu, 2004). Clinical isolates from Iran hospitals displayed lower susceptibility values, amikin in 28%, ceftazidime in 28%, gentymicin, 40% and imipenem en 38% than those found in this study (Golshani et al., 2012).

Resistance to antimicrobial agents may have low power of discrimination and is often associated with mobile genetic elements (transposons, plasmids). Antibiogram changes may also reflect spontaneous point mutations such as those that have been observed for fluoroquinolones. Some isolates that are epidemiologically linked and are genetically indistinguishable may show different antimicrobial susceptibilities due to the acquisition of new genetic material in time or loss of plasmids. Conversely, unrelated isolates may have indistinguishable resistance profiles, which may

represent the acquisition of the same plasmid by multiple species (Tenover et al., 1997).

The 64113 isolate (20) can be considered as a multidrug resistant strain (MDR) for its resistance to 4 antibiotics used in this study. Several studies worldwide have reported the presence of multiresistant strains (Salma, 2010; Kerr and Snelling, 2009; Mauldin et al., 2010; Tumbarello et al, 2011). It has been shown that resistance of P. aeruginosa to antimicrobial agents is due to synergy between MDR efflux pumps and the low permeability of the outer membrane (Tam et al., 2010). The resistance may also result from the production of metallo -lactamases, which may be encoded on a plasmid or on the chromosome (Livermore, 2001, Aendekerk et al., 2005, Luna de Araujo, et al., 2012).

Methods of detection in selective media are prolonged, very unreliable and require a quick and accurate diagnosis for an effective treatment of infections caused by this bacterium, and to control the spread of it in the hospitals as well (Salman et al., 2013, Xu et al., 2004).

Molecular methods for the rapid detection of P. aeruginosa, have been developed, specially the polymerase chain reaction (PCR), which is highly sensitive and specific. The ERIC-PCR technique, the16S rDNA and oprL genes were used in this study. The 16S rDNA gene has been widely used in taxonomy to identify the phylogeny of species of bacteria (Woese, 1977, Whitman et al., 1998). Selective amplification of 16S rDNA by PCR gene has been used to differentiate species of Pseudomonas from clinical and environmental samples (Salman et al., 2013). In this study the 16S rDNA gene specific for the Pseudomonas genus was used for the 37 isolates obtaining a 618 bp amplification fragment in all of them as previously reported (Spilker, et al., 2004). The oprL gene has been used for the identification of P. aeruginosa clinical isolates. The results of this study are consistent with previous studies since the expected 504-bp amplification fragment in all isolates was obtained (De Vos et al., 1997, Hassan et al., 2012, Khattab et al., 2015).

Studies have reported that the ERIC-PCR technique is among the most useful molecular methods to establish the variability among isolates of P. aeruginosa (Jena et al., 2015 in press). This technique detected the differences in the number and distribution of repetitive sequences in the isolates. The most prominent bands were repeated in all the isolates (350 bp, 450bp, 700 bp and greater than 1000 bp) and other studies report similar bands (Wolska and Szweda, 2008). The control strain P. aeruginosa ATCC 27853 displayed a similar profile to the isolates while P. stutzeri and P. putida gave a completely different profile showing the utility of this technique to discriminate against P. aeruginosa. No correlation was observed between the profiles obtained by ERIC-PCR and resistance patterns. None of the isolates showed resistance to at least three of the four antibiotics used pooled in the same clade. These results may suggest that the resistance in these isolates responded more to genes located on extrachromosomal elements.

This study demonstrated the utility of the 16S rDNA, oprL genes and the ERIC-PCR technique for the easy and specific identification of P. aeruginosa, indicating that it can be used as a routine diagnosis in Panamanian hospitals.

Table 1

P. aeruginosa Isolates growth on CAA and AC media

	Cepa	AC	CAA Pioverdina	Source
C-	31548P. stutzeri	+ low growth	_	Blood culture
C-	26110P.putida	+ low growth	_	Blood culture
C+	ATCC27853	+	+	Positive Control
1	60018	+	+	Endotracheal aspirate
2	22913	+	+	Blood
3	60136	+	+	Soft tissue (Ulcer)
4	74393	+	+	Blood culture-Catheter
5	71196	+	+	Soft tissue(Nasal)
6	72872	+	+	Soft tissue (Left ear)
7	61869	+	+	Soft tissue (Right eye)
8	45806	+	+	Blood culture
9	60569	+	+	Bronchial secretion
10	63691	+	+	Pleural fluid
11	59264	+	+	Peripheral Blood
12	65287	+	+	Periferal blood
13	65410	+	+	Urine culture
14	61757	+	+	Blood
15	27450	+	+	Peripheral blood
16	74508	+	+	Soft tissue
17	59026	+	+	Peripheral blood
18	63095	+	+	Bronchial secretion
19	65402	+	+piov-pioc	Soft tissue (Ear Intern)
20	64113	+	+	Soft tissue (eye)
21	65467	+	+	Wound (right ear)
22	62953	+	+	Peripheral blood
23	78029	+	+	Soft tissue Pleural
24	74343	+	+	Catheter blood culture
25	60111	+	+	Abdominal wound
26	65875	+	+	Peripheral blood
27	73116	+	+	Abscess
28	73908	+	+	Soft tissue (Ear)
29	69566	+	+	Endotracheal aspirate
30	70314	+	+	Peripheral blood
31	78467	+	+	Ear intern
32	59959	+	-	Peripheral blood
33	75936	+	+	
34	65372	+	+	Urine culture
35	75977	+		Central venous catheter
36	74956	+	+	Peripheral blood
37	69343	+	+	Peripheral blood

Table 2

Antimicrobial susceptibility profile of Pseudomonas aeruginosa isolates from the Hospital del Niño, Panamá between years 2013 and 2014

Cepas		Antibióticos			
		Ceftazidime	Imipenem	Gentymicin	Amikin
C-	31548 *P. stutzeri (aislado)*	S	S	S	S
C-	26110 *P. putida(aislado)*	S	S	R	S
C+	ATCC27853	S	S	S	S
1	60018	R	R	S	R
2	22913	S	S	S	S
3	60136	I	R	R	R
4	74393	S	S	S	S
5	71196	S	S	S	S
6	72872	S	S	S	S
7	61869	S	S	S	S
8	45806	S	S	S	S
9	60569	S	ND	ND	R
10	63691	S	S	S	S
11	59264	S	S	S	S
12	65287	S	S	S	S
13	65410	S	S	S	S
14	61757	S	S	S	S
15	27450	S	S	S	S
16	74508	S	S	R	R
17	59026	S	S	S	S
18	63095	R	S	S	S
19	65402	S	S	S	S
20	64113	R	R	R	R
21	65467	S	S	ND	ND
22	62953	ND	ND	ND	ND
23	78029	S	S	S	S
24	74343	S	S	S	S
25	60111	S	R	S	S
26	65875	S	S	S	S
27	73116	S	S	S	R
28	73908	S	S	S	S
29	69566	S	S	ND	S
30	70314	S	S	R	R
31	78467	S	S	S	R
32	59959	S	S	S	S
33	75936	I	S	S	S
34	65372	S	S	S	S
35	75977	R	S	S	ND
36	74956	R	R	S	S
37	69343	I	R	R	R

R: Resistant, **I**: Intermediate **S**: Sensitive **ND**: undetermined

Table 3

Susceptibility pattern of P. aeruginosa isolate to different antibiotics (Number of strains)

Antibiotic	Susceptible	Intermediate	Resistant	No determined
ceftazidime,	28 (76%)	3 (8%)	5 (14%)	1 (2%)
imipenem	29 (78%)	0	6 (16%)	2 (5%)
gentymicin	27 (73%)	0	6 (16%)	4 (11%)
amikin	25 (68%)	0	9 (24%)	3 (8%)

Figure 1

PCR Amplification of 16S rDNA gene of P. aeruginosa isolates (lanes 2-11). Lane 1 is a 100 bp DNA ladder (Promega)

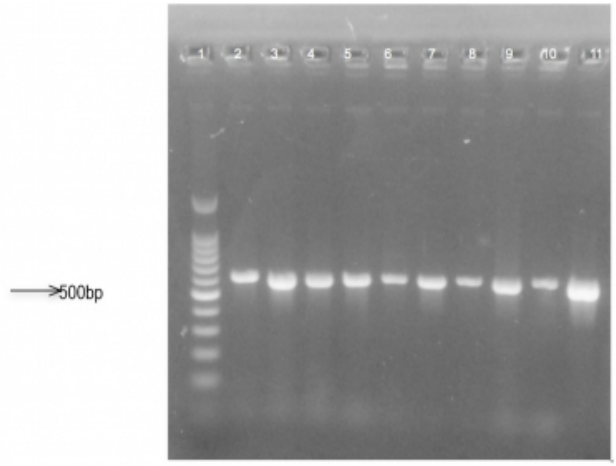


Figure 2

PCR Amplification of oprL 16S gene of P. aeruginosa isolates (lanes 2-10).). Lane 1 is a 100 bp DNA ladder (Promega)

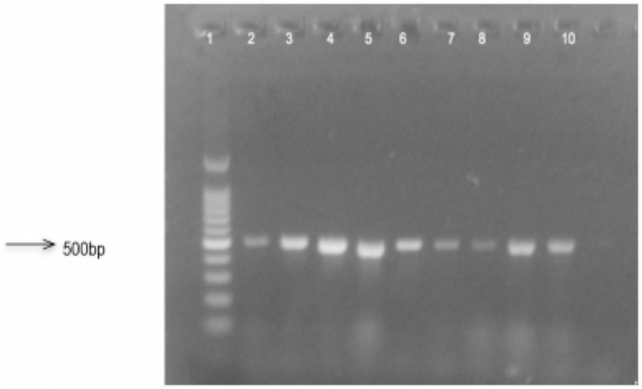


Figure 3

ERIC-PCR fingerprints of P. aeruginosa. Lane1: pst1, Lane 2: P. aeruginosa ATCC 27853; Lane 3-12: clinical isolates, Lane 13: 100bp DNA ladder; lanes 14-20: clinical isolates, Lane 21: P. stutzeri, lane 22: P. putida

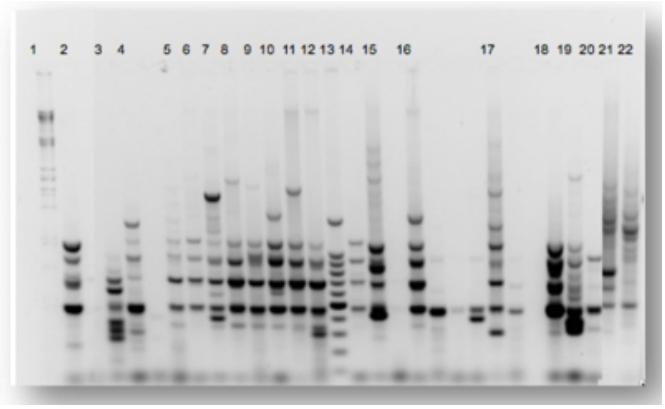
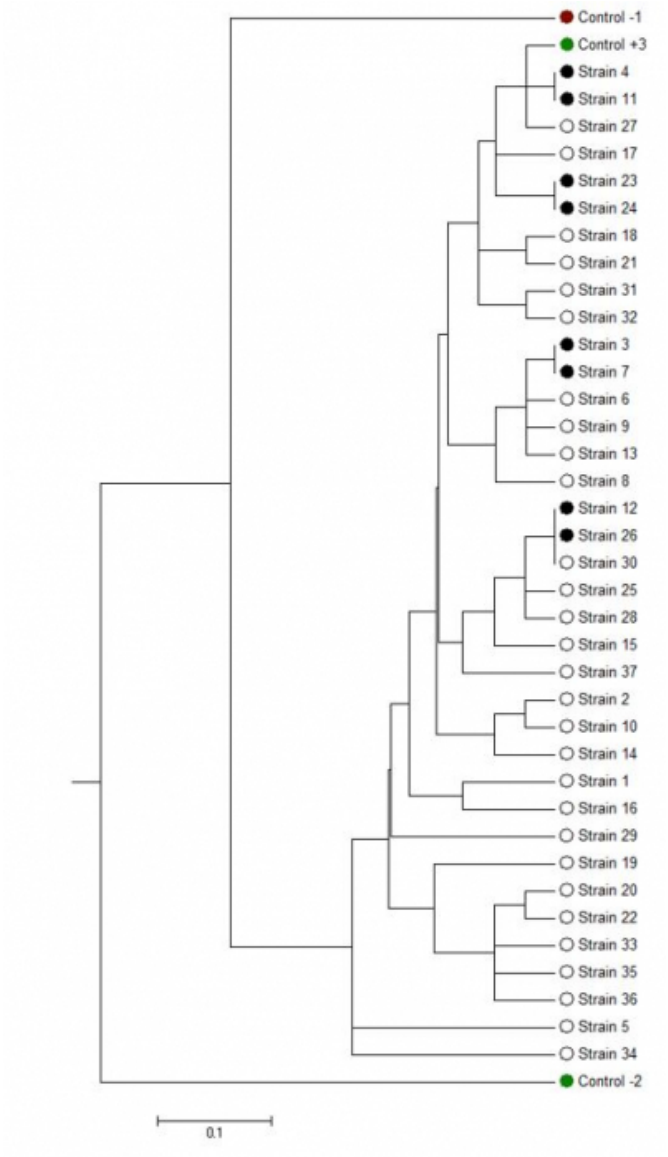


Figure 4

UPGMA based on the Nei genetic standard distance of the P aeruginosa isolates.



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