Genetic characterization of *Pseudomonas aeruginosa* isolated from contact lenses and other sources by RAPD analysis

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Abstract:
Pseudomonas aeruginosa (24 isolates) collected from contact lens storage cases, contact lenses and contact lenses wearer in Saudi Arabia. PCR amplification of all isolates revealed one fragment with 230 bp that represented 16S rDNA gene. A total of 48 amplified DNA fragments (from 3500 to 90 bp) were observed using three RAPD primers; B-01, B-11 and B-14.Whereas, 42 fragments were polymorphic and the other 6 amplified fragments were commonly detected among all *Pa* isolates. The three primers showed a mean polymorphism of 87%, whereas, the polymorphic % B-01 primer was higher (89%) followed by primer B-11 and B-14 with 88 and 83%, respectively. The constructed UPGMA dendrogram showed two main clusters; the first included 10 isolates with a medium bootstrap 83%, the second included the remaining 14 isolates with two sub-clusters. The first sub-cluster divided to two branches; the first contained isolates 21 & 23 and the second was divided more to two sub-branches. The second sub-cluster was divided to isolate 2 that occupied unique branch and two other branches that divided again to several sub-branches. Isolate 8 revealed most high similarity with isolate 7(77%) followed by isolates 18 and 17 (74%). Isolates 3 & 1 and 22 & 15 showed similar percentage (72%). Isolates 7 and 3 displayed (70%). Conclusion: high DNA polymorphism occurrence of amplified fragments &Variable genetic similarities of *Pa* isolates (24) was noticed. The fluctuation of genetic similarity percentages of each of the isolates with others revealed the divergent genetic backgrounds of different serotypes.

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***Key words*:** *Pseudomonas aeruginosa* isolates, contact lens and contact lenses wearer, 16S rDNA gene, RAPD-PCR

1.INTRODUCTION:

Sight-threatening microbial keratitis associated with contact lens wear remains a serious concern for patients, eye-care practitioners, and the contact lens industry. Several decades of research and some major advances in lens and solution technology have not resulted in a decline in disease incidence (Fleiszig *et al*., 2010).

Contact lens wearer continues to be a significant risk factor for the development of acute sight-threatening corneal infections; microbial keratitis (Ibrahim *et al.* 2009; Edwards *et al.*, 2009).

Pseudomonas aeruginosa is a Gram-negative opportunistic pathogen implicated in sight-threatening occular infectious diseases such as keratitis (Willcox 2007; Green *et al.*, 2008a). For more than 20 years in this field, Pearlman *et al.*, (2008) have worked toward understanding why the corneas of contact lens wearers are more susceptible to infection. However, the widespread use of contact lenses is now recognized as an increasingly common risk factor for development of corneal infection in otherwise healthy eyes (Green *et al.* 2008b).

P. aeruginosa has remained the most common cause of contact lens-related keratitis, accounting for 60–70 % of culture-proven cases (Cheng *et al.*, 1999). *P. aeruginosa* is also one of the most commonly isolated organisms in non-contact lens-related ocular trauma events that lead to keratitis (Parmar *et al.*, 2006). *P. aeruginosa* keratitis is considerably more common in contact lens wearers compared with non-contact lens wearers, presumably because of the altered ocular environment. Biofilms produced by *P. aeruginosa* are thought to be the main cause of persistent ocular infections associated with contact lens wearer (Costerton *et al.*, 1999) through attachment to contact lens and contact lens storage case surfaces (McLaughlin-Borlace *et al.*, 1998). Bacterial contamination of lenses and storage cases has been reported even in association with good compliance with care and hygiene regimens. Biofilm-associated *P. aeruginosa* contamination is found in both contact lens cases and disinfectants with rates varying between 24 and 81% (Zegans *et al.*, 2002).

Phenotypic traits expressed in biofilms are partially responsible for the emerging resistance against antimicrobial therapy (del Pozo_and Patel

2007) of contact lens-related keratitis. In addition, emergence of multi-drug resistance in *P. aeruginosa* strains (Rossolini and Mantengoli 2005) becomes a major concern when antibiotics such as fluoroquinolones are used as monotherapeutic agents. Choy *et al.,* (2008) suggested that *P. aeruginosa* isolates from different infection origins may have different characteristics. Seventeen eyes (63%) lost more than one line of visual acuity with a resultant permanent medical downgrading in duty capability in nine cases. Increased awareness of the health risks of contact lens wear together with standardized treatment regimens based on improved pathogen detection using molecular diagnostics have improved outcomes (Musa *et al.,* 2010).

Typing of bacterial isolates has been used for decades for studying local outbreaks e.g. nosocomial outbreaks, as well as, for national and international surveillance when monitoring newly emerging (resistant) clones, e.g. for pathogens such as *P. aeruginosa* (Inglis *et al.,* 2010). During the last decades, genotyping techniques (DNA fingerprinting) such as RAPD analysis have largely replaced phenotypic techniques, such as serotyping, phage susceptibility typing and protein SDS-PAGE. With the development of faster, cheaper and more automated sequencing capacity, sequence-based typing, such as multilocus sequence typing (MLST)is gradually replacing these DNA fingerprinting techniques. Whereas, MLST is a reference approach for large scale surveillance and for population biology studies, there remains a need for rapid, less laborious and cheap approaches, such as RAPD, on a local scale. Although the interrun reproducibility of this approach is known to be limited, it still offers the possibility of studying within a single run the genotypic relatedness of a limited number of isolates that might possibly belong to a single outbreak e.g. in a hospital or hospital ward (Deschaght *et al.,* 2011). We aimed in this study to identify *Pseudomonas aeruginosa* strains isolated from soft contact lenses belong to healthy persons, patients with contact lens-associated red eyes (CLARE), asymptomatic wearer (CLSCaw) and patients with keratitis by 16S rDNA gene and studying the genetic similarity and variations of the isolates using PCR-RAPD analysis.

2.MATERIALS AND METHODS:

2.1. Materials:

2.1.1.Bacterial strains and growth condition

A collection of 24 *Pseudomonas aeruginosa* clinical isolates were obtained from contact lens storage cases, contact lenses and contact lenses wearer between November 2010 and December 2011 in Saudi Arabia as shown in Table (1). All strains were stored in tryptic soy broth (TSB) containing 30% glycerol at -80°C. Isolates were inoculated on chocolate agar plates and incubated overnight at 37°C.

All the strains were identified by 16S rDNA gene, colony´s morphology, Gram staining, mobility characteristic of polar flagellation, pigment production, fluorescence, and phenotypic analysis with API 20 NE identification kit (bioMérieux, Marcy l'Etoile, France). Cells were stored at 4°C for further experiments.

2.1.2.Primers:

A pair of primer was designed as forward(5′-CGACGATCCGTAACTGGTCT-3′) and reverse(5′-CCGGTGCTTATTCTGTTGGT-3′) with a final product size of 230 bp. RAPD analysis was performed using three 10-mer random primers (Operon Technologies, Alameda, Calif.), Table (2) showed their names and sequences

Table 1. Contact lens-related and non-contact-lens-related P. aeruginosa (Pa)

No. of *Pa* isolates	Sources	The case of the source
7	Soft contact lenses	Used by healthy persons
4	Soft contact lenses	Patients with contact lens-associated red eyes (CLARE)
1	Eyes of contact lenses wearers	Asymptomatic wearer (CLSCaw)
12	Eyes of contact lenses wearers	Patients with keratitis

Table 2. Names and sequences of RAPD primers used for PCR analysis

Name	Sequences
B-01	5′ TGC GCC CTT C ′3
B-11	5′ GTA GAC CCG T ′3
B-14	5′ GTA GAC CCG T ′3

2.2. Methods:

2.2.1.DNA extraction and PCR amplification of 16S rDNA gene

Genomic DNA was prepared from 18 h cultures in an exponential phase in Luria-Bertani medium. DNA extraction was performed according to the method of Ben Haj Khalifa *et al.,* (2010). Aliquots of 10 ml of bacterial culture were harvested by centrifugation at 12,000 rpm for 15 min at 4°C and washed once in sterile distilled water.

The pellets were resuspended in 400 µl of lysis buffer containing 2% glucose, 50 mM Tris-HCl (pH 8.0), 25 mM EDTA, 3 mg/ml lysozyme and 200 mg/ml RNase. The cell suspension was incubated for 1 h at 37°C.

PCR amplification was carried out in a DNA thermocycler (Biometra, Germany) for 30 cycles each. The PCR reaction was carried out in a final volume of 25 µl with 1X PCR buffer containing 10

mM Tris-HCl, 25 mM $MgCl_2$, 1 µl of template DNA, 0.2 mM deoxynucleoside triphosphate, 2 µM (each) primer and 0.5 U of *Taq* DNA polymerase (Promega). PCR conditions of 16S rDNA and RAPD amplification consisted of initial denaturation at 95°C for 2 min followed by 95°C for 1 min, annealing to primers at 54°C for 1 min (16S rDNA) and 50°C for 1 min (RAPD) and extension at 72°C for 1 min with a final extension step at 72°C for 5 min. PCR-amplified products were separated using agarose gel electrophoresis in 1% TBE buffer and stained with 0.2 µg/ml ethidium bromide. Amplified fragments were detected and photographed under UV light. In order to rapidly identify the 24 *Pseudomonas aeruginosa* clinical isolates, the 16S rDNA gene was amplified using accession JN377436 obtained from the NCBI GenBank.

2.2.2.Genetic analysis

RAPD fragments were scored as present (+) or absent. The data was used for similarity-based analysis using the program MVSP (version 3.1b) from www.kovcomp.com. RAPD analyses were analyzed using the Nei genetic similarity index (Nei and Li 1979) based on the equation: Similarity = 2Nab/(Na+ Nb)

3.RESULTS:

Results in (Table 1) illustrated the origin of the isolated twenty four strains; one of them was isolated from patients with endophthalmitis, and 4 from contact lenses belongs to a patient with contact lens-associated red eye (CLARE). Twelve strains from patients with keratitis strains from consecutive patients attending King Khaled Eye Hospital in Riadh, Saudi Arabia over a 12-month period. The remaining five strains were isolated from contact lens cases belonging to asymptomatic wearers (CLSCaw).

PCR amplification of 16S rDNA in *Pseudomonas aeruginosa* clinical isolates

PCR amplification of the 24 *P. aeruginosa* clinical isolates revealed one fragment with 230 bp that represented the 16S rDNA gene (Fig. 1).

Genetic characterization of *P. aeruginosa* (*Pa*) isolates by RAPD analysis

A total of 48 amplified DNA fragments ranging in size from 3500 to 90 bp were observed using the three random amplified polymorphic DNA (RAPD) primers; B-01, B-11 and B-14, whereas 42 fragments were polymorphic and the other 6 amplified fragments were commonly detected among the 24 *Pa* isolates (Table 3).

The three primers showed a meanpolymorphism of 87%, whereas the polymorphic percentage of primer B-01 was higher (89%) followed by primer B-11 and B-14 with 88 and 83%, respectively.

Primer B-01 resulted in 19 fragments, 17 of which were polymorphic with sizes ranging from 2000 to 100 bp; two fragments were common among the 24 isolates (Fig. 2 and Table 3). The total number of amplified fragments of the isolates varied considerably. For instance, in isolates (3 and 7) 14 fragments with different molecular sizes were amplified while 6 fragments were amplified in isolates (13, 14 and 21). The remaining isolates were intermediate. RAPD analysis revealed that the amplified PCR products of most of the 24 isolates vary in molecular size patterns even with the equal total fragments.

Primer B-11 revealed 17 fragments, 15 of which were polymorphic with sizes ranging from 3500 to 90 bp (Fig. 2 and Table 3). The total fragment numbers of the 24 isolates varied significantly in their amplified fragments: whereas isolate 11 revealed the highest with 13 fragments, followed by isolates 9 and 10 with 12, while six isolates revealed two.

Primer B-14 revealed 12 fragments, 10 of which were polymorphic with sizes ranging from 750 to 110 bp and two fragments with 250 and 190 bp were commonly detected among all isolates (Fig. 2 and Table 3).

The total number of fragments of the 24 isolates varied substantially: for example, isolates 3, 6, 9, 11 and 12 showed ten, while isolates 21 and 22 showed four. Although, several isolates revealed equal total fragments they noticeably difference in their molecular sizes. For instant, isolates (3, 6, 9, 11 and 12) with 10 fragments were also quite differences due to appearance and disappearance of some fragments. Similar observation was also detected in isolates (1, 2, 5, 8 and 10) with nine fragments, isolates (4, 7 and 20) with eight, isolates (15 and 17) with seven, isolates (13, 14 and 24) with six and isolates (21 and 22) with four.

Genetic similarity of *P. aeruginosa* isolates using UPGMA dendrogram

Genetic similarity between each two pairs of the 24 *P. aeruginosa* isolates was performed using the Nei similarity index on the basis of RAPD amplified fragments using the three random primers (Table 4).

Genetic similarity between the 24 *P. aeruginosa* isolates was calculated from the amplified fragment data using un-weighted pair group method with averages (UPGMA).

The constructed UPGMA dendrogram of the three primers showed two main clusters, whereas the first includes 10 isolates with a medium bootstrap 83% and the second includes the remaining 14 isolates with two sub-clusters.

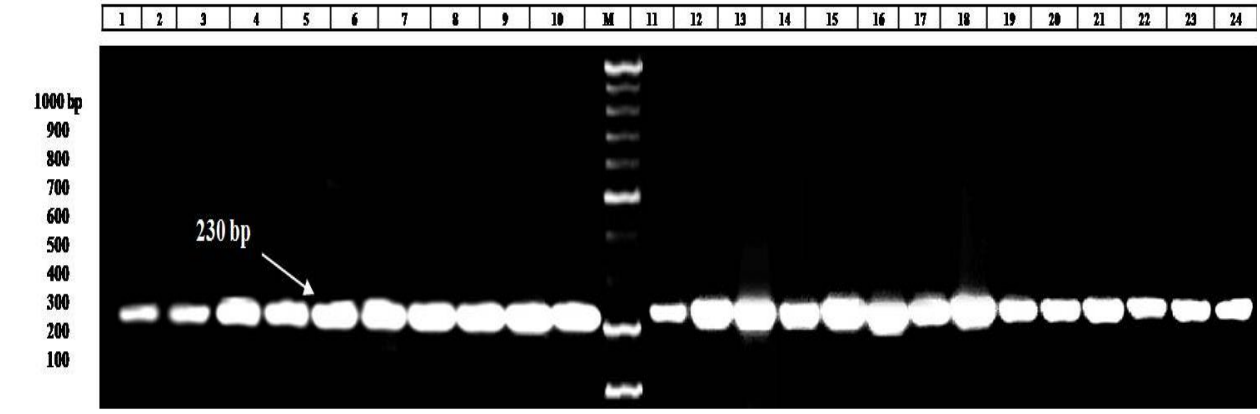

Fig. 1. PCR amplified products of 16S rDNA gene of the 24 *P. aeruginosa* isolates using the designed primers with expected size 230 bp.

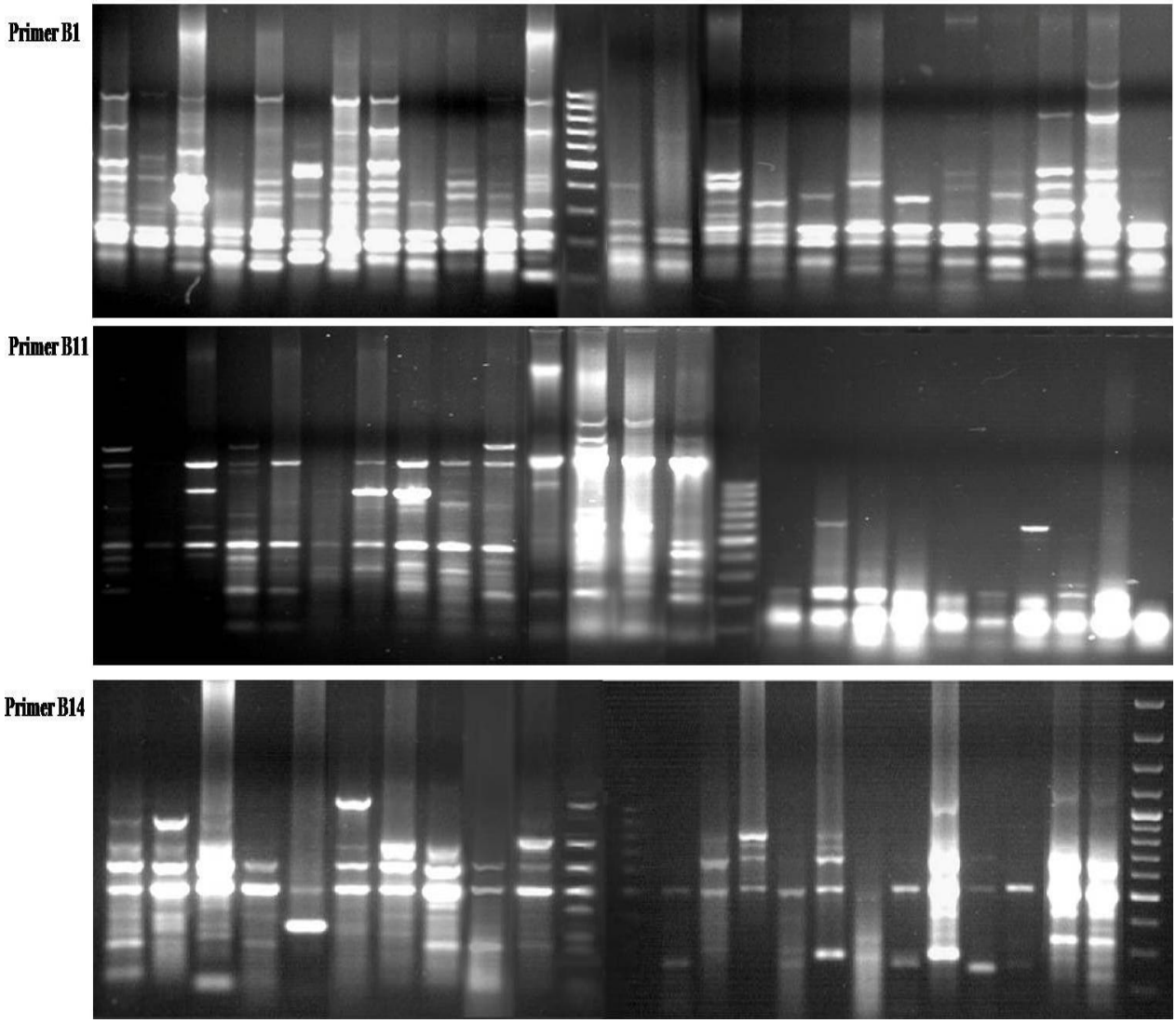

Fig. 2. RAPD amplified products of the 24 *P. aeruginosa* isolates using three random primers; B1, B11 and B1

Table 3. ***RAPD analysis of variable (polymorphic) fragments of the 24 P. aeruginosa isolates using three random primers; B1, B11 and B14***

Primer name	No	Ms (bp)	P%	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
	1	2000		+	+	+		+		+	+			+	+											+	
	2	1600			+	+				+																	
	3	1500				+																					
	4	1000		+	+	+		+		+	+			+	+			+							+	+	
	5	900				+	+	+	+	+	+			+													
	6	750			+	+			+																		
	7	690		+	+	+			+	+	+		+	+	+		+	+	+	+	+				+	+	+
	8	600	89	+	+	+		+		+	+		+	+	+			+	+			+	+	+	+	+	
B1	9	550		+																	+						
	10	500		+		+	+	+	+	+	+	+	+	+				+	+	+	+		+	+	+	+	+
	11	440			+	+				+	+	+	+	+	+			+	+	+	+	+	+	+	+		
	12	390		+	+	+	+	+		+	+		+	+				+			+	+	+		+	+	
	14	260																+	+	+							+
	16	180		+	+	+	+	+	+	+	+			+	+	+	+	+	+			+	+		+	+	
	17	160						+		+		+		+		+	+	+	+	+	+	+	+				+
	18	130					+			+		+			+	+	+	+	+	+	+	+	+	+	+	+	+
	19	100										+			+	+				+		+	+				+
	T=19			10	11	14	7	10	7	14	11	7	7	12	10	6	6	12	10	9	9	9	10	6	10	10	8
	1	3500		+	+								+	+	+												
	2	3000		+	+	+	+	+	+	+	+	+	+	+	+			+							+		
	3	2000										+		+	+								+				
	4	1500			+				+				+	+		+							+				
	5	1300			+	+				+	+	+															
B11	6	1000								+	+	+	+														
	7	820	88				+	+						+													
	8	750					+	+	+					+	+												
	9	560		+		+	+	+	+	+	+	+	+	+	+			+							+		
	11	450		+		+		+			+	+	+		+												
	12	350					+	+	+	+	+	+	+	+	+			+									
	13	290					+	+	+	+	+	+	+	+	+			+									
	14	270					+		+		+	+	+	+		+											
	15	220			+						+					+			+				+				
	16	150			+		+	+				+	+	+	+	+			+			+					
	T=17			6	8	6	10	10	9	8	11	12	12	13	11	6	2	6	4	2	2	3	5	2	4	2	2
	1	750								+	+		+	+	+												
	2	650				+		+	+			+		+	+								+				
	3	550		+	+	+			+				+		+			+		+							
	4	410	83	+	+	+	+	+	+	+		+	+	+	+		+	+		+			+				
	5	340		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+		+		+
B14	7	230		+	+	+	+	+	+		+	+	+	+						+	+	+	+			+	
	8	210		+	+	+	+		+	+	+	+		+	+	+							+				
	10	160		+	+	+	+	+		+	+	+	+	+	+	+	+	+	+				+	+	+	+	+
	11	130				+		+	+	+	+	+			+	+	+	+	+	+	+	+					+
	12	110		+		+	+	+	+	+	+	+	+	+		+								+		+	+
	T=12			9	9	10	8	9	10	8	9	10	9	10	10	6	6	7	5	7	5	5	8	4	4	5	6
Total=48			*87	25	28	30	25	29	26	30	31	29	28	35	31	18	14	25	19	18	16	17	23	12	18	17	16

Table 4. ***Genetic similarity percentages of the 24 isolates based on RAPD products of three random primers.***

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
2	61																						
3	**72**	66																					
4	52	40	49																				
5	59	43	64	69																			
6	50	42	60	65	57																		
7	56	51	**70**	56	62	50																	
8	60	51	**70**	56	62	54	**77**																
9	43	37	53	61	54	54	55	60															
10	61	51	53	56	54	54	55	64	60														
11	60	55	60	69	**70**	62	63	67	55	68													
12	56	51	56	47	58	50	59	55	60	55	67												
13	33	38	**32**	47	37	41	39	39	47	34	36	39											
14	39	36	38	39	39	38	45	**32**	36	**31**	**30**	41	57										
15	56	47	57	52	59	50	**70**	56	47	56	51	60	38	56									
16	38	42	40	38	41	**32**	47	43	38	38	36	43	58	65	63								
17	36	**32**	38	**31**	**31**	39	37	33	41	36	**28**	37	44	55	56	64							
18	41	33	40	37	36	36	42	38	38	38	**32**	**31**	40	58	52	59	**74**						
19	36	41	38	40	44	30	41	37	41	36	34	41	57	55	50	64	62	65					
20	42	47	44	42	42	37	43	43	47	39	47	43	58	44	47	58	50	52	63				
21	37	**29**	36	37	**32**	**27**	39	34	38	38	**31**	**30**	41	44	42	55	45	47	45	48			
22	59	48	55	48	47	38	58	53	39	48	49	49	37	52	**72**	61	46	55	52	54	58		
23	62	45	52	45	48	34	50	50	**32**	41	46	37	39	48	50	50	42	50	48	50	61	67	
24	38	**27**	36	38	38	37	44	35	43	34	**29**	35	62	**71**	48	62	68	63	52	48	59	50	52

+= present of fragment, T=total fragments, P%= polymorphism %, *Average polymorphism %, Ms= DNA ladder with 100 bp

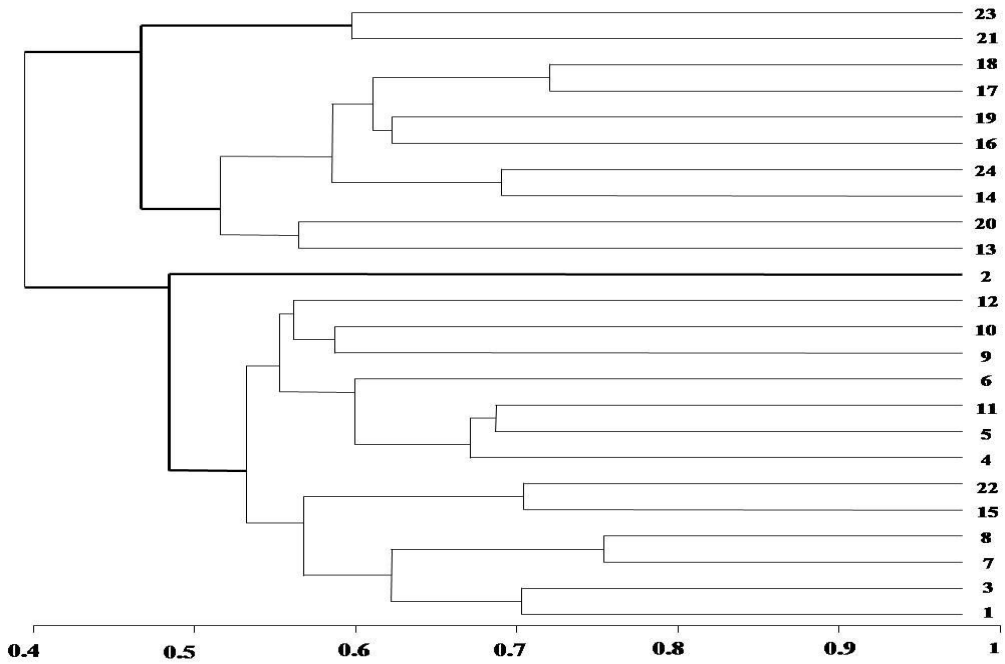

Fig 3. ***Dendrogram represented the genetic relationships among the 24 isolates using UPGMA cluster analysis of Nei's genetic similarity coefficients generated from three RAPD primers.***

Some pairs displayed 70% such as isolates 7 and 3, isolates 8 and 3, isolates 11 and 5, isolates 15 and 7.Consequently, the 24 *P. aeruginosa* clinical isolates revealed high DNA polymorphism using the three RAPD primers; either in the occurrence of amplified fragments or in the variable genetic

similarities of each isolate with the others. Despite the fact that they should display the most narrow and low variation due to the genomic structure of the same 24 *P. aeruginosa* isolates and the structure of the 10 mer-RAPD primers. Eventually, the fluctuation of genetic similarity values each of the 24 isolates with others using the three primers evidently revealed the divergent genetic backgrounds of such isolates with their high DNA polymorphism patterns.

4. DISCUSSION

The polymer type used to construct the contact lens may influence subsequent bacterial adhesion events. Contact lenses made from nonionic polymers with high water content may carry higher risks of bacterial contamination (Yen *et al*., 2003).

RAPD analysis recently emerged as a reliable means of typing several bacterial species, including *P. aeruginosa* (Kersulyte *et al.,* (1995); Mahenthiralingam *et al.,* (1996) and Mereghetti *et al.*, (1998). Moreover, Giordano *et al.,* (2001) characterized *P. aeruginosa* strains by three phenotyping methods: biotyping, antibiotyping and serotyping. Its aims were to evaluate their typing capacity in relation to various isolate profiles. The use of RAPD-PCR makes it possible to identify non-serotyped strains, and shows the necessity of this molecular typing technique for typing *P. aeruginosa* strains from patients with CF.

The obtained results were agreed with Hafiane and Ravaoarinoro (2011) who developed RAPD analysis for the routine typing of *P. aeruginosa* strains isolated from the sputum of cystic fibrosis patients (CFP) that are frequently difficult to type by conventional typing methods. They also showed that RAPD typing characterized 30 distinct genotypes and two small clusters of strains were observed among isolates with each primer. Strains belonging to one cluster were present in two (6%) of the 35 strains. Strains belonging to the other cluster were present in three (8%) of the 35 strains. The occurrence of these clusters indicates that cross-infection may occur. Their results indicated that only the RAPD method can establish a clonal relation whereas the other methods may only reflect phenotypical differences, and thus are inadequate to type these strains.

RAPD typing of bacteria is important for monitoring newly emerging pathogens and for examining local outbreaks. Accordingly, Deschaght *et al.,* (2011) evaluated the RAPD technique in combination with melting curve analysis (McRAPD) of the amplified DNA fragments to genotype isolates from five Gram-negative species, i.e. *Achromobacter xylosoxidans*, *Acinetobacter baumannii*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Stenotrophomonas maltophilia*. RAPD typing of the 88 *P. aeruginosa* blood isolates gave 54 different RAPD fingerprints, 46 of which contained only one strain. The dominant RAPD type amongst the remaining 8 fingerprints contained 14 strains. There was little correlation between the presumed focus of the bacteremia and RAPD grouping although 5 of the 14 strains in the dominant RAPD type were thought to have a respiratory focus. The 75 CF isolates were likewise separated into 41 different RAPD types with 29 containing a single strain and the most dominant containing 12 (Wareham and Curtis, 2007).

The epidemiology of *P. aeruginosa* infections and colonization was studied prospectively on a 12-bed medical intensive care unit. *P. aeruginosa* strains isolated from patients and water samples were analyzed by RAPD-PCR typing. During the 6-month period, 60 of 143 (42%) water samples contained *P. aeruginosa* at various levels ranging from 1 to >100 colony-forming units per 100 ml sample. Genotypically, water samples contained 8 different colonotypes. Nine patients had infections due to *P. aeruginosa* and 7 patients were colonized. Isolates from patients showed a similar distribution of genotypes as did tap water isolates and strains of identical genotype as patient strains had been isolated previously from tap water outlets in 8 out of 16 (50%) infection or colonization episodes (Trautmann *et al.,* 2006).

The study of Campbell *et al.,* (2000) employed 600 samples to evaluate RAPD typing and found that it had a high degree of reproducibility with 98.5% yielding the same banding patterns for each triplicate. Epidemiologically related isolates, such as multiple isolates from individual patients and those from each of five sibling pairs, were appropriately "read" as identical by RAPD. Isolates that were epidemiologically unrelated, such as those from the environmental and the IATS type strains, each had a unique RAPD pattern. This method was highly reproducible and was able to differentiate apparently unrelated strains. RAPD typing is a robust, simple, and highly reproducible method that should be useful in clarifying the epidemiology of *P. aeruginosa* in patients with CF.

RAPD typing is best suited to situations in which a large number of isolates are to be evaluated. Results from RAPD analysis can be used in conjunction with the more cumbersome PFGE method to resolve and confirm the difference or identity among smaller numbers of isolates when an ambiguous result is obtained with RAPD.

Genetic fingerprinting of 30 *Pseudomonas aeruginosa* (*Pa*) isolates from three types of nosocomial infection cases from two Osun State Teaching Hospitals was compared using RAPD-PCR markers (Akanji *et al.,* 2011). Ten out of 50 operon primers tested showed polymorphism with reproducible results among the isolates and produced 131 bands of which 74 were polymorphic with sizes ranging between 200 and 3000 bp. Cluster analysis using the 74 polymorphic markers

classified the 30 *P. aeruginosa* isolates into two (*PgA* and *PgB*) genetic groups. Ben Haj Khalifa *et al.*, (2010) characterized 96 clinical isolates of *P. aeruginosa* recovered in a Tunisian teaching hospital during a 16-month period using RAPD analysis. Genotyping showed 83 RAPD types and the isolates showing the same serotype could show different genotypes.

Conclusion and perspectives

The identification and discrimination of 24 individual *Pseudomonas aeruginosa* isolated from contact lens wearers, non-contact lens wearer revealed that they are novel strains with different serovars and each isolate could be characterized by unique RAPD pattern.

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