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Full Length Research Paper

Epidemiology of *Pseudomonas aeruginosa* in Intensive Care Unit and Otolaryngology Department of a Tunisian hospital

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Ninety four clinical isolates of *Pseudomonas aeruginosa* cultured from patients admitted to the Intensive Care Unit (ICU) (N= 37) and Otolaryngology (ORL) (N= 57) during one year (2001-2002) at the Rabta hospital (Tunisia) have been investigated by using serotyping, pyocintyping, drug susceptibility, M13-PCR and PFGE typing. Result shown that most of the isolates at the ICU belonged to serotype O:12 (11/37) that showed high resistance to commonly used antimicrobials (-lactamins, aminosids, and quinolone) and a predominance for pyocinotype P10. Despite the frequent occurrence of identical serotypes and pyocinotypes, most of the isolates represent unique RAPD-M13 genotype (88/94). PFGE typing detected three distinct clusters amongst the O12 isolates, suggesting a clonal relatedness among multiresistant O12 isolates. This study illustrates the importance of phenotypic and genotypic epidemiological surveillance of predominant O12 serotype clones in such service in local hospital.

Key words: *Pseudomonas aeruginosa*, epidemiology, clonal diffusion, multidrug resistance.

INTRODUCTION

P. aeruginosa is an opportunistic pathogen that is able to cause severe invasive diseases in critically ill and immunocompromised patients (Deplano et al., 2005). It is a common pathogen in hospitals and particularly in intensive care units (ICU) and shows a high phenotypic diversity (Fonseca et al., 2007). Moreover, high rates of resistance to antibiotics and frequent multidrug-resistance (MDR) are associated with nosocomial *P. aeruginosa* strains (Di Martino et al., 2002). It has been associated with sporadic or clustered cases of infection generally confined to single hospitalization units. In contrast, it has much more rarely been involved in large hospital outbreaks or interhospital spreads (Pellegrino et al., 2002; Pitt et al., 1989) . Propagation of such clones proceeds through complex routes that may involve, for example, the hands of health care personnel, environmental reservoirs, medical equipment, or reagents. A European survey on the prevalence of nosocomial infection in ICU patients showed that *P. aeruginosa* was one of the most frequent pathogens isolated from 29% of ICU-acquired infections (Vincent, 2000). Therefore, genomic diversity may allow this bacterium to expand its pathogenic potential (Head and Yu, 2004). Infection epidemiology has been investigated by means of several phenotypic and genotypic methods (Fegan et al., 1991; Liu et al., 1983).

The present study aimed the investigation of phenotypic and molecular epidemiology of *P. aeruginosa* isolates in ICU and ORL ward of the Rabta hospital with special emphasis on multiresistant *P. aeruginosa* O:12 isolates. The correlations between antibiotic resistant pattern, pyocinotypes, siderotypes, and RAPD- M13 profiles in relation to serotypes of the isolates were also investigated. PFGE typing was used to suspect clonal relatedness among O:12 serotype *P. aeruginosa* isolates in ICU.

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Table 1. Serotype frequency distribution in ICU and ORL wards.

Serotype	ICU	ORL
O:11	9	7
O:16	1	2
O:4	1	6
O:9	0	4
O:6	4	7
O:10	1	5
O:1	3	5
O:3	2	2
O:12	11	0
O:7	0	1
O:2	1	2
O:14	0	1
NAM	4	11
PAM	0	1
PAG	0	3

MATERIALS AND METHODS

Bacterial strains

During the period of September 2001 and September 2002, 94 isolates of *P. aeruginosa* were collected from patients admitted to the intensive care unit (N= 37) and otolaryngology ward (ORL) (N= 57) of the Rabta Hospital. The isolates were initially characterized on the basis of colonial morphology, positive oxidase reaction and motility. They were identified as *P. aeruginosa* by conventional methods on the basis of their production of pyoverdine on King B medium and other chemical characters in the API20NE system (BioMérieux, France).

Serotyping

Isolates were serotyped by slide agglutination according to the international serogrouping schema for *P.* aeruginosa (Head and Yu, 2004; Liu et al., 1983).

Antibiotic susceptibility

Antimicrobial susceptibility testing was carried out on Müeller-Hinton agar by a standard agar diffusion method, using ticarcillin, ceftazidime, ciprofloxacin, piperacillin, tobramycin, amikacin, cefsulodin and imipenem. It was interpreted according to the guidelines of the CA-SFM (2008). For purposes of this study, resistance to at least three of the following four drugs (ceftazidime, imipenem, tobramycin, ciprofloxacin) was considered multidrug resistance (MDR).

Pyocintyping

This was carried out according to a previously described method (Fyfe et al., 1984). This method identifies 105 main types and 25 subtypes on the basis of different inhibition patterns observed against thirteen indicator strains.

RAPD-M13 fingerprinting

The genomic DNA was prepared using a rapid method for gram-negative bacteria (Chen and Kuo, 1993). RAPD-M13 fingerprinting was performed as previously described (Miteva et al., 1998). M13-PCR products were analyzed by 2% agarose gel electrophoresis at 5 V/cm. The DNA molecular weight 100bp Ladder was used as a size standard. Positions of M13 patterns bands were transformed into a binary character matrix. Pairwise distance matrices were compiled using percent similarity. Phylogenetic tree was created by the UPGMA average cluster analysis using MVSP Version 3.1 (Kovach, 1999).

PFGE

PFGE typing was performed for eleven O12 serotype *P. aeruginosa* isolates. Genomic DNA from *P. aeruginosa* was prepared by the procedure described previously (Grundmann et al., 1995). In each case, the plug was digested with 20 U *Spe* I restriction enzyme. PFGE was performed using a CHEF DRII system (Biorad). Running conditions consisted of two ramps in sequence (ramp A consisted of an initial switch time of 0.5 s, a final switch time of 25 s, and a run time of 20 h; ramp B consisted of an initial switch time of 30 s, a final switch time of 60 s, and a run time of 4 h) . Gel pictures were interpreted using Gelpro 3.1 software for windows (media cybernetics). The peak positions of PFGE patterns were analysed. The UPGMA linkage was applied using MVSP Version 3.1. Potential clonal relatedness was determined at an 80% level of similarity, corresponding to a maximum six-band difference (Goering and Tenover, 1997).

RESULTS

Serotyping

The *P. aeruginosa* isolates were O-serotyped immediately after isolation (Table 1). Serotyping generated an overall number of 12 different types. Remarkable differences in the incidence of individual O- serotypes were observed by comparing isolates from ICU and ORL Ward. Serotype O:12 was most common in ICU, representing 11/37 (29.73%) of all ICU isolates. Most of the strains at the ORL ward belonged to serotypes O:11, O:6 (7/57; 12.28% each) and O:4 (6/57; 10.53%). Isolates with serotypes O:9, O:7 and O:14 were detected at the ORL ward but they were absent at the ICU. Those with serotype O:12 most represented in the ICU were absent at the ORL ward. Thus, only five major serotypes O:11, O:6, O:12, O:1 and O:4 were representative for 53/94 (56.38%) of *P. aeruginosa* isolated in the Rabta hospital with predominance for O:11 serogroup (16/94; 17.02%).

Antibiotic susceptibility

The results of drug susceptibility testing were shown in Figure 1. ORL ward isolates were highly susceptible to antimicrobials tested. *P. aeruginosa* isolates showed high resistance to commonly used antimicrobials especially in ICU. Antibiotic susceptibility results suggested that

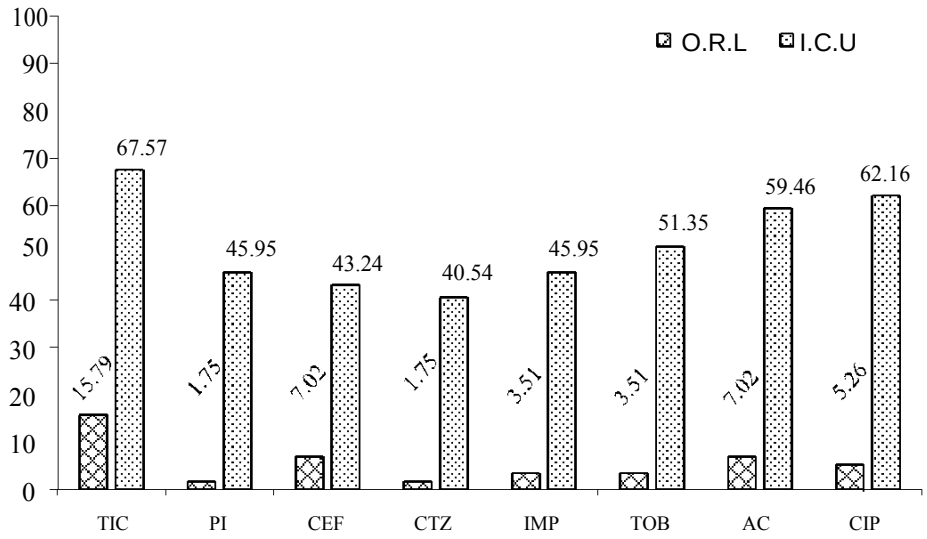


Figure 1. Resistance (%) of *P. aeruginosa* isolated from ICU and ORL at Rabta hospital to various antibiotics. TIC, ticarcillin; PI, piperacillin; CEF, cefsulodin; CTZ, ceftazidime; IMP, imipenem; TOB, tobramycin; AC, Amikacin; CIP, ciprofloxacin.

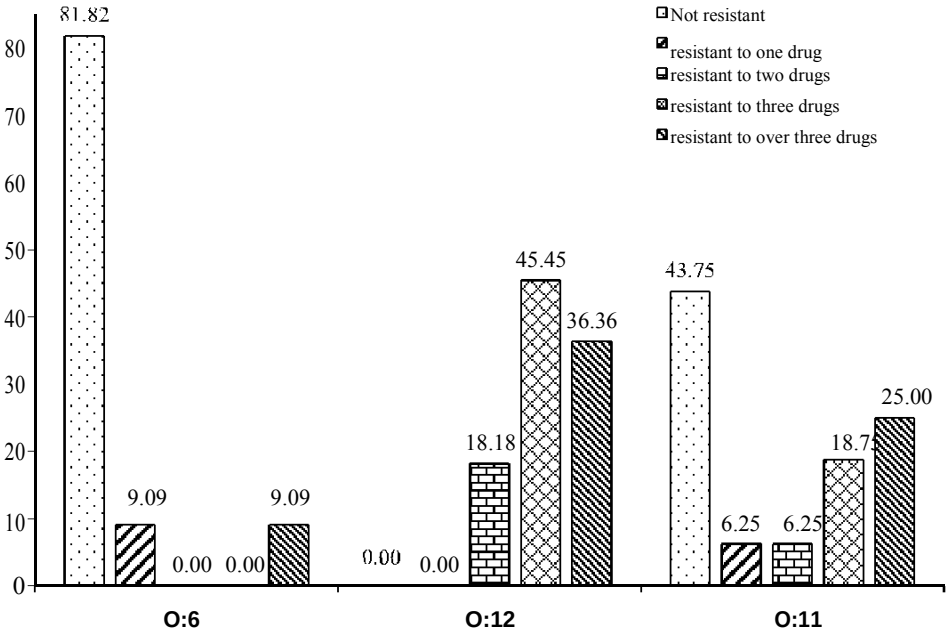


Figure 2. The number of anti- pseudomonal drugs (ceftazidime, imipenem, tobramycin, ciprofloxacin) to which predominant *P. aeruginosa* serotypes (O:6, O:12 and O:11) were

ceftazidime was the most effective to isolated strains.

To evaluate the extent of multidrug resistance, the number of individual drugs (ceftazidime, imipenem, tobramycin, ciprofloxacin) to which *P. aeruginosa* were resistant was assessed. ICU *P. aeruginosa* isolates were highly multidrug resistant compared to ORL ward isolates. Only (7/37) of ICU isolates were sensitive to all of this drugs and (18/37) were considered multidrug resistant. However, (36/57) ORL *P. aeruginosa* isolates were sensitive to all used drugs and no one was multidrug resistant (MDR) . Analysis of antibiograms by serotype show the extend of multidrug resistance of the most prevalent serotypes (O:11, O:6 and O:12) as represented in Figure 2. The results show that susceptibility to all drugs used was represented by 9/11 and 7/16 respectively for O:6 and O:11 isolates. None O:12 serotype *P. aeruginosa* isolates were susceptible to all these drugs. Whereas, multidrug resistance was

represented by 1/11, 7/16 and 9/11 respectively for O:6, O:11 and O:12 *P. aeruginosa* isolates.

Pyocintyping

The *P. aeruginosa* isolates were subdivided into 21 pyocinotypes as shown in Figure 3. The most represented pyocinotypes are P10 (41/94), P1 (5/94) and P105, P17 and P6 (3/94 each). The non-pyocinotypable (NT) isolates that produce pyocins with inhibition profile inexistent in the pyocinotypes table of Gillies and Govan (1966) are represented by 18/94 isolates. The results show 1/94 atypical (AT) isolate with no pyocin production. Among the most represented pyocinotypes P10 and 2/41 are non-subpyocinotypables. Whereas, 39/41 can be subdivided into 6 subpyocinotypes.

RAPD-M13 fingerprinting

A total of 90 different profiles were obtained by RAPD-M13 fingerprinting. Most profiles (88/90) were unique, but 2 profiles were common each for 3 strains that were not necessarily from the same ward. The different M13 profiles were compared by numerical methods and the resultant dendrogram based on the similarity percentage between isolates, shows a high degree of genetic diversity as represented in Figure 3. Seven major groups were defined at the 70% similarity level.

PFGE typing

Genomic relatedness of the eleven 012 *P. aeruginosa* isolates was investigated by comparing the macrorestriction profiles of *Spe*I- digested genomic DNA analyzed by PFGE. The obtained PFGE profiles were not identical but identify three cluster (designated I to III), suggesting clonal relatedness represented in Figure 4.

DISCUSSION

Serotyping of *P. aeruginosa* has been subject to several studies (Ben Slama et al., 2001; Bouhaddioui et al., 2002; Jerboui, 1997; Nour, 1990) that show the predominance in Tunisia of serotypes O:1, O:6, O:11 and O:10 at hospital environment. These serotypes dominated during the same period in European, Asiatic and African countries (Bert et al., 1994; Kezzal and Rahal, 1986; Shahcheraghi et al., 2003; Vanhoof et al., 1993; Vieu et al., 1987). Permanent investigation by serotyping allows us to suspect quickly epidemic dissemination when rarely encountered serotype appears within several patients of the same ward or the same hospital. The O:12 serogroup was the most frequent in the ICU ward, contrary to previous studies reporting that O:12 serogroup was absent or rarely encountered in Algeria (0.25%), Mauritania (1.5%) and in Tunisia (4.6% or absent) (Ben Slama et al., 2001; Bouhaddioui et al., 2002). Its expansion seems to be caused by the multiple antibiotics resistances (Kezzal and Rahal, 1986; REUSSIR, 2001).

Thus, generally, serotype O:12 proved to be the most resistant to antibiotics tested in the present study. Moreover, these results are in agreement with previous observations on the relatively frequent isolation of multiresistant serotype O:12 strains in some European countries (Tzouvelekis et al., 1989), in Egypt (Vieu et al., 1984) and in the Indian sub-continent (Rukmini Devi and Bhaskaran, 1984). Finally, it should be pointed out that O:12 serotype strains predominant in ICU are associated with a particular virulent phenotype enhancing their ability to colonize the ICU patients and cause infection (REUSSIR, 2001). These strains were suspected of belonging to a single outbreak, suggesting nosocomial transmission of *P. aeruginosa* 0:12 and cross-infection within this ward.

The differences in proportion of antibiotic resistance between the two hospital wards investigated may be due to the application of different policies in treatment of patients or differences in the management of the ICU and ORL wards. This high resistance to antibiotics indicates the improper use of antibiotics at hospitals, as shown in many studies (Shahcheraghi et al., 2003; Struelens 1998).

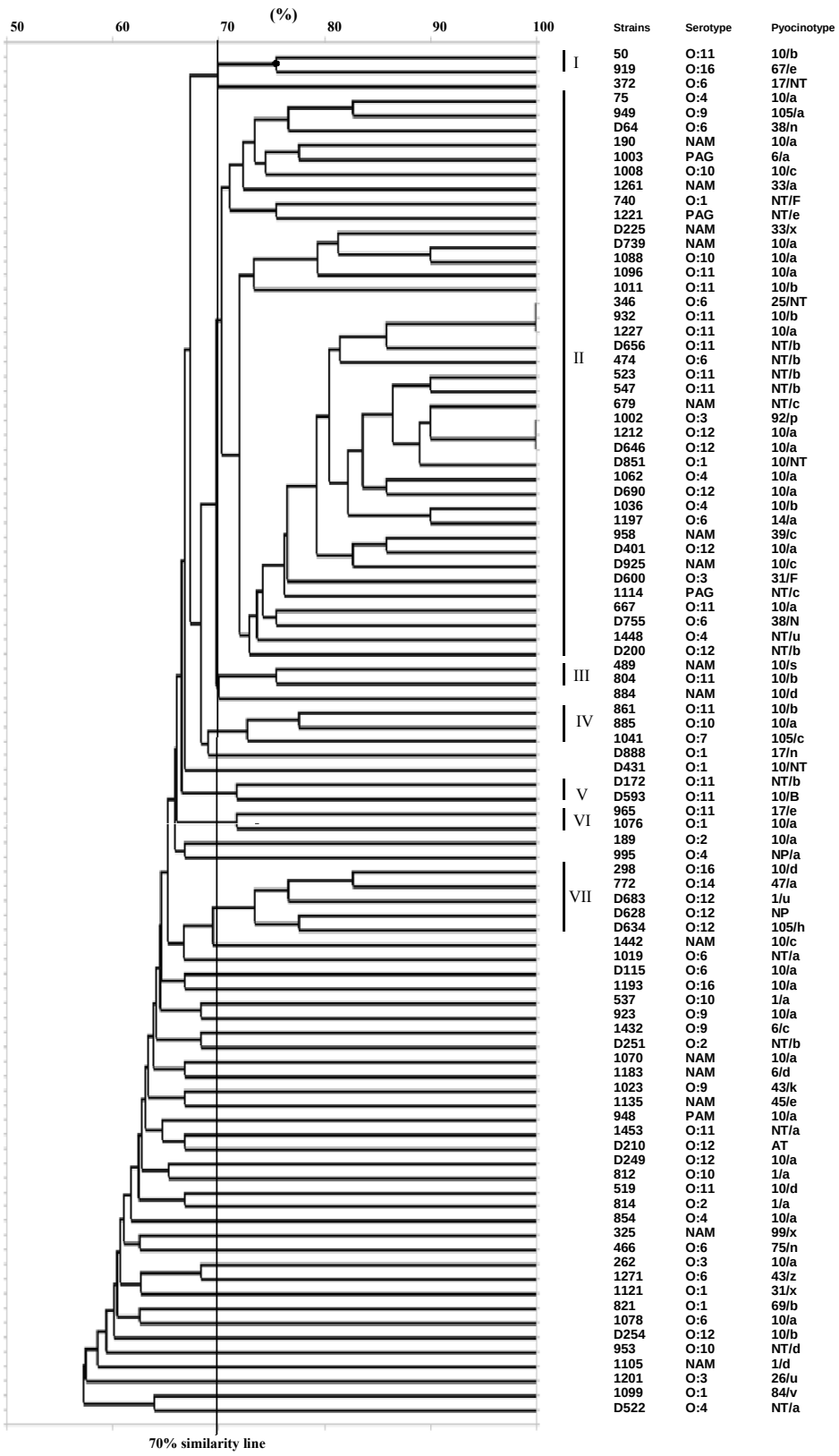
P. aeruginosa pyocintyping has been the object of several previous studies. In Tunisia, the most predominant pyocinotypes between 1987/1988 were P1 (40%) and P10 (30%) (Bouhaddioui et al., 2002). In Europe, the same pyocinotypes (P1, P3, P5 and P10) were dominant at hospitals and environment with frequencies between 58 and 89% as previously reported (Orsi et al., 1994; Poh et al., 1988).

It is interesting to note that among the prevalent serotypes O:11, O:12 and O:6 *P. aeruginosa*, high genetic heterogeneity could be documented based on RAPD-M13 typing in accordance with a previous reports on clinical *P. aeruginosa* isolates (Fonseca et al., 2007; Morales et al., 2004).

The majority of the strains from serotype O:12 are distributed by two major clusters. Moreover, these results indicate that RAPD-M13 typing can differentiate between the dominant multidrug resistant O:12 isolates in ICU as reported by (Elaichouni et al., 1994). The results of the present work also show that the phenotypic clusters of the isolates were not congruent with M13-PCR clonal lineages.

As a result, it can be assumed that clusterization by use of RAPD-M13 was poorly significant. Analysis of clinical isolates by other molecular techniques can be helpful in studying the epidemiology of outbreak strains, and in confirming their clonality (De Vos et al., 1997).

Comparing the PFGE fingerprints and antibiotic



	Strains	Serotype	Pyocinotype
I	50	O:11	10/b
	919	O:16	67/e
	372	O:6	17/NT
II	75	O:4	10/a
	949	O:9	105/a
	D64	O:6	38/n
	190	NAM	10/a
	1003	PAG	6/a
	1008	O:10	10/c
	1261	NAM	33/a
	740	O:1	NT/F
	1221	PAG	NT/e
	D225	NAM	33/x
	D739	NAM	10/a
	1088	O:10	10/a
	1096	O:11	10/a
	1011	O:11	10/b
	346	O:6	25/NT
	932	O:11	10/b
	1227	O:11	10/a
	D656	O:11	NT/b
	474	O:6	NT/b
	523	O:11	NT/b
	547	O:11	NT/b
	679	NAM	NT/c
	1002	O:3	92/p
	1212	O:12	10/a
	D646	O:12	10/a
	D851	O:1	10/NT
	1062	O:4	10/a
	D690	O:12	10/a
	1036	O:4	10/b
	1197	O:6	14/a
	958	NAM	39/c
	D401	O:12	10/a
	D925	NAM	10/c
	D600	O:3	31/F
	1114	PAG	NT/c
	667	O:11	10/a
	D755	O:6	38/N
	1448	O:4	NT/u
	D200	O:12	NT/b
III	489	NAM	10/s
	804	O:11	10/b
	884	NAM	10/d
IV	861	O:11	10/b
	885	O:10	10/a
	1041	O:7	105/c
	D888	O:1	17/n
	D431	O:1	10/NT
V	D172	O:11	NT/b
	D593	O:11	10/B
VI	965	O:11	17/e
	1076	O:1	10/a
	189	O:2	10/a
	995	O:4	NP/a
VII	298	O:16	10/d
	772	O:14	47/a
	D683	O:12	1/u
	D628	O:12	NP
	D634	O:12	105/h
	1442	NAM	10/c
	1019	O:6	NT/a
	D115	O:6	10/a
	1193	O:16	10/a
	537	O:10	1/a
	923	O:9	10/a
	1432	O:9	6/c
	D251	O:2	NT/b
	1070	NAM	10/a
	1183	NAM	6/d
	1023	O:9	43/k
	1135	NAM	45/e
	948	PAM	10/a
	1453	O:11	NT/a
	D210	O:12	AT
	D249	O:12	10/a
	812	O:10	1/a
	519	O:11	10/d
	814	O:2	1/a
	854	O:4	10/a
	325	NAM	99/x
	466	O:6	75/n
	262	O:3	10/a
	1271	O:6	43/z
	1121	O:1	31/x
	821	O:1	69/b
	1078	O:6	10/a
	D254	O:12	10/b
	953	O:10	NT/d
	1105	NAM	1/d
	1201	O:3	26/u
	1099	O:1	84/v
	D522	O:4	NT/a

Figure 3. Computed dendrogram derived from digitized RAPD-M13 patterns for 94 *P. aeruginosa* strains. The dendrogram was constructed by cluster analysis by UPGMA. The scale at the top of this figure shows the percentage similarity. The line in the dendrogram denotes the threshold 70% homology for defining groups of genetic similarity. Roman numerals indicate clusters with 70% homology. NT- nonpyocinotypable; AT- atypical.

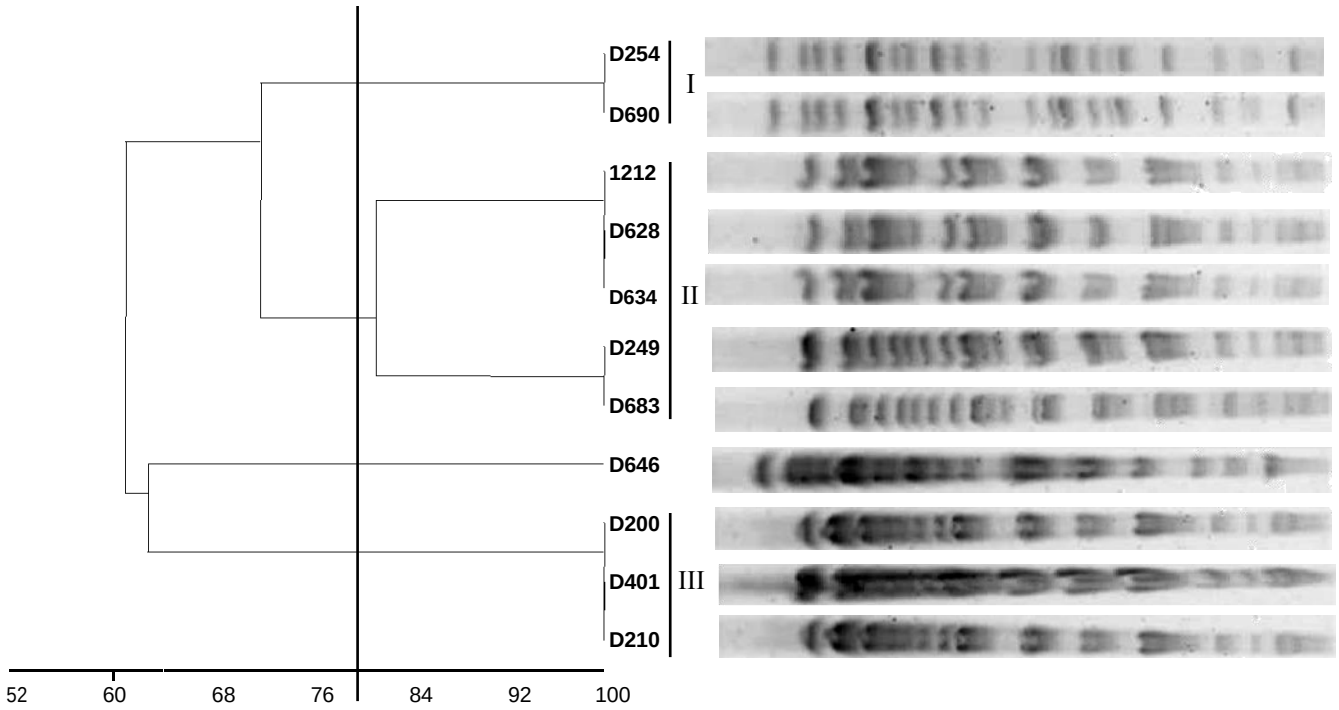


Figure 4. Pulsed Field Gel Electrophoresis banding patterns after SpeI digestion of O12 serotype *P. aeruginosa* isolates. The dendrogram is based on a cluster analysis of the unweighted-pair group method with percent similarity. The cutoff at the 80% level of genetic similarity is indicated by a vertical line.

resistance patterns, the multiresistant O12 isolates are shown to be clonal (cluster II). PFGE analysis confirmed clonality for O12 *P. aeruginosa* isolates between patients and over time. The extent of clonal dissemination within the hospital was estimated by determining the ratio of infected patients to clones. The results presented higher values in ICU of the Rabta hospital, between 2 to 5 (5 infected patients per clone), denoting the occurrence of epidemic or endemic infections with 012 *P. aeruginosa* isolates during the study period.

These results were in agreement with previous studies, which showed that the O12 serotype *P. aeruginosa* has a long-recognized association with multiresistance and that a single MDR strain could persist in the same patient for many months and cause recurrent episodes of clinical infection (Hsueh et al., 1998; Nordmann and Poirel, 2002).

These findings are consistent with previous studies on Outbreaks of serotype O12 *P. aeruginosa* in Italy, Greece, and Korea showing their tight clonality (Crespo et al., 2004). It has been previously proposed, that the uniformity of characters of multiresistant O:12 *P. aeruginosa* in Europe is suggestive of a common origin for these strains (Pitt et al., 1989). Previous finding, described the relatively high frequency of isolation of serotype 12 in Italy, showed to be resistant to a large number of antibiotics. They were in particular responsible for extensive and life-threatening outbreaks (Giammanco et al., 1985).

Conclusion

The present work showed a high phenotypic diversity of the studied clinical strains of *P. aeruginosa.* A specific cluster associated with multidrug resistant 012 serotype isolates, and PFGE genogroups was found suggesting the occurrence of epidemic or endemic infections with 012 *P. aeruginosa* isolates during the study period. Thus, the results of the current study demonstrated that both the management and the policy of treatment should be revised at ICU and ORL wards at Rabta hospital in order to lower the burden of resistant bacteria from the environment. Such measures may also contribute to reducing the spread of resistance genes to other opportunistic microorganisms. This study illustrates the importance of phenotypic and genotypic surveillance to perform infection control. Indeed, *P. aeruginosa* strains have been recognized as a major public health problem, and therefore, active surveillance is needed to detect and prevent the dissemination of these O12 serotype epidemic clones.

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