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Epidemiology and virulence of VIM-4 metallo-beta-lactamase-producing *Pseudomonas aeruginosa* isolated from burn patients in eastern Algeria

Samah Meradji [a], *Abouddihaj Barguigua* [b,c], *Mohamed cherif Bentakouk* [d], *Kaotar Nayme* [b,c], *Khalid Zerouali* [c], *Dekhil Mazouz* [e], *Houria Chettibi* [a], *Mohammed Timinouni* [b,*]

[a] *Biochemistry and Applied Microbiology Laboratory, Badji Mokhtar Faculty of Sciences, Department of Biology, Annaba University, Box 12 Sidi Amar, 23000 Annaba, Algeria*
[b] *Molecular Bacteriology Laboratory, Institut Pasteur du Maroc, 1 Place Louis Pasteur, 20360 Casablanca, Morocco*
[c] *Microbiology Laboratory, Faculty of Medicine and Pharmacy, 1 Street Hospital, 20360 Casablanca, Morocco*
[d] *Resuscitation Center and Burns Treatment, University Hospital Ibn Rochd, 23000 Annaba, Algeria*
[e] *Microbiology Laboratory, University Hospital Dorban, 23000 Annaba, Algeria*

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ABSTRACT

In this study, we investigated the prevalence of carbapenem-resistant *Pseudomonas aeruginosa* (CRPA) in burn patients from eastern Algeria, CRPA virulence factors and the molecular epidemiology of CRPA. The overall prevalence of CRPA was 48.38%. Seven (46.66%) isolates were metallo-β-lactamases (MBL) producers and contained the MBL genes bla_{VIM-4} ($n = 6$) and bla_{VIM-2} ($n = 1$). Risk factors for CRPA infection were urinary catheter use and intubation ($p = 0.008$). A high percentage of virulence factors (86.6% of these isolates were able to produce protease; 73.3% of isolates has DNase; and 66.6% were haemolysin positive) was observed in CRPA isolates. Among the seven MBL-producing isolates, four had the same clonal profile. The class 1 integrons, which contained the *aadA7* gene cassette, were detected in six isolates. The 16SrRNA methylase gene, *rmtB*, was detected in one strain. All CRPA isolates were biofilm formers. A study on the kinetics of biofilm production revealed that biofilm production increased when the concentration of imipenem or ciprofloxacin and the incubation time increased. This is the first study to report the presence of VIM-4-producing *P. aeruginosa* from North Africa and also of the high prevalence of CRPA isolates. Based on our study of burn unit patients, the high percentage of *P. aeruginosa* with virulence factors and multi-drug resistance is alarming.

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* *Corresponding author*. Tel.: +212 05 22 43 44 50; fax: +212 05 22 26 09 57.
E-mail address: mohammed.timinouni@pasteur.ma (M. Timinouni).

Abbreviations: CRPA, carbapenem-resistant *Pseudomonas aeruginosa*; CSPA, carbapenem-susceptible *P. aeruginosa*; MBLs, metallo-β-lactamases; MDR, multidrug-resistant; MICs, minimum inhibitory concentrations.

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1. Introduction

Burns are an increasing and growing public health problem worldwide especially in developing economies such as Algeria [1]. *Pseudomonas aeruginosa* is a life-menacing nosocomial pathogen that appears as a leading cause of critical infections in burn patients. The treatment of *P. aeruginosa* infections are constantly difficult to treat due to limited susceptibility to antimicrobial drugs and the appearance of antibiotic resistance during therapy. Multidrug resistance, which is engendered by a diversity of resistance mechanisms, leaves insufficient alternatives for treatment in some patients [2].

Carbapenems are selective drugs used for the treatment of multidrug-resistant (MDR) strains [3]. However, the increasing frequency of carbapenem-resistant *P. aeruginosa* (CRPA) has recently become a serious concern worldwide. Among the mechanisms of acquired resistance to imipenem, Class B metallo-β-lactamases (MBLs) induce resistance to all β-lactams except aztreonam. Worldwide, several types of MBLs have been determined in *P. aeruginosa* isolates (IMP, VIM, SPM, GIM, SIM, AIM, FIM and NDM) [4]. Of the MBLs, VIM types are the most frequent [4–7]. The clinically important MBL families VIMs have been reported worldwide, in different geographical areas including Mediterranean region, European countries [5,6] and from African countries [8–10]. Specifically, blaVIM-2 has appeared as the most prominent MBL variant worldwide [5,6,8–10]. Algeria is a northern country in Africa, where recent antibiotic resistance data indicate a worrying situation. Indeed, the last ten years have been marked by the appearance and extended of new resistance genes in particular in northern Africa [8,9]. *P. aeruginosa* is not only one of the most frequent nosocomial pathogen with acquired antimicrobial resistance traits, but it is also a model pathogen with great adaptability and metabolic versatility [11–13]. The pathogenesis of *P. aeruginosa* is multifactorial and dependent on a larger virulence factors. It relies on the following traits of pathogenicity including a complex arsenal of soluble molecules (such as toxins, enzymes and exopolysaccharides) and cellular structures (such as pili and secretion systems) with complementary effects and the production of virulence factors depends on multiple regulation circuits [14,15]. Among the numerous of virulence determinants of *P. aeruginosa*, biofilm formation is the most important because it is strongly correlated with resistance to antibiotic chemotherapy [16–18]. The high resistance and tolerance of biofilm embedded bacteria which can be 1000-fold more resistant to antibiotics than planktonic cells to virtually any antimicrobial is related to several factors or combinations of factors. These include restricted penetration of antimicrobials into a biofilm, decreased growth rate, and expression of several resistance genes [16]. Considering that the biofilm is a critical factor for the efficacy of antimicrobials and the spread of drug resistance, our first aim was to determine to the prevalence of *P. aeruginosa* isolated from burn unit patients from Ibn Sina Annaba hospital in eastern Algeria and determine the molecular characteristics of various strains and to determine risk factors related with imipenem resistance and virulence factors. Our second aim was to assess the effect of imipenem and ciprofloxacin on *P. aeruginosa* biofilm formation, which can conduct to strategies for the prevention and investigation of *P. aeruginosa* treatment.

2. Material and methods

2.1. Setting

The study was performed at a state-wide adult burns service unit located at Ibn Sina Annaba Hospital (279 beds) affiliated with a tertiary referral centre in Annaba University Hospital (Annaba city, eastern Algeria).

2.2. Ethics

Ethics approval was obtained from the hospital's human research and ethics committee.

2.3. Data collection

Between April 2014 and January 2015 we prospectively enrolled all patients admitted to the Department of Burns at Annaba University Hospital in Algeria. All the patients infected with *P. aeruginosa* were included in the study. Demographic features, clinical conditions during hospitalization and concomitant diseases of the included patients were retrospectively reviewed and analyzed.

Demographic and clinical information were obtained from the patient medical record, including gender, age, duration of hospitalization, burn cause and degree, comorbidities (previous surgical interventions, presence of chronic pulmonary, cardiac, diabetes mellitus, central nervous system and other diseases), use of invasive devices, previous antibiotic use, and previous hospitalizations.

The analysis was limited to the first episode. According to the Center for Disease Control criteria, a nosocomial infection was considered if the infection was not evident until >48 h of hospitalization [19]. Previous hospitalization was defined as an inpatient stay at Annaba University hospital or another hospital for at least 2 days during the 3 months preceding the index hospitalization.

Prior antibiotic exposure was defined as the exposure to antimicrobial agents for at least 3 consecutive days within three months of the isolation of the organism onset. The following classes of antibiotics were recorded: aminoglycosides, carbapenems, third-generation cephalosprins, fluroquinolone, and macrolide.

2.4. Antibiotic susceptibility testing

Antimicrobial drug susceptibility was determined using the disk diffusion method on Mueller-Hinton (MH) agar plates (Bio-Rad, Marnes-la-Coquette, France) according to recommendations from the Clinical and Laboratory Standards Institute (CLSI, 2012) [20]. Thirteen antibiotics were tested, including ticarcillin, ceftriaxon, piperacillin, cefepime, ticarcillin/clavulanic acid, ceftazidim, imipenem, aztreonam, amikacin, gentamicin, ciprofloxacin, levofloxacin, and colistin. *P. aeruginosa* ATCC 27853 was used as a wild-type susceptible control.

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Minimum inhibitory concentrations (MICs) of imipenem were determined using Etest-strips (AB BioMerieux, France), as described by the manufacturer's instructions.

2.5. Phenotypic detection of carbapenemase production

Carbapenemase production was detected phenotypically using a modified Hodge test by using an imipenem disc (10 μg) as described by the CLSI [20]. The detection of metallo-β-lactamase production was also performed by the combined-disc test using two imipenem discs one (10 μg per disc), and another disc containing 10 μL of 0.1 M (292 μg) anhydrous EDTA (Sigma Chemicals, St. Louis, MO, USA). The discs were placed 25 mm apart on a MH agar plate. An increase in the zone diameter of >4 mm around the imipenem-EDTA disc compared with that of the imipenem disc alone was considered positive for MBL production.

2.6. Phenotypic detection of AmpC and ESBL production in CRPA isolates

Extended spectrum β-lactamase (ESBL) production was detected by the double-disc synergy test (DDST) using clavulanic acid-ticarcillin (20/10 mg) and ceftazidim (30 mg) and aztreonam (30 mg) on MH agar as described previously [8,21]. Phenotypic detection of ESBLs can be obscured by chromosomal AmpC cephalosporinase in *P. aeruginosa*; hence DDST with cloxacillin was performed. Cloxacillin (250 μg/mL; Sigma, St Louis, MO, USA) was added to MH agar to inhibit cephalosporinase activity [8,9]. AmpC overproduction was confirmed according to the method of Touati, Diene [8], Sefraoui, Berrazeg [9]. Isolates were considered positive for AmpC overproduction when there was at least a two-fold dilution difference between the MICs of ceftazidime and imipenem alone and the MICs of ceftazidime and imipenem with cloxacillin [8,9].

2.7. Preparation of DNA template for PCR

A few colonies of an overnight culture of *P. aeruginosa* isolates on Luria Bertani agar (Bio-Rad, Marnes-la-Coquette, France) were suspended in 500 μL of DNase- and RNase-free water (Invitrogen, Paisley, UK). The suspension was boiled at 100 °C for 10 min in a thermal block (Polystat 5, Bioblock Scientific, France), then centrifuged at 19,000 × *g* for 5 min. A 1-μL aliquot of the supernatant was used as the DNA template for PCR.

2.8. Detection of β-lactamase-encoding genes

All CRPA isolates were screened by PCR for the following carbapenemase genes: bla_{VIM}, bla_{IMP}, bla_{GES}, bla_{KPC}, bla_{OXA-58}, bla_{OXA-40}, bla_{OXA-23} and bla_{OXA-51} and others β-lactamases: bla_{TEM}, bla_{SHV}, bla_{SPE-1} and bla_{OXA-1}, as described previously [22–24]. CRPA resistance to ceftazidim was further tested using primers specific for plasmid-mediated AmpC β-lactamase genes as described by Pérez-Pérez and Hanson [25].

2.9. Detection of class 1 integrons

PCR was performed using primers CS-3′ and CS-5′ to amplify different fragment sizes of class 1 integrons. The primers, PCR conditions and reaction mixtures used were as described previously [22].

2.10. Detection of plasmid-mediated quinolone resistance genes and aminoglycoside resistance determinant genes

The assessment of plasmid-mediated quinolone resistance (PMQR) and aminoglycoside resistance determinants in CRPA stains was conducted as described previously [24,26]. All CRPA strains were screened by multiplex PCR for *qnr* genes (*qnrA*, *qnrB*, *qnrD*, *qnrC* and *qnrS*). PCR amplification of *aac(6′)-Ib* (encoding aminoglycoside 6′-N-acetyltransferase type Ib), *aac(3)-II* (encoding 3-N-aminoglycoside acetyltransferase genes) and 16S RNA methylase genes (*armA*, *rmtA*, *rmtB* and *rtmC*) was performed using primers that amplified all variants.

2.11. Sequencing of resistance genes

All amplified products were sequenced to validate their identities. Both strands of the purified amplicons were sequenced with a Genetic Analyzer 3130 × 1 sequencer (Applied Biosystems, Foster City, CA, USA) with the same primers used for PCR amplification. The nucleotide and deduced protein sequences were analyzed with software available on the National Center for Biotechnology Information website (www.ncbi.nlm.nih.gov).

2.12. Genotyping of MBL-producing P. aeruginosa by pulsed-field gel electrophoresis

Pulsed-field gel electrophoresis (PFGE) analysis was performed according to the standardized protocol developed by Durmaz et al. [27] using *SpeI*. The Dice similarity coefficient was calculated between pairs of lanes, and then strains were grouped using the dendrogram construction tool Dendro UPGMA (Biochemistry and Biotechnology Department, Rovirai Virgili University, Tarragona, Spain) (http://genomes.urv.cat/UPGMA/index.php). The isolates were considered to be genetically related if the Dice coefficient of correlation was 80% or greater.

2.13. Virulence phenotypes study

Proteolytic activity. Casein hydrolysis was tested on tryptone casein soy (TCS) agar plates (Sanofi Diagnostics Pasteur) containing 10% (w/v) skimmed milk (Difco, Barcelona, Spain) and streptomycin at 50 g/mL. After centrifugation at 13,000 × *g* for 10 min, 100 μL of each suspension was placed in 5-mm-diameter wells cut into TCS agar and incubated at 37 °C for 24 h. This test was performed in triplicate. The presence of a transparent zone around the wells indicated protease activity and the diameter of the transparent zone reflected the intensity of the exo-enzyme released [28].

Haemolytic activity. Haemolytic activity was measured to assess the presence of virulence factors associated with haemolysis. Briefly, strains were tested for haemolytic activity on agar base (Oxoid, France) supplemented with 5% sheep erythrocytes. 5 μL of each suspension was streaked onto the plates and incubated at 37 °C for 24 h. This test was carried out

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in triplicate. The presence of a clear colourless zone surrounding the colonies indicated haemolytic activity [28,29].

DNase test agar. DNase test agar was used to identify bacteria capable of producing DNase, an exoenzyme. The test agar contained an emulsion of DNA and peptides as a nutrient source. Bacterial colonies that secrete DNase hydrolyze the DNA in the medium into smaller fragments; when plates are flooded with HCl, this results in clearing around the bacterial colony [30].

Motility study. This test was carried out in triplicate. The degree of motility of the tested isolates was variable; a cutoff of 1 cm of motility was chosen to distinguish between highly motile (>1 cm) and less motile (<1 cm) strains.

- ''*Swimming*'' *test*. Tryptone swim plates (1% tryptone, 0.5% NaCl and 0.3% agar) were inoculated with bacteria using a sterile toothpick and incubated for 16 h at 25 °C. Motility was assessed qualitatively by examining the circular turbid zone formed by bacterial cells migrating away from the point of inoculation [29].
- ''*Swarming*'' *test*. Swarm plates were composed of 0.5% Bacto Agar and 8 g of nutrient broth/litre (both from Difco, Detroit, MI, USA) supplemented with 5 g of dextrose/litre and dried overnight at room temperature (30 °C). Cells were point inoculated with a sterile toothpick, and the plates were incubated at 30 °C for 24 h. Motility was then assessed qualitatively by examining the circular turbid zone formed by bacterial cells migrating away from the point of inoculation [29].
- ''*Twitching*'' *test*. The *P. aeruginosa* colony tested was stab-inoculated (approximately 3-mm depth) into an agar plate. After incubation at 30 °C for, the agar was carefully removed and the zone of motility at the agar/plate interface was visualized and measured by staining with crystal violet [29].

2.14. *Biofilm assay*

A slightly modified version of the microtitre plate assay developed by O'Toole and Kolter [31] was used for the biofilm assay. Briefly, 4 μL of overnight culture was inoculated into 100 μL of LB in a 96-well culture-treated polystyrene microtitre plate (Nunc) with different concentrations of imipenem (4–128 μg/mL) and ciprofloxacin (0.5–8 μg/mL). Control plates did not contain antibiotics. We then studied the effect of imipenem and ciprofloxacin on the biofilm-forming ability of one isolate from each MBL-producer clone (Pa.4 for clone A, Pa.12 for clone B, Pa.10 for clone C and Pa.27 for clone D).

All assays were performed in triplicate. After regular time intervals of two hours of incubation at 37 °C, surface-adherent biofilm formation was measured by staining bound cells for 15 min with a 0.5% (w/v) crystal violet. After rinsing with distilled water, bound dye was released from stained cells using 95% ethanol. The number of bound cells was determined by measuring the optical density (OD) at 550 nm.

As a control, sterile medium was used to determine background OD. The cut-off OD (ODc) was defined as three standard deviations above the mean OD of the negative control. According to the results of the microtiter plate test, the isolates were classified into the four following categories based on the OD: non-biofilm producers (OD test < ODc), weak biofilm producers (ODc < OD < 2× ODc), moderate biofilm producers (2× ODc < OD < 4× ODc), and strong biofilm producers (4× ODc < OD).

3. Data analysis

To identify variables associated with carbapenem-resistant *P. aeruginosa*, a risk factor analysis was performed using a case–control study format. Demographics and hospitalization variables from patients infected with *P. aeruginosa* and CRPA patients were compared with non-infected patients and carbapenem-susceptible *P. aeruginosa* (CSPA) patients, respectively. Data were entered into a database using the SPSS 20.0 for Windows (SPSS Inc., Chicago, USA). The χ^2 test and independent samples *t* tests were used for categorical and continuous variables, respectively. A stepwise multivariate logistic regression was conducted to examine the association of risk factors, while controlling for potential confounders. The logistic model included all variables for which $p < 0.1$ was obtained in the multivariate analysis. $p < 0.05$ was considered significant.

4. Results

A total of 50 patients were included in this study and the distribution was 48% female (F) and 52% male (M) (M:F ratio of 1.08). Thirty patients presented with *P. aeruginosa* infection (60%; 95% CI: 46.4–73.6) with 15(50%) patients with CRPA infection. The median age was 39.5 in the CRPA group and 44 in the CSPA group, with no significant difference, using parametric (for mean) or non-parametric (for median) tests. The duration of hospitalization was different between the groups (CSPA and CRPA), although it had a tendency to be prolonged in the CSPA group (Table 1). A comparison between infected and non-infected patients revealed that the duration of hospitalization was statistically different and 90–120 days for patients infected with *P. aeruginosa*.

In the group of patients infected with *P. aeruginosa*, most burns were due to flames by gas explosion (46.7%). Burns due to hot water and electricity were relatively common at 26.7 and 23.3%, respectively. The most common burn sites included the upper limbs, head/neck and chest. Among the CRPA isolates, 12 (70.6%) were isolated from hospitalized patients who underwent surgery such as skin grafting at the intensive care unit. Even though several variables were compared (CRPA vs. CSPA), urinary catheter use and intubation were statistically significant. In the multivariate analysis, none of the risk factors for CRPA were identified. However, the comparison between infected and non-infected patients revealed that previous use of macrolide antibiotics and polymicrobial infection were statistically significant. The mortality of patients with *P. aeruginosa* infection was 46.7% for patients with CRPA and 33.3% for patients with CSPA, without statistical significance.

Virulence studies on all *P. aeruginosa* strains were performed to verify the virulence factors profile. Virulence factors from all *P. aeruginosa* strains isolated including CRPA and CSPA isolates are shown in Table 2. Swimming and swarming motility types were exhibited by all tested strains

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Table 1 – Characteristics of patients infected with carbapenem-resistant and carbapenem-susceptible *P. aeruginosa*.

Factors	Patients infected with *P. aeruginosa*, % (*n*)				Non infected patients with *P. aeruginosa*, % (*n*)	*p*-value
	CRPA	CSPA	*p*-value	All isolates		
Gender						
Male	66.7 (10)	46.7 (7)	NS	53.3 (16)	50 (10)	NS
Female	33.3 (5)	53.3 (8)	NS	46.7 (14)	50 (10)	NS
Age (years)						
Mean	44.6	35.1	NS	39.7	46.76	NS
SD	20.44	20.12	NS	20.51	20.57	NS
Hospitalization stay (days)						
[1–30]	33.3 (5)	13.3 (2)	NS	23.3 (7)	40 (8)	NS
[30–60]	13.3 (2)	33.3 (5)	NS	23.3 (7)	40 (8)	NS
[60–90]	0	6.7 (1)	NS	3.3 (1)	15 (3)	NS
[90–120]	20 (8)	46.7 (7)	NS	51.6 (16)	5 (1)	0.0006
Burn cause						
Electricity	26.7 (4)	20 (3)	NS	23.3 (7)	10 (2)	NS
Gas explosion	46.7 (7)	46.7 (7)	NS	46.7 (14)	55 (11)	NS
Hot water	20 (3)	33.3 (5)	NS	26.7 (8)	35 (7)	NS
Petrol	6.7 (1)	6.7 (1)	NS	6.7 (2)	0	NS
Burn degree						
First + second-degree (deep)	6.7 (1)	0	NS	3.3 (1)	5 (1)	NS
Second-degree (deep)	26.7 (4)	40 (6)	NS	33.3 (10)	40 (8)	NS
Second + third-degree (deep)	53.3 (8)	60 (9)	NS	56.7 (17)	50 (10)	NS
Third-degree (deep)	6.7 (1)	0	NS	3.3 (1)	5 (1)	NS
Second-degree (superficial)	6.7 (1)	0	NS	3.3 (1)	5 (1)	NS
Previous antibiotics use						
Aminoglycoside	80 (12)	46.7 (7)	NS	63.3 (19)	70 (14)	NS
Macrolide	0	20 (3)	NS	10 (3)	40 (8)	0.017
Fluroquinolone	66.7 (10)	66.7 (10)	NS	66.7 (20)	45 (9)	NS
3rd Generation Cephalosporin	6.7 (1)	33.3 (5)	NS	20 (6)	25 (5)	NS
Carbapenem	33.3 (5)	20 (3)	NS	26.7 (8)	5 (1)	NS
Invasive devices						
Intubation	26.7 (4)	26.7 (4)	NS	26.7 (8)	5 (1)	NS
Intubation + Urinary catheter	60 (9)	13.3 (2)	0.008	36.7 (8)	15 (3)	NS
Surgical intervention						
Yes	60 (9)	68.7 (11)	NS	66.7 (20)	40 (8)	NS
No	40 (6)	26.6 (4)	NS	33.3 (10)	65 (12)	NS
Polymicrobial infection						
Yes	93.3 (14)	80 (12)	NS	86.7 (26)	5	<0.0001
No	6.7 (1)	20 (3)	NS	13.3 (4)	95	NS
Mortality						
Yes	46.7 (7)	33.3 (5)	NS	40 (12)	NA	NA
No	53.3 (8)	66.7 (10)	NS	63.3 (19)	NA	NA

NS, non-significant, CRPA, carbapenem-resistant *P. aeruginosa*, CSPA, carbapenem-susceptible *P. aeruginosa*.

and the twitching motility type was exhibited by 80.6% (86.6% CRPA vs. 75% CSPA) of strains tested. CSPA isolates expressed haemolytic (75% CSPA vs. 66.6% CRPA) and DNase activity (81.2% CSPA vs. 73.3% CRPA) more frequently. In contrast, CRPA isolates had greater protease production (86.6% CRPA vs. 81.2% CSPA). Only 62% of all strains were able to produce all three virulence factors.

All *P. aeruginosa* isolates were able to adhere to the surface of polystyrene and form a biofilm. A comparison between CRPA and CSPA isolates revealed that kinetics of biofilm-forming ability was very heterogeneous (Fig. 1). CSPA isolates were stronger biofilm formers than CRPA isolates. CRPA isolates were more moderate and weak biofilm formers than CSPA isolates in almost all incubation times except the 6-h incubation time where 50% of CSPA isolates were significantly more frequent ($p = 0.04$) moderate biofilm formers than CRPA isolates (Fig. 1).

Antimicrobial susceptibility testing was assessed using the disc diffusion method on 30 clinical isolates of *P. aeruginosa*. Of all the isolates tested, resistance for β-lactam antibiotics was 100% (ticarcillin), 96.7% (piperacillin), 96.7% (ceftazidime), 96.7% (cefepime), 93.5% (ceftriaxone), 70.9% (ticarcillin/clavulanic acid) and 32.2% (aztreonam). For non-β-lactam antibiotics, the resistance was 80.6% (gentamicin), 48.4% (amikcin), 22.5% (ciprofloxacin), 22.5% (levofloxacin) and 29% (colistin). Overall, a significantly greater number of CRPA

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Table 2 – The distribution of virulence factors of carbapenem-resistant and carbapenem-susceptible *P. aeruginosa* isolates.

Virulence factor	CRPA isolates % (*n*)			CSPA isolates % (*n* = 16)	All strains (*n* = 31)
	CP (*n* = 7)	Non-CP (*n* = 8)	All strains (*n* = 15)		
Haemolytic	71.43 (5)	62.5 (5)	66.6 (10)	75 (12)	70.9 (22)
Proteolysis	85.71 (6)	87.5 (7)	86.6 (13)	81.2 (13)	83.7 (26)
DNase	85.71 (6)	62.5 (5)	73.3 (11)	81.2 (13)	77.4 (24)
Twitching	100 (7)	62.5 (5)	86.6 (13)	75 (12)	80.6 (25)
Swimming	100 (7)	100 (8)	100 (15)	100 (16)	100 (31)
Swarming	100 (7)	100 (8)	100 (15)	100 (16)	100 (31)
Biofilm formation	100 (7)	100 (8)	100 (15)	100 (16)	100 (31)

CP, carbapenemase producers; Non-CP, non-carbapenemase producers; CRPA, carbapenem-resistant *P. aeruginosa*; CSPA, carbapenem-susceptible *P. aeruginosa*.

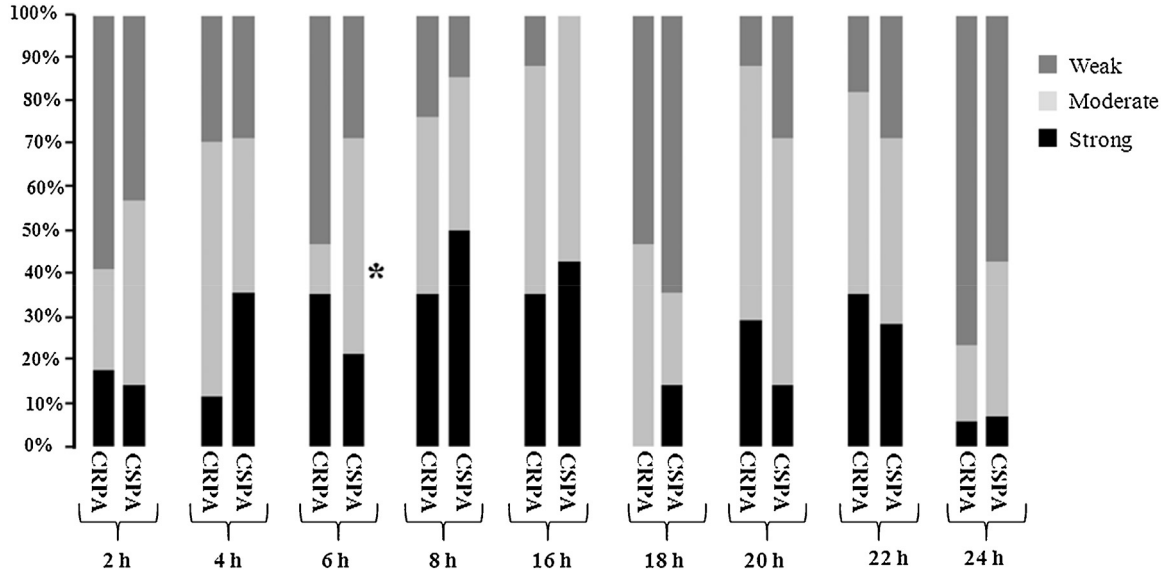

Fig. 1 – Comparison of the biofilm-forming ability between carbapenem-resistant *P. aeruginosa* isolates and carbapenem-susceptible *P. aeruginosa* isolates. * $p < 0.05$. CRPA: carbapenem-resistant *P. aeruginosa*, CSPA: carbapenem-susceptible *P. aeruginosa*.

isolates were resistant to ticarcillin/clavulanic acid, ciprofloxacin, amikacin, gentamicin and colistin than CSPA isolates (Table 3).

For all CRPA isolates, the MIC of imipenem was >32 μg/mL. Among the 15 CRPA isolates, seven (46.6%) isolates tested positive for MBL and carbapenemase using the MBL screen with EDTA and a modified Hodge test, respectively. PCR and sequencing analysis on CRPA isolates revealed a bla_{VIM-4} gene in six strains and a bla_{VIM-2} gene in one strain of an MBL-positive isolate. The characteristics of VIM-positive isolates and patients infected by these strains are presented in Fig. 2. None of the isolates that tested negative for MBL and carbapenemase gave positive PCR results for the carbapenemase-encoding genes bla_{KPC}, bla_{OXA-58}, bla_{OXA-40}, bla_{OXA-23} and bla_{OXA-51}. Synergy tests using clavulanic acid with ticarcillin-, aztreonam-, and ceftazidime-containing disks did not give evidence of inhibition of aztreonam and ceftazidime resistance for all CRPA and CSPA isolates, indicating ESBL was not produced. AmpC over-expression has been previously reported to correlate highly with ceftazidime resistance. We found that AmpC over-expression was present in 70.96% of CRPA isolates. These results were confirmed by the detection of plasmid-mediated AmpC β-lactamase genes by PCR. The 16S rRNA methylase, *rtmB* was detected in one isolate, Pa.27. The class 1 integron (*Int1*) was detected in all MBL-producing isolates, except the Pa.27 isolate, as a 1-kb PCR amplicon. Sequence analysis of these amplicons revealed the presence of the *aadA7* gene cassette.

In the genotyping analysis, the DNA fingerprint patterns of the seven MBL-producing isolates revealed four distinct clones, clones A, B, C and D (Fig. 2). Three of these clones (clones A, C and D) were represented by a single isolate. Clone B was represented by four isolates (Pa.8, Pa.9, Pa.11 and Pa.12). Clone A was identified in strain Pa.4, which was isolated in August 2014 from a 42-year-old male. This patient was hospitalized in the intensive care unit with 21% degree of his body burned at the head and chest. Two isolates (Pa.8 and Pa.9) with clone B were isolated on the same day in September 2014 from two patients- a 19-year-old male with 60% degree of burns and a 21-year-old female who was hospitalized for skin graft abscesses. The two other strains (Pa.11 and Pa.12) obtained in October 2014 were isolated from a male and

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Table 3 – Comparison of antimicrobial susceptibility between carbapenem-resistant *P.aeruginosa* and carbapenem-susceptible *P. aeruginosa*.

Antibiotics	Resistance ratios, % (no. resistant strains)				
	CRPA			CSPA ($n = 16$)	All strains ($n = 31$)
	CP ($n = 7$)	Non-CP ($n = 10$)	All strains ($n = 15$)		
TIC	100 (7)	100 (10)	100 (17)	100 (14)	100 (31)
TCC	100 (7)*	90 (9)*	94.12 (16)**	42.86 (6)	70.97 (22)
PIP	100 (7)	90 (9)	94.12 (16)	100 (14)	96.77 (30)
CAZ	100 (7)	90 (9)	94.12 (16)	92.86 (13)	93.55 (29)
ATM	100 (7)***	80 (8)***	47.06 (8)	14.29 (2)	32.26 (10)
FEP	100 (7)	90 (9)	94.12 (16)	100 (14)	96.77 (30)
CRO	100 (7)	90 (9)	94.12 (16)	100 (14)	96.77 (30)
LEV	0	60 (6)**	35.29 (6)	7.14 (1)	22.58 (7)
CIP	0	60 (6)**	35.29 (6)***	7.14 (1)	22.58 (7)
AK	85.71 (6)	90 (9)	88.24 (15)***	0	48.39 (15)
GN	100 (7)***	100 (10)***	100.00 (17)**	57.14 (8)	80.65 (25)
Cs	14.29 (1)	70 (7)**	47.06 (8)*	7.14 (1)	29.03 (9)

* $p < 0.01$.
** $p < 0.001$.
*** $p < 0.0001$.
CP, carbapenemase producers, Non-CP, non-carbapenemase producers, CRPA, carbapenem-resistant *P. aeruginosa*, CSPA, carbapenem-susceptible *P. aeruginosa*, TIC, ticarcillin, CRO, ceftriaxon, PIP, piperacillin, FEP, cefepime, TCC, ticarcillin/clavulanic acid, CAZ, ceftazidim, ATM, aztreonam, AK, amikacin, GN, gentamicin, CIP, ciprofloxacin, LEV, levofloxacin; Cs, colistin.

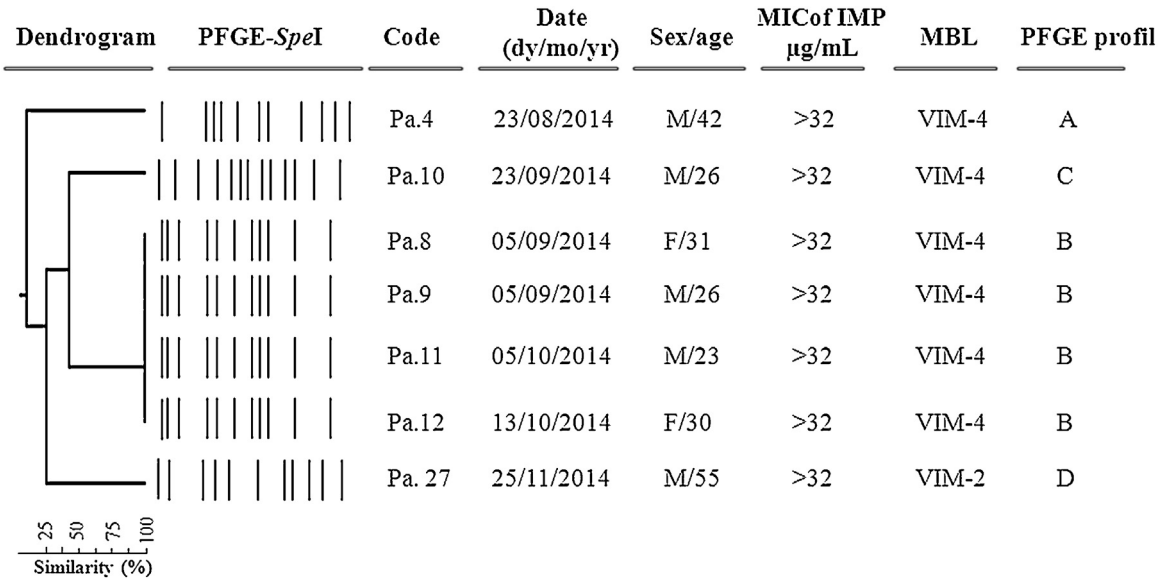

Fig. 2 – Representative *SpeI* pulsed-field gel electrophoresis (PFGE) profiles of MBL-producing *P. aeruginosa* isolates. The dendrogram was generated with Dendro UPGMA (http://genomes.urv.cat/UPGMA/index.php). The PFGE profile, sex and age of patients infected, and MBL enzyme are indicated.

female with 60% and 98% degree of burns, respectively. The Pa.27 isolate included in clone D was isolated in November 2014 from a patient with 78% degree of burn.

The adherence and biofilm-forming ability of *P. aeruginosa* on different surfaces at hospitals are believed to be the primary source of infection and diffusion of epidemic strains. A strain's ability to form a biofilm on surfaces and the detachment of cell clumps from biofilm aggregates was probably responsible for cross-contamination during the study period. Since most patients were receiving antibiotics, we assessed the behaviour of biofilm from MBL-producing *P. aeruginosa* isolates in the presence of antibiotics.

We then studied the effect of imipenem and ciprofloxacin on the biofilm-forming ability of one isolate from each MBL-producer clone (Pa.4 for clone A, Pa.12 for clone B, Pa.10 for clone C and Pa.27 for clone D). In the presence of sub-MICs of ciprofloxacin, the biofilm biomass, as measured by the OD, decreased and there was an inverse relationship between OD and antibiotic concentration (Fig. 3). However, when the concentration of imipenem increased, the biofilm biomass

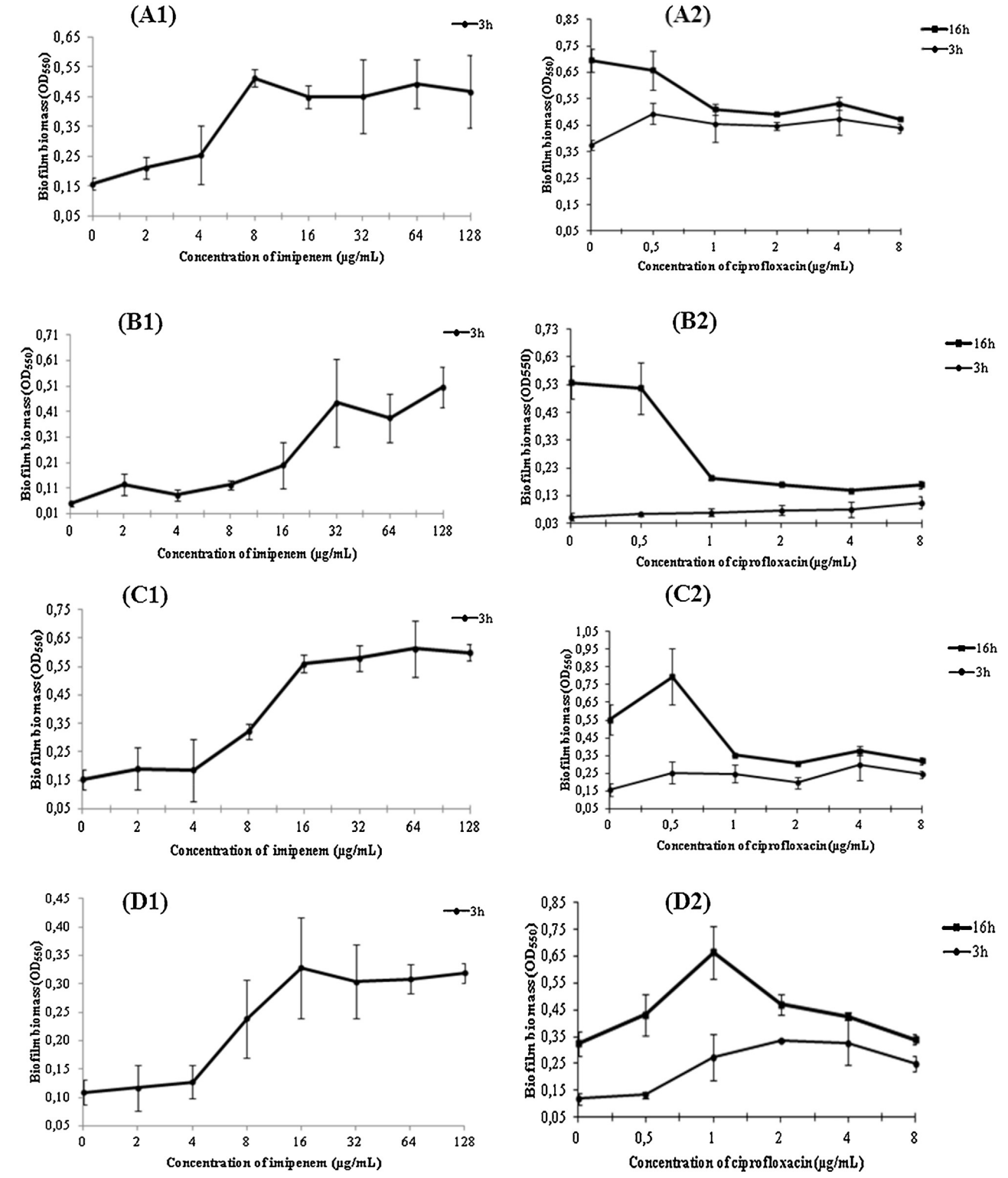

Fig. 3 – Quantification of the biofilm biomass by crystal violet staining (OD_{550}) in the presence of increasing concentrations of imipenem and ciprfloxacin in microtitre plates after 3 h of incubation for imipenem and 3 or 16 h for ciproloxacin. (A1), (A2): Pa.4 isolate; (B1), (B2): Pa.12 isolate; (C1), (C2):Pa.10 isolate and (D1), (D2): Pa. 27 isolate.

also increased (Fig. 3). Since for imipenem, all concentrations tested were at least 4-fold lower than the MIC, we can infer that sub-inhibitory concentrations of this antibiotic enhance biofilm formation.

The effects of different concentrations of imipenem and ciprofloxacin on the kinetics of *P. aeruginosa* biofilm production are presented in Fig. 4. With imipenem and ciprofloxacin, the biofilm biomass increased when the concentration of

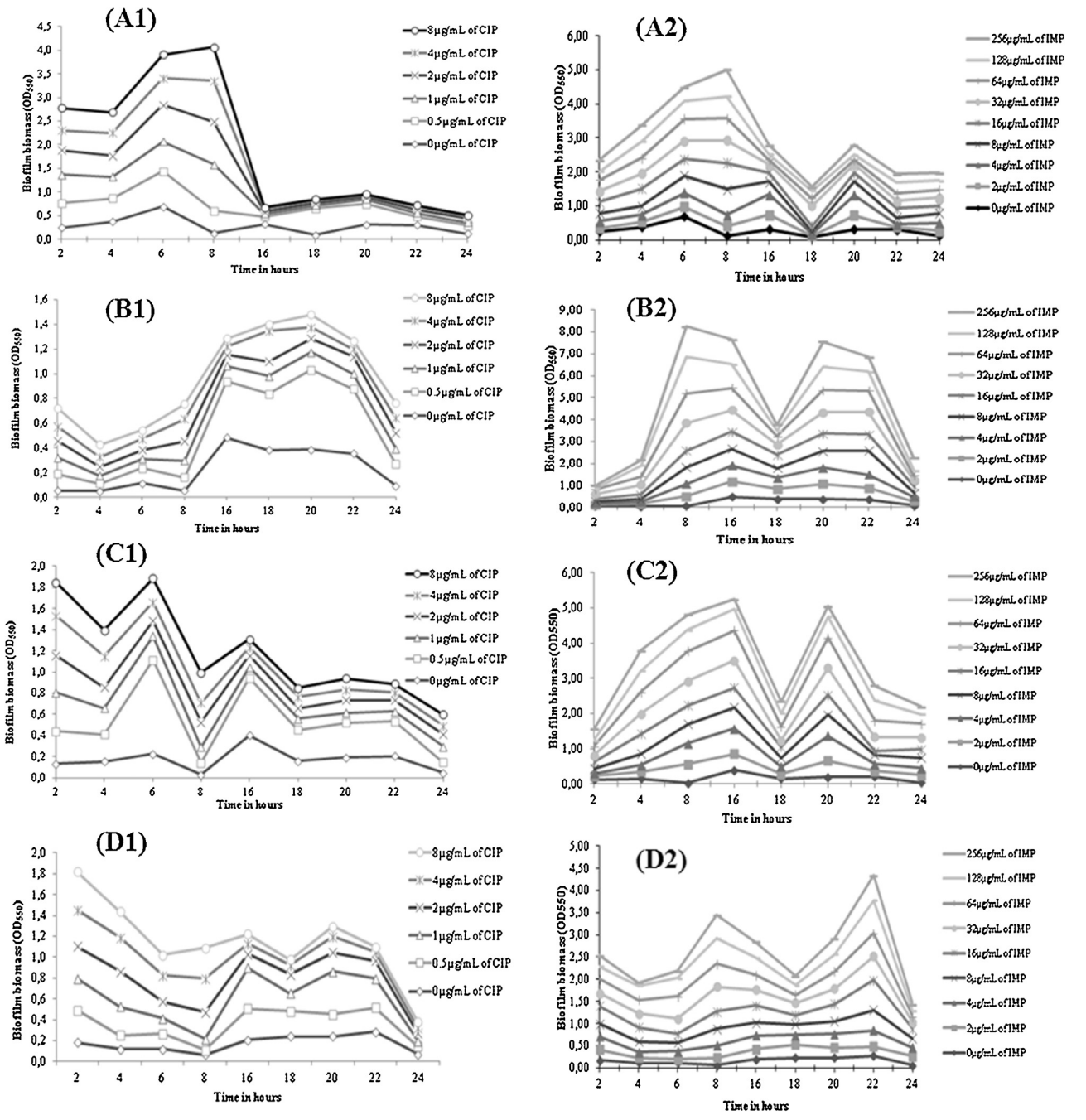

Fig. 4 – Adhesion kinetics of *P. aeruginosa* strains cultivated in the presence and absence of different concentrations of imipenem and ciprofloxacin in microtitre plates. (A1), (A2): Pa.4 isolate; (B1), (B2): Pa.12 isolate; (C1), (C2):Pa.10 isolate and (D1), (D2): Pa.27 isolate.

imipenem and ciprofloxacin and the incubation time increased, this was linked by the time required for maximal adhesion, which ranged from 8 to 22 h.

5. Discussion

It have been given that Burn wards harbour MDR isolates of *P. aeruginosa* which can colonize burn patients and lead to infection [32,33]. Our study showed a high prevalence of *P. aeruginosa* infections (62%) among burn patients compared with studies from others countries, such as Morocco (15.1%) [34], Tunisia (15%) [35], Egypt (19.8%) [36], Iran (37.5%) [37] and India (59%) [38]. This difference might be attributed to antibiotics abuse, different hospital strategies for the management of infections, hygiene and geographic climatic. In the present study, the administration of macrolide antibiotics, longer hospitalization and the presence of polymicrobial infections, especially infection with *Staphylococcus* species, were the greatest risk factors for *P. aeruginosa* infections in the burn unit.

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Treatment of *P. aeruginosa* infection is frequently complicated by limited susceptibility to antimicrobial drugs and the spread of antibiotic resistance during therapy. Carbapenems have been the drug of choice for the treatment of infections caused by MDR *P. aeruginosa* strains [39]. In latest years, Algeria has been configured as one of countries with the highest rates of antimicrobial resistance [8,23,40]. In our study, there were high rates of resistance to all commercially available antimicrobial drugs among *P. aeruginosa* isolated from Annaba Hospital. 48.38% of CRPA isolates were carbapenem resistant; this limits the treatment options in hospitals. The percentage of CRPA isolates varied by geographic region, specimen source, and the presence of selective pressure from antibiotics [41]. In Algeria, Drissi et al. [40] concluded that *P. aeruginosa* isolates had the highest resistance to imipenem (35%) in a study conducted during the period 2005–2007. Also, Sefraoui et al. [23] showed that among *P. aeruginosa* strains, 39.32% were resistant to imipenem in a study conducted during the period 2009–2012. However, the CRPA frequency shown in the studied hospitals during the period 2014–2015 was higher than those reported in Algeria. Among neighbouring countries, such as Libya, Tunisia and Egypt, the incidence of imipenem resistant *P. aeruginosa* ranged from 24.2 to 39.34% [42–44].

Resistance to carbapenem in *P. aeruginosa* is due to multiple mechanisms with or without the production of carbapenemase [39]. Diminished expression or mutational loss of the specific porin OprD and carbapenem hydrolyzing enzymes-carbapenemase are the most common mechanism of resistance to carbapenems [39,45]. In the present study, 46.6% of CRPA isolates were MBL producers with bla_{VIM-4} and bla_{VIM-2} type detected. A few MBL-producing *P. aeruginosa* isolates have been documented in Africa, particularly in North Africa. In Algeria, VIM-19 was the first carbapenemase identified, especially in *Enterobacteriaceae* species [46]. Recently, Touati et al. [8] was the first to identify bla_{VIM-2} in clinical isolates of *P. aeruginosa* from eastern Algeria. A second description of VIM-2-producing *P. aeruginosa* throughout the year was reported in western Algeria [9]. Our study was the first to report the detection of VIM-4-producing *P. aeruginosa* in North Africa. This discovering may indicate the current emergence of MBLs in clinically relevant Gram-negative strains throughout Northern Africa.

Worldwide reports of VIM-4 class MBL isolates in *P. aeruginosa* and *Enterobacteriaceae* are illustrated in Fig. 5. As bla_{VIM} genes are carried on gene cassettes in class 1 integrons, they can be disseminated rapidly. Bacteria producing VIM-4 have been disseminated worldwide, in different geographical (Greece, Italy, Sweden, Hungary, Poland, Belgium, Tunisia, USA and Australia) and were associated to different species (*P. aeruginosa*, *Pseudomonas putida*, *Aeromonas* spp., *Enterobacter cloacae*, *Klebsiella pneumoniae* and *Acinetobacter baumannii* [47].

Previous studies have investigated the incidence of aminoglycoside resistance mechanisms from different countries [39,48,49]. In the current study, our finding indicates that a higher rate of gentamicin resistance was found in CRPA (100%). This was much higher than the levels previously reported in Annaba hospitals (28.09%) [23]. The class I integron with *aadA7* gene cassette were found in six CRPA isolates. These class I integrons may appear as an important tool in the emergence of antimicrobial resistance and spread of MDR *P. aeruginosa* [50]. The *aadA7* family of enzymes in these strains provide resistance to tobramycin and amikacin (subfamily I) or gentamicin (subfamily II). *P. aeruginosa* aminoglycoside resistance was primarily mediated by transferable aminoglycoside-modifying enzymes, followed by 16 rRNA methylase [39,51,52]. In the current study, *rmtB* gene was identified in one MBL-producing isolate. To the best of our knowledge, this finding is the first description of the *rmtB* gene in Algeria and North Africa, especially in MBL-producing *P. aeruginosa*. *rmtB* gene is associated with mobile genetic elements, such as

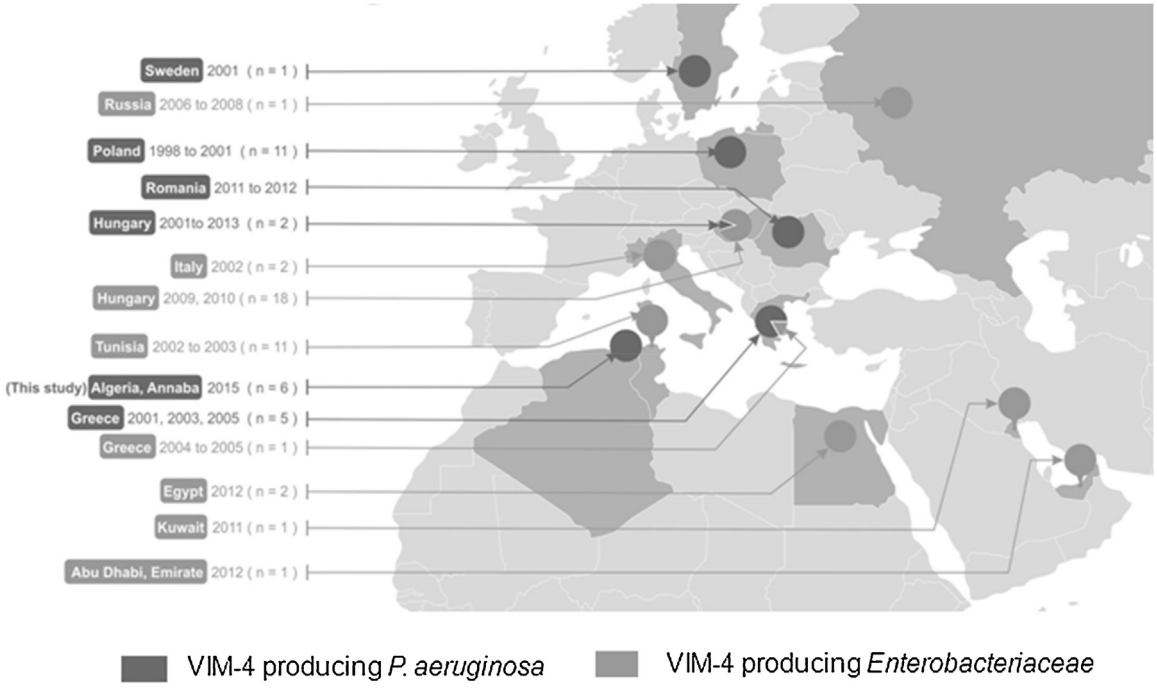

Fig. 5 – Geographical distribution of VIM-4 producing *P. aeruginosa* and *Enterobacteriaceae* isolates worldwide.

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transposons [39,51,52], that provide the means to spread horizontally to other strains and species.

In this study, we used PFGE to examine the clonality of MBL-producing *P. aeruginosa* isolated from the burn unit. Among seven MBL producers, clonal diffusion was observed in four strains. This clonal diffusion of MDR strains suggests that hospitals need to develop better strategies to prevent and control infections. This involves screening and identification of colonized or infected patients, treatment of infected patients, and implementation of preventative geographic isolation. The risk factors for acquiring CRPA were supported by results of previous studies demonstrating that the host condition, infection control practices, and antimicrobial use can be related [53–55]. Regarding invasive procedures, in our study, urinary catheter use and intubation were the only risk factors related with CRPA infection. This is due to the fact that *P. aeruginosa* tends to form a biofilm on the surface of urinary catheters, which increases the possibility of dissemination of bacteria to the other body locations, causing infection [56,57]. In our study, 100% of the clinical isolates of *P. aeruginosa* were able to form biofilm on polystyrene substrate and the best biofilm producers were CRPA strains, suggesting biofilm formation is a mechanism that promotes resistance chemotherapeutic agents [58]. This was not surprising, given that *P. aeruginosa* tends to make biofilm on the surface of urinary catheters increased the possibility of dissemination of bacteria to the urinary tract, causing infection. In addition, several studies found that 80–100% of *P. aeruginosa* strains isolated from different anatomical sites and catheters were able to form biofilm on polystyrene microtiter plates. Furthermore, these studies found that MDR-producing *P. aeruginosa* isolates were associated with an increased ability to form biofilm, but reduced motility, pigment production and in vitro fitness [59–63].

In this study, we examined the effect of sub-MIC concentrations of imipenem and ciprofloxacin on the kinetics of biofilm formation of MBL-producing *P. aeruginosa*. *P. aeruginosa* isolates cultivated with these antibiotics in microtitre plates had significantly greater biofilm production compared with the same isolates cultivated in the absence of these antibiotics. These results are in accordance with the previous observations by Linares et al. [64] who found that sub-MIC concentrations of tobramycin, ciprofloxacin and tetracycline induced *P. aeruginosa* biofilm formation by approximately 2-fold as determined by crystal violet staining. The induction of *P. aeruginosa* biofilm formation by imipenem and ciprofloxacin was previously observed to be associated with an increase in alginate production. The mechanism of biofilm induction involves co-regulatory networks that confer β-lactam resistance, the alginate production pathway (*algD*, *algA*), quorum-sensing and virulence factor production, with AmpR and AmpC playing a central role [65]. In addition to biofilm formation, at least 60% of clinical strains of *P. aeruginosa* had DNase, haemolytic and proteolytic activity. These enzymatic activities are important virulence factors during *P. aeruginosa*–host interplay, and contribute to the establishment of the infectious process [66]. Bacterial motility is one of virulence factor that organisms use to initiate colonization and trigger their pathogenic [66]. Our study showed that at least 86.6% of CRPA isolates exhibited twitching, swimming and swarming motility. Consequently, the treatment of burn patients infected by theses MDR isolates is a difficult task and a real problem that needs to be solved.

6. Conclusion

Many MBLs have been described in bacteria from many genera and species worldwide. Algeria, like many countries, is now facing the threat of the emergence of carbapenem-resistant *P. aeruginosa*. To the best of our knowledge, this is the first study to report a VIM-4-producing strain of *P. aeruginosa* in Algeria and the production of a VIM-4 MBL type enzyme in *P. aeruginosa* in North Africa. The detection of endemic clonal dissemination of MBL producing isolates in the burn unit and the presence of highly virulent MDR *P. aeruginosa* isolates suggests that the risk for serious infection is high in burn unit patients. Therefore strict measures should be developed to control the spread of these pathogens in the hospital setting.

Conflicts of interest

The authors declare no conflicts of interest.

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