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Original article

Antibiotic resistance and virulence properties of *Pseudomonas aeruginosa* strains from mechanically ventilated patients with pneumonia in intensive care units: comparison with imipenem-resistant extra-respiratory tract isolates from uninfected patients

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Abstract

We investigated the epidemiology of antibiotic resistance and virulence properties among *Pseudomonas aeruginosa* clinical isolates collected in 1999 from patients hospitalized in the intensive care units of the centre hospitalier d'Orléans, in France. We compared the totality of the strains from mechanically ventilated patients with pneumonia (33 non-duplicate isolates, group 1) to 15 randomly chosen, imipenem-resistant, extra-respiratory tract isolates, collected from non-infected patients hospitalized in the same units (group 2). The isolates were serotyped, typed by random amplified polymorphic DNA (RAPD), and screened for their pneumocyte cell adherence, cytotoxicity, and antibiotic resistance. A total of 35 RAPD profiles were found, and only two profiles were encountered in both groups, demonstrating a high genetic diversity. 84.8% of the group 1 and 93.3% of the group 2 isolates adhered to A549 cells. Three non-exclusive adhesive patterns were observed: a diffuse adhesion in 38 isolates, a localized adhesion in 14 isolates, and an aggregative adhesion in seven isolates. 78.8% of the group 1 and 93.3% of the group 2 isolates were cytotoxic. Considering all 48 isolates, there was a strong and statistically significant correlation between cytotoxicity and adherence. Among the three dominant serotypes, O:12 isolates were in majority avirulent, but the great majority of O:1 and all the O:11 isolates were found adherent and cytotoxic. Gentamicin was the least active antibiotic for both groups, and ceftazidime was the most active antibiotic for group 1 and amikacin for group 2. The penicillinase production phenotype was significantly correlated with a decrease in *P. aeruginosa* virulence. © 2002 Éditions scientifiques et médicales Elsevier SAS. All rights reserved.

Keywords: Pseudomonas aeruginosa; Respiratory; Infection; Epidemiology; Antibiotic; Adherence; Cytotoxicity

1. Introduction

Pseudomonas aeruginosa is an opportunistic pathogen that can cause acute infections in patients with immunosuppression, burns or cystic fibrosis [1,2]. *P. aeruginosa* is the most common Gram-negative organism associated with nosocomial pneumonia [3]. Patients infected by this species are more likely to develop multiple organ failure and to die than patients with other types of pneumonia. Moreover, nosocomial *P. aeruginosa* strains exhibit high rates of resistance to antibiotics and are frequently multidrug resistant [4–10]. This high incidence of resistance causes several therapeutic complications and is associated with treatment failure and death. Thus, surveillance cultures of antibiotic-resistant *P. aeruginosa* strains from the rectum, stomach, oropharynx, trachea, or skin are usually done on admission and during hospitalization in intensive care units (ICUs) [11,12].

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Ventilator-associated pneumonia is almost always preceded by colonization of the upper respiratory tract with the causative microorganism [3]. Respiratory tract colonization can originate from endogenous sources such as the intestine or the stomach, but it predominantly comes from exogenous sources such as contaminated equipment and other, colonized, patients [13]. Even in non-epidemic settings, cross-colonization seems to play an important part in the epidemiology of colonization and infection with *P. aeruginosa*.

The first step in colonization of the respiratory tract is adherence to host tissues. *P. aeruginosa* recognizes receptors on epithelial cells and mucin [14,15]. Type IV pili and non-pilus adhesins are involved in this adherence process, which has been studied in vitro using human epithelial cell-lines like A549 cells [16–19]. The high mortality rate of *P. aeruginosa* pneumonia seems to be secondary to the ability of some strains to cause necrosis of the lung epithelium and to disseminate into the circulation rapidly [20,21]. Several extracellular virulence determinants may contribute to cytotoxicity, necrosis, invasion and dissemination [1].

We report here the epidemiological investigation of multidrug resistance and in vitro virulence phenotypes (pneumocyte cell adherence and cytotoxicity) among the *P. aeruginosa* strains isolated from respiratory tract infections in the ICUs of the centre hospitalier d'Orléans, France, during one year. We compared these strains to imipenem-resistant, extra-respiratory tract isolates collected from non-infected patients hospitalized in the same ICUs during the same period.

2. Materials and methods

2.1. Bacterial strains

A total of 33 *P. aeruginosa* strains, corresponding to all 1999 non-duplicate respiratory tract (R.T.) isolates responsible for pneumonia from 33 mechanically ventilated patients hospitalized in ICUs of the centre hospitalier d'Orléans, France (group 1), were compared to 15 imipenem-resistant, extra-respiratory tract (E.R.T.) isolates collected during the same period from non-infected patients hospitalized in the same ICUs (group 2). Diagnosis of *P. aeruginosa* pneumonia was based on quantitative cultures obtained from protected specimen brush samples ($>10^3$ CFU/ml), bronchoalveolar lavage samples ($>10^4$ CFU/ml), or endotracheal aspirates ($>10^7$ CFU/ml, >25 leucocytes, and <25 epithelial cells per observation field of a photonic microscope at a magnification 400×). The imipenem-resistant strains were isolated from nasal, rectal and skin (armpit or groin) specimens by culture on agar plates containing imipenem at a concentration of 8 mg/l. These imipenem-resistant strains were randomly chosen from among all the *P. aeruginosa* strains isolated in 1999 from surveillance cultures realized systematically on ICU admission excluding group 1 patients. The isolates were identified on the basis of typical morphology by Gram-negative staining, a positive oxidase reaction, and conventional biochemical tests using the API 20NE system (API BioMérieux, Lyon, France). The susceptibility to antimicrobial agents was determined by the disk diffusion method and interpreted according to the recommended French standards (Comité de l'antibiogramme de la Société Française de Microbiologie, http://www.sfm.asso.fr/). The intermediately resistant isolates were grouped with the resistant ones. Five β-lactam resistance phenotypes were distinguished according to sensitivity to ticarcilline, piperacilline, ceftazidime and aztreonam (4, 8, 9): type 1 (SSSS), type 2 (RRSS), type 3 (RRRR), type 4 (RSSR), type 5 (RRSR), corresponding, respectively, to wild-type, penicillinase production, cephalosporinase overproduction, permeability modification, penicillinase production and permeability modification.

2.2. Serotyping

Serotyping was performed by slide agglutination using commercially available O antisera (Sanofi Diagnostic Pasteur, Marnes-la-Coquette, France) as recommended by the manufacturer. All agglutinations were repeated at least two times to assess reproducibility.

2.3. RAPD analysis

Bacterial cells were cultivated in TS broth at 37 °C for 20 h. Genomic DNA was extracted by lysozyme and proteinase K treatment, and purified by phenol chloroform extraction and ethanol precipitation. DNA concentrations were determined by absorbance at 280 and 260 nm and adjusted to a final concentration of 40 ng/μl in Tris HCl 50 mM + EDTA 1 mM. RAPD reaction mixtures were set up and incubated exactly as described by Mahenthiralingam et al. [22], with primer 272 (5'-AGCGGGCCAA-3'). RAPD products (one-third of each reaction mixture) were then separated by electrophoresis in 1.5% agarose gels, with 0.5X TBE running buffer, containing ethidium bromide. As previously described, isolates that differed by two or more prominent bands were considered sufficiently divergent to warrant separate strain designation [23]. Duplicated RAPD analysis was performed for each strain to assess reproducibility. The results obtained with this RAPD protocol have been previously shown to be exactly correlated to the strain types found by *Spe*I macrorestriction analysis followed by pulse-field gel electrophoresis [22].

2.4. Adherence assay and cytotoxicity determination

Bacterial adherence and cytotoxicity experiments were carried out with the human A549 pneumocyte cell-line (ATCC CCL 165). Monolayers of epithelial cells were grown at 37 °C in HAM F12 medium (Eurobio, Les Ulis, France) containing 10% (v/v) fetal calf serum, 2 mM

glutamine, and antibiotics (200 U of penicillin and 50 mg of streptomycin per liter, respectively), on 24-well Falcon tissue culture plates (Becton Dickinson Labware, Oxnard, CA). Before adhesion tests, cells were washed with phosphate-buffered saline (PBS; pH 7.2). Bacteria were grown for 48 h in TS broth at 37 °C. Bacterial cells were resuspended at 5×10^8 bacteria per milliliter in the HAM F12 medium, added to the tissue culture, and incubated for 3 h at 37 °C. After five washes with PBS, cells were fixed in methanol, stained with 20% Giemsa (v/v), and examined microscopically under oil immersion. An adhesion index representing the average number of bacteria per cell and a cytotoxicity index representing the percentage of morphologically altered A549 cells were determined by examining 100 cells. Each adhesion and cytotoxicity result represents the mean of three separate experiments.

2.5. Statistical analysis

The correlation between adhesion and cytotoxicity indices was assessed using Spearman's rank-order (r_s) and Kendall's tau (τ) correlation coefficients. The statistical significance of differences was evaluated with equal-variance Student's *t*-test, following variance test with Fisher *F*-statistics. *P*-values below 0.05 were considered significant.

3. Results

3.1. Antibiotic resistance

The comparison of the two groups' rates of resistance of isolates to selected antipseudomonadal antibiotics is shown in Table 1. Among the 33 *P. aeruginosa* R.T. isolates, 28 were resistant to at least one class of the antibiotics tested. Twenty-three (69.7%) were multidrug resistant, i.e., resistant to antibiotics belonging to two or more distinct classes, 36.4% being resistant to β-lactams, aminoglycosides, and quinolones and 18.2% being resistant to only the first two compound families. The respective frequencies of the β-lactam resistance phenotypes were (Table 2): 57.6% (type 1), 21.2% (type 2), 12.1% (type 3), 6.1% (type 4) and 3.0% (type 5). The most active antimicrobial was ceftazidime (resistance in 12.1% of the isolates), and the least active antibiotic was gentamicin (resistance in 63.6% of the isolates). Resistance rates to imipenem, ciprofloxacin, norfloxacin and ticarcilline were high, with resistance in 57.6, 34.8, 54.5 and 42.4% of the isolates, respectively.

Among the 15 imipenem-resistant *P. aeruginosa* E.R.T. isolates collected from non-infected patients, rates of resistance were increased for ticarcilline, ceftazidime, aztreonam, gentamicin, netilmicin and ciprofloxacin, decreased for amikacin and norfloxacin, and very similar to R.T. isolates for piperacilline and tobramycin. The most active antimicrobial was amikacin (resistance in 6.7% of the isolates), and the least active antibiotic, except imipenem,

Table 1
Rates of resistance to selected antipseudomonadal antibiotics

Antibiotic	Percentage of isolates with resistance status[a]	
	R.T.[b]	E.R.T.[c]
Ticarcilline	42.4	53.3
Piperacilline	36.4	33.3
Ceftazidime	12.1	20
Aztreonam	21.2	40
Imipenem	57.6	100
Gentamicin	63.6	93.3
Tobramycin	27.7	26.7
Amikacin	21.2	6.7
Netilmicin	21.2	73.3
Ciprofloxacin	34.8	40
Norfloxacin	54.5	46.7

[a] Intermediately resistant and resistant isolates.
[b] R.T., respiratory tract isolates.
[c] E.R.T., extra-respiratory tract isolates.

was gentamicin (resistance in 93.3% of the isolates). The respective frequencies of the β-lactam resistance phenotypes were: 54.2% (type 1), 18.7% (type 2), 12.5% (type 3), 10.4% (type 4) and 4.2% (type 5).

3.2. In vitro adherence and cytotoxicity

Adhesion was considered positive if the adhesion index (mean number of bacteria per cell) was >1. As shown in Table 2, 28 (84.8%) *P. aeruginosa* R.T. isolates adhered to the pneumocyte cell-line A549, with high adhesion index value (more than five bacteria per cell) for 10 of them. Fourteen (93.3%) E.R.T. isolates were adherent, and five of them were highly adherent. Adherence was not significantly different between R.T. and E.R.T. isolates ($P = 0.46$).

Three patterns of adhesion, diffuse, localized and aggregative, were observed (Fig. 1). In the diffuse pattern, adherent bacteria were randomly and individually dispersed at the A549 cell surface. The localized and aggregative patterns were characterized by the formation of adherent microcolonies corresponding to small or huge clusters of bacteria, respectively. The tested strains revealed predominantly diffuse-type adherence, since 38 isolates expressed this adherence phenotype. The localized adhesion pattern was found in 14 isolates, and the aggregative pattern was found in seven isolates. Twelve isolates expressed both diffuse and localized patterns, and six isolates expressed both diffuse and aggregative patterns.

Cytotoxicity was considered positive if the cytotoxicity index (C.I., percentage of morphologically altered A549 cells) was >10%. For the control monolayers of pneumocyte cells, the C.I. was $7.93 \pm 1.43\%$. As shown in Table 2, 26 (78.8%) *P. aeruginosa* R.T. isolates were cytotoxic for the pneumocyte A549 cells, with high C.I. value (more than 50% of morphologically altered cells) for 20 of them. Fourteen (93.3%) E.R.T. isolates were cytotoxic, and 10 of them were highly cytotoxic. Cytotoxicity was not significantly different between R.T. and E.R.T. isolates ($P = 0.38$).

Table 2
Properties of the 48 *P. aeruginosa* clinical isolates

Bacterial strains	Origin[a]	β-Lactam resistance phenotype[b]	Serogroup[c]	RAPD type	Adhesion index[d]	Adhesion pattern[e]	Cytotoxicity index[f]
ER61964	R.T.	3	N.T.	1	2.77 (0.27)	D	66.6 (9.3)
ER72755	R.T.	1	1	2	11.97 (7.97)	D L	79.9 (5.4)
ER97314	E.R.T.	3	1	3	4.57 (0.46)	D L	58.4 (9.5)
ER76825	R.T.	1	1	3	2.71 (0.33)	D	44.1 (4.1)
ER87356	R.T.	1	1	4	3.91 (1.51)	D	86.2 (1.6)
ER86940	R.T.	5	1	5	3.08 (1.92)	L	6.7 (3.7)
ER93506	R.T.	3	1	6	4.88 (1.22)	D	78.6 (18.5)
ER63259	R.T.	1	3	7	5.68 (1.46)	D L	73.6 (26.3)
ER65564	R.T.	1	4	8	3.97 (0.22)	L	60.0 (23.8)
ER68916	R.T.	1	4	9	2.78 (0.21)	D L	20.1 (5.5)
ER65609	R.T.	1	4	10	5.50 (1.59)	D L	68.6 (18.6)
ER86676	R.T.	3	5	11	11.60 (6.21)	D A	42.3 (7.0)
ER78997	R.T.	2	5	12	0.20 (0.11)	–	9.3 (3.1)
ER69084	R.T.	1	6	13	4.88 (1.10)	D L	78.1 (13.9)
ER83950	R.T.	1	6	14	3.65 (0.55)	D	80.8 (9.4)
ER96426	R.T.	1	6	15	1.55 (0.44)	–	74.3 (17.6)
ER77126	R.T.	4	9	16	2.88 (0.39)	D L	100 (0)
ER65566	R.T.	1	10	17	4.25 (0.85)	D	78.9 (15.3)
ER74171	R.T.	1	11	18	4.42 (1.01)	D	96.5 (3.5)
ER96805	R.T.	1	11	19	16.53 (3.50)	D A	78.9 (7.9)
ER65673	R.T.	1	11	20	13.50 (5.29)	D L	56.6 (3.6)
ER67081	R.T.	1	11	21	8.14 (1.60)	D L	80.7 (19.3)
ER99146	R.T.	1	11	22	9.56 (0.36)	D A	100 (0)
ER98980	R.T.	4	11	23	1.86 (0.05)	D	38.2 (10.3)
ER91887	R.T.	2	11	24	13.78 (0.94)	D A	57.9 (36.6)
ER84081	R.T.	3	12	25	0.32 (0.13)	–	6.2 (1.2)
ER86371	R.T.	1	1	26	4.66 (0.54)	D	36.2 (3.2)
ER96904	R.T.	1	1	26	15.52 (5.49)	A	59.5 (14.1)
ER72565	R.T.	2	1	27	2.11 (0.17)	D	6.9 (0.8)
ER64194	R.T.	1	1	27	7.70 (1.70)	D	100 (0)
ER97030	E.R.T.	2	12	27	2.13 (1.04)	D	28.7 (4.9)
ER88377	R.T.	2	12	27	2.48 (0.28)	D	44.7 (14.5)
ER93610	R.T.	2	12	27	0.77 (0.14)	–	7.0 (0.4)
ER63204	R.T.	2	12	27	0.75 (0.14)	–	10 (0.1)
ER91886	R.T.	2	12	27	0.54 (0.07)	–	9.1 (0.1)
ER98513	E.R.T.	1	1	28	4.46 (1.46)	D	84.4 (6.0)
ER69360	E.R.T.	1	1	28	6.0 (0.85)	D	87.1 (12.9)
ER74520	E.R.T.	1	1	28	4.39 (0.14)	D	59.4 (18.8)
ER92581	E.R.T.	1	5	28	3.96 (0.55)	D	56.6 (4.9)
ER65026	E.R.T.	1	N.T.	28	7.57 (2.57)	D	21.6 (0.8)
ER99833	E.R.T.	1	1	29	14.69 (5.67)	D A	100 (0)
ER78976	E.R.T.	1	4	30	2.80 (1.42)	D L	82.3 (7.0)
ER99832	E.R.T.	4	4	31	4.43 (1.21)	D L	36.8 (4.5)
ER64949	E.R.T.	3	5	32	5.06 (1.69)	D L	23.55 (4.3)
ER69005	E.R.T.	4	10	33	3.80 (1.55)	D	80.3 (8.2)
ER96635	E.R.T.	4	10	33	3.89 (0.83)	D	86.4 (13.7)
ER99210	E.R.T.	5	11	34	10.92 (5.33)	D A	65.2 (4.4)
ER82483	E.R.T.	2	12	35	0.61 (0.08)	–	9.1 (1.1)

[a] R.T., respiratory tract isolates; E.R.T., extra-respiratory tract isolates.

[b] Five β-lactam resistance phenotypes were distinguished according to sensitivity to ticarcilline, piperacilline, ceftazidime, and aztreonam: type 1 (SSSS), type 2 (RRSS), type 3 (RRRR), type 4 (RSSR), type 5 (RRSR), corresponding, respectively, to wild-type, penicillinase production, cephalosporinase overproduction, permeability modification, penicillinase production, and permeability modification.

[c] N.T., not typeable.

[d] The adhesion index, representing the average number of bacteria per cell, was determined by examining 100 cells. Each adhesion result represents the mean of three separate experiments. Standard errors are indicated within brackets.

[e] D, diffuse; L, localized; A, aggregative.

[f] The cytotoxicity index, representing the percentage of morphologically altered A549 cells, was determined by examining 100 cells. Each cytotoxicity result represents the mean of three separate experiments. Standard errors are indicated within brackets.

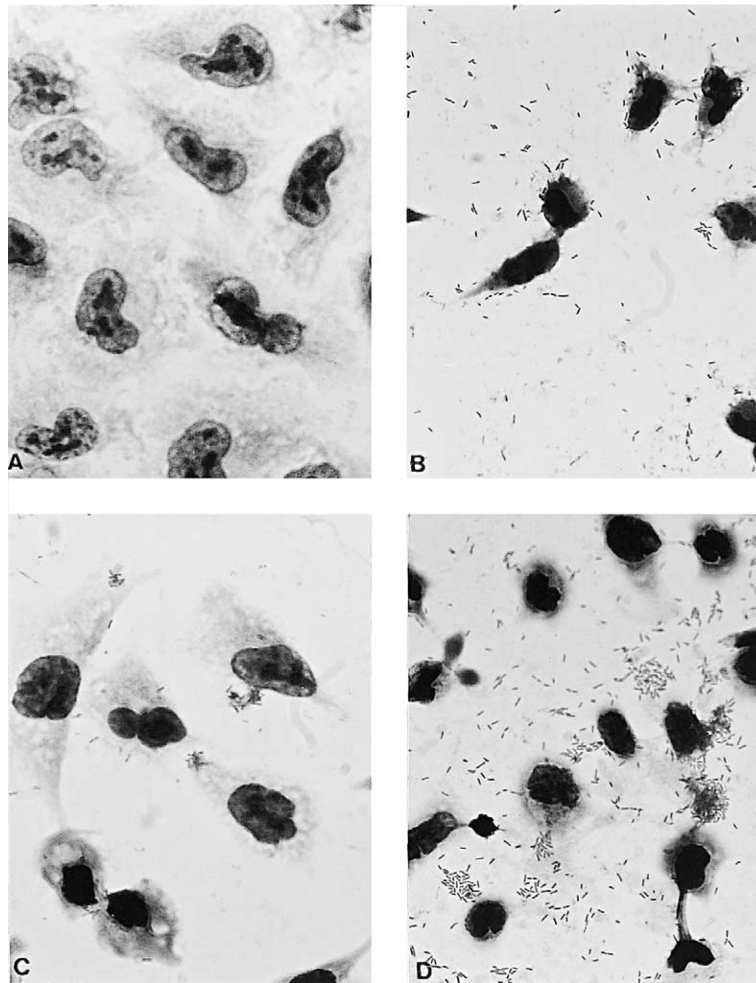

Fig. 1. Light micrographs of Giemsa-stained A549 cells, showing the three types of *P. aeruginosa* adherence patterns, after a 3-h incubation of 5×10^8 bacteria with A549 cells: A549 pneumocyte cells alone (A), diffuse adhesion (bacteria scattered over the cell surface) (B), localized adhesion (small clusters) (C), and aggregative adhesion (huge clusters of adhering bacteria) (D).

Considering all 48 isolates, adhesion indices were significantly positively correlated to cytotoxicity indices, both by Spearman's ($r_s = 0.50024$, $P = 0.00015$) and Kendall's ($\tau = 0.3201$, $\sigma_\tau^2 = 0.00995$, $P = 0.0007$) rank correlation tests, demonstrating a strong and statistically significant correlation between cytotoxicity and adherence.

3.3. Serotyping and RAPD analysis

Table 2 lists the serogroup and RAPD fingerprint of all the *P. aeruginosa* clinical isolates included in this study. Nine serogroups were found, and two isolates were non-serotypeable because they did not agglutinate with the serum used. The predominant serotypes were O:1, O:11 and O:12, representing 29.2, 16.7 and 14.6%, respectively, of serotypeable isolates. Thirty-five genotypes corresponding to unique RAPD types were found, indicating a high degree of genetic diversity. Five strains, representing 18 isolates, corresponding to genotypes 3, 26, 27, 28 and 33, were shown to be epidemic since they were isolated from at least two unrelated patients. Isolates belonging to genotypes 3 and 27 were encountered in both respiratory and extra-respiratory groups. There was no systematic relationship between RAPD profiles and serogroups. For example, ER72565 and ER88377, assigned to the same RAPD profile, had different serogroups (O:1 and O:12, respectively), and all the strains of the O:11 serogroup had different RAPD fingerprints.

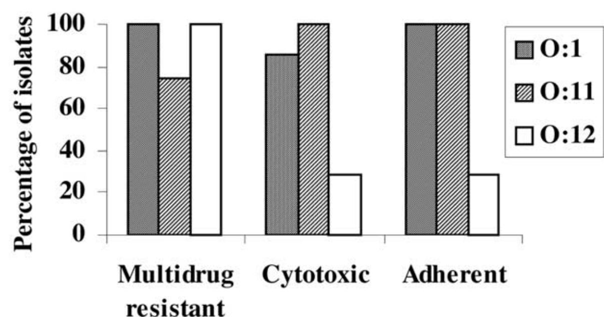

Fig. 2. Rates of multidrug-resistant, cytotoxic, and adherent isolates among the three major serotypes O:1, O:11 and O:12.

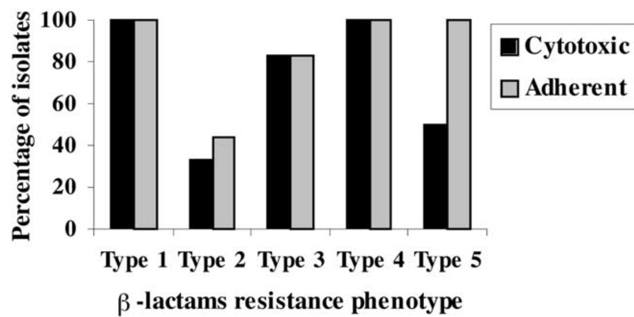

Fig. 3. Correlation between β-lactam resistance phenotypes, cytotoxicity and adherence. Five β-lactam resistance phenotypes were distinguished according to sensitivity to ticarcilline, piperacilline, ceftazidime and aztreonam: type 1 (SSSS), type 2 (RRSS), type 3 (RRRR), type 4 (RSSR), type 5 (RRSR), corresponding, respectively, to wild-type, penicillinase production, cephalosporinase overproduction, permeability modification, penicillinase production and permeability modification. The isolates expressing β-lactam resistance phenotype 2 were significantly less cytotoxic and less adherent than the rest of the isolates considered as a whole ($P = 0.0001$ and $P = 0.014$, respectively).

3.4. Correlation between antibiotic resistance, serotyping and in vitro virulence

The rates of multidrug-resistant, cytotoxic and adherent isolates among the three major serotypes O:1, O:11 and O:12 are presented in Fig. 2. All the *P. aeruginosa* isolates of the O:1 and O:12 serogroups, and six of the eight O:11 isolates were multidrug resistant. 85.7% of the O:12, 42.9% of the O:1, and 37.5% of the O:11 isolates were resistant to antibiotics belonging to the β-lactam, aminoglycoside and quinolone classes. All the O:1 and O:11 isolates were adherent, and all the O:11 and 85.7% of the O:1 isolates were cytotoxic. Among the O:12 isolates, 71.4% were not adherent and not cytotoxic.

The correlation between the β-lactam resistance phenotypes, cytotoxicity and adherence is presented in Fig. 3. The higher incidences of cytotoxicity and adherence were encountered among *P. aeruginosa* isolates belonging to β-lactam resistance patterns 1, 4 and 3. All the isolates expressing the β-lactam resistance phenotypes 1 and 4, 83.3% of the type 3, 44.4% of the type 2 and the two type 5 strains adhered to A549 cells. The respective frequencies of cytotoxicity expression among bacterial strains of the β-lactam resistance patterns 1, 2, 3, 4, and 5 were 100, 33.3, 83.3, 100 and 50%. The isolates expressing the β-lactam resistance phenotype 2 were significantly less cytotoxic and less adherent than the rest of the isolates considered as a whole ($P = 0.0001$ and $P = 0.014$, respectively).

4. Discussion

P. aeruginosa is an opportunistic pathogen that is an important cause of nosocomial respiratory tract infections and, more particularly, acute pneumonia in patients hospitalized in ICUs. There is experimental and epidemiological evidence for the existence of multiple mechanisms leading to colonization, and induction of cytotoxicity, pathology, and mortality during *P. aeruginosa* infections in vivo [11,20,21,24,25]. The use of mouse models of infection has shown that in vitro cellular toxicity could predict *P. aeruginosa* virulence in lung infections [20], and mutants decreased in virulence in vivo have been shown to be also decreased in adherence to epithelial cells in vitro [21]. A very recent study demonstrated that there was a significant correlation between the type III secretory protein phenotype, patient death, and higher toxicity in cellular and murine models of *P. aeruginosa* infections [25]. Nevertheless, little is known about the in vitro virulence status of *P. aeruginosa* strains responsible for respiratory tract infections in ICUs, and a correlation between adherence and cytotoxicity among these strains remains speculative. The present study was a comparison of the incidence of antibiotic resistance, adherence and cytotoxicity between *P. aeruginosa* strains responsible for respiratory tract infections and *P. aeruginosa* surveillance strains from extra-respiratory tract swabs, isolated in ICUs. We wanted to determine the virulence status of colonizing and pneumonia-causing strains, to examine the existence of correlations between adherence and cytotoxicity, and between virulence and antibiotic resistance.

Forty-eight *P. aeruginosa* isolates, corresponding to the totality of the non-replicate *P. aeruginosa* clinical isolates collected in 1999 from respiratory tract infections in patients hospitalized in the ICUs of the Orléans Hospital Center (33 isolates), and 15 imipenem-resistant isolates from surveillance cultures from non-infected patients performed during the same period and in the same ICUs were studied. We found high incidences of antibiotic resistance in both groups, which is consistent with the ICU origin of the strains tested [5,6]. Nevertheless, the majority of the isolates expressed wild-type β-lactam resistance phenotype 1. Of the three dominant serogroups, O:1, O:11 and O:12, O:12 had the highest degree of multidrug resistance. Several outbreaks of infections caused by *P. aeruginosa* O:12 isolates have been reported; a multi-resistant O:12 strain has been shown to spread in different countries in Europe [26,27]. The great majority of the O:12 isolates of the present study belong to the same RAPD type, indicating that one strain may have spread in the ICUs of the Orléans Hospital Center during the year 1999. This strain was sometimes assigned to the O:1 serotype, a phenomenon of serotype variation previously described after antibiotic treatment [28]. The nosocomial transmission of one *P. aeruginosa* strain and cross-infection within this hospital may be limited since RAPD typing demonstrated a high degree of diversity among all the isolates tested, suggesting the occurrence of mainly independent infectious episodes. Nevertheless, since two strains representing nine isolates were present in both groups, cross-acquisition, cross-colonization and cross-infection may have occurred. Genetic diversity, polyclonal endemicity of colonization and cross-colonization have

been previously described among *P. aeruginosa* strains isolated in ICUs [7,11,12,29,30], but the incidence of cross-acquisition and colonization in the epidemiology of *P. aeruginosa* in ICUs seems to be variable between hospitals.

We found high frequencies of in vitro virulence phenotypes (adherence and cytotoxicity) among both group 1 and group 2 isolates, and the incidence of virulence properties was not statistically different between the two groups. Moreover, there was a strong and statistically significant correlation between adherence and cytotoxicity among the totality of the isolates, indicating that expressing both virulence properties may be essential for *P. aeruginosa* during infections, and that surveillance isolates may have the same infectious potential as pneumonia-causing isolates. Rajan et al. [31] recently demonstrated that only fully virulent *P. aeruginosa* capable of coordinately expressing both adhesins and cytotoxins were able to induce apoptosis in respiratory epithelial cells. Moreover, adhesion and expression of the type III secretion system has also been shown to be necessary for efficient apoptosis of Chang epithelial cells by *P. aeruginosa* [32]. Hostacka and Majtan [33] previously showed high incidence (68.8%) of bacterial culture filtrate toxicity in a rabbit model vascular permeability activity assay correlated to Vero cell cytotoxicity among *P. aeruginosa* strains isolated from urine samples of patients after kidney transplantation. In a different study, Bartkova and Ciznar [34] showed that 89% of *P. aeruginosa* strains obtained from different origins (stool, urine, wounds, otitis, sputum, and sewage water) adhered to undifferentiated epithelial-like HeLa cells. They described the localized and diffuse adherence patterns and showed that Hela cells were a more suitable model for the differentiation of *P. aeruginosa* strains according to the mentioned types of adherence than CHO and Vero cells. Our results give evidence that the A549 pneumocyte cell-line is also a suitable model to determine the adhesive pattern of *P. aeruginosa* strains. Moreover, we were able to differentiate another adhesion pattern, termed aggregative. This aggregative pattern was different from the localized one, since it was characterized by the formation of adherent microcolonies after 60 min of bacterial interaction with pneumocyte cells and by the growth of these adherent microcolonies during the interaction time, growth that was absent for the localized adhering strains (data not shown). These adhesion patterns have been defined by analogy with diarrheagenic *Escherichia coli* and nosocomial *Klebsiella pneumoniae* strains [35,36]. Unlike *K. pneumoniae* strains, which mainly expressed the aggregative pattern, the *P. aeruginosa* respiratory tract isolates expressed mainly diffuse adhesion. In *K. pneumoniae* strains, the localized adhesion phenotype has been associated with multidrug-resistant isolates producing the CAZ-5/SHV-4 β-lactamase, but we found no association between the adhesion patterns and antibiotic resistance phenotypes among the *P. aeruginosa* strains tested. Independently from the adherence patterns, the lowest incidence of virulence properties was encountered among *P. aeruginosa* isolates belonging to β-lactam resistance pattern 2 corresponding to penicillinase production. Since five out of seven type 2 β-lactam resistance phenotype isolates corresponded to the same strain, this association could be explained by the spread of a less virulent clone producing a penicillinase. An experimental study has recently demonstrated a correlation between acquired resistance to antibiotics and virulence in *P. aeruginosa* in a mouse model of acute pneumonia [37]. Among four isogenic variants of a *P. aeruginosa* O:12 strain that differed in their resistance phenotypes to various β-lactam antibiotics, clones overexpressing a chromosomal type 1 β-lactamase were less virulent and produced lower amounts of extracellular virulence factors. Nevertheless, a direct implication of mutations responsible for β-lactam resistance in the reduction of *P. aeruginosa* virulence factor expression remains to be demonstrated.

In conclusion, the study of the epidemiology of antibiotic resistance and virulence properties of *P. aeruginosa* strains isolated during one year in the ICUs of the Orléans Hospital Center showed a high genetic diversity and high incidences of antibiotic resistance and virulence properties independently of their origin (R.T. or E.R.T.). Penicillinase production was shown to be correlated with a decrease in *P. aeruginosa* virulence.

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