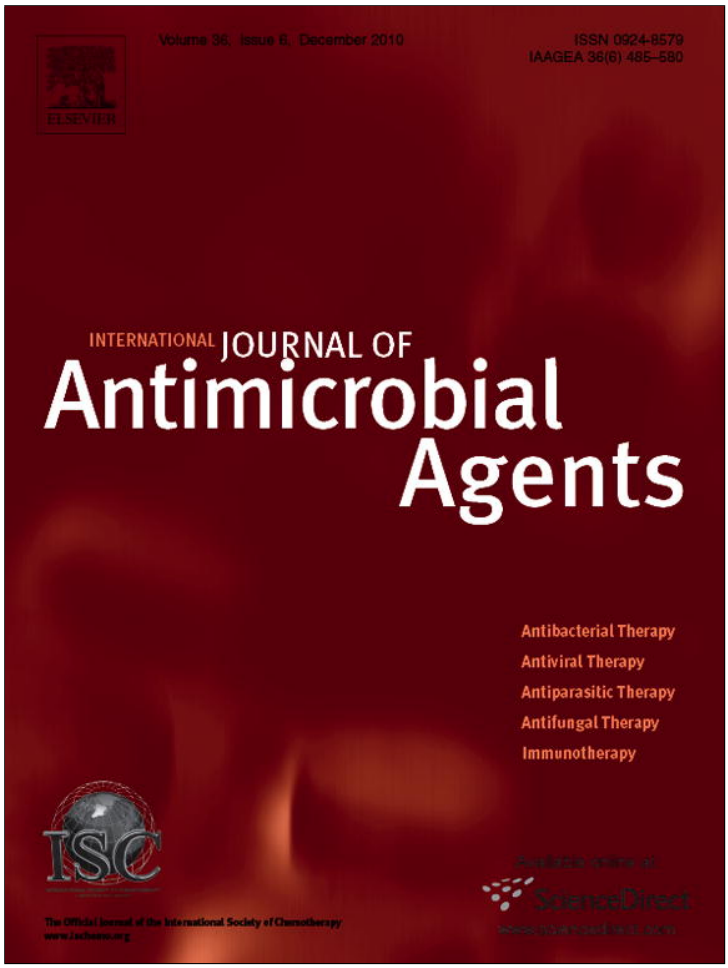

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In vivo development of antimicrobial resistance in *Pseudomonas aeruginosa* strains isolated from the lower respiratory tract of Intensive Care Unit patients with nosocomial pneumonia and receiving antipseudomonal therapy

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ABSTRACT

Pseudomonas aeruginosa causes severe nosocomial pneumonia in Intensive Care Unit (ICU) patients, with an increased prevalence of multiresistant strains. We examined the impact of the use of antipseudomonal antibiotic(s) on the susceptibility of *P. aeruginosa* isolated from ICU patients with clinically suspected hospital-acquired pneumonia collected in five teaching hospitals (110 non-duplicate initial isolates; 62 clonal pairs of initial and last isolates during treatment). Minimum inhibitory concentrations (MICs) were determined for amikacin, ciprofloxacin, meropenem, piperacillin/tazobactam (TZP), cefepime and ceftazidime (used in therapy) as well as five reporter antibiotics (aztreonam, colistin, gentamicin, piperacillin and ticarcillin) using Clinical and Laboratory Standards Institute (CLSI) methodology. Susceptibility was assessed according to European Committee on Antimicrobial Susceptibility Testing (EUCAST) and CLSI breakpoints. Resistance rates prior to treatment exceeded 25% for cefepime, ceftazidime, piperacillin, ticarcillin and aztreonam (EUCAST and CLSI) and for gentamicin, TZP and colistin (EUCAST only). The highest rates of cross-resistance were noted for ceftazidime and cefepime and the lowest rate for amikacin. Mean MIC values were systematically higher in isolates from patients previously exposed (1 month) to the corresponding antibiotic. For clonal pairs, a systematic increase in MIC between initial and last isolates (significant for amikacin, cefepime, meropenem and TZP) was noted. There was a significant correlation between the use of antibiotics (adjusted for respective proportional use of each drug) and loss of susceptibility at the population level when using EUCAST breakpoints. The high level of resistance of *P. aeruginosa* in ICU patients with nosocomial pneumonia as well as its further increase during treatment severely narrows the already limited therapeutic options. Further observational studies and the development of early diagnosis for resistant isolates are warranted.

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1. Introduction

Pseudomonas aeruginosa is a major nosocomial pathogen [1]. One of its preferential niches is the respiratory tract of Intensive Care Unit (ICU) patients with severe co-morbidities and receiving antibiotic treatment(s), resulting in so-called hospital-acquired pneumonia (HAP), especially in patients with impaired host defences [2–5]. The need for early appropriate antibiotic treatment in these patients [6] is substantiated by the observation of a direct correlation between increase in mortality rates and the delay with which such treatment is initiated [7–9]. *Pseudomonas aeruginosa* has a remarkable ability to develop resistance to most antimicrobial agents through multiple mechanisms. In this context, the last decades have witnessed the rapid and worldwide emergence of multidrug resistance in *P. aeruginosa*, with strains developing acquired resistance to almost all available classes of antipseudomonal antibiotics, including broad-spectrum penicillins, cephalosporins, carbapenems, aminoglycosides and fluoroquinolones (see [10] for a first historical report regarding imipenem, [11] for a review of 173 studies until the early 1990s and [9,12–14] for selected more recent reports). Acquisition of resistance is multifactorial, with mechanisms as varied as changes in membrane permeability, active efflux, production of antibiotic-degrading enzymes, and target mutations [14–17]. Infections with resistant strains are a major concern because they increase the risk of therapeutic failure [18,19] and are associated with secondary bacteraemia [20] and a considerable increase in mortality, length of hospital stay and overall health costs [9,21,22].

The emergence of multidrug-resistant (MDR) phenotypes makes epidemiological surveillance of resistance increasingly essential for the appropriate choice of empirical antibiotic regimens. Longitudinal surveillance may be even more important since there is increasing evidence that *P. aeruginosa* is capable of developing resistance to antibiotics during treatment [12,23–25].

In the present study, the level of in vitro resistance of *P. aeruginosa* isolates obtained at the onset of therapy and during treatment (clonal pairs) from patients with clinically suspected nosocomial pneumonia for which microbiological cultures strongly suggested that *P. aeruginosa* was the causative organism and who, accordingly, were treated with antipseudomonal antibiotics was assessed. A high rate of initial resistance to all antibiotics used in this set-up was observed, except for amikacin, as well as an increase in resistance of the same clonal isolates during treatment in relation to the global use of antibiotics in this population.

2. Materials and methods

2.1. Overall study design, patient selection, clinical analysis, record of antibiotic prescription and use, and time frame

The protocol of this observational study (no deviation from the standard of care of patients), as approved by the Ethical Committee of the Faculty of Medicine, Université catholique de Louvain (Brussels, Belgium), was to enrol prospectively patients with a clinical diagnosis of nosocomial pneumonia (defined as not present or incubating at the time of admission to the hospital and occurring >48 h later) based on clinical findings (fever, increase in volume of bronchial secretions, inflammatory syndrome with leukocytosis) along with the appearance of new radiographic infiltrates [after exclusion of other non-infectious causes of chest infiltrates such as alveolar haemorrhage due to trauma or other causes unrelated to infection (such as drug toxicity or acute respiratory distress syndrome)] and showing the presence of *P. aeruginosa* in endotracheal aspirates, bronchoalveolar lavage or puncture samples such as pleural fluid, empyema or blood cultures. Cultures were quantitative in some centres and semiquantitative in others [i.e. grading of bacterial growth as heavy (+4), moderate (+3), light (+2) or rare (+1) according to the growth density following streaking of the culture plates in four quadrants]. When multiple microorganisms were present, the role of *P. aeruginosa* as the likely aetiological pathogen was only retained if it appeared as the predominant organism. Cystic fibrosis patients were excluded. A complete retrospective analysis of the clinical charts was made to collect information on prior and current antibiotic regimens (during the pneumonia episode) as well as overall treatment outcome. Since suboptimal therapies are considered to promote the emergence of resistance, the quality of the treatments used was examined in terms of dosages and schedules of administration and was compared with (i) those recommended in the corresponding official Belgian labelling (also known as Summary of Product Characteristics) for severe infections and (ii) those based on accepted pharmacokinetic/pharmacodynamic criteria for optimised therapy foe the corresponding antibiotics [26–28].

2.2. Sample collection

Sample collection was performed in five Belgian teaching hospitals (four in the Brussels region and one in Wallonia region) and was initiated in 2006, although most samples were collected during the period 2007–2009. A sample was obtained at the time of initial diagnosis (D0 samples) for all enrolled patients (104 patients; 110 initial isolates). For 69 patients, a second (or more) subsequent sample(s) could be obtained during the course of therapy (range 1–123 days; mean 23 days; median 17.5 days) based on the decision of the clinicians to perform such additional sampling as part of their standard of care. Bacterial identification was carried out locally using standard microbiological methods, after which isolates were frozen in cryovials at −80 °C for transfer to the co-ordinating laboratory (Université catholique de Louvain).

2.3. Isolates used for the study, reference strains, minimum inhibitory concentration (MIC) determination and susceptibility criteria

Each isolate received by the co-ordinating laboratory was checked for purity and for the presence of a single clone based on colony morphology. When needed, identification was checked using a commercial gallery (API® 20 NE; bioMérieux, Marcy l'Etoile, France) and by ability to grow at 42 °C. *Pseudomonas aeruginosa* reference strains ATCC 27853 and PAO1 [29] were used as internal quality controls. MICs were determined by geometric microdilution in cation-adjusted Mueller–Hinton broth (BD Diagnostics, Franklin Lakes, NJ) according to Clinical and Laboratory Standards Institute (CLSI) recommendations [30]. Susceptibility categorisation was assessed according to current susceptibility and resistance breakpoints of the European Committee on Antibiotic Susceptibility Testing (EUCAST) [31] and the CSLI [32].

2.4. Determination of clonality

Clonality of successive isolates obtained from individual patients was assessed by repetitive extragenic palindromic–polymerase chain reaction (REP-PCR). *Pseudomonas aeruginosa* isolates were cultured overnight at 37 °C on Luria–Bertani agar (Sigma-Aldrich, St Louis, MO) plates. Total bacterial DNA was extracted using an UltraClean™ Microbial DNA Isolation Kit (MO BIO Laboratories Inc., Carlsbad, CA) as detailed in the manufacturer's protocols. REP-PCR profiles of the *P. aeruginosa* isolates were obtained using a DiversiLab™ system (bioMérieux). The PCR mixture (25 μL final volume) contained 11.5 μL of sterile distilled water, 1.25 μL of GeneAmp® 10× PCR buffer I (Applied

Biosystems, Life Technologies, Carlsbad, CA), 9 μL of REP-PCR MM1 (bioMérieux), 1 μL of Primer Mix (bioMérieux), 0.25 μL (1.25 U) of AmpliTaq® DNA polymerase (Applied Biosystems) and 2 μL of template DNA. Thermal cycles included an initial denaturation at 94 °C for 2 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 50 °C for 30 s and extension at 70 °C for 90 s, and a final extension at 70 °C for 3 min. REP-PCR profiles were obtained using microfluidic DNA chips (DiversiLab™ LabChip®; bioMérieux) and an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA) according to the manufacturers' instructions. REP-PCR fingerprinting profiles were compared using the Web-based DiversiLab software v.3.3.4 (bioMérieux), which uses the Pearson correlation coefficient and the outweighed pair group method.

A threshold criterion of 95% similarity was used, corresponding to two or less peak differences in the whole electrophoresis pattern. The same method was used to assess the clustering of initial (D0) samples in either the much conserved clonal serotype O12 or in one of four serotype O11 clonal complexes (CCs) (F, G, H and I).

2.5. Materials for laboratory studies

Gentamicin, amikacin, ticarcillin, piperacillin, ciprofloxacin and colistin (polymyxin E) were obtained from Sigma-Aldrich (St Louis, MO); aztreonam and cefepime (Bristol-Myers Squibb, Brussels, Belgium), meropenem (AstraZeneca, Brussels, Belgium), ceftazidime (GlaxoSmithKline, Genval, Belgium) and piperacillin/tazobactam (TZP) (Wyeth Pharmaceuticals, Ottignies-Louvain-la-Neuve, Belgium) were obtained as the corresponding branded products registered for intravenous administration and complying with the provisions of purity and content of the European Pharmacopoeia. All other chemicals were of analytical grade and were obtained from E. Merck AG (Darmstadt, Germany) or Sigma-Aldrich. All culture media were from BD Diagnostics.

2.6. Statistical methods

Statistical analyses were performed using GraphPad® Prism software v.4.3 and GraphPad InStat v.3.06 (GraphPad Software Inc., La Jolla, CA) and using the online statistical calculator from the Saint John's University (Collegeville, MI) [33] for testing the normality of the MIC distributions included in each comparison [two-sample Kolmogorov–Smirnov test of normality, with calculation of cumulative probabilities (KS *P*)].

3. Results

3.1. Overall study design, characteristics of samples and patients, general clinical data and treatments

Fig. 1 shows the origin and mode of selection of the isolates analysed in this study. From an initial number of 144 patients [identified as being hospitalised in the ICUs of the participating hospitals and from whom a *P. aeruginosa* strain had been isolated (233 non-duplicate isolates)], 104 patients (199 non-duplicate isolates) were retained as fulfilling the clinical and radiological criteria for suspicion of nosocomial pneumonia and with *P. aeruginosa* likely to be the main aetiological agent. From these 104 patients, 110 non-duplicate initial isolates were obtained (referred to as D0 samples). To exclude biases due to the potential presence of epidemic multiresistant clones, all isolates were genotyped by the semi-automated REP-PCR-based DiversiLab method. Whether isolates clustered in the very conserved serotype O12 clone or in one of four serotype O11 CCs (F, G, H and I), known to be the most frequently involved in outbreaks caused by MDR *P. aeruginosa* strains, was also analysed [34]. Only two isolates could be associated with

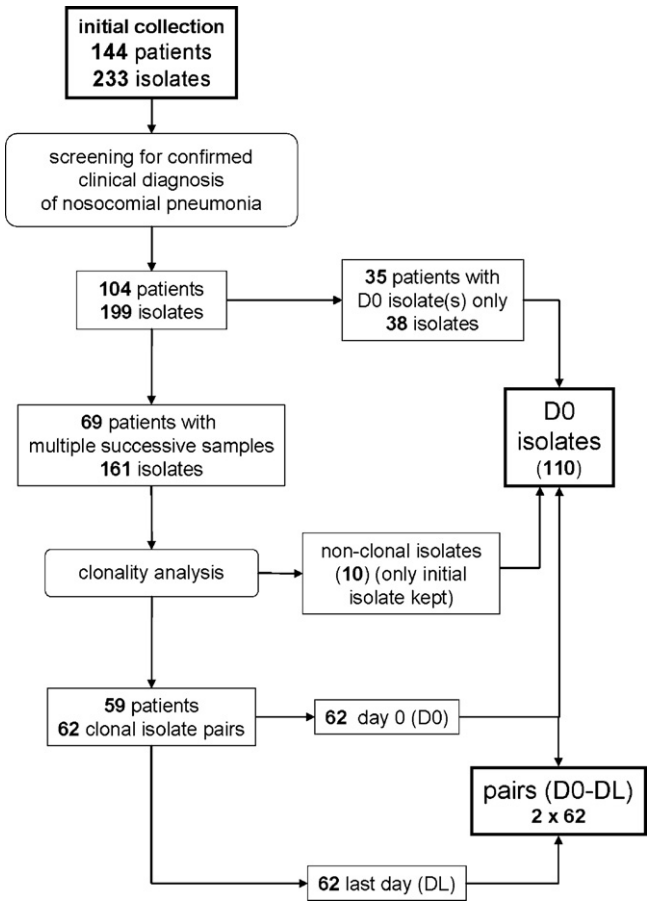

Fig. 1. Flow diagram identifying the origin of the isolates used in the present study. The initial collection consisted of non-duplicate isolates of *Pseudomonas aeruginosa* obtained from patients hospitalised in the Intensive Care Units of the participating institutions and with a suspicion of nosocomial pneumonia. All cases were screened for clinical evidence of nosocomial (hospital-acquired) pneumonia (including ventilated patients) and the corresponding first samples (collected before initiation of antipseudomonal therapy) constituted the initial (D0) isolates. Samples obtained during antipseudomonal therapy (from patients with successive positive samples) were subjected to clonality analysis (together with the corresponding initial sample) to constitute clonal pairs of first (D0) and last (DL) isolates.

the epidemic MDR O12 clone, but they were isolated in different hospitals at a 2-year interval. Six isolates could be associated with one of the O11 CCs, but again these were from different hospitals and obtained at several months interval.

For 69 patients, successive isolates were obtained during antipseudomonal treatment, resulting in 62 confirmed clonal pairs of an initial and a last isolate (the latter being referred to as DL samples). Patients were mostly adults (only three patients were <18 years of age and excluding them did not change the results of the analyses) and were very often ventilated at the time of the diagnosis (Table 1). For patients from whom clonal pairs could be obtained: (i) approximately one-half received monotherapy only; (ii) in general, amikacin (often for a short period only and always in combination with another antibiotic), meropenem, cefepime and TZP were preferentially prescribed; and (iii) approximately one-third of the patients died, but only one-half of them from the infection. It was also observed that in all patients, initial antibiotic dosages and schedules were (i) those recommended for severe infections and (ii) according to their pharmacokinetic/pharmacodynamic properties (see details in Table 1). Analysis of the clinical records performed independently of the attending clinicians showed no systematic bias in the way follow-up samples were obtained between patients and centres.

Table 1
General characteristics of patients, treatments and outcomes.

Total population (N = 104)					
	Min.	GM	Mean ± S.D.	Median	Max.
Age (years)	1.2	54.1	60.0 ± 19.3	63.1	85.0
Ventilated (No. of patients)					
Yes	74				
No	30				

Patients with clonal pairs (n = 59)	
Antibiotics with antipseudomonal potential (initial treatment[a]) (no. of patients)	
AMK	29
CIP	11
MEM	28
TZP	31
FEP	29
CAZ	4

Assessment of adequateness of initial therapy[b]				
	No. of patients	No. of adequate antibiotics/total	% (no.) of patients with adequate therapy[c] based on breakpoints of:	
			EUCAST	CLSI
Monotherapy	26	1/1	57.7 (15)	73.1 (19)
2 antibiotics	14	2/2	71.4 (10)	85.7 (12)
		1/2	28.6 (4)	14.3 (2)
3 antibiotics	13	3/3	38.5 (5)	46.2 (6)
		2/3	30.8 (4)	30.8 (4)
		1/3	23.1 (3)	23.1 (3)
4 antibiotics	1	4/4	0.0 (0)	0.0 (0)
		3/4	100 (1)	100 (1)
Clinical outcome (no. of patients)[d]				
Alive	41			
Dead				
Pneumonia	9			
Other causes	9			

GM, geometric mean; S.D., standard deviation; AMK, amikacin; CIP, ciprofloxacin; MEM, meropenem; TZP, piperacillin/tazobactam; FEP, cefepime; CAZ, ceftazidime; EUCAST, European Committee on Antimicrobial Susceptibility Testing; CLSI, Clinical and Laboratory Standards Institute. Patients with clonal pairs (n = 59).

[a] Typical initial treatments (for adults): AMK, 15 mg/kg every 24 h; CIP, 200–400 mg every 12 h; MEM, 2 g every 8 h; TZP, 4 g every 6–8 h; FEP, 2 g every 8–12 h; CAZ, 2 g every 8–12 h.

[b] Considering only patients having received one (or several) of the six antipseudomonal antibiotics examined in this study (n = 54 patients); based on the minimum inhibitory concentration of the initial isolate(s) and using EUCAST or CLSI criteria for non-resistant organisms [susceptible (S) or intermediate (I); see breakpoints in Table 2].

[c] Figures indicate the percentage of patients with an isolate non-resistant to the drug prescribed in the case of monotherapy, or to one, two, three or four of the antibiotics prescribed in case of multiple drug therapy.

[d] Assessed after 90 days following the collection date of the first isolate, except for two patients (alive) for whom the observation period was extended to 202 days and 213 days, respectively.

3.2. Susceptibilities of initial isolates

Table 2 shows the susceptibility patterns and $MIC_{50/90}$ values (MICs for 50% and 90% of the organisms, respectively) of the 110 isolates obtained from the first sample (D0), with susceptibility categorisation according to EUCAST and CLSI criteria (see Supplementary Fig. 1 for a graphical representation of the cumulative MIC distribution). With respect to the main antibiotics used for therapy in the institutions surveyed, resistance (based on EUCAST breakpoints) exceeded 25% for TZP, cefepime and ceftazidime, was ca. 20% for meropenem and ciprofloxacin and was only 8% for amikacin. More than 25% of the isolates were resistant by EUCAST criteria to all other antibiotics tested for epidemiological purposes. Of note, MICs of colistin were all in a narrow range (1–4 mg/L), i.e. at the limit of the EUCAST breakpoints. There was a high level of cross-resistance between TZP on the one hand and ceftazidime and cefepime on the other hand (in ca. 75% of the TZP-resistant isolates; see Supplementary Table 1). Isolates resistant to colistin according to EUCAST were also often resistant to ciprofloxacin, meropenem, cefepime and ceftazidime (33–42% of colistin-resistant isolates).

Fig. 2 shows the impact of previous exposure (up to 1 month) to five antibiotics on the MIC of the initial *P. aeruginosa* isolates (ceftazidime was excluded because of the small number of patients). For all antibiotics except amikacin, MIC values were systematically higher when patients had been previously exposed to the corresponding antibiotic (with geometric means approaching or even exceeding the EUCAST susceptibility breakpoint). However, this decrease in susceptibility was statistically significant for meropenem and TZP only.

3.3. Changes in susceptibilities during exposure to antipseudomonal antibiotics and clinical outcomes

Fig. 3 shows the change in susceptibility of clonal isolates between the initial (D0 isolate) and last day (DL isolate) of treatment. The MIC of all antibiotics increased, with the differences reaching statistical significance for all antibiotics (for ciprofloxacin, by considering $\log_2$ transformed data only, probably due to the low number of samples). When assessing each clone individually, it was found that MIC values of most antibiotics increased by two- to four-fold compared with the initial value (1–2 $\log_2$ dilutions) (Fig. 4). Excluding patients with <5 days of antipseudomonal treatment (8 of 59 patients) did not modify the results. A retrospective case–control study was performed to identify whether a MIC

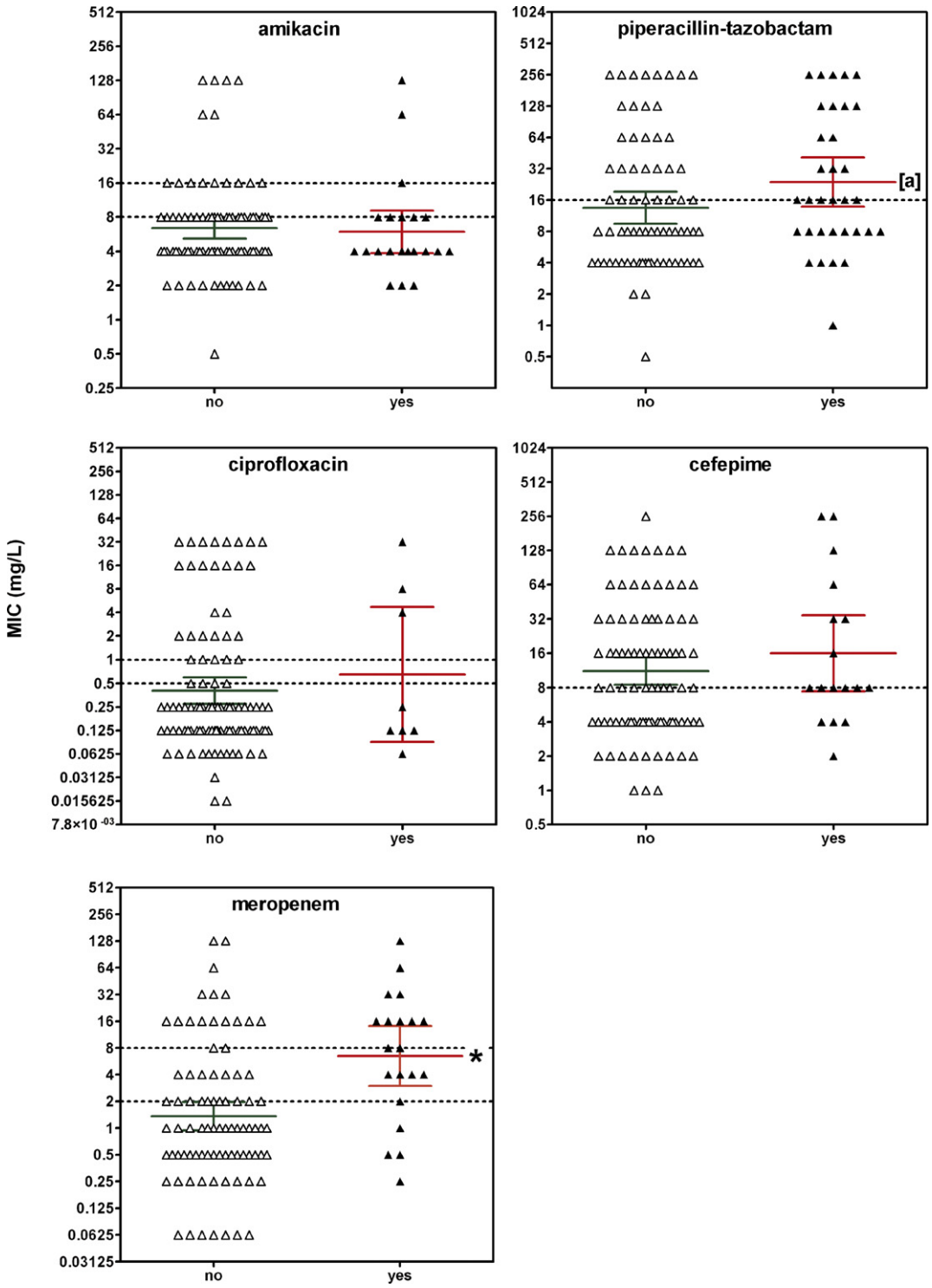

Fig. 2. Minimum inhibitory concentrations (MICs) of five antibiotics used in empirical antipseudomonal therapy against initial isolates, stratified between patients having either not received (no) or received (yes) the corresponding drug within 1 month prior to collection of the isolate. The scatter dot-plots show the individual values with their geometric mean and 95% confidence interval. The number of isolates was (no/yes, respectively): amikacin, 87/23; ciprofloxacin, 102/8; meropenem, 90/20; piperacillin/tazobactam (TZP), 77/33; and cefepime, 93/17. The two dotted lines in each graph show the susceptible (S) (lowest line) and resistant (R) (highest line) European Committee on Antimicrobial Susceptibility Testing (EUCAST) breakpoints of the corresponding antibiotic (MIC ≤ S indicates susceptible; MIC > R indicates resistant; and MIC > S and ≤ R indicates intermediate; there is no intermediate category for TZP and cefepime). For the statistical analysis, the distributions were subjected to normality test [Kolmogorov–Smirnov (KS)] and were found to be unlikely to be normally distributed when using the raw data, but normally distributed for meropenem [KS P (yes group only) = 0.65] and possibly normally distributed for TZP [KS P (yes group only) = 0.22)] when using their $\log_2$ transformed data. The differences in MICs between each of the two sets of samples (no vs. yes) were therefore examined both by a parametric test [unpaired t-test (two-tailed) with Welch's correction] and a non-parametric test (Mann–Whitney) and were found to be: (i) significant for meropenem by Mann–Whitney ($P = 0.0009$) when considering raw data and by both tests ($P = 0.008$ and 0.0009, respectively) when considering their $\log_2$ transforms (marked as *); (ii) close to significance ($P = 0.0579$ by Mann–Whitney both for raw and $\log_2$ transformed distributions) for TZP (marked as [a]), but not for the other antibiotics ($P > 0.06$ for both tests and both for raw and $\log_2$ transformed data).

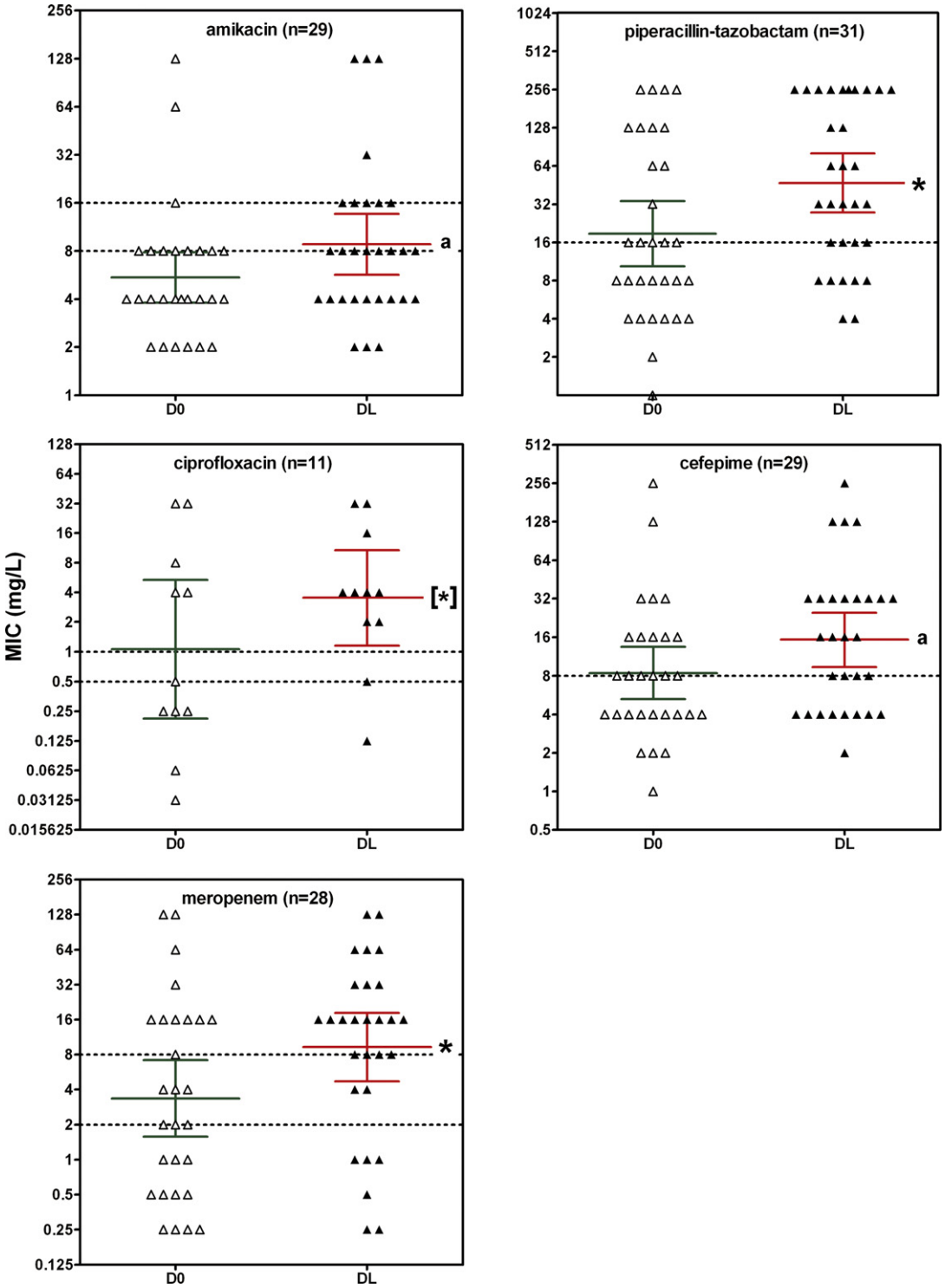

Fig. 3. Changes in the minimum inhibitory concentrations (MICs) of five antibiotics used in empirical antipseudomonal therapy against the isolate identified before onset of therapy (D0) versus the last isolate (DL) collected from the same patient during treatment with the corresponding antibiotics and showing clonal similarity with the first isolate. The scatter dot-plots show the individual values with their geometric mean and 95% confidence interval. The two dotted lines in each graph show the susceptible (S) (lowest line) and resistant (R) (highest line) European Committee on Antimicrobial Susceptibility Testing (EUCAST) breakpoints of the corresponding antibiotic [MIC ≤ S indicates susceptible; MIC > R indicates resistant; and MIC > S and ≤ R indicates intermediate; there are no intermediate categories for piperacillin/tazobactam (TZP) and cefepime]. For the statistical analysis, the distributions were subjected to normality test [Kolmogorov–Smirnov (KS)] and were found to be unlikely to be normally distributed when using the raw data, but normally distributed for ciprofloxacin (KS $P > 0.59$), consistent with a normal distribution for meropenem, TZP and cefepime (KS $P = 0.23$–0.41) and unlikely to be normally distributed for amikacin [KS P (at D0) = 0.05) when using their $\log_2$ transformed data. The differences in MICs between each of the two sets of samples (D0 vs. DL) was therefore examined both by a parametric (two-tailed paired t-test) and a non-parametric (Wilcoxon matched-pair test) using both raw data and their $\log_2$ transforms. Differences were found to be significant both for raw and $\log_2$ transformed data by both tests (marked as *) for meropenem ($P = 0.011$ and 0.002) and TZP ($P = 0.028$ and 0.008) and by Wilcoxon test only (marked as **a**) for amikacin ($P = 0.013$) and cefepime ($P = 0.009$). For ciprofloxacin, the difference was not statistically significant ($P > 0.05$ for both tests) when considering raw data, but was significant ($P < 0.02$ for both tests) when using their $\log_2$ transforms (marked as [*]).

Table 2
MIC_{50} and MIC_{90} values and susceptibility patterns (based on EUCAST and CSLI criteria) of the initial isolates ($n = 110$) of *Pseudomonas aeruginosa* from patients with clinically suspected nosocomial pneumonia and enrolled in the study.

Antibiotic	$MIC_{50/90}$ (mg/L)	% non-susceptible isolates according to:			
		EUCAST		CLSI	
		Breakpoint[a] (≤S/R>) (mg/L)	%I/R[b]	Breakpoint[c] (≤S/R≥) (mg/L)	%I/R[b]
AMK[d]	4/16	8/16	9/8	16/64	1/7
CIP[d]	0.25/8	0.5/1	7/23	1/4	4/18
MEM[d]	1/16	2/8	12/24	4/16	3/24
TZP[d]	8/128	16/16	**34**[f]	64[g]/128	7/12
FEP[d]	8/64	8/8	**46**[f]	8/32	17/**30**
CAZ[d]	4/64	8/8	**39**[f]	8/32	6/**33**
GEN[e]	2/64	4 /4	**26**[f]	4/16	10/15
PIP[e]	8/128	16/16	**36**[f]	64[g]/128	0/**26**
TIC[e]	64/512	16/16	**86**[f]	64/128	0/**39**
ATM[e]	8/32	1/16	**68**/**30**	8/32	20/**30**
CST[e]	2/4	4/4	4.5[f]	2/8	26/0

$MIC_{50/90}$, minimum inhibitory concentrations for 50% and 90% of the organisms, respectively; EUCAST, European Committee on Antibiotic Susceptibility Testing; CLSI, Clinical and Laboratory Standards Institute; S, susceptible; R, resistant; I, intermediate; AMK, amikacin; CIP, ciprofloxacin; MEM, meropenem; TZP, piperacillin/tazobactam; FEP, cefepime; CAZ, ceftazidime; GEN, gentamicin; PIP, piperacillin; TIC, ticarcillin, ATM, aztreonam; CST, colistin.

[a] From the EUCAST website (http://www.eucast.org); breakpoints (clinical; organisms with MIC > S and ≤ R are considered intermediate).

[b] Figures in bold indicate situations in which resistance to a given antibiotic exceeds 25% of isolates based on the corresponding criteria (EUCAST or CLSI).

[c] From CLSI [35]; breakpoints (clinical; isolates with MIC > S and < R are considered intermediate).

[d] Antibiotics commonly used for antipseudomonal treatment.

[e] Antibiotics used for epidemiological purposes in the context of the present study.

[f] No intermediate category of clinical breakpoints for this antibiotic.

[g] According to the CLSI, the S category for TZP or PIP relates to high-dose therapy for serious infections and monotherapy is associated with treatment failure in serious infections.

increase could be correlated with administration of the respective antibiotic. Whilst each antibiotic treatment was associated with an odds ratio >1 for MIC increase, this was statistically significant for amikacin only. An attempt to link the decrease in susceptibility to the duration of exposure to any specific antibiotic, or to all of them, did not yield significant results because of the low number of patients in each subgroup.

Table 3 shows that the decreased susceptibility observed during treatment caused marked increases in the proportion of isolates categorised as intermediate or resistant using EUCAST breakpoints, with all of them except amikacin exceeding a threshold of 25%. There was a significant correlation between the proportional use of each antibiotic and the loss of susceptibility at the whole population level. An apparent loss of susceptibility for colistin (based on the EUCAST breakpoint for resistance of >4 mg/L) was also documented. Cross-resistance was also increased (not shown), but this did not reach statistical significance because of the too small number of isolates.

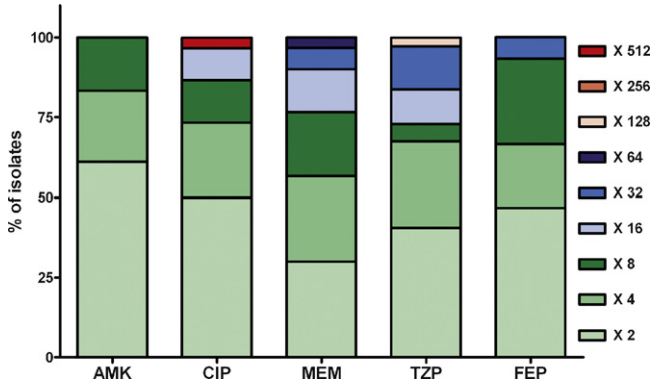

Fig. 4. Increases in the minimum inhibitory concentrations (MICs) of five antibiotics used in empirical antipseudomonal therapy between the first isolate (D0) and last isolate (DL) collected from the same individual patient. The *y*-axis shows the percentage of clonal pairs with a given increased MIC [from $2 \times (1 \log_2$ dilution) to $512 \times (9 \log_2$ dilutions) the value of the D0 isolate] out of all those showing an increased MIC ($n = 18$ for AMK, $n = 30$ for CIP, $n = 30$ for MEM, $n = 37$ for TZP and $n = 30$ for FEP). AMK, amikacin; CIP, ciprofloxacin; MEM, meropenem; TZP, piperacillin/tazobactam; FEP, cefepime.

Patients who died from the pneumonia ($n = 9$) (see Table 1) had not been more exposed to inappropriate antipseudomonal antibiotics during treatment [in terms of proportion of active antibiotics received (12 of 15)] than the general population.

4. Discussion

The present study represents one of the first recent efforts to document systematically the loss of susceptibility of *P. aeruginosa* isolates to antipseudomonal antibiotics when used in the treatment of clinically suspected HAP for which *P. aeruginosa* was considered the putative causative organism. Making a diagnosis of pneumonia in the ICU is notoriously difficult [36] since radiographic signs of chest infiltrates as well as microbiological analysis both lack specificity. Furthermore, collection of deep invasive specimens by bronchoscopy is often not feasible in these mechanically ventilated patients because of their unstable condition. Because this study was observational, it was not possible to obtain true quantitative cultures from all patients as this would have exceeded the current standard of care. Thus, we are left with some degree of uncertainty about the true pseudomonal nature of the infection in some episodes. However, since all enrolled patients were treated with antipseudomonal antibiotics, the main goal of our study, which was to examine the increase in resistance of *P. aeruginosa* in patients (i) from the onset of their antipseudomonal treatment and (ii) for whom clonal pairs could be isolated during this treatment was actually reached, irrespective of whether the true causative organism was *P. aeruginosa*. Potential biases due to the presence of known multiresistant epidemic clones were explicitly excluded, analysing in detail the MIC shifts occurring for clonal pairs obtained during exposure to antipseudomonal antibiotics and applying the interpretative criteria of EUCAST. Access to follow-up samples was limited by the decision of the clinician as to whether to perform a second or more subsequent samplings during therapy owing to ethical and practical considerations. Whilst this may have led to lack of samples from patients with a rapid fatal outcome (which could have heralded gross antibiotic failure), the converse may also be true, i.e.

Table 3
Comparative susceptibility of clonal isolates obtained from 59 patients with a clinical diagnosis of nosocomial pneumonia before initiation of treatment (D0) and during antipseudomonal treatment (DL).

Antibiotic	Use (%)	Non-susceptible isolates according to:				Loss of susceptibility (%) during treatment[b] and correlation with antibiotic use	
		EUCAST (%I/R)[a]		CLSI (%I/R)[a]			
		D0	DL	D0	DL	EUCAST	CLSI
AMK[c]	22.0	1.6/11.3	11.3/16.1	0.0/11.3	4.8/11.3	14.5	4.8
CIP[c]	8.3	4.8/**25.8**	4.8/**35.5**	3.2/22.6	6.5/**29.0**	9.7	9.7
MEM[c]	21.2	12.9/22.6	14.5/**35.5**	1.6/22.6	6.5/**35.5**	14.5	17.7
TZP[c]	23.5	**33.9**[e]	**53.2**[e]	0.0/17.7	0.0/**32.3**	19.5	14.6
FEP[c]	22.0	**40.3**[e]	**53.2**[e]	12.9/**27.4**	8.1/**45.2**	14.5	12.9
CAZ[c]	3.0	**35.5**[e]	**46.8**[e]	8.1/**27.4**	8.1/**38.7**	11.3	11.3
						$r = 0.89$[f] ($P = 0.03$)	$r = 0.27$[f] ($P = 0.66$)
GEN[d]		21.0[e]	**29.0**[e]	8.1/12.9	12.9/16.1	8.0	8.0
PIP[d]		**35.5**[e]	**54.8**[e]	0.0/24.2	0.0/**33.9**	19.4	9.7
TIC[d]		**87.1**[e]	**91.9**[e]	0.0/**37.1**	0.0/**62.9**	4.8	25.8
ATM[d]		71.0/24.2	53.2/**43.5**	24.2/24.2	21.0/**43.5**	1.6	16.1
CST[d]		4.3[e]	**37.1**[e]	24.6/0	24.2/0	32.8	0

EUCAST, European Committee on Antimicrobial Susceptibility Testing; CSLI, Clinical and Laboratory Standards Institute; I, intermediate; R, resistant; AMK, amikacin; CIP, ciprofloxacin; MEM, meropenem; TZP, piperacillin/tazobactam; FEP, cefepime; CAZ, ceftazidime; GEN, gentamicin; PIP, piperacillin; TIC, ticarcillin, ATM, aztreonam; CST, colistin.

[a] See breakpoints in Table 2; figures in bold indicate situations in which resistance to a given antibiotic exceeds 25% of isolates based on the corresponding criteria (EUCAST or CLSI).

[b] % of isolates moving from S to I or R between Day 0 and Day ≥3.

[c] Antibiotics actually used for treatment.

[d] Antibiotics used for epidemiological or resistance mechanism-uncovering purposes.

[e] EUCAST has no intermediate category for these antibiotic/*P. aeruginosa* combinations.

[f] Non-parametric correlation (Spearman rank) between the % of use of each antibiotic (% of all antibiotic prescriptions) in the whole population (AMK, 24.0; CIP, 9.6; MEM, 20.2; FEP, 15.4; and CAZ, 3.8) and the increase in % of isolates with change in susceptibility (moving from S to I, I to R, or S to R) for the corresponding antibiotic.

patients with rapid improvement, perhaps due to antibiotic(s), are less likely to yield more than an initial sample.

The data show that (i) empirical therapy, assuming that *P. aeruginosa* is the causative organism, is often inappropriate in terms of choice of antibiotic (with pre-exposure to the same antibiotic being a detrimental element, except for amikacin) and (ii) increase in resistance occurs for all antibiotics during exposure.

Treatment of pneumonia caused by *P. aeruginosa* is difficult, with crude mortality rates reaching 40% or higher [37]. In the present series, the mortality rate of patients for whom successive clonal samples could be obtained reached ca. 30%, with inability to control the infection being the main likely cause of death for approximately one-half of these patients. In contrast to an earlier report in which rates of primary resistance of *P. aeruginosa* in the ICU were relatively low [38], here it was found, as in another recent study [39], that MDR organisms are frequent at the very early onset of the disease. Although not designed to provide a true epidemiological estimate, the present study clearly shows that the clinician's choice of active antibiotics has become increasingly narrow when *P. aeruginosa* is amongst the causative organisms. Thus, combined empirical therapy, although still a matter of debate [40–42], may now be the only available option to ensure a reasonable coverage if *P. aeruginosa* is considered to be the aetiological agent. As was the case in this study, combination therapy is actually often used in daily practice and is advocated as being essential to obtain a satisfactory response [39,43]. A first main conclusion from this study is, therefore, that significant efforts must be deployed to accelerate the early assessment of bacterial susceptibility in order to decrease the risk of therapeutic failure [44] whilst at the same time avoiding unnecessary use of wide-spectrum combinations.

As anticipated from previous recent studies [38,39,45], a clear trend towards an increase in resistance of the initial isolates during treatment was also observed. In addition, results from the present study demonstrate that therapeutic choices if *P. aeruginosa* is amongst the target organisms are narrowed down considerably when EUCAST interpretative criteria are endorsed. Although the decreased susceptibilities observed in the present study were often not statistically significant, this should not undermine the conclusions. The antibiotic doses and treatment schedules used (all at or close to the maximum values set forth in the respective labellings; see footnote a in Table 1) as well as the frequent use of combinations were actually expected to decrease the risk of emergence of resistance and/or selection of less susceptible subpopulations [16,26–28,46–48]. For obvious ethical reasons, a study in which a significant proportion of patients would be treated in a suboptimal fashion is, nowadays, impossible to design in a prospective way given what we know about optimisation of antibiotic use. Thus, the trends we see may actually be the only, but important, signals heralding the risks associated with antibiotic therapy of pseudomonal infection. Of note, short-course amikacin therapy (to minimise the risk of nephrotoxicity) may have contributed to the maintenance of its overall activity towards initial isolates.

Because *P. aeruginosa* isolates collected from hospitalised patients may originate from multiple sources, it is often difficult to distinguish between emergence of resistance within the original population from the acquisition of another less susceptible strain. Clonal analysis of successive isolates of *P. aeruginosa* was previously used to address this issue [38,49]. The method used here targets highly conserved non-coding repetitive sequences [50] and thus ensures a higher level of reliability. This study therefore provides overwhelming evidence that the decrease in susceptibility of *P. aeruginosa* observed in patients receiving antipseudomonal antibiotics may really take place within the original bacterial population. Although the precise mechanisms that cause these changes in susceptibility still need to be studied in detail, the moderate increases in MIC would suggest a predominant role of increased efflux or decreased porin permeability [51,52]. A second main conclusion of this study is therefore that close monitoring of susceptibility testing should be performed during treatment since even minor changes may result in a change of susceptibility categorisation when using EUCAST breakpoints. Based on the most likely underlying antibiotic resistance mechanism, they also may lead to cross-resistance between structurally very different antibiotic classes [52–54]. Both considerations should lead to important reassessment of the ther-

apeutic strategies. In this context, the present data on colistin are interesting as they rationalise the recent EUCAST breakpoint (resistant >4 mg/L) adaptation. At Day 0 we were mainly confronted with an essentially wild-type population (few patients if any had received colistin), and yet the former EUCAST breakpoint (resistant >2 mg/L) would have categorised almost one-half of this population as resistant. The rise in colistin MIC observed in DL isolates is a reason for concern as this drug was almost never used for treatment. More broadly speaking, these results, and those of many other studies, clearly call for the design and use of new molecules with a lesser propensity to trigger the emergence of resistance.

Throughout this study we were faced with the difficulty of choosing appropriate criteria, namely those of EUCAST or CLSI, for categorising isolates as susceptible, intermediate or resistant to the antibiotics under study. In vitro criteria of this kind are only useful as long as they provide reasonably accurate predictive information about the clinical outcome of therapy. However, this study was not designed to validate their accuracy for prediction of clinical outcomes. Further studies focusing on specific antibiotics will be needed to allow for a rational and final choice between these two common sets of interpretative criteria.

In summary, this study demonstrates that resistance of *P. aeruginosa* to commonly recommended antipseudomonal antibiotics is an every-day reality in the present environment of ICUs and that current standard therapies do not prevent an increase in resistance during exposure to these antibiotics. Whilst only *P. aeruginosa* was studied here, a similar situation may prevail for other Gram-negative bacteria causing nosocomial pneumonia, such as *Klebsiella* spp., *Enterobacter* spp. or *Acinetobacter baumannii*. These are indeed emerging rapidly, in part as a consequence of prior antibiotic therapy [55], and share the capacity of becoming resistant to many first-line antibiotics. Observational studies coupled to the development of early diagnostic methods therefore seem warranted.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ijantimicag.2010.08.005.

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