

Prevalence and spread of *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* strains in patients with hematological malignancies

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SUMMARY

The aim of the study was to determine the prevalence of *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* strains in patients with acute leukemias, to assess their clinical significance, and to define the sources and ways of their spread using genetic analysis. Thirty-four patients were investigated during the observed period. Twenty-one strains of *Pseudomonas aeruginosa* and 35 strains of *Klebsiella pneumoniae* were isolated from patient samples. In the case of *Pseudomonas aeruginosa*, 47.6% of strains were identified as pathogens and caused infection. By contrast, only 4 isolates (11.4%) of *Klebsiella pneumoniae* could be regarded as etiological agents of bacterial infection. Based on the obtained results, *Klebsiella pneumoniae* strains are assumed to be of mostly endogenous origin. In the case of *Pseudomonas aeruginosa* strains, the proportion of identical strains detected in various patients was higher and exogenous sources were more significant. In addition, our results confirmed the ability of *Pseudomonas aeruginosa* strains to survive on a particular site in the hospital for a longer time.

KEY WORDS: Gram-negative bacteria, Source, Dissemination, Hemato-oncology

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INTRODUCTION

The introduction of penicillin into medical practice and the subsequent development of other antibiotics raised hopes that bacterial diseases would no longer be a problem. To a great disappointment, the expectations were not met. Dr. Harold Neu (1992) in his Science article warned of an alarming increase in bacterial resistance to antimicrobials. At the turn of the 21st century, we

are witnessing an extremely dangerous situation - a dramatic increase in infectious diseases caused by bacteria with new and extensive resistance phenotypes (Paterson and Bonomo, 2005; Peterson, 2005). Therefore, bacterial infections continue to pose a serious and rising problem in current medicine.

Bacterial infections threaten particular persons with impaired function of the immune system. This may be illustrated in patients with hematological malignancies in whom the myelosuppressive and immunosuppressive effects of cytostatics contribute to further weakening of the body's defense mechanisms.

The subsequent infections of various etiologies (especially bacterial and fungal) significantly influence the prognosis of patients. The significant bacterial pathogens in these patients are the

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strains of *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* whose importance increases as their resistance to antibiotics rises (Paterson *et al.*, 2004; Fanci *et al.*, 2001). Certain strains of the above-mentioned species, the so-called epidemic clones, are enormously successful at spreading within hospitals.

Therefore, it is obvious that identical bacteria may be spread horizontally among patients of a particular department by environmental vectors, such as health workers' hands. Modern molecular biology methods, such as pulsed-field gel electrophoresis of macrorestriction fragments of genomic DNA, enable identification of the epidemic clones, thus significantly contributing to the traditional methods of bacterial surveillance (Gori *et al.*, 1996).

The presented work aimed at determining the prevalence of *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* strains in patients with severe neutropenia over 2 months, assessing their clinical significance, and defining the sources and ways of spread of these strains by genetic analysis.

MATERIAL AND METHODS

The Department of Hemato-Oncology (DHO) at the Teaching Hospital in Olomouc, Czech Republic is a tertiary care facility providing comprehensive treatment, including intensive high-dose chemotherapy and allogeneic stem cell transplantation. Treatment of acute leukemia patients in the study including induction and consolidation chemotherapy was performed at a 16-bed intensive care unit where vital functions may be monitored.

Patients were nursed in two-bed rooms equipped with shower and toilet facilities. Physicians and nurses wore face masks and washed their hands with disinfectant soap and solutions before entering the rooms. The patients received a normal hospital diet. Water and air were neither conditioned nor filtered. The rooms were routinely cleaned twice daily with disinfectants in accordance with the hospital guidelines.

No standard antibiotic prophylaxis was given to patients treated for febrile neutropenia according to an established protocol.

Strains of *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* were isolated from clinical material (sputum, blood and urine samples; throat, rectal, urethral and vaginal swabs) obtained from patients treated at the above-mentioned unit over a 2-month period (5 January - 5 March, 2007). From each patient, one strain of *Pseudomonas aeruginosa* or *Klebsiella pneumoniae* was included, isolated from individual clinical specimens during two weeks.

Over the same time period, samples were collected three times from surfaces and objects in rooms where patients were hospitalized. The samples were taken with sterile cotton swabs soaked in sterile MH broth (Becton Dickinson), either from a 10x10 cm area in two directions perpendicular to each other or from entire smaller objects. Subsequently, the swabs were put into MH broth containing 1% glucose (Becton Dickinson) and placed in an incubator for primary culture.

The isolated strains were identified by standard microbiological procedures using the Vitek 2 (bioMérieux) automated system. The antibiotic susceptibility was assessed by the standard microdilution method according to the CLSI (2007).

The breakpoint values were set as follows: 1 mg/L for ciprofloxacin; 2 mg/L for ofloxacin, levofloxacin and tetracycline; 4 mg/L for cefazolin, cefuroxime, cefotaxime, ceftazidime, cefepime, meropenem, tobramycin, gentamicin, chloramphenicol and colistin; 8 mg/L for cefoperazone, cefoperazone/sulbactam, ampicillin/sulbactam and amikacin; 16 mg/L for piperacillin/tazobactam; and 32 mg/L for cotrimoxazole. These are breakpoints used in the Czech Republic. Strains with minimum inhibitory concentration (MIC) of a given antibiotic higher than the above-mentioned breakpoints were classified as resistant. Reference strains *Escherichia coli* ATCC 25922, *Escherichia coli* ATCC 35218 and *Pseudomonas aeruginosa* ATCC 27853 served for protocol quality control. The MIC₅₀ and MIC₉₀ values were defined as minimum concentrations at which 50% and 90%, respectively, of isolates were inhibited by a given antibiotic.

In all *Klebsiella pneumoniae* strains with an MIC of at least one of the tested third-generation cephalosporins (cefotaxime, ceftazidime or cefoperazone) higher than 1 mg/L, a modified dou-

ble-disc synergy test (DDST) was carried out to determine ESBL production. Jarlier's DDST was modified by including a disc with cefepime and by simultaneous use of a disc with ceftazidime and clavulanic acid (Jarlier *et al.*, 1988; Tzelepi *et al.*, 2000; Pitout *et al.*, 2003). AmpC beta-lactamases were detected by an AmpC disc test using discs with cefotaxime, ceftazidime, ceftazidime with clavulanic acid, and their combinations with 3-aminophenylboronic acid, an AmpC beta-lactamase inhibitor.

The above-mentioned antibiotic discs containing 400 µg of the acid were used to detect production of the enzymes if inhibition zones were extended by more than 5 mm when compared with discs without 3-aminophenylboronic acid (Yagi *et al.*, 2005).

Phenotypic testing for ESBL production was supplemented with detection of genes encoding the appropriate beta-lactamases. The isolates were inoculated onto meat-peptone agar and incubated aerobically for 16 h at 37°C.

Bacterial DNA was obtained by suspending 1-2 grown colonies in 100 L of water and warming to 99° C for 15 min.

The supernatant containing the bacterial DNA was used for PCR detection of the *bla*_{TEM}, *bla*_{SHV}, *bla*_{OXA} and *bla*_{CTX-M} genes with the following primers: TEM-F (5'-ATGAGTATTCAACATTTC-CG-3'), TEM-R (5'-CCAATGCTTAATCAGT-GAGC-3'), SHV-F (5'-CTTTACTCGCTTTATCG-3'), SHV-R (5'-TCCCGCAGATAAATCACCA-3'), OXA-1F (5'-ACACAATACATATCAACTTCGC-3'), OXA-1R (5'-AGTGTGTTTAGAATGGTGATC-3'), OXA-2F (5'-TTCAAGCCAAAGGCACGATAG-3'), OXA-2R (5'-TCCGAGTTGACTGCCGGGTTG-3'), OXA-10F (5'-CGTGCTTTGTAAAAGTAGCAG-3'), OXA-10R (5'-CATGATTTTGGTGGGAATGG-3'), CTX-MU1 (5'-ATGTGCAGYACCAGTAARGT-3') and CTX-MU2 (5'-TGGGTRAARTARGTSACCA-GA-3') (Arlet *et al.*, 1995; Chanawong *et al.*, 2000; Steward *et al.*, 2001; Pagani *et al.*, 2003).

Detection of plasmid-encoded AmpC beta-lactamases was performed by multiplex PCR using the following set of primers: MOXMF (5'-GCT-GCTCAAGGAGCACAGGAT-3'), MOXMR (5'-CACATTGACATAGGTGTGGTGC-3'), CITMF (5'-TGGCCAGAACTGACAGGCAAA-3'), CITMR (5'-TTTCTCCTGAACGTGGCTGGC-3'), DHAMF (5'-AACTTTCACAGGTGTGCTGGGT-3'), DHAMR (5'-CCGTACGCATACTGGCTTTGC-3'),

ACCMF (5'-AACAGCCTCAGCAGCCGGTTA-3'), ACCMR (5'-TTCGCCGCAATCATCCCTAGC-3'), EBCMF (5'-TCGGTAAAGCCGATGTTGCGG-3'), EBCMR (5'-CTTCCACTGCGGCTGCCAGTT-3'), FOXMF (5'-AACATGGGGTATCAGGGAGATG-3') and FOXMR (5'-CAAAGCGCGTAACCG-GATTGG-3') (Perez-Perez & Hanson, 2002).

The resulting PCR products were subjected to electrophoretic separation in 1.5% (w/v) agarose gel. Visualization of the PCR products was completed by staining with ethidium bromide (Sigma-Aldrich) (1 µg/mL).

Molecular biology typing was carried out by pulsed-field gel electrophoresis (PFGE) of DNA fragments digested with *Xba*I restriction endonuclease (Girlich *et al.*, 2000; Pournaras *et al.*, 2003). Genomic DNA was isolated according to procedures described by Shi *et al.* (1996). PFGE in 1.2% gel was run for 24 hours at 6 V/cm and pulse times of 2-35 s.

After PFGE, the gel was stained with ethidium bromide (Sigma-Aldrich) (1 µg/mL) and visualized under UV illumination. The acquired restriction maps of individual isolates were compared using GelCompar II software and 1.5% tolerance Dice coefficients and their relationships were determined.

Clinical significance of the isolated strains of *Pseudomonas aeruginosa* and *Klebsiella pneumoniae*, or distinction between colonization and being an etiological agent of infection, was based on the assessment of all available clinical and laboratory investigations.

RESULTS

During the above-mentioned period (5 Jan - 5 Mar, 2007), a total of 34 patients with acute leukemia receiving combined cytostatic therapy were investigated. In all patients, the administered chemotherapy resulted in the development of severe neutropenia.

From the collected clinical samples, 21 strains of *Pseudomonas aeruginosa* and 35 strains of *Klebsiella pneumoniae* were isolated. The number of patients in whom the *Klebsiella pneumoniae* strains were isolated was 24, with repeated detection from either different clinical samples or identical material collected with a time interval of more than 14 days in 5 patients.

The *Pseudomonas aeruginosa* strains were detected in 12 patients, with 41.7% of cases being repeatedly detected from either different clinical samples or identical material collected with a time interval of more than 14 days (Table 1).

A total of 90 samples were collected from surfaces and objects in the DHO, and 6 isolates of *Pseudomonas aeruginosa* were detected (Table 2). These strains were repeatedly isolated from two rooms, with different time intervals. No *Klebsiella pneumoniae* strains were detected in this type of collected samples.

Susceptibility of the species to individual antibiotics was assessed. As seen from the results listed in Table 3, the *Klebsiella pneumoniae* isolates were most susceptible (100%) to colistin and meropenem. Higher susceptibility was also observed to amikacin (94.3%).

Production of ESBL was neither phenotypically nor genotypically confirmed. AmpC beta-lactamases were detected in 7 (20.0%) strains. Using multiplex PCR, a single product of 405 bp (DHA-like) was observed in all the cases. In *Pseudomonas aeruginosa*, the highest suscepti-

TABLE 1 - Analysis of detection of *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* strains in patients with hematological malignancies.

	<i>Klebsiella pneumoniae</i>		<i>Pseudomonas aeruginosa</i>	
Number of patients with a single isolate detected	19		7	
Number of patients with multiple isolates detected	Patient CL	5 Sputum (10) + throat swab (3) + rectal swab (12)	Patient KJ	5 Urine (twice; 38-day time interval) (10, 21)
	Patient FJ	Sputum (5) + blood (6) + urine (25) + rectal swab (15)	Patient LJ	Throat swab (3) + rectal swab (4)
	Patient HJ	Sputum (26) + throat swab (24) + rectal swab (17)	Patient PJ	Blood (6) + vaginal swab (5) + urethral swab (9)
	Patient KM	Throat swab (8) + rectal swab (1)	Patient VV	Sputum (2) + throat swab (7)
	Patient ZR	Sputum (twice; 26-day time interval) (7, 20) + throat swab (23) + rectal swab (30)	Patient ZR	Sputum (twice; 20-day time interval) (14, 19) + throat swab (15) + rectal swab (twice; 17-day time interval) (16, 20)

NB: The numbers in brackets correspond with the sample (isolate) numbers in the dendrogram (Figures 1 and 2).

TABLE 2 - Detection of *Pseudomonas aeruginosa* strains in samples collected from surfaces and objects in the DHO.

Date of collection	Number of swabs	Number of positive results	Site of collection
1 Feb 2007	30	2	Room A - showerhead Room B - bath sponge
13 Feb 2007	30	3	Room A - showerhead Room A - bathroom sink Room B - bathroom sink
5 Mar 2007	30	1	Room A - showerhead

bility (100%) was observed to amikacin and colistin, and higher activity (71.4%) to ceftazidime and gentamicin.

In the case of *Pseudomonas aeruginosa*, 10 isolates (47.6%) were identified as pathogens causing infections, most commonly pneumonia (28.6%), bloodstream infection (19.1%) and urinary tract infection (9.5%). By contrast, only 4 isolates (11.4%) of *Klebsiella pneumoniae* could

be regarded as etiological agents of bacterial infection, namely bloodstream infection in two patients and pneumonia or urinary tract infection in the remaining two.

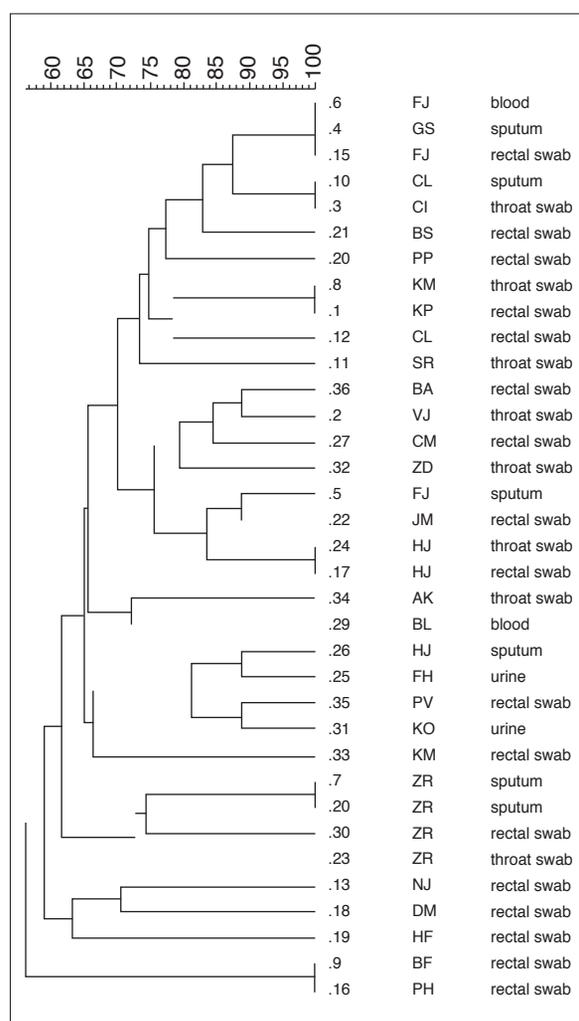
Whereas the *Klebsiella pneumoniae* strains were most frequently cultured from rectal (48.6%) and throat (22.9%) swabs, the strains of *Pseudomonas aeruginosa* were most commonly isolated from throat and rectal swabs (both 23.8%) and from

TABLE 3 - Susceptibility of *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* isolates to antimicrobial agents, including MIC ranges, MIC₅₀ and MIC₉₀.

Antibiotic	<i>Klebsiella pneumoniae</i>				<i>Pseudomonas aeruginosa</i>			
	MIC range (mg/L)	MIC ₅₀ (mg/L)	MIC ₉₀ (mg/L)	Susceptibility (%)	MIC range (mg/L)	MIC ₅₀ (mg/L)	MIC ₉₀ (mg/L)	Susceptibility (%)
Ampicillin/sulbactam	0.5 - ≥128	16	128	54.3	-	-	-	-
Piperacillin/tazobactam	0.25 - ≥128	4	128	77.1	1 - ≥512	4	128	61.9
Cefazolin	1 - ≥128	4	128	51.4	-	-	-	-
Cefuroxime	0.5 - ≥128	2	16	60.0	-	-	-	-
Ceftazidime	0.125 - ≥32	0.25	8	77.1	0,5 - ≥64	1	32	71.4
Cefotaxime	0.125 - ≥32	0.25	16	77.1	-	-	-	-
Cefoperazone	0.125 - ≥32	0.5	32	77.1	1 - ≥64	4	32	61.9
Cefoperazone/sulbactam	0.125 - ≥32	0.5	32	77.1	-	-	-	-
Cefepime	0.063 - 2	0.125	0,5	80.0	-	-	-	-
Meropenem	0.063 - 1	0.063	0,25	100	0.25 - 64	2	16	61.9
Gentamicin	0.125 - ≥64	0.25	8	85.7	0.25 - ≥64	2	32	71.4
Tobramycin	0.125 - ≥64	0.5	16	71.4	-	-	-	-
Amikacin	0.25 - 16	0.5	2	94.3	0.25 - 1	0.5	1	100
Ciprofloxacin	0.063 - ≥32	0.125	8	74.3	0.125 - ≥32	0.25	32	61.9
Ofloxacin	0.063 - ≥32	0.125	16	74.3	0.25 - ≥32	1	32	61.9
Levofloxacin	0.063 - ≥32	0.125	8	74.3	0.25 - ≥32	1	16	61.9
Chloramphenicol	1 - ≥64	8	32	62.9	-	-	-	-
Tetracycline	2 - ≥64	8	16	8.6	-	-	-	-
Cotrimoxazole	0.25 - ≥256	0.5	64	80.0	-	-	-	-
Colistin	0.125 - 2	0.5	2	100	0.25 - 4	1	2	100

TABLE 4 - Clinical material from which the *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* strains were isolated.

Clinical material	<i>Klebsiella pneumoniae</i>		<i>Pseudomonas aeruginosa</i>	
	Total number	Percentage	Total number	Percentage
Rectal swab	17	48.6	5	23.8
Throat swab	8	22.9	5	23.8
Sputum	6	17.1	3	14.3
Blood	2	5.7	3	14.3
Urine	2	5.7	3	14.3
Urethral swab	0	0	1	4.8
Vaginal swab	0	0	1	4.8



sputum, blood and urine samples (all 14.3%) (Table 4).

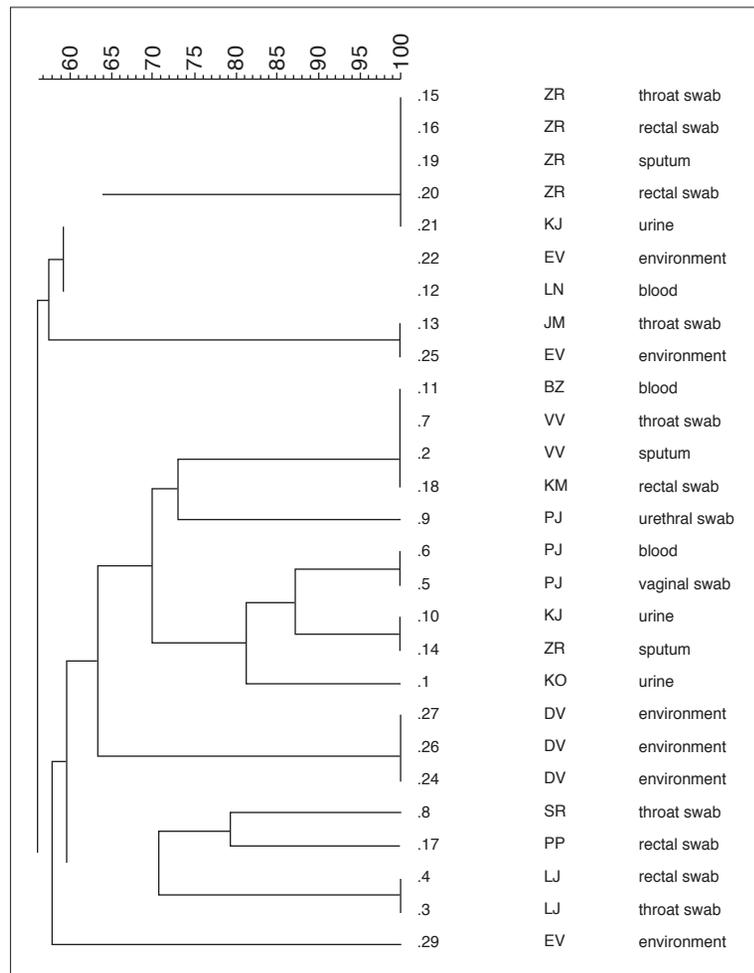
PFGE of the *Klebsiella pneumoniae* strains identified 5 pairs and 1 triad of identical strains (Figure 1). Four of the pairs were observed in a single patient, either from different clinical samples or from identical material collected with a time interval of more than 14 days. Therefore, it is obvious that in Patient ZR (Table 1), the two strains isolated from sputum with a 26-day time interval were identical. In two cases (1 pair and 1 triad), the identical bacterial strain was found in different patients.

The triad was from two patients, with the strains being isolated from blood and a rectal swab in the first of them and from sputum only in the other. In both patients, these strains were responsible for infection, namely bloodstream infection and pneumonia. In another pair of patients, the strains were isolated from rectal swabs and were just colonizing.

In the case of the *Pseudomonas aeruginosa* strains, 1 group of five, 1 group of four, 1 group of three and 4 pairs of identical strains were identified (Figure 2). Three identical isolates were collected from the same place in the DHO, a show-

FIGURE 1 - Dendrogram of the *Klebsiella pneumoniae* isolates. Key: Horizontal axis - percentage of strain similarities, vertical axis - isolate numbers, patient initials, materials.

FIGURE 2 - Dendrogram of the *Pseudomonas aeruginosa* isolates. Key: Horizontal axis - percentage of strain similarities, vertical axis - isolate numbers, isolate numbers, patient initials, materials.



erhead in the room facilities. However, these strains were not detected in patients' clinical samples. One pair consisted of a surface isolate (bathroom sink) and that from a patient (throat swab), in whom colonization only was confirmed. Two isolate pairs were from identical patients but different biological material.

In one patient, the strain was isolated from rectal and throat swabs and found to be only colonizing. In the other, the strain isolated from blood and a urethral swab was responsible for bloodstream infection.

Another pair was observed in two different patients. Very likely, the same strain was transmitted from one patient to the other one, causing urinary infection in one and pneumonia in the other. These patients exchanged another strain as well. This was isolated five times in one patient, first colonizing him (the strain was

detected twice in a rectal swab and once in a throat swab of Patient ZR, Table 1) and then causing his pneumonia (repeated isolation from sputum). The same strains were also detected in other patients' urine, causing no clinically apparent infection. Four identical isolates were detected in 3 patients, namely in sputum and a throat swab of one with pneumonia, in blood of another patient with bloodstream infection and in a rectal swab of the third one without clinically apparent infection.

DISCUSSION

Infectious diseases caused by *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* strains pose a serious threat to patients with hematological malignancies (Paterson *et al.*, 2004; Fanci *et al.*,

2001). Therefore, it is reasonable to analyze the clinical significance of these strains and their resistance to antimicrobial agents. Results of the presented study reveal that in 35% of patients hospitalized in the DHO during the study period, strains of *Pseudomonas aeruginosa* were isolated from the investigated clinical material. Most frequently, these were isolates from rectal and throat swabs, together accounting for 48%. In the case of *Klebsiella pneumoniae*, the strains were isolated from 71% of the hospitalized patients, with 49% being obtained from rectal swabs and 23% from throat swabs.

Of particular interest is the assessment of clinical significance of the isolated strains. Nearly 50% of the *Pseudomonas aeruginosa* strains were clinically significant, i.e. causing clinically apparent diseases, in particular pneumonia (29%) and bloodstream infection (19%).

By contrast, only 11% of the *Klebsiella pneumoniae* isolates could be regarded as etiological agents of diseases, mostly bloodstream infection. In their work, Fanci *et al.* (2008) documented that in acute leukemia patients under 60 years of age, 18% of sepsis cases were due to gram-negative bacteria (mainly *Escherichia coli* and *P. aeruginosa*); in those over 60 years, the proportion was 29%.

The assessment of susceptibility of the investigated species to antibiotic agents produced relatively favorable results. In *Pseudomonas aeruginosa*, the efficiency of tested antibiotics reached at least 60%.

Similarly, the *Klebsiella pneumoniae* strains showed high susceptibility. Also positive is the fact that no ESBL-positive strains of *Klebsiella pneumoniae* were detected. On the other hand, strains producing AmpC beta-lactamases were observed which accounted for 20% of all isolates. Direct sequencing in the next study will determine whether the DHA-1, DHA-2 or newly discovered DHA-3 types of plasmid-mediated AmpC beta-lactamase are present (Gaillot *et al.*, 1997; Fortineau *et al.*, 2001; Wu *et al.*, 2005). These strains present a potential threat to patients due to possible failure of antibiotic therapy (Pai *et al.*, 2004).

Generally it may be assumed that all potentially pathogenic bacteria may play a role in the etiopathogenesis in patients with hematological malignancies. The infections are often endoge-

nous, with their reservoir being the patients' own microflora of the intestine, oral cavity and skin. Other disease-causing agents may be environmental, i.e. exogenous, microorganisms. The results of molecular genetic analysis determining individual clones may help characterize the prevalence of individual clones and thus probable representation of endogenous and exogenous infections.

Furthermore, practical measures in the antibiotic policy may stem from the data. Whereas in the case of large numbers of clones and strains with unique restriction profiles, the selection pressure of antibiotics is applied, hygienic and anti-epidemic measures are more valuable in smaller numbers of clones. Based on the obtained results, *Klebsiella pneumoniae* strains are assumed to be of mostly endogenous origin. Only in two patients, severe bloodstream infection and pneumonia caused by an identical strain were detected, suggesting transmission by an environmental vector.

In the environment of the observed ward, the strains were not detected and the possible route of transmission is therefore subject to speculation. However, the potential role of health personnel may not be ruled out. In the case of *Pseudomonas aeruginosa* strains, an identical strain causing infection was detected in two patients, responsible for urinary tract infection in one and for pneumonia in the other. In another three patients, an identical strain was also detected, causing bloodstream infection in one and pneumonia in another patient. Therefore, these strains are of exogenous origin. This was proved by positive detection of the *Pseudomonas aeruginosa* strains in samples collected from surfaces and objects, and even a case of confirmed identical environmental and clinical isolates. The results clearly show the ability of *Pseudomonas aeruginosa* strains to survive on a particular site in the hospital (a shower head) for more than 60 days.

For hospitalized patients, the hospital setting is an important source of nosocomial pathogens. Despite the shorter time interval and smaller group of strains, the study results confirmed that identical bacterial strains are capable of spreading both among patients and between patients and the environment. Although a significant contribution of environmental strains to the devel-

opment of nosocomial infections was not detected, strict adherence to hygiene and epidemiology precautions is obvious as this may help to limit the rate of nosocomial infections due to strains from exogenous sources.

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