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A potential role of aminoglycoside resistance in endemic occurrence of *Pseudomonas aeruginosa* strains in lower airways of mechanically ventilated patients

Q14 Julianna Mózes,^a, Ildikó Szűcs,^b, Dávid Molnár,^a, Péter Jakab,^a, Ebrahimi Fatemeh,^a, Mária Szilasi,^b,
 5 László Majoros,^a, Piroska Orosi,^c, Gábor Kardos,^{a,*}

6 a Department of Medical Microbiology, Medical and Health Science Center, University of Debrecen, H-4032 Debrecen Nagyerdei krt. 98, Hungary

^b Department of Pulmonology, Medical and Health Science Center, University of Debrecen, H-4032 Debrecen Nagyerdei krt. 98, Hungary

^c Department of Hospital Hygiene and Infection Control, Medical and Health Science Center, University of Debrecen, H-4032 Debrecen Nagyerdei krt. 98, Hungary

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40 **1. Introduction**

Healthcare-associated infections represent the most serious 41 problem of present-day medicine. Pseudomonas aeruginosa is a 42 significant pathogen in such infections considering its natural 43 44 resistance to many antimicrobials and its predisposition for acquired resistance as well (Navon-Venezia et al., 2005). It causes the most 45 severe problems in intensive care units (ICUs), especially among 46 mechanically ventilated patients, where it is among the first 47 48 pathogens colonizing the respiratory tract (Navon-Venezia et al., 2005). Accordingly, ventilator-associated pneumonia (VAP) is fre-49quently caused by P. aeruginosa. Moreover, increased mortality was 50shown even in patients not meeting the criteria for VAP, but with high 5152burden of *P. aeruginosa* in the airways (Zhuo et al., 2008).

53While outbreaks of multiresistant P. aeruginosa cause significant 54morbidity and mortality together with increased healthcare-associ-55ated cost and are therefore readily investigated and reported, the driving forces behind an endemic pattern are less well studied 56(Deplano et al., 2005; Navon-Venezia et al., 2005; Sader et al., 1993). 57Hypothetically, the selective advantage of a pathogen in a certain 58setting may derive from its virulence and/or from its resistance to 59 antimicrobials used. 60

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ABSTRACT

Altogether, 98 *Pseudomonas aeruginosa* isolates from a 5-bed intensive care unit were fingerprinted with 25 pulsed-field gel electrophoresis and tested for aminoglycoside resistance genes aac(6')-lb, aac(3'')-lIa, ant(2'')- 26 *Ia*, *armA*, *rmtA*, and *rmtB* and integrons and virulence genes/operons *phzI*, *phzII*, *phzM*, *phzS*, *apr*, *IasB*, *plcH*, *plcN*, 27 *pilA*, *algD*, *toxA*, *exoS*, *exoT*, *exoY*, and *exoU*. Two major clusters were identified (49 and 19 isolates), harbouring 28 aac(6')-lb, bla_{PSE-1} , and ant(3'')-la genes or ant(2'')-la gene, respectively, on a class I integron. Most virulence 29 genes except for *exoU* and *pilA* were found. Only 1 isolate of the minor cluster (8 isolates) and 1 of the 22 30 sporadic isolates carried integrons (without gene cassettes); virulence profile was highly variable. Comparing 31 the resistance and virulence patterns of endemic and sporadic isolates suggests that integron-borne 32 aminoglycoside resistance is more closely associated with the frequency than virulence. Consequently, 33 aminoglycoside usage may have played a role in maintenance of the endemic clones. 34

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Antibiotic usage frequently serves as a driving force both in 61 outbreaks and in endemicity; outbreaks of multiresistant *P*. 62 *aeruginosa* occurred multiple times as a consequence of antibiotic 63 overuse. For example, outbreaks of carbapenem- or ceftazidime- 64 resistant strains associated with carbapenem or third generation 65 cephalosporin use have been reported several times (e.g., El Amari 66 et al., 2001; Hsueh et al., 2005). Though the consequences of the 67 selective pressure exerted by other antibiotic groups are less well 68 documented, they may be important, especially the frequently used 69 fluoroquinolones and aminoglycosides. This is especially plausible in 70 case of aminoglycosides, where resistance is frequently associated 71 with resistance integrons, genetic structures which were shown to 72 be associated with nosocomial *P. aeruginosa* strains (Ruiz-Martínez 73 **Q2** et al., 2011).

Despite a number of virulence factors described, the virulence 75 mechanisms underlying the high mortality of VAP caused by *P*. 76 *aeruginosa* are poorly understood. Type 3 secretion system and its 77 effectors, especially the exotoxins *exoS* and *exoU*, may be important, 78 but the role of other virulence-associated genes is less unequivocal 79 (Berra et al., 2010). 80

The present work is concerned with the ecology of *P*. 81 *aeruginosa* in a non-outbreak situation, i.e., to find and describe 82 differences between successful clones and sporadic isolates in 83 terms of carriage of virulence factors, aminoglycoside resistance 84 genes, and integron-associated gene cassettes at a 5-bed ICU with 85 a pulmonology profile. 86

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^{*} Corresponding author. Tel.: +36-52-255-425; fax: +36-52-255-424. *E-mail address:* kg@med.unideb.hu (G. Kardos).

2

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J. Mózes et al. / Diagnostic Microbiology and Infectious Disease xxx (2013) xxx-xxx

87 **2. Materials and methods**

88 2.1. Setting of ICU

The ICU involved is a newly constructed 5-bed ICU in a building 89 previously also dedicated to healthcare. The ICU is directly connected to 90 the pulmonology clinic of a tertiary care center (university clinic); 91 therefore, the most common admission diagnosis is respiratory failure 92 or pneumonia; the most common underlying disease is end-stage 93 94 chronic obstructive pulmonary disease (COPD) or lung cancer. The patient population of the ICU is the elderly in general, as suggested by 95 96 the underlying diseases. The ICU also accepts patients directly from other departments (surgery, internal medicine, etc.) if they are 97 diagnosed with a pulmonology-related illness. Pediatric patients are 98never admitted to the department. 99

100 The study was conducted between September 2008 and February 2010 (546 days); during this period, the occupancy index was 85.6%, 101 102and the average ICU stay was 16 (8-33) days. The microbiological 103 monitoring was intensive; critically ill (e.g., intubated) patients were 104 sampled frequently; even daily sampling was performed in some cases. Besides P. aeruginosa, Acinetobacter baumannii, ESBL-producing, and Q3105 ESBL-negative Klebsiella pneumoniae were the most frequently isolated 106 107 bacteria. Fungal colonization was also frequent, commonly with Candida glabrata. Unexpected increases in the number of P. aeruginosa isolations 108 were not detected. Environmental samples (samples from suckers, 109 110 moisturizers, hospital furniture, fomites used in healthcare, tap surface)

t1.1 Table 1

Patient characteristics.

and tap water samples collected each month during the study period 111 consistently yielded no Gram-negative bacteria. 112

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2.2. Collection of bacterial isolates

Altogether, 98 isolates of P. aeruginosa were included isolated from 114 37 patients; patient characteristics are shown in Table 1. The majority 115 (87) of isolates originated from the ICU from 28 patients; however, 11 116 isolates of 11 patients from other wards of the clinic (oncology, 117 pulmonology, and rehabilitation) were also tested. Two patients were 118 sampled both in the ICU and in other wards. Most isolates were 119 cultured from lower airway samples (bronchial washing, sputum, or 120 tracheal aspirate); colony-forming unit (CFU) numbers varied 121 between 10^2 and $>10^5/mL$. Three samples originated from the upper 122 airways, 3 from intravenous cannula and 1 from pleural fluid. 123 Antibiotic susceptibility was determined by the CLSI disk diffusion 124 method against imipenem, meropenem, piperacillin + tazobactam, 125 ceftazidime, cefepime, ciprofloxacin, amikacin, gentamicin, and 126 tobramycin. Species identification was confirmed by PCR specific for 127 P. aeruginosa (Spilker et al., 2004). 128

2.3. Determination of genotype

Pulsed-field gel electrophoresis (PFGE) was used to assess genetic 130 relatedness. Approximately 4×10^8 CFU of bacteria was gently mixed 131 with 2% low melting point SeaPlaque agarose (Lonza) containing 1% 132 Q4

t1.3	Patient	Sex	Year of birth	Ward(s)	Number of hospitalization episodes	Outcome	Main diagnosis	Cumulative (cumulativ	Clusters encountered				
								Amikacin	Gentamicin	Streptomycin	Tobramycin		t1.
t1.5	BGn	F	1923	ICU	1	Died	Respiratory failure	1,0				А	-
t1.6	BI	Μ	1936	ICU	2	Died	COPD	19,0				Α	
t1.7	BM	F	1962	ICU	1	Died	COPD	17,0				С	
t1.8	BG	Μ	1951	ICU	1	Died	Lung cancer, COPD	8,0				В	
t1.9	CsI	Μ	1937	Pulmonology	1	Survived	COPD	12,0				С	
t1.10	DA	Μ	1960	ICU	1	Survived	Pneumonia	14,0				Α	
t1.11	DSn	F	1928	Pulmonology	1	Survived	Pneumonia					None ^a	
t1.12	DnHI	F	1934	ICU	1	Died	Pneumonia					None	
t1.13	DY	Μ	1943	ICU, rehabilitation	1	Survived	COPD	11,0				Α	
t1.14	JGn	F	1928	ICU	1	Survived	COPD					С	
t1.15	KIB	Μ	1926	ICU	7	Died	Respiratory failure	15,5				A	
t1.16	FÁ	Μ	1928	Pulmonology, ICU	1	Survived	COPD					None	
t1.17	FJ	Μ	1969	Pulmonology	1	Survived	Pneumonia					None	
t1.18	HJ	Μ	1947	Oncology	2	Survived	Lung cancer	14,0				None	
t1.19	IJ	Μ	1950	Pulmonology	2	Died	Tuberculosis, COPD	22,0	13,6	83,4		None	
t1.20	JSn	F	1920	ICU	1	Died	Respiratory failure					С	
t1.21	KMn	F	1939	ICU	1	Died	Respiratory failure					В	
t1.22	KG	Μ	1950	ICU	1	Survived	COPD					A and B	
t1.23	KI	Μ	1942	ICU	7	Survived	COPD					A and C	
t1.24	KJ	Μ	1944	Pulmonology	1	Survived	COPD					None	
t1.25	MM	Μ	1969	ICU	1	Died	Respiratory failure	4,0				С	
t1.26	MJ	Μ	1990	ICU	1	Survived	COPD					В	
t1.27	MF	Μ	1953	Rehabilitation	1	Survived	COPD					None	
t1.28	MZ	Μ	1947	ICU	1	Died	Lung cancer	5,0	4			None	
t1.29	MB	Μ	1946	ICU	1	Survived	COPD	13,0				None	
t1.30	NGy	Μ	1932	ICU	3	Died	Lung cancer	8,5				В	
t1.31	NI	Μ	1938	ICU	6	Survived	COPD	15,0				B-related	
t1.32	Oz	F	1939	ICU	1	Died	COPD					None	
t1.33	PSn	F	1944	Oncology	2	Survived	COPD					None	
t1.34	PJn	F	1942	ICU	1	Survived	COPD					В	
t1.35	PL	Μ	1941	ICU	10	Survived	COPD				22,0 ^b	С	
t1.36	SLn	F	1942	ICU	1	Survived	Respiratory failure	7,0	1,6			С	
t1.37	SF	Μ	1948	ICU	1	Died	COPD	17,0				Α	
t1.38	SzS	Μ	1957	ICU	5	died	COPD	18,0				В	
t1.39	SzG	М	1944	ICU	3	Survived	COPD	17,0				A and B	
t1.40	TJ	М	1940	ICU	1	Survived	COPD					В	
t1.41	VFn	F	1940	Endoscopy	1	Survived	Pneumonia					None	
													-

F = female; M = male.

t1.43 ^a These patients only harboured *P. aeruginosa* isolates belonging to smaller clusters of with unique patterns.

t1.44 ^b Inhalational use.

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sodium-lauril-sarcosil and 1 mg/mL Proteinase K (Fermentas). Plugs 133 134were incubated overnight in 100 mmol/L EDTA containing 0.2% sodium deoxycholate, 1% sodium lauril-sarcosil-sulphate and 1 mg/ 135mL Proteinase K. On the following day, they were washed for 15 136 minutes with distilled water, then for 1 hour with Tris-EDTA buffer 137 containing 0.35 mg/mL PMSF, and finally 3 times for 1 hour with Tris-**05**138 EDTA buffer. Plugs were stored in Tris-EDTA at 4 °C. Before digestion 139with restriction enzyme, plugs were washed for 1 hour in the buffer 140 recommended by the manufacturer. Digestion of plugs was per-141 formed with the enzyme SpeI using the buffer and conditions 142143recommended by the manufacturer. Separation of fragments was **06**144 performed in a CHEF DRIII machine (BioRad), in 1% SeaKem Gold agarose (Lonza) at 14 °C. The gel ran at 6 V/cm, with a reorientation 145 angle of 120°, switch times were ramped between 5 s and 30 s for 15 146hours followed by a 30 to 90 s ramping for 7 hours. Gels were stained 147 148 with ethidium bromide and visualized under UV light. DNA banding patterns were analysed with the Fingerprinting II software (BioRad), 149using the Dice coefficient and the UPGMA method with an 150optimization of 0.5% and a position tolerance of 1-1.5%. Similarity of 151at least 80% was considered as the threshold of probable genetic 152153relatedness to define clusters.

Isolates belonging to the same cluster were isolated from a patient
 at the ICU and later in another ward were considered to represent an
 exportation event. Conversely, importation is defined when a non-ICU
 isolate precedes a related isolate found in the ICU.

158 2.4. Aminoglycoside resistance and integron analysis

DNA was extracted by heat treatment; a loopful of bacteria was 159heated to 98 °C in TE buffer (100 mmol/L Tris, 10 mmol/L EDTA) for 15 **O7**160 minutes, and the supernatant was used as a template. Aminoglycoside 161 modifying enzyme genes aac(6')-Ib, ant(2'')-Ia and aac(3)-IIa were 162 sought for using the methods described by Frana et al. (2001). 163 Presence of *armA*, *rmtA*, and *rmtB* genes coding for aminoglycoside 164 resistance methylases was tested using the PCR assays reported by 165Bogaerts et al. (2007). Integron carriage was assessed by the PCR 166assays of Mazel et al. (2000). Amplification and sequencing of the 167variable regions were performed for all integron-carrying isolates 168 169 outside the clusters as well as for selected isolates representing the main PFGE clusters as described by White et al. (2001). Sequences 170 were handled using CLC DNA Workbench 4.0 (CLC Bio), and genes **08**171 172were identified by a GenBank search. After restriction site identifica-173 tion, the identity of integrons within clusters A and B was confirmed 174 by restriction fragment length polymorphism analysis of the variable 175regions using XbaI and XhoI (Fermentas), respectively, as recommended by the manufacturer. 176

177 2.5. Detection of virulence genes.

The genes of virulence factors exotoxin A (*toxA*), alginate (*algD*), type 3 secretion system effectors (*exoT*, *exoS*, *exoU*, *exoY*), phospholipases (*plcH*, *plcN*), proteases (*apr*, *lasB*), type IV pili (*pilA*), phenazin synthesis (*phzI*, *phzII*, *phzS*, *phzM*), and pyoverdin receptors (*fpvA-II*, *fpvA-II*, *fpvA-III*) were sought for by means of previously published PCR assays (de Chial et al., 2003; Finnan et al., 2004; Lanotte et al., 2004).

184 2.6. Antibiotic consumption.

Consumption of antibiotics (fluoroquinolones as a group, strepto-185mycin, amikacin, gentamicin, and tobramycin) was expressed in the 186 number of defined daily doses (DDDs) per 100 bed-days each year 187 from 2005 to 2009 using the MS Excel application ABC Calc version 188 3.1. (Monnet 2006). Data on antibiotics used to treat individual 189 190 patients were collected from the patient records and expressed in number of DDDs used. Cumulative administered aminoglycoside 191 doses per patient are shown in Table 1. Patients were also grouped 192

according to the genotype of *P. aeruginosa* harboured (clusters versus 193 isolates outside the main clusters), and the differences in total 194 consumption of antibiotics as well as in aminoglycoside consumption 195 between these groups were analysed by Kruskal-Wallis test and post-196 hoc pairwise comparisons with Bonferroni correction using the Past 197 3.0 software (Hammer et al., 2001). The patient with multidrug-198 resistant tuberculosis (IJ) receiving prolonged treatment with 199 amikacin and streptomycin was excluded from the statistical analysis. 200

3. Results

3.1. Aminoglycoside resistance and type of integrons 202

Out of the 6 aminoglycoside resistance genes sought, only the 203 modifying enzyme genes aac(6')-*Ib* and ant(2'')-*Ia* were found in the 204 isolates (see below); aac(3')-*Ila*, *armA*, *rmtA*, and *rmtB* genes were 205 never detected. Only class I integrons were detected. 206

3.2. Genetic relatedness

Among the 98 isolates tested, 3 main similarity clusters (A-C) were 208 identified; 22 further isolates belonged to 3 smaller clusters (2–3 209 isolates) or had unique patterns (Fig. 1.) Cluster A included 49 isolates 210 from 12 patients; this cluster was present throughout almost the whole 211 study period. Multiple patients carried the strain simultaneously during 212 certain periods, and returning patients were found colonized at 213 multiple hospitalization events. One isolate was isolated at the 214 rehabilitation ward from a former ICU patient indicating exportation. 215

Antibiotic susceptibility of the isolates within the cluster was 216 highly variable. While all isolates were uniformly resistant to 217 ciprofloxacin, amikacin, gentamicin, and tobramycin, resistance to 218 other agents was different sometimes even in case of isolates found in 219 the same day, ranging from susceptibility to all beta-lactam agents 220 tested to extensive resistance leaving colistine as the only therapeutic 221 option. All isolates harboured a class I integron with a gene cassette 222 array aac(6')-Ib, bla_{PSE-1} , and ant(3')-Ia (In99 according to the 223 integron nomenclature at the Integrall database, http://integrall.bio. 224 ua.pt). The cluster was characterized by presence of ferripyoverdin 225 receptor type III and lack of *pilA* and *exoU* genes; all other virulence 226 genes were found.

Cluster B included 16 isolates and was present throughout the 228 study period. Though 9 isolates from a single patient dominated this 229 cluster, it affected 11 patients altogether. Antibiotic susceptibility was 230 characterized by ciprofloxacin, gentamicin, and tobramycin resis- 231 tance, but resistance to beta-lactams was less extended as compared 232 to cluster A; only 5 isolates were resistant to a beta-lactam agent, 233 resistance to all antipseudomonal beta-lactams was not observed. 234 These isolates also carried a class I integron harbouring a single 235 ant(2")-Ia gene (In159). Virulence gene composition was similar to 236 that of cluster A, except for the presence of ferripyoverdin receptor 237 type I instead of type III. Three further isolates were highly similar to 238 this cluster, showing the same virulence profile and carrying the same 239 integron (Fig. 1).

Cluster C included 8 isolates and was present at the ICU from 241 March to December 2009 affecting 6 patients. One isolate (July 2009) 242 was isolated from a patient in the pulmonology ward (potential 243 importation/exportation). Antibiotic susceptibility of the cluster was 244 characterized by imipenem resistance observed in all isolates, except 245 the pulmonology ward isolate. Besides imipenem resistance, 3 isolates 246 (from the same patient) showed a somewhat variable resistance to 247 beta-lactams, ciprofloxacin, and aminoglycosides (Fig. 1). One of these 248 isolates carried class I integrons carrying no gene cassettes (In0). 249 Curiously, another isolate harboured an aac(6')-Ib gene; other 250 aminoglycoside resistance genes were not detected in the cluster. 251 Except *exoS*, all virulence genes sought for were present. 252

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J. Mózes et al. / Diagnostic Microbiology and Infectious Disease xxx (2013) xxx-xxx

	FGE-Spel	- number	ward	patient ID	isolation date	sample	resistance profile
		968	ITO	K.I.	12.01.2010.	bronchus	imi, mem, cip, gen, tob
L		1335	ITO	Sz.G.	17.01.2010.	bronchus	imi, mem, cip, gen, tob
		155	ITO	K.G.	04.01.2010.	bronchus	imi, mem, taz, cip, gen, tob
H		19948	ITO	к.і.в.	17 09 2009	capule	imi, mem, taz, cip, gen, tob
4		1586	ITO	Sz.G.	20.01.2010.	bronchus	imi, mem, taz, cip, gen, tob
		20551	ITO	B.I.	23.09.2009	bronchus	imi, mem, taz, cip, amik, gen, tob
		20798/2	ITO	K.I.B.	27.09.2009	bronchus	imi, mem, cip, gen, tob
🛽 h 🛛		20219	ITO	B.I.	20.09.2009	bronchus	imi, gen, tob
		21411	ITO	K.I.B.	04.10.2009	bronchus	imi, mem, taz, cip, gen, tob
		19615	ITO	B.I.	14.09.2009	bronchus	imi, taz, cip, gen, tob
		133	ITO	K.I.B.	03.01.2010	bronchus	imi, mem, taz, fep, cip, gen, tob
1 20		6271/1	ITO	B.Gn.	20.03.2009	bronchus	imi, cip, gen, tob
		6394	ITO	B.Gn.	22.03.2009	bronchus	imi, cip, gen, tob
- '		6038	ITO	B.Gn.	17.03.2009	bronchus	cip, gen
		3313	ITO	Sz.G.	09.02.2010	bronchus	imi, cip, gen, tob
		326/1	ITO	K.I.	05.01.2010	bronchus	imi, mem, taz, cip, gen, tob
L [3]		1796	ITO	Sz.G.	22.01.2010.	bronchus	imi, mem, taz, cip, gen, tob
Ь		19473/1	ITO	K.I.B.	10.09.2009	bronchus	imi, mem, taz, fep, cip, gen, tob
1.1		19946/1	ITO	K.I.B.	16.09.2009	bronchus	taz, cip, gen, tob
		19617	ITO	K.I.	14.09.2009	trachea	imi, taz, cip, gen, tob
		19616	ITO	S.F.	14.09.2009	trachea	imi,cip, gen, tob
		24296	ITO	S. Ln.	08.11.2009	bronchus	imi, mem, cip, amik, gen, tob
		25291	ITO	D. A.	20.11.2009	bronchus	imi, mem, taz, caz, fep, cip, gen, tob
🔤 h 🖂		24539	ITO	D. A.	10.11.2009	bronchus	taz, caz, fep, cip, gen, tob
/ h		24038	ITO	D. A.	08.11.2009	bronchus	cip, amik, gen, tob
		25897	ITO	D. A.	27.11.2009	bronchus	imi, mem, taz, caz, fep, cip, gen, tob
		24873	ITO	D. A.	15.11.2009	bronchus	imi, mem, taz, caz, fep, cip, gen, tob
	I III III IIIIII II II II <u>I</u>	25692	ITO	D. A.	24.11.2009	bronchus	imi, mem, taz, caz, fep, cip, gen, tob
		1336	ITO	K.I.	17.01.2010.	bronchus	imi, mem, taz, cip, gen, tob
		34	ITO	K.I.B.	01.01.2010	bronchus	cip, gen, tob
		1974	ITO	K.G.	24.10.2010.	bronchus	imi, cip, gen, tob
		1176	ITO	Sz.G.	15.01.2010.	bronchus	imi, taz, cip, gen, tob
		325	ITO	K.G.	05.01.2010.	bronchus	imi, cip, gen, tob
		2606	ITO	K.I.	31.01.2010	bronchus	imi, cip, gen, tob
ll .∈		28020	ITO	D.Y.	27.02.2009	sputum	imi, mem, cip, gen, tob
		27821	ITO	M.M.	22.12.2008	canule	imi, mem, taz, fep, cip, gen, tob
		3230	ITO	D.Y.	11.02.2009.	throat	imi, mem, taz, cip, gen, tob
		12170	ITO	P.L.	08.06.2009	bronchus	imi, cip, gen, tob
		21182/1	ITO	Sz.G.	30.09.2009	bronchus	cip, gen, tob
		20067	ITO	B.I. Sz G	18.09.2009	bronchus	imi, mem, taz, cip, gen, tob
		720		52.G.	10.01.2010.	bronchus	imi, mem, cip, gen, tob
		19019/1	ITO	K.I.	06.09.2009	bronchus	imi,amik
		18840/2	ITO	K.I.	03.09.2009	bronchus	imi,amik, tob
		17150	pulmonology	F.J.	11.08.2009	pleura	imi, mem, cip, amik, gen, tob
		14848	ITO	J.Sn.	12.07.2009	throat	- imi. gen
		19795	rehab	M.F.	09.09.2008	canule	cip
	1 11. 10 10 11 10 1 10 4 11	25425/2	ITO	M.J.	22.11.2009	bronchus	-
		8659/2	ITO	M.B.	19.04.2009	bronchus	·
		19767	pulmonology	F.A.	15.09.2009	bronchus	
		19910	nulmonology	F.A.	16.09.2009	bronchus	caz, rep, cip
		1337	ITO	0.Z.	18.01.2010.	bronchus	
		8392	ITO	M.Z.	16.04.2009	bronchus	imi, mem, taz, caz, fep, cip, gen, tob
n		20799/2	ITO	B.I.	27.09.2009	bronchus	imi, mem, cip, amik, gen, tob
		19035	ITO	Dn. H.I.	06.09.2009	bronchus	taz, caz, fep, cip, amik, tob
· · · · · · · · · · · · · · · · · · ·		18842	ITO	Dn. H.I.	03.09.2009	bronchus	cip
			paintonology	K I	12 01 2010	sputum	
		5762	ITO	K.J. B.M.	12.01.2010. 15.03.2009	sputum bronchus	- imi
r 1		5762 15124	ITO ITO	K.J. B.M. J.Sn.	12.01.2010. 15.03.2009 15.07.2009	sputum bronchus bronchus	- imi imi
L L		5762 15124 8393/2	іто іто іто	K.J. B.M. J.Sn. K.I.	12.01.2010. 15.03.2009 15.07.2009 15.04.2009	sputum bronchus bronchus bronchus	- imi imi imi, mem, amik, gen, tob
L L		5762 15124 8393/2 14894	ITO ITO ITO pulmonology	K.J. B.M. J.Sn. K.I. Cs.I.	12.01.2010. 15.03.2009 15.07.2009 15.04.2009 13.07.2009	sputum bronchus bronchus bronchus sputum	- imi imi, mem, amik, gen, tob
		5762 15124 8393/2 14894 6040 6300	ITO ITO ITO pulmonology ITO	K.J. B.M. J.Sn. K.I. Cs.I. J.Gn.	12.01.2010. 15.03.2009 15.07.2009 15.04.2009 13.07.2009 17.03.2009 22.022	sputum bronchus bronchus bronchus sputum bronchus	- imi imi, mem, amik, gen, tob - imi imi
		5762 5762 15124 8393/2 14894 6040 6390 1975/2	ITO ITO ITO pulmonology ITO ITO	K.J. B.M. J.Sn. K.I. Cs.I. J.Gn. K.I	12.01.2010. 15.03.2009 15.07.2009 15.04.2009 13.07.2009 17.03.2009 22.03.2009 24.10.2010	sputum bronchus bronchus sputum bronchus bronchus bronchus	- imi imi, mem, amik, gen, tob - imi imi imi mem, taz cip, nan tob Cluster
		5762 15124 8393/2 14894 6040 6390 1975/2 19227	ITO ITO ITO pulmonology ITO ITO ITO ITO	K.J. B.M. J.Sn. K.I. Cs.I. J.Gn. J.Gn. K.I.	12.01.2010. 15.03.2009 15.07.2009 15.04.2009 13.07.2009 22.03.2009 24.10.2010 08.09.2009	sputum bronchus bronchus bronchus sputum bronchus bronchus bronchus bronchus	- imi imi, mem, amik, gen, tob - imi imi imi imi, mem, taz, cip, gen, tob cluster imi
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		5762 15124 8393/2 14894 6040 6390 1975/2 19227 3071 28158/2	ITO ITO Pulmonology ITO ITO ITO ITO ITO ITO	K.J. B.M. J.Sn. K.I. Gs.I. J.Gn. J.Gn. K.I. <u>F.Sn</u> K.I.B.	12.01.2010. 15.03.2009 15.07.2009 15.04.2009 17.03.2009 22.03.2009 24.10.2010. 08.09.2009 10.02.2009 22.12.2009	sputum bronchus bronchus sputum bronchus bronchus bronchus <u>bronchus</u> sputum bronchus	imi imi imi, mem, amik, gen, tob - imi imi imi, mem, taz, cip, gen, tob caz, fep, cip, gen, tob
		5762 15124 8393/2 14894 6040 6390 1975/2 19227 3071 28158/2 28429 323067	ITO ITO Pulmonology ITO ITO ITO ITO ITO ITO ITO ITO	K.J. B.M. J.Sn. K.I. Cs.I. J.Gn. K.I. K.I. P.Sn K.I.B. K.G.	12.01.2010. 15.03.2009 15.07.2009 15.04.2009 17.03.2009 22.03.2009 24.10.2010. 08.09.2009 20.03.2009 24.10.2010. 08.09.2009 20.02.2009 22.12.2009 28.12.2009 28.12.2009	sputum bronchus bronchus sputum bronchus bronchus bronchus sputum bronchus bronchus	- imi imi imi, mem, amik, gen, tob - imi imi imi, mem, taz, cip, gen, tob cip, gen, tob cip, gen, tob cip, gen, tob cip, gen, tob cip, gen, tob
		5762 15124 8393/2 14894 6040 6390 1975/2 19227 3071 28158/2 28429 27818/1 28575	ITO ITO pulmonology ITO ITO ITO Oncology ITO ITO ITO ITO ITO	K.J. B.M. J.Sn. K.I. Cs.I. J.Gn. J.Gn. K.I. K.I. B. P.Sn K.I.B. K.G. Sz.S. K.G.	12.01.2010. 15.03.2009 15.07.2009 15.04.2009 13.07.2009 22.03.2009 22.03.2009 22.03.2009 24.10.2010. 08.09.2009 10.02.2009 12.212.2009 12.12.2009 15.12.2009 15.12.2009 15.12.2009	sputum bronchus bronchus bronchus bronchus bronchus bronchus bronchus bronchus bronchus bronchus	- imi imi imi, mem, amik, gen, tob - imi imi imi, mem, taz, cip, gen, tob ciz, fep, cip, gen, tob cip, gen, tob cip, gen, tob cip, gen, tob cip, gen, tob
		5762 15124 8393/2 14894 6040 6390 1975/2 19227 3071 28158/2 28429 27818/1 28575 4979	ITO ITO pulmonology ITO ITO ITO ITO ITO ITO ITO ITO ITO ITO	K.J. B.M. J.Sn. K.I. Cs.I. J.Gn. J.Gn. K.I. B. K.I.B. K.G. Sz.S. K.G. K.G.	12.01.2010. 15.03.2009 15.07.2009 15.04.2009 13.07.2009 24.03.2009 24.03.2009 24.10.2010. 08.09.2009 10.02.2009 22.12.2009 19.12.2009 19.12.2009 10.12.2009 30.12.2009	sputum bronchus bronchus bronchus bronchus bronchus bronchus bronchus bronchus bronchus bronchus bronchus bronchus	- imi imi imi, mem, amik, gen, tob - imi imi imi - caz, fep, cip, gen, tob cip, gen, tob cip, gen, tob cip, gen, tob cip, gen, tob
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		5762 15124 8393/2 14894 6040 6390 1975/2 1975/2 28158/2 28458/2 28458/2 28575 3611 3744 28575 3611 3744 28639 4061 3971 32160/1 327/1 28160/1 337/1 338/1	ITO ITO pulmonology ITO ITO ITO ITO ITO ITO ITO ITO ITO ITO	K.J. B.M. B.M. J.Sn. J.Sn. J.Gn. J.Gn. J.Gn. J.Gn. K.I. K.J. K.G. K.G. K.G. M.J. M.J.	12.01.2010. 15.03.2009 15.07.2009 15.07.2009 17.03.2009 22.03.2009 22.03.2009 22.03.2009 22.10.2010. 08.09.2009 22.12.2009 23.12.2009 23.01.2.2009 23.01.2.2009 23.01.2.2009 11.02.2010 15.02.2010 15.02.2010 16.02.2010 16.02.2010 16.02.2010 16.02.2010 22.12.2009 05.01.2010 05.01.2010 17.01.2010.	sputum bronchus sputum bronchus sputum bronchus	imi imi imi imi imi imi imi caz, fep, cip, gen, tob cip, gen, tob
		5762 15124 8393/2 14894 6040 6390 1975/2 19227 28158/2 28458/2 28459 3071 28575 4079 3611 3744 28575 4079 3611 3744 28639 4061 3771 28158/2 28639 4061 3771 28158/2	ПТО ПТО риlmonology ПТО ПТО ПТО ПТО ПТО ПТО ПТО ПТО	K.J. B.M. J.Sn. K.I. Cs.I. J.Gn. J.Gn. K.I. K.I.B. P.So. K.I.B. K.I.B. K.G. K.G. K.G. K.G. K.G. K.G. K.G. K	12.01.2010. 15.03.2009 15.07.2009 15.07.2009 13.07.2009 22.03.2009 22.03.2009 22.03.2009 22.03.2009 22.12.2009 22.12.2009 23.12.2009 23.12.2009 23.12.2009 23.12.2009 23.12.2009 11.02.2010 15.02.2010 15.02.2010 15.02.2010 16.02.2010 16.02.2010 16.02.2010 16.02.2010 16.02.2010 15.01.2010 26.12.2009 27.12.2009 26.12.2009 27.12	sputum bronchus sputum bronchus sputum bronchus	imi imi imi imi imi imi imi imi imi imi
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		5762 15124 8393/2 14894 6040 6390 19227 28158/2 24459 28059/1 3611 3744 28575 4979 28059/1 3611 3744 28639 4061 2271 28160/1 327/1 28160/1 327/1 28160/1 327/1 28162 2819	ITO ITO pulmonology ITO ITO ITO ITO ITO ITO ITO ITO	K.J. B.M. B.M. J.Sn. J.Sn. J.Sn. J.Sn. J.Gn. J.Gn. J.Gn. J.Gn. K.I. K.B. K.G. K.G. K.G. M.J. N.J. N.I. N.I.	12.01.2010. 15.03.2009 15.07.2009 15.07.2009 17.03.2009 12.03.2009 22.03.2009 22.03.2009 22.10.2010. 05.09.2009 22.12.2009 20.12.2009 20.12.2009 20.12.2009 15.02.2010 15.02.2010 15.02.2010 15.02.2010 15.02.2010 15.02.2010 15.02.2010 15.02.2010 15.02.2010 15.02.2010 15.02.2010 15.02.2010 15.02.2010 15.02.2010 15.02.2010 15.02.2010 22.12.2009 15.02.2010 22.12.2009 25.12.2009 25.12.2008 26.1	sputum bronchus bronchus sputum bronchus	imi imi imi imi imi imi imi imi
		5762 15124 8393/2 14894 640 630 1975/2 1975/2 28158/2 28158/2 28458/2 28458/2 28575 3611 3744 26639 4061 3871 28160/1 327/1 250 1338/1 4541 26152 28019 12833	ПТО ПТО риlmonology ПТО ПТО ПТО ПТО ПТО ПТО ПТО ПТО	K.J. B.M. B.M. J.Sn. J.Sn. J.Sn. J.Sn. J.Gn. J.Gn. J.Gn. J.Gn. K.I. K.J. K.I. P.Sn. K.I. K.G. K.G. K.G. K.G. K.G. K.G. K.G. K.G. K.G. K.G. N.G. T.J. N.J. N.J. N.J. N.J. I.J. I.J.	12.01.2010. 15.03.2009 15.07.2009 15.07.2009 17.03.2009 22.03.2009 22.03.2009 22.03.2009 22.03.2009 22.12.2009 22.12.2009 22.12.2009 23.01.22009 23.01.22009 23.01.22009 10.02.2010 15.02.2010 15.02.2010 15.02.2010 15.02.2010 15.02.2010 15.02.2010 15.02.2010 15.02.2010 15.02.2010 15.02.2010 15.02.2010 15.02.2010 15.02.2010 15.02.2010 15.02.2010 15.02.2010 22.12.2009 05.01.2010 17.01.2010 17.01.2010 22.12.2009 20.12.2008 20.12.2009 20.12.2008 20	sputum bronchus sputum bronchus sputum bronchus	imi imi imi imi, mem, amik, gen, tob imi imi imi caz, fep, cip, gen, tob cip, gen, tob
		5762 15124 8393/2 14894 6040 6330 1977/2 19227 3071 28158/2 2429 27818/1 28659 3611 3744 28659 3671 28669 3071 28659 3744 28659 327/1 28619 28151 28152 28333 14893 28018	ПО ПО ринолоюду ПО ПО ПО ПО ПО ПО ПО ПО ПО ПО	K.J. B.M. B.M. J.Sn. J.Sn. J.Sn. K.I. Cs.I. J.Gn. J.Gn. J.Gn. K.I. K.B. K.G. K.G. K.G. M.J. NI. N.I. I.J. N.I. NI.	12.01.2010. 15.03.2009 15.07.2009 15.07.2009 13.07.2009 22.03.2009 22.03.2009 22.03.2009 22.03.2009 22.12.2009 22.12.2009 23.12.2009 23.12.2009 23.12.2009 23.12.2009 23.12.2009 11.02.2010 15.02.2010 15.02.2010 15.02.2010 15.02.2010 15.02.2010 15.02.2010 15.02.2010 15.02.2010 15.02.2010 15.02.2010 15.02.2010 15.02.2010 15.02.2010 15.02.2010 15.02.2010 15.02.2010 15.02.2010 15.02.2010 22.12.2008 20.12.2008 20.12.2008	sputum bronchus sputum bronchus sputum bronchus	imi imi imi, mem, amik, ger, tob imi imi imi, mem, taz. cip, gen, tob cip, gen, tob
		5762 15124 8393/2 14894 6040 6390 1975/2 19227 28158/2 28429 27818/1 28575 4079 28059/1 3611 3744 28639 4061 337/1 28162 1338/1 28152 1338/1 28152 14893 28018 14893 28018 14893 28018 14893 28018 14893 28018 14893 28018 14893 28018 14893 28018 14893 28018 14893 28018 14894 1484 1484 1484 1484 1484 1484 1484 1484 1485	ПТО ПТО риlmonology ПТО ПТО ПТО ПТО ПТО ПТО ПТО ПТО	K.J. B.M. B.M. J.Sn. J.Sn. J.Sn. K.I. Cs.I. J.Gn. J.Gn. K.I. K.I. P.Sn. K.I. K.I. K.G. K.G. K.G. K.G. K.G. K.G. K.G. K.G. K.G. K.G. K.G. K.G. K.G. N.G. K.G. N.J. N.I. N.I. N.I. N.I. N.I. N.I. N.I. V.Fn.	12.01.2010. 15.03.2009 15.07.2009 15.07.2009 13.07.2009 22.03.2009 22.03.2009 22.03.2009 22.03.2009 22.10.2010. 08.09.2009 22.12.2009 23.12.2009 23.12.2009 23.12.2009 23.01.2010 15.02.2010 17.01.2008 25.12.2008 20.1	sputum bronchus	imi imi imi imi imi imi imi imi

Fig. 1. PFGE patterns and clusters. Clusters are shown in frames; the additional 3 isolates aligned to cluster B are framed with a dashed line. Arrows point to potential importation/ exportation events.

Out of the 22 isolates found in 1 or 2 patients (small clusters and 253 254unique patterns), 9 were susceptible to all antipseudomonas antibiotics tested, and only 1 harboured aminoglycoside resistance 255genes, an aac(6')-Ib gene. (Other aminoglycoside resistance genes 256were not detected in these 22 isolates.) A single isolate harboured an 257empty integron (In0). Virulence gene distribution was very hetero-258geneous; only phzII, apr, exoT, and plcH genes were present in all 259isolates; however, the other genes, except pilA and exoU, were found 260in the majority (>16) of isolates. A single isolate harboured all 15 261 262 tested virulence determinants.

263 Out of the 39 patients, 24 had at least 1 isolate belonging to one of 264 the main clusters; all 24 were hospitalized in the ICU. Ten of the remaining 18 patients (with isolates with unique patterns or 265belonging to small clusters) were not admitted to the ICU. Ten 266 patients had more than 1 strain; these sometimes were carried 267268 simultaneously, i.e., isolates belonging to the different strains were isolated only 1 to 3 days apart. In returning patients, both main 269clusters A and B harbouring class I integrons and aminoglycoside 270resistance genes were represented. 271

272 3.3. Antibiotic consumption

Antibiotic consumption of the pulmonology clinic between 2005 273274and 2009 was above the university average (97.76 versus 38.78 DDDs/ 275100 bed-days); the most common antibiotics were macrolides, amoxicillin + clavulanic acid, and fluoroquinolones (average yearly 276consumptions were 29.78, 26.58 and 16.56 DDDs/100 bed-days, 277 respectively). Aminoglycoside consumption similarly was above the 278 university average, 7.46 (5.79-9.90) versus 1.89 (1.91-2.51); beta-279lactam therapy was frequently potentiated by aminoglycosides. 280 Aminoglycosides used included streptomycin, netilmycin, tobramy-281 cin, gentamicin, amikacin (average consumptions 0.26, 0.02, 1.42, 282 2.80, and 2.96 DDDs/100 bed-days). A switch from tobramycin to 283 284gentamicin in 2006–2007 and then partial replacement of gentamicin by amikacin in 2008–2009 were observed. Fluoroquinolone usage was 285relatively frequent, 16.58 (12.3-20.7) DDDs/100 bed-days by average, 286287but a marked decrease and the lowest value were observed in 2009 288 (Fig. 2).

Out of the patients included in the study, only a single patient did 289not receive any antibiotics. Broad-spectrum beta-lactams (piperacillin 290+ tazobactam and carbapenems) were the most frequently adminis-291 292tered antibiotics. Amikacin was the most frequently used aminoglyco-293side; 19 of the 37 patients, i.e., 16 of the 28 ICU and 3 of the 11 non-ICU 294patients (one of them for multidrug-resistant tuberculosis) received amikacin (Table 1). Gentamicin was given to 3 patients only; 295296streptomycin, only to the tuberculotic patient; and 1 patient received 297inhalational tobramycin. Other aminoglycosides were not used.



Fig. 2. Aminoglycoside and fluoroquinolone consumption rates of the Department of Pulmonology.

Statistical analysis demonstrated that patients harbouring isolates 298 belonging to cluster A received significantly more amikacin than 299 patients harbouring isolates from other clusters or with unique 300 fingerprints (P = 0.046). As overall antibiotic consumptions (as well 301 as consumption of beta-lactams and fluoroquinolones) were compa-302 rable among clusters (P > 0.5), this was not caused by increased use of 303 antibiotics in general. 304

4. Discussion

Pattern of isolations and presence of *P. aeruginosa* strains for 306 months suggest an endemic occurrence of clusters A and B, which 307 were transmitted between multiple patients, recolonized returning 308 patients, and persisted for the whole study period. Disappearance of 309 any of the strains from the ICU was not confirmed during the study 310 period. The majority of the ICU isolates (74/87) belonged to clusters A 311 and B, which were present throughout the study period; isolates of 312 cluster C were found less frequently but continuously. Transmission of 313 strains between the ICU and other wards was also detected, but most and an unique patterns and were not clonal.

Similar endemic *P. aeruginosa* epidemiology was reported by Foca 316 et al. (2000) in a neonatal intensive care unit, where the source was 317 the healthcare personnel, and patients hospitalized for prolonged 318 periods served as a putative reservoir. Similar findings were reported 319 by Bergmans et al. (1998) and Suarez et al. (2011) in case of adult 320 patients. As the environment repeatedly proved to be negative for *P.* 321 *aeruginosa*, the reservoir of the strains must have been the healthcare 322 personnel or the patients (e.g., patients KI and SzG for cluster A in 323 January-February 2010 or patient KI for cluster B in the same period) 324 in the present study as well. Cross-transmission between patients was 325 reported to be the major route for colonization earlier (Bergmans 326 et al., 1998; Boyer et al., 2011).

It is also interesting that *P. aeruginosa* isolates of the same patient 328 isolated a few days apart were found to be genetically distinct. This 329 may be explained by the simultaneous colonization by multiple 330 strains (Ortega et al., 2004; Pirnay et al., 2003). Unfortunately, the 331 proportion of patients colonized with multiple strains at the same 332 time could not be assessed from the data in the present study. As 333 routine susceptibility testing is based on a single colony of a 334 homogeneous culture of a species, the possibility of simultaneous 335 presence of multiple strains, possibly with different susceptibilities, 336 must be acknowledged and considered by clinicians in treatment 337 decisions. The impact of colonization with multiple strains on 338 outcome is to be examined by upcoming studies.

Virulence pattern of the isolates was curious; it was not 340 unequivocal that a successful strain (i.e., one that is present in 341 multiple patients for prolonged periods) carries more virulence 342 factors than sporadic isolates (isolated from only 1 or 2 samples of 343 1 or 2 patients), as could be expected. For example, both frequently 344 isolated strains carried the gene exoS coding for a type 3 secretion 345 system effector shown to be important for VAP pathogenesis (Berra 346 et al., 2010), but not exoU, which is considered the most harmful 347 effector for host cells (Berra et al., 2010; Veesenmeyer et al., 2009). In 348 contrast, some sporadic isolates did carry exoU. Moreover, a sporadic 349 isolate with a wider array of virulence factors than that of the endemic 350 strains was also found. Though presence of a virulence gene is not 351 necessarily equal to its expression in efficient amounts, present data 352 suggest that virulence patterns are not in direct association with 353 success as a colonizer or pathogen. Though one could argue that 354 findings on colonizing isolates are not necessarily applicable to 355 pathogenic isolates, it should also be considered that high mortality 356 was reported in heavily colonized ventilated patients without 357 clinically proven VAP (Zhuo et al., 2008). 358

Integron carriage is strongly associated with aminoglycoside 359 resistance (Poole 2005). In accordance, the majority of isolates in 360

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J. Mózes et al. / Diagnostic Microbiology and Infectious Disease xxx (2013) xxx-xxx

the present study carrying a modifying enzyme gene tested was alsoclass I integron positive.

Both of the 2 most prevalent clones carried integrons and integron-363 borne aminoglycoside resistance genes, but only 1 of 21 isolates not 364 belonging to major clusters carried an integron, which, in turn, did not 365 harbour any gene cassettes. High variability was observed regarding 366 resistance to beta-lactam agents among isolates of clusters A and B; 367 only aminoglycoside and ciprofloxacin resistance was common to all 368 isolates. A probable explanation may be a synergistic effect of the 369 integron-borne bla_{PSE-1} cassette and a putatively overexpressed efflux 370 371 pump (cluster A) as reported by Peña et al. (2009) or overexpression of 372 both the chromosomal *ampC* lactamase and efflux pumps (cluster B) as reported by Deplano et al. (2005). Though in many reports the 373 suspicion of clonal spread is raised by a particular resistance pattern 374 shared by the isolates (Hsueh et al., 2005; Lolans et al., 2005), these 375 376 data show that isolates may be related even when this is not seen. In such situations, an epidemiologically important strain may not be 377 identified, underlining the importance of surveillance studies in 378 settings where *P. aeruginosa* is a frequent pathogen. 379

380 The association between aminoglycoside resistance and clonality 381 suggests a role of aminoglycosides in the maintenance and/or spread 382 of successful clones. The pattern of antibiotic consumption, i.e., 383 steadily increasing amikacin usage in the clinic and the statistically 384 significant association of amikacin administration and carriage of 385 cluster A by the same patients points to the role of amikacin usage in 386 the emergence and maintenance of cluster A with integron-borne resistance against amikacin and tobramycin. The role of antibiotic 387 consumption in provocation of resistance is well-known (Hsueh et al., 388 2005; Iosifidis et al., 2008; Loeffler et al., 2003), and it was reported as 389 a driving force for outbreaks as well (Falagas and Kopterides, 2006; 390 Suarez et al., 2011). In accordance with the above assumption, 391 antibiotic usage was shown to be a risk factor for cross-transmission 392 in case of *P. aeruginosa* as well (Boyer et al., 2011). 393

The present report shows a complex dynamics of *P. aeruginosa*, 394 characterized by continuous presence of at least 2 genetically 395unrelated clones suggesting intensive transmission, which were 396 carrying aminoglycoside resistance genes on class I integrons in an 397 endemic epidemiological situation. The data collected indicate that 398 antibiotic usage may also be and sometimes may be the main driving 399 400 force for maintenance of not only outbreaks but endemic strains as 401 well. Antibiotic resistance in certain situations may be more 402 important than virulence in spread of successful clones.

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