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ARTICLE

Molecular analysis of population structure and antibiotic resistance of *Klebsiella* isolates from a three-year surveillance program in Florence hospitals, Italy

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Abstract We report the results of a three-year surveillance program of *Klebsiella* spp. in six hospitals in Florence (Italy). A total of 172 *Klebsiella* isolates were identified and typed by AFLP: 122 were *K. pneumoniae* and 50 were *K. oxytoca*. Most *K. pneumoniae* (80%) and *K. oxytoca* (93%) showed unrelated AFLP profiles. Beside this heterogeneous population structure, we found five small epidemic clonal groups of *K. pneumoniae*. Four of these groups were involved in outbreak events, three of which occurred in neonatal ICUs. The fifth clonal group spread in three different wards of two hospitals. Only one non-epidemic clonal group of *K. oxytoca* was detected. The frequencies of isolates with multiple antibiotic resistances increased with time; at the end of the study period, most *K. pneumoniae* were resistant to all the antibiotics tested. A PCR analysis of seven ertapenem resistant isolates was unable to detect any of the major genes known to underlie carbapenem resistance in *K. pneumoniae*.

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Introduction

Since the 1950s, gram-negative bacteria (GNBs) have been recognized agents of nosocomial infections, but it is only since the late 1970s and early 1980s, with the selection of multiple-resistant organisms paralleling the widespread use of antibiotics, that GNBs have become a real concern [1]. The major GNB pathogens associated with nosocomial infections are saprophytic or commensal microbes, such as *Klebsiella* spp. *K. pneumoniae*, normally found in the human intestines and feces, is an increasingly important invasive pathogen in pneumoniae, bloodstream infections, wound or surgical site infections in healthcare settings of different countries [2]. The ability of this organism to spread rapidly often leads to nosocomial outbreaks, especially in neonatal units [3–5]. *K. oxytoca* is another medically important *Klebsiella* sp. [6].

Patients most at risk for *Klebsiella* infections are those receiving treatment for other conditions and have devices like ventilators or intravenous catheters, or are taking long courses of antibiotics. Some *K. pneumonia* strains are contemporary resistant to one or more classes of first line antimicrobial agents, and sometime to all but one or two commercially available antimicrobial agents. These multidrug resistance (MDR) strains show an increasing prevalence in nosocomial settings, and are a major health problem. Most notable are *K. pneumonia* strains producing extended spectrum β-lactamases (ESBLs), like different variants of the TEM, SHV, and CTX-M β-lactamases; these strains show resistance to all β-lactam antibiotics except carbapenems [7, 8]. The threat to patient safety becomes awesome when, in addition to first line antibiotics, the microorganism develops resistance to carbapenems, that are one of the last lines of defense against gram-negative infections [9, 10]. Carbapenem resistance

in *K. pneumoniae* is mainly related to carbapenem hydrolyzing β-lactamases that, following the amino acid sequence homology-based Amber classification, can be divided into different classes: class B, metallo β-lactamases (MBLs); class D, expanded spectrum oxacillinases; and class A, carbapenemases [11, 12]. Carbapenem-resistant *K. pneumoniae* (CRKP) strains have been associated with increased morbidity and mortality, length of stay, and increased cost [13]. CRKPs are resistant to almost all available antimicrobial agents [14]. The risk for dissemination of CRKPs is increased by a mechanism of resistance based on the production of a class A carbapenemase enzyme encoded by the transposon-carried bla_{KPC} gene [15, 16].

With the emergence and spread of MDR *Klebsiella* arises the need for aggressive detection and control strategies. In healthcare settings, *Klebsiella* bacteria can spread through person-to-person contact and from patient-to-patient on the hands of healthcare personnel. Studies utilizing molecular genotyping can be applied to GNB in order to understand their epidemiology in greater detail, for example, to identify the source and prevalence of an outbreak strain, and to devise rational interventions to control the epidemic, but also to define their population structure and dynamics during non-outbreak periods. In this study, we describe outbreak events caused by *K. pneumonia* clones and the population structure of nosocomial *Klebsiella* spp. by AFLP typing. Moreover, *K. pneumoniae* isolates were investigated for the emergence of MDR phenotype and its genetic origin.

Materials and methods

Surveillance system, specimen collection, and phenotypical analysis of bacterial isolates

From July 2006 to December 2008, a surveillance program of nosocomial infections was applied in 19 high-risk wards. Surveillance system, hospitals and wards, as well as the criteria to define hospital (HAI) and community (C) infection, and outbreaks, have been described previously [17, 18]. *Klebsiella* spp. were identified using the automated Vitek2 system (bioMérieux, Marcy l'Etoile, France). According to the recommendations of the Clinical and Laboratory Standards Institute (CLSI), isolates were tested for antimicrobial susceptibility by Vitek 2 System and Etest and screened for carbapenemase production by Hodge tests [19]. All patients positive for *Klebsiella* spp. were included in the study. *Klebsiella* strains from the same patient (duplicates) were included only if different by typing analysis. For further analysis, only one strain per duplicate was used.

AFLP analysis

AFLP analysis was performed as described by Vos et al. [20] with slight modification [16]. Specifically, *Eco*RI and *Mse*I restriction enzymes were used to digest genomic DNA; after ligation of EcoRI and MseI adaptors, PCR amplification was performed with EcoRI-T (6-carboxy-fluorescein-5′- GACTGCGTACCAATTCT) and MseI-T (5′-GATGAGTCCTGAGTAAT) primers, with a selective T at their 3′ ends. Amplified fragments from 60 to 280 bp were considered for AFLP profile analysis by GeneMapper 4.0 software (Applied Biosystems). Cluster analysis of the profiles was performed by unweighted pair group method with arithmetic mean (UPGMA) and Numerical Taxonomy and Multivariate Analysis System NTsys-pc v.2 software (Exeter Software), and percentage of similarity calculation by Dice correlation coefficient. AFLP genomic fingerprinting was used to classify strains to both species and subspecies levels [21]. Reproducibility of AFLP analysis was assessed by comparing different profiles obtained with replicates of *K. pneumoniae* reference strain ATCC 10031. To determine the discriminatory power of AFLP, Simpson's index of diversity (*D*) [22] with 95% confidence intervals was calculated.

Mathematical and statistical methods: PCA and AMOVA

Principal component analysis (PCA) was used to determine the general pattern of variation between isolates of each species, as previously described [17].

The analysis of molecular variance (AMOVA) was used to estimate the genetic differences among populations (corresponding to AFLP clusters) and among individuals within *K. pneumoniae* populations. The internal variability of each clonal group was calculated using the Euclidean distances between all possible combinations of AFLP patterns taken in pairs [23]. The genetic structure of bacterial populations was investigated by an analysis of the variance framework as reported by Dalmastri et al. [24]. Pairwise genetic distance (*F*st) values [25] and their corresponding *P* values were calculated to quantify the differentiation between all pairs of populations. AMOVA and *F*st values generation were performed using Arlequin version 3.1 software [26].

Phenotypic and molecular analyses of antibiotic resistance

K. pneumoniae isolates were phenotypically investigated, as above reported, for resistance to different antibiotics or combination antibiotics (Table 2) including: amikacin (AMK), levofloxacin (LEV), trimethoprim (TRI), ampicillin (AMP), piperacillin/tazobactam (PIP/TAZ) ampicillin/sulbactam (AMP/SUL), cefepime (CFP), ceftazidime

(CFT), cefazoline (CFZ), imipenem (IMP), ertapenem (ERT), meropenem (MER) and aztreonam (AZT). Molecular analysis of resistance was performed by PCR amplification on a One Personal thermocycler (Euroclone, Italy): bla_{SHV} and bla_{TEM} [27], bla_{KPC} [28], bla_{IMP}, bla_{VIM}, bla_{GIM}, bla_{SIM}, and bla_{SPM} [29], bla_{OXA48} [11], bla_{GES} [30]. FIPP 1 strain [31] was used as positive control for bla_{SHV}, bla_{TEM} and bla_{KPC}; other reference strains were *Klebsiella* SIM, *Pseudomonas* GIM, *Pseudomonas* SPM (kindly provided by Dr. Neil Woodford, Health Protection Agency, London, UK), and the *E.coli* strains SI-pVIM-1, SI-pIMP-1, SI-pGES-1 and SI-pOXA-48 (kindly provided by Professor Rossolini, University of Siena, Italy).

Results

Patient data and bacterial isolates

During the study period, among isolates collected from 165 patients, the Vitek 2 system identified 172 *Klebsiella* spp. isolates, assigning 124 of them to *K. pneumoniae* and 48 to *K. oxytoca* (see Materials and Methods for the selection criteria of patients and bacterial isolates). The specimens from which the isolates were more often obtained were bronchoaspirates (30%), blood (9%), pharyngeal swabs (28%), urine (8%), ocular swabs (6%), and central venous catheter (3%). Based on the answers to a clinical questionnaire for infection/colonization data, we had 21% infections and 62% colonizations, whereas 17% of the questionnaires were unanswered.

AFLP analysis

The overall similarity between the AFLP profiles of our isolates was 47%, which was in the range reported by Savelkoul et al. [20] for isolates of the same genus. At species level, AFLP grouped 122 isolates (71%) as *K. pneumoniae* and 50 (29%) as *K. oxytoca*, suggesting that Vitek 2 had previously misidentified two *K. oxytoca* isolates as *K. pneumoniae*. Most *K. pneumoniae* isolates (80%) showed unrelated AFLP profiles (singletons), thus highlighting the high variability of strains circulating in the monitored wards (Fig. 1); even isolates from the same patient varied (not shown). The remaining 20% of *K. pneumoniae* isolates grouped in five small clusters with a level of similarity ≥ 79% (Fig. 1) and, on the grounds of epidemiological concordance criteria (see Outbreak investigation paragraph below), they were regarded as clonal groups. Like *K. pneumoniae*, most *K. oxytoca* isolates (94%) remained ungrouped, and only one *K. oxytoca* clonal group (KO1) was detected (Fig. 1).

The genetic relatedness of *Klebsiella spp*. isolates shows that AFLP has very good discriminatory power (D=99%, with a confidence interval of 87–100%). Reproducibility of AFLP analysis was higher than 85% (data not shown).

Outbreak investigation

From July 2006 to January 2009, four *K. pneumoniae* outbreaks occurred, caused by different clonal groups (Table 1). Isolates of each outbreak showed a high genetic relatedness (≥79% of similarity; Fig. 1). The first outbreak (clonal group KP1) lasted from September to December 2006 in H1-W24, and four patients were involved. An environmental investigation, performed in H1-W24 demonstrated the presence of a KP1 strain from an inadequately disinfected basin where the neonates were washed (Table 1). The second outbreak (clonal group KP2) occurred between February and May 2007 in two adjoining neonatal sub-intensive units of the same hospital (Table 1), which shared patients and hospital personnel; this outbreak involved nine patients. The third outbreak (clonal group KP4) occurred in a neonatal ICU (H2-W3) from July to September 2008. The KP8 isolates involved in the last outbreak (December 2008) were collected from the adult ICU H1-W10. After hospital measures were introduced such as intensification of standard cross-infection precautions, including cleaning and disinfection of patients' room to control cross-contamination of patients, no more isolates belonging to these clonal groups were collected in the monitored wards.

Concerning *K. oxytoca*, two of the three isolates of clonal group KO1 were collected from July to September 2006 in the neonatal ICU H2-W3 so that a cross contamination could be hypothesised; the third KO1 isolate was collected from H1-W13 in May 2007.

Space/time distribution of *Klebsiella* isolates

Clonal groups KP1, KP2, KP4 and KP8 were present only in the ICUs where the corresponding outbreaks occurred and only during the outbreak (Table 1; Fig. 2); Kp7, not responsible for any outbreak, spread in three different wards of two hospitals (H1-W1, H1-W2, H3-W1), during five weeks. *K. pneumoniae* singletons were from all monitored hospitals, during the entire study period (Fig. 2).

The population structure of *K. oxytoca* appeared similar to that of *K. pneumoniae*: singletons predominated, denoting a high degree of heterogeneity. *K. oxytoca* clonal group KO1, unlike *K. pneumoniae* ones, lasted longer (about ten months).

Different *Klebsiella* strains were isolated from the same kind of specimen of the same infected patient. Such mixed

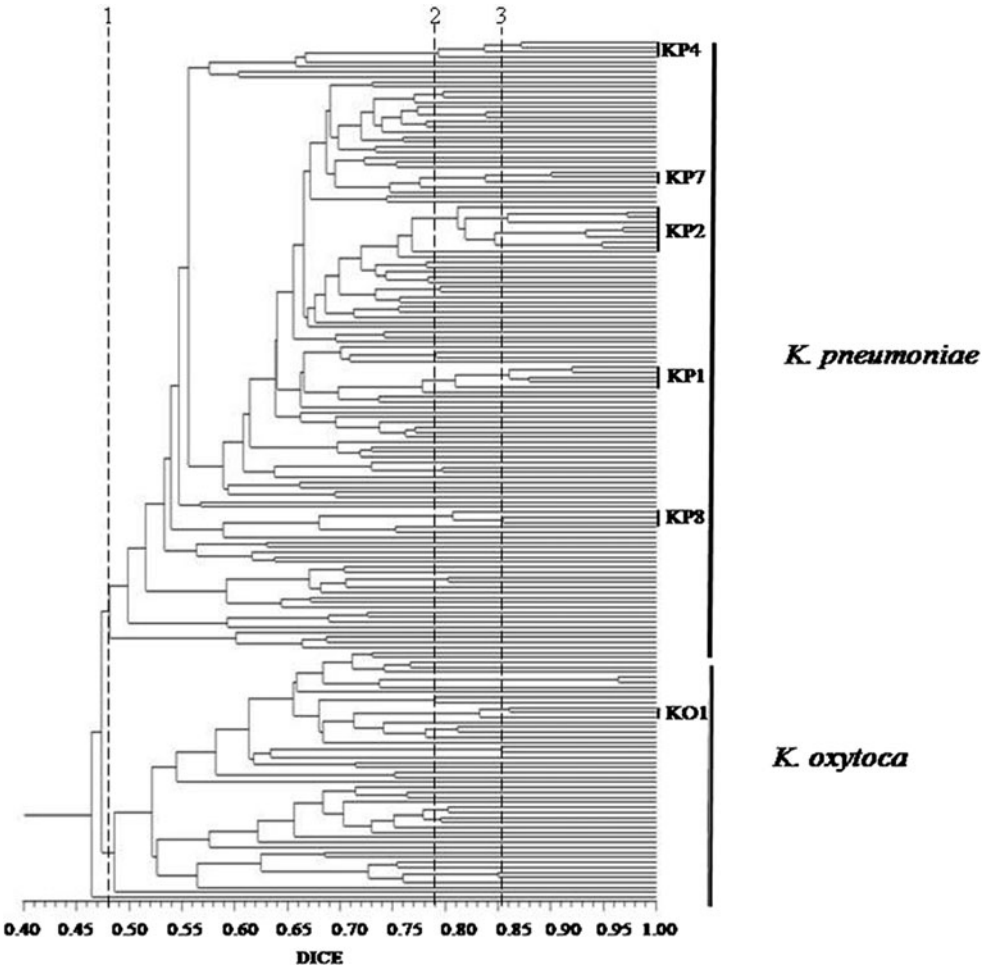

Fig. 1 AFLP analysis dendrogram of *Klebsiella* spp. isolates. KP1, KP2, KP4, KP7, and KP8 are clonal groups of *K. pneumoniae*; KO1 is a *K. oxytoca* clonal group. Percentage values of DICE coefficient of similarity are shown under the dendrogram. *Vertical dashed lines 1*, *2* and *3* indicate cut-off values belonging to species (48%), clonal group (79%) and reproducibility of the AFLP method (85%), respectively

infective populations were found for both *K. pneumoniae* (four patients) and *K. oxytoca* (one patient).

PCA results

In order to better investigate the relatedness of isolates, AFLP profiles were subjected to PCA. Despite the low total variance contained in the first two components (10.7%), the PCA confirmed the results obtained by UPGMA analysis; clonal groups differentiated from each other and from singletons (not shown).

Genetic variability among *K. pneumoniae* isolates

AMOVA analysis of AFLP data showed that the genetic variability of the five *K. pneumoniae* clonal groups was equally due to inter- (50.2%) and intra-clonal group (49.8%) differences. When the level of genetic divergence among pairs of clonal groups was calculated by *F*st statistic, and expressed as mean percentages of pairwise difference for each group versus the others, KP1 and KP8 showed the highest values (53.8% and 53.5%, respectively), KP2 the lowest (48.8%), whereas KP4 and KP7 showed an intermediate value (50.8%). Overall, these results confirmed the high genetic difference between groups, and the high genetic variability within the groups.

Antibiotic resistance of *K. pneumoniae* isolates

As expected, Vitek 2 results show that all *K. pneumoniae* were intrinsically resistant to ampicillin, and 67% of them were resistant only to this antibiotic (profile 1, Table 2); the remaining 33% of isolates grouped in 18 different antibiotic profiles. Isolates belonging to clonal groups KP1, KP4 and KP7 showed unique profiles, whereas Kp2 and Kp8 showed profiles in common with some singletons. The frequency of isolates with multiple antibiotic resistances increased with time, i.e. most of the isolates collected during the first 12 months were resistant only to penicillin, whereas in the last four months isolates resistant to all classes of antibiotics tested were reported (Fig. 3, Table 2). All isolates of the KP8 clonal group and four singletons had a MIC breakpoint ≥8 μg/ml for ertapenem (not shown). Two ertapenem resistant singletons were positive to Hodge test, suggesting carbapenemase production. However, when

Table 1 Outbreak events and epidemiological characteristics of involved isolates

Outbreak event	Patient	Sample collection date	Hospital ward	Infection (IN)/ colonization (C)	AFLP profile
1	1	27/09/2006	H1-W24	-	KP1
	2	09/10/2006		IN	
	3	02/11/2006		C	
	4	23/11/2006		C	
	sink	01/12/2006		Environment	
2	1	29/03/2007	H1-W11	IN	KP2
	2	11/04/2007		IN	
	3	26/04/2007		C	
	4	22/05/2007		C	
	5	24/02/2007	H1-W24	IN	
	6	10/04/2007		C	
	7	26/04/2007		IN	
	8	04/05/2007		IN	
	9	19/05/2007		IN	
3	1	03/06/2008	H2-W3	-	KP4
	2	11/06/2008		IN	
	3	18/07/2008		IN	
	4	26/08/2008		IN	
4	1	15/12/2008	H1-W10	IN	KP8
	2	17/12/2008		-	
	3	19/12/2008		-	

H1-W24 and H1-W11, neonatal sub-intensive unit; H2-W3, neonatal intensive unit; H1-W10, adult intensive unit

A dash (-) indicates an unanswered clinical questionnaire

the presence of the major carbapenem hydrolyzing β-lactamases genes was investigated by PCR, all the ertapenem resistant isolates were negative.

Discussion

In this study, AFLP has been applied to investigate the intraspecific population structure of *Klebsiella* spp. isolates collected during three years of an epidemiological surveillance program of nosocomial infections. As reported in other studies [32], *Klebsiella* spp. populations show a high degree of heterogeneity. One non-epidemic clonal group of *K. oxytoca* was detected; whereas five small clonal groups of *K. pneumoniae* emerged, four of which were epidemic clones strictly associated with outbreak events. One outbreak was in an adult ICU, but the other three occurred in neonatal ICUs, confirming the major concern for *K. pneumoniae* infection in these patients [33, 34]. Hospital environment was investigated to locate suspected sources of clones associated with outbreaks; in the outbreak in which clone KP1 was involved, a KP1 isolate was identified in a basin of a room where neonates were washed. After the introduction of strict preventive measures, no more isolates

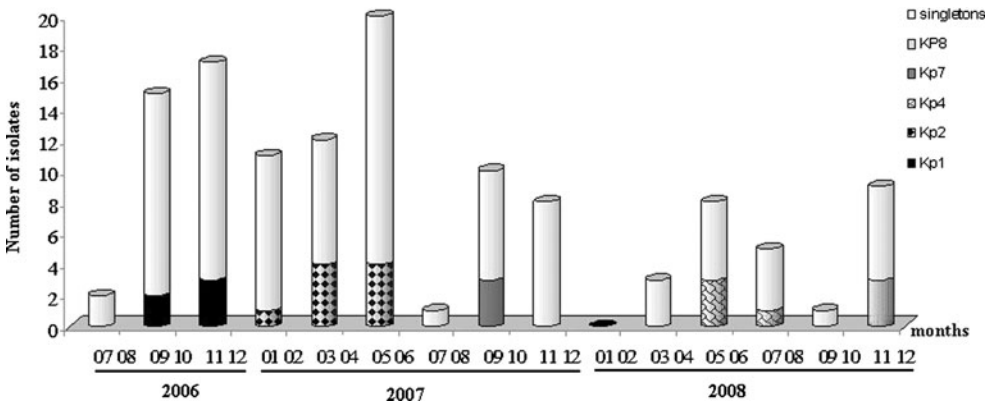

Fig. 2 Time distribution and number of *K. pneumoniae* isolates belonging to clonal groups and singletons

Table 2 Antimicrobial susceptibility profiles of *K. pneumoniae* isolates

ANTIBIOTIC PROFILE	Antibiotics[a]											N° isolates	
	AMP	AMK	AMP SUL	AZT	PIP TAZ	CFP	CFZ	CFT	LEV	ERT	TRI	singletons	Clonal strains[b]
1	■											69	-
2	■		■									3	-
3	■					■						2	-
4	■		■			■						-	5 (KP1)
5	■		■			■				■		1	-
6	■		■			■		■				1	-
7	■		■	■		■	■	■				3	-
8	■	■	■			■		■				-	4 (KP4)
9	■		■	■	■	■		■				4	9 (KP2)
10	■		■	■		■	■	■				2	-
11	■		■	■	■	■	■	■				2	-
12	■		■	■		■	■	■	■			2	-
13	■	■	■	■		■	■	■				1	-
14	■		■	■		■	■	■			■	2	-
15	■		■	■		■	■	■	■		■	2	-
16	■		■	■	■	■	■	■	■		■	1	-
17	■	■	■	■		■	■	■	■		■	1	-
18	■	■	■	■	■	■	■	■	■		■	-	3 (KP7)
19	■	■	■	■	■	■	■	■	■	■	■	2	3 (KP8)

[I] Ampicillin (AMP), amikacin (AMK), ampicillin/sulbactam (AMP/SUL), aztreonam (AZT), piperacillin/tazobactam (PIP/TAZ), cefepime (CFP), cefazoline (CFZ), ceftazidime (CFT), levofloxacin (LEV), ertapenem (ERT), and trimethoprim (TRI). No isolate was resistant to imipenem and meropenem (not reported). [II] In brachets are reported the corresponding clonal group.

belonging to this clone were collected, denoting that the rapid and accurate identification of outbreak strains is important in inhibiting cross contamination [33].

K. pneumoniae isolates can be grouped in 19 antibiotic resistance profiles. During the study period, we observed an increased frequency of the MDR phenotype in singletons

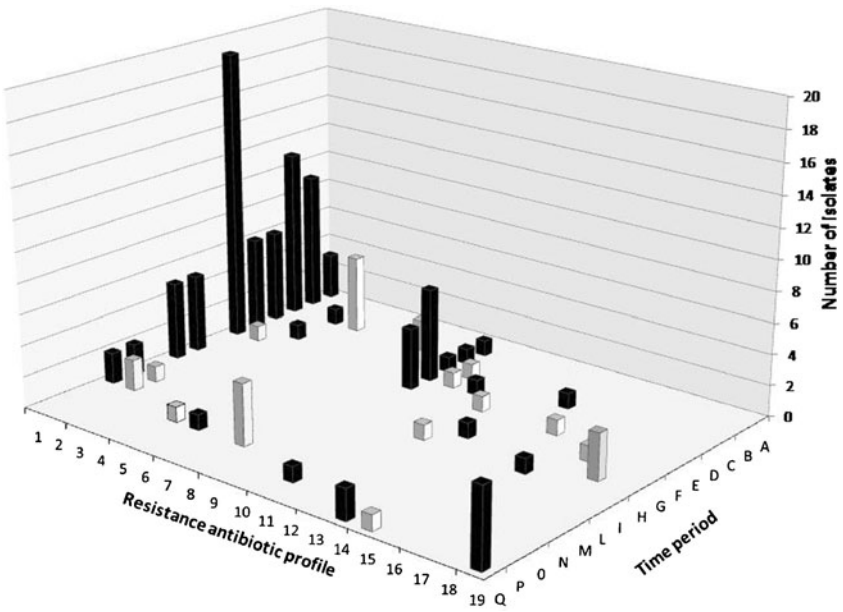

Fig. 3 Time distribution (intervals of two months, A–Q, from July–August 2006 to November–December 2008) of *K. pneumoniae* resistant isolates with different antibiotic profiles (see Table 2). Mean number of patients per day in the monitored wards was 1,500

and clonal group strains. The mode of transmission of resistance genes could be horizontal transfer, contributing to further adaptation and expansion (outbreak events) of MDR strains in the hospital setting. The concern about the high spreading propensity of MDR strains is particularly strong when CRKP strains are involved. CRKP strains have been detected in different European countries [16], and in October 2008 the first Italian isolation of a CRKP strain was reported, isolated in a ward of one of the hospital (H1) monitored during the present study [31]. The bla_{KPC-3} gene was identified as responsible for carbapenem resistance in this strain.

Seven of the *Klebsiella* strains in this study (5.7%), the entire KP8 clonal group and four singletons, showed resistance to ertapenem. Ertapenem resistance in *K. pneumoniae* is rare [35]. Different studies reported that, in *K. pneumoniae*, ertapenem resistance can be mediated by KPC carbapenemases [36, 37], whereas other studies stated that it is rarely mediated by true carbapenemases [35, 38]. In the last case, the resistance is associated to the presence of ESBL genes combined with deficiency in the expression of outer membrane proteins (OMPs). In our ertapenem resistant strains, the search for the major genes responsible for carbapenem resistance was always negative, supporting the occurrence of a resistance mechanism not linked to true carbapenemases.

Monitoring and surveillance, and molecular typing of strains with multiple resistances are necessary procedures to control the emergence of MDR strains in hospital settings and the occurrence of related outbreak events. The possibility of exploring the population biology of ESBL-harboring *K. pneumoniae* in a single institution may be essential to obtain an understanding of the evolution of the highly complex interplay among genes, plasmids, and clones [39].

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