Journal of Medical Microbiology (2014), **63**, 000–000 DOI 10.1099/jmm.0.082818-0

Effect of carbapenem consumption patterns on the molecular epidemiology and carbapenem resistance of *Acinetobacter baumannii*

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This study investigated the molecular epidemiology of *Acinetobacter baumannii* in the University of Debrecen in relation to antibiotic consumption. Overall and ward-specific antibiotic consumption was measured by the number of defined daily doses (DDD) per 100 bed-days between 2002 and 2012. Consumption was analysed against the number of *A. baumannii* positive patients per 100 bed-days, number of isolates per positive sample, and proportion of carbapenem resistant *A. baumannii*, using time-series analysis. Altogether 160 *A. baumannii* isolates from different wards were collected and analysed. Carbapenemase genes $bla_{OXA\text{-}23\text{-}like}$, $bla_{OXA\text{-}24\text{-}like}$, $bla_{OXA\text{-}48\text{-}like}$, $bla_{OXA\text{-}51\text{-}like}$, $bla_{OXA\text{-}58\text{-}like}$ and integrons were sought by PCR. Relatedness of isolates was assessed by PFGE. Prevalence and carbapenem resistance of *A. baumannii* were statistically associated with carbapenem consumption. Prevalence data followed carbapenem usage with three quarterly lags ($r=0.51–0.53$, $P<0.001$), and meropenem and ertapenem, but not imipenem usage affected prevalence. Colistin usage, in turn, lagged behind prevalence with one lag ($r=0.68–0.70$, $P<0.001$). Six clusters were identified; the neurology ward with the lowest carbapenem consumption was associated with the carbapenem-susceptible cluster, as well as with the carbapenem-susceptible isolates in the cluster with variable susceptibility. Wards with high carbapenem usage almost exclusively harboured isolates from carbapenem-resistant clusters. All clusters were dominated by isolates of one or two wards, but most wards were represented in multiple clusters. Increases in prevalence and carbapenem resistance of *A. baumannii* were associated with usage of meropenem and ertapenem but not of imipenem, which led to the spread of multiple clones in the University.

Received 21 August 2014
Accepted 23 September 2014

INTRODUCTION

Acinetobacter baumannii, the clinically most important species in the *A. baumannii-Acinetobacter calcoaceticus* complex, represent a major nosocomial problem, especially due to the frequency of the multidrug resistant phenotype. Recently, extensively resistant clones susceptible only to polymyxins have emerged worldwide, for which even carbapenems, formerly the drug group of choice against *A. baumannii*, remain ineffective (Zarrilli *et al.*, 2004). The emergence of carbapenem resistance is linked to plasmid-borne class D BlaOXA-23-like and BlaOXA-58-like carbapenemases. Though most clinically important *A. baumannii* subpopulations harbour a naturally occurring chromosomal BlaOXA-51-like carbapenemase, this alone is not sufficient for the carbapenem resistant phenotype (Poirel & Nordmann, 2006; Walther-Rasmussen & Høiby, 2006). These carbapenemases are associated with the insertion sequences ISAba-1, -2 and -3 (Karunasagar *et al.*, 2011; Tsakris *et al.*, 2008), and their production has been linked to certain pandemic clones (Ansaldi *et al.*, 2011; Merkier *et al.*, 2008; Tsakris *et al.*, 2008; Villalón *et al.*, 2013).

Emerging carbapenem resistance in *A. baumannii* has been shown by several authors to be a sequel to third generation cephalosporin and carbapenem usage (Cisneros & Rodríguez-Baño, 2002; Goel *et al.*, 2011; Iosifidis *et al.*, 2008, Manikal *et al.*, 2000; Ogutlu *et al.*, 2014). This is attributed both to acquisition of mechanisms providing carbapenem resistance

Abbreviations: DDD, defined daily doses; ICU, intensive care unit; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight.

and to spread of carbapenem resistant clones (Goel *et al.*, 2011; Manikal *et al.*, 2000; Ogutlu *et al.*, 2014; Poirel & Nordmann, 2006).

The aim of the present study was to investigate the molecular epidemiology of *A. baumannii* and its carbapenem resistance in a Hungarian tertiary care centre, in relation to changes in antibiotic consumption.

METHODS

Antibiotic consumption and frequency of *Acinetobacter* spp. Quarterly antibiotic consumption [number of defined daily doses (DDD) per 100 bed-days] used by inpatient departments of the University was monitored between 2002 and 2012, based on the data of the University Pharmacy using the MS Excel application ABC Calc Version 3.0. (Monnet 2006). Department-specific data were collected between 2004 and 2012 (ICU-specific data were not available).

The annual frequency of *Acinetobacter* isolations in the University was characterized by the number of patients positive for *A. baumanii* per 100 bed-days, the proportion of positive samples of inpatients yielding *A. baumannii*, the proportion of *A. baumannii* among the isolated Gram-negative aerobic/facultatively anaerobic bacteria, and by the proportion of *A. baumanii* isolations out of positive blood culture samples. Antibiotic resistance data of non-duplicate (from one patient only the first isolate was considered) isolates were collected from the database of the Laboratory for Bacteriological Diagnostics serving the University.

The relationship between antibiotic consumption in the University and changes in isolation frequency, as well as antibiotic resistance of *Acinetobacter* spp., was analysed by linear regression. In the case of antibiotics where the consumption data showed significant correlation with prevalence or resistance data, after trend removal cross-correlation analysis was performed using quarterly time lags. The lag with the highest significant correlation coefficient was considered as the most probable delay antibiotic consumption precedes or follows changes in resistance or prevalence. Out of the available drugs in the carbapenem group, the effect of imipenem, meropenem and ertapenem (introduced in 2004) was analysed, as doripenem was introduced in the year of the beginning of the study period and was used less than other carbapenems. 1

Prevalence of carbapenem-susceptible and -resistant *A. baumannii* at different wards was compared using a chi-squared test; pairwise comparisons were performed by means of a Fisher exact test. Consumption of different antibiotic classes at different wards was compared by means of a Kruskal–Wallis test. Statistical tests were performed using PAST 3.0. (Hammer *et al.*, 2001).

Bacterial isolates. A total of 160 isolates were collected between November 2010 and May 2011 at the University of Debrecen, and the majority (n=135) of isolates originated from different intensive care units (ICUs). The carbapenem resistance rate in this collection was 79.4 % (127/160). Most isolates were cultured from bronchial (73/160), blood (18/160), canule (17/160), wound (14/160) and tube (11/160) samples. The remaining isolates originated from urine (6/160), throat (5/160), catheter tip (4/160), decubitus (3/160), pleura (2/160), sputum (2/160), drain (2/160), abscess (2/160), cerebrospinal fluid (1/160) and outer ear (1/160). These isolates were chosen randomly; they represent 31.9 % of all *A. baumanii* isolates recovered and 46.9 % of patients infected with *A. baumannii* during the study period. The isolates originated from the ICU wards of the first and second departments of internal medicine (18 isolates from 14 patients, and 14 isolates from 12 patients, respectively), neurology (18 isolates from 13 patients), surgery (19 isolates from 13 patients), pulmonology (53 isolates from 10 patients) and neurosurgery (seven isolates of four patients), and from the perinatal (14 isolates from six patients) and paediatric (seven isolates from four patients) ICUs of the department of paediatrics, as well as from eleven other wards of the University (24 isolates from 22 patients). These represent 17.6 %, 43.8 %, 62.1 %, 23.5 %, 51.0 %, 25.9 %, 25.0 %, 36.9 % and 28.9 % of isolates from the department, respectively, and 41.2 %, 63.2 %, 81.3 %, 43.3 %, 62.5 %, 28.6 %, 40.0 %, 44.4 % and 37.9 % of all patients from whom *A. baumannii* was isolated at the department. All wards with ten or more *A. baumannii*-positive patients were represented.

Samples were incubated overnight at 37 °C on blood and eosin methylene blue agar plates, and isolates were identified by biochemical tests and by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) Biotyper (Bruker Daltronics). Susceptibility to imipenem, meropenem, piperacillin + tazobactam, colistin, ciprofloxacin, sumetrolim, doxycycline, tigecycline, amikacin, tobramycin and gentamicin was determined using the Clinical and Laboratory Standards Institute (CLSI, 2010) disc diffusion method. Isolates were stored frozen until analysis. 2 3

Detection of carbapenemase, aminoglycoside and tetracycline resistance genes. Isolates were grown overnight at 37 °C on blood agar. A loopful of bacteria was heated to 98 °C in TE buffer (100 mM Tris, 10 mM EDTA) for 15 min and centrifuged at 11 000 ***g*** for 3 min; the supernatant was used as a template for PCR analysis. Stock solutions were kept at −70 °C and working solutions were renewed after every five freeze–thaw cycles.

The carbapenemase genes $bla_{\text{OXA-23-like}}$, $bla_{\text{OXA-24-like}}$, $bla_{\text{OXA-48-like}}$, $bla_{\text{OXA-51-like}}$ and $bla_{\text{OXA-58-like}}$ were sought using the methods of Woodford *et al.* (2006). The association of bla_{OXA} and the insertion sequences IS*Aba-1* was also assessed (Turton *et al.*, 2006).

The genes coding for the aminoglycoside-modifying enzymes *aac(3)-IIa*, *aac(6′)-Ib*, *ant(2′)-Ia*, *ant(3′)-Ia*, *aph(3′)-Ia* and *aph(3′)-VIa* were tested as described previously (Frana *et al.*, 2001; Lévesque *et al.*, 1995; Vila *et al.*, 1999). Genes *armA*, *rmtA*, *rmtB* encoding 16S rRNA methylases were sought using the method reported by Bogaerts *et al.* (2007).

Analysis of integrons and integron-associated gene cassettes. The occurrence of resistance integrons was studied by integrase-specific PCRs (Mazel *et al.*, 2000). Variable regions were amplified as described by White *et al.* (2001). At least two representative amplimers were purified using the Isolate PCR and gel kit (Bioline), and sequenced using the Sanger method with primer walking when necessary. CLC DNA Workbench 4.0 (CLC Bio) was used to assemble and analyse sequences, and genes were identified by GenBank (http://www.ncbi.nlm.nih.gov) search. The identity of integrons with variable regions of the same size was confirmed by restriction fragment length polymorphism analysis using *Xba*I, *Hin*dIII and *Dde*I (Fermentas), as determined by restriction site analysis of the fully sequenced representatives. Restriction analysis was performed as recommended by the manufacturer.

Determination of the genotype. Genetic relatedness among isolates was analysed by PFGE. Plugs were prepared as previously described (Mózes *et al.*, 2014) and digested with with *Apa*I. A CHEF DRIII machine (Bio-Rad) was used to separate the fragments in 1 % SeaKem Gold agarose (Lonza) at 14 °C. Electrophoresis was performed at 6 V cm^{-1}, with a reorientation angle of 120°, and switch times were ramped between 2 s and 35 s for 20 h. DNA banding patterns were analysed with the Fingerprinting II software (Bio-Rad) using the Dice coefficient and the unweighted pair group method with averages (UPGMA). Setting of optimization and position tolerance was 1 %

and 1.5 % respectively; similarity of at least 90 % was considered as the threshold of probable genetic relatedness.

RESULTS

Association of prevalence and resistance data with antibiotic consumption

A. baumannii was isolated from approximately 1 % of all positive samples submitted during the years 2000 to 2008, but in 2009 and 2010 a sudden increase in the isolation rates from all positive samples (to 2.3 % and 2.5 %, respectively) was detected. A similar increase was found in positive blood samples (from 1–2 % to 4.4 %). In parallel, the proportion of carbapenem-resistant isolates increased from 6.2 % to 63.8 % from 2000 to 2010, and increased further to 73.0 % in 2011.

The antibiotic consumption of the University showed an increasing trend from 2006. This was mainly attributable to the increased consumption of broad-spectrum antibiotics, third generation cephalosporins, fluoroquinolones and, from 2008, carbapenems. In cross-correlation analysis, increases in carbapenem consumption followed the trend in third generation cephalosporin ($r=0.63$, $P<0.001$ at the -9 quarterly lag corresponding to the period roughly two years earlier), but replaced piperacillin + tazobactam usage ($r=-0.54$, $P<0.001$ at $+4$ quarterly lag corresponding to the period a year later). Significant temporal correlation between carbapenem usage and consumption of other antibiotic classes was not found, except for polymyxins, which lagged behind carbapenem usage with five quarterly lags (slightly more than a year, $r=0.80$, $P<0.001$).

The prevalence as well as the carbapenem resistance of *A. baumannii* showed association with consumption of third generation cephalosporins, carbapenems and colistin, but not with any other antibiotic classes. Consumption of third generation cephalosporins preceded the increase in prevalence of *A. baumannii* among positive samples and among isolated Gram-negative bacteria with 13 (16 in case of blood samples) quarterly lags (three to four years; r=0.55-0.77, $P<=0.001$ in all cases), as well as the increase in prevalence of *A. baumannii*-positive patients (with 14 quarterly lags, three and a half years; $r=0.58$, $P<0.001$). However, this association was indirect, as carbapenem usage followed the consumption of third generation cephalosporin with nine quarterly lags (see above).

Increasing carbapenem consumption led to an increase in prevalence of *A. baumannii* in all positive samples, as well as in positive blood samples, with a delay of three quarterly lags (nine months; $r=0.53$, $P<0.001$ and $r=0.51$, $P<0.001$, respectively). When analysing the increasing prevalence of *A. baumannii* among Gram-negative bacteria, the delay was slightly longer (five quarterly lags; $r=0.54$, $P<0.001$ and $r=0.62$, $P<0.001$, respectively). Increasing prevalence was not explained by the consumption of any other antibiotic group tested (fluoroquinolones, piperacillin + tazobactam or third generation cephalosporins). The effect on the number of patients with *A. baumannii* positive culture was comparable (four quarterly lags; $r=0.46$, $P=0.002$).

Carbapenem resistance of *A. baumannii* showed a similar correlation with carbapenem usage, also with a delay of nine months ($r=0.43$, $P=0.005$ at the -3 quarterly lag), but not with usage of any other drug classes.

Out of the different carbapenem drugs, meropenem showed temporal association with prevalence (eight to four lags; r=0.45-0.49, $P=0.002$-0.006) and carbapenem resistance (eight lags; $r=0.44$, $P=0.007$) of *A. baumannii* or with the number of positive patients (two lags; $r=0.39$, $P=0.01$). Ertapenem showed a similar effect, but with shorter delays. Prevalence lagged behind ertapenem usage with one to five quarterly lags (r=0.54–0.58, $P<0.001$), and carbapenem resistance and the number of positive patients per 100 bed-days with six ($r=0.42$, $P=0.009$) and four ($r=0.56$, $P<0.001$) lags, respectively. These variables were not influenced by imipenem consumption (Fig. 1.). [4]

Increasing colistin consumption followed the increase in prevalence and carbapenem resistance of *A. baumanni* with a delay of one quarterly lag (r=0.68-0.70, $P<0.001$ for prevalence variables and $r=0.58$, $P<0.001$ for carbapenem resistance).

Ward-specific differences in antibiotic consumption, prevalence and carbapenem resistance

Examining carbapenem consumption among the wards with the highest isolation rates of *A. baumannii* revealed that usage in the department of surgery and pulmonology was above the University average, while the first department of internal medicine, paediatrics and neurology consumed fewer carbapenems (Fig. 2). The department of [5] surgery consumed significantly more carbapenems than other wards ($P=0.001$–0.018), while the neurology department consumed significantly fewer carbapenems than any other wards analysed ($P=0.010$–0.026).

Meropenem was the most popular carbapenem accounting for 56.7 % of yearly carbapenem cumulative doses (36.2–92.5 %), followed by imipenem with 26.9 % (6.8–42.8 %) and ertapenem with 11.8 % (0–28.7 %). Doripenem usage was very low (4.6 %; 0–12 % of yearly carbapenem cumulative doses). The preferred carbapenems were different among wards; meropenem was less preferred in surgery and pulmonology departments (37.5 % and 33.5 % of all carbapenem DDDs consumed, respectively) than in other departments (54.6–89.0 % of all carbapenem DDDs consumed). This difference was significant in all pairwise comparisons ($P=0.002$–0.041). Ertapenem was popular both in surgery and pulmonology departments (22.4 % and 29.7 % of all carbapenem DDDs consumed, respectively), but not in any other departments (0.0–1.3 % of all carbapenem DDDs consumed; $P=0.004$–0.034). Imipenem

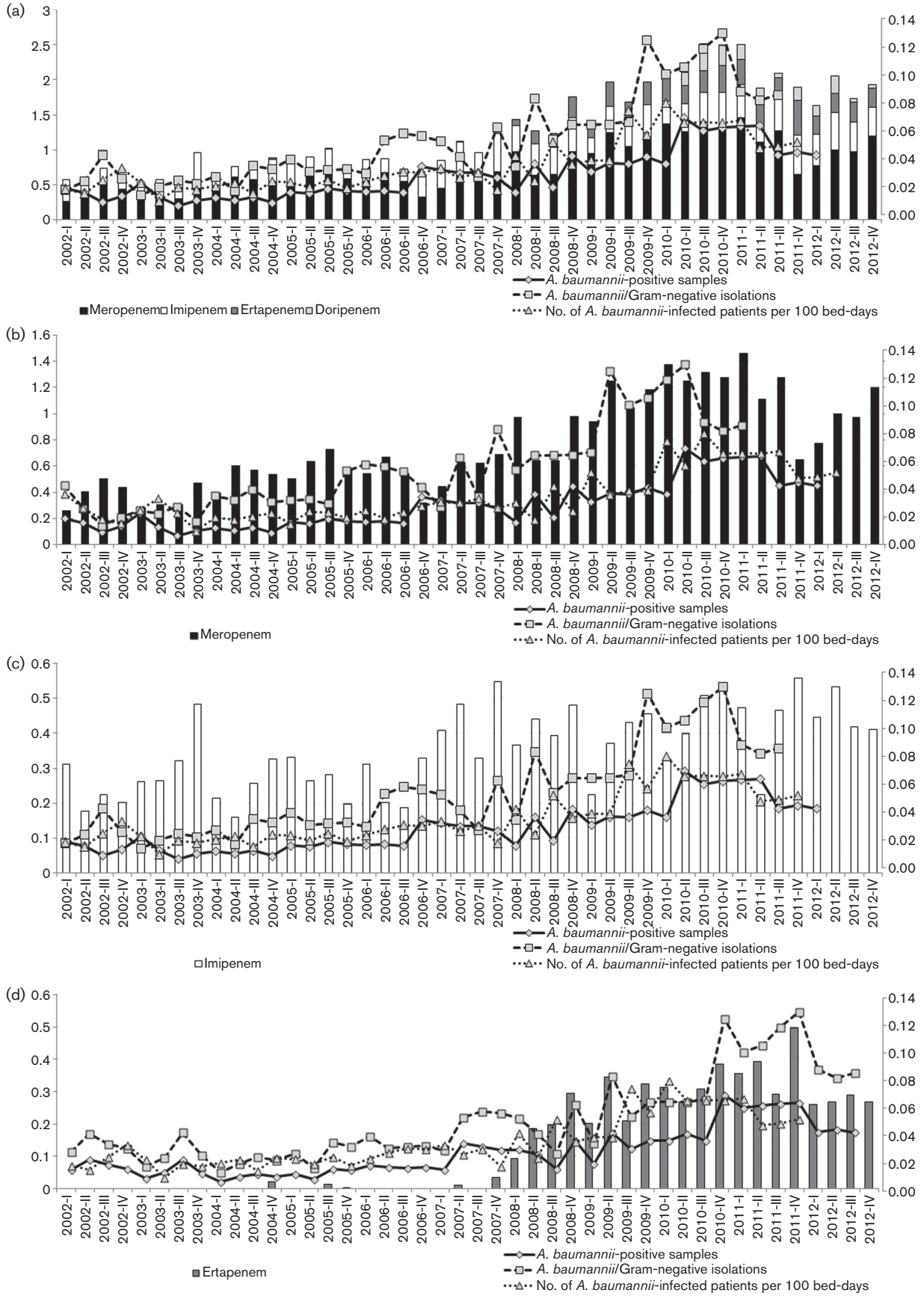
(a)
Meropenem Imipenem Ertapenem Doripenem
A. baumannii-positive samples
A. baumannii/Gram-negative isolations
No. of A. baumannii-infected patients per 100 bed-days
(b)
Meropenem
A. baumannii-positive samples
A. baumannii/Gram-negative isolations
No. of A. baumannii-infected patients per 100 bed-days
(c)
Imipenem
A. baumannii-positive samples
A. baumannii/Gram-negative isolations
No. of A. baumannii-infected patients per 100 bed-days
(d)
Ertapenem
A. baumannii-positive samples
A. baumannii/Gram-negative isolations
No. of A. baumannii-infected patients per 100 bed-days

Fig. 1. Distribution of use rates of all carbapenems (A), meropenem (B), imipenem (C) and ertapenem (D) between 2002 and 2012. Prevalence data are depicted with the delay revealed by the time-series analysis.

usage was important in the surgery and paediatric departments, and doripenem usage was invariably low.

Overall aminoglycoside usage was highest in the pulmonology department (P=0.005–0.045 in pairwise comparisons), where the isolates harbouring the aminoglycoside resistance methylase gene *armA* were relatively frequent (see below).

Regarding the number of *A. baumannii* positive patients per 100 bed-days in each year, different wards (except the second department of internal medicine) showed similar trends, with a sharp increase between 2005 and 2010, than a small decrease in 2011 (Fig. 3).

Carbapenem resistance in *A. baumannii* was highly prevalent in the surgery ICU, pulmonology ICU, first internal medicine ICU, perinatal ICU and neurosurgery ICU, as well as in other wards, but was significantly lower in the neurology ICU (P>0.001–P=0.002 in pairwise comparisons), and in the paediatric ICU during the study period (P=0.001–0.029 in pairwise comparisons). Cross-correlation between ward-specific consumption and prevalence/resistance data were not significant.

Resistance genes and integrons

As expected, all isolates carried the chromosomal weak carbapenemase gene $bla_{OXA\text{-}51}$ and the insertion sequence ISAba-1. $bla_{OXA\text{-}23}$ and $bla_{OXA\text{-}24}$ were present in 78.1 % (125/160) and 1.2 % (2/160), respectively, and $bla_{OXA\text{-}48}$ and $bla_{OXA\text{-}58}$ were not detected. Carbapenem susceptible isolates carried only the $bla_{OXA\text{-}51}$ gene, and carbapenem resistance was linked to carriage of $bla_{OXA\text{-}23}$ or $bla_{OXA\text{-}24}$.

Genes coding for amikacin resistance, *aph(3′)-VIa* and *aac(6′)-Ib*, were detected in 90.6 % (145/160) and 56.9 % (91/160) of the isolates, respectively. *aph(3′)-Ia* was found in 68.7 % (110/160), *ant(3′)-Ia* in 77.5 % (124/160) of isolates, and *ant(2′)-Ia* was not detected. Out of the tested aminoglycoside methylase genes only *armA* could be detected (11.9 %, 19/160), and *rmtA* and *rmtB* genes were not present. Two or more aminoglycoside resistance genes were harboured simultaneously in all isolates. Prevalence of tetracycline resistance genes *tetA*, *tetB* and *tetD* was 11.9 % (19/160), 6.9 % (11/160) and 39.4 % (63/160), respectively, and *tetC* was not encountered. Isolates carrying *tetD* were always positive for *tetA* as well.

Class I integrons were found in the majority of isolates (93.7 %, 150/160), and class II and III integrons were not present.

Identification and characterization of *A. baumannii* clusters

PFGE distinguished six clusters (A1, A2, B, C1, C2, D), a pair of isolates with identical patterns and two isolates with unique profiles. Clusters A1 and A2, as well as clusters C1 and C2, showed somewhat similar profiles (with similarities of 84.6 % and 86.3 %, respectively).

Cluster A1 contained 26 isolates from 19 patients. The majority of the isolates originated from the neurology ICU/stroke ward (15/26) and from the paediatric ICU (7/26). The isolates of this cluster were carbapenem susceptible, but uniformly resistant to ciprofloxacin, amikacin and tobramycin. All isolates harboured the $bla_{OXA\text{-}51}$ gene, the ISAba-1 sequence and a class I integron with the gene cassette array *aac(6′)-Ib*; hypothetical protein; $bla_{OXA\text{-}20}$ (In426). The genes *aph(3′)-Ia*, *aph(3′)-VIa*, *tetA* and *tetD* were detected in 76.9 % (20/26), 50.0 % (13/26), 73.0 % (19/26) and 42.3 % (11/26) of the isolates, respectively.

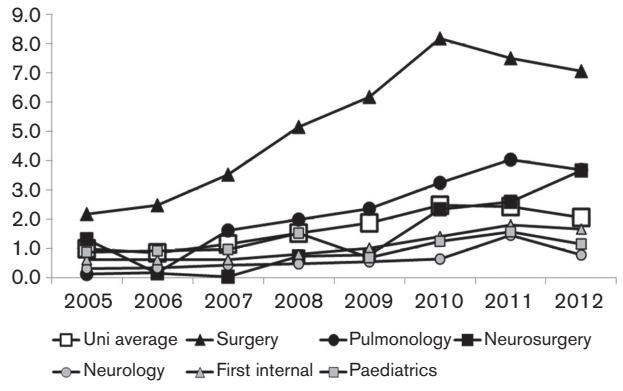

Fig. 2. Carbapenem consumption (DDD per 100 bed-days) in the different departments.

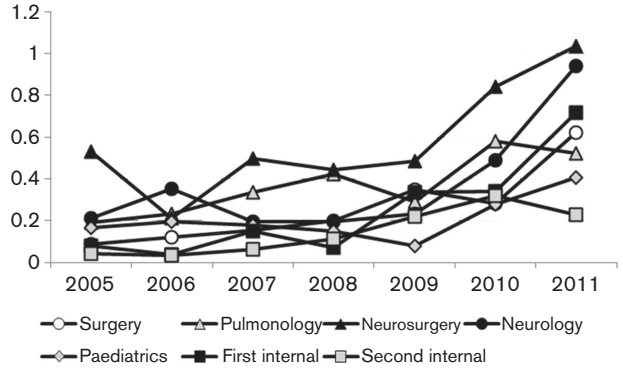

Fig. 3. Changes in the occurrence of *A. baumannii*-positive patients (number of *A. baumannii*-positive patients per 100 bed-days) in the different departments.

Other bla_{OXA}, tetracycline or aminoglycoside resistance genes were not found in the cluster.

Cluster A2 contained seven isolates from four patients. Six of the seven isolates in this cluster originated from the surgery ICU, only one isolate originated from the neurosurgery ICU. They were found to be susceptible only to doxycycline, tigecycline and polymyxins showing resistance to all other antibiotic groups, excepting the neurosurgery isolate. Beside bla_{OXA-51}, all of them carried the $bla_{OXA-23-like}$ gene. They were positive for the *aph(3′)-Ia* and *aph(3′)-VIa* genes, but not for any other aminoglycoside or tetracycline resistance genes tested. None of the isolates harboured integrons.

Cluster B included 19 isolates from 11 patients. The majority of the isolates (14/19) in the cluster were originated from the perinatal ICU, and all tested isolates from the perinatal ICU belonged to this cluster. Antibiotic susceptibility was characterized by carbapenem and aminoglycoside resistance. Both bla_{OXA-51} and $bla_{OXA-23-like}$ carbapenemases, *aph(3′)-Ia*, *aph(3′)-VIa* and *ant(3′)-Ia* were detected in all members. They uniformly carried a class I integron with a gene cassette array of *aac(3)-Ia*; hypothetical protein; hypothetical protein; *ant(3′)-Ia* (In561).

Cluster C1 contained 12 isolates of 10 patients, all except one from the first internal medicine ICU. Ten isolates were susceptible to doxycycline, tigecycline and polymyxin-B, one isolate was susceptible to tigecycline and polymyxin-B, and one was susceptible only to polymyxins. Genes $bla_{oxa-51-like}$ with ISAba-1, $bla_{oxa-23-like}$, *ant(3′)-Ia* and *aph(3′)-VIa* were identified in all isolates. Other tested resistance genes (*armA*, *rmtA*, *rmtB*, *tetA*, *tetB*, *tetC*, *tetD*, *tetE)* were not found. All isolates were class I integron positive, and the gene cassette array in eleven isolates was *aac(3)-Ia*; hypothetical protein; hypothetical protein; *ant(3′)-Ia* (In561). Curiously, the remaining isolate carried an integron with a sole *ant(3′)-Ib* gene (In127).

Cluster C2 contained 29 isolates from 24 patients. The origin of the isolates was varied, with eleven, six, six and two isolates from the surgery ICU, neurosurgery ICU, neurology ICU/stroke and first internal medicine ICU departments, respectively, and the remaining four isolates from four other wards. Antibiotic susceptibility was highly diverse within the cluster; while all isolates from neurology were susceptible to carbapenems, isolates from other departments showed susceptibility only to doxycycline, tigecycline and polymyxins or only to polymyxins. The resistance gene pattern was similar to that of isolates of cluster C1 except the $bla_{oxa-23-like}$ gene, which was absent in the six carbapenem susceptible isolates from neurology. The class I integron carried by all isolates was also identical to that found in Cluster 1.

Cluster D included 63 isolates, 50 of which originated from the pulmonology ICU, the other 13 isolates were from different wards of the second internal medicine department (eight isolates), first internal medicine ICU (three isolates), surgery ICU (two isolates) and various other wards (three isolates). The majority of the isolates were resistant to all drugs except polymyxins and tigecycline. Isolates within this cluster had the highest number of resistance genes; all isolates carried $bla_{oxa-23-like}$, $bla_{oxa-51-like}$ and ISAba-1, *aac(6′)-Ib*, *aph(3′)-Ia*, *ant(3′)-Ia*, *aph(3′)-VIa*, *aac(3′)-Ia* and *tetB* genes. Moreover, 30.2 % (19/63) of the isolates in the cluster carried a 16S rRNA methylase gene *armA*. All isolates carried a class I integron with a variable region containing *aac(6′)-Ib*; *catB8*; *ant(3′)-Ia* gene cassette array (In439). Curiously, the integrase gene of this integron was not amplifiable with the primers widely used to detect class I integrase.

Four isolates did not belong to any cluster. One from the surgery ICU was similar to cluster A2 (showing a similarity of 86.6 %), but carried an additional *aac(6′)-Ib* gene. An identical pair of isolates from different departments (pulmonology ICU and first internal medicine ICU) showed extensive drug resistance (susceptible only to tigecycline and colistin). These two isolates carried $bla_{oxa-24-like}$ carbapenemase besides $bla_{oxa-51-like}$ and ISAba-1, but none of the aminoglycoside or tetracycline resistance genes tested. They were also integron negative. The fourth isolate was also extensively resistant, and was similar to cluster D regarding resistance genes, except it was lacking the *aph(3′)-Ia*, the *armA* and the *tetB* genes. It also carried the integron found in cluster D.

Distribution of the clusters among different wards

Isolates originating from the neurology department (except for a single isolate) and from the paediatric ICU were susceptible to carbapenems; all other wards were dominated by carbapenem-resistant *A. baumannii* ($P<0.001$). Neurology ICU and paediatric ICU had a significantly lower number of patients with carbapenem-resistant than with carbapenem-susceptible *A. baumannii* ($P<0.001$–$P=0.002$ and $P=0.001$–0.03, respectively) as compared with other wards in pairwise comparisons.

All clusters were dominated by isolates from one or two wards. Cluster A1 was dominated by isolates from neurology and from the paediatric ICU; all except one isolate from cluster A2 originated from the surgery ICU; the majority of isolates in cluster B were found in the perinatal ICU; isolates from the first internal ICU dominated cluster C1; surgery ICU and neurology were the most frequent source for isolates in cluster C2 and the pulmonology ICU was the main site for isolates in cluster D. Cluster distribution among different wards was significantly different ($P<0.0001$). Eight of the 17 patients with *armA* positive isolates originated from the pulmonology ICU ($P<0.001$–$P=0.036$ in pairwise comparisons with other wards).

DISCUSSION

Prolonged outbreaks or endemic occurrence by multi-resistant pathogens is often provoked and maintained by

selection pressure exerted by antibiotic consumption. This has been demonstrated in the case of Gram-negative as well as Gram-positive pathogens (Bergman *et al.*, 2009; Bronzwaer et al., 2002; Harthug *et al.*, 2000; Rogues et al., 2007), including several cases of multidrug resistant *Pseudomonas aeruginosa* (Rogues et al., 2014; Suarez *et al.*, 2011), vancomycin-resistant enterococci (Harthug *et al.*, 2000) and *A. baumannii* (Paul *et al.*, 2005). Some of these are associated with clonal spread and maintenance of a single multiresistant clone (Giannouli *et al.*, 2010), while in other cases resistance is acquired by the pathogens during the outbreaks (Corbella *et al.*, 2000; Harthug *et al.*, 2000). Stopping the outbreaks very frequently necessitated restriction of the usage of the provoker antibiotic, (Suarez *et al.*, 2011), further confirming the importance of antibiotic usage as a driving force.

The choice of antibiotic is often motivated by the concern about drug resistance, leading to increased usage of broad-spectrum antibiotics, which in turn provokes another outbreak, creating the resistance spiral. The present study reports such a situation, when concern about extended-spectrum beta-lactamase (ESBL) producers provoked by the consumption of third cephalosporins (unpublished data) led to increase in carbapenem usage with an approximately two-year lag, which brought about increased prevalence and carbapenem resistance in *A. baumannii*. A similar relationship has also been reported previously (Goel *et al.*, 2011; Xu *et al.*, 2013). Increasing colistin use, in turn, was linked to increasing *A. baumannii* prevalence, thus representing a further turn in the resistance spiral, and a threat to emergence of non-treatable pandrug-resistant *A. baumannii*.

Increased prevalence and carbapenem resistance was directly linked to carbapenem consumption at the ward level. In the neurology department, where carbapenem consumption was significantly lower than in any other department analysed, the majority of *A. baumannii* isolates were carbapenem-susceptible, while carbapenem resistance is a concern in almost all other departments.

The usage of different carbapenems showed different effects; while imipenem consumption seemed to be less important as a cause for carbapenem resistance, meropenem and especially ertapenem consumption was associated with increased prevalence of *A. baumannii*. This corresponds well to the observation that imipenem is slightly more effective against *A. baumannii* than meropenem (MacGowan *et al.*, 1995; Villar *et al.*, 1997). Ertapenem was also shown as a provoker of carbapenem resistance in *P. aeruginosa*, at least *in vitro* (Vainio *et al.*, 2013). Although data were inconclusive on the differential role of the different carbapenems at the ward level, the surgery and pulmonology departments, where ertapenem is a preferred carbapenem, are heavily burdened. These data raise the possibility that not only increased carbapenem consumption is important in the causality of the spread of carbapenem resistance, but also the pattern of the particular drugs used may also play an important role. This issue needs to be addressed by upcoming studies, as this information may be crucial in optimizing the choice of carbapenems.

Analysis of the molecular epidemiology revealed that carbapenem resistance could not be linked to a single clone; it was rather due to acquisition of BlaOXA-23-like carbapenemases, similarly to previous findings (Corbella *et al.*, 2000; Giannouli *et al.*, 2010; Zarrilli *et al.*, 2004). All resistant clones harboured this gene; moreover, the resistant isolates in cluster C2 differed from susceptible isolates in the cluster by the carriage of the $bla_{OXA\text{-}23\text{-}like}$ carbapenemase gene. Interestingly, these susceptible isolates originated from, and were exclusively found in, samples from the neurology department, the ward with the lowest carbapenem consumption, suggesting that antibiotic use may have directly facilitated horizontal acquisition of resistance genes (Couce & Blázquez, 2009; Hawkey & Jones, 2009; Valenzuela *et al.*, 2007). The majority of patients harbouring isolates that carried the aminoglycoside resistance methylase gene *armA* were patients of the pulmonology department, the one with the highest aminoglycoside consumption, similarly suggesting the role of antibiotic consumption in horizontal gene transfer. This assumption is supported by the known genome plasticity of *A. baumannii* (Imperi *et al.*, 2011).

Interestingly, most clusters were dominated by isolates from one or two wards, but many clusters were represented in multiple wards by a few isolates, suggesting that the ward-specific strains are continuously transferred to other wards, creating a polyclonal endemicity pattern.

In conclusion, low carbapenem use was associated with carbapenem susceptibility even within a single *A. baumannii* clone, while the spread of carbapenem resistance of *A. baumannii* was clearly linked to meropenem and ertapenem but not to imipenem consumption. This points to non-equivalence of even very similar drugs not only in terms of efficacy but also as provokers of drug resistance.

ACKNOWLEDGEMENTS

The expert technical assistance of Éva Székely is gratefully acknowledged. The authors thank István Kraszits for providing raw data on antibiotic consumption. J. Mózes and G. Kardos were supported by TÁMOP 4.2.4. A/2-11-1-2012-0001 'National Excellence Program – Elaborating and Operating an Inland Student and Researcher Personal Support System'. The project was subsidized by the European Union and co-financed by the European Social Fund.

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