SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PHD)

Investigating mechanisms of multidrug resistance in Gram-negative pathogens: genomic epidemiology of carbapenem-resistant *Acinetobacter baumannii*

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UNIVERSITY OF DEBRECEN
DOCTORAL SCHOOL OF PHARMACEUTICAL SCIENCES

DEBRECEN, 2022

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The PhD Defense takes place at the lecture hall of Department of Emergency Care and Oxyology, Faculty of Medicine,University of Debrecen, from 1 p.m., on 12th of January, 2023

Introduction

Nosocomial or healthcare-associated infections are a major problem worldwide. In hospital care, 7 out of every 100 patients in developed countries and 10 in developing countries become infected. Indeed, in different hospital wards, such as intensive care units (ICU), burns wards and neonatal wards, infection rates may differ. The most frequent and serious infections occur in ICUs, and in these wards the infection rate can reach 20.6% alone. High incidence rates are also associated with increased mortality. According to the World Health Organisation, the death rate in infant wards may be as high as 75% in the poorest regions (South-East Asia, Sub-Saharan Africa). However, even in wealthy regions, the rate is between 3.5% and 12.0%. The spread of nosocomial pathogens and the outbreaks are multifactorial processes. The pathogen can be a member of the normal flora of inpatients, which may as an opportunistic pathogen can cause severe infection when the immune system is weakened. Another source of infections is the hospital environment itself. Transmission may occur through hospital staff, equipment, or direct contact with another infected patient. In addition, the potential presence of pathogens in the water supply increases the chance of transmission, which can be reduced by following proper hygiene guidelines (hand disinfection, proper use of mouth masks, etc.).

The problem is exacerbated by the presence of multidrug resistant (MDR) pathogens. These bacteria found in the hospital environment and can be resistant to a wide range of antibiotics or even to disinfectants. Many of them are able to survive on abiotic surfaces for long periods (>100 days) and also resistant to desiccation. The so-called ESKAPE pathogens (*Enterococcus* species, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter species*) are responsible for most of the nosocomial infections. Infections caused by non-fermenting, aerobic, Gram-negative bacteria have become a major clinical challenge in recent decades.

Aims

Since 2010, our working group has been carrying out molecular epidemiological studies of multidrug resistant *A. baumannii* resistance, complemented by analysis of antibiotic use. This study presents the results after 2012 and compares *A. baumannii* isolates from faecal samples with clinical isolates from the same period. Our preliminary investigations suggested the emergence of a new carbapenem-resistant *A. baumannii* strain at the Clinical Centre of the University of Debrecen. Therefore, we wanted to find an answers to the following questions:

- Did a strain change occur?
- Did the resistance of the new strain differ from the previously dominant clones, either genomically or phenotypically?
- What might be the reason for the strain switch?
- Does antibiotic use play a role in strain switching?
- How far do new and old strains diverge?
- Can asymptomatic carriers appear?

Materials and methods

Acinetobacter baumannii clinical isolates

The clinical *Acinetobacter baumannii* isolates investigated were collected at the Clinical Center of the University of Debrecen between 2012 and 2017. A total of 521 isolates were tested: 74 in 2012; 118 in 2013; 128 in 2014; 136 in 2016 and 65 isolates were collected until April 2017. Isolates were selected that only one isolate from each patient was included in the study year. The samples originated from the inpatient and intensive care units of the clinics. In terms of the site of isolation, 44.7% (233/521) of the isolates were from bronchus samples, followed by wound (38/521), trachea (33/521), urine (36/521) and blood (37/521) samples. Identification of clinical isolates was performed by MALDI-TOF-MS (Matrix-Associated-Laser-Desorption/Ionization Time-of-Flight Mass-Spectrometry) using a MALDI Biotyper (Bruker Daltonics). The resistance profiles (genotypic and phenotypic) of *A. baumannii* strains collected and tested in 2012, 2013, 2014, 2016 and 2017 were also compared with our working group's previous results of *A. baumannii* isolates from 2010/2011.

Faecal *A. baumannii* isolates

In another study of our working group, faecal samples received for routine diagnostic culturing were also inoculated on eosin-methylene blue medium containing 2 mg/L cefotaxime to detect multiresistant Gram-negative bacteria. Bacterial colonies cultured on the media were identified by MALDI-TOF-MS and *A. baumannii* colonies were isolated. The isolates were collected between January 2017 and April 2019. A total of 7806 stool samples from different inpatient wards of the University of Debrecen Clinical Centre were analysed.

Antibiotic susceptibility testing

The antibiotic susceptibility testing of both clinical and faecal *A. baumannii* isolates was performed by the Kirby-Bauer disk diffusion method according to the current annual recommendation of the European Committee on Antimicrobial Susceptibility Testing (EUCAST) to the following antibiotics: imipenem, meropenem, ciprofloxacin, amikacin, tobramycin, gentamicin. In all cases, colistin sensitivity was determined by microdilution assay (Merlin Diagnostics, Germany).

Antibiotic consumption and changepoint analysis

Monthly antibiotic consumption data and incidence densities of infections were collected monthly from 2005 to 2017. Consumption data were calculated in defined daily dose (DDD), with the number of DDDs per 100 occupied bed-days (OBD). Infection incidence density was characterized as the number of inpatient infections per 100 bed-days. The time series were tested for the presence of a trend using the Mann-Kendall test. Significant changes in the time series of incidence density of carbapenem-susceptible and carbapenem-resistant strains and in the time series of carbapenem consumption data were examined by changepoint analysis. By comparing the changepoints found in each time series, we searched for correlations between antibiotic use and the incidence of carbapenem-susceptible and carbapenem-resistant *Acinetobacter baumannii* (CRAb).

Changepoints were searched for in each time series and the temporal distance of changepoints found in different time series were analyzed. For the changepoint analysis, we examined the deviations of the mean by the Pruned Exact Linear Time (PELT) method with the cpt.mean command in the changepoint R package. First, we searched for the penalty value where the increase in the number of changepoints in a given time series is no longer exponential (Changepoints for a Range of Penalties (CROPS) method), and then we used this penalty value to determine the changepoints. The test was parameterised by suppressing the occurrence of periods shorter than six months (periods between two changepoints). If the changepoints of two time series were close in time, a relationship was assumed to exist between them. The changepoint analysis was performed with the help of the supervisor.

Granger causality test

The causality between asymptomatic carriage and *A. baumannii* isolates from clinical samples was investigated by the Granger causality test. The Granger causality test is based on a prediction quality comparison: a time series is tested alone and then another time series is included. If the forecast quality is better in the latter case, the first time series is Granger-caused the other, indicating a possible relationship. The Granger causality test was performed following Anil et al. using the baseline data (without differencing and Box-Cox transformation).

For the Granger causality test, we collected the prevalence of carbapenem-susceptible and resistant *A. baumannii* from inpatient faecal samples and nosocomial infections by month

per 100 days of care between 2017 and 2019. Granger causality was tested between pairs of *A. baumannii* incidence time-series from infection and from faecal origin.

The Granger causality test was performed with the assistance of supervisor.

Resistance genes

The carbapenem resistance genes $bla_{OXA\text{-}23\text{-}like}$, $bla_{OXA\text{-}40\text{-}like}$ and $bla_{OXA\text{-}51\text{-}like}$ and the *ISAba-1* insertion sequence were screened by polymerase chain reaction (PCR) using the methodology described by Woodford et al. and Turton et al. The occurrence of aminoglycoside resistance genes (*aac(6')-Ib, aph(3')-Ia and armA*) were investigated by PCR using the method described by Frana et al., Vila et al. and Bogaerts et al. The reactions were optimized in our previous work.

Whole genome sequencing (WGS)

For whole genome sequencing, 9 isolates representing all *A. baumannii* pulsotypes (A1; A2; B; C1; C2; D) and individual isolates from the 2010/11 study were selected. Isolates with >90% similarity in pulsed-field gel electrophoresis were considered as belonging to a pulsotype. We selected 14 isolates from the 2017 clinical isolates and 15 isolates of faecal origin based on differences in resistance phenotype (susceptible to all antibiotics tested; resistant to amikacin but susceptible to tobramycin; susceptible to gentamicin and colistin only; susceptible to ciprofloxacin and colistin only; resistant to all antibiotics tested except colistin) and gene expression (carbapenemases and presence of the *armA* gene) for whole genome analysis.

Genomic DNA of all isolates was extracted using the DNeasy UltraClean Microbial Kit (Qiagen, Hilden, Germany). Libraries were generated using Nextera DNA Flex library preparation kit (Illumina, San Diego, USA) and sequenced on MiSeq platform (Illumina) using MiSeq Reagent Kit v2 (300 cycles) (Illumina) according to the manufacturer's instructions. Using the resulting FASTQ files, draft genomes were assembled de novo using Velvet assembler integrated into Ridom SeqSphere+ software (Ridom GmbH, Munich, Germany). The cgMLST analysis was performed using SeqSphere+ software (Ridom) based on the *A. baumannii* cgMLST version 1.0 schema. The antibiotic resistance genes were identified using ResFinder v3.9 (cge.cbs.dtu.dk/services/ResFinder/) and the Comprehensive Antibiotic Resistance Database (CARD) v3.1.0 (card.mcmaster.ca/analyze). Raw sequences were uploaded to the NCBI BioProject database (BioProject ID: PRJNA671692).

Whole genome sequencing was performed with the assistance of Dr. Ákos Tóth and his colleagues (National Center for Public Health).

Time-kill assay

In time-kill assays, the effects of meropenem, imipenem and colistin were tested against both clinical and faecal isolates. The initial bacterial inoculum was 10^6 CFU/ml, antibiotic concentrations were 16 and 128-1024 mg/L for carbapenems (imipenem, meropenem) and 2-32 mg/L for colistin in Mueller-Hinton broth (Lab M Limited, Heywood, UK.). Each experiment included a positive (antibiotic-free) and a negative (antibiotic and bacteria-free) control. The reaction volume was 5 mL, and was incubated at 37 °C. At 2; 4; 6; 8; 10; 12 and 24 hours of incubation a serial dilution was performed of each tubes and plating onto solid Mueller-Hinton medium was performed (Lab M Limited, Heywood, UK). Colonies grown on the solid media were counted after 24 h incubation at 37 °C. The kill rate (k) was determined as $\mathbf{N_t = N_0 \times e^{-kt}}$, where $\mathbf{N_t}$ is the number of viable bacterial colonies at time **t**, $\mathbf{N_0}$ is the number of viable bacterial colonies in the initial medium, ***k*** is the kill rate and **t** is the incubation time. Negative and positive *k* values indicate growth and death, respectively. Bactericidal effect was defined as a reduction of >99.9% compared to the initial viable bacterial count. All tests were performed in at least two parallel experiments.

Results

Antibiotic consumption:

Carbapenem consumption increased steadily between 2005 and 2018 ($p<0.001$, meropenem use increased fourfold between the two endpoints. The average consumption was 0.64 DDD/100 OBD in 2007 and 2.69 DDD/100 OBD in 2017. Imipenem consumption was similar to meropenem in 2007 (mean consumption 0.48 DDD/100 OBD) but did not change markedly over the study period (mean consumption 0.80 DDD/100 OBD in 2017). Meropenem consumption significantly exceeded imipenem consumption.

Changepoint analysis

We found six changepoints in the study where the prevalence of CRAb strains increased significantly (the mean of the time series changed). We found a temporal association between increased CRAb prevalence and carbapenem usage in two cases: between February and June 2009 and October and December 2014.

Granger causality

Faecal carriage of CRAb in inpatients was associated with increased prevalence of clinical CRAb ($F = 15.84$; $p < 0.001$), but the presence of CRAb in faeces of inpatients did not increase the prevalence of CRAb ($F = 0.03$, $p = 0.855$) in clinical samples. Conversely, neither the asymptomatic carriage of carbapenem-susceptible *A. baumannii* was causally related to the prevalence of carbapenem-susceptible *A. baumannii* in clinical samples ($F = 2.15$, $p = 0.155$), nor backwards ($F = 0.13$, $p = 0.726$).

Clinical isolates

Antibiotic susceptibility of clinical isolates

Rate of resistance was above 75% for all tested antibiotics except colistin. Carbapenem resistance increased steadily throughout the study ($p<0.001$). More than 90% of isolates were CRAb. Resistance to ciprofloxacin and gentamicin remained very high throughout the study, while resistance to amikacin and tobramycin decreased significantly between 2016-2017 ($p<0.001$). Resistance to colistin was sporadic.

Resistance genes among the clinical isolates

The prevalence of aminoglycoside resistance genes decreased over the study period. The prevalence of the dominant *aph(3')-VIa* phosphotransferase gene decreased from 90.6% to 6.2% ($p<0.001$). A similar pattern was observed in case of *aac(6')-Ib* acetyltransferase with a significant decrease between study endpoints (56.9% vs. 27.7%). Methyltransferase *armA* prevalence showed no significant difference between the two endpoints, but there was a significant decrease in gene prevalence between 2014 and 2016 ($p<0.001$).

All isolates were harbouring the $bla_{OXA\text{-}51\text{-}like}$ gene, demonstrating that all investigated isolates were *A. baumannii*. The predominant carbapenemase gene between 2010 and 2014 was $bla_{OXA\text{-}23\text{-}like}$, but the prevalence of this gene family significantly decreased in 2016 and 2017 ($p<0.001$). The prevalence of $bla_{OXA\text{-}40\text{-}like}$ carbapenemases showed an opposite trend, while the gene family was almost absent at the beginning of the study, its prevalence significantly increased in 2016 ($p<0.001$) and was even higher in 2017 ($p<0.001$).

Whole genome sequencing of clinical isolates

In 2010, the carbapenemases of the $bla_{OXA\text{-}23\text{-}like}$ gene family were identified as $bla_{OXA\text{-}23}$ genes in all sequenced strains, representing all major pulse types, and ST1; ST2 and ST45 were found.

All isolates harbouring solely the $bla_{OXA\ 23\text{-}like}$ acquired carbapenemase gene from 2017 belonged to ST2. The isolates carrying the $bla_{OXA\text{-}40\text{-}like}$ gene family (75.0% in 2016 and 92.3% in 2017) belonged to ST636 and ST492, these strains encoded the $bla_{OXA\text{-}72}$ gene and $bla_{OXA\text{-}72}$ and $bla_{OXA\text{-}23}$ genes together encoded by three ST636 strains. The genetic environment of $bla_{OXA\text{-}23}$ gene in case of ST636 and ST492 isolates were identical to ST1 and ST45 from 2010 (e.g. the aph(3')-VIa gene). No other types of acquired carbapenemase genes were found in the sequenced genomes.

The ST636 isolates were genetically very uniform, with a distance of ≤ 4 alleles, whereas the ST2 strains were more diverse, the difference reached 20 alleles between some strains, thus could be classified into distinct subgroups.

Time-kill analysis of clinical isolates

The investigated isolates were clinically resistant to both meropenem and imipenem, the minimum inhibitory concentration (MIC) was >32 mg/L, except for two isolates encoding only the $bla_{OXA\text{-}51\text{-}like}$ gene (MIC=2 mg/L in both cases). Against the only $bla_{OXA\text{-}23}$ carriers (ST2), meropenem showed concentration-dependent bactericidal activity between 128 and

1024 mg/L (mean k=0.48; 0.13-0.59), whereas the *bla*$_{OXA-72}$ carriers (ST636; ST492) were not killed at 256 mg/L (mean k= -0.27; -0.49 to -0.01), bactericidal effects were between 512 and 1024 mg/L (mean k=0.14; 0.00 to 0.28), and against isolates carrying both carbapenemases, *bla*$_{OXA-23}$ and *bla*$_{OXA-72}$, only bacteriostatic effects were observed at 1024 mg/L (mean k= -0.14; 0.00 to -0.25). Meropenem at 32 mg/L was bactericidal against isolates carrying only the chromosomally encoded *bla*$_{OXA-51-like}$ gene (mean k=0.08; 0.05-0.10).

Imipenem showed concentration-independent bactericidal activity between 16 and 128 mg/L against strains encoding the carbapenemase *bla*$_{OXA-72}$ (mean k=0.17; 0.05-0.31) and *bla*$_{OXA-23}$ (mean k=0.36; 0.19-0.57). For strains encoding both carbapenemases together (ST636), the bactericidal concentration was 128 mg/L (mean k=0.43; 0.16-0.71). Similarly to meropenem, lower concentrations (16-32 mg/L) were ineffective, but imipenem proved slightly more effective at 16 mg/L against the only *bla*$_{OXA-72}$ carriers (ST636; ST492).

The effect of colistin was isolate rather than strain dependent, but colistin showed bactericidal activity against both *bla*$_{OXA-23}$, *bla*$_{OXA-72}$ and *bla*$_{OXA-23}$/*bla*$_{OXA-72}$ harbouring strains at <2 mg/L concentrations.

Faecal isolates

Distribution and phenotypic resistance of faecal isolates

During the first study period (2011-2013), only one *A. baumannii* isolate (0.02%) was detected in faecal samples (n=4067). Between January 2017 and April 2019, 7806 stool samples were analysed and 55 (0.15%) *A. baumannii* isolates found (24 in 2017; 25 in 2018; 6 in 2019). Out of these 55 isolates, 30 were from paediatrics, 15 from internal medicine and 10 from various other clinics (e.g. pulmonary, neurosurgery). Among the *A. baumannii* isolates 15 were CRAb (resistant to imipenem and meropenem), 17 were resistant to ciprofloxacin, 9 to amikacin and tobramycin, and 13 to gentamicin.

Whole genome sequencing of faecal isolates

For whole genome analysis, 16 faecal isolates were selected based on antibiotic susceptibility and PCR assays. Fifteen of the sequenced isolates were positive for at least one acquired carbapenemase, and one was found to carry neither a *bla*$_{OXA-23-like}$ nor a *bla*$_{OXA-40-like}$ gene but was phenotypically resistant to the tested carbapenems.

Among the sequenced isolates, only *bla*$_{OXA-72}$ carrier ST636 (n=9) and ST492 (n=2) were detected. Two ST636 isolates encoded *bla*$_{OXA-72}$/*bla*$_{OXA-23}$ together. The two ST492

strains also carried the aminoglycoside resistance *armA* gene. Further analysis of the environment of the *bla*$_{OXA-72}$ gene revealed that ST636 and ST492 isolates carried plasmids pMAL-1-like and pA105-2-like, respectively. Only three isolates did not belong to the newly released sequence types (ST492, ST636), ST132 encoded *aac(6')-Ib-cr* and *bla*$_{OXA-120}$, the ST45 harboured *bla*$_{OXA-20}$ gene. In case of new sequence type the *bla*$_{OXA-106}$ gene was found.

The WGS confirmed that, similar to the ST636 CRAB isolates of clinical origin, the faecal ST636 strains are genetically very close, with only 4 allelic distances detected.

Comparison of clinical and faecal isolates

The resistance rates of clinical isolates from 2017 were significantly higher for imipenem (95.4% vs 27.3%; p<0.001), meropenem (95.4% vs 27.3%; p<0.001), ciprofloxacin (96.9% vs 30.9%; p); <0.001) and gentamicin (96.9% vs 23.6%; p<0.001). This difference was not significant for amikacin and tobramycin, but these antibiotics also had higher resistance rates among clinical isolates.

The MIC of imipenem and meropenem was >32 mg/l in all cases. Time-kill analysis showed no difference in growth kinetics between faecal and clinical strains in the presence of carbapenems. Meropenem was bactericidal at 128 mg/L against ST492 isolates, but no bactericidal effect was ever found against ST636; neither against *bla*$_{OXA-40-like}$ nor against isolates carrying *bla*$_{OXA-40-like}$ and *bla*$_{OXA-23-like}$ carbapenemases. Imipenem at a dose of 256 mg/L was bactericidal against all tested isolates.

The prevalence of the *bla*$_{OXA-40-like}$ carbapenemase gene was significantly higher in clinical *A. baumannii* isolates (76.9% vs. 36.4; p<0.001), two isolates carried only *bla*$_{OXA-23-like}$ gene, while there were no strains in the faeces that carried only the *bla*$_{OXA-23-like}$ carbapenemase. The prevalence of isolates carrying both carbapenemases simultaneously was also significantly higher among clinical isolates (15.4% vs 3.6%; p<0.05). There was no significant difference in case of the *armA* aminoglycoside resistance gene (9-2% vs 3.6%).

In both clinical and faecal ST636 and ST492 isolates, the *bla*$_{OXA-72}$ carbapenemase gene was carried on a pMAL-1-like plasmid, with one clinical isolate found to carry the complete pMAL-1 plasmid with 99.99% identity in a single contig (GeneBank ID: KX230793.1). In all cases, the *bla*$_{OXA-23}$ gene was linked to the *Tn2008* (GeneBank ID: LN877214.1) transposon.

Discussion

In the present study, whole genome analysis confirmed the strain switch in case of CRAb, whereby isolates belonging to the previously dominant *bla*$_{OXA\text{-}23\text{-}like}$ carrying sequence types ST2 and ST45 were replaced by strains ST636 and ST492, which encoded *bla*$_{OXA\text{-}40\text{-}like}$ carbapenemase, but could not completely displace the former strains. The evolution associated with antibiotic use towards increasingly resistant bacterial strains is a well-known process. In the case of *A. baumannii*, the newly emerging strains show an increasing prevalence worldwide, consistent with the evolutionary processes mentioned above, as the enzymes encoded by the *bla*$_{OXA\text{-}51\text{-}like}$ genes, which have intrinsically poor hydrolyzation capabilities, were first replaced by encoded *bla*$_{OXA\text{-}23\text{-}like}$ and *bla*$_{OXA\text{-}58\text{-}like}$, respectively, and then by clones carrying *bla*$_{OXA\text{-}40\text{-}like}$ carbapenemases, which are most efficient in hydrolyzing carbapenems.

Adaptation to the hospital environment may not only be in the form of antibiotic resistance, although it is a fact that rapid and effective resistance to antibiotics is one of the main drivers of this change. In a Korean study, a correlation was found between increased fluoroquinolone consumption and the prevalence of third-generation cephalosporin and ciprofloxacin resistant *E. coli* and *K. pneumoniae*. Similar results were obtained by Mamoon et al. for fluoroquinolone usage and the prevalence of ESBL-producing *E. coli*. Although the acquisition of a resistance mechanism may have a significant fitness cost, while avoiding antibiotic use provides a survival advantage, selection pressure due to antibiotic will result in the emergence, spread and persistence of increasingly resistant bacteria. In addition to the acquisition of resistance mentioned above, virulence and transmission factors also play an important role in the spatial (interclass) and temporal exchange of strains.

The spread of increasingly resistant *A. baumannii* is a well-documented process in the literature, associated with the increasing use of third generation cephalosporins and carbapenems. The latter link is supported by the changepoint analysis presented in this study. This process is part of a resistance spiral, the increase in carbapenem use may be associated with the spread of ESBL-producing bacteria and the consequent increase in carbapenem use, which our working group has statistically confirmed on national data. In addition, a study of CRAb isolates before 2012 also showed a link between carbapenem consumption and increased prevalence of resistant strains.

The time-kill experiments showed the above process, the resistance to individual agents within the antibiotic group may differ, even if only to a small extent. The killing

kinetics of meropenem and imipenem were different; meropenem showed less efficacy against the newly released isolates ST636 and ST492 than imipenem. This difference may have been a selection advantage for the new *bla*$_{OXA-72}$ positive strains. The antibiotic consumption data show that meropenem use in clinic wards increased fourfold between 2007 and 2017, while use of imipenem remained relatively unchanged.

It is important to note that the benefit of ST636 and ST492 isolates at clinically achievable concentrations is not demonstrated but may still be an important factor in the selection competition between strains. A similar explanation can be given, for example, for the success of the H30Rx *E. coli* pandemic clones against other fluoroquinolone resistant *E. coli* ST131 subclones. Again, in this case, the subclone with the highest level of resistance proved to be the most successful in the competition between clinically resistant clones with different levels of resistance. These results, highlight the importance of correct antibiotic use, which means not only the use of an antibacterial agent that is effective against the pathogen, but also the balanced use of members of a group with small differences in their spectrum. Thus, even strains with clinically high levels of resistance can be replaced by more resistant new clones under high selection pressure, because achieving higher levels of resistance can be evolutionarily advantageous.

Increased faecal carriage coincided with strain switching, with ST636 and ST492 sequence types carrying the *bla*$_{OXA-72}$ carbapenemase gene found in faecal CRAb isolates, while ST2 isolates carrying only the *bla*$_{OXA-23}$ gene, previously dominant in clinical isolates, were absent.

It is important, that all of the faecal CRAb isolates were from patients with no other clinical specimens showing either carbapenem-resistant or susceptible *A. baumannii*. (Unfortunately, systematic investigation of gut colonization in infected patients was not possible.) Previous studies have investigated colonisation by carbapenem-resistant *A. baumannii* strains, but these were performed in intensive care units and severe infection caused by *A. baumannii* in patients were present. Our results on prevalence were similar to experiments by Dijkshoorn et al. where the prevalence of *A. baumannii* in healthy adult population is estimated to be between 0.8 and 1.0%, but data is lacking about patients not previously infected with CRAb. Li et al. reported a similar faecal prevalence of 1.48% (74/5000) in a follow-up study of hospitalized patients, but the manuscript did not indicate whether the patients were infected with *A. baumannii* or colonized asymptomatically.

Our results suggests that the newly emerged strains not only increased the number of infections, but also the number of those who colonized without developing disease, which was

not the case for the previously prevalent strains. The ability to colonization may therefore be strain-specific and, in addition to greater resistance to meropenem, may have contributed to the success of strains carrying *bla*$_{OXA-72}$.

The Granger-casuality test indicated that there is a correlation between increased CRAb prevalence and the appearance of carbapenem-resistant strains in faecal samples, however a similar causality in case susceptible isolates to the antibiotic group was not found. The prevalence of infections caused by resistant strains affected the occurrence of carriage, whereas carriage did not increase the occurrence of infections, i.e. increased carriage may be a consequence of increased prevalence of infections, for example as a result of higher environmental contamination due to more patients.

From a clinical point of view, the depletion of asymptomatic colonization of the intestinal tract with CRAb isolates is a major challenge, as these patients may transmit the pathogen to healthcare workers and the hospital environment (e.g. patient bed) may also be contaminated. This is supported by the fact that the Granger-causality test suggests that the rising number of colonized patients is explained by the rising number of infected patients. The contaminated hospital environment or colonized nursing staff may result in subsequent colonization and even invasive infection of critically ill patients arriving later. As colonization of the colon by a multi-resistant pathogen is not an indication for antibiotic therapy, nor does antibiotic therapy eliminate colonization, screening and isolation of patients still appears to be the most effective method against spread.

Antibiotic stewardship efforts generally aim to reduce the consumption of a group of antibiotics by treating equally the same spectrum of drug within the group. The present study highlights the fact that related compounds are not necessarily equivalent and may favour the spread of certain resistant strains and/or the development of higher levels of resistance in already resistant strains or even colonization of the gut flora. On the contrary, the versatile use of individual compounds within an antibiotic group may hinder the spread of resistance by reducing the uniform selection pressure. If total antibiotic restriction is not feasible, such an approach may be beneficial.

New results:

- Changepoint analysis found correlation between increased antibiotic use and CRAb prevalence in two cases where increase in antibiotic consumption was followed by an increase in the prevalence of resistant strains.
- Among clinical CRAb isolates, strain replacement occurred between 2014 and 2016. The previously dominant ST2 strains encoding the $bla_{OXA\text{-}23}$ carbapenemases were suppressed by the newly identified ST636 and ST492 strains encoding the $bla_{OXA\text{-}72}$ gene.
- The resistance of the new strains was different: meropenem showed concentration-dependent killing effect, isolates carrying $bla_{OXA\text{-}72}$ were more resistant. In the case of imipenem, the antibiotic showed concentration-independent activity against both carbapenemase producers.
- The prevalence of *A. baumannii* was increased in faecal samples and CRAb isolates were also present at the same time as the strain replacement. The previously dominant ST2 or only $bla_{OXA\text{-}23}$ encoding isolates carbapenemase were found in faecal samples. All CRAb isolates belonged to sequence types ST636 and ST492.
- The results of Granger causality analysis indicate that increased prevalence of CRAb infection induced the appearance of faecal carriage rather than backwards. This correlation was absent in case of carbapenem susceptible isolates.

Summary

In case of the CRAb strains, *bla*$_{OXA\text{-}23\text{-}like}$ carbapenemase was the dominant gene between 2010 and 2014 at the University of Debrecen Clinical Centre. The prevalence of the isolates carrying the gene decreased in 2016 and *bla*$_{OXA\text{-}40\text{-}like}$ encoding *A. baumannii* isolates became dominant by 2017. Whole-genome sequencing was used to demonstrate strain replacement. The carbapenem consumption has increased fourfold in the last 10 years, predominantly due to an increase in meropenem use. Our hypothesis was that the strain switch is probably due to the pattern of antibiotic consumption, mainly uniform carbapenem use. The strain replacement coincided with the appearance of asymptomatic carriage of *A. baumannii* inpatients.

Differences in resistance between clinical strains carrying the *bla*$_{OXA\text{-}23}$ and *bla*$_{OXA\text{-}72}$ (the latter belonging to the *bla*$_{OXA\text{-}40\text{-}like}$ group) carbapenemase genes were investigated in time-kill assays with carbapenems (meropenem and imipenem; 16, 128-1024 mg/L) and colistin (2-32 mg/L); the curves were used to calculate killing rates (k).

Meropenem showed a bactericidal effect between 256-1024 mg/L (k=0.346-0.859) against the *bla*$_{OXA\text{-}23}$ (ST2; ST45) carriers dominant in 2010, whereas this effect for *bla*$_{OXA\text{-}72}$ (ST636, ST492) carriers was shown only with 512-1024 mg/L concentrations (k=0.156-0.421). In contrast, imipenem showed a concentration-independent bactericidal effect between 128-1024 mg/L against any isolates (k=0.106-0.639). The effect of colistin was isolate-dependent.

Between 2017 and 2019, 55 *A. baumannii* isolates were identified from 7806 faecal samples (0.15%). Carbapenem resistance was less common in faecal isolates than in clinical isolates, collected from the same period. Only the *bla*$_{OXA\text{-}40\text{-}like}$ carbapenemase gene family was detectable among the faecal isolates (36.4%), there were no isolates carrying only the *bla*$_{OXA\text{-}23\text{-}like}$ gene, and the proportion of isolates encoding both carbapenemases was 3.6%. In case of the clinical isolates, the prevalence of the *bla*$_{OXA\text{-}40\text{-}like}$ gene was 76.9%, while the prevalence of the *bla*$_{OXA\text{-}23\text{-}like}$ gene was 3.1%. The two genes were encoded by 15.4% of the isolates.

The difference in meropenem resistance may have contributed to the spread of *bla*$_{OXA\text{-}72\text{-}}$ carrier strains. This difference was not detected with imipenem, highlighting the dangers of uniform antibiotic use. There is a Granger-causality between the clinical prevalence of CRAb isolates and the incidence of faecal carriage of the new carbapenem-resistant ST636 and ST492 isolates, but this is not detectable in case of the sensitive isolates.

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Registry number: DEENK/414/2022.PL
Subject: PhD Publication List

Candidate: Bence Balázs
Doctoral School: Doctoral School of Pharmacy
MTMT ID: 10054316

List of publications related to the dissertation

1. **Balázs, B.**, Tóth, Z., Nagy József, B., Majoros, L., Tóth, Á., Kardos, G.: Faecal Carriage of Carbapenem-Resistant Acinetobacter baumannii: comparison to Clinical Isolates from the Same Period (2017-2019).
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   Antibiotics. 10 (2), 1-12, 2021.
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List of other publications

3. Nagy, B. J., **Balázs, B.**, Benmazouz, I., Gyüre, P., Kövér, L., Kaszab, E., Bali, K., Lovas-Kiss, Á., Damjanova, I., Majoros, L., Tóth, Á., Bányai, K., Kardos, G.: Comparison of Extended-Spectrum Beta-Lactamase-Producing Escherichia coli Isolates From Rooks (Corvus frugilegus) and Contemporary Human-Derived Strains: A One Health Perspective.
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4. Forgács, L., Borman, A. M., Kovács, R. L., Balázsi, D., Tóth, Z., **Balázs, B.**, Chun-Ju, C., Kardos, G., Kovács, I., Majoros, L.: In Vivo Efficacy of Amphotericin B against Four Candida auris Clades.
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DOI: http://dx.doi.org/10.1093/cid/ciy1086
IF: 8.313

Total IF of journals (all publications): 43,753
Total IF of journals (publications related to the dissertation): 9,753

The Candidate's publication data submitted to the iDEa Tudóstér have been validated by DEENK on the basis of the Journal Citation Report (Impact Factor) database.

01 September, 2022