The role of resistance plasmids influencing gastrointestinal colonization of multidrug resistant *Klebsiella pneumoniae* in murine model

PhD thesis book

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1. Introduction

Antimicrobial resistance (AMR) is a major global health challenge, arising when microorganisms develop mechanisms to evade the effects of antimicrobial drugs, leading to ineffective treatments, prolonged illnesses, and increased mortality. Horizontal gene transfer (HGT) is a pivotal mechanism in bacterial evolution, enabling the acquisition of advantageous traits such as antibiotic resistance through the transfer of genetic material across species.

CTX-M-type β-lactamases represent a rapidly expanding class of ESBLs that confer resistance to a broad spectrum of β-lactam antibiotics, particularly cephalosporins. CTX-M-15, a member of the CTX-M-1 group, being the most widespread and clinically significant variant. Carbapenemases are β-lactamases that degrade carbapenems, presenting critical clinical challenges due to limited treatment options. The global spread of ESBL-producing *Enterobacteriaceae* has driven increased reliance on carbapenems, subsequently accelerating the emergence of carbapenemase-producing *Enterobacteriaceae* (CPE). Among these, OXA-48-like carbapenemases are particularly prevalent.

The gastrointestinal tract (GIT) serves as a critical reservoir for multidrug-resistant (MDR) *Klebsiella pneumoniae*. GIT colonization often precedes invasive infections. The immune defense of the GIT relies on defensins and secretory IgA to maintain intestinal homeostasis and prevent pathogenic colonization. Defensins, including α-defensins secreted by Paneth cells and β-defensins expressed by epithelial cells, exhibit broad-spectrum antimicrobial activity and recruit immune cells to enhance mucosal immunity. Secretory IgA prevents pathogen adherence and promotes the removal of microbes, limiting the colonization of MDR Enterobacteriaceae.

2. Objectives

Our aims were

- to investigate the colonization dynamics of a multi-resistant, high-risk clone of *K. pneumoniae*
- to detect and identify bacterial resistance plasmids which are transferred via conjugation and to gain transconjugant strains
- to detect the alteration of local antibacterial response in the gut following colonization
- to investigate the adaptive humoral immune response by determining the fecal IgA antibody content
- to investigate the non-specific antibacterial response of the gut mucosa by measuring the fecal levels of small antimicrobial peptides
- to detect the alteration of the gut microbiota by 16S metagenomic analysis

3. Methods

3.1. Bacterial strains

We acquired a multidrug-resistant, high-risk variant of *K. pneumoniae* ST15 that was isolated from a clinical sample (MDR-KP) at the National Center for Public Health and Pharmacy in Budapest, Hungary. An azide-resistant *E. coli* J53 laboratory strain (EC) served as the acceptor strain in the conjugation experiments. Transconjugant strains were generated.

3.2. Antibacterial susceptibility testing

To investigate the resistance of the original and transconjugant strains, their susceptibility was determined by broth microdilution methodology in accordance with the EUCAST (European Committee on Antimicrobial Susceptibility Testing) guidelines version 14.0 (www.eucast.org).

3.3. Conjugation assay

Conjugation assays were performed using the broth mating technique in Luria-Bertani (LB) broth (Sigma-Aldrich, USA). MDR-KP served as the donor strain, while EC as the recipient strain.

3.4. Whole genome sequencing

Bacterial strains were characterized by whole genome sequencing. Generated data were further analysed by the SEED and RAST softwares.

3.5. Animal studies

Male C57BL/6 mice aged 6 to 8 weeks were used. To facilitate the colonization of introduced bacterial strains within the GIT, the mice were pre-treated with 0.5 mg/L ampicillin (Sandoz) in their drinking water. Colonization was achieved through orogastric gavage. Fresh fecal samples were collected at specific time points—Days 5, 10, and 14 post-colonization.

3.6. Determination of the fecal germ count of mice

The quantification of fecal shedding of the colonized bacterial strains was performed by determining the bacterial germ count in freshly collected fecal samples with selective media for each strain.

3.7. Determination of total IgA and defensin levels in stool by ELISA

The levels of total IgA, murine β-defensin 3, and murine α-defensin 5 were quantified from mouse fecal samples using commercially available ELISA kits (MyBiosource, San Diego, CA, USA; product codes: MBS7725462, MBS7725303, and MBS7725358, respectively).

3.8. Microbiome composition with 16S metagenomic analysis

To assess the impact of different colonizing bacterial strains on the composition of the gastrointestinal microbiota in mice, fecal samples were collected on Day 14 post-colonization. The bacterial 16S rRNA gene's V3–V4 region was sequenced, data were further analyzed by CosmosID-HUB software version 2.0.

4. Results

4.1. Characterization of the antibiotic susceptibility of the donor, recipient and transconjugant strains used in this study

This study analyzed whole-genome sequencing data of four bacterial strains (MDR-KP, EC, EC-CTXM, and EC-OXA) to investigate the role of mobile genetic elements in antibiotic resistance. The multidrug resistant, clinical isolate of *Klebsiella pneumoniae* (MDR-KP) harbored four plasmids (IncF(I)B, IncF(II)K, ColpVC, and IncL) and multiple resistance genes, including *blaSHV-28* (chromosomal), *blaCTX-M-15* (plasmid-borne), and *blaOXA-1* and *blaOXA-162* (both plasmid-borne).

Conjugation experiments demonstrated plasmid-mediated gene transfer to the recipient *E. coli* J53 strain (EC), producing two transconjugant strains. EC-CTXM acquired an IncF(II)K plasmid carrying *blaCTX-M-15* within a class 1 mobile genetic element and fluoroquinolone resistance genes, while EC-OXA obtained an IncL plasmid with *blaOXA-162* embedded in a Tn1999.2 transposon. Microdilution assays revealed that both transconjugants exhibited elevated MIC values for specific antibiotics, reflecting moderate resistance conferred by the transferred genes.

4.2. Fecal shedding of the colonized strains

The highest bacterial load was observed in the MDR-KP group, reflecting the enhanced colonization ability associated with its extensive resistance gene repertoire. The EC-CTXM and EC-OXA groups demonstrated higher CFU counts than the EC group, suggesting that plasmid-borne resistance genes (blaCTX-M-15 and blaOXA-162) improved colonization potential. The EC group, lacking plasmids, showed the lowest bacterial load. (Figure 2).

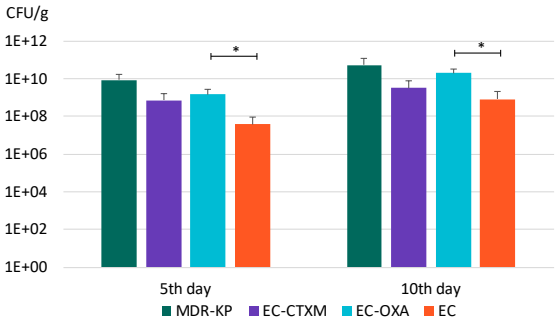

Figure 1. The gastrointestinal colonization rate characterized by germ count in the feces. Statistical differences are marked with * $p < 0.05$; ** $p < 0.01$; and *** $p < 0.001$

4.3. Fecal IgA level of the experimental groups

Baseline IgA content averaged 5.89 mg/g, with no significant change observed in the control group (6.26 mg/g) or the EC group, indicating that the plasmid-free, non-virulent strain elicited minimal humoral immune response despite high-density colonization. In contrast, strains harboring resistance plasmids significantly increased IgA levels by day 10. The MDR-KP strain doubled IgA levels to 13.67 mg/g, while the transconjugant strains, EC-CTXM and EC-OXA, induced a fourfold increase to 22.68 mg/g and 23.52

mg/g, respectively. This heightened response in transconjugants suggests that plasmid-borne resistance genes significantly enhance immunogenicity, even in strains with low innate immune-stimulating capacity. The lower IgA increase in the MDR-KP group may reflect the combined effects of its plasmids and chromosomal factors.

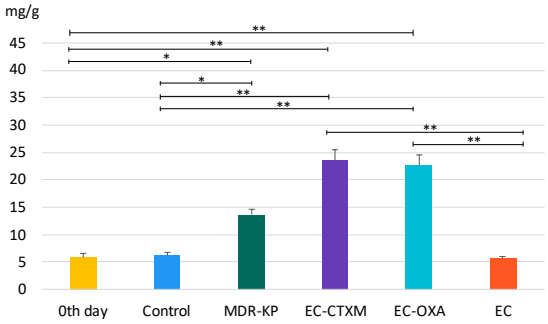

Figure 2. The IgA level in feces of mice colonized by different bacteria. Statistical differences are marked with * $p < 0.05$; ** $p < 0.01$; and *** $p < 0.001$

4.4. Fecal β-defensin 3 level of the experimental groups

Baseline mBD3 levels were low (196.63 pg/g) and remained unchanged in the control group after 10 days. Colonization with resistant strains significantly increased mBD3 levels, with the highest induction observed in the EC-CTXM group (1825 pg/g), followed by the MDR-KP group (1640 pg/g). These findings suggest that the plasmid carrying the blaCTX-M-15 gene strongly enhances mBD3 production. In contrast, the EC-OXA group exhibited mBD3 levels comparable to baseline (309 pg/g), indicating a minimal effect of the plasmid carrying blaOXA-162. The plasmid-free EC strain moderately increased mBD3 levels (898 pg/g).

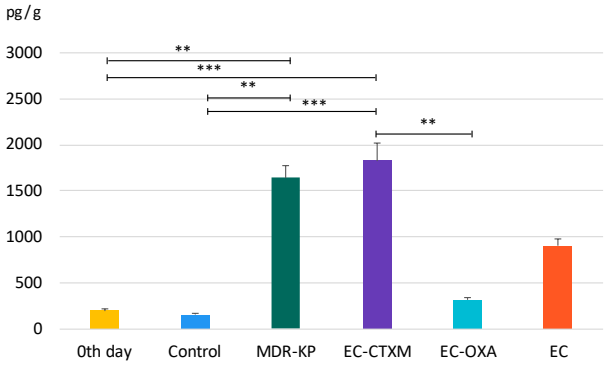

Figure 3. The β-defensin-3 level in feces of mice colonized by different bacteria. Statistical differences are marked with * $p < 0.05$; ** $p < 0.01$; and *** $p < 0.001$

4.5. Fecal α-defensin 5 level of the experimental groups

The antibacterial response to gastrointestinal colonization was assessed through murine α-defensin 5 (mAD5) levels in fecal samples. Baseline mAD5 levels averaged 12.6 pg/g, with no significant change observed in the non-colonized control group by day 10. In contrast, the EC group, colonized with the plasmid-free E. coli strain, exhibited the highest mAD5 induction, averaging 172 pg/g, followed by the MDR-KP group at 125 pg/g. These increases were statistically significant. Colonization with transconjugant strains carrying resistance plasmids resulted in modest mAD5 increases that were not statistically significant, with levels averaging 104 pg/g in the EC-CTXM group and 50 pg/g in the EC-OXA group.

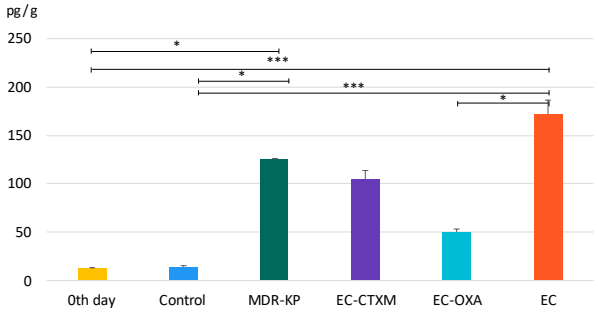

Figure 5. The alfa-defensin-5 in feces of mice colonized by different bacteria. Statistical differences are marked with * $p < 0.05$; ** $p < 0.01$; and *** $p < 0.001$

4.6. Alterations in the fecal microbiota composition in the experimental groups

Alpha diversity indices (CHAO1 and Simpson) calculated from 16S metagenomic data revealed no statistically significant differences between experimental groups, though diversity was uniformly low due to antibiotic administration, favoring successful colonization. Analysis of microbiota composition showed distinct patterns at taxonomic levels, with notable differences among experimental groups. *Proteobacteria* and *Bacteroidota* were the dominant phyla in groups colonized by resistant strains (MDR-KP, EC-CTXM, and EC-OXA), while *Firmicutes* predominated in the EC group.

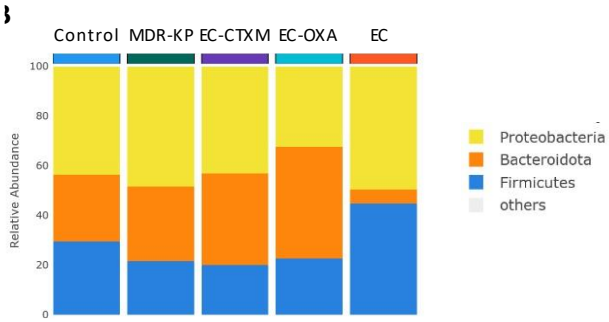

Figure 6. Average values of relative abundances at the phylum level were calculated for samples from the same treatment groups.

At the family level, *Muribaculaceae* was significantly enriched ($p < 0.05$) in the EC-OXA group, suggesting a correlation between *Muribaculaceae* abundance and the presence of the OXA-162 plasmid. Conversely, *Lachnospiraceae*, prevalent in the EC group, demonstrated a potential protective role against colonization by resistant strains and the dissemination of CTX-M-15-containing plasmids.

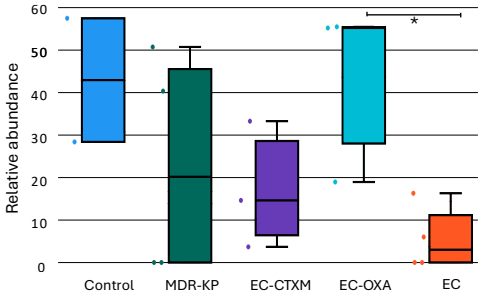

Figure 7. The relative abundance of *Muribaculaceae* family in each group. Statistical difference is marked with * $p < 0.05$

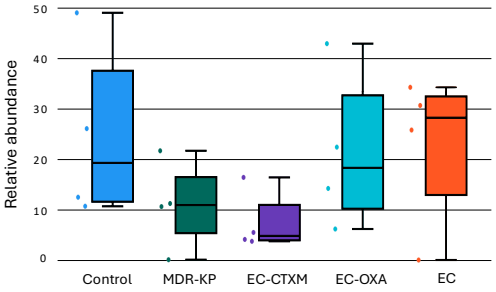

Figure 8. The relative abundance of *Lachnospiraceae* family in each group

5. Conclusions

Our study utilized a murine model to investigate the multifactorial nature of intestinal colonization by the MDR strains. CTX-M-15- and OXA-162-producing *Klebsiella pneumoniae* ST15 high-risk clone served as good model for this. The findings indicate that colonization within the gastrointestinal tract is not solely attributable to the high-risk MDR clone itself but is also significantly influenced by the presence of resistance plasmids, specifically the IncFII(K) and IncL plasmids. These plasmids play a critical role in modulating colonization efficiency and persistence.

Additionally, several host and microbial factors contribute to the colonization dynamics of this MDR strain. The levels of IgA and antimicrobial peptides, such as mBD3 and mAD5, were found to influence colonization, highlighting the interplay between host immune responses and pathogen persistence. Furthermore, the composition of the intestinal microbiota emerged as another key determinant, underscoring the complex interactions between microbial community structure and the ability of MDR K. pneumoniae to establish and maintain colonization. These findings collectively emphasize the intricate

and multifaceted mechanisms underlying the colonization of high-risk MDR clones in the GIT.

This study is, to our knowledge, the first to comprehensively evaluate gut microbiome dynamics alongside IgA production and defensin levels during colonization by an MDR K. pneumoniae high-risk clone. Our results underscore the critical roles of IgA and β-defensin 3 in mediating colonization and plasmid dissemination. These findings highlight that plasmids carrying resistance genes contribute significantly to the spread of high-risk clones worldwide, with implications extending beyond antimicrobial resistance.

As new scientific findings, our studies demonstrated resistance plasmids, IncFII(K) encoding blaCTX-M-15 gene and IncL plasmids encoding blaOXA-162 can alter not only the colonization dynamics of host bacterial strain after conjugation, but also cause alteration in the induction of antimicrobial responses. Both plasmids were potent activator of the specific immunity. However, the IncFII(K) plasmid induces mBD3 and in less extent mAD5 production likely to the host MDR-KP strain, the IncL plasmid had a strong inhibitory effect on both, mBD3 and mAD5 production.

Further research is needed to elucidate the direct effects of defensins on resistance plasmids or their indirect effects through microbiota modulation. Future studies should also investigate other MDR high-risk clones of K. pneumoniae and E. coli harboring diverse resistance plasmids and genes in colonization models to expand our understanding of these mechanisms.

6. Bibliography of the candidate's publications

6.1. Publications related to the PhD thesis

1. Stercz B, Domokos J, Dunai ZA, Makra N, Juhasz J, Ostorhazi E, Kocsis B, Szabo D. The roles of a multidrug-resistant *Klebsiella pneumoniae* high-risk clone and its resistance plasmids on the gastrointestinal colonization and host-defense effectors in the gut. Antibiotics (Basel). 2024;13:698. **Impact factor (expected):4.3**
2. Stercz B*, Farkas FB*, Tóth Á, Gajdács M, Domokos J, Horváth V, Ostorházi E, Makra N, Kocsis B, Juhász J, Ligeti B, Pongor S, Szabó D. The influence of antibiotics on transitory resistome during gut colonization with CTX-M-15 and OXA-162 producing *Klebsiella pneumoniae* ST15. Sci Rep. 2021;11:6335. **Impact factor: 4.997** *Authors contributed equally.

6.2. Publications not related to the PhD thesis

1. Kelemen J, Sztermen M, Dakos EK, Budai J, Katona J, Szekeressy Zs, Sipos L, Papp Z, Stercz B, Dunai ZsA, Kocsis B, Juhasz J, Michelisz F, Daku Zs, Domokos J, Szabó D. Complex Infection-Control Measures with Disinfectant Switch Help the Successful Early Control of Carbapenem-Resistant Acinetobacter baumannii Outbreak in Intensive Care Unit. Antibiotics (Basel). 2024;13:869. **Impact factor (expected): 4.3**
2. Monyók Á, Mansour B, Vadnay I, Makra N, Dunai ZsA, Nemes-Nikodém É, Stercz B, Szabó D, Ostorházi E. Change in Tissue Microbiome and Related Human Beta Defensin Levels Induced by Antibiotic Use in Bladder Carcinoma. Int J Mol Sci. 2024;25:4562. **Impact factor (expected)**: **4.9**

3. Szabo D, Ostorhazi E, Stercz B, Makra N, Penzes K, Kristof K, Antal I, Rethelyi JM, Zsigmond RI, Birtalan E, Merkely B, Tamas L. Specific nasopharyngeal Corynebacterium strains serve as gatekeepers against SARS-CoV-2 infection. Geroscience. 2023;45:2927-2938. **Impact factor: 5,3**
4. Mansour B, Monyók Á, Gajdács M, Stercz B, Makra N, Pénzes K, Vadnay I, Szabó D, Ostorházi E. Bladder Tissue Microbiome Composition in Patients of Bladder Cancer or Benign Prostatic Hyperplasia and Related Human Beta Defensin Levels. Biomedicines. 2022;10:1758. **Impact factor: 4,7**
5. Farkas E, Tarr R, Gerecsei T, Saftics A, Kovács KD, Stercz B, Domokos J, Peter B, Kurunczi S, Szekacs I, Bonyár A, Bányai A, Fürjes P, Ruszkai-Szaniszló Sz, Varga M, Szabó B, Ostorházi E, Dóra Sz, Horváth R. Development and In-Depth Characterization of Bacteria Repellent and Bacteria Adhesive Antibody-Coated Surfaces Using Optical Waveguide Biosensing. Biosensors (Basel). 2022;12:56. **Impact factor: 5,4**
6. uksakiet K, Stercz B, Tóth G, Jaikumpun P, Gróf I, Tengölics R, Lohinai ZsM, Horváth P, Deli MA, Steward MC, Dobay O, Zsembery A. Bicarbonate Evokes Reciprocal Changes in Intracellular Cyclic di-GMP and Cyclic AMP Levels in Pseudomonas aeruginosa. Biology (Basel). 2021;10:519. **Impact factor: 5,168**

7. Juhász J, Ligeti B, Gajdács M, Makra N, Ostorházi E, Farkas FB, Stercz B, Tóth Á, Domokos J, Pongor S, Szabó D. Colonization Dynamics of Multidrug-Resistant 5,168Klebsiella pneumoniae Are Dictated by Microbiota-Cluster Group Behavior over Individual Antibiotic Susceptibility: A Metataxonomic Analysis. Antibiotics (Basel). 2021;10:268. **Impact factor: 5,222**
8. Tarcsai KR, Kapran I, Hídvégi M, Stercz B, Nagy K, Ongrádi J. Fermentált búzacsíra-kivonat (Avemar) antivirális hatásának vizsgálata macska AIDS modellben. Hungar Vet J. 2020;142:731-741. **Impact factor: 0,220**
9. Jaikumpun P, Ruksakiet K, Stercz B, Pállinger É, Steward M, Lohinai Zs, Dobay O, Zsembery Á. Antibacterial Effects of Bicarbonate in Media Modified to Mimic Cystic Fibrosis Sputum. Int J Mol Sci. 2020;21:8614. **Impact factor: 5,924**
10. Ongrádi J, Chatlynne LG, Tarcsai KR, Stercz B, Lakatos B, Pring-Åkerblom P, Gooss D, Nagy K, Ablashi DV. Adenovirus Isolated From a Cat Is Related to Human Adenovirus 1. Front Microbiol. 2019;10:1430. **Impact factor: 4,236**
11. Dobay O, Laub K, Stercz B, Kéri A, Balázs B, Tóthpál A, Kardos Sz, Jaikumpun P, Ruksakiet K, Quinton PM, Zsembery Á. Bicarbonate Inhibits Bacterial Growth and Biofilm Formation of Prevalent Cystic Fibrosis Pathogens. Front Microbiol. 2018;9:2245. **Impact factor: 4,259**
12. Ongrádi J, Ablashi DV, Yoshikawa T, Stercz B, Ogata M. Roseolovirus-associated encephalitis in immunocompetent and immunocompromised individuals. J Neurovirol. 2017;23:1-19. **Impact factor: 3,228**

13. Kovesdi V, Stercz B, Ongradi J. Kurthia gibsonii as a sexually transmitted zoonosis: From a neglected condition during World War II to a recent warning for sexually transmitted disease units. Indian J Sex Transm Dis AIDS. 2016;37:68-71.
14. Mathesz A, Valkai S, Ujvarosy A, Aekbote B, Sipos O, Stercz B, Kocsis B, Szabo D, Der A. Integrated optical biosensor for rapid detection of bacteria. Optofluid Microfluid Nanofluid. 2015;2:15-21.
15. Mathesz A, Valkai S, Sipos O, Stercz B, Kocsis B, Szabo D, Der A. Integrated optical sensor for rapid analysis of biological samples. Orv Hetil. 2015;156:2116-2119. **Impact factor: 0,291**
16. Ongrádi J, Stercz B, Kövesdi V, Nagy K, Chatlynne L. Isolation of kurthia gibsonii from non-gonorrheal urethritis: Implications for the pathomechanism upon surveying the literature. Acta Microbiol Immunol Hung. 2014;61:79-87. **Impact factor: 0,778**
17. Stercz B, Perlstadt H, Nagy K, Ongradi J. Immunochemistry of adenoviruses: Limitations and new horizons of gene therapy. Acta Microbiol Immunol Hung. 2013;60:447-459. **Impact factor: 0,78**
18. Stercz B, Nagy K, Ongrádi J. Adenovirus infections in immunocompromised patients. Orv Hetil. 2012;153:1896-1904.
19. Ongrádi J, Stercz B, Kövesdi V, Vértes L. Immunosenescence and vaccination of elderly: II. New strategies to restore age-related immune impairment. Acta Microbiol Immunol Hung. 2009;56:301-312.
20. Ongrádi J, Stercz B, Kövesdi V, Vértes L. Immunosenescence and vaccination of the elderly: I. Age-related immune impairment. Acta Microbiol Immunol Hung. 2009;56:199-210.