UNIVERSITY OF PÉCS

Doctoral School of Biology and Sportbiology

Occurrence of antimicrobial pharmaceuticals and characterization of β-lactamases in Gram-negative pathogens from wastewater

PhD Thesis

Mutuku Christopher Sikuku

Supervisor:

Zoltán Gazdag, PhD

Pécs, 2023

INTRODUCTION

The antibiotics frequently administered to animals and humans persist in the environment through accumulation, transformation, and deposition. They are regarded as emergent micropollutants of concern whose long-term exposure can produce significant eco-toxicological effects even at minute concentrations. Their consumption patterns influence the extent of their environmental contamination where an increase in consumption, especially during the cold season when the frequency of infections is higher, elevates their occurrence in environmental systems (Wang et al. 2020). The occurrence of these antimicrobial pharmaceuticals, their metabolites and diverse bacteria in aquatic media has the potential to select for resistant bacteria (Kumar et al. 2019) . According to Directive 91/271/EEC, no specific restrictions are imposed for hospital sewage effluents that can be discharged into urban wastewater sewerage networks without previous treatment. This implies that many countries across Europe release untreated hospital effluents carrying antimicrobials into the urban wastewater stream for co-treatment with the municipal wastewater at the urban wastewater treatment plant prior to discharge into the environment. The antimicrobials in the wastewater systems may exert a continuous selective pressure leading to the evolution of antibiotic resistant bacteria through adaptation, vertical and horizontal transfer of resistance determinants. Some of these bacteria such as, *Escherichia coli*, *Klebsiella* species and *Enterobacter* form part of bowel microbiota and are human opportunistic pathogens, while others such as *Pseudomonas aeruginosa* is an environmental organism that is associated with hospital-acquired infections. These bacteria have developed resistance to multiple antimicrobials, including enzymatic degradation of the broad-spectrum β-lactams that are commonly used in clinical practice. Among the most common degrading enzymes are acquired extended-spectrum serine β-lactamases (ESBLs), which inactivate the oxyimino cephalosporins (ceftriaxone, ceftazidime, cefepime) and aminopenicillins (amoxicillin and ampicillin), as well as metallo-β-lactamases (MBLs), which degrade most of the β-lactams, including the lifesaving carbapenems.

AIMS

The main aim was to isolate and characterize the extended-spectrum β-lactamase (ESBL) and carbapenemase-producing Gram-negative opportunistic pathogens belonging to the order Enterobacterales and *Pseudomonas aeruginosa* from hospital effluents, urban wastewater and wastewater treatment plant (influent, activated sludge reactor and digested sludge) as well as to

determine the presence and concentration of selected antibiotic compounds in the same wastewater samples.

In order to achieve this aim, the study was guided by the following objectives.

1. Determine the presence and concentration of various antibiotic compounds in hospital effluents, wastewater treatment plant and urban wastewater.
2. Isolate ESBL and carbapenenamase-producing members of Enterobacterales (*Escherichia coli*, *Klebsiella* spp, *Enterobacter,* and *Citrobacter* species) and *Pseudomonas aeruginosa* from the wastewater.
3. Investigate the antimicrobial resistance profiles and determine the presence of ESBLs and metallo-β-lactamases (MBLs) among the broad-spectrum β-lactam resistant isolates phenotypically.
4. Molecular genotyping of common ESBLs (CTX-M, TEM, and SHV*)* and carbapenamases (IMP, VIM, NDM, SPM, KPC, OXA-48) among the broad-spectrum β-lactams ($3^{rd.}$ generation cephalosporins and carbapenems) resistant isolates of Enterobacterales and *P. aeruginosa.*
5. Comparative sequence analysis of the plasmid DNA of selected multiple antibiotic-resistant isolates of *Escherichia coli, Klebsiella* spp. and *C. freundii* to identify the gene variants of the ESBL and other plasmid encoded resistance mechanisms.
6. Sequence and identify the bla_{VIM} gene variant prevalent in carbapenems-resistant isolates of *P. aeruginosa* and possible mutations among the VIM sequences of selected isolates.

MATERIALS AND METHODS

Study sites and sampling strategy

This study was carried out in the city of Pécs, in southwest Hungary. Wastewater samples were drawn from four hospital wastewater discharge points, H1 (387 beds), H2 (106 beds), H3 (127 beds), and H4 (348 beds), a discharge point of a nursing home for the elderly (NH, 490 beds), urban wastewater sewer lines (UWW1-2), and a WWTP **(Figure 1**). A 30 mL sample was collected every 15 minutes by lowering a flask into the wastewater flow over a period of 4 h and the aliquots pooled to constitute 480 mL composite sample in sterile 500 mL glass bottles. Samples were

transported on ice to the laboratory and stored at 4^0C before assaying within 6 hours. The WWTP processes wastewater from the central business district, health care facilities, domestic wastewater, and some storm runoff and serves a population equivalent to slightly over 200000 inhabitants. The study was conducted during 2019-2020 with each sampling spot visited thrice.

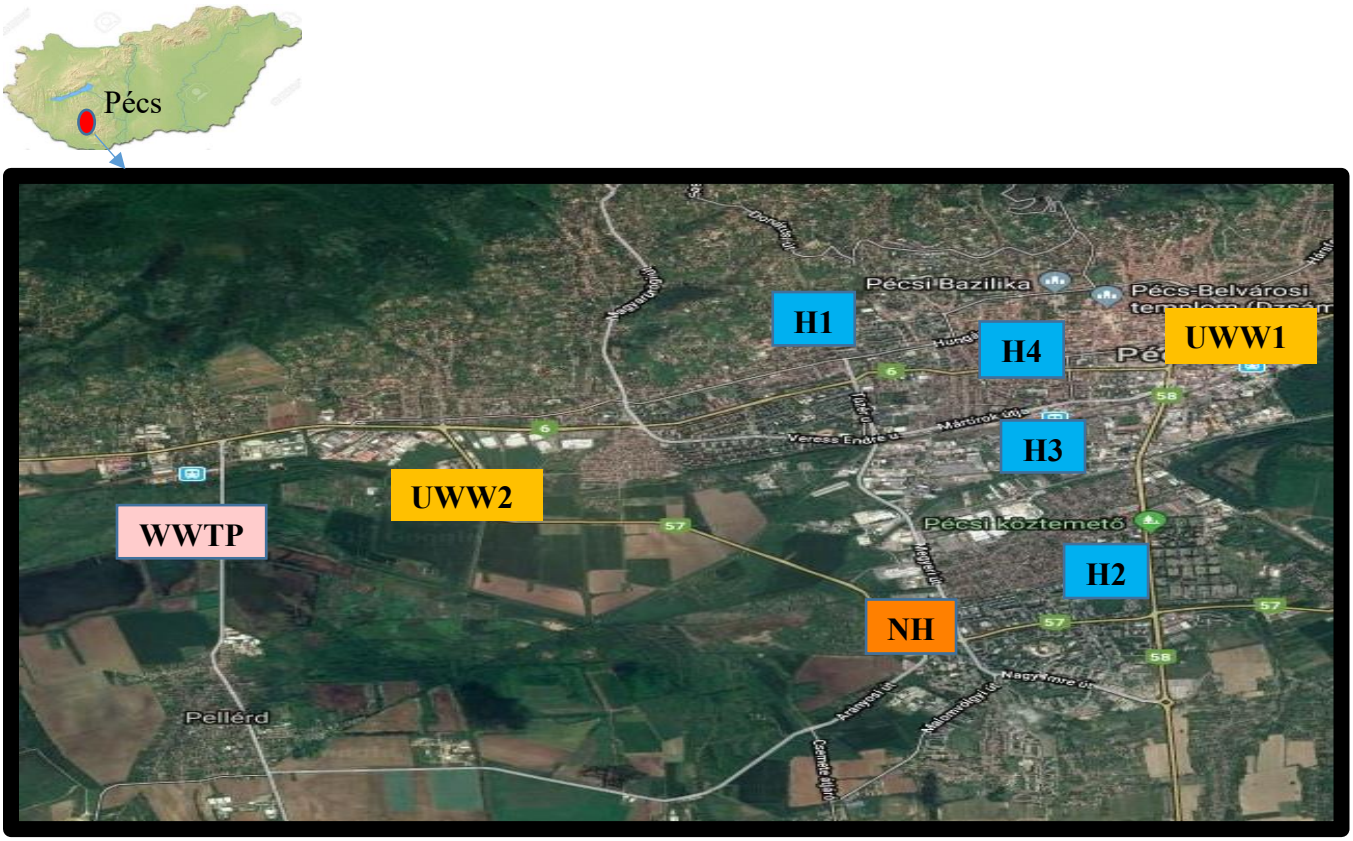


Figure 1. A section of the map of the city of Pécs showing the sampling locations. **Study sites:** H1-H4: hospital effluent, NH: nursing home, UWW: urban wastewater, WWTP: wastewater treatment plant.

Analysis of antibiotic molecules was done by LC-MS. Isolation of ESBL and carbapenemase-producing Enterobacterales was done on Eosin methylene blue agar supplemented with antibiotics while isolation of *P. aeruginosa* was done on antibiotics supplemented Cetrimide nalidixic agar Strain identification; Matrix assisted laser desorption ionization-time of flight mass spectrometer (MALDI-TOF/MS). Antibiotic resistance profiles was perfomed by Standardized disk diffusion method (EUCAST, 2018). For Enterobacterales, 10 antibiotics impregnated discs; ceftriaxone (CRO, 30 µg), ceftazidime (CAZ, 10 µg), cefotaxime (CTX, 30 µg), cefpodoxime (CPD, 10 µg),

cefoxitin (FOX, 30 µg), imipenem (IMP, 10 µg) and meropenem (MEM, 10 µg), gentamicin (GN, 10 µg), ciprofloxacin (CIP, 5 µg) and sulfamethoxazole/trimethoprim (SXT, 1.25/23.75 µg) (Oxoid, Wesel, Germany) were used, whereas for *P. aeruginosa*, 5 antibiotics impregnated discs, ceftazidime (CAZ. 10 µg), imipenem (IMP, 10 µg), meropenem (MEM, 10 µg), gentamicin (GN, 10 µg) and ciprofloxacin (CP, 5 µg) (Oxoid, Wesel, Germany) were used. Phenotypic detection of ESBL and carbapenemases was done by combined disks test (CDT) - cefotaxime/clavulanic acid (CTC, 30/10 µg, CTC 40), cefpodoxime/clavulanic acid (10/10 µg, CD 01), ceftazidime/clavulanic acid (CZC 20), meropenem/ethylenediamine tetraacetic acid (MEM/EDTA 10/292) and imipenem/ethylenediamine tetraacetic acid (IMP/EDTA 10/292) beta-lactamase inhibitors.
Molecular typing of ESBL and carbapenemases was done by plasmid DNA isolation using the alkaline lysis method followed by PCR amplification of template DNA for detection of enzyme families. Sequencing of Enterobacterales plasmids was performed by NGS whereas *P. aeruginosa* PCR products were sequenced by Sanger technique.

NEW SCIENTIFIC RESULTS

We established the presence of antimicrobial pharmaceuticals namely, macrolides (azithromycin, clarithromycin), fluoroquinolones (ciprofloxacin, norfloxacin and ofloxacin), sulfonamides (sulphadiazine, sulfamethoxazole) and trimethoprim at significantly varied concentrations in wastewater from the various sources, with trimethoprim and fluoroquinolones being the dominant compounds, mainly from the hospital drainage systems.

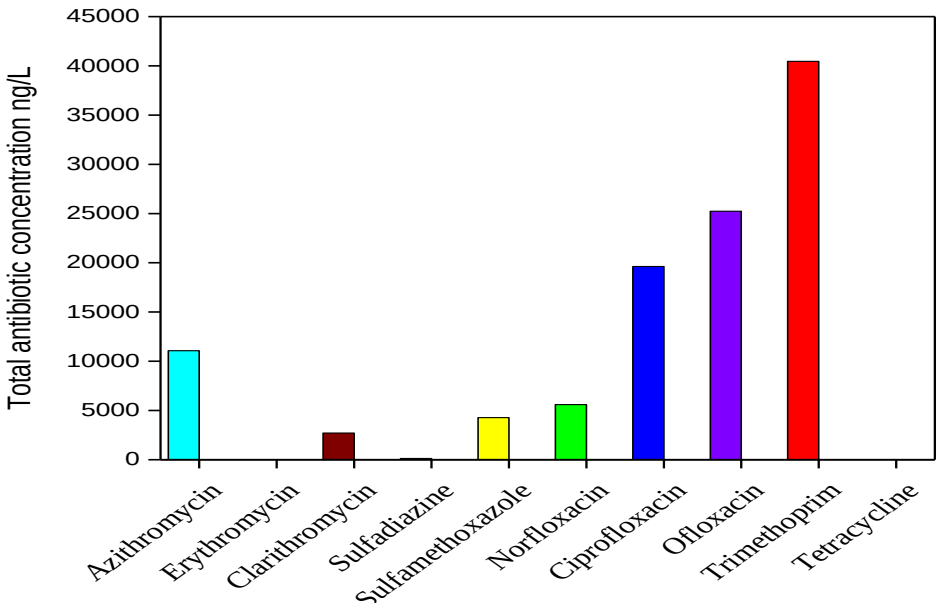


Figure 2. The total load for each antibiotic showing the elevated content trimethoprim and fluoroquinolones (ciprofloxacin and ofloxacin).

We determined a 2 fold higher cfu count of the Gram-negative bacteria resistant to broad spectrum cephalosporin (ceftriaxone) in hospital and nursing home effluents compared to that of wastewater treatment plant and the urban wastewater, while the cfu count of carbapenem (imipenem) resistant Gram-negative bacteria was 8 fold higher in the hospital and nursing home effluents than that of the wastewater treatment plant and the urban wastewater. However, in comparison, resistance to ceftriaxone was higher than that of imipenem in samples across all the locations.

We established a widespread multidrug resistance among enteric bacteria and *P. aeruginosa* in wastewater, especially from the hospital and sanitary sources and the resistance rate was enriched at the biological reactor and further during mesophilic digestion of the sewage sludge.

We established a positive correlation between the concentration of fluoroquinolone residues (ciprofloxacin and ofloxacin) in wastewater and resistance to ciprofloxacin in *P. aeruginosa* isolates which points to possible selection of resistance created by antimicrobials in wastewater systems.

We established a similar resistance rate of β-lactam antibiotics among Enterobacterales from environmental sources (although these drugs are considered the backbone of antibiotic therapy

making them the most widely used antibiotics in clinical practice) as other classes of antimicrobials, namely fluoroquinolones, aminoglycosides and sulfonamides, which can be linked to simultaneous transmission of plasmid encoded genes. However, carbapenems (imipenem and meropenem) remain the most potent antimicrobials against enteric bacteria with carbapenem resistance in *P. aeruginosa* observed to be on the upward trend.

We determined the presence of multiresistant members of Enterobacterales harboring plasmid-mediated extended-spectrum β-lactamases mainly of TEM, CTX-M and SHV types in wastewater. The broad-spectrum β-lactamase producers occurred at a higher frequency in hospital effluents than in wastewater from other sources. Their presence can be attributed either to the development of resistance in the source population, and/or its build-up in the environment through selection pressure as well as resistance dissemination of the phenotype via horizontal gene transfer.

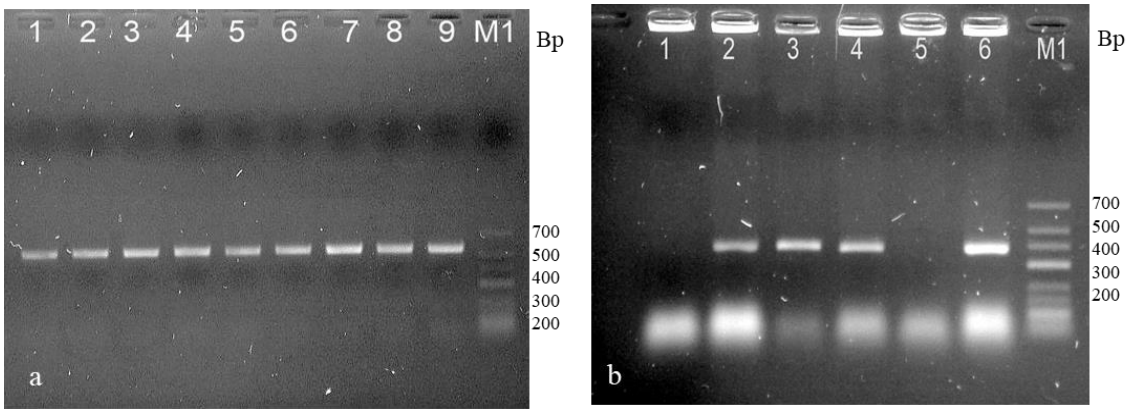
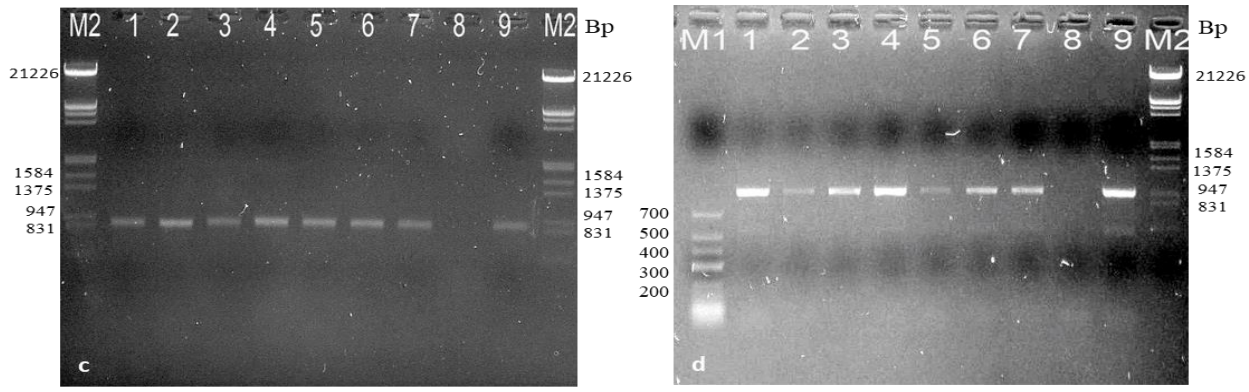


Figure 3 a-d. Gel images of *E. coli* and *Klebsiella* spp. showing the β-lactamase genes, $bla_{CTX\text{-}M}$, bla_{VIM}, bla_{SHV}, and bla_{TEM}. (a) Lanes 1- 4, *E. coli*, Lanes 5 and 6 *K. pneumoniae*, Lanes 7 and 8 *K. oxytoca*, all strains carrying plasmid borne $bla_{CTX\text{-}M}$ gene with product size 544 bp. Lane 9, positive control strain *K. pneumoniae* 722, and (b). Lanes 1 and 5 *K. oxytoca* strains with missing bla_{VIM} gene, Lanes 2, 3, and 4, *K. oxytoca* strains carrying chromosomally borne bla_{VIM} gene demonstrated by colony PCR, product size 390 bp, Lane 6, positive control strain *K. pneumoniae* 1745, M1, Low range ladder molecular weight marker (700bp).



(c) Lanes 1 - 7, *K. pneumoniae* spp carrying plasmid borne bla_{SHV} gene (*Nhe*I digested), product size 865 bp, lane 8 distilled water, lane 9, positive control strain, *K. pneumoniae* MGH 78578, (d). Lanes 1- 4, *E. coli*, lanes 5 -7, *Klebsiella* spp carrying plasmid borne bla_{TEM} gene, product size 963 bp. lane 8, *Klebsiella* spp lacking bla_{TEM} gene, Lane 9, positive control strain *K. pneumoniae* 722, M2, Lambda phage DNA- *Eco*RI/*Hind*III molecular weight marker (21226 bp), M1, Low range ladder molecular weight marker (700 bp).

We established that, metallo-β-lactamase VIM-4 was the main enzyme responsible for the inactivation of the carbapenems in *P. aeruginosa* from the wastewater. The reporting of bla_{VIM-4} in isolates of *P. aeruginosa* from wastewater and the same gene variant previously reported from clinical isolates from one of the healthcare facilities in the city points to a direct connection between resistance in clinical isolates and their environmental counterparts. On the other hand, detection of bla_{NDM} in one of the *P. aeruginosa* isolates indicates the possibility of the spread of this gene in the environment.

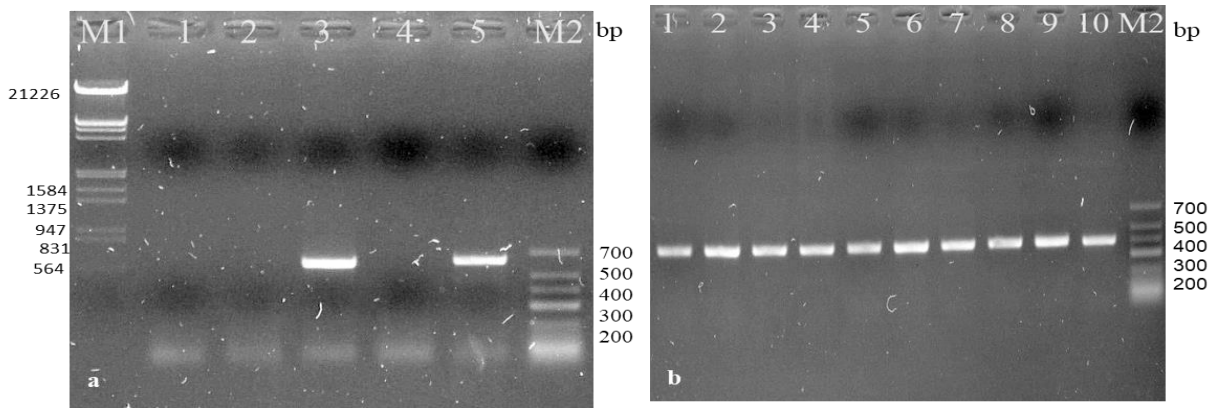


Figure 4. Genomic DNA of *P. aeruginosa* isolates, carrying bla_{NDM} gene and bla_{VIM} gene. (a) Lane 3, Genomic DNA of *P. aeruginosa* carrying bla_{NDM} gene, product size 621 bp, lanes 1, 2, and 4,

P. aeruginosa lacking the gene, lane 5, *Klebsiella pneumoniae* 131946 positive control strain, (b) Lanes 1-9, Genomic DNA of *P. aeruginosa* carrying bla_{VIM} gene, product size 390 bp, lane 10, *Klebsiella pneumoniae* 1745 positive control strain, M1, Lambda phage DNA- *EcoRI/HindIII* (21226 bp), M2, Low range ladder (700 bp)- molecular weight markers.

We established that *E. coli* isolates from wastewater harbor ESBL of mainly $bla_{\text{CTX-M-27}}$ whereas *Klebsiella* spp, harbor mainly $bla_{\text{CTX-M-15}}$, $bla_{\text{CTX-M-30}}$, and $bla_{\text{SHV-12}}$ variants.

We observe that continuous exposure of the environment to antimicrobial residues may lead to the emergence of resistant strains, and soil amendment with sewage sludge carrying resistant determinants may result in the buildup of environmental reservoirs of ESBL and carbapenemase producing bacteria emanating from the pool of resistance genes circulating in the broad microbial population entering the ecosystem.

Overall, this study demonstrates the presence of antimicrobial pharmaceuticals in wastewater and abundance of multidrug resistant enteric bacteria and *P. aeruginosa* in the same environment. This wastewater has direct connection to human sources, especially the clinical wastewater where the resistance prevalence is higher. The hospital effluents discharged into municipal wastewater network without pretreatment present an ideal vehicle for the carriage of bacteria of both human and environmental origin harboring antibiotic resistance genes. A proportion of these antibiotic-resistant Gram-negative bacteria is enriched at the biological reactor as wastewater treatment progresses. This potentiates a public health threat posed by the disposal of non-decontaminated digested sewage sludge into the environment for soil amendment because it may expose antibiotic resistant bacteria and their resistance genes to human and animal hosts.

Therefore, it is desirable to disinfect such hospital wastewater and subsequently decontaminate the digested sewage sludge prior to disposal. These would be feasible management strategies to minimize the risk of environmental contamination with antibiotic resistant bacteria and to slow the progression of antimicrobial resistance in the environment. The findings of this study demonstrate that monitoring wastewater of anthropogenic origin presents a promising strategy for generating valuable data which can be correlated to the prevalence of clinically important resistant bacteria from the source population and may provide a cheaper alternative in regions (especially low-income countries) facing challenges that limit clinical surveillance.

Publications

Publications related to the thesis

Mutuku, C.; Melegh, S.; Kovacs, K.; Urban, P.; Virág, E.; Heninger, R.; Herczeg, R.; Sonnevend, Á.; Gyenesei, A.; Fekete, C,; Gazdag, Z. Characterization of β-Lactamases and Multidrug Resistance Mechanisms in Enterobacterales from Hospital Effluents and Wastewater Treatment Plant. *Antibiotics* **2022**, 11, 776. https://doi.org/10.3390/ *Antibiotics*11060776 (Q1; IF_{2021}: 5.222) Number of independent citations: 1.

Mutuku, C. Gazdag, Z. Melegh, S. Occurrence of antibiotics and bacterial resistance genes in wastewater: resistance mechanisms and antimicrobial resistance control approaches. *World Journal of Microbiology and Biotechnology* (2022) 38:152 https://doi.org/10.1007/s11274-022-03334-0 (Q2; IF_{2021}: 4.253) Number of independent citations: 5.

Mutuku, C. Kutasy B, Urban P, Melegh S, Herczeg R, Gazdag Z and Virág E. Plasmid sequence dataset of multidrug-resistant Enterobacterales isolated from hospital effluents and wastewater treatment plant. *Applied Microbiology, Data in Brief* (2022) https://doi.org/10.1016/j.dib.2022.108736 (Q4; IF_{2021}:1,38).

Mutuku, **C**. Beta-lactam resistance in Enterobacterales. *Gastroenterology: Medicine and Research* (2022) 7: 1. Paper: 000653, 4p https://***DOI:****10.31031/GMR.2022.07.000653* (IF_{2021}: 0.929).

Manuscripts in preparation related to the thesis

Mutuku C, Melegh S, Maász A, Kutasy B, Maász G, Kovács K, Kulágin D, Kálmán N, Virág E, Sonnevend Á, Hadzsiev K, Galambos I, Fekete C, Gazdag Z · Municipal wastewater containing clinical effluents harboring antimicrobial pharmaceuticals disseminates carbapenemase-producing *Pseudomonas aeruginosa***.**

Mutuku C, Kovacs K, Melegh S, Meszéna R, Sonnevend A, Fekete C, Gazdag Z. Untreated hospital wastewater discharge disseminates vancomycin resistant enterococci (VRE) into the Environment.

Conference abstracts related to the thesis

Mutuku C., Kovacs K., Melegh S., Heninger R., Kulagin D., Urban Peter., Mestyan Gyula., Sonnevend A., Fekete C., Gazdag Z. (2020). Multiple Antimicrobial Resistance and Molecular Characterization of β-lactamases in enteric bacteria from Hospital Effluents and Wastewater Treatment Plant. Medical Conference for PhD Students and Experts of Clinical Sciences, MEDPECS2020, P.70

Mutuku C., Kovács K., Melegh S., Biro A., Boros V., Heninger R., Urban P., Sonnevend Á., Fekete Csaba., Gazdag Z. (2020). Detection of multidrug resistance and prevalence of extended spectrum β-lactamase (ESBL) producing Enterobacteriaceae from hospital effluents and wastewater treatment plants. XVIII. Szentagothai multidiszciplinaris konferencia es hallgatoi versant JSMC-2020, P.188

Mutuku C., Kovács K., Melegh S., Urban P., Sonnevend Á., Fekete C., Gazdag Z. (2021). Antimicrobial resistance and prevalence of extended spectrum and metallo-β-lactamases in *Pseudomonas aeruginosa* from clinical and municipal wastewater. 18th and 19th János SZENTÁGOTHAI Multidisciplinary Conference and Student Competition, JSMC-2021, P. 26

Mutuku C., Kovács K., Melegh S., Urban P., Sonnevend Á., Fekete C., Gazdag Z. (2021). Detection of Vancomycin Resistant Enterococci (VRE) from Hospital Effluents. Online Medical Conference for PhD Students and Experts of Clinical Sciences, MEDPECS2021, P. 107

Other Publications

Mutuku C, Okemo P and Boga H. 2014. Metal pollutants distribution within the Lake Victoria basin and their influence on native and transient microbial flora. *ARPN Journal of Agricultural and Biological Sciences, Jabs* 9(4)*:*127-133

Mutuku C, Okemo P and Boga H. 2014. Association of Metal Tolerance with Multidrug Resistance among Environmental Bacteria from Wetlands of Lake Victoria Basin. *Agriculture and Biology Journal of North America (ABJNA)* 5 (1): 24 – 32. doi:10.5251/abjna.2014.5.1.24.32

Onyango BO, Mbaluto CA, **Mutuku CS,** Otieno DO. 2016. Molecular characterization of wood ear mushrooms (*Auricularia sp*.) from Kakamega Forest in Western Kenya. *Current Research in Environmental & Applied Mycology, CREAM*, 6(1):51-60.

Impact factor of publications related to the thesis: 11.784

Impact factor of all publications: 12.017

All citations: 50

Independent citation: 5