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VIM-1-producing *Enterobacter asburiae* with mobile colistin resistance genes from wastewaters

Iva Sukkar[1,2], Adam Valcek[1,3,4] and Monika Dolejska[1,2,5,6*]

Abstract

Background Wastewaters are considered as important players in the spread of antimicrobial resistance, thus affecting the health of humans and animals. Here, we focused on wastewaters as a possible source of carbapenemase-producing Enterobacterales for the environment.

Methods A total of 180 presumptive coliforms from hospital and municipal wastewaters, and a river in the Czech Republic were obtained by selective cultivation on meropenem-supplemented media and tested for presence of carbapenemase-encoding genes by PCR. Strains carrying genes of interest were characterized by testing antimicrobial susceptibility, carbapenemase production and combination of short- and long- read whole-genome sequencing. The phylogenetic tree including publicly available genomes of *Enterobacter asburiae* was conducted using Prokka, Roary and RAxML.

Results Three VIM-producing *Enterobacter asburiae* isolates, members of the *Enterobacter cloacae* complex, were detected from hospital and municipal wastewaters, and the river. The bla_{VIM-1} gene was located within a class 1 integron that was carried by different F-type plasmids and one non-typeable plasmid. Furthermore, one of the isolates carried plasmid-borne colistin-resistance gene *mcr-10*, while in another isolate chromosomally located *mcr-9* without colistin resistance phenotype was detected. In addition, the analysis of 685 publicly available *E. asburiae* genomes showed they frequently carry carbapenemase genes, highlighting the importance of this species in the emergence of resistance to last-line antibiotics.

Conclusion Our findings pointed out the important contribution of hospital and community wastewaters in transmission of multi-drug resistant pathogens.

Keywords Antimicrobial resistance, Carbapenemase, *mcr*, Environment

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Background

Enterobacter species is widely presented in the soil and water environment. Although it is a part of commensal gut microbiota it also shows pathogenic potential for humans, animals and plants [1, 2]. Members of *Enterobacter cloacae* complex (ECC) including *Enterobacter asburiae* represent a common cause of nosocomial infections such as pneumonia, urinary tract infections or septicaemia, especially in immunocompromised patients [1, 3].

Intrinsic resistance of *Enterobacter* spp. to penicillins, first- and second-generation cephalosporins along with often-reported multi-drug resistance profile of hospital acquired ECC strains leaves limited options for treatment [1]. Therefore, an increasing number of worldwide reports of carbapenemase-producing ECC including their presence in the environment represents a serious threat to public health. Furthermore, plasmid-encoding resistance to another last-resort antibiotic colistin have been recently reported in ECC strains from different niches [4, 5].

Wastewaters are considered an important player in the spread of antimicrobial resistance, thus affecting the health of people and animals [6]. To combat antimicrobial resistance, the monitoring of their spread even outside the hospital settings is crucial. Here, we focused on hospital and municipal wastewaters as a possible source of carbapenemase-producing Enterobacterales for the environment.

Materials and methods

Sampling sites, selective cultivation and carbapenemase detection

A total of six water samples including raw hospital sewage, inflow and outflow of the University hospital Brno Bohunice wastewater treatment plant (WWTP; 49°10′ 49.4′′N 16°34′ 20.7′′E), inflow and outflow of municipal WWTP Brno Modrice (49°07′ 52.9′′N 16°37′ 57.7′′E), and the river Svratka (49°07′ 49.3′′N 16°37′ 38.0′′E) taken upstream of the municipal WWTP were collected in autumn 2016 in the city of Brno, Czech Republic as described previously [7]. The samples were diluted by serial ten-fold dilution method or concentrated using filtering (0.22 µm; Sigma-Aldrich, US) and plated on Brilliance™ *E. coli*/coliform Selective Agar (Oxoid, UK) with addition of 0.125 mg/L meropenem and 100 mg/L $ZnSO_4$ to select for carbapenemase-producing Enterobacterales. Approximately 30–40 colonies per sample were subcultured on MacConkey agar (Oxoid, UK). A total of 180 presumptive coliform isolates were obtained and subsequently tested for carbapenemase-encoding genes bla_{IMP}, bla_{KPC}, bla_{NDM}, bla_{OXA-48} and bla_{VIM} by PCR [8]. Positive isolates were purified, species identified using MALDI-TOF MS (Microflex LT, Bruker Daltonics, Germany) and subjected to further typing.

Phenotyping of carbapenemase-producing isolates and transferability of genes encoding carbapenemase production

Isolates were tested for carbapenemase production by Mastdiscs combi Carba plus D73C (Mast Diagnostics, UK). Minimum inhibitory concentration (MIC) to 24 antimicrobials was determined by broth dilution method using commercially available Mikrolatest MIC plates (Erba Lachema, Czech Republic). MICs values were evaluated according to EUCAST (European Committee on Antimicrobial Susceptibility Testing) Clinical Breakpoint Tables v.13.0, 2023 [9], and CLSI (Clinical and Laboratory Standards Institute) M100, 2020 [10] for tetracycline and cefoperazone. No breakpoints have been established for cefoperazone-sulbactam.

The transferability of plasmids carrying the carbapenemase gene was tested using conjugation at 37 °C into plasmid-free rifampin- and sodium azide-resistant *Escherichia coli* MT102 recipients [11] on Luria-Bertani agar plates with sodium azide (100 mg/L), rifampin (25 mg/L), meropenem (0.125 mg/L) and $ZnSO_4$ (100 mg/L).

Whole-genome sequencing and data assembly

Genomic DNA was isolated by the NucleoSpin tissue kit (Macherey-Nagel, Germany) and Nanobind CBB Big DNA kit (Circulomics, USA), and subjected to short- and long-read sequencing, respectively. Libraries for short-read sequencing were prepared by the Nextera XT DNA Library Preparation Kit (Illumina, Inc., USA) and sequenced on the MiSeq platform (2×250 bp paired-end sequencing, MiSeq Reagents Kits v2, Illumina). SQK-RBK004 rapid barcoding 1D kit was used for libraries preparation for long-read sequencing on a flow cell (FLO-MIN106 R9.4 SpotON) running for 48 h on MinION instrument (Oxford Nanopore Technologies, ONT, Oxford, UK).

The short reads were quality- ($Q \leq 20$) and adaptor-trimmed using Trimmomatic. The long reads were basecalled and demultiplexed using Guppy (https://nanoporetech.com/), quality ($Q \leq 15$) and length (2000 bp) trimmed using NanoFilt. The long reads were *de novo* assembled using Flye followed by polishing using medaka (https://github.com/nanoporetech/medaka) and three rounds of Pilon.

Genomic data analysis

Genomic sequences were analyzed using BLAST algorithm, ResFinder 4.1 (http://www.genomicepidemiology.org/) for the content of antimicrobial resistance genes and PlasmidFinder 2.1 (http://www.genomicepidemiology.org/) for plasmid replicons in combination with

Geneious 9.1.8 software. Cut-off for positive detection of resistance genes and plasmid replicons was set up for at least 90% identity and coverage to reference sequences. Comparison of genetic regions was visualized by clinker [12] and edited in Inkscape v1.2 (https://inkscape.org/). Species identification of *Enterobacter* spp. isolates was performed using pairwise average nucleotide identity (ANI) with a≥96% cut-off and in silico digital DNA-DNA hybridization (dDDH) with a≥70% cut-off as recommended [2]. Sequence type (ST) of isolates was determined using Public databases for molecular typing and microbial genome diversity (PubMLST) for *Enterobacter cloacae* (https://pubmlst.org/organisms/enterobacter-cloacae).

Phylogenetic analysis

Phylogenetic analysis included 672 *Enterobacter asburiae* genomes available from GenBank database (as 26th of July 2024, 37 atypical genomes and 33 genomes derived from metagenomes were excluded from the analysis), three genomes obtained in our study and 13 *E. asburiae* genomes from human clinical isolates originating in the Czech Republic [13]. The open reading frames were predicted from FASTA files using Prokka [14] and the GFF files were used to create the core-genome multi-FASTA alignment using Roary [15] with default settings (except for --group_limit 70000). The single nucleotide polymorphisms (SNPs) were extracted from the multi-FASTA alignment using SNP-sites [16] and used to calculate the phylogenetic tree using RAxML [17], with the general time-reversible (GTR) model supported by 500 bootstraps. The relatedness between isolates was determined based on SNP difference obtained from the core-genome alignment using snp-dists (https://github.com/tseemann/snp-dists). Resulting tree topology was midpoint rotted and visualized via iTOL v6 [18] and edited in Inkscape v1.2 (www.inkscape.org).

Results

E. asburiae isolates producing VIM carbapenemase

Three *E. asburiae* isolates carrying bla_{VIM-1} were detected in inflow to the hospital WWTP (BP16m1), and in the inflow (MP4m4) and the outflow (MO13m20) of the municipal WWTP. Isolates BP16m1 and MP4m4 belonged to novel sequence type ST2057 while the isolate MO13m20 was assigned to ST484. All three isolates showed metallo-beta-lactamase production and multi-drug resistance phenotype, but they differed in the content of antibiotic resistance genes and plasmid replicons (Fig. 1). They carried between 9 and 23 resistance genes encoding up to 10 antimicrobial classes including aminoglycosides, beta-lactams, formaldehyde, fosfomycin, macrolides, phenicols, quinolones, sulfonamides, tetracyclines and trimethoprim (Fig. 1). Interestingly, MP4m4 carried chromosomally located *mcr-9* which did not confer colistin resistance while BP16m1 was resistant to colistin and contained plasmid-encoded *mcr-10*. In BP16m1 and MP4m4, two different plasmids were detected while MO13m20 carried 13 plasmids. The horizontal transfer of bla_{VIM-1} via conjugation was successful only in MP4m4.

Phylogenetic relatedness of *E. asburiae* global collection

Phylogenetic relatedness of 688 *E. asburiae* genomes including our three isolates showed high genetic variability (SNPs in the range 0-18111). They belonged to 112 different STs with ST252 (n=75, SNPs in the range 0–77), ST24 (n=59, SNPs in the range 0-3481) and ST484 (n=56, SNPs in the range 0-23019) being the most prevalent (Table S1, Figure S1). Unfortunately, 95 isolates have not been assigned to any known ST mainly because of a new allelic combination which points out the low attention paid to typing of *E. asburiae* so far.

The phylogenetic analysis showed relatedness between BP16m1 and MP4m4 isolates (4 SNPs) but not to any other genomes. Although MO13m20 clustered with other 52 isolates of ST484 (Figure S1), subsequent phylogenetic tree consisting of only *E. asburiae* ST484 (Figure S2) did

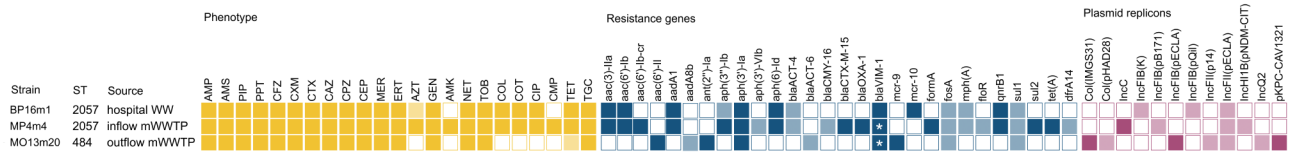

Fig. 1 Characteristics of three VIM-1-producing *E. asburiae* isolates. *gene bla_{VIM} in two copies. WW, wastewater; mWWTP, municipal wastewater treatment plant. Phenotype: Deep yellow color indicates resistance; light yellow color indicates intermediate resistance. MICs interpretation according to EUCAST Clinical Breakpoint Tables v.13.0 valid from 2023-01-01 and CLSI M100, 2020 for tetracycline and cefoperazone. No breakpoints have been established for cefoperazone-sulbactam (not included in the Fig. 1). AMP, ampicillin; AMS, ampicillin-sulbactam; PIP, piperacillin; PPT, piperacillin-tazobactam; CFZ, cefazolin; CXM, cefuroxime; CTX, cefotaxime; CAZ, ceftazidime; CPZ cefoperazone; CEP, cefepime; MER, meropenem; ERT, ertapenem; AZT, aztreonam; GEN, gentamicin; AMK, amikacin; NET, netilmicin; TOB, tobramycin; COL, colistin; COT, trimethoprim-sulfamethoxazole; CIP, ciprofloxacin; CMP, chloramphenicol; TET, tetracycline; TGC, tigecycline. Resistance genes and plasmid replicons: deep blue and violet color indicates 100% length coverage and identity to the reference sequence, light blue and violet color indicates length coverage and identity below 100% but at least 90% to the reference sequence

not showed any close relatedness between MO13m20 and the other isolates (SNPs in the range 1407–21532). Majority of ST484 strains were of clinical origin and came from various geographic areas. They all carried the same variant of $bla_{\text{ACT-6-like}}$, *fosA* encoding resistance to fosfomycin and 92.9% (52/56) of them had *mcr-9* (Figure S2). Analysis of beta-lactamase gene content showed that strains of the same ST carry identical variants of bla_{ATC}; for ST24 ($n=59$) $bla_{\text{ATC-1}}$ or $bla_{\text{ATC-1-like}}$ was typical while ST252 ($n=75$) carried $bla_{\text{ATC-3}}$ or $bla_{\text{ATC-3-like}}$ (Figure S1).

Occurrence of carbapenemase genes among 688 *E. asburiae* genomes was high (39.5%, 272/688; Table S1), suggesting this species is frequently associated with clinically relevant resistance mechanisms. However, this association could have been caused by selective cultivation of these isolates on carbapenem-supplemented media as performed in our study or due to the focus on genomes carrying carbapenemases in most studies. Twenty-five isolates (3.6%, 25/688) carried bla_{VIM}, namely variants $bla_{\text{VIM-1}}$ ($n=18$), $bla_{\text{VIM-2}}$ ($n=6$) and $bla_{\text{VIM-4}}$ ($n=4$). Two different variants of bla_{VIM} were detected in three isolates and detection of two copies of the same bla_{VIM} variant was found only in two isolates from our study. VIM-producing isolates originated from clinical samples (France, Germany, Poland, Italy and the Czech Republic) and wastewaters (the Czech Republic, or the location was not specified). The other most frequently detected carbapenemases among *E. asburiae* isolates included KPC-2 (8%, 55/688), NDM-1 (5.8%, 40/688), KPC-3 (4.1%, 28/688), IMP-1 (4.5%, 31/688) and OXA-48 (2.8%, 19/688).

A total of 217 (31.5%, $n=688$) isolates from the *E. asburiae* collection contained *mcr* genes. One hundred and sixty-nine isolates (24.6%) carried *mcr-9* while only sixty-three isolates (9.2%) harbored *mcr-10*, of which in fifteen isolates a combination of *mcr-9* and *mcr-10* was detected. Ninety-six isolates with *mcr-9* and nineteen isolates with *mcr-10* carried also a carbapenemase gene, mainly $bla_{\text{KPC-2}}$ (32/217), $bla_{\text{NDM-1}}$ (17/217) and $bla_{\text{OXA-48}}$ (11/217).

Genetic surrounding of $bla_{\text{VIM-1}}$

In all three *E. asburiae* isolates $bla_{\text{VIM-1}}$ was the first cassette of a class 1 integron followed by two cassettes conferring resistance to aminoglycosides, conserved segment encoding resistance to quaternary ammonium compounds (*qacEΔ1*) and sulfonamide resistance gene (*sul1*) (Fig. 2). In BP16m1, the integron was surrounded by IS*26* and IS*4321*carried by IncFIB(K)/IncFIB(pQil) plasmid (non-typeable by RST; 138894 bp). Isolate MP4m4 showed very similar genetic composition including IS*26* and IS*4321* but $bla_{\text{VIM-1}}$ was detected in two copies next to each other and the whole structure was part of IncFIB/FII plasmid (F-:A-:B70; 299792 bp). On the contrary, in MO13m20, *aac(6')-II* was inserted between two copies of $bla_{\text{VIM-1}}$ on non-typeable plasmid (26050 bp). Only three plasmids with identical nucleotide sequence encoding the replication protein of p8MO13m20_VIM were detected in the GenBank, namely pROUE1 (*Pseudomonas putida*, MK047608), pbAMD1 (*Aminobacter niigataensis*, OX341520) and pKLC102 (*Pseudomonas aeruginosa*, AY257539). The presence of the IS*Pa17* in the proximity of $bla_{\text{VIM-1}}$ and the IR sites suggests its involvement in the mobilization of $bla_{\text{VIM-1}}$, similarly as in pJB12 [19]. Isolate ENCL48212 with $bla_{\text{VIM-1}}$ on pKPC-CAV1193-like plasmid (Fig. 2) from a clinical *Enterobacter hormaechei* was recently detected in the Czech Republic [4].

Genetic comparison of plasmids encoding $bla_{\text{VIM-1}}$

The plasmids p1BP16m1_VIM (accession nr. CP129515) and p2MP4m4_VIM (accession nr. CP129499) were BLASTn searched in the GenBank, however neither the similarity nor the size of the hits corresponded to queries, making the plasmids p1BP16m1_VIM and p2MP4m4_VIM unique.

In contrast, the plasmid p8MO13m20_VIM (accession nr. CP129508) was similar to multiple plasmids from various species when applying an arbitrary identity threshold of >90% with the query coverage of >70%. The size of the query (26050 bp) was taken into account as well, and hits exceeding 100 kb were excluded. While the plasmid pbAMD1 originating from *A. niigataensis* did not encode any resistance genes, the other examined plasmids (pNOR-2000, pJB12, pJB35, pVIM_Pse435, pCOOP-101 and pCfr12-3) encoded some of the variants of bla_{VIM}. However, only the plasmid pAX22 (*Achromobacter denitrificans*, Italy) carried the $bla_{\text{VIM-1}}$ variant. The circular comparison of p8MO13m20_VIM and above-mentioned plasmids with corresponding metadata is shown in Figure S3.

Genetic context of *mcr-10*

In BP16m1, *mcr-10* gene was flanked by *xerC* encoding site-specific recombination upstream and IS*3* family IS*Ec36* element downstream. The association of *xerC* with *mcr-10* was observed in all *E. asburiae* isolates carrying *mcr-10* (identity of *mcr-10* to reference *mcr-10* was set up ≥99.9%) from the global collection. This genetic structure was previously described on IncFIB-FII plasmid pECL981-1 (accession nr. CP048651) originating from *Enterobacter roggenkampii* (hospital sewage water, China, 2019) and was suggested to play a role in mobilization of *mcr-10* [20].

The plasmids p2BP16m1_mcr10 (accession nr. CP129516, 165059 bp) of IncFII(pECLA) replicon (non-typeable by RST) was BLASTn searched in the GenBank, however only a single non-typeable plasmid pSTW0522-51-1 (accession nr. AP022432, 159829 bp) obtained from

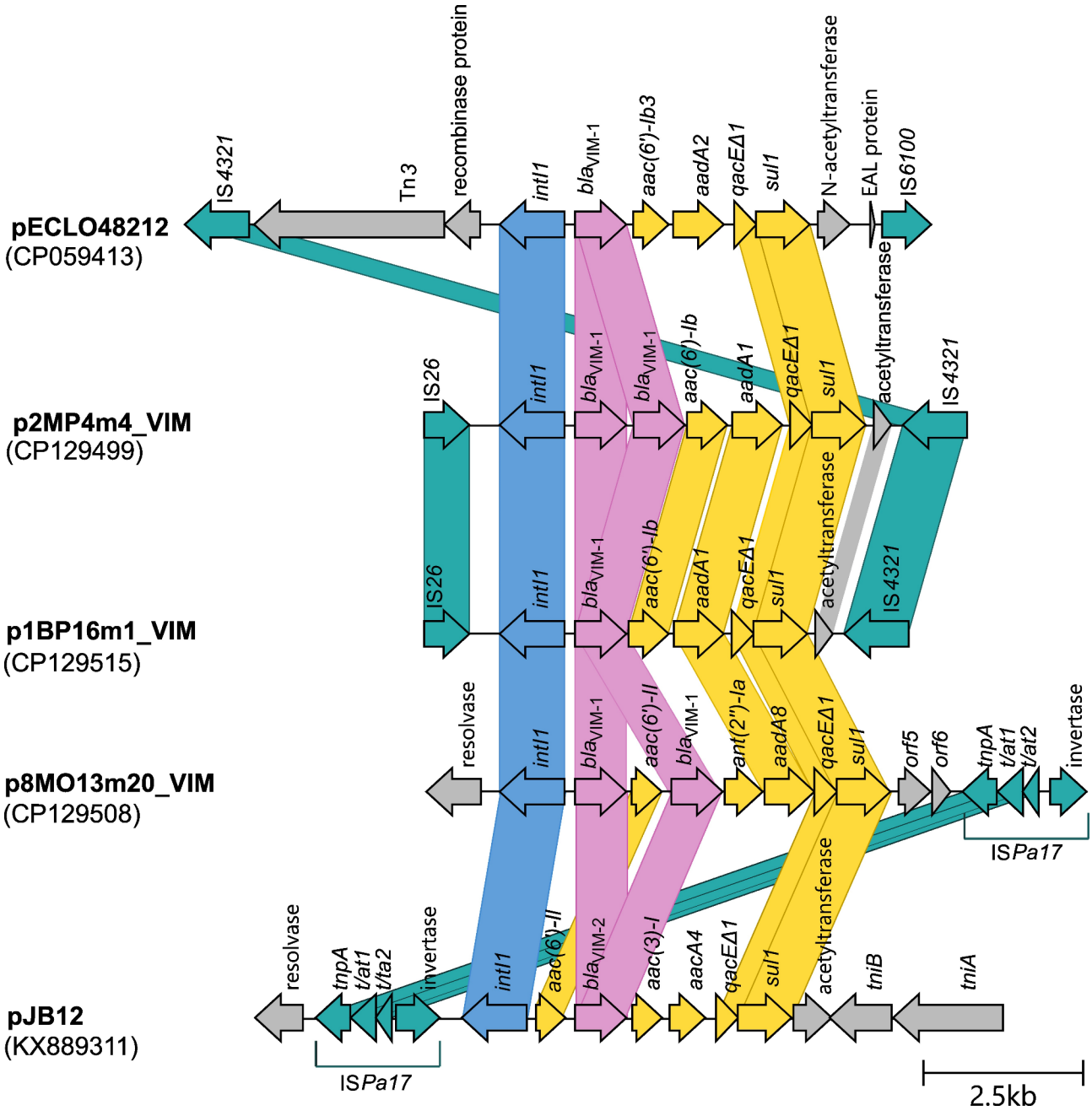

Fig. 2 Comparison of genetic surrounding of bla_{VIM} gene. Links are based on 90% nucleotide identity. Purple arrows indicate bla_{VIM}, yellow arrows resistance genes, green arrows insertions sequences, blue arrows *intI1* and grey is used for other features

Enterobacter kobei of hospital sewage in Japan was similar (63% query coverage, 99.1% identity). p2BP16m1_mcr10 carried also genes *aac(3)-IIa* and *qnrB1* encoding resistance to aminoglycosides and quinolones, respectively, while no other resistance genes were detected on pSTW0522-51-1. Further hits were either of coverage lower than 60% or of incomparable size (266951 bp and more).

Discussion

The resistance to last-line antibiotics is growing concern for patient health worldwide. In the Czech Republic, emergence of carbapenem resistance in hospital settings has been mainly associated with *Klebsiella pneumoniae*, *E. coli*, *Citrobacter* spp. and *Acinetobacter baumannii* [21–23] while reports of carbapenem-resistant *Enterobacter* spp. isolates are scarce [24]. Only two studies with carbapenemase-producing *E. asburiae* isolates of Czech clinical origin were published so far, including isolate

Easb-36567cz producing IMI-2 [25] and isolate CZ863 producing GES-5 [26] in 2016 and 2020, respectively.

In this study, three VIM-1-producing *E. asburiae* isolates (1,7%, $n=180$), two of them co-harboring *mcr* genes, were detected in community and hospital wastewaters. Recent reports of carbapenemase-producing bacteria in the environmentl are not rare [27, 28], geographically copying occurrences of carbapenemase variants present in clinical settings. In our previous study focused on transmission routes of *E. coli* from patients via wastewater to the environment in the Czech Republic [29], low occurrence of carbapenem-resistant bacteria was also observed. Only seven GES-5- or OXA-244-producing *E. coli* isolates (1.7%, $n=408$) were obtained using selective cultivation on antibiotic-supplemented media. Despite the high phylogenetic diversity among the isolates in the above mentioned study, related *E. coli* clones producing extended spectrum beta-lactamase CTX-M-15 from different sources were detected. Here, we also reported phylogenetically related carbapenemase-producing *E. asburiae* isolates from hospital sewage and inflow to municipal WWTP, highlighting wastewater as an important pathway for antibiotic resistance spread.

In addition to resistance to carbapenems, resistance to another last-line drug colistin mediated by *mcr* genes, was observed in *E. asburiae* strains. Our recent national surveillance showed that the occurrence of *mcr* genes in human clinical isolates is rare as it was detected only in 3.8% isolates resistant to colistin [13]. Most *mcr*-positive isolates belonged to *E. coli* (44/73) with *mcr-1* variant or *Enterobacter* spp. (24/73) with *mcr-9*. On the contrary, *mcr* genes were frequently found in the animal sector during national monitoring of slaughter animals and meat [30]. To best of our knowledge, no study exploring the spread of plasmid-mediated colistin resistance in the environment has been published so far in the Czech Republic.

There is some evidence of increasing importance of ECC in human infections [3]. However, the frequency of *E. asburiae* in these infections have not been determined yet probably due to the difficulty to distinguish particular species within ECC without whole-genome sequencing followed by appropriate data analysis [2]. Using comparative genomics of a global collection of 688 *E. asburiae* genomes, we observed that more than a third of *E. asburiae* isolates carry carbapenemase genes, highlighting the clinical importance of this species. Besides common resistance to carbapenems, the carriage of the intrinsic AmpC beta-lactamase gene bla_{ACT} was common through the global *E. asburiae* collection and a link between particular bla_{ACT} variant and the isolate genotype was observed. *E. asburiae* ST252 was the most common genotype. The common association of this ST with $bla_{\mathrm{ACT-3}}$ has been previously reported [31, 32]. *E. asburiae* ST24 was the second most common ST, isolates were of human or hospital wastewater origin from different countries and associated with $bla_{\mathrm{ACT-1}}$. *E. asburiae* ST484 isolates with $bla_{\mathrm{ACT-6}}$ was previously detected in Czech patients throughout years 2012 to 2020, with more isolates of this resistance profile from the recent years [13]. Furthermore, these strains also carried *mcr-9* along with fosfomycin resistance determinants as described in this study. A single clinical isolate of *E. asburiae* ST484 encoding $bla_{\mathrm{ACT-6}}$ and *mcr-9* was identified in 2018 in Japan [33]. This suggests strong connection of $bla_{\mathrm{ACT-6}}$ to ST484 in the Czech Republic, but not limited to it as was also observed in the global *E. asburiae* collection.

Conclusions

The dissemination of *E. asburiae* with clinically important resistance to carbapenems and colistin via wastewaters was reported within this study. Phylogenetic analysis of available *E. asburiae* genomes has shown their so far low representation in public genomic databases probably due to the complicated species identification of *Enterobacter* spp. Nevertheless, a considerable occurrence of *E. asburiae* strains with resistance to last-resort drugs was observed pointing out their increasing importance for public health.

Abbreviations

A. niigataensis	*Aminobacter niigataensis*
ANI	Average Nucleotide Identity
CLSI	Clinical and Laboratory Standards Institute
EUCAST	European Committee on Antimicrobial Susceptibility Testing
E. asburiae	*Enterobacter asburiae*
E. coli	*Escherichia coli*
GTR	General Time-Reversible
dDDH	digital DNA-DNA Hybridization
ECC	*Enterobacter cloacae* Complex
MIC	Minimum Inhibitory Concentration
SNPs	Single Nucleotide Polymorphisms
ST	Sequence Type
WWTP	Wastewater Treatment Plant

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12864-024-10780-7.

Supplementary Material 1

Supplementary Material 2

Supplementary Material 3

Supplementary Material 4

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Author contributions

IS performed laboratory experiments, analysed, and visualized genomic data, wrote original draft. AV analysed genomic data, was responsible for

genome annotation and participated in writing of original draft. MD designed and supervised the study, edited manuscript, and participated in obtaining funding.

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Data availability
The complete genomes are available under BioProject PRJNA973937, accession numbers CP129500 - CP129513 for strain MO13m20, CP129514 - CP129516 for BP16m1 and CP129497 - CP129499 for MP4m4.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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