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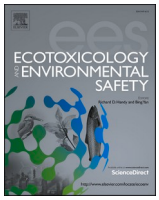

Bacterial contamination in public transport during COVID-19 pandemic: Characterization of an unusual *Staphylococcus aureus* isolate tolerant to vancomycin

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ABSTRACT

Public transport represents a potential site for the transmission of resistant pathogens due to the rapid movement of large numbers of people. This study aimed to investigate the bacterial contamination of frequently touched surfaces in the public transport system operating in the proximity of the biggest Czech hospital during the coronavirus pandemic despite extensive cleaning and disinfection efforts. In June and September 2020, samples from the metro trains, ground transport and stationary objects were collected, enriched and cultured. The antimicrobial susceptibility was tested by broth microdilution. *Staphylococcus aureus* isolates exhibiting inconclusive results of vancomycin susceptibility testing were retested by broth macrodilution and subjected to whole genome sequencing. All *S. aureus* isolates were tested for vancomycin heteroresistance (hVISA). A total of 513/542 (94.6 %) samples were culture-positive with higher frequency in September ($p = 0.004$). *S. aureus* was the most frequent opportunistic bacterial pathogen found (3.7 %, 20/542) followed by Enterobacterales spp. (1.8 %, 10/542). No methicillin-resistant *S. aureus* (MRSA), extended-spectrum beta-lactamase producers (ESBL) or carbapenemase-producing bacteria were detected. Resistance to clinically relevant drugs was rare except for resistance to ampicillin (67 %, 8/12), cefuroxime (42 %, 5/12) in Enterobacterales and chloramphenicol (90 %, 18/20), penicillin (45 %, 9/20), and erythromycin (20 %, 4/20) in *S. aureus*. One *S. aureus* isolate was shown to be resistant to vancomycin (8 mg/L) by forming large visible cell aggregates. Population analysis profile-area under the curve ratio (PAP-AUC) testing did not confirm the hVISA phenotype, but mutations in the hVISA phenotype-related gene *vraR* and other genes related to cell wall synthesis (*fmtB*) and intercellular adhesion (*sasC*) were found. Our study shows that in the COVID-19 pandemic, despite the intensive use of disinfectants, public transport was a source of opportunistic bacterial pathogens including *S. aureus* with unusual vancomycin resistance phenotype that could be easily missed by standard susceptibility testing.

1. Introduction

The spread of antimicrobial-resistant bacteria represents one of the most serious global challenges today (CDC. Antibiotic Resistance Threats in the United States, 2019; Tacconelli et al., 2018). Following the COVID-19 pandemic, there were significant changes in the epidemiology of antimicrobial-resistant bacteria causing invasive human infection in the Czech Republic. Between 2019 and 2022, the incidence of methicillin-resistant *Staphylococcus aureus* (MRSA) infections dropped by 29.7 % (3.06–2.15 per 100,000 population) while the incidence of carbapenem-resistant *Klebsiella pneumoniae* increased by 156.3 % (0.09–0.24). (ECDC, 2023). However, the environmental spread of antimicrobial resistance is much less documented. Growing evidence suggests that among urban environments, the public transport system can serve as an important means for the transmission of resistant pathogens, including MRSA (Conceicao et al., 2013; Lutz et al., 2014) but also newly emerging *mcr-1*-producing colistin-resistant Enterobacterales (Shen et al., 2018). Several studies associated the presence of resistant bacteria with proximity to healthcare facilities (Shen et al., 2018; Mendes et al., 2015; Zou et al., 2019).

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Intensive disinfection could lead to the selection of resistant bacteria in the environment as the biocide resistance genes and antimicrobial resistance genes are frequently co-localized on the same mobile genetic elements (Basiry et al., 2022). Indeed, the presence of resistant bacteria in indoor environments correlates with the presence of biocide residues (e.g. Triclosan) or increased confinement and cleaning (Mahnert et al., 2019; Fahimipour et al., 2018). Specifically, the correlation between triclosan and quinolone resistance was observed (Fahimipour et al., 2018). The presence of biocides has been also reported to increase the incidence of resistance to clinically relevant antibiotics by promoting biofilm formation, through mutations in efflux pumps and porins (Merchel Piovesan Pereira, Wang, and Tagkopoulos, 2021).

During the COVID-19 pandemic, the regular deep cleaning of public transport has been introduced and frequent hand hygiene was recommended. It is unknown if the increased use of disinfectants had a possible effect on the selection of antimicrobial-resistant bacteria in public transport. Thus the aim of our study was to investigate bacterial contamination on frequently touched surfaces in the public transport system including the metro trains, buses, trams and stationary objects around public transport stops operating in the proximity of the large Czech hospital during the COVID-19 pandemic.

2. Material and methods

2.1. COVID-19 hygiene measures

During the initial wave of the COVID-19 pandemic in Europe in March 2020, strict hygiene measures and free movement restrictions were applied in Prague public transport including wearing a face mask, keeping a distance and an availability of hand disinfection at metro stations. Metro trainsets were disinfected whenever arrived at the depot and in addition, the trainsets were disinfected with ozone once a week. The cleaning of areas such as handrails and stop buttons in buses and trams was enhanced by the polyhexamethylenguanidine-based disinfection (DisiClean AIR and DisiClean Home, Wero Water Service). The ticket machines and elevator handrails were cleaned with ethanol-based disinfection every day (Prague Public Transit Co., personal communication).

2.2. Sample collection and cultivation

In June (n = 263) and September (n = 279) 2020, samples from the metro trains (line A, n = 198), ground transport (buses and trams, six lines each, n = 200) operating near the Motol University Hospital, Prague, Czech Republic and stationary objects in public transport stations from 11 various locations in Prague city centre (n = 144) were collected during working hours not directly after cleaning, totalling 542 samples (Supplementary Table 1).

The sampling was performed by wiping an area of approximately 10 × 10 cm from frequently touched surfaces with a sterile dacron swab. In metro trains and ground transport vehicles, the handrails and stop buttons were wiped. In stations, the swabs of escalators handrails, ticket machines, lift buttons, and disinfection dispensers were collected. The swabs were immediately put into the enrichment thioglycolate broth (Oxoid), used in clinical microbiology to capture a wide variety of bacteria including both anaerobes and aerobes and transported to the laboratory. Samples were incubated at 37 °C for 24 hours and afterwards, 10 μl of enriched culture was inoculated onto (a) chromogenic Brilliance™ UTI Clarity™ agar (Oxoid) to culture Enterobacterales spp. and *Pseudomonas* spp., (b) mannitol salt agar (Oxoid) to culture staphylococci and (c) Columbia blood agar (Oxoid) for other common culturable bacteria. Subsequently, all agar plates were cultured at 37 °C for 24–48 hours.

2.3. Species identification

Suspected colonies grown on agar plates were identified using Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF/MS), Biotyper v 3.1 (Bruker Daltonics). All Enterobacterales spp., *Enterococcus* spp., *Pseudomonas* spp., *Acinetobacter* spp., *Moraxella* spp., *S. aureus*, clinically important *Streptococcus* spp., and *Aerococcus* spp. were grouped together because of the low capture of individual group and were considered opportunistic pathogens for further analysis. Common skin commensals (e.g. coagulase-negative staphylococci) and environmental bacteria (e.g. *Bacillus* spp.) were not further tested for antimicrobial susceptibility. In *S. aureus* isolates, *spa* typing (Harmsen et al., 2003) was performed as described previously and the genes for methicillin resistance *mecA* and Panton-Valentine leukocidin *lukSF-PV* (PVL) were screened by qPCR (Okolie et al., 2015).

2.4. Antimicrobial susceptibility

The susceptibility of isolates to clinically relevant antimicrobials was tested using the broth microdilution method (SENSILAtest G-I, G-II, G+, STAPHY; MIKROLATEST®, Erba Lachema) with EUCAST breakpoints. (The European Committee on Antimicrobial Susceptibility Testing. Breakpoint tables for interpretation of MICs and zone diameters. Version 10.0).

2.5. Analysis of vancomycin susceptibility of S. aureus

A heterogeneous vancomycin-intermediate *S. aureus* (hVISA) phenotype was tested by the Brain Heart Infusion (BHI) screen agar method (Castro et al., 2020). Briefly, 0.5 McFarland inoculum density was prepared in saline and four 10 μl-droplets from bacterial suspension were dropped by a pipette onto BHI agar plates with casein (Sigma-Aldrich) supplemented with 4 mg/L of vancomycin. Plates were incubated at 37 °C for 48 hours. The result was considered positive if two or more colonies were found in at least one droplet.

Isolates positive by the BHI screen agar method were re-tested by the population analysis profile-area under the curve ratio (PAP-AUC) method for hVISA confirmation (Satola et al., 2011). Briefly, a 100 μl of 0.5 McFarland suspension of tested isolate in BHI broth was plated onto BHI agar with vancomycin (0; 1; 2; 3; 4 and 8 mg/L) and incubated at 37 °C. After 48 hours, colony-forming units (CFU) were counted. Then, $\log_{10}$ CFU/mL was plotted against the vancomycin concentrations to determine AUC. For AUC calculation and visualisation, GraphPad Prism (v10.2.0) software was used. If the ratio AUC_{strain}/AUC_{Mu3} was between 0.9 and 1.3, the strain was considered as hVISA. hVISA Mu3 (ATCC 700698) and vancomycin-susceptible *S. aureus* (VSSA, ATCC 25923) were used as controls.

Simultaneously, *S. aureus* isolates showing inconclusive results of vancomycin susceptibility testing due to the presence of skip-well phenotype when tested by broth microdilution, were retested by the macrodilution method in the presence of 0; 4; 8 and 16 mg/L of vancomycin in triplicate (Vaudaux et al., 2010).

2.6. Whole-genome sequencing of S. aureus isolate with hVISA phenotype

The DNA was extracted from antibiotic-free culture and from the culture supplemented with 8 mg/L of vancomycin during broth microdilution testing by using ChargeSwitch™ gDNA Mini Bacteria Kit (Thermo Fisher Scientific), quantified using Qubit™ dsDNA HS Assay Kit (Thermo Fisher Scientific). The purity was measured using NanoDrop (Thermo Fisher Scientific). The DNA sequencing library was prepared by Nextera XT DNA Library Preparation Kit (Illumina), according to manufacturers' instructions and sequenced on MiSeq sequencer (Illumina, Macrogen).

Long reads sequencing was performed using Ligation Sequencing Kit,

#SQK-LSK109 (Oxford Nanopore Technologies) and Flongle AMY533 (Oxford Nanopore Technologies). To acquire hybrid assembly, long reads were assembled using Flye v2.9.1 (Kolmogorov et al., 2019) and then polished by long reads with Medaka v1.7.2 (ONT, 2021) and by short reads with Polypolish0.5.0 (Wick and Holt, 2022).

Multi-locus Sequence Type (MLST) was determined using MLST 2.0 where sequences are compared to the database using BLASTn (Larsen et al., 2012). ResFinder 4.1 (Bortolaia et al., 2020) and VirulenceFinder 2.0 (Joensen et al., 2014) with a minimum identity threshold of 90 % and a minimum length coverage of 60 % were used for the identification of resistance and virulence genes, respectively. Then, MobileElementFinder was used for the identification of mobile genetic elements (Johansson et al., 2021).

A single-nucleotide polymorphism (SNP) analysis was performed using Snippy v4.6.0 (Seemann, 2015) with a minimum mapping quality of 60, minimum coverage to call an SNP of 10, minimum variant frequency of 0.9 and minimum variant quality of 100. The genome of ST45 *S. aureus* (MCRF184, accession no. CP014791) and *S. aureus* isolate exposed to 8 mg/L were used as a reference.

2.7. Statistical analysis

The prevalence of all the bacterial groups in public transport was estimated with 95 % confidence intervals. Differences between groups were evaluated using Chi-square or Fisher's exact test with Holm's correction for categorical variables. For *post hoc* tests, Fisher's exact test with Holm's correction was used and $p < 0.05$ was considered statistically significant. Statistical tests were performed in R v. 2021.09.1.

3. Results

3.1. Bacterial contamination of surfaces in public transport during June and September 2020

Out of a total of 542 samples, 513 (94.6 %) were culture-positive (Table 1) and 673 bacterial isolates were acquired. Bacterial culture positivity was significantly higher in September (273/279; 97.9 %) compared to June (240/263; 91.3 %; $p = 0.004$). Details of statistical comparison between sampling locations at different time points are given in Tables 2 and 3 and Supplementary Table 2.

S. aureus and Enterobacterales were detected in 3.7 % (20/542) and 1.8 % (10/542) of the samples, respectively. The 20 isolates of *S. aureus* were assigned to 17 different *spa* types with the most frequent *spa* type t267 (3/20, 15 %) (Supplementary Table 1). No isolate carried PVL. Other opportunistic bacterial pathogens were rarely detected, Table 1 (Supplementary Table 1).

The lift buttons (16.0 %; 4/25) and the ticket machines (13.2 %; 10/76) were most frequently contaminated by opportunistic bacterial pathogens. Isolation of these bacteria was negatively correlated with the presence of environmental bacteria (83.7 % vs. 16.3 %, $p < 0.001$), but not with the presence of skin commensals ($p = 0.47$) as 79 % (34/43) of opportunistic bacterial pathogens grew in the presence of skin commensals. Colonization of stationary objects by skin commensals was more frequently observed in June (53/67 vs. 44/77; $p = 0.03$), while environmental bacteria were more frequently detected during September (79/263 vs. 148/279; $p < 0.001$; Supplementary Table 3).

3.2. Antimicrobial resistance of acquired isolates

No extended-spectrum beta-lactamase producers (ESBL) or carbapenem resistance in Enterobacterales and methicillin resistance in *S. aureus* isolates were detected. Overall, resistance to clinically relevant drugs was rare except for resistance to ampicillin (67 %, 8/12), cefuroxime (42 %, 5/12) in Enterobacterales and resistance to chloramphenicol (90 %, 18/20), penicillin (45 %, 9/20) and erythromycin (20 %, 4/20) in *S. aureus* (Table 4; Supplementary Tables 4 and 5).

When tested by microdilution, the skip-well (i. e. paradoxical growth in a higher concentration of the drug while inhibited by its lower concentration) was detected in three *S. aureus* isolates upon vancomycin exposure; in two of them, it breached the resistance breakpoint. As vancomycin resistance is rare in *S. aureus*, a detailed analysis of vancomycin susceptibility in all *S. aureus* isolates was performed (Table 4).

3.3. Detection of vancomycin resistance in S. aureus

All 20 *S. aureus* isolates were tested for hVISA phenotype. Using BHI screen agar plates supplemented with 4 mg/L of vancomycin, six of 20 *S. aureus* isolates (30 %) were tested positive for hVISA phenotype, including all isolates exhibiting skip well phenotype (Table 4). However, the PAP-AUC method did not confirm these isolates as an hVISA (Fig. 1, Supplementary Table 6). When retested by macrodilution method, one *S. aureus* isolate (STAU_60) originating from the handrail in metro trains

Table 1
Summary of culture-positive samples and isolates obtained during June and September 2020 from public transport.

Group	Genus/Species	No. of samples June (%) n = 263	No. of samples September (%) n = 279	Total no. of samples (%) n = 542
Gram-positives	*Aerococcus viridans*	1 (0.4)	0 (0)	511 (94.3)**
	Environmental bacteria	79 (30.0)	148 (53.0)	
	Enterococccus spp.*	3 (1.1)	2 (0.7)	
	Skin commensals	202 (76.8)	197 (70.6)	
	Staphylococcus aureus	13 (4.9)	7 (2.5)	
	Streptococcus lutetiensis	1 (0.4)	0 (0)	
Gram-negatives – Enterobacterales spp.	*Cronobacter* sp.	1 (0.4)	0 (0)	10 (1.8)***
	Enterobacter bugandensis	1 (0.4)	0 (0)	
	Escherichia hermannii	0 (0)	1 (0.4)	
	Leclercia adecarboxylata	0 (0)	2 (0.7)	
	Klebsiella aerogenes	1 (0.4)	0 (0)	
	Klebsiella oxytoca	0 (0)	2 (0.7)	
	Pantoea spp.	3 (1.1)	0 (0)	
	Pseudescherichia vulneris	0 (0)	1 (0.4)	
Gram-negatives – other	*Acinetobacter pittii*	1 (0.4)	1 (0.4)	8 (1.5)
	Moraxella osloensis	2 (0.8)	0 (0)	
	Pseudomonas luteola	2 (0.8)	2 (0.7)	
Total positive samples		**240 (91.3)**	**273 (97.8)**	**513 (94.6)**

The number represents the number of samples containing gram-positive/gram-negative isolates
* *Enterococcus* spp. includes isolates of *E. faecalis* (n = 2), *E. faecium* (n = 2) and *E. hirae* (n = 1).
** 142 samples contained two isolates of gram-positives.
*** 2 samples contained two isolates of *Enterobacter* spp.

Table 2
Comparison of bacterial contamination between June and September in different types of sampling sites Statistically significant differences are highlighted in red.).

	Summary				Stop-button				Handrail			
	June (%)	September (%)	p value	Adjusted p value	June (%)	September (%)	p value	Adjusted p value	June (%)	September (%)	p value	Adjusted p value
Summary occurrence	91.25 (240/263)	97.85 (273/279)	**0.0013**	**0.0039**	91.84 (90/98)	97.03 (98/101)	0.1962	0.3924	91.84 (90/98)	100.00 (101/101)	**0.0030**	**0.0119**
Metro trains	86.46 (83/96)	97.06 (99/102)	**0.0133**	0.0534	83.33 (40/48)	94.12 (48/51)	0.1656	0.6624	89.58 (43/48)	100.00 (51/51)	**0.0239**	0.0958
Ground transport	97.00 (97/100)	100.00 (100/100)	0.2462	0.4975	100.00 (50/50)	100.00 (50/50)	1.0000	1.0000	94.00 (47/50)	100.00 (50/50)	0.2424	0.7273
Stationary objects	89.55 (60/67)	96.10 (74/77)	0.1885	0.3770	-	-	-	-	-	-	-	-
Skin commensals	76.81 (202/263)	70.61 (197/279)	0.1239	0.1463	74.49 (73/98)	73.27 (74/101)	0.9722	0.9722	77.55 (76/98)	78.22 (79/101)	1.0000	1.0000
Metro trains	64.58 (62/96)	66.67 (68/102)	0.8738	0.8738	60.42 (29/48)	64.71 (33/51)	0.8157	0.8157	68.75 (33/48)	68.63 (35/51)	1.0000	1.0000
Ground transport	87.00 (87/100)	85.00 (85/100)	0.8385	0.8385	88.00 (44/50)	82.00 (41/50)	0.5754	1.0000	86.00 (43/50)	88.00 (44/50)	1.0000	1.0000
Stationary objects	79.10 (53/67)	57.14 (44/77)	**0.0087**	**0.0260**	-	-	-	-	-	-	-	-
Environmental bacteria	30.04 (79/263)	53.05 (148/279)	**0.0000**	**0.0000**	27.55 (27/98)	52.48 (53/101)	**0.0006**	**0.0023**	30.61 (30/98)	49.50 (50/101)	**0.0101**	**0.0303**
Metro trains	35.42 (34/96)	46.08 (47/102)	0.1675	0.5024	31.25 (15/48)	45.10 (23/51)	0.2266	0.6624	39.58 (19/48)	47.06 (24/51)	0.5843	1.0000
Ground transport	23.00 (23/100)	56.00 (56/100)	**0.0000**	**0.0000**	24.00 (12/50)	60.00 (30/50)	**0.0006**	**0.0023**	22.00 (11/50)	52.00 (26/50)	**0.0037**	**0.0149**
Stationary objects	32.84 (22/67)	58.44 (45/77)	**0.0037**	**0.0147**	-	-	-	-	-	-	-	-
Opportunistic bacterial pathogens	10.27 (27/263)	5.73 (16/279)	0.0732	0.1463	10.20 (10/98)	1.98 (2/101)	**0.0325**	0.0974	10.20 (10/98)	6.93 (7/101)	0.5671	1.0000
Metro trains	10.42 (10/96)	4.90 (5/102)	0.2313	0.5024	8.33 (4/48)	1.96 (1/51)	0.1955	0.6624	12.50 (6/48)	7.84 (4/51)	0.5169	1.0000
Ground transport	10.00 (10/100)	4.00 (4/100)	0.1658	0.4975	12.00 (6/50)	2.00 (1/50)	0.1117	0.3352	8.00 (4/50)	6.00 (3/50)	1.0000	1.0000
Stationary objects	10.45 (7/67)	9.09 (7/77)	1.0000	1.0000	-	-	-	-	-	-	-	-

was able to grow in the presence of vancomycin up to the concentration of 8 mg/L (MIC >8 mg/L) forming large visible aggregates approximately 1 mm in diameter (Fig. 2).

The DNA of the STAU_60 isolate was extracted from an antibiotic-free culture as well as from the culture supplemented with 8 mg/L of vancomycin (STAU_60van) to rule out the option that the phenotype was caused by a spontaneous mutation arising during susceptibility testing. Both STAU_60 and STAU_60van were subjected to short-read sequencing (Illumina). In addition, the STAU_60van isolate was sequenced using long-read sequencing (MinION) to obtain the circular genome using hybrid assembly. Short reads from STAU_60 were mapped to complete genome of STAU_60van.

No genetic differences or SNPs in coding regions were detected between STAU_60 and STAU_60van. Both isolates belonged to the same ST6949 of the clonal complex CC45. Both isolates were found to carry the cadmium resistance genes (*cadD* and *cadX*) and *blaZ* gene present on the rep16 plasmid (Inc18 family) leading to resistance to ampicillin, penicillin, piperacillin and amoxicillin (Supplementary Table 7). Mutation in the GrlA protein (I45M) was detected but susceptibility to ciprofloxacin was preserved. VirulenceFinder showed the presence of genes

Table 3
Comparison of bacterial contamination between different types of sampling sites in June and September Statistically significant differences are highlighted in red.

	June					September					June + September				
	Metro trains (%)	Ground transport (%)	Stationary objects (%)	p value	Adjusted p value	Metro trains (%)	Ground transport (%)	Stationary objects (%)	p value	Adjusted p value	Metro trains (%)	Ground transport (%)	Stationary objects (%)	p value	Adjusted p value
Summary occurrence	86.46 (83/96)	97.00 (97/100)	89.55 (60/67)	0.0281	0.0842	97.06 (99/102)	100.00 (100/100)	96.10 (74/77)	0.1562	0.4687	91.92 (182/198)	98.50 (197/200)	93.06 (134/144)	0.0087	0.0261
Skin commensals	64.58 (62/96)	87.00 (87/100)	79.10 (53/67)	0.0009	0.0035	66.67 (68/102)	85.00 (85/100)	57.14 (44/77)	0.0002	0.0006	65.66 (130/198)	86.00 (172/200)	67.36 (97/144)	0.0000	0.0000
Environmental bacteria	35.42 (34/96)	23.00 (23/100)	32.84 (22/67)	0.1403	0.2806	46.08 (47/102)	56.00 (56/100)	58.44 (45/77)	0.1981	0.4687	40.91 (81/198)	39.50 (79/200)	46.53 (67/144)	0.4025	0.8050
Opportunistic bacterial pathogens	10.42 (10/96)	10.00 (10/100)	10.45 (7/67)	0.9938	0.9938	4.90 (5/102)	4.00 (4/100)	9.09 (7/77)	0.3945	0.4687	7.58 (15/198)	7.00 (14/200)	9.72 (14/144)	0.6363	0.8050

Table 4
Staphylococcus aureus MICs (mg/L) to antimicrobials by microdilution, and vancomycin resistance testing.

Isolate	PEN (≥0,25)	COX (≥8)	ERY (≥4)	CLI (≥1)	LIZ (≥8)	CMP (≥16)	TET (≥4)	CIP (≥2)	T / S (≥8/152)	GEN (≥2)	NFT (≥128)	VAN (≥4)	hVISA (screen)	VAN (macrodilution) **	PAP-AUC
30	**> 4**	4	1	0,12	4	**16**	1	0,25	0,12/ 2,38	0,5	16	1	neg.	N/A	N/A
35	0,12	2	1	0,12	4	**32**	1	1	0,06/ 1,19	0,5	16	2	neg.	N/A	N/A
60	**> 4**	4	2	0,12	4	**16**	1	0,5	0,12/ 2,38	0,5	32	**4***	**positive**	16	0.6
73	**4**	2	1	0,25	4	**16**	1	0,5	0,06/ 1,19*	0,5	32	1	neg.	N/A	N/A
77	0,06	2	0,5	0,12	2	**16**	0,5	0,25	0,06/ 1,19	**8***	32	**8***	**positive**	≤ 4	0.5
96	0,06	2	1	0,12	4	**16**	1	0,5	0,12/ 2,38	0,25	16	2	neg.	N/A	N/A
109	**2**	2	0,5	0,12	4	**16**	1	1	0,5/9,5	0,25	16	1	neg.	N/A	N/A
132	0,12	4	**> 8**	0,25	4	**32**	1	0,5	0,06/ 1,19	0,25	16	2	**positive**	N/A	0.5
181	**> 4**	2	1	0,12	4	**16**	1	0,5	0,12/ 2,38	0,5	32	1	neg.	N/A	N/A
192	**> 4**	4	1	0,5	4	**16**	1	0,5	0,12/ 2,38	0,5	16	1	neg.	N/A	N/A
196	0,12	4	**> 8**	0,5	4	**16**	1	1	0,06/ 1,19	0,5	32	1	neg.	N/A	N/A
233	0,06	2	1	0,25	4	**16**	**4**	0,5	0,06/ 1,19	1	16	1	neg.	N/A	N/A
249	0,06	1	2	0,25	4	**16**	2	1	0,5/9,5	1	16	2	**positive**	N/A	0.7
277	**> 4**	2	1	0,12	4	**16**	**4**	1	0,06/ 1,19	**2**	32	2	neg.	N/A	N/A
352	**4**	2	1	0,25	4	**16**	2	0,5	0,06/ 1,19	**2**	32	2	**positive**	N/A	0.5
357	0,06	2	**≥ 8**	0,12	4	8	0,5	0,5	0,12/ 2,38	0,25	16	1	neg.	N/A	N/A
393 A	**> 4**	2	0,5	0,25	2	**16**	0,5	0,5	0,03/0,6	1	32	1	neg.	N/A	N/A
404	0,06	4	1	**1**	4	**16**	**> 8***	1	0,12/ 2,38	0,5	32	1	neg.	N/A	N/A
409	≤ 0,03	1	0,5	0,25	2	8	0,5	0,5	0,06/ 1,19	0,25	16	0,5	neg.	N/A	N/A
448	**0,25**	4	**≥ 8**	0,25	4	**16**	1	0,5	0,12/ 2,38	**2***	16	**> 16***	**positive**	≤ 4	0.6

PEN – penicillin; COX - cefoxitin; ERY - erythromycin; CLI - clindamycin; LIZ - linezolid; CMP - chloramphenicol; TET - tetracycline; CIP - ciprofloxacin; T/S – trimethoprim/sulfamethoxazole; GEN - gentamicin; NFT – nitrofurantoin; VAN - vancomycin; N/A - not analyzed, PAP- AUC - population analysis profile-area under the curve

Breakpoints are indicated in brackets (mg/L). Numbers in bold indicate MIC values above the resistance breakpoint (EUCAST breakpoint tables v10.0)

Isolates with PAP-AUC ratio (AUC_{strain}/AUC_{Mu3}) between 0.9 and 1.3, were considered as hVISA. None of the isolates was a hVISA strain.

* skip-well phenomenon observed

** For vancomycin macrodilution susceptibility of the isolates was tested in the presence of 0; 4; 8 and 16 mg/L of vancomycin.

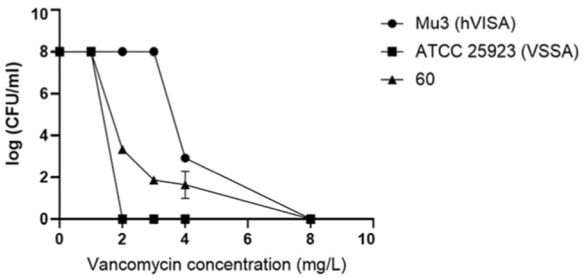

Fig. 1. Population analysis profile curves of vancomycin-resistant isolate. Isolates include reference hVISA Mu3 strain, reference strain for antibiotic susceptibility ATCC 25923 and isolate acquired from the metro train handrail (STAU_60) which was able to grow in the presence of vancomycin up to the concentration of 8 mg/L forming large visible aggregates.

Fig. 2. Aggregates forming by *Staphylococcus aureus* (STAU_60) in the presence of 8 mg/L of vancomycin.

for aureolysin (*aur)*, staphylokinase (*sak)*, complement inhibitor (*scn)*, gamma-hemolysin components (*hlgA*, *hlgB*, *hlgC)* and enterotoxins (*seg*, *sei*, *sem*, *sen*, *seo*, *seu)*.

The SNP analysis comparing the STAU_60 and STAU_60van isolates with reference strain from the same clonal complex CC45 (MCRF184, accession no. CP014791) revealed 341 non-synonymous SNPs. Two amino acid substitutions in the hVISA phenotype-associated proteins VraT (G156E) and VraR (A44T) were detected. No mutations in the other hVISA phenotype-associated proteins including VraS, GraR, GraS, WalKR, LytSR, SaeS, MprF, MsrR and RpoB were found.

However, STAU_60 and STAU_60van carried several mutations possibly related to the resistance phenotype (aggregates) compared to the MCRF184 reference strain. These mutations were found in genes coding proteins related to cell wall synthesis, division and cell-to-cell interactions (Supplementary Table 8), including intercellular adhesion protein SasC (I541L, S168R), capsular polysaccharide synthesis enzyme Cap5C (K206E), polysaccharide biosynthesis protein EpsC (A395V, V577F), a transcriptional regulator of biofilm formation AraC (I696N), cell division proteins YggT (Q55P), FtsA (G331S), FtsK (A1079V) and YtgP (S372P), clumping factors ClfA (S133L) and ClfB (S95R, D604fs) and cell wall biosynthesis protein FmtB (A133V, G1365D).

Moreover, in short reads, a heterogeneous signal in some genes was detected, indicative of the possible co-existence of several different sequence variants. In such sequences, it was not possible to identify the amino acid with a 75 % threshold, and the signal was therefore ambiguous. These genes included genes coding for the adhesin SdrC, protein A, clumping factors ClfA and ClfB, collagen-binding factor Cna, elastin-binding factor EbpS and cell wall biosynthesis protein FmtB (Supplementary Table 9, Fig. 3).

4. Discussion

There is a lack of data on the spread of antimicrobial resistance in an urban environment including public transport due to the episodic nature of most urban environmental studies, lack of systematic surveillance and disparity of surveillance protocols (Cave, Cole, and Mkrtchyan, 2021; Berendonk et al., 2015). Moreover, these studies are disproportionally focused on staphylococci and MRSA, while much less attention is paid to multidrug-resistant gram-negative bacteria (Cave, Cole, and Mkrtchyan, 2021). In our study, we searched for bacterial contamination of public transport in Prague, Czech Republic, by gram-negative and gram-positive antimicrobial-resistant opportunistic pathogens during the time of increased hygiene measures initiated by the COVID-19 pandemic.

In June 2020, 94.6 % of samples in our study were positive for bacterial contamination while in September 2020 it was 97.9 % ($p = 0.004$). As the swabs were not taken immediately after disinfection, the effect of intensified hygiene measures to prevent microbial contamination seems to be limited. The increase in bacterial culture positivity in September 2020 might be explained by the loosening of extraordinary measures due to a low number of COVID-19 cases, and decreased adherence of passengers to hygiene rules (i.e. lower frequency of hand disinfection). The frequency of cleaning and the disinfectants used remain the same (Prague Public Transit Co., personal communication).

Overall, *S. aureus* (3.7 % of all samples) was the most common

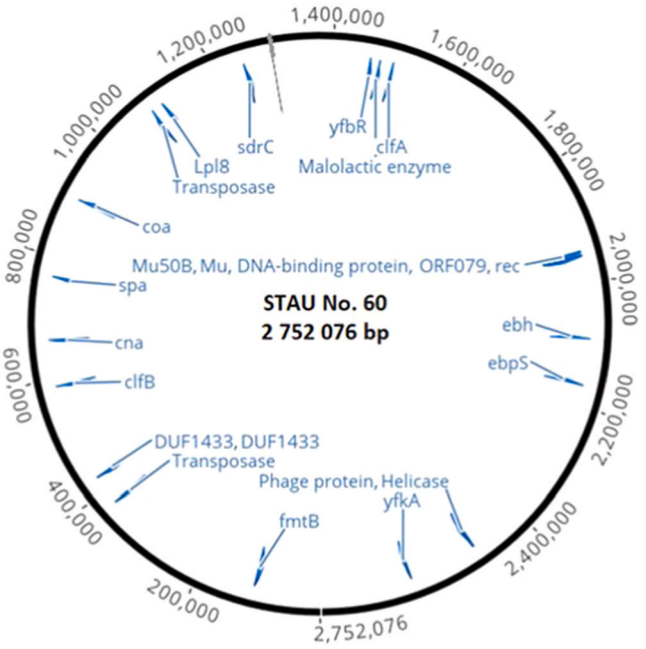

Fig. 3. Genome of *Staphylococcus aureus* (STAU_60) with highlighted genes containing heterogenenous sequences.

opportunistic pathogen in this study. No *S. aureus* isolate was found to be methicillin-resistant. Similarly to our study, but not during the COVID-19 pandemic, no MRSA isolate was found in the Lyon (France) metro (Gaymard et al., 2016) or samples from public transportation, hotels and hospital public areas in London (Otter and French, 2009). On the other hand, in Porto (Portugal), 71.4 % (80/112) of buses and 64.6 % (51/79) of trains contained MSSA isolates and 16.1 % (18/112) of buses, and 8.9 % (7/79) of trains carried MRSA (Mendes et al., 2015). A high prevalence of MSSA and MRSA was detected in public transportation in Columbus (Ohio, U.S.A.) where 68 % (27/40) of buses were positive for *S. aureus* and 63 % (25/40) of the buses were contaminated by MRSA (Lutz et al., 2014).

In this study, we found the presence of Enterobacterales in 1.8 % (10/542) of samples, in addition, 0.7 % (4/542) and 0.4 % (2/542) of samples contained *Pseudomonas luteola* and *Acinetobacter* spp., respectively. While no carbapenem- or colistin-resistant nor ESBL-producing isolate was found in our study, antibiotic-resistant gram-negative bacteria were recently found in public transport in China and shared bikes in Beijing metro stations (Shen et al., 2018)(Zou et al., 2019). An Italian study performed during the COVID-19 pandemic found the most frequent subway contaminants to be coagulase-negative staphylococci, less frequently Enterobacterales (D'Accolti et al., 2023). Interestingly, in the context of the observation of the negative correlation between the presence of *Bacillus* spp and pathogenic bacteria in this study D'Accolti et al. proved that *Bacillus* sp. based probiotic disinfection could inhibit the growth of pathogenic bacteria (D'Accolti et al., 2023), possibly due to production of fengycins, a quorum sensing disruptors, by *Bacillus* sp. (Piewngam et al., 2018).

From available studies, gram-negative contamination of inanimate surfaces and objects is rather low (Chavignon et al., 2021; Allan et al., 2018; Kahsay, Asgedom, and Weldetinsaa, 2019). Compared to gram-positives like staphylococci, the gram-negative bacteria require a sufficiently humid environment for its survival and their persistence quickly fades with decreasing humidity and increasing temperature (Williams et al., 2005; Kramer, Schwebke, and Kampf, 2006). A recent study reported large differences in the microbiome and public transport resistome between cities of different geographical locations (Leung et al., 2021), further studies are needed to identify specific factors influencing the circulation of antimicrobial-resistant bacteria in public transportation.

As the skip-well phenotype observed in three *S. aureus* isolates could be caused by the presence of the resistant subpopulation, i. e. heteroresistance (Landman, Salamera, and Quale, 2013), we performed testing for vancomycin heteroresistance (hVISA). The prevalence of hVISA among human clinical isolates ranges between 0.2 % and 17.3 % but there is high variability in methods and protocols used (Shariati et al., 2020). In this study, six isolates were suspected of hVISA after BHI screening agar testing (Castro et al., 2020) but following PAP-AUC testing (Satola et al., 2011) did not confirm the hVISA phenotype. Interestingly, when the macrodilution, using a higher volume of bacterial culture, was used to confirm the MIC results, one isolate STAU_60 originating from the hospital-serving metro line was able to grow up to $>$ 8 mg/L of vancomycin forming visible clusters. The formation of aggregates is most likely the mechanism of decreased susceptibility to vancomycin in analysed isolate as this phenotypic behaviour together with biofilm production is a way of bacterial protection from the action of antibiotics (Haaber et al., 2012). The cluster formation which could be manifested only during the planktonic growth, explains observed negative results of the plate-based PAP-AUC method for hVISA.

S. aureus with decreased susceptibility to vancomycin belonged to *spa* type t065 (CC45) which was found previously among MSSA (5 %, 5/100) isolated from Czech patients suffering from cystic fibrosis (Tkadlec et al., 2015).

WGS analysis comparing the isolate before (STAU_60) and after vancomycin exposure isolated from media containing 8 mg/L of vancomycin (STAU_60van) did not identify any genetic difference. However, a comparison with closely related vancomycin-susceptible *S. aureus* MCRF184 revealed STAU_60 to carry SNP leading to amino acid substitution in genes VraR (A44T) and VraT (G156E) implied in hVISA phenotype. No mutation was found in other genes associated with hVISA like *graS* and *graR* (Bakthavatchalam et al., 2019; Howden et al., 2010). Interestingly, the STAU_60 isolate had two mutations in the gene coding for surface protein C (SasC) which was previously described as involved in cell aggregates forming (Zhu, Liu, and Sun, 2020). Lack of genetic difference between isolates before and after vancomycin exposure could be result of changes on the level of gene expression regulation possibly in the response to the vancomycin as a cell wall stressor. However, such analysis was beyond the scope of current study.

The hVISA phenotype can be unstable due to fluctuation between the proportion of hVISA and VSSA during the infection. In the study of Miller *et al.*, *S. aureus* isolate was able to rapidly evolve resistance to vancomycin, ceftaroline and daptomycin within a month following treatment with these drugs (Miller et al., 2021). Another example of the unstable clinically important phenotype of *S. aureus* is small colony variants (SCV). The SCVs, often rapidly reverting to the wild-type phenotype, are mainly characterised by the ability to escape the immune system (Kahl, Becker, and Loffler, 2016), but the increased resistance to vancomycin and other cell wall active antibiotics was described among isolates forming SCV phenotype (Vaudaux et al., 2002; Tsuji et al., 2008). Tolerance to cell wall active antibiotics could be possibly linked to the upregulation of proteins associated with the cell wall or cellular adhesion observed in SCV isolates (Zhou et al., 2022). Interestingly, we observed multiple mutations in genes participating in intercellular interactions and cell wall synthesis (e.g. SasC or FmtB) in the STAU_60 isolate. However, a heterogeneous signal detected in the place of some of these mutations implies possibly the presence of more subpopulations different in their vancomycin susceptibility. Such an isolate can be easily missed during routine microbiological testing leading possibly to treatment failure that was previously associated with vancomycin heteroresistance (Howden et al., 2010).

The study has some limitations. There are various protocols for surface screening and as such the protocol (i.e. using dry swabs) in this study could lead to under- or overrepresentation of certain bacterial species compared to other studies. Thioglycolate broth used in the enrichment step supports preferentially the growth of anaerobic bacteria and as such the frequency of strictly aerobic bacteria could be slightly underestimated. Moreover, the lack of the pre-moistening step and replicate samples for each area could lead to a lower microbial recovery (Rawlinson, Ciric, and Cloutman-Green, 2019). Environmental variables (i.e. temperature, and humidity) and the exact time from the last cleaning could influence microbial abundance but these variables were not collected. The effect of detected mutations in vancomycin-resistant *S. aureus* was not experimentally confirmed i.e. by complementation of existing mutation by the introduction of vector carrying wild-type allele, as it was beyond the scope of the present study.

5. Conclusions

Despite the increased hygiene measures during the COVID-19 pandemic, our study shows public transport as a possible source of opportunistic bacterial pathogens like Enterobacterales or *S. aureus*. While the bacteria were mostly susceptible to tested antimicrobials, we detected the presence of vancomycin-resistant *S. aureus* with an unusual resistance phenotype that could be easily missed by standard susceptibility testing. A follow-up after the COVID-19 pandemic study would be needed to detect changes in the level of bacterial contamination in public transport.

CRediT authorship contribution statement

Jan Tkadlec: Writing – review & editing, Supervision, Project administration. **Pavel Drevinek:** Writing – review & editing,

Supervision. **Marie Brajerova:** Investigation. **Vaclav Capek:** Formal analysis. **Marcela Krutova:** Writing – review & editing, Validation, Supervision, Investigation, Conceptualization. **Eva Smelikova:** Writing – original draft, Investigation.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2024.117624.

Data Availability

All data used in the manuscript are included in the manuscript itself, in the Supplementary files or accessible in public databases through provided identifiers (sequencing data)

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