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Biofilm removal in hospital sink drains drives unintended surges in antibiotic resistance

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The prevalence and proliferation of antimicrobial-resistant bacteria is considered one of the critical issues of our time. Wastewater is a habitat for complex microbial communities where bacteria share antimicrobial-resistance genes through horizontal gene transfer. Hospital wastewater plumbing systems are an ideal reservoir for environmental and pathogenic bacteria to interface and exchange antimicrobial-resistance genes. Replacement of contaminated plumbing may be the most intuitive and widely deployed response to the detection and colonization of highly-resistant potentially pathogenic bacteria in hospital sink drains. In this study, we analyzed sink-drain biofilms from six intensive-care patient rooms using shotgun metagenomic sequencing and microbial culture. We show an evident shift in biofilm community structure toward increased abundance of Enterobacteriaceae following plumbing replacement. Higher resistome load and abundance of clinically relevant resistance and typically encountered mobile genes in the newly replaced plumbing was also observed. Taken together, these finding suggest that exchanging contaminated plumbing for new plumbing may actually have the unexpected consequence of increased abundance of Enterobacterales and antimicrobial-resistance genes in the sink drains. Disruption of preexisting complex environmental biofilms may result in an unintended microbial population shifts and a potential subsequent increase in the amount of antimicrobial-resistant Enterobacterales which are targeted for elimination.

Antimicrobial resistance has emerged as a major threat to human health globally[1]. Some of the most concerning antimicrobial resistance is driven by the ability of pathogenic bacteria to exchange antimicrobial-resistance genes. Enterobacterales which can acquire carbapenem resistance genes via horizontal gene transfer are among the most clinically relevant antimicrobial resistant bacteria, with associated infections resulting in high morbidity and mortality[2]. Highly resistant Enterobacterales are of the greatest concern because they not only cause human infections but can also thrive in the environment[3,4] and have shown to transmit from sink drains to patients[5]. Wastewater has been identified as a domain which can facilitate mixing of diverse bacteria enabling the exchange of some of the most consequential antimicrobial-resistance genes[6]. This is particularly influenced by compounds which may be present in wastewater such as antibiotics, disinfectants, antimicrobials, and heavy metals, which can exert selection pressures even in low concentrations or in their derivative forms.

The microbiology of the built environment is heavily influenced by the occupancy and behavior of that environment[7,8]. Hospitals represent a habitat harboring some of the most resistant bacteria carried by patients with heavy antimicrobial usage and a high proportion of human pathogens compared to other built environments[9]. Hospital environment, especially wastewater plumbing systems are an ideal reservoir where environmental and pathogenic bacteria interface and exchange antimicrobial-resistance genes[10,11]. Further, highly antibiotic-resistant pathogens and opportunistic pathogens are now frequently detected in the wastewater plumbing of hospital sinks and toilets with reports of transmission to vulnerable

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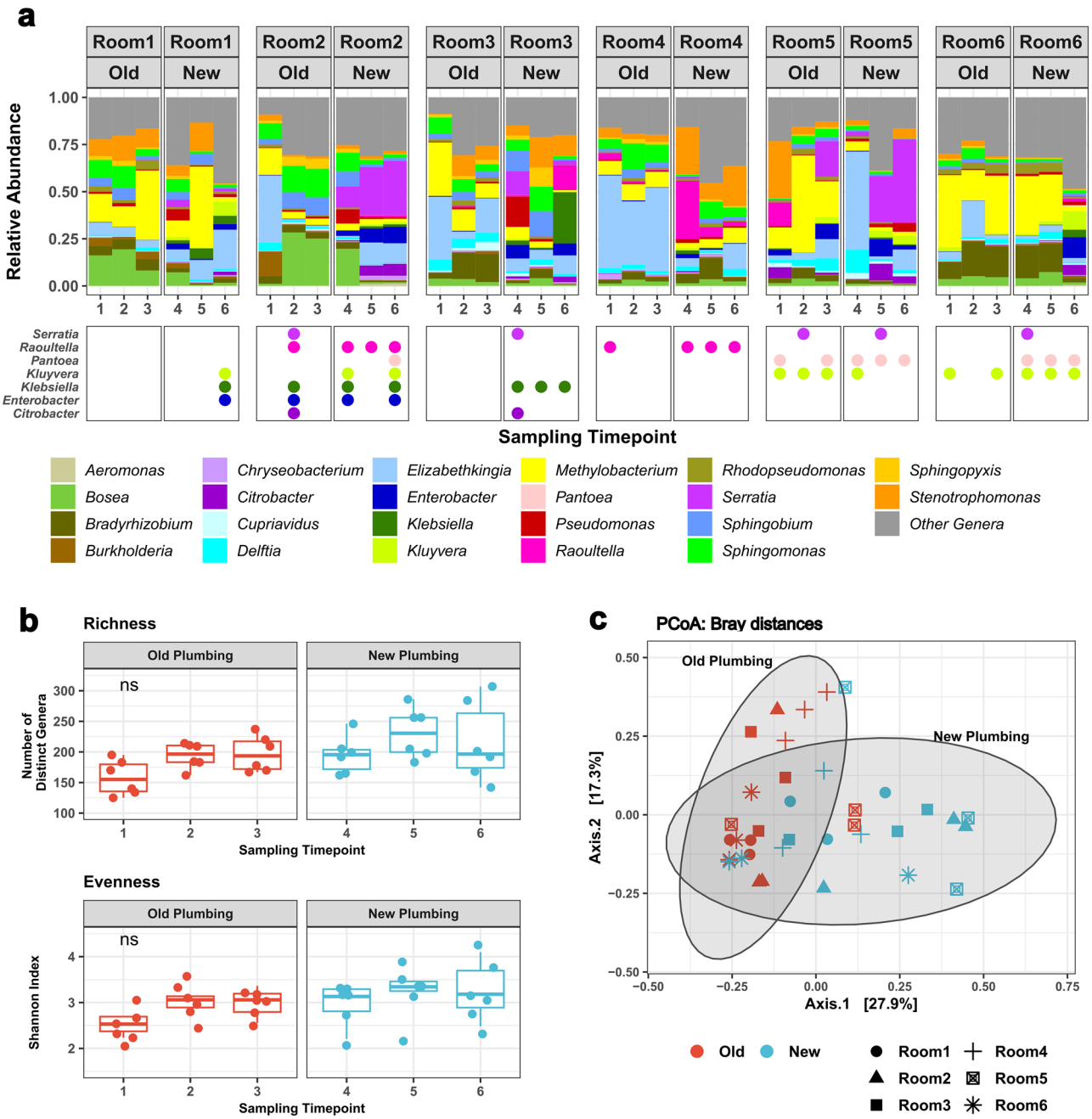

Fig. 1 | Microbial community composition in sink microbiomes. a Top panel: Relative abundance of bacterial genera in the sink microbiomes grouped by old (time points 1, 2, 3) and new plumbing (time points 4, 5, 6) for each room. Abundances of select genera are shown as stacked bars, with remaining low abundance genera aggregated into *Other genera*. Bottom panel: Cultured CPE isolates from sink-drain biofilms. **b** Boxplots showing alpha diversity of the microbial community, represented as richness and evenness (Shannon index), stratified by old plumbing (time points 1, 2, 3) and new plumbing (time points 4, 5, 6). The horizontal box lines represent the median and the inter-quartile range. No evidence of change observed over time (Kruskal-Wallis; p value > 0.05). **c** Beta diversity at genus-level among microbial communities of sink metagenomes, explored by principal coordinate analysis of pairwise Bray-Curtis dissimilarity indices (ellipse indicate 95% confidence interval of samples belonging to old and new plumbing). Axis 1 and 2 represent the axes that explain the greatest amount of variation.

patients[3]. Environmental reservoirs such as sinks in patient care areas contaminated with highly-resistant bacteria are frequently targeted for mitigation under the auspices of patient safety. Intervention measures have included replacement of plumbing[12–17], and/or the use of chemical disinfectants such as bleach[14], acetic acid[15,18], and hydrogen peroxide[13]. Plumbing replacement is a widely implemented intervention strategy and was found to be a relatively more effective method in mitigating outbreaks[4]. However, duration of follow-up after many intervention studies has been short, and the longer-term impact on plumbing-associated bacterial communities remains unevaluated[14–16,19–22].

Results

Changes in sink microbial communities using culture-based assay

The culture data highlights that, sink drains were positive for multiple species of CPE (Fig. 1a bottom panel) more frequently in old plumbing (39%; 7/18) than in newly replaced plumbing (89%; 16/18). Two of the six sinks (Room1 and Room3) that were CPE-negative by culture at all sampling time points for three months prior to plumbing replacement, were detected to be positive for CPE after plumbing replacement. All six sinks with newly replaced plumbing tested positive for CPE at least once with one

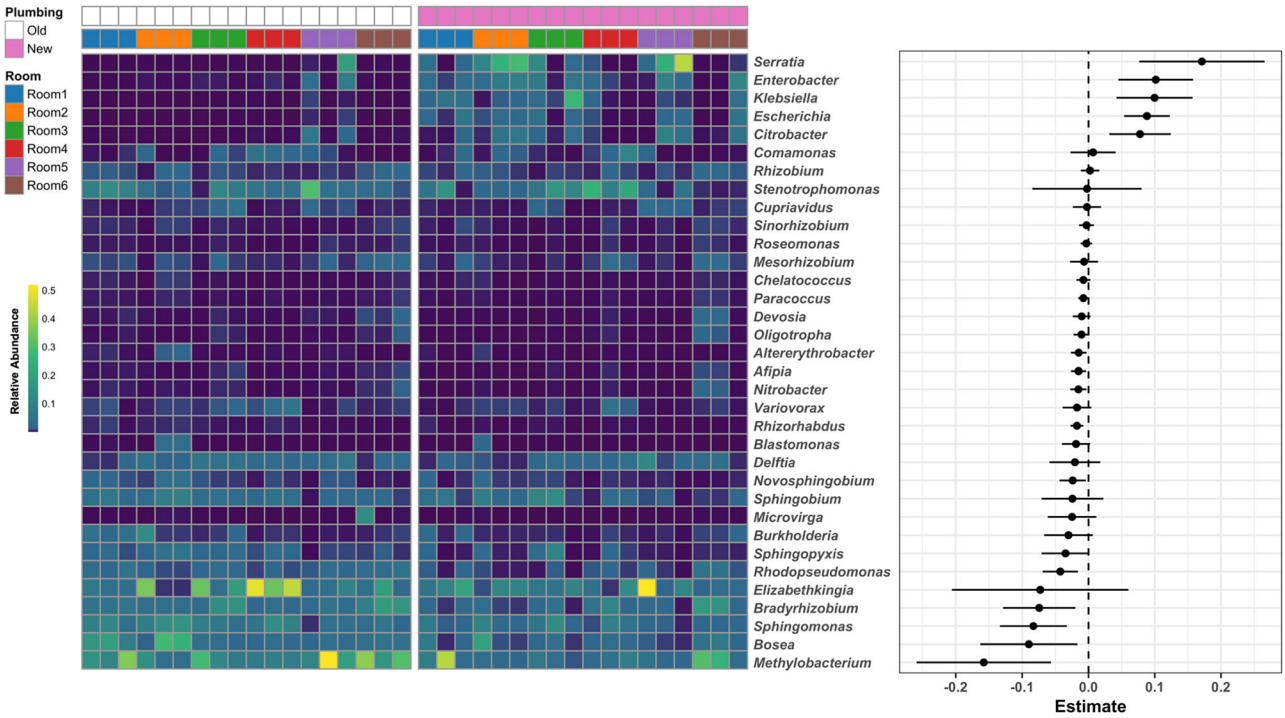

Fig. 2 | Relative abundance (left panel; heat map) and estimates using linear mixed effects modeling (right-panel; points indicate estimate values, with line showing 95% CI) for significantly differentially abundant genera in new sink plumbing compared to old plumbing samples. Samples are grouped by old and new plumbing, with samples collected from same room further grouped together (as indicated by annotation bars at top). (See Supplementary Table S2 for estimates, 95%CI, *p*-values, and q-values for each taxa).

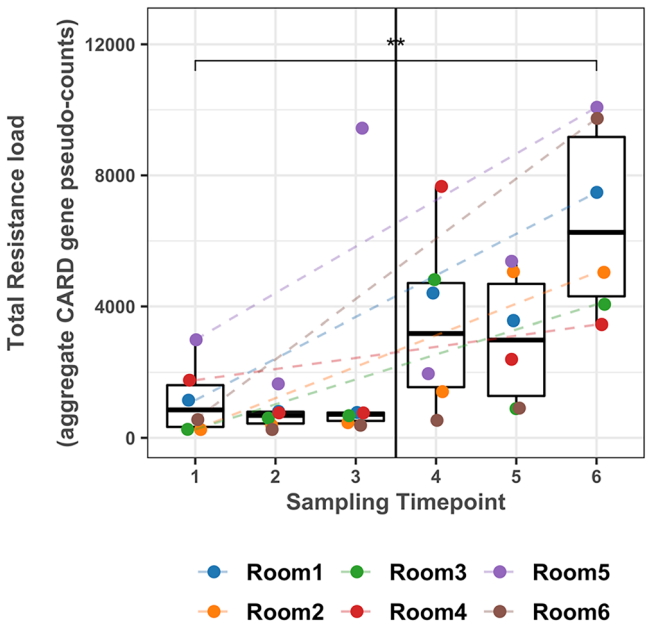

Fig. 3 | Box plots showing total sum abundances of resistance genes (aggregate pseudo-counts; see Methods for details) in sink biofilms; stratified by old plumbing (time points 1, 2, 3) and new plumbing (time points 4, 5, 6). The horizontal box lines represent the median and the inter-quartile range. There is a significant increase in total resistome load in old plumbing samples collected at time point 1 compared to new plumbing samples collected at time point 6 (two-sided paired Wilcoxon test; $n = 12$; $p = 0.002$).

or more CPE species. In all but one room (Room 1), CPE isolates were cultured immediately after plumbing replacement (sampling timepoint-4) and were persistently detected in the subsequent months. Occurrence of CPE detection in sink drains was significant higher ($p = 0.006$) after plumbing replacement. Prevalent CPE species included *Serratia sp., Raoultella sp., Pantoea sp., Kluyvera sp., Enterobacter sp., and Citrobacter sp.*, which were all found to carry bla_{KPC} as confirmed by PCR. Cumulative incidence rate of CPE isolates per sampling event increased from 0.89 pre a to 1.83 post-plumbing exchange.

Effect of plumbing replacement on sink microbiome profile

Taxonomic diversity obtained from metagenomic analysis revealed a largely dissimilar and variable community composition and structure across sinks, samples, and time points (Fig. 1a top panel). At genus-level, replacing the plumbing showed no significant changes in the community richness and evenness (Fig. 1b, c). Despite no significant changes in the overall diversity, there was a significant increase in the Enterobacterales population post-plumbing replacement including genera commonly associated with nosocomial infections and antimicrobial resistance such as *Serratia, Enterobacter, Klebsiella* and *Citrobacter* (Fig. 2 and Fig. S1). In contrast, the relative abundance of slow-growing, native dwellers of water environments like *Methylobacterium, Bosea, Sphingomonas* and *Bradyrhizobium* were negatively associated with plumbing exchange.

Effect of plumbing replacement on sink resistome profile

The shift in bacterial community structure towards Enterobacterales after plumbing replacement was positively associated with the significant increase in total resistance gene load (Fig. 3). Across all six rooms the total resistome load at the sink drain increased following the replacement of plumbing.

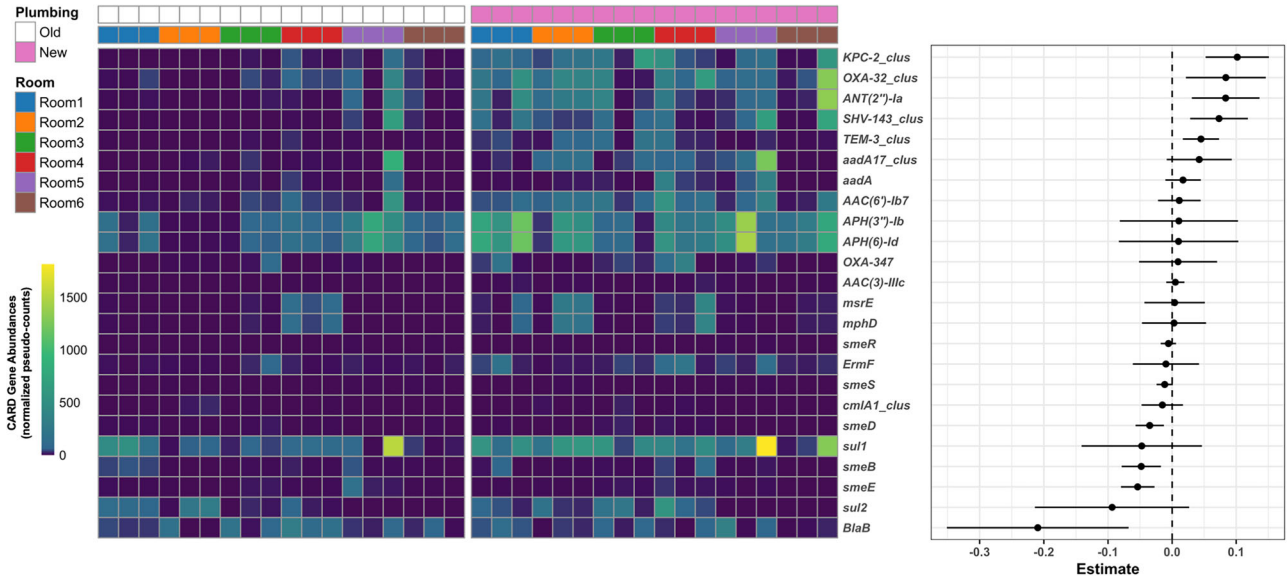

Fig. 4 | Relative abundance (left panel; heat map) and estimates using linear mixed effects modeling, (right-panel; points indicate estimate values, with line showing 95% CI) for significantly differentially abundant antimicrobial-resistance genes in new sink plumbing compared to old plumbing samples. Samples are grouped by old and new plumbing, with samples collected from same room further grouped together (as indicated by annotation bars at top). The annotation bar to the left indicates the resistance class for resistance gene. (See Supplementary Table S2 for estimates, 95%CI, *p* values, and *q*-values for each gene).

Relative abundances of genes conferring resistance to beta-lactams (bla_{KPC-2}, bla_{OXA-32}, and bla_{TEM-3} clusters) and aminoglycosides that are mostly plasmid-borne and frequently found in CPE, were significantly higher in the new plumbing (Fig. 4). As shown by the pairwise analysis (Fig. S2), relative abundance of these genes and Enterobacterales were strongly correlated. New plumbing with noticeably higher bla_{KPC} abundance corresponded with a remarkably higher abundance of *Klebsiella, Enterobacter* and *Serratia*.

Discussion

Having experienced a prolonged outbreak of carbapenemase-producing Enterobacterales (CPE) associated with wastewater plumbing contamination and persistence in our institution, we set out to monitor the dynamics around removal of sink plumbing and recolonization of the in situ biofilm in the newly replaced sink drains. We studied six intensive care room hand wash sinks, all of which had similar design. Sink drains were sampled at monthly intervals for three months before and after plumbing exchange with an envision to understand the effect this intervention would have on the sink microbiome. Paired bacterial culture and shotgun metagenomics of the collected drain biofilm was performed at monthly cadence to track the microbiome shift. It has been established in many recent reports that sink-drain biofilm are one of the main sources of CPE transmission to patients admitted to ICUs[23–26]. Number of sinks and the time frame were relatively small which is an inherent limitation of the study but, we wanted to understand at the microbiome level the recolonization of resistance pathogens like CPE immediately and then over time. It is critical to understand what taxa (and resistance genes) re-establish in newly replaced sink drains and how quickly they establish given the context of hospital ICU rooms and the intervention which is often implemented.

To assess whether variation due to sampling and subsequent shotgun sequencing could influence the observed taxonomic or resistome patterns, we quantified and compared the DNA yields between old and new plumbing samples did not differ significantly (Welch's $p = 0.134$), indicating comparable biomass recovery across groups. We also evaluated the metagenomic capture using Nonpareil coverage (Fig. S3), which demonstrated uniformly high sequencing coverage for all metagenomes, with mean estimated coverage of 0.975 (SD 0.016) in old plumbing and 0.966 (SD 0.016) in new plumbing, and no significant difference between groups (Welch's $p = 0.098$). The tightly overlapping Nonpareil curves confirm that sequencing effort was sufficient to capture the vast majority of microbial diversity in both groups.

Selective culture methods used for the screening, exclusively detect CPE colonizing the sink drains. The effect of plumbing replacement on total viable bacteria was not measured. This is reflected in the differences observed between culture profile and relative abundance from shotgun metagenomics (Fig. 1a- top and bottom panels). While selective culture enriches for CPE, shotgun metagenomics provides a comprehensive microbial profile of the biofilm. Detection of specific bacterial taxa using culture methods doesn't necessarily translate to higher relative abundance in the microbiome and vice versa. Using culture, *Klebsiella, Kluyvera, Pantoea, Raoultella* were most frequently detected CPE genera in the newly replaced sink drains. Isolates belonging to these genera frequently carrying the carbapenemase gene, bla_{KPC}, have been isolated from patients and plumbing in this setting[23,24] and, all the CPE identified from culturing plumbing biofilm in this study were positive for bla_{KPC}. We speculate that a few factors may be driving the recolonization of Enterobacterales in the new hospital sink plumbing after the exchange; the influx of bacteria from sink usage, antimicrobial selective pressure, microbiome composition of the biofilm in the connected wastewater plumbing causing retrograde transmission and perhaps the differential growth rates of various species colonizing the biofilm. For example, slower growing genera such as *Methylobacterium*[27], *Sphingomonas*[28] and *Bradyrhizobium*[29] shifted to significantly less abundant after exchange with increases in faster growing Enterobacterales[30] (*Serratia, Enterobacter, Klebsiella* and *Citrobacter*) (Fig. 2). Based on the significant differentially abundant genera in old plumbing viz., *Methylobacterium, Bosea, Sphingomonas*, are associated with relatively slower growth rates compared to Enterobacterales which may have benefitted their populations in the recolonized biofilm in the new plumbing. Additionally, carbapenemase genes that they harbor may have provided the selective advantage under the antibiotic pressure.

In the microbiome genera- Serratia, Enterobacter and Citrobacter were found to be positively associated with bla_{KPC} and other clinically consequential antimicrobial-resistance (AMR) genes (Fig. S2 -cluster # 2) and in contrast *Methylobacerium, Rhodopseudomonas, Nitrobacter, Bosea* are negatively associated. In the context of sink-drain biofilms, biotic communities that are native to wastewater environments may protect against

AMR by maintaining a high level of microbial biodiversity, which may employ various direct and indirect mechanisms to outcompete and inhibit the growth of resistant and pathogenic bacteria[31]. Additional studies will be necessary to evaluate relationship between biotic community colonizing sinks and resistant pathogen populations.

As evident from alpha and beta diversity of the microbial communities of old and new (Fig. 1b, c), shift in microbiome profile was not significant across the sinks. This is in contrast to the shift in microbiome diversity seen in the setting of disinfection of hospital surfaces where there was a decrease in diversity[9]. In another study where chemical intervention was deployed in colonized hospital sink drains, significant increase in microbiome diversity in the intervention and post-intervention phase was observed[32]. In a longitudinal study (>8 yrs), hospital sink microbiomes were found to have stable composition with low turnover index as compared to other surfaces in the hospital environment which have opportunities of human microbiome seeding[33]. Increased relative abundance of genera belonging to Enterobacterales namely, Serratia, Enterobacter, Klebsiella and Citrobacter was nevertheless observed in the newly replaced pluming (Fig. 2). Studies have demonstrated rapid recolonization with drug-resistant potential pathogens after hospital plumbing exchange[15,16,26], and this systematic study supports those findings, but highlights in much greater detail the associated wider microbiome shifts that occur over short timeframes when sink drains are replaced. To investigate the potential effect of external factors, we compared the room attributes and patient factors that might have affected the microbial community in the sink-drain biofilms. Whilst power was limited, there were no statistically significant differences in factors that directly or indirectly impact the sink usage in the rooms before and after plumbing replacement (Table S1). Environmental conditions such as room temperature, faucet water temperature and relative humidity in these rooms across the study timeframe were maintained to the hospital operational standards, and therefore should have negligible effect on the microbiome.

Coinciding with the increase in Enterobacterales, the total resistome load was found to be significantly higher in newly replaced plumbing (Fig. 3). Re-emergence and detection of antimicrobial resistance including bla_{KPC}, bla_{NDM} in newly replaced sink drains and p-traps has been reported on multiple instances[15,16,23,26,34] and often associated with one or more Enterobacterales species. And similar to the present study, reappearance and/or detection was within a few weeks or some instances few months following sink intervention.

Although replacing drain plumbing may seem like an intuitive response when antimicrobial-resistant pathogens are identified, this may have the unintended consequence of destabilizing a microbial community, disrupting colonization resistance, and thus promoting the successful overgrowth of the antimicrobial resistant pathogens which were originally targeted for removal. Rather than relying solely on plumbing replacement, mitigation strategies may be more effective when focused on preventing pathogen establishment in the first place and limiting dispersal from sinks to patients for example, by using barriers. Altering the structure of the sink-drain microbiome alters the function of the community, which likely reflect changes in the ecologic, resistance, and metabolic functions of the microbiome and thereby facilitate colonization with non-native potentially more drug-resistant, pathogenic communities.

We postulate that the rapid recolonization of newly replaced plumbing by Enterobacterales is analogous to human gut dysbiosis[35] after antibiotic exposure or ecological dysbiosis following natural disasters[36] or an invasive event, where a disrupted natural population may be replaced by a less desirable invasive population[37]. The imbalance in case of plumbing replacement is particularly pronounced, especially attributed to the complete loss of biotic communities that was established over prolonged time and a stable bacterial community could potentially prevent colonization of resistant pathogens. A low-diversity community is less resilient to further disturbances and often less functionally robust. Stable microbial communities that characterize a balanced ecosystem suppress but when disturbed, may create ecological void, leaving niches open for the emergence of pathogenic and antibiotic-resistant microbes to colonize and proliferate via transmission to newer interlinked ecosystems[31]. Disruptions in microbial communities can also promote the exchange of mobile genetic elements, allowing antimicrobial resistance genes to spread rapidly and widely further allowing for the promotion and spread of antimicrobial resistant bacteria.

Methods

Sample collection and processing

Sink drain biofilms were prospectively collected regularly at a monthly interval from six patient rooms in medical and neurosurgical intensive care units for three months before and after the plumbing fixtures (drain, tailpipe, P-trap, trap-arm) were replaced. Both the old and the new sink plumbing fixtures used in the study were made of brass material with 17-Gauge thickness, 1-1/4 inch diameter that are commercially available (P/N 760-1 & 701-1, Dearborn Brass®-Oatey, Cleveland, Ohio). Patient rooms are required to maintain within the guidelines as set forth in (American Society for Health Care Engineering) ASHE Standard 170-2017, Ventilation of Health Care Facilities, which states "Patient rooms must 70-75 °F with a relative humidity range of 30-60%". All six rooms in this study were maintained to these standards.

Pre-sterilized wooden applicator sticks (SKU#: 807, Puritan Medical Products LLC., Guilford, ME) were inserted 1 inch below the sink drain and swirled in a circular motion against the luminal surface, equating to approximately 25 cm^2 area to collect representative biofilm samples. Collected biofilm was re-suspended into 1 ml saline solution and vortexed thoroughly to homogenize the biofilm biomass. All sink-drain biofilm samples across this study were collected and processed using the same protocols. Aliquots of homogenized biofilm suspension were processed for culture~~d~~ to quantify carbapenem-resistant organisms by directly plating serial dilutions on Colorex KPC agar (North East Laboratories, Waterville, Maine), as well as after enrichment in tryptic soy broth containing 10 µg ertapenem disc, and subsequent plating on Colorex KPC agar. Species identification of cultured isolates was performed using the VITEK2 (Biomerieux, Durham, NC); isolates were also screened for bla_{KPC} by PCR as previously described[23]. Total DNA was extracted from ~~the~~ 200 µl of biofilm suspension samples using the PowerSoil DNA Isolation kit (QIAGEN) following the manufacturer's protocol. After quantification using picogreen, the extracted DNA was used to construct shotgun metagenomic libraries using the Nextera XT kit (Illumina, Inc.) following the manufacturers' protocols. Sequencing was performed using the Illumina HiSeq 4000, multiplexing six samples per lane to generate approximately 40 million 150 paired-end reads per sample.

Metagenomic analyses

To estimate metagenome coverage and diversity, we used Nonpareil3 (v3.5.5)[38]. Low-quality portions of sequences were trimmed using TrimGalore[39]. Specifically, we discarded reads below an average Phred score of Q25 and a length cut-off of 75 bp, and the first 13 bp of Illumina adapters. Estimation of taxonomic composition of all metagenomics samples was performed with Kraken[40] using NCBI's RefSeq bacterial and viral genomes as reference database (accessed: 02/2018). For each taxonomic level, relative abundances were estimated using Bracken[41]. To remove spurious and low-abundance assignments, only genera with >0.01% relative abundance was considered for downstream diversity and statistical analysis.

For AMR gene abundance estimation, the filtered metagenomic reads were mapped using bbmap[42] against the Comprehensive Antibiotic Resistance Database (CARD, v2.0.1)[43]. As short reads do not provide sufficient resolution to distinguish highly similar AMR gene homologs, we pre-clustered the CARD database ($n = 2448$ sequences) at 90% identity and only retained one representative sequence for each cluster ($n = 1074$ sequences). The aligned BAM files were used for gene counting, normalization and abundance estimation using the approach implemented within ResPipe[44]. Briefly, gene cluster counts were generated for reads that mapped with 100% sequence identity to representative sequence, followed by pseudo-

abundance estimation by normalizing using the gene length as well as lateral coverage of the gene sequence by reads.

Statistical analyses

Within-sample richness and evenness (alpha diversity) of microbial communities were determined by computing the species count and Shannon Index, respectively, using the *vegan* package (v2.5-6)[45] in R (v3.5.2). Pairwise compositional dissimilarity (beta diversity) was quantified using the Bray-Curtis index. To visualize the dissimilarity between samples, we employed Principal Coordinates Analysis on Bray distances, implemented within the *phyloseq* package in R. For differential abundance analysis, genus-level taxonomic relative abundance profiles were converted to arcsine square-root transformed proportions and random effects model estimates were calculated, using room as a random effect to account for within-room heterogeneity. P values obtained from random effects models were corrected for multiple comparisons by computing the False Discovery Rate, with resulting q-values < 0.1 considered statistically significant. Similarly, resistance gene counts were analysed using random effects models directly on the pseudo-counts. Hierarchical all-against-all association testing was performed to find grouped linear associations between genus-level abundances and AMR gene abundances, using *HALLA*[46].

Room attributes collection

For each environmental sampling event, attributes from all six rooms and data on antibiotic usage was gathered from our health system data warehouse (Table S1). Attributes that could potentially have an influence on sink usage and in turn on drain biofilm microbial community (CRE positive patients and antibiotic selective pressure) were included. Data from the three months before and after plumbing replacement for each room were aggregated. Data was collected from the infection prevention and control database established under the University of Virginia Institutional Review Board protocol 18393. Data analysis and patient chart review was done under protocols 18776.

Data availability

The metagenomic sequences are available at NCBI's Sequence Read Archive (SRA) under the BioProject ID: PRJNA587635.

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Author contributions

A.J.M., S.M.K., and K.E.B. conceived and planned the interventions and executed the laboratory work. S.M.K., H.I.P., H.S.G., N.S., K.V., and A.J.M. developed and refined the microbiome analysis methods and analyzed the microbiome data. S.M.K., H.I.P., H.S.G., N.S., A.S.W., D.W.C., K.V., and A.J.M. reviewed the statistical approaches and contributed to the interpretation of the results. S.M.K., H.I.P., H.S.G., N.S., and A.J.M. took the lead in writing the manuscript. All authors provided critical feedback and helped shape the research, analysis, and manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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