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WASHINGTON UNIVERSITY IN ST. LOUIS

McKelvey School of Engineering
Department of Biomedical Engineering

Dissertation Examination Committee:
Gautam Dantas, Chair
Megan Baldrige
Michael Brent
Carey-Ann Burnham
Mike Vahey

# Mechanisms and Prevention of Antibiotic Resistant Organism Spread in Hospitals

by

Erin P. Newcomer

A dissertation presented to
the McKelvey School of Engineering
of Washington University
in partial fulfillment of the
requirements for the degree
of Doctor of Philosophy

December 2023
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Erin Newcomer<br>*Washington University in St. Louis*<br>*December 2023*

*Pour mon chéri, Jacob, et mes chats chéris.*

ABSTRACT OF THE DISSERTATION

Mechanisms and Prevention of Antibiotic Resistant Organism Spread in Hospitals

by

Erin P. Newcomer

Doctor of Philosophy in Biomedical Engineering

Washington University in St. Louis, 2023

Professor Gautam Dantas, Chair

Our quest as a species to treat and prevent disease has led us to develop medical care that would have been inconceivable even one century ago. However, as we learn to overcome previous roadblocks, new hurdles that we hadn't even considered continue to present themselves. Once we learned to understand the germ theory of disease, we sought treatments for infectious diseases, which led us to the development of antibiotics. Antibiotics are responsible for saving countless lives and have ushered in the advances in other fields of medicine like cancer treatment and surgery. Now, antibiotic resistance threatens our ability to treat disease once more. Healthcare environments like hospitals have become an epicenter for antibiotic resistance, due to the condensed nature of both antibiotic usage and highly susceptible patients. Combatting healthcare-associated infections (HAIs) is a current challenge in infectious disease treatment, and the incidence of antibiotic resistance only complicates our efforts. In this thesis, I first study two different mechanisms by which antibiotic resistant organisms (AROs) transmit and cause disease in a healthcare setting. Then, I investigate the impacts of a proposed environmental hygiene intervention to reduce the burden of AROs in patient rooms.

In Chapter 2, we investigated reservoirs of long-term AROs colonization on hospital surfaces (Sukhum, Newcomer *et al*. 2022. Communications Medicine). This study included samples from

the last three months of an old ward's operation, two weeks prior to patient and staff move-in in a new ward, and for the new ward's first year of operation. We collected environmental samples from many surfaces such as sink drains, floors, nurse call buttons, etc, and selectively cultured these samples for AROs. We also collected isolates from patient stool and patient blood stream infections. We found ARO burden to be the highest in sink drains and did not find any increase in sink drain isolates over the year of sampling. *Pseudomonas aeruginosa* and *Stenotrophomonas maltophilia* were our most frequently identified organisms, and we utilized whole-genome sequencing methods to track how they colonized sink drains longitudinally throughout the study. Concerningly, we found one strain of *P. aeruginosa* (of ST1894) that was found before patient and staff move-in, spread to all sinks sampled, and caused 3 patient bloodstream infections across the study. This work highlights how sink drains can function as a reservoir for AROs, and should be a target for future infection prevention interventions.

In Chapter 3, we characterized how *Clostridioides difficile* contaminates hospital surfaces and virulence in current prevalent strains (Newcomer, Fishbein *et al*. 2023. *In submission*). From two wards over 6 months, we selectively cultured *C. difficile* from patient stool and rectal swabs and swabs from surfaces in their rooms. Using genomics analyses, we found some bedrail and keyboard contamination came from a source other than the in-room patient. Despite this, we found no evidence of *C. difficile* transmission that led to *C. difficile* infection CDI, suggesting current infection prevention measures focused on CDI patients may be sufficient. However, though hypervirulent strains may be more likely to cause CDI, we found that more total CDI cases were attributable to non-hypervirulent strains. We identified two patients that were *C. difficile* carriers and were later diagnosed with CDI with the same strain. Thus, we used genomics analyses to further interrogate virulence of clade 1 isolates and identified new associations between *C. difficile*

toxin and binary toxin regulator genes. These data describe how contamination persists in the hospital setting, and how future work must focus on how the accessory genome may contribute to the transition from colonization to CDI.

In Chapter 4, I began to investigate the potential of sink hygiene intervention for reducing ARO burden in hospital sink drains (Newcomer, *et al.* 2023. *In submission*). We used 10% bleach wipes to wipe sink surfaces, and then pumped a foamed peracid-based disinfect into sink drains to break down bacterial biofilms. This multi-phasic study began with a baseline period of no interventions, followed by two rounds of intervention and baseline. Rooms either received no intervention and just normal cleaning practices, a low-frequency intervention (1x/week), or a high-frequency intervention (5x/week). We collected swabs from sink surfaces and selectively cultured for total growth, gram-negative growth, and unique AROs. This intervention successfully reduced both total microbial and gram-negative bacteria recovered from sink drains when compared to baseline phases. It also significantly reduced the proportion of sink drains yielding *P. aeruginosa* and *S. maltophilia* at both intervention frequencies. These results are promising for the potential of this intervention, though future work is necessary to quantify its impact on HAI rates.

Finally, in Chapter 5 I utilized whole-genome sequencing to understand the genomic impacts of the intervention described in Chapter 4 (Newcomer *et al*. 2023. *In preparation*). I used strain tracking methods to identify closely related strain groups of *P. aeruginosa* and *S. maltophilia*, and found the same strains described in Chapter 2 colonizing the same sink drains over 3 years later. The number of unique strains did not significantly decrease during intervention phases, but after intervention there were significantly more new strains of *P. aeruginosa* isolated from intervention rooms than from control rooms. In contrast, the *S. maltophilia* strains present largely stayed the same even after intervention phases. Finally, I found no significant increases in the number of

antibiotic resistance genes present in *P. aeruginosa* or *S. maltophilia* strains during or after interventions. The strains found in intervention rooms also did not appear to acquire mutations more frequently in any specific genes than strains found in control rooms. These findings provide insight into the mechanisms behind long-term colonizing AROs and support the safety of this intervention with respect to antibiotic resistance or mutational adaptations.

# Chapter 1. Introduction

## 1.1 On the historical understanding of medicine and disease

For as long as humans have existed, we have needed medicine. Prehistoric people experienced diseases such as Salmonella[1], Tuberculosis[2], and leprosy[2], and various infections due to injuries. Though rudimentary by today's standards, some medicines, procedures, and practices that were used as early as 60,000 years ago to combat these illnesses[3]. Medical practice in these communities was guided by shamans, or witch doctors, who largely gathered plant- and earth-based medicines for their treatments. Some evidence suggests ancient peoples used yarrow, a plant in the Asteraceae family with astringent properties, to reduce bleeding in wounds, a practice that is still used today[3,4].

Further development of medicinal theory through the ages brings us to late 5th century BCE, when Ancient Greek physician Hippocrates proposed the humoral theory of disease[5]. This theory proposed that the human body contained four humors, or fluids: black bile, yellow bile, blood, and phlegm. If the humors became imbalanced, this would result in disease. Each of these humors was ascribed associations with traits, seasons, body parts, and stages of life to explain their impact; black bile, for example, was associated with melancholy and the cold and dry of winter, while yellow bile with choleric and the heat of summer[6,7]. Unfortunately, these beliefs guided much of medicine through the 19th century CE, resulting in a myriad ill-conceived treatments. Doctors strongly believe that a rebalancing of the humors would cure most diseases, and utilized bloodletting and purging (through emetics or laxatives), which likely were more harmful than beneficial[8].

By the 19th century CE, new theories for the causes behind disease gained traction. Driven by a series of cholera epidemics in London from 1831-1866, a different idea fascinated both scientists

and the public: the miasmatic theory of disease[9]. Closely associated with the humors, Hippocrates also wrote on the subject: this theory contended that miasmas, or 'infected air' (often associated with bad smells emanating from rotting meat, vegetation, or molds) caused disease[9]. Cholera, which we now know is caused by the bacterium *Vibrio cholera*, causes acute diarrhea and was responsible for large epidemics and significant mortality[10]. In an era of rapid growth and industrialization, London grew from a population of 1 million in 1800 to 6.9 million people by 1900[11]. At the time, most homes were built without flush toilets. Waste disposal systems drained directly into the Thames River, and were vastly inadequate to properly handle an increasingly dense and overcrowded population, resulting in several cholera epidemics[10,12]. The palpable increase in foul smells paralleled these frequent epidemics, causing suspicion of the smells themselves and interest in the miasmatic theory.

## 1.2 On early developments in infection prevention and germ theory

A famous story from the time of those cholera outbreaks marks a noteworthy development in our understanding of disease: that of John Snow, an anesthetist who noticed that many cholera patients all used the same water pump on Broad Street in London. He convinced the local government to remove the pump handle, thus forcing locals to go to other sources to retrieve water. This quickly led to a cessation of the epidemic, and Snow used this to point attention towards drinking water contamination from poorly managed sewage. Though not quite developed into a theory on how disease works, it pointed towards contamination rather than the popular miasma theory of 'infected air'[9].

Another story of significant contribution to the growing understanding of disease concerns Ignaz Semmelweis, a Hungarian obstetrician[13]. His story began in 1846, when he was assigned in today's

terms as a Chief Resident in Obstetrics at the Vienna General Hospital. At this hospital, mothers birthed children at one of two clinics; only medical students were instructed at the first, and only midwives were instructed at the second. During this time, ‘childbed fever’ (now called puerperal fever) was a common malady, afflicting many mothers with a mortality rate of approximately 10 percent. The cause of childbed fever was, at the time, attributed to many different causes and often blamed on the mothers; some suggested the shame of being viewed by doctors led to the disease[13].

Semmelweis quickly noted that while the first clinic maintained similar mortality rates to other clinics in Europe, the second clinic had merely a third the mortality rate but did not understand why. When a colleague died after receiving a cut while conducting an autopsy, Semmelweis noted that this colleague suffered from symptoms very similar to those of childbed fever, though he was a man and couldn’t have been exposed in the same way that birthing mothers were. Semmelweis concluded that there must be the same cause for both his colleague’s death and childbed fever, which he dubbed ‘cadaveric particles’. He then went on to explain that the medical students in the first clinic regularly conducted autopsies, while the midwives in the second clinic did not, which explained the difference in the two clinics. Though medical students and midwives both washed with soap and water previously, he implemented mandatory handwashing with a chlorine solution prior to examination of mothers, and the mortality rate due to childbed fever in both clinics fell to nearly 1.3 percent[13].

Finally, by late in the 19$^{th}$ century, the world was prepared to accept the currently accepted theory of disease: germ theory. The earliest contribution in this field was in 1665, when scientists Robert Hooke and Antoni van Leeuwenhoek described microscopic ‘animacules’, or little animals, in visualized in an early microscope[14]. These animacules would later be identified as the group of fungi named Mucurmycetes[15]. However, the existence of these organisms was not connected to

disease until centuries later, when French chemist Louis Pasteur proved that food spoiled due to the presence of microscopic bacteria in the 1860s[16]. He proposed what we know today as germ theory: that these bacteria could cause infection and disease, rather than spontaneous disease due to miasma, humors, or other causes. This theory was finally proven by scientist Robert Koch in the late 1800s. Koch observed rod-shaped bacteria in the blood of cows that died of anthrax and was able to infect mice by injecting them with the infected blood[17]. He would later go on to develop Koch's Postulates, which are four criteria still used today to determine causation between microbes and disease.

## 1.3 On the rise and fall of antibiotics

The development of antibiotics is arguably one of the greatest medical breakthroughs of the 20th century[18]. After the popularization and widespread acceptance of germ theory, the search was on to combat bacterial pathogens. The first anti-infective agent is largely credited to Paul Ehrlich in 1910, who developed a synthetic drug called salvarsan for the treatment of *Treponema pallidum* infection, or syphilis[18]. However, this development was soon after surpassed by another discovery in 1928: penicillin. Alexander Fleming, a bacteriologist at St. Mary's Hospital in London, identified an area around an invading *Penicillium* fungus in which bacteria did not grow[19]. Fleming named the active agent responsible for the antibacterial effect penicillin, but was unable to isolate it. Though it took many years, scientists Ernst Chain and Howard Florey finally isolated penicillin in 1939, and in 1941 it was shown to considerably benefit a patient with a severe infection. The Nobel Prize in Medicine and Physiology in 1945 was awarded to Fleming, Chain, and Florey for their efforts, and their work stimulated the discovery of many antibiotic classes during the 'golden age of antibiotic discovery' of the mid-20th century[18,19]. Antibiotics have been credited with saving

millions of lives from infectious diseases each year, and ushered in the development of many modern procedures such as cancer treatment, organ transplants, and safer surgeries[18].

However, as we have learned from the work of Sir Isaac Newton in physics, "whenever one object exerts a force on another object, the second object exerts an equal and opposite on the first"[20]. As quickly as penicillin and other new antibiotics began to be mass-produced and used clinically, we identified antibiotic resistance that foiled the new treatments[21]. Now, resistance has been identified against nearly all antibiotics that have been developed, complicating treatment and at times resulting in treatment failure[18]. Though it seems ironic that immediately after use of antibiotics we find these resistances, it's largely because we hadn't known to look for them before. Thinking back to Alexander Fleming's discovery of penicillin, we knew that the *Penicillium* fungi naturally produced penicillin to combat bacterial growth[19]. It is then only natural that bacteria themselves would develop mechanisms to resist this antibiotic activity, and studies have since found antibiotic resistance dating back over 30,000 years[22]. The problem of antibiotic resistance continues to grow: in 2013, the CDC declared that humans are now in a 'post-antibiotic era', and in 2014 the World Health Organization warned that the antibiotic resistance crisis is becoming dire[21]. An estimated 1.27 million deaths were attributed to bacterial antibiotic resistance in 2019, and now reports of pan-resistant bacteria with no treatment options are becoming increasingly common[21,23].

Despite these dire warnings, we are unlikely to return to a state like the pre-antibiotic era. Though antibiotic resistance can occur naturally without our intervention, we know that the misuse and over prescription of antibiotics stimulates increased resistance[24]. Any time bacteria are exposed to an antibiotic they can develop resistance, and unnecessary exposures lead to additional resistance. Interventions such as antibiotic stewardship programs emphasize adhering to appropriate treatment

and dosing with antibiotics, and have been shown to successfully reduce the incidence of antibiotic resistance as well as improve patient outcomes[25].

## 1.4 On healthcare-associated infections

Today, we benefit from the developments of many infectious disease scientists, physicians, and care providers before us. We understand germ theory and the mechanisms behind how pathogens cause disease, we have antibiotics to treat bacterial infections, and we have even developed tools and protocols to prevent these diseases. However, even within this age of modern medicine, new challenges arise. One such challenge is the incidence of healthcare-associated infections (HAIs), which are infections that are acquired while receiving care in a hospital or other healthcare facility[26]. These infections are an unfortunate side effect of medical care, affecting nearly 1.7 million patients yearly in the United States alone, contributing to 98,000 deaths yearly[26]. Prevalence rates differ between countries, but on average HAI incidence is around 7.5% of hospitalized patients in high-income countries, and 5.7-19.2% in low- and middle-income counties[27].

There are several factors involved in why HAIs happen, both concerning the patients themselves as well as the healthcare environment. First, hospitalized patients tend to carry risk factors that increase their susceptibility to infection, including severe illness or a compromised immune system[28]. Further, many treatments that are critical for patient care, like chemotherapies or antibiotics, can leave a patient more susceptible to infections[29]. For example, native bacteria, fungi, and other microorganisms make up a healthy 'gut microbiome' in humans that lends the host protection against *C. difficile* infection (CDI). Treatments like antibiotics can damage the gut microbiome and leave the patient more susceptible to CDI[29].

In addition to patients being more at risk of infection, the hospital or healthcare environment also creates increased risk for transmission of pathogens through two distinct mechanisms[30]. One method is by using surfaces like bedrails, floors, staff hands, and equipment, as an intermediate vector for transmission. *C. difficile* is a bacterial pathogen transmitted through the fecal-oral route, i.e., *C. difficile* spores from fecal matter contaminate a surface, and then enter a new patient through the mouth[31]. The high density of patients at risk for CDI in hospitals results in a cycle of greater *C. difficile* burden and more frequent CDI[32]. Alternatively, pathogens that cause HAIs can be acquired directly from the hospital environment itself, rather than another patient. Recent reports have specifically called out plumbing systems, including water, faucets, sinks, and ice machines, for seeding outbreaks of infections[33]. The bacteria responsible for these outbreaks, such as *Pseudomonas aeruginosa*, *Legionella pneumophilia*, *Stenotrophomonas maltophilia,* and *Burkholderia* species have been dubbed opportunistic premise plumbing pathogens (OPPPs). These OPPPs may be common in the outside environment in water sources or soil, but can opportunistically cause infections in already immune-compromised patients[34].

To compound the risk of HAIs, there is increasing concern for the risk of HAIs that are caused by antibiotic resistant organisms (AROs)[35]. As described above, these organisms make HAIs more difficult to treat by reducing the number of treatment options. In some cases, AROs can resist all antibiotic options and cause ‘untreatable’ infections[21]. Many of the AROs responsible for HAIs are termed Urgent and Serious threats by the CDC, and are a top priority for infection prevention and treatment research[36].

## 1.5 On this body of work

Our ability to understand disease and provide care for other humans stands as a testament to the generations of curious minds who have labored for solutions. As shown over the millennia, every

little piece of the puzzle of human health builds upon the last, and what we learn leads to astonishing results. This thesis compiles my contributions to this endless lesson. Though we have come a long way since the days of Drs. Semmelweis and Snow, we are still learning how bacteria utilize environments to spread and cause disease. We have solved some contamination problems with interventions such as hand washing or water treatment, but now struggle with new challenges like *C. difficile* and OPPP colonization of sink drains. Eventually, we may look back at our current practices in the same way we laugh at historical care providers for not doing something as simple as washing their hands. This is the nature of progress, and I hope my work contributes to it in some way. In Chapters 2 and 3, I investigate the two different mechanisms of ARO transmission in hospitals using genomic analyses. Then, in Chapters 4 and 5, I investigate the impacts of a proposed intervention to reduce ARO burden in hospital sink drains. Finally, I conclude with the future goals and next steps of these projects that will be continued by other lab members. I hope my work will continue to benefit patients and be built upon like so many before me, and I will continue my passion for contributing to this field throughout my career in public health.

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# Chapter 2

# Antibiotic-resistant organisms establish reservoirs in new hospital built environments and are related to patient blood infection isolates

The contents of this chapter are adapted from a manuscript published in *Communications Medicine*:

Sukhum KV*, **Newcomer EP***, Cass C, Wallace MA, Johnson C, Fine J, Sax S, Barlet MH, Burnham CAD, Dantas G, Kwon JH. Antibiotic resistant organisms establish reservoirs in new hospital built environments and show high relatedness to patient blood infection isolates. 2022, Communications Medicine, DOI: 10.1038/s43856-022-00124-5.

* = equal contribution

## 2.1 Abstract

Healthcare-associated infections due to antibiotic-resistant organisms pose an acute and rising threat to critically ill and immunocompromised patients. To evaluate reservoirs of antibiotic-resistant organisms as a source of transmission to patients, we interrogated isolates from environmental surfaces, patient feces, and patient blood infections from an established and a newly built intensive care unit. We used selective culture to recover 829 antibiotic-resistant organisms from 1594 environmental and 72 patient fecal samples, in addition to 81 isolates from blood cultures. We conducted antibiotic susceptibility testing and short- and long-read whole genome sequencing on recovered isolates. Antibiotic-resistant organism burden is highest in sink drains compared to other surfaces. *Pseudomonas aeruginosa* is the most frequently cultured organism from surfaces in both intensive care units. From whole genome sequencing, different lineages of *P. aeruginosa* dominate in each unit; one *P. aeruginosa* lineage of ST1894 is found in multiple sink drains in the new intensive care unit and 3.7% of blood isolates analyzed, suggesting movement of this clone between the environment and patients. These results highlight antibiotic-resistant organism reservoirs in hospital built environments as an important target for infection prevention in hospitalized patients.

## 2.2 Plain Language Summary

Patients in hospitals often have a suppressed immune system, putting them at increased risk of infection by bacteria that are resistant to antibiotics, some of which may come from sources in the hospital environment. We sampled multiple different surfaces in an established and a newly built intensive care unit and collected patient infection samples. We tested bacteria in these samples for their resistance to antibiotics and sequenced the genetic code of the bacteria to identify relationships between environmental and patient infections. We found the most antibiotic resistant

organisms in hospital sink drains. Our sequencing data revealed strains of a certain kind of bacteria could form reservoirs and survive in sink drains and also cause patient infections. These results highlight the importance of removing these antibiotic resistant organism reservoirs to prevent infections.

Sukhum, Newcomer et al. evaluate reservoirs of antibiotic-resistant organisms within the built environment and patient samples from an established and a newly-built intensive care unit. The authors demonstrate colonization of sink drains and other sites and show relatedness between environmental reservoirs and patient infections.

## 2.3 Introduction

Healthcare-associated infections (HAIs) are a global challenge, posing a particularly acute threat in intensive care units (ICUs) where critically ill and immunocompromised patients are at elevated risk for infection during their stay[1,2]. Worldwide, HAIs are responsible for an estimated 2.5 million infections every year and are associated with increased morbidity, mortality, and healthcare costs[1,3–5]. The COVID-19 pandemic is associated with further expansion of hospitalized critically-ill individuals[6]. HAIs due to AROs in the ICU can be difficult to treat due to limited treatment options; available options are also associated with toxicity, are poorly tolerated by patients, and may exhibit negative interactions with other drugs[1,7,8].

Many studies and initiatives have focused on trying to limit HAIs through surveillance, prevention, and intervention[1,9,10]. Recent studies have used culture-independent metagenomic sequencing of hospital surfaces to generate an important catalog of the diversity and composition of their resident microbial communities[11–15]. However, metagenomic characterizations are limited in their ability to track viable, antibiotic-resistant strains and remain ambiguous to whether the taxa discovered

on surfaces are environmental- or patient-derived, and/or associated with infections in patients. To better understand relationships between viable antibiotic-resistant organisms (ARO) in the built environment and critically-ill patients, we must determine 1) what hospital surfaces are acting as ARO reservoirs, i.e., surfaces where an organism can be cultured from multiple time points; 2) what are the spatial and temporal dynamics of reservoir colonization; and 3) whether viable ARO strains colonizing the hospital built environment can also be detected from human clinical infections.

There are multiple models proposed for ARO reservoir colonization and transmission in hospitals (Figure 2.9.1a)[1,16–18]. A prominent model is that AROs are shed from colonized patients, frequently through fecal contamination, to surfaces, instruments, and shared equipment in patient rooms (Figure 2.9.1a)[19,20]. High-touch hospital surfaces can act as intermediate ARO reservoirs, and transmission may occur from these reservoirs through patients, healthcare staff, and visitors[10,20–23]. Another model is that AROs are seeded from microbial communities which persistently colonize hospital built environments, particularly plumbing sources, where biofilms form and can act as a reservoir for potential pathogens (Figure 2.9.1a)[24–27]. These models are not mutually exclusive. ARO reservoirs are likely dependent on a given facility's history and modes of transmission likely interact within a hospital[28]. To better understand the colonization and transmission of AROs in the hospital built environment, we leveraged a unique opportunity to sample a newly-built stem cell transplant and oncology (SCT) ICU both before patient and staff occupancy and for one year after ICU establishment. This allowed us to identify and track persistent colonization of sink drains by AROs that began prior to patient and staff occupancy, a facet that has not been characterized in previous studies. As immunocompromised cancer patients demonstrate prolonged duration of ARO shedding and are at high risk of HAIs, the SCT ICU is a

critical environment to study ARO surface colonization and transmission[29–32]. Additionally, we compared this new ICU environment (new ICU) with environmental samples from the established SCT ICU previously housing these patients and staff (old ICU). While previous studies have longitudinally tracked surface and patient samples within an ICU, they have been limited in their ability to discern the impact of the facility built environment from the population of patients and healthcare workers in the facility. Here, the same patients and healthcare providers transitioned between the old and new buildings across the study period, allowing for a direct comparison between their ARO communities.

To track ARO transmission events between patients and ICU surfaces, we collected remnant fecal samples from patients in the SCT ICU who had laboratory studies ordered on fecal samples and isolates from positive blood cultures ordered as part of routine clinical care during the same collection period. From this unique collection of environmental and patient samples, we used selective microbiologic culturing and whole-genome sequencing (WGS) to identify AROs, assess antibiotic resistance, and track strains across time and location.

We found ARO contaminants were rare on most ICU surfaces but prevalent in sink drains in both ICUs, with the old ICU having significantly higher ARO burden in sink drains than the new ICU. AR Enterobacterales, which are frequently associated with fecal contamination, were rarely found on surfaces. In both ICUs, *Stenotrophomonas* spp. and *Pseudomonas* spp. were the two most frequently collected genera; however, different lineages dominated each ICU. *Stenotrophomonas maltophilia* strains formed months-long reservoirs in sink drains in the new ICU with no evidence of strains association with bloodstream infections during our study time period. In contrast, *Pseudomonas aeruginosa* strains formed persistent reservoirs for most of the year in the new ICU in multiple sink drains and showed evidence of shared strains across environmental

samples and patient blood cultures. These results provide evidence that sink drains in the healthcare environment can serve as ARO reservoirs that are associated with human clinical infections.

## 2.4 Methods

2.4.1 Sample collections and culturing

Environmental and fecal samples received a non-human subjects determination by the Institutional Review Board (IRB) of Washington University (201712083). Blood culture clinical isolate collection was reviewed and approved by IRB (201901053) and by the Siteman Cancer Center Protocol Review and Monitoring Committee. We received IRB approval and Siteman Cancer approval for clinical isolates from patients. The IRB granted a waiver of informed consent for the collection of these specimens because they had been collected as part of routine clinical care. We sampled 6 SCT ICU (old ICU) rooms 3 times over the course of 1 month in the old building from December 2017 – January 2018. At each time point, nine surfaces were sampled using Eswab collections (Copan) pre-moistened with molecular water: the foam dispenser, the gown and glove storage area, the bedside rail, the nursing call button, the room floor, the light switch, the computer, the in-room sink handles, and the in-room sink drain. Three swabs were held together to simultaneously sample each surface. We also collected 2 samples of 15 mL in-room sink water directly from the faucet: 1 sample was collected immediately after turning the faucet on, and 1 sample was collected after allowing the water to run for 2 min.

We sampled 6 SCT ICU (new ICU) rooms and communal SCT ICU areas every other week for 5 months and then every month for 1 year in the new building for a total of 21 samplings (Figure 2.9.1b). Samples were collected twice during the first week of sample collections in the new ICU building: the first after construction terminal clean and the second after custodial terminal clean.

Both time points collected were before patients and staff had entered the ICU. At each time point, the same nine patient room surfaces as described above were sampled plus an additional 3 surfaces: the sofa from the patient room, the bathroom toilet from adjoining bathroom, and the sink drain from the adjoining bathroom. We also collected 15 mL of in-room sink water and bathroom sink water. At each time point, we also sampled four communal surfaces: the housekeeping closet drain, the family area floor, the soiled utility room drain, and the vending machine. For each time point in both buildings, we obtained remnant de-identified fecal specimens that had been submitted to the clinical microbiology laboratory for *C. difficile* testing from patients in the same unit as surface swab collection.

Eswab specimens from surfaces, water samples and fecal samples were cultured the same day of sampling. Eswab specimens were vortexed and 90 µL of eluate was used for culture inoculation per plate/test condition. For fecal specimens, 90 µL of specimen was used for culture inoculation. For water samplings, 100 µL of vortexed water sample was used for culturing. All samples were inoculated to each of the following culture medium: Sheep's blood agar (Hardy), VRE chromID (bioMerieux), Spectra MRSA (Remel), HardyCHROM ESBL (Hardy), MacConkey agar with cefotaxime (Hardy), Cetrimide agar (Hardy), and Sabouraud dextrose + chloramphenicol (Hardy). Plates were incubated at 35 °C in an air incubator and incubated up to 48 h prior to discard if no growth (up to 7 days for sabouraud dextrose + chloramphenicol). Two colonies of each colony morphotype were subcultured and identified using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALD-TOF MS) with the VITEK MS system. All isolates recovered were stored at −80 °C in TSB with glycerol.

Isolates recovered from standard-of-care blood cultures during the same time frame of the surface sampling were recovered from frozen stocks in the clinical microbiology laboratory.

2.4.2 Antimicrobial susceptibility testing

Antimicrobial susceptibility testing (AST) was performed using Kirby Bauer disk diffusion, interpreted according to CLSI standards[33]. AST was performed on gram negative bacilli using ampicillin, cefazolin, cefotetan, ceftazidime, ceftriaxone, cefepime, meropenem, ciprofloxacin, levofloxacin, piperacillin-tazobactam, ceftolozane-tazobactam, ceftazidime-avibactam, ampicillin-sulbactam, trimethoprim-sulfamethoxazole, gentamicin, amikacin, fosfomycin, colistin, aztreonam, doxycycline, minocycline, and nitrofurantoin and antimicrobials were interpreted/reported as appropriate for the specific species. We also performed a carbapenamase inactivation assay on all Enterobacterales and *Pseudomonas* isolates that were resistant or intermediate to meropenem or imipenem.

2.4.3 Short read sequencing

Total genomic DNA was extracted from cultured isolates using the Bacteremia kit (Qiagen, Gernmantown, MD, USA) and DNA was quantified using the PicoGreen dsDNA assay (Thermo Fisher Scientific, Waltham, MA, USA). A total of 5 ng/µL was used as input for Illumina sequencing libraries with the Nextera kit (Illumina, San Diego, CA, USA). The libraries were pooled and sequenced on a NextSeq HighOutput platform (Illumina) to obtain 2x150bp reads. The reads were demultiplexed by barcode and had adapters removed with Trimmomatic[34]. Reads are available under BioProject PRJNA741123 (http://www.ncbi.nlm.nih.gov/bioproject/741123). Processed reads were assembled into draft genomes using SPAdes v3.11.0[35]. Assemblies were assessed for quality using Quast v3.2[36] and checkM v1.0.13[37]. Assemblies were considered to have passed quality standards if completeness was greater than 90% and contamination was below 5%. We used Prokka on the assembled genomes to identify and annotate open reading frames[38].

2.4.4 Long read sequencing

Isolates were streaked from frozen stocks onto LB agar and allowed to grow at 37 °C for 48 h prior to extraction. Lawns were scraped from plates into nuclease free water. Genomic DNA was extracted using the bacteremia kit (Qiagen, Gernmantown, MD, USA), with the modification of limiting the vortex step to 2 min to preserve DNA fragment length. A total of 1 ug DNA from each isolate was used as input for library preparation using the Oxford Nanopore ligation sequencing kit and native barcode expansion kits (Oxford Nanopore Technologies, Oxford Science Park, OX4 4DQ, UK). Libraries were pooled and sequenced on a MinION flow cell (Oxford Nanopore Technologies, Oxford Science Park, OX4 4DQ, UK). Raw reads were preprocessed using Filtlong v0.2.0[39] with parameters *–min_length 1000 –keep-percent 95 –target_bases 650000000*. Hybrid assemblies were created by assembling long read sequencing data in Flye v2.8.1[40] and polished with short reads from Illumina sequencing[41]. Assemblies were assessed for quality using Quast v3.2[36] and checkM v1.0.13[37]. Reads are available under BioProject PRJNA741123 (http://www.ncbi.nlm.nih.gov/bioproject/741123).

2.4.5 Genomic taxonomic identification

Following draft assembly, we determined taxonomic identification by ANI, MASH, and MLST. Species were determined if the genome had >75% aligned bases and >95% ANI with the type genome. Assembled genomes were considered to be the same genomospecies if they had >95% pairwise match but no >95% match with a type genome. We compared all assembled genomes against all assembled genomes and all type genomes using dnadiff[42]. If no species were identified, we used Mash to determine genera by comparing assembled genomes against all NCBI reference genomes[43]. After all phages were removed, genera were considered to be the same as the hit/hits with the highest identity. MLST was determined using mlst v2.4[44,45].

2.4.6 Phylogenetic analyses

To create core genome alignments, the gff files produced by Prokka were used as input in Roary[46]. Roary alignments were used to create an approximate maximum likelihood tree with FastTree[47]. Branch length precision was rounded to 0.0001 substitutions per site. The output newick files were visualized and annotated with isolate source data using ggtree (R)[48,49]. Roary pangenome sequences were further annotated using EggNOG v5.0[50].

2.4.7 Isolate groupings based on SNP pairwise distances

Snippy v4.4.3[51] was used to map forward and reverse reads for isolates to the type strain complete genome assembly and to call SNPs. To determine groups, we compared pairwise SNP distances between each isolate pairs of the same species. Isolates were grouped into perfectly reciprocal groups at every pairwise distance cutoff between isolates using igraph[52]. The SNP distance cutoff was set at the lowest SNP value where number of groups plateaued for many thousands of SNPs, indicating that the members of these groups are much more closely related to one another than other isolates.

2.4.8 Antibiotic-resistant gene identification and analyses

We identified acquired antibiotic resistance mutations against aminoglycosides, amphenicols, β-lactam, folate pathway inhibitors, fosfomycin, macrolides/lincosamides/streptogramins, quinolones, rifamycin, tetracycline, and vancomycin using ResFinder[53].

2.4.9 Bayesian phylogenetic analysis of molecular sequences using BEAST 2

Group 1 isolates were long-read sequenced and quality filtered as described above, and the core genome alignment was constructed as above. The core genome alignment was composed of 5964 core genes out of 6986 total genes, which we used as input genes for our time-measured phylogenetic analysis in BEAST v2.6.5[54]. The core genome alignment was converted to a Nexus

file using MEGA X[55]. We used BEAUti v2.6.5 from the BEAST v2.6.5[54] software package to convert the Nexus file into a.xml file for input into BEAST. We chose to use the HKY site model because it allows for some flexibility in substitution rate for different types of substitutions, and catches most major biases[56]. We also used a strict clock model because our sequences are all from the same hospital within just over a year of each other, so we have no reason to suspect different substitution rates for different lineages[56]. Tip dates were determined as the number of days between each sample and the first sample collected. Model diagnostic information and parameter distribution were viewed using Tracer v1.7.2[57]. Individual trees were visualized using FigTree v1.4.4[58] and the consensus tree was visualized using DensiTree v2.2.7[59].

#### 2.4.10 Statistics and reproducibility

Comparative statistics between old and new building samples were normalized by number of samplings. Generalized linear mixed models were used for significance testing, with Room and Week as random effects. In Figure 2.9.2c,d, isolate frequencies were collapsed by Room and then averaged. Error bars indicate standard error. For all main text phylogenetic trees, branches with less than 80% bootstrap support were collapsed, and branches with 80–90% bootstrap support were labeled as such. Supplementary Figures containing phylogenetic trees (Figures 2.10.1c,d, 1.11.2, and 2.10.3a) have a minimum resolution of 0.00055.

## 2.5 Results

#### 2.5.1 AROs were collected and cultured from ICU surfaces, fecal samples, and clinical blood cultures in an old and new ICU

To test models of ARO reservoir colonization and transmission in a hospital built environment setting, we collected 1594 surface samples and 72 patient fecal samples at 24 time points from 6 ICU rooms in 2 buildings. Full metadata for 829 collected isolates has been included as Supplementary Data 1. The first building was the SCT ICU that was located in a well-established

hospital building, the old ICU. The second was a newly constructed SCT ICU (new ICU); after construction was completed on the new ICU, the same staff and patients from the old ICU were all relocated to the new ICU. The old ICU rooms were sampled 3 times, with a week between samplings, during the final month of ICU occupancy (Figure 2.9.1b). New ICU rooms were sampled twice (two days apart) after the completion of construction while the rooms were unoccupied, then once every other week for the first 5 months of patient and staff occupancy ($n = 11$ samplings), then once every month for the rest of the first year of occupancy ($n = 8$ samplings) (Figure 2.9.1b). For both ICUs, we swabbed 10 high-touch ICU surfaces (with an additional 4 surfaces from attached bathrooms in new ICU rooms). We also obtained remnant fecal samples submitted for routine *Clostridioides difficile* testing as well as isolates recovered from standard-of-care blood cultures from patients in the ICU. We utilized selective microbiologic culture on surface and fecal samples to enrich for and culture AROs, including 1) organisms that form colonies on antibiotic media, which we later assessed for resistance phenotypes by antibiotic susceptibility testing (AST), and 2) organisms that are inherently resistant to antibiotics, including *Pseudomonas*, *Stenotrophomonas*, and *C. difficile*[60–62]. Results from AST can be found in Supplementary Data 2. Blood culture isolates were recovered in the clinical laboratory as part of routine clinical methods (i.e., not selectively cultured for ARO) and were retrospectively obtained for during 46 different weeks of the study spanning 61 weeks total. We recovered 566 AROs from surface environmental samples and 164 AROs from fecal samples, and we obtained 99 isolates from clinical blood cultures in the clinical microbiology laboratory.

2.5.2 Sink drains had a high ARO burden compared to other ICU surfaces

To identify potential ARO surface reservoirs, we measured ARO burden (defined as number of different ARO isolates/morphotypes per samples collected) on different surfaces. Cultured bacteria

were identified using VITEK MS matrix assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) (bioMerieux). ARO burden was significantly higher in sink drains than on other ICU room surfaces in both the old and new ICUs (Figure 2.9.2a, GLMM: $p < 0.001$, marginal $R^2 = 0.942$, conditional $R^2 = 0.945$). All other ICU room surfaces had at least a 6-fold lower ARO burden (mean old ICU sink drains: 4.02 isolates/sample collection, mean old ICU other surfaces: 0–0.64 isolates/sample collection, mean new ICU sink drains: 1.59–1.72, mean new ICU other surfaces: 0–0.21). ICU sink water had low ARO burden (mean range of 0–0.02 isolates/sample collection) (Figure 2.9.2a).

*Pseudomonas* was the most frequently detected genus, comprising 235/696 (33.8%) of all isolates cultured from the new ICU and 48/133 (36.1%) from the old ICU (Figure 2.9.2b). The second most frequently identified genus was *Stenotrophomonas* (115/696 (16.5%) in the new ICU and 13/133 (9.8%) in the old ICU). Both genera were found primarily in sink drain samples (215/283 (76.0%) of *Pseudomonas* and 114/128 (89.1%) of *Stenotrophomonas*). Enterobacterales made up 77/696 (11.1%) and 20/133 (15.7%) of all isolates from the new and old ICUs, respectively, but only 7/97 (7.2%) were isolated from surface samples (Figure 2.9.2b). *Candida* spp. isolates were identified in both the new ICU (64/696 (9.2%)) and old ICU (12/133 (9.0%)) with isolates primarily coming from fecal samples (50/76 (65.8%)) and room floor (10/76 (13.2%)). Gram positive AROs, including genera *Enterococcus*, *Staphylococcus*, and *Clostridium*, were found in both the new ICU (63/696 (9.0%)) and old ICU (23/133 (17.3%)). *Clostridium* was recovered from in fecal samples ($n = 5$). *Staphylococcus* and *Enterococcus* were found primarily in blood and fecal samples (52/81 (64.2%) of *Staphylococcus* and *Enterococcus*) and never found in sink drains. (Figure 2.9.2b). These data suggest that in both buildings, sink drains are areas of

substantial concern since they persistently yield cultures of *Pseudomonas* spp. and *Stenotrophomonas* spp., which both include strains capable of causing human infection[63,64].

2.5.3 ARO burden did not increase after patients and staff move in or over one year of sampling in new ICU

Next, we compared ARO burden across ICUs, patient and staff occupancy, and time points. Since there were large differences in ARO burden across surfaces, we separated environmental samples into 2 groups: sink drains and other (Figure 2.9.2c, d). First, we compared ARO differences between the old ICU and new ICU before and after patient occupancy. We found ARO burden was higher in the old ICU than in the new ICU in sink drains (Figure 2.9.2c, GLMM $p < 0.001$, $R^2 = 0.59$) but not on other surfaces (Figure 2.9.2d, GLMM $p > 0.05$, $R^2 = 0.07$). Further, there was no difference in ARO burden before and after patient occupancy (Figure 2.9.2c, d, GLMM $p > 0.05$). When we compared ARO burden in sink drains over time, we found no significant differences between the first week of collection after patients' occupancy in the new ICU and any other time point collected (Figure 2.9.2e, Wilcoxon signed-rank test $p > 0.05$). The same was true for other surface collections (Figure 2.9.2f, Wilcoxon signed-rank test $p > 0.05$), although ARO burden for other surfaces had high variation across weeks (mean range 0.02–0.35). Together, this suggests that there were environmental-associated differences in ARO burden between the old and new ICUs, and that ARO burden did not change after patient occupancy in the new ICU nor significantly increase or decrease during 1 year of collections.

2.5.4 No evidence of AR Enterobacterales reservoirs on surfaces in either ICU

To determine taxa-specific patterns in reservoir colonization, we performed WGS of Enterobacterales, *Pseudomonas*, and *Stenotrophomonas* isolates from environmental, fecal, and blood samples from both ICUs. AR Enterobacterales are some of the most feared AROs for HAIs[8] and many are associated with human fecal colonization[65–67]. We collected 97 isolates from

4 genera of Enterobacterales: *Escherichia*, *Klebsiella*, *Citrobacter*, and *Enterobacter* (Figure 2.10.1A). Isolates were recovered primarily from fecal samples (45/97 (46.4%) of Enterobacterales) and from blood cultures (45/97 (46.4%) of Enterobacterales) (Figure 2.10.1A). *Escherichia coli* was the most frequently detected Enterobacterales species (37/97 (38.1%)), followed by *Klebsiella pneumoniae* (18/97 (18.6%)) (Figure 2.10.1A). Notably, from 1594 surface samples over 24 time points, there were only 7 instances of an Enterobacterales isolate being cultured from an ICU surface sample (Figure 2.10.1B). Of the 7 isolates, 2 were different morphotypes of *Citrobacter freundii* isolated from the same sample with high average nucleotide identity (ANI) (99.99%), suggesting closely-related organisms or morphovariants. Apart from those 2 *C. freundii* isolates, no 2 surface Enterobacterales were the same species and no 2 Enterobacterales were found on the same surface twice (Figure 2.10.1B). These data suggest AR Enterobacterales do not represent ARO reservoirs on any of the sampled ICU surfaces, despite being present in many patient fecal samples.

To determine within species isolate similarity, we compared strain genomes and antibiotic resistance profiles across the two most frequent Enterobacterales species: *E. coli* and *K. pneumoniae*. When we compared multi-locus sequence typing (MLST) profiles of *E. coli* isolates, we found one instance of shared sequence type (ST131) between a surface isolate and a blood or fecal isolate. In a core genome phylogenetic tree, we found no phylogenetic clustering based on isolate type or ICU, except for 3 different *E. coli* morphotype isolates all taken from the same fecal sample and sharing 99.98% ANI (Figure 2.10.1C). To determine if antibiotic resistance profiles vary by sample type or location, we determined phenotypic susceptibility and identified antibiotic resistance genes (ARGs) using Resfinder[68,69]. By Kirby Bauer disk diffusion, interpreted according to Clinical and Laboratory Standards Institute (CLSI) standards, 2/37 blood *E. coli* isolates were

not resistant or intermediate resistant to any tested antibiotics. AST profiles varied across the *E. coli* isolates with isolates frequently resistant to ampicillin (23/37), cefazolin (20/37), ciprofloxacin (19/37), and levofloxacin (19/37) (Figure 2.10.1C). We found 9/37 *E. coli* isolates were resistant to cefepime, including the 1 surface isolate, and no *E. coli* isolates were resistant to meropenem. We found 24/37 *E. coli* isolates were resistant to multiple antibiotics with 20 isolates resistant to four or more drugs. *E. coli* isolates harbored various ARGs (Figure 2.10.1C), but ARG profile did not vary by sample type or location.

In *K. pneumoniae* isolates, we also found no phylogenetic clustering based on isolate type or ICU building (Figure 2.10.1D). Only one *K. pneumoniae* isolate, which was recovered from patient blood culture, demonstrated meropenem resistance, but it was negative for carbapenemase activity using the Carbapenem Inactivation Method[33]. 3/18 *K. pneumoniae* isolates were resistant to cefepime. 10/18 *K. pneumoniae* isolates were resistant to multiple drugs with 7 isolates resistant to four or more antibiotics. *fosA*, *oqxA*, and *oqxB* were found in a majority of isolates, 14/18 (78%), 17/18 (94%), and 17/18 (94%) respectively (Figure 2.10.1D). Together, these data show that while AR Enterobacterales were recovered from fecal specimens and can be a cause of blood stream infection in patients in the ICUs, these isolates were rarely found on surfaces, with no clear relationships between source of isolation and MLST, building, or antibiotic resistance. This suggests patient fecal contamination of sampled surfaces in these ICUs was rare and did not lead to ARO reservoir formation.

2.5.5 Stenotrophomonas maltophilia strains are found persistently across one year of sampling in single ICU rooms

While *S. maltophilia* is predominantly found in environmental water sources, the species is an emerging pathogen associated with HAIs, particularly in immunocompromised patients; these

infections are associated with substantial case fatality rates[64], primarily because of the intrinsic antimicrobial resistance of this microorganism and the vulnerable patient population that it affects. *Stenotrophomonas* spp. were isolated from every week sampled, except for the first week of collection in the old ICU, although the ratio of *Stenotrophomonas* isolates to all collected isolates varied over time (Figure 2.9.3a). Among 128 isolates identified as *S. maltophilia* by MALDI-TOF MS, ANI species identification and MASH genus identification typed them as 54 *S. maltophilia* isolates, 1 *S. lactiubi*, and 53 *Stenotrophomonas* spp. (not otherwise specified) in 9 genomospecies groupings (Figure 2.9.4b). When we compared MLST and core genome phylogeny of *S. maltophilia* isolates, we found that sequence type and phylogenetic clades were not shared across ICUs (Figure 2.9.3c, d, Figure 2.10.2). Only two sequence types were identified on the same respective surface over multiple weeks, suggesting that these surfaces acted as reservoirs (Figure 2.9.3c, blue sequence types). *S. maltophilia* of ST27 was found 9 times over 35 weeks, and *S. maltophilia* of ST1 was found 13 times over the course of a year (56 weeks), including before patient and staff occupancy (Figure 2.9.3c). Both sequence types remained in the same room, with no evidence of crossover between rooms in the new ICU (Figure 2.9.3c). Phenotypic susceptibly demonstrated no isolates with trimethoprim-sulfamethoxazole or minocycline resistance, one isolate with levofloxacin resistance, and 34/54 isolates with colistin resistance.

2.5.6 *P. aeruginosa* were diverse and found persistently across one year of sampling and in all 6 new ICU rooms

While commonly found in the environment, *Pseudomonas* spp. have a long history of causing HAIs[60,70–73]. Studies have shown that *P. aeruginosa* reservoirs established in hospital built environments can lead to infections and outbreaks[74–78]. However, it is unclear when these reservoirs became established, relative to patient or staff occupancy of the healthcare environment, and how pervasively *Pseudomonas spp*. may colonize ICU surfaces. We recovered

more *Pseudomonas* spp. isolates than any other genus during our collections (Figure 2.9.2b). MALDI-TOF MS identified 283 *Pseudomonas* spp. isolates. *Pseudomonas* spp., and particularly *P. aeruginosa*, isolates were collected at every time point in the study period, including before patient occupancy (Figure 2.9.4a, b). After ANI species identification and MASH genus identification, we found 155 *P. aeruginosa* isolates, 71 *Pseudomonas* spp. isolates in 13 genomospecies groupings, and 54 isolates from other *Pseudomonas* species (Figure 2.9.4c). Most *P. aeruginosa* isolates were from surface samples (80%); 11% were from fecal samples; and 9% were from blood cultures (Figure 2.9.4c). We did not find overlap between any other *Pseudomonas* spp. isolates from patient blood cultures and environmental samples.

When we compared MLST and core genome phylogeny of *P. aeruginosa* isolates, we find that isolates from different ICUs fall into different clades and strain types (Figure 2.9.4d,e, Figure 2.10.3). To understand the genomic context of *P. aeruginosa* isolates, we compared the genomes of isolates recovered from surface and patient sampling with 172 reference *P. aeruginosa* genomes downloaded from NCBI (Supplementary Data 3). Reference genomes were phylogenetically diverse and fell into 3 categories: (i) isolates from clinical infections, (ii) AR isolates from the CDC with known antibiotic resistance, and (iii) environmental isolates that had been collected from water and waste projects. The isolates we collected from both the old and new ICUs spanned most of the diversity of *P. aeruginosa* with no distinct clustering between collected ICU surface isolates and environmental, clinical, or AR isolates (Figure 2.9.4d). Although there were no distinct clades based on isolate building or surface source, we do find that our isolates form a number of clades with highly-related surface isolates (Figure 2.9.4d). These frequently corresponded with sequence type. There were two cases of overlap in sequence type between the old and the new building (Figure 2.9.4e). ST17 was found in sink drains in both the old and new

ICU and found in a blood culture in the new ICU. ST170 was found in surface samples in the old ICU and a patient fecal sample in the new ICU (Figure 2.9.4e). Notably, *P. aeruginosa* of ST1894 was recovered from the same sink drain beginning before patient occupancy and continuing through for the full year of collection in the new ICU. This repeated isolation of ST1894 suggests that it may have established a continuous reservoir in this room in the new ICU. Furthermore, isolates of *P. aeruginosa* of ST1894 were also recovered from sink drains in all 6 sampled ICU rooms and were found across 5 or more time points in 5/6 sampled ICU rooms (Figure 2.9.4e), suggesting this colonization and persistence is more widespread. Finally, we found that 3 blood culture isolates (3.7% of all blood culture isolates tested) also belonged to ST1894, which prompted a higher resolution comparative analysis of all ST1894 strains, due to its potential to contaminate the environment and be associated with bloodstream infections.

2.5.7 Antibiotic resistance in *P. aeruginosa* isolates varies between the two ICUs

To determine if antibiotic resistance profiles vary by location, we determined phenotypic susceptibility using antibiotic susceptibility testing (AST) and identified ARGs in assembled genomes using Resfinder[53]. *P. aeruginosa* are defined as AROs because of their intrinsic resistance to many aminoglycosides, tetracyclines, β-lactams, and quinolones;[60,79] we performed ASTs for 14 antibiotics for all *Pseudomonas* isolates to measure acquired resistances to β-lactams, cephalosporins, carbapenems, penicillins, fluoroquinolones, aminoglycosides, and polymyxins. AST profiles were similar across *P. aeruginosa* of the same sequence type (Figure 2.9.5). *P. aeruginosa* isolates of ST1894 were largely not resistant to the antibiotics tested. *P. aeruginosa* isolates of ST282 were resistant to meropenem (11/15) and gentamicin (15/15). *P. aeruginosa* isolates of ST308 were resistant to meropenem (6/8), imipenem (5/8), ciprofloxacin (8/8), levofloxacin (8/8), and gentamicin (8/8). As different sequence types dominated the different

ICUs and resistance profiles were similar across sequence types, we found trends in resistance to be different between the two ICUs with isolates from the old ICU having a higher percentage of resistance to meropenem and imipenem than *P. aeruginosa* isolates from the new ICU (new ICU: 7% imipenem, 7% meropenem and old ICU: 40% imipenem, 55% meropenem) (Figure 2.9.5).

Much like the AST profiles, the ARG profiles also appeared to be linked to sequence type (Figure 2.9.5). Nearly all *P. aeruginosa* isolates carried the resistance genes *aph(3')-IIb* (153/155), *blaPAO* (154/155), *catB7* (151/155), and *fosA* (155/155). Isolates from ST282 were the only identified to contain the aminoglycoside resistance genes *aac(6')-IIb* (15/15) and *aadA1b* (15/15), which could explain the phenotypic resistance to gentamicin. All isolates from ST 1894 carried the β-lactam resistance gene *blaOXA-396* (52/52), while it was less common in other sequence types (35/103). *P. aeruginosa* is also capable of developing resistance to carbapenems (meropenem, imipenem), fluoroquinolones (ciprofloxacin, levofloxacin), and aminoglycosides (gentamicin) by chromosomal point mutations, rather than acquisition of ARGs[80,81].

<u>2.5.8 *P. aeruginosa* Group 1 strain was found across 1 year of sampling and in both environmental and patient samples</u>

While MLST has been used previously to describe strains and outbreaks, it is limited to a small number of genes or alleles and does not enable genome-resolved understanding of strain relatedness. Accordingly, here we utilized WGS data for each *P. aeruginosa* isolate to perform an in-depth analysis of similarity across genomes. We calculated pairwise SNP distances by mapping quality filtered short-reads from all *P. aeruginosa* isolates to a high-quality, long-read sequencing-assembled genome of the first temporal occurrence of ST1894, with a mean of 89.8% of reads mapped to the genome. We then used a grouping technique on *P. aeruginosa* isolates to find fully

reciprocal groups[82]. We compared pairwise SNP distances between *P. aeruginosa* isolate pairs and iterated through each unique SNP distance cutoff to filter the isolate pairwise network list (Figure 2.9.6a, b). For each SNP cutoff, we determined the number of complete subgraph groups, defined by each node in the group was connected to every other node in the group, and isolates per group. The number of *P. aeruginosa* groups rose initially from 3 to 18 groups as SNP distances increased from 0 to 377 SNPs. After a peak at 756 SNPs with 20 groups, the total number of groups slowly decreased to a plateau of 14 groups at 2743 SNPs (Figure 2.9.6c). From this, we determined an appropriate SNP cutoff that separated closely-related isolates from other groups was 2743. Using this definition, *P. aeruginosa* isolates fell into 14 groups, with the largest group (Group 1) including 53 isolates (Figure 2.9.6d). Only three groups had isolates that spanned patient and environmental isolates: Group 1, Group 6, and Group 12. Group 1 had no more than 11 SNPs between isolates and included isolates from blood cultures and environmental samples. 52/53 of the isolates in Group 1 were ST1894, and the remaining isolate was unidentified but had 5/6 alleles identical to ST1894. The isolates in this group persistently and pervasively colonized new ICU sink drains and were cultured from sink drains 49 times across 56 weeks (Figure 2.9.6f). Aside from sink drains, Group 1 was also found in 3 patient blood cultures, 1 of which was isolated from a different ward in the same building (Figure 2.9.6f). 1 isolate from Group 1 was isolated from the gown and glove personal protective equipment box located just outside the room. All isolates within this group were within 11 short-read SNPs of each other. Group 2 (ST17) was found once in a sink drain in the old ICU, 7 times in the bathroom sink drain of Room 5 in the new ICU, and once in a blood culture isolate. Group 12 (ST241) was found once in a sink drain in the new ICU, and once in a blood culture isolate. This highlights 3 instances where a sink drain isolate was found within the same genome-resolved group as a blood culture isolate of a patient in the ICU.

When we compared the accessory genomes of our cultured isolates and reference *P. aeruginosa* genomes, we found a mean of 4018 (range 3221–5003) accessory genes per genome. Group 1 isolates have a mean of 3947 (range 3885–4022) accessory genes, suggesting average accessory genome size. To compare variation in accessory genomes across *P. aeruginosa* we used a principal component analysis (PCA). We found distinct clustering between Group 1 isolates and the rest of the *P. aeruginosa* isolates (PERMANOVA: $p < 0.001$) (Figure 2.9.6e). There were 36 accessory genes with high loading scores on PC1 that are unique to Group 1, of which only 7 could be characterized by EggNOG (Supplementary Data 4, Supplementary Data 5)[50].

### 2.5.9 *P. aeruginosa* Group 1 isolate lineages clustered by room

SNP analysis from short reads allows us to accurately estimate genomic relatedness and group highly-related genomes, but obtaining fully-resolved genomes is necessary to identify transmission and reservoir persistence in sink drains. Our short-read data indicated <11 SNPs between Group 1 isolates, which is well within previously established probable transmission for *P. aeruginosa*[83]. Indeed, our phylogenomic tree building grouped isolates similarly to this analysis (Figure 2.9.4d, Figure 2.10.3). To investigate reservoir formation of *P. aeruginosa* Group 1 isolates over time at higher genomic resolution, we obtained long-read sequencing data for the 53 isolates in Group 1. We created hybrid assemblies of each genome (assemblies had between 1–11 and an average of 4.32 contigs) and found the core genome to consist of 4863 genes out of 9714 total genes. By examining the accessory genome, we identified 4 additional isolates that were responsible for a large portion of the accessory genome and removed them from analysis, as they were unlikely to be part of the same lineage. The remaining 49 Group 1 isolates consisted of 5964 core genes from 6986 total genes.

In our time-measured phylogenetic analysis using BEAST[54], we created a consensus tree of estimated time since most recent common ancestor (TMRCA) using the Group 1 core genome (Figure 2.10.4). As we do not have isolates collected this far back, confidence in branch divisions is low (Figure 2.10.5) and the TMRCA of 8034 days was largely driven by one isolate, which was removed from further analysis. The remaining isolates formed a consensus tree (Figure 2.9.6g) with a TMRCA of 2752 days with a 95% highest posterior density interval (HPD) of 1523–4362 days. 38/48 of these isolates were taken from the in-room sink drains; 7 were found in the sink drains from the attached bathroom; and 3 were isolated from blood infections.

The majority (40/48) of these isolates are contained under Node 1 and have a TMRCA of 778 days with a 95% HPD of 488–1122 days. This clade displays 3 unique evolutionary patterns. Descending from Node 2, eight isolates cluster together. 7/8 of these isolates were taken from the in-room sink drains in Room 1 within the first 2 weeks of the study, suggesting the diversity shown represents heterogeneity within a given sink rather than in-room evolution. We also found two likely instances of within room evolution, marked by Nodes 3 and 4. These isolates branch off from one lineage as time progresses, suggesting an evolving, single reservoir. Outside of these main features, the remaining isolates under Node 1 were from mixed rooms and collection weeks with low confidence in the branching (Figure 2.10.4). The commingling and low confidence in branching suggests strain exchange between rooms of a common pandemic strain throughout the sampling period.

Interestingly, one of the blood isolates clustered with an environmental isolate within the clade under Node 1, but the other two were further removed from the bulk of Group 1. While all isolates were collected from patients in this ward at some point during their stay, this isolate was collected two days before the patient moved into Room 5. The other two blood isolates were further removed

from Node 1 environmental isolates. However, there are possibly different evolutionary pressures within each sample type, which could drive different mutation rates. The overall close relation of the blood and surface isolates implies direct correlation within the duration of the study period and potentially presents a great risk to patient safety.

## 2.6 Discussion

The process of ARO reservoir colonization of the hospital built environment is dependent on complex interactions, and transmission events to vulnerable patients are not well understood[27,84,85]. In this study we investigated the microbiologic changes in a new SCT ICU before and after patient or staff occupancy and tracked ARO strains cross ICU surfaces and patients. We identified a mechanism of ARO colonization development that occurred prior to patient or staff move-in, which could promote the necessity of future surveillance investigations. We compared these colonization patterns to equivalent microbial sampling in the corresponding old SCT ICU during its final month of occupancy, before patients and staff moved from there to the new ICU, to obtain a unique comparison of distinct hospital built environments following relocation of patients and hospital staff. We found ARO reservoirs were rare on most hospital surfaces apart from sink drains. Non-sink drain surfaces showed no difference in ARO burden between the two buildings, but sink drains in the old ICU had a significantly higher burden than those in the new ICU. Further, reservoir colonization and transmission varied by taxa and between buildings, with evidence in *P. aeruginosa* of shared strains across multiple sinks and human clinical infections in the new ICU.

Recent studies have focused on better understanding and characterizing the hospital microbiome using metagenomics[11–15]. These characterizations find correlations between samples of hospital surfaces, patients, and staff, particularly in skin- and gut-associated taxa such as Enterobacterales and *Staphylococcus*, suggesting the microbiomes of humans and the hospital built environment

influence each other[11–15]. Further, strain tracking using metagenomic analyses indicates that similar strains may be present on surfaces over time, suggesting potential reservoir colonization on surfaces[11,13]. Building on these studies, we focused on high-resolution, temporal, genomic and phenotypic investigation of viable AR strains which colonize or infect surfaces and patients in ICUs. We found that AROs isolated from patient stools were rarely found on ICU surfaces, and with the exception of sink drains, we do not find persistent reservoir colonization of most ICU surfaces. In contrast we found multiple instances of ARO reservoir colonization of ICU sink drains, with highly-related strains of these AROs also recovered from patient blood cultures.

We found AROs more frequently in sink drains in the old ICU compared to the new ICU. There are many possible reasons for these differences, including: building material and layout differences, water sources, natural history, and extended time for establishment and accumulation of AROs[86–89]. In the new ICU, AROs were found before patient or staff occupancy, and ARO burden in both sink drains and other surfaces did not significantly change after 1 year of patient occupancy. This baseline level of ARO burden in an ICU suggests that patients are not the primary source of AROs found on surfaces nor do they cause significant increases in ARO burden during the first year of ICU establishment. Further, these results have important implications for remediation strategies that involve removing or rebuilding infrastructure and suggests such strategies may not always be successful.

When comparing ICU room surfaces, we found AROs more frequently on sink drains and rarely on any other ICU room surface. While studies in low to medium income countries have found high ARO burdens on hospital surfaces, our results are consistent with other studies in the United States (US) that have found low ARO burden on ICU surfaces and high ARO burden in sink drains[82,90]. While it is possible that our sampling methods may miss some AROs, the sparsity and

inconsistency of AROs on surfaces suggests that most surfaces other than sink drains are not acting as persistent reservoirs for AROs. It may also be that some AROs do not survive well on dry ICU surfaces where they cannot easily form biofilms[88,90]. However, other studies have found ARO colonization on these types of surfaces for long periods, suggesting that colonization is possible[82,91–93]. Instead, high standards of cleaning, self-disinfecting equipment, and special training in high income countries such as the US may be effective at removing and limiting ARO reservoirs on most commonly-touched surfaces[91,94,95]. While national standards and studies have suggested protocols for cleaning many hospital surfaces[95], there are no standardized protocols for cleaning sink drains. This may lead to variable and inconsistent decontamination of these areas compared to other commonly-touched surface areas. Further, sink drains are often difficult to clean as liquid disinfectant is less effective when poured down the drain without coating the drain surface, and the drains are often covered by a drain cover and cannot easily be wiped down or scrubbed[96,97].

Reservoir colonization by AROs in sink drains appears to be specific to particular taxa. While we cultured a wide diversity of AROs from sink drains, only two species had strains that formed reservoirs in sink drains: *S. maltophilia* and *P. aeruginosa*. These results corroborate previous work identifying *Pseudomonas* spp. and *Stenotrophomonas* spp. as capable of long-term colonization of sink drains[74–77]. In contrast, we did not find evidence of persistent colonization of sink drains by Enterobacterales species, which have commonly been associated with hospital built environment outbreaks[17,28,88,98–100]. It is possible these organisms were present but weren't isolated because they were not resistant to the antibiotics used in selective culturing.

*S. maltophilia* is an environmental organism that is emerging as a serious concern for HAIs and other infections[64]. For our purposes, we defined reservoirs to mean surfaces where at least 2

isolates from the same sequence type were isolated from consecutive samplings. We found reservoirs of *S. maltophilia* in at least 3 sink drains. *S. maltophilia* ST1 established reservoirs in two surfaces of the same ICU room, suggesting a similar source or the spread of one strain type to a different location. However, we find little evidence of strain transfer to sink drains in other rooms in the same ICU, and no evidence of transmission to patients. In fact, while we found 3 *Stenotrophomonas* isolates in blood cultures, when we used ANI to identify species, none of these were identified as *S. maltophilia*. This may have broad clinical applications as poor identification of blood isolates could potentially lead to inappropriate treatment. However, even though we find no evidence of transmission of *S. maltophilia* sink strains to patients, since *S. maltophilia* has been shown to be a pathogen in immunocompromised patients, it is still important to identify methods to remove sink drain reservoirs of these organisms.

*P. aeruginosa* has long been characterized as an opportunistic pathogen that inhabits environmental sources, particularly water sources, as well as the human gut[60,70–73]. When compared to diverse *P. aeruginosa* genomes from other studies, we found no distinct clustering with environmental or clinical isolates, suggesting that our isolates are not coming from a strictly environmental strain pool. Instead, the strains we characterized were phylogenetically diverse, indicating that the adaptations necessary to survive in sink drains in the ICU are not restricted to a single clade. Further, there was limited apparent transfer of surface isolates between ICUs as patients and staff moved from one location to the other, as strains were unique between surfaces in the old and new ICU.

Remarkably, the genomic diversity of *P. aeruginosa* isolates from ST1894 in sink drains was incredibly low, even after one year or sampling. *P. aeruginosa* ST1894 was first described in 2014 in a cystic fibrosis patient in Spain (Isolate RC19, id:2398)[44]. Since only the MLST was done for

this isolate and not WGS, it is impossible to determine if this isolate and our ST1894 isolates have similar ancestry. The ST1894 isolates from our study are not only capable of surviving well in sink drains but also of colonizing multiple sink drains; our collection scheme documents Group 1 *P. aeruginosa* of ST1894 first being cultured from a single room, but after 17 weeks of sample collection, was found in all six ICU rooms samples. Our initial short read-based WGS approach provided the resolution to cluster isolates into groups based on whole-genome SNP distances, which has been the mainstay for transmission dynamics up until this point[83,101,102]. However, our long-read sequencing analysis elucidated the more nuanced relationships necessary for transmission and reservoir colonization dynamics. Specifically, our phylogenetic analysis with high-quality hybrid assemblies indicates key cases of a ST1894 strain inhabiting one sink drain before patients move into the hospital, and then spreading and exchanging between all rooms sampled. Our sampling illuminated the diversity and evolution of this lineage across time and space during the course of the study period. We also found evidence of 3 instances where this strain was found in blood cultures from hospitalized patients, highlighting ST1894 as an urgent threat to this healthcare facility and associated immunocompromised patients. The bias for these isolates originating from the in-room sink drains rather than the bathroom sink drains also suggests that the water source system, which is common to all drains, is not a likely source of reservoir contamination. This, in association with the patient sample that was collected outside the sampling ward, lead us to suspect this strain may be more widespread in this healthcare system than our sampling area. Our genomic analyses indicate that *P. aeruginosa* ST1894 has a very unique accessory genome compared to other *P. aeruginosa*, thus leaving a long list of candidate genes that might explain its prevalence in sink drains. Further investigation into these genes and other

similar strains will help us better understand the genomic evolution that might have allowed for its environmental pathogenicity.

Globally, antibiotic resistance in *P. aeruginosa* isolates is a growing concern, with infection mortality rates of 33–71% in carbapenem-resistant infections[81]. *P. aeruginosa* is capable of both intrinsic chromosomal modifications and acquisition of mobile ARGs that encode resistance to all classes of antibiotics currently used in *P. aeruginosa* treatment. However, carbapenem resistance in *P. aeruginosa* has only been acquired through the acquisition of mobile ARGs, most commonly metallo--lactamases (MBLs) and are typically encoded on plasmids, integrons, and mobile cassettes[81]. In general, carbapenem resistance was rare in *P. aeruginosa* isolates collected in the new ICU, while it was common in *P. aeruginosa* isolates collected in the old ICU. *P. aeruginosa* ST1894 was generally susceptible to the suite of antibiotics we tested against, with only 2 instances of resistance observed. Fortunately, this means there are currently a number of viable antibiotic treatment options against the existing reservoirs of ST1894 in our healthcare system. However, the presence of other *Pseudomonas spp*. with much higher AR burdens in this same hospital environment, and the known ease of resistance transmission in *Pseudomonas spp*., emphasizes the risk that this widely disseminated ST1894 reservoir could evolve into a greater ARO threat.

Despite our success in identifying multiple reservoirs with our current methods, it is plausible that we are under sampling the genomic diversity and persistent colonization through the cross section of time points sampled. For example, in our identification of reservoirs by Group 1 of *P. aeruginosa* (Figure 2.9.6f), we believe the strain was likely still present even when it appears to skip certain weeks. Even with selection of multiple isolates per selective plate, further work could improve these methods, such as a metagenomics based approach, and reveal additional reservoirs.

It is intriguing that many AROs were found in sink drains even prior to patient relocation to this unit. Previous work has suggested sources of contamination such as patient or hospital staff carriage of *P. aeruginosa*[103], or diffusion through water pipes[103,104], but these don't address contamination identified prior to patient or staff move-in. Other studies have identified water contamination as a potential source[75,76,105], but our sampling did not indicate water as the source of these AROs. Further research is necessary to understand the origins of the strains. Regardless of their origins, these findings highlight the need for a more thorough decontamination procedures, both during the terminal clean and regular operation of ICU facilities.

In conclusion, our investigation of ARO reservoirs allowed us to assess and compare models of colonization and transmission in an old and new hospital built environment with the same patient and staff populations, including before and after patient or staff occupancy. Our approach of selective microbiologic culture combined with WGS analyses provide for a detailed analysis of ARO variation across one year of sampling in an SCT ICU. Together these data provide a high-resolution characterization of AROs in the hospital built environment, highlighting that SCT ICU sink drains are a major reservoir for AROs with direct links to patient infections. Most pressingly, the surprisingly rapid development of *P. aeruginosa* colonization and association with patient infections emphasizes the need for future work to decrease the spread of AROs in hospital built environments, completed by efforts towards decolonizing and eliminating sink drain ARO reservoirs.

## 2.7 Supplementary Information

Supplementary information and tables can be found in the full text of this manuscript.

## 2.8 Funding and Declarations

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## 2.9 Figures

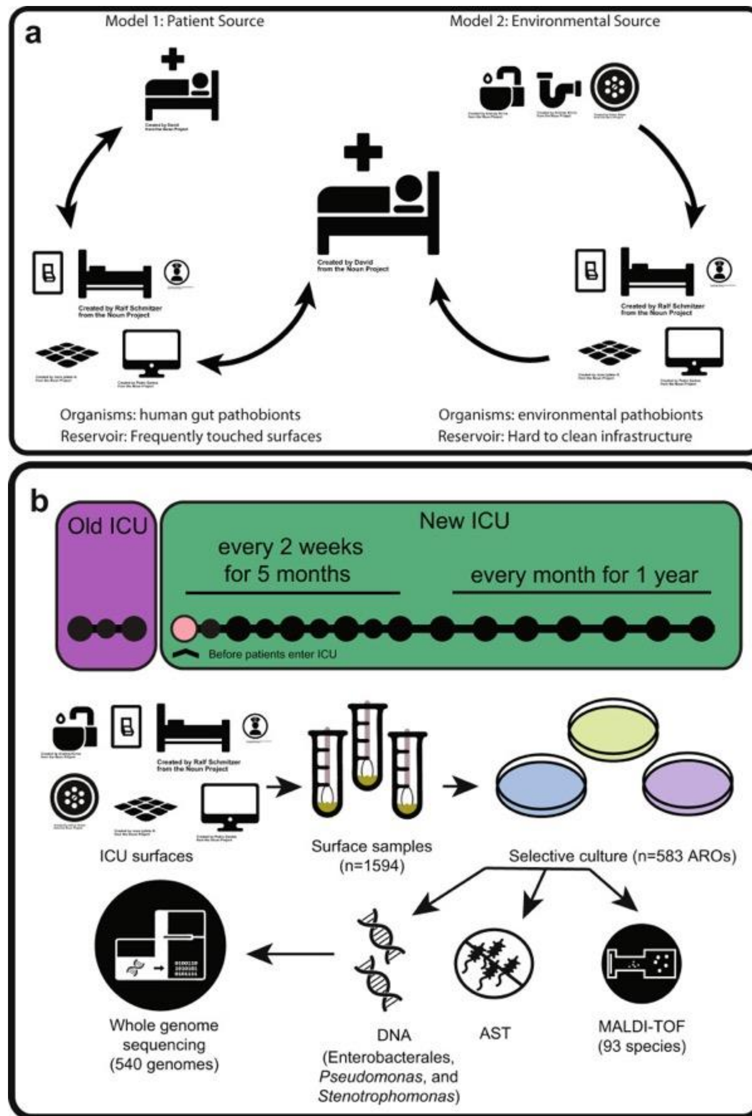


**Figure 2.9.1 ARO reservoir colonization models and sample processing scheme. a** Two models of reservoir colonization. Model 1 shows antibiotic-resistant organism (ARO) transmission from patients to hospital surfaces and then to other patients. Model 2 shows ARO transmission from environmental reservoirs to hospital surfaces to patients. **b** Sample collection time points and sample processing scheme from surface collections to WGS. In sample collection scheme, large circles represent months with small circles representing 2-week sampling within months. Purple indicates old intensive care unit (ICU) collections, green indicates new ICU collections, and pink indicates collections taken before patients enter the building in the new ICU. Icons labeled as such were acquired from nounproject.com, and other icons were used with permission from D'Souza, Potter et al.[82]. AST antibiotic susceptibility testing, MALDI-TOF matrix-assisted laser desorption/ionization-time of flight mass spectrometry.

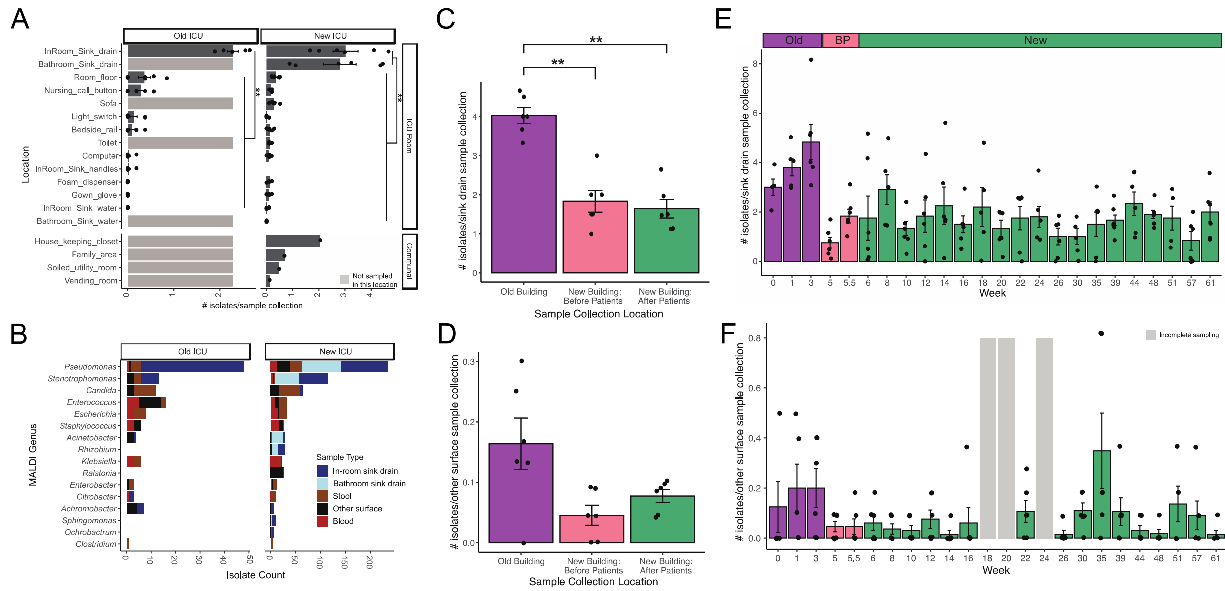


**Figure 2.9.2 Variation in isolate collection location, identity, and timing across all sampling.** Error bars indicate standard error of intensive care unit (ICU) rooms. ** indicates generalized linear mixed-modeling (GLMM) p-value <0.01. **a** In-room and bathroom sink drains have significantly more isolates per collection than other surface locations in both the old and new ICU buildings (n = 566 surface isolates). Locations in light gray were not collected in old ICU. **b** Genus of matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF) species identification of all collected isolates in both the new and old ICU. Other Surface includes all other surfaces that are not in-room or bathroom sink drain. **c** Variation in number of isolates collected per bathroom or in-room sink drain sample collection by building (excludes fecal and communal samples, n = 429). **d** Variation in number of isolates per other surface sample collection by building (excludes sink drain, fecal, and communal samples, n = 137). **e** Variation in number of isolates per bathroom or in-room sink drain sample collection for all time points, n = 429. f Variation in number of isolates collected per other surface sample collection across all time points (excludes sink drain, fecal, and communal samples, n = 137). Gray bars indicate weeks with incomplete sampling of surfaces. BP before patient and staff move-in.

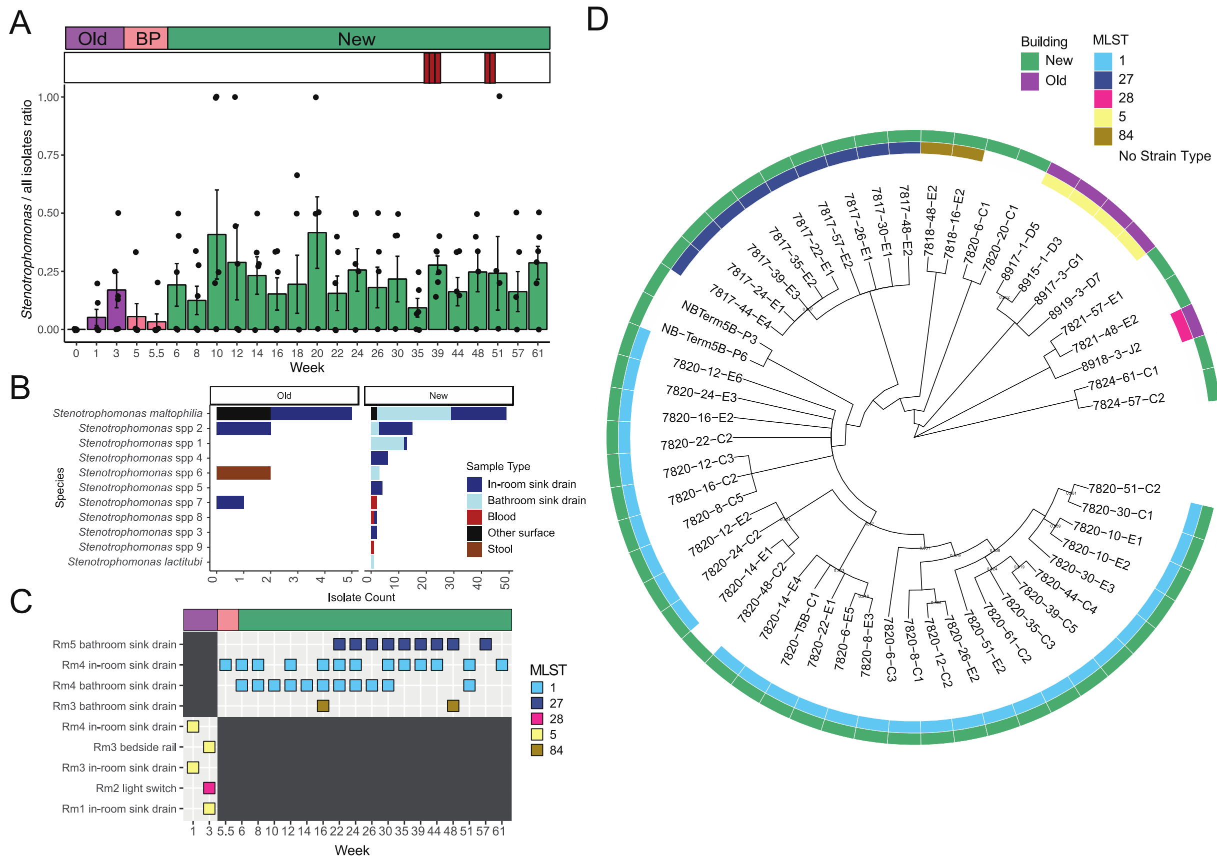


**Figure 2.9.3 Timing, identity, and phylogenetics of Stenotrophomonas isolates. a** Ratio of Stenotrophomonas isolates to all isolates across all time points (n = 128 Stenotrophomonas isolates). Error bars indicate standard error of intensive care unit (ICU) rooms. Red bars indicate collection timing of Stenotrophomonas blood culture isolates. **b** Identity of all collected Stenotrophomonas genomes by >95% average nucleotide identity (ANI) to reference genome by sample collection type (n = 128 isolates). Other Surface indicates all other surface/water genomes apart from in-room and bathroom sink drain. All genomes were identified as Stenotrophomonas by MASH. Stenotrophomonas various genomospecies includes all different genomospecies that did not share >95% ANI with a reference genome. **c** Time point mapping of shared S. maltophilia MLST groups by sample collection location. d Cladogram built from a core genome alignment of S. maltophilia genomes. Branches with less than 80% bootstrap support are collapsed. Branches with bootstrap values between 80–95% are labeled. BP before patient and staff move-in.

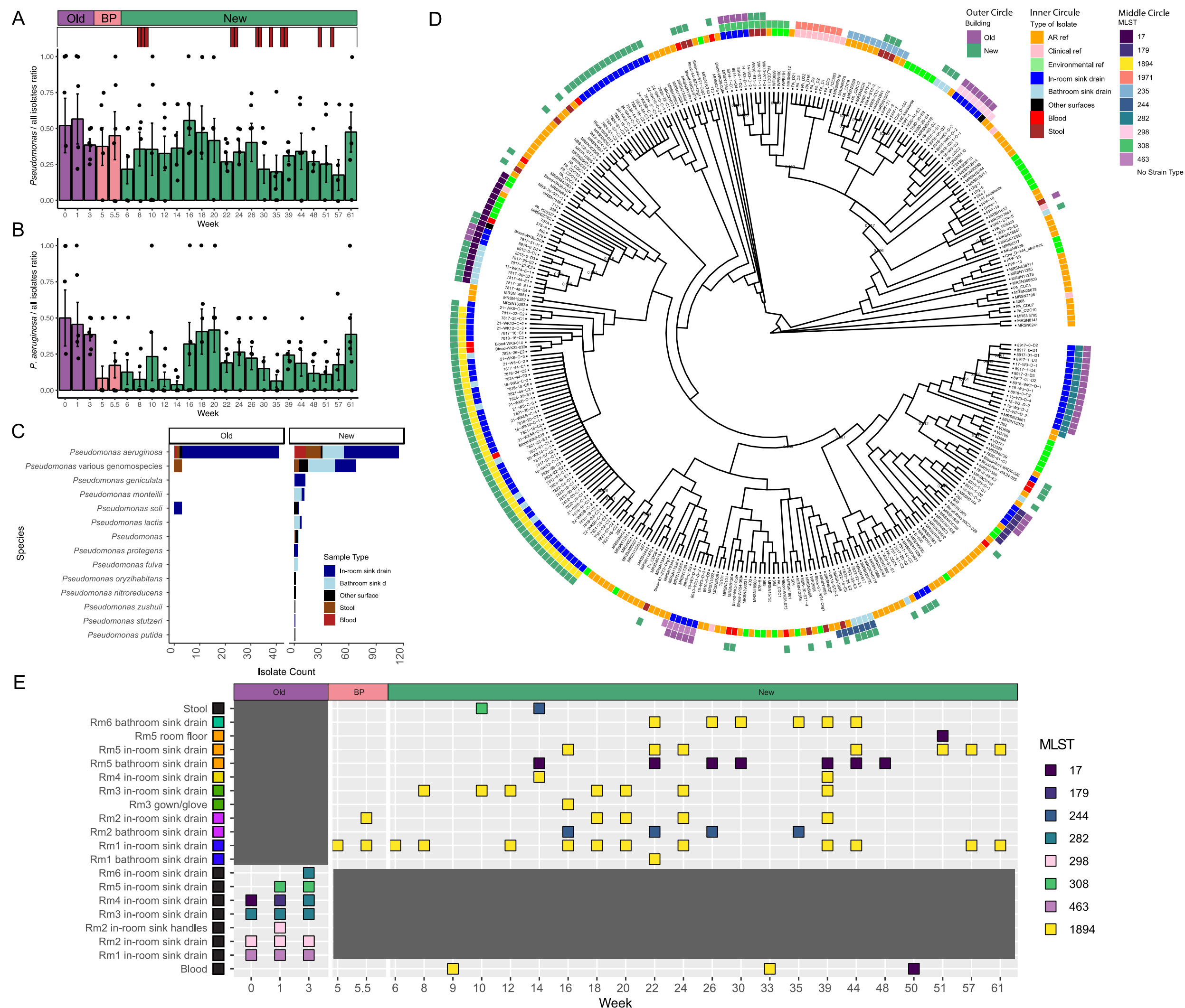


**Figure 2.9.4 Timing, identity, and phylogenetics of Pseudomonas spp. isolates. a** Ratio of Pseudomonas spp. to all isolates across all time points (n = 283 Pseudomonas isolates). Error bars indicate standard error. Red bars indicate collection timing of Pseudomonas aeruginosa blood culture isolates. **b** Ratio of P. aeruginosa to all isolates across all time points (n = 155 P. aeruginosa isolates). Error bars indicate standard error. **c** Identity of all collected Pseudomonas spp. genomes by >95% ANI to reference genome by sample collection type. Other indicates all other surface/water genomes apart from in-room and bathroom sink drain. All genomes were identified as Pseudomonas spp. by MASH. Pseudomonas various genomospecies includes all different genomospecies that did not share >95% ANI with a reference genome. **d** Cladogram from a core genome alignment of P. aeruginosa genomes. Branches with less than 80% bootstrap support are collapsed. Branches with bootstrap values between 80–95% are labeled. Reference P. aeruginosa genomes included antibiotic-resistant (AR) isolates, clinical isolates, and environmental isolates. Reference MLST is included if it shares a MLST with collected isolates. **e** Time point mapping of top 8 MLST P. aeruginosa groups by sample collection location. BP before patient and staff move-in.

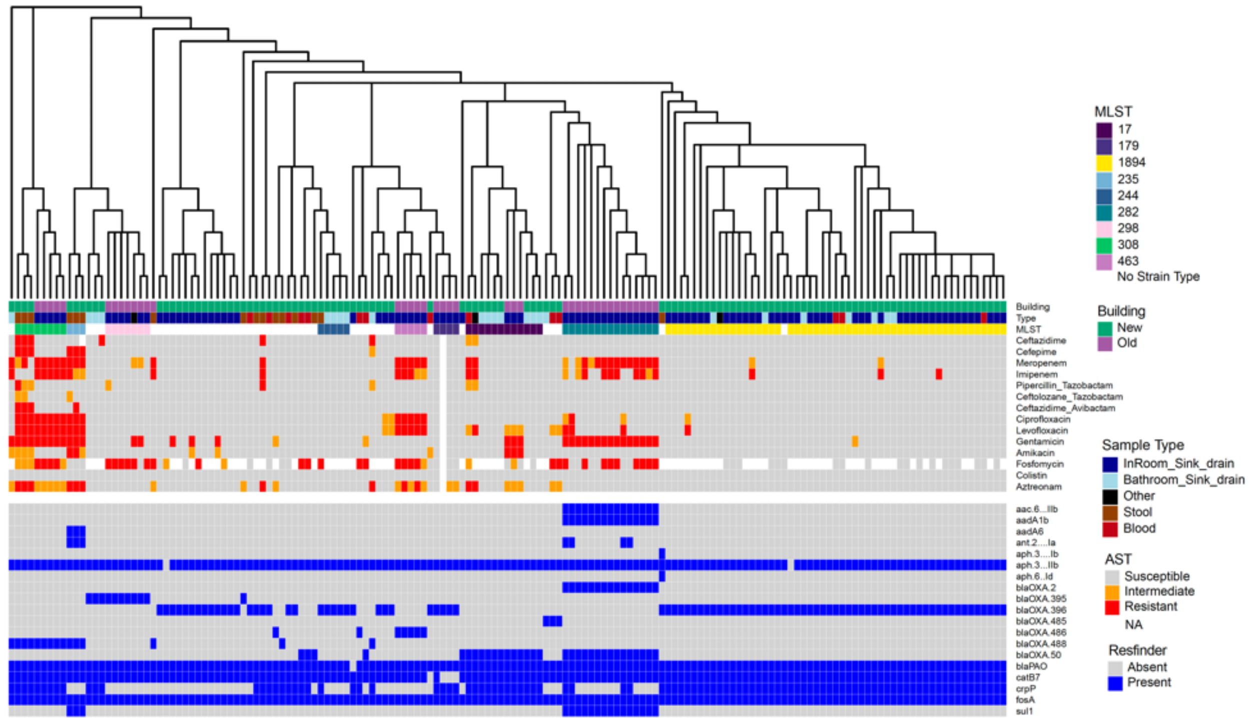


**Figure 2.9.5 Phenotypic and genotypic antibiotic resistance of collected *P. aeruginosa* isolates.** Phylogenetic tree is from a core genome alignment. Phenotypic resistance determined by antibiotic susceptibility testing (AST). Genotypic resistance determined by Resfinder[53].

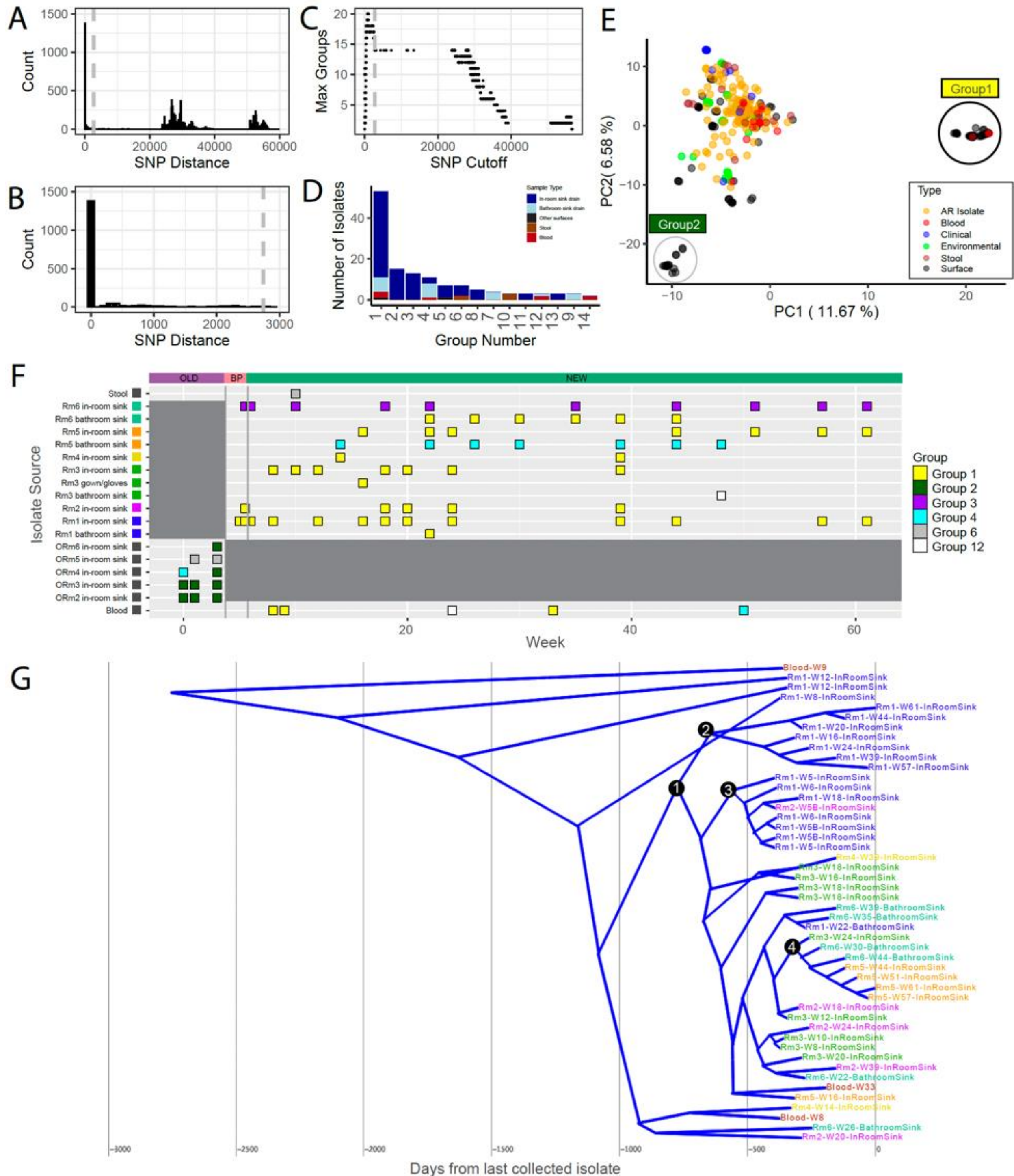


**Figure 2.9.6 Highly-related genomic groups of *P. aeruginosa* across locations and time.** **a** Histogram of pairwise single nucleotide polymorphism (SNP) distances between *P. aeruginosa* genomes indicate three modes of pairwise distances. The first corresponds to highly-related genomic groups. We define group SNP threshold as pairwise distances that fall before 2743 (gray dashed line). **b** Zoomed in histogram of pairwise SNP distances between *P.*

*aeruginosa* genome with a cut off at 3000 SNPs show only highly-related genomic groups. **c** Max groupings by SNP cut off show pairwise groups plateau at 2743 SNPs. **d** Number of isolates per highly-related genomic group. Other surfaces includes all other surfaces that are not in-room or bathroom sink drain. **e** First two components of principal component analysis (PCA) of the accessory genome of all *P. aeruginosa* genomes. Black circle encloses all Group 1 *P. aeruginosa* genomes. Gray circle encloses all Group 2 *P. aeruginosa* genomes. **f** Time point mapping of top 4 *P. aeruginosa* highly-related groups and highly-related groups that shared isolates between patient and surface samplings. **g** Time-measured phylogenetic analysis consensus tree of $n = 48$ Group 1 *P. aeruginosa* isolates depicted using DensiTree v2.2.7[59]. Nodes labeled with black circles. Node 1 marks the main clade with a time since most recent common ancestor (TMRCA) of 778 days. BP before patient and staff move-in.

# 2.10 Supplementary Figures

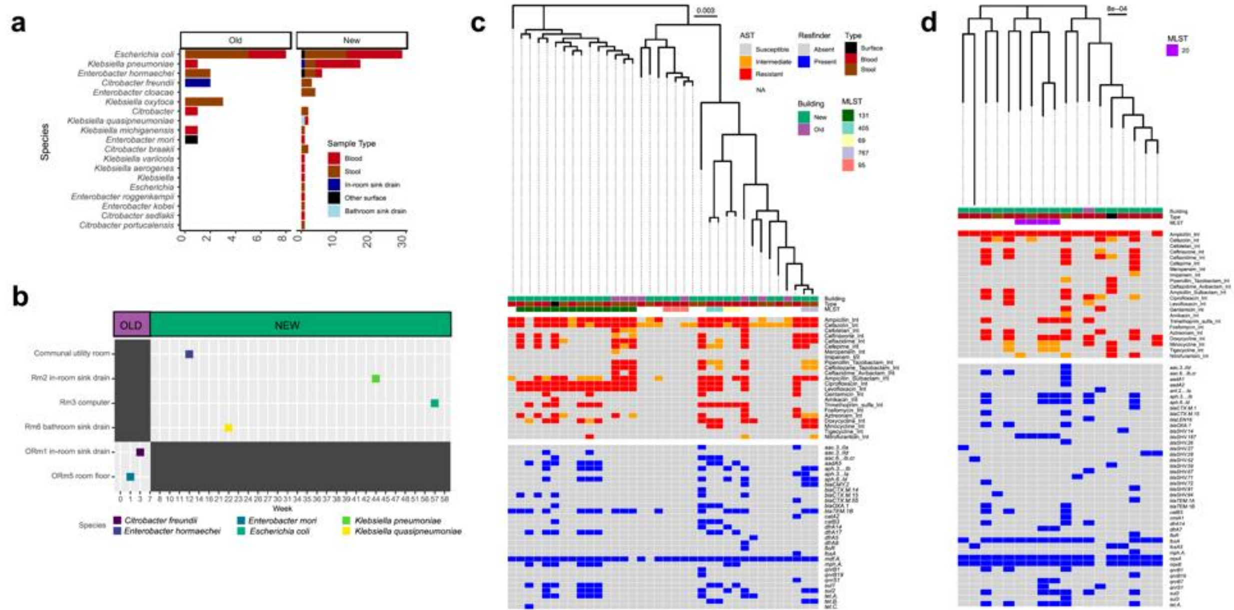


**Figure 2.10.1 Identity, timing, and resistance of Enterobacterales isolates. a** Identity of all collected Enterobacterales genomes by >95% average nucleotide identify (ANI) to reference genome, colored by sample collection type. Other indicates all other surface/water genomes apart from in-room and bathroom sink drain. All genomes were identified to genus by MASH. **b** Time point mapping of all antibiotic-resistant (AR) Enterobacterales isolates cultured from surface samples by sample collection location. Dark grey boxes indicate no surface collections. Purple point indicates 2 morphotypes of *Citrobacter freundii*. **c** Phenotypic and genotypic antibiotic resistance of collected *E. coli* isolates. Phylogenetic tree is from a core genome alignment. Branches with low bootstrap values are shown and tree has resolution of 0.00055. Phenotypic resistance determined by antibiotic susceptibility testing (AST). Genotypic resistance determined by Resfinder[1]. **d** Phenotypic and genotypic antibiotic resistance of collected *K. pneumoniae* isolates. Phylogenetic tree is from a core genome alignment and has resolution of 0.00055. Phenotypic resistance determined by AST. Genotypic resistance determined by Resfinder[1].

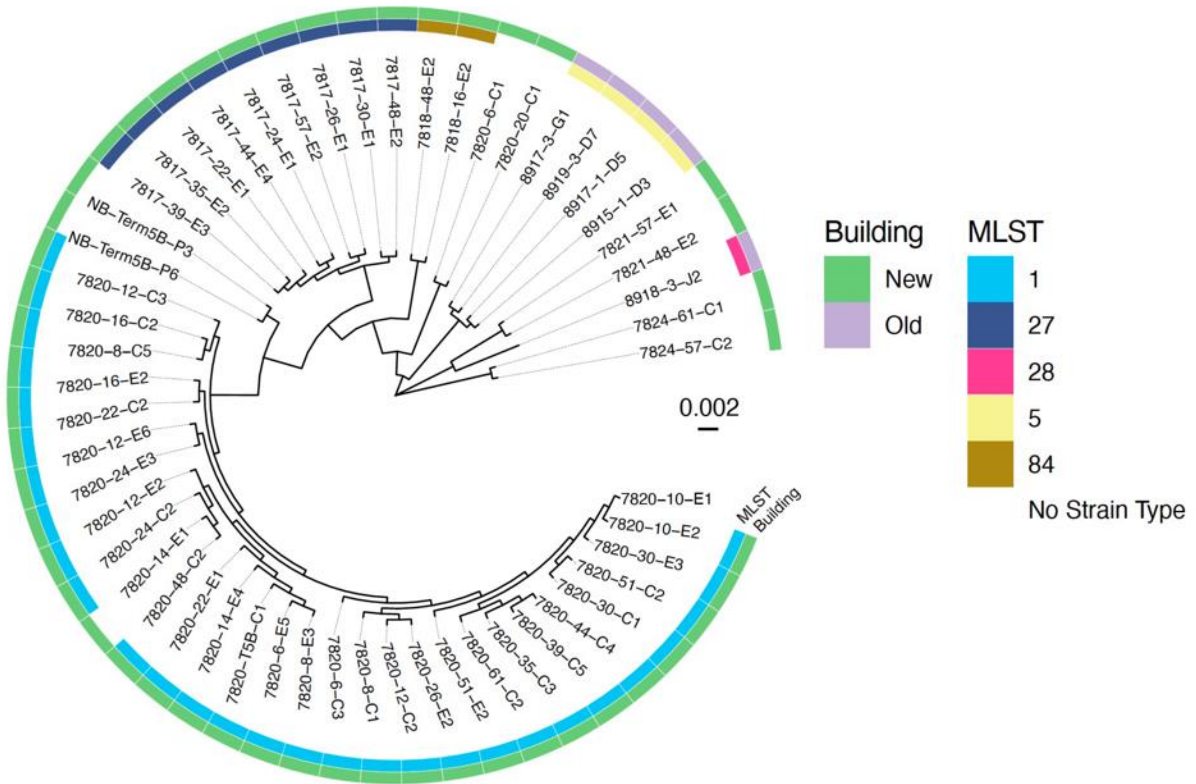


**Figure 2.10.2 Phylogram built from core genome alignments of S. maltophilia genomes.** Branches with low bootstrap values are shown, minimum resolution of 0.00055.

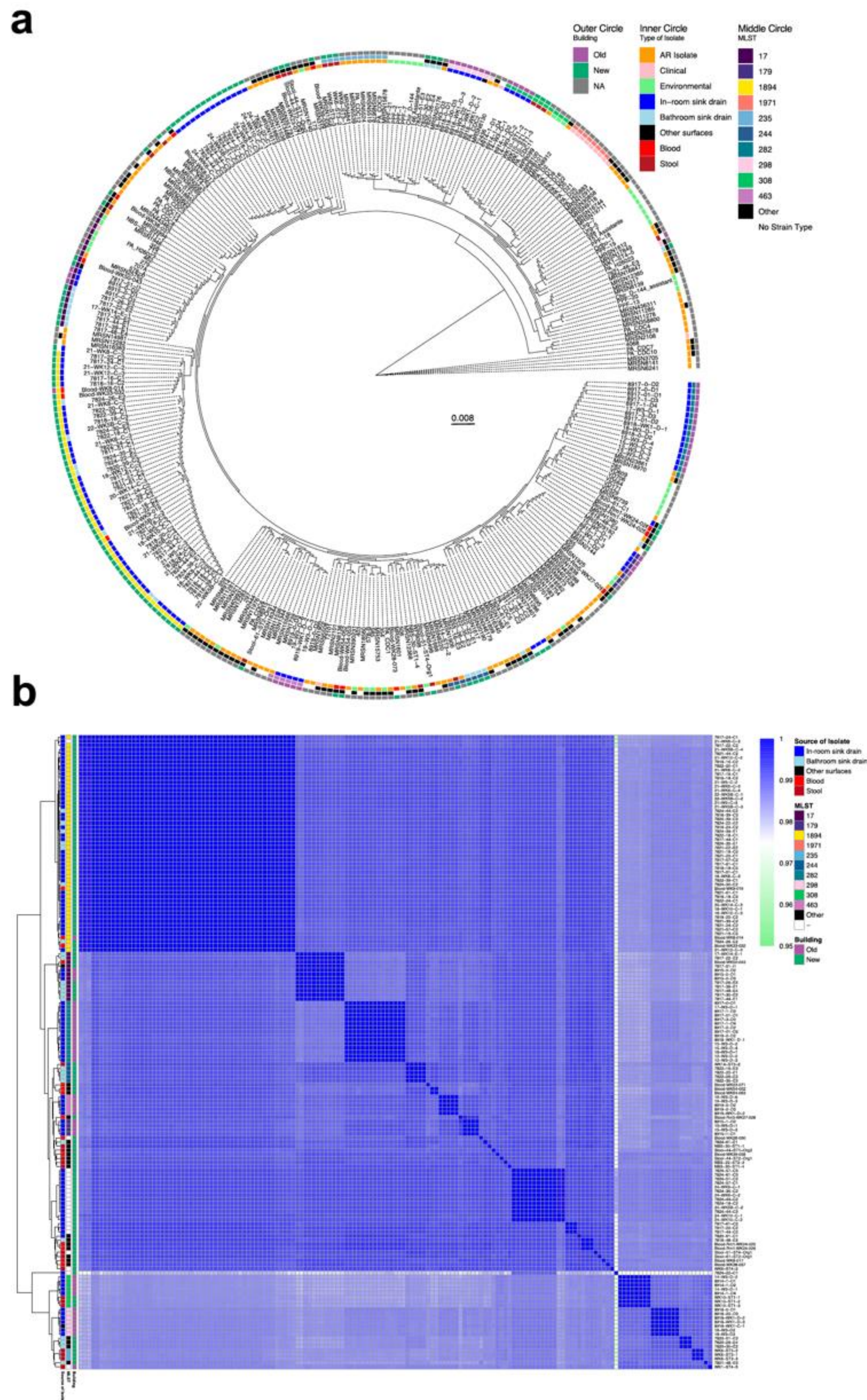


**Figure 2.10.3 Average nucleotide identity (ANI) and phylogenetics of *P. aeruginosa* genomes.** **a** Phylogram built from core genome alignments of *P. aeruginosa* genomes. Branches with low bootstrap values are shown, minimum resolution of 0.00055. **b** Average nucleotide identify (ANI) heatmap of all *P. aeruginosa* genomes.

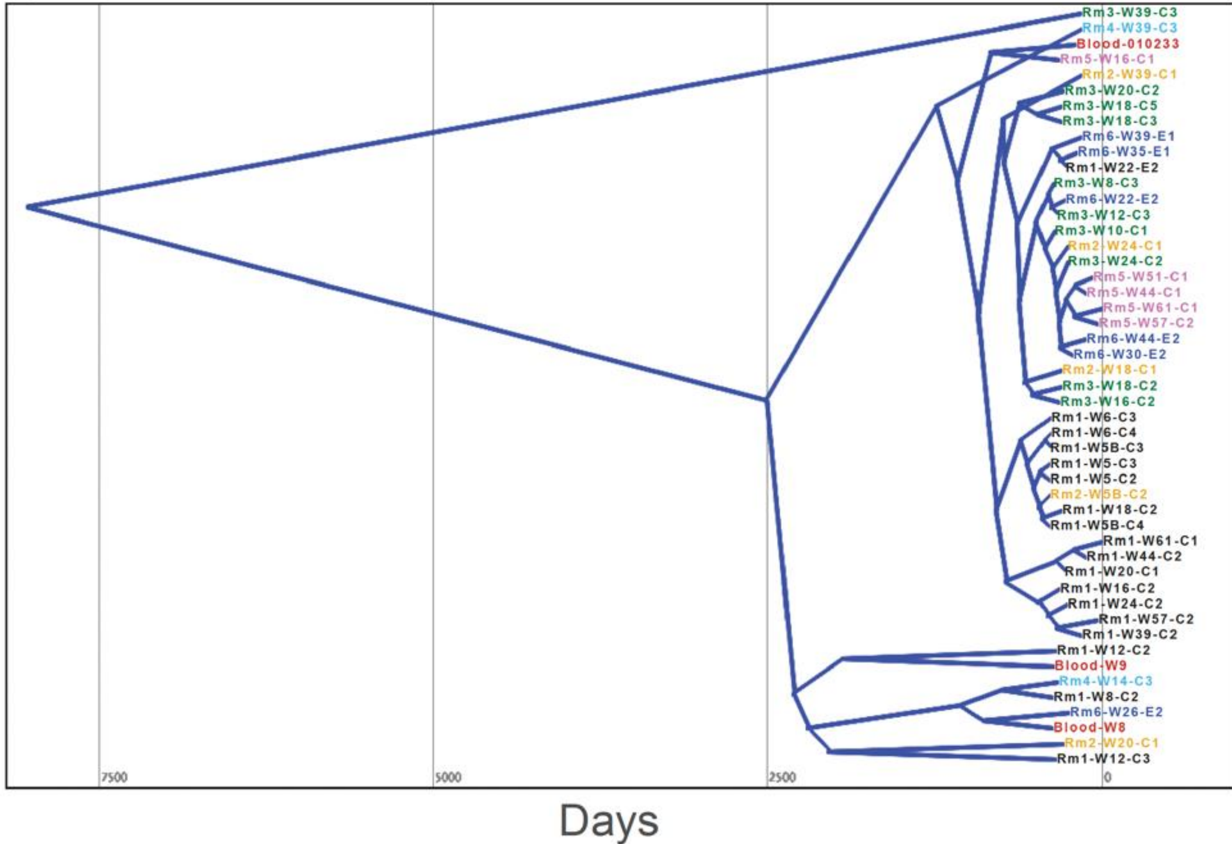


**Figure 2.10.4 Time-measured phylogenetic analysis consensus tree of Group 1 *P. aeruginosa* isolates.** Tree of n=49 isolates depicted using DensiTree v2.2.72. 4 Group 1 isolates were removed from this tree because they represented a significant portion of the accessory genome and were unlikely part of the same lineage. The most distant isolate (Rm3-W39-C3) was excluded from further analysis.

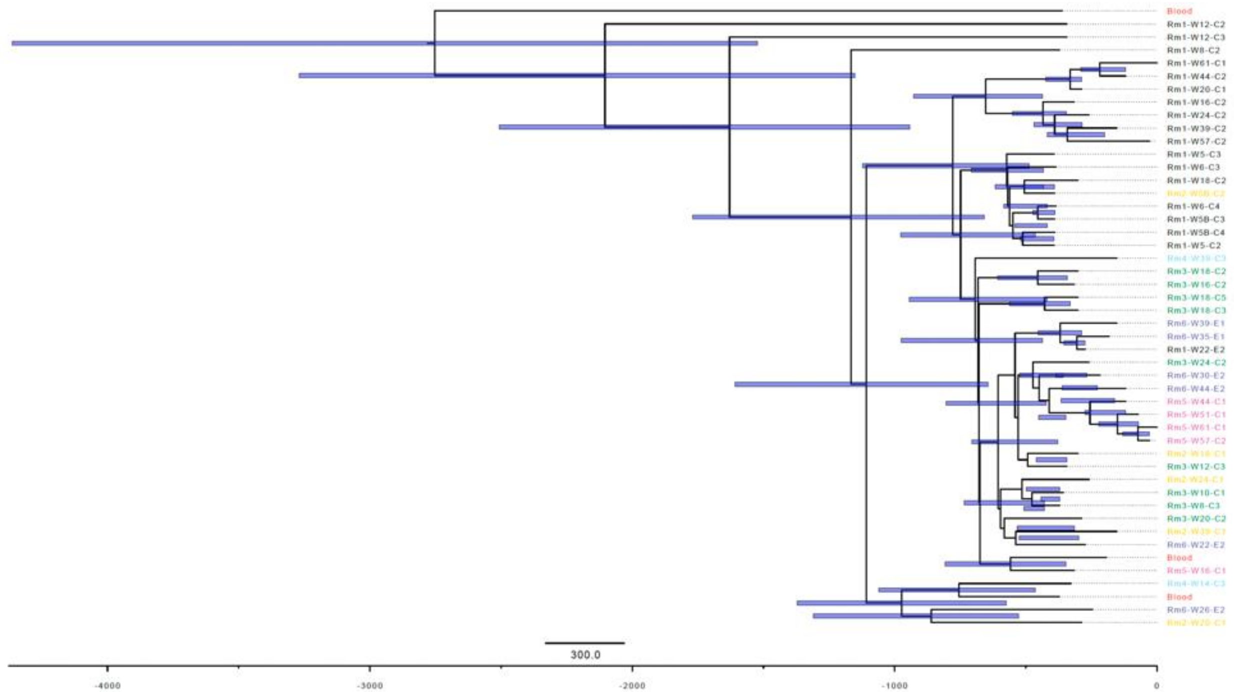


**Figure 2.10.5 Time-measured phylogenetic analysis consensus tree of Group 1 P. aeruginosa isolates.** Tree of n=48 isolates depicted using FigTree v1.4.4[3]. 4 Group 1 isolates were removed from this tree because they represented a significant portion of the accessory genome and were unlikely part of the same lineage. One isolate was removed because it was highly divergent from all other remaining isolates. Error bars represent the 95% highest posterior density interval (HPD) interval for node height. Uncertainty is higher for earlier time since most recently ancestors (TMRCAs) because they are outside of the sampling range.

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# Chapter 3

# Genomic surveillance of *Clostridioides difficile* transmission and virulence in a healthcare setting

The contents of this chapter are adapted from a manuscript that is currently in submission:

**Newcomer EP***, Fishbein SRS*, Zhang K, Hink T, Reske K, Cass C, Iqbal ZH, Struttmann EL, Dubberke ER, Dantas G. Genomic surveillance of *Clostridioides difficile* transmission and virulence in a health-care setting. 2023, *In submission*.

* = equal contribution

## 3.1 Abstract

*Clostridioides difficile* infection (CDI) is a major cause of healthcare-associated diarrhea, despite the widespread implementation of contact precautions for patients with CDI. Here, we investigate strain contamination in a hospital setting and genomic determinants of disease outcomes. Across two wards over six months, we selectively cultured *C. difficile* from patients (n=384) and their environments. Whole-genome sequencing (WGS) of 146 isolates revealed that most *C. difficile* isolates were from clade 1 (131/146, 89.7%), while only one isolate of the hypervirulent ST1 was recovered. Of culture-positive admissions (n=79), 19 (24%) of patients were colonized with toxigenic *C. difficile* on admission to the hospital. We defined 25 strain networks at ≤ 2 core gene SNPs; 2 of these networks contain strains from different patients. Strain networks were temporally linked (p<0.0001). To understand genomic correlates of disease, we conducted WGS on an additional cohort of *C. difficile* (n=102 isolates) from the same hospital and confirmed that clade 1 isolates are responsible for most CDI cases. We found that while toxigenic *C. difficile* isolates are associated with the presence of *cdtR*, nontoxigenic isolates have an increased abundance of prophages. Our pangenomic analysis of clade 1 isolates suggests that while toxin genes (*tcdABER and cdtR*) were associated with CDI symptoms, they are dispensable for patient colonization. These data indicate toxigenic and nontoxigenic *C. difficile* contamination persists in a hospital setting and highlight further investigation into how accessory genomic repertoires contribute to *C. difficile* colonization and disease.


## 3.2 Background

*Clostridioides difficile* infection (CDI) is one of the most common healthcare-associated infections (HAIs) in the US and is the leading cause of healthcare-associated infectious diarrhea[1,2]. Since the early 2000s, *C. difficile* research has focused largely on hypervirulent strains, such as PCR ribotype

027[1,3-6], which were responsible for hospital-associated CDI outbreaks. Strains of ribotype 027 were responsible for 51% and 84% of CDI cases in the US and Canada in 2005, respectively[1,4,5]. Since then, other circulating strains have emerged as the prevalent strains causative of CDI, such as 078 and 014/020[7-9]. One report indicated that the prevalence of PCR ribotype 027 decreased from 26.2% in 2012 to 16.9% in 2016[9]. As the landscape of *C. difficile* epidemiology continues to evolve, we must update our understanding of how various strains of this pathogen evolve, spread, and cause disease.

In addition to the changing prevalence of CDI-causing *C. difficile* strains, their transmission dynamics also appear to be evolving. In the late 1980s, it became clear that patients with active CDI shed spores onto their surroundings, leading to *C. difficile* transmission and future CDI events in the healthcare setting[1]. Because of this, patients with active CDI are placed on contact precautions to prevent transmission to susceptible patients, which has been successful in reducing rates of CDI[2,10]. Nevertheless, while epidemiological estimates indicate that 20-42% of infections may be connected to a previous infection, multiple genomic studies fail to associate a CDI case to a previous case[11-13]. This suggests other potential sources of disease development in the hospital environment. Indeed, while asymptomatic carriers of *C. difficile* have not been a significant focus of infection prevention efforts, studies have shown these carriers do shed toxigenic *C. difficile* spores to their surroundings that could cause disease[14]. Though carriers have not been consistently identified as major transmitters of *C. difficile* that causes CDI, recent work has suggested that patients carrying *C. difficile* asymptomatically may be at elevated risk for development of CDI[15]. Correspondingly, it is critical to both confirm this finding in another setting, and understand the genomic factors that may influence the transition from carrier to CDI manifestation in hospitalized patient populations. Correspondingly, it is critical to understand if *C. difficile* carriers are major

contributors to new *C. difficile* acquisition or CDI manifestation in hospitalized patient populations.

*C. difficile* strains are categorized into five major clades and three additional cryptic clades. These clades encompass immense pangenomic diversity with many mobilizable chromosomal elements[16,17], including numerous temperate phages that have potential influences over *C. difficile* toxin expression, sporulation, and metabolism[18]. Two major toxin loci, not required for viability, encode large multi-unit toxins that independently augment the virulence of *C. difficile*. Epithelial destruction and CDI have largely been attributed to the presence of pathogenicity locus (PaLoc) encoding toxins TcdA and TcdB. In addition, an accessory set of toxins (CdtA and CdtB) encoded at the binary toxin locus, may worsen disease symptoms[19]. Yet, many nontoxigenic strains of *C. difficile* have been documented and are adept colonizers of the GI tract, even without the PaLoc[20]. As there has been continued debate about strain-specific virulence attributes[21-23], it is important to investigate the extent of strain-level pangenomic diversity and consequences of such diversity on host disease[24,25].

The purpose of this study was to evaluate the role of *C. difficile* strain diversity in colonization outcomes and hospital epidemiology. By sampling patients (n=384) and their environments for six months in two leukemia and hematopoietic stem cell (HCT) transplant wards at Barnes-Jewish Hospital in St. Louis, USA, we used isolate genomics to identify environmental contamination of both toxigenic (TCD) and nontoxigenic (NTCD) *C. difficile* by carriers and CDI patients, and corresponding transmission between both patient groups. Integration of isolate genomic data and CDI information from this prospective study with isolate genomic data from a complementary retrospective study of asymptomatic vs symptomatic *C. difficile* colonization in the same hospital[26,27] indicated that the clade 1 lineage, containing both toxigenic strains and nontoxigenic

strains, dominates circulating populations of *C. difficile* in this hospital. Further, this lineage revealed novel clade-specific genetic factors that are associated with CDI symptoms in patients.

## 3.3 Methods

### 3.3.1 Study Design

This prospective observational study took place in the leukemia and hematopoietic stem cell transplant (HCT) wards at Barnes-Jewish Hospital (BJH) in St. Louis, Missouri, United States. Each ward consisted of two wings with 16 beds; on the acute leukemia ward we enrolled from both wings (32 beds) and on the HCT ward we enrolled on one wing (16 beds). The wards were sampled for 6 months from January 2019-July 2019 (acute leukemia) and 4 months from March 2019-July 2019 (HCT). These units are located 2 floors apart in the same building. Colonized on admission was defined as: 1. Having a *C. difficile* culture positive specimen collected before calendar day 3 of admission, or 2. Having a history of an earlier *C. difficile* culture positive specimen of the same strain collected during a previous hospitalization. Acquisition was considered indeterminate when the earliest culture positive specimen was collected on calendar day 4 or later during admission. For EIA positive admissions, new acquisitions were defined as having toxigenic culture negative specimen that preceded the EIA positive clinical stool collection during the same admission. EIA positive admissions were classified as indeterminate if the patient did not have any stool or rectal swab culture results available prior to the collection of the EIA positive stool sample. EIA positive admissions were defined as colonized on admission or pre-existing colonization if the patient had a toxigenic culture positive specimen that preceded the EIA positive clinical stool during the same admission.

### 3.3.2 Sample collection, selective culture, and isolate identification

Patients and their environments were sampled upon admission to a study ward and then weekly until discharge. Per hospital standards, bleach is used for daily and terminal discharge cleaning. From each patient, a stool specimen and/or rectal swab was collected as available. Remnant fecal samples from the BJH microbiology laboratory that were obtained during routine clinical care for *C. difficile* testing were also collected. Stool samples and rectal swabs collected on enrollment were refrigerated for up to 3 hours before processing. Specimens from all other timepoints were stored in at -80°C in tryptic soy broth (TSB)/glycerol before processing. Environmental samples were collected from bedrails, keyboards, and sink surfaces using 3 E-swabs (Copan). If a surface was unable to be sampled, a swab was taken from the IV pump or nurse call button as an alternative. Swab eluate were stored at -80°C until processing.

Broth enrichment culture for *C. difficile* in Cycloserine Cefoxitin Mannitol Broth with Taurocholate and Lysozyme (CCMB-TAL) (Anaerobe Systems, Morgan Hill, CA) was performed on all admission specimens and checked for growth at 24 hours, 48 hours, and 7 days after inoculation. If *C. difficile* was isolated, all other specimens collected from that patient and their surroundings were also cultured on Cycloserine-Cefoxitin Fructose Agar with Horse Blood and Taurocholate (CCFA-HT) agar (Anaerobe Systems). Colonies resembling *C. difficile* (large, spreading, grey, ground glass appearance) were picked by a trained microbiologist and sub-cultured onto a blood agar plate (BAP). Growth from the subculture plate was identified using Matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS) (bioMerieux, Durham, NC). Upon identification, sweeps of *C. difficile* BAPs were collected in tryptic soy broth (TSB) and stored at -80C for sequencing. If both rectal swab sample and stool sample produced a *C. difficile* isolate, the stool isolate was preferentially used for analysis over the rectal swab isolate. The discharge / last specimen collected for an admission was also cultured

for *C. difficile* if *C. difficile* was not isolated from the admission specimen. If *C. difficile* was isolated from the discharge/last specimen collected, then all specimens from that admission were also cultured for *C. difficile*.

*C. difficile* toxin enzyme immunoassay (EIA) was conducted as part of routine clinical care based on clinical suspicion of CDI. To be diagnosed with *C. difficile* infection (CDI), a patient must have been EIA+ for *C. difficile* toxin (Alere TOX A/B II); those who weren't tested (due to no clinically significant diarrhea) or tested EIA- and were culture-positive for *C. difficile* were considered *C. difficile* carriers. Episodes of carriage or CDI are defined as the time from the first culture-positive specimen from a patient to the last culture-positive specimen during a given hospital admission.

### 3.3.3 Short read sequencing and *de novo* genome assembly

Parameters used for computational tools are provided parenthetically. Total genomic DNA from *C. difficile* isolates was extracted from frozen plate scrapes using the QIAamp BiOstic Bacteremia DNA Kit (Qiagen) and quantified DNA with the PicoGreen dsDNA assay (Thermo Fisher Scientific). DNA from each isolate was diluted to a concentration of 0.5 ng/µL for library preparation using a modified Nextera kit (Illumina) protocol[28]. Sequencing libraries were pooled and sequenced on the NovaSeq 6000 platform (Illumina) to obtain 2 × 150 bp reads. Raw reads were demultiplexed by index pair and adapter sequencing trimmed and quality filtered using Trimmomatic (v0.38, SLIDINGWINDOW:4:20, LEADING:10, TRAILING:10, MINLEN:60)[29]. Cleaned reads were assembled into draft genomes using Unicycler (v0.4.7)[30]. Draft genome quality was assessed using Quast[31], BBMap[32], and CheckM[33], and genomes were accepted if they met the following quality standards: completeness greater than 90%, contamination less than 5%, N50 greater than 10,000 bp, and less than 500 contigs >1000bp.

### 3.3.4 Isolate characterization and typing

A Mash Screen was used to identify likely related genomes from all NCBI reference genomes[34]. Average nucleotide identity (ANI) between the top three hits and the draft assembly was calculated using dnadiff[35]. Species were determined if an isolate had >75% alignment and >96% ANI[36] to a type strain, and were otherwise classified as genomospecies of the genus level taxonomy call.

In silico multilocus sequence typing (MLST) was determined for all *C. difficile* and genomospecies isolates using mlst[37,38]. Isolate contigs were annotated using Prokka[39] (v1.14.5, -mincontiglen 500, -force, -rnammer, -proteins GCF_000210435.1_ASM21043v1_protein.faa[40]). *cdtAB* was determined to be a pseudogene if there were three hits to *cdtB*, indicating the damaged structure of the pseudogene[41]. *C. difficile* clade was determined using predefined clade-MLST relationships described in Knight, et al[16].

3.3.5 Phylogenetic analyses

The .gff files output by Prokka[39] were used as input for Panaroo (v1.2.10)[42] to construct a core genome alignment. The Panaroo alignment was used as input to construct a maximum-likelihood phylogenetic tree using Fasttree[43]. The output .newick file was visualized using the ggtree (v3.4.0)[44] package in R. Cryptic clade isolates were determined as such based on phylogenetic clustering with cryptic clade reference isolates.

3.3.6 SNP analyses and network formation

We identified pairwise SNP distances between isolates identified as the same MLST type. The isolate assembly with the fewest number of contigs in an MLST group was chosen as a reference for that MLST group. Cleaned reads were aligned to their respective reference and SNP distances were calculated with snippy[45]. Pairwise SNP distances between isolates were calculated by merging VCF files with bcftools[46] and a custom script. Only SNPs within the core genome of each MLST group were considered, thus core MLST SNPs were used for strain network determination.

A cutoff of <=2 core MLST SNPs was used to define strain networks, as has been used previously to account for strain variation[15,47].

### 3.3.7 Phage identification and clustering

Isolate genomes were analyzed with Cenote-Taker 2[48] to identify contigs with end features as direct terminal repeats (DTRs) indicating circularity, and inverted linear repeats (ITRs) or no features for linear sequences. Identified contigs were filtered by length and completeness to remove false positives. Length limits were 1,000 nucleotides (nt) for the detection of circularity, 4,000 nt for ITRs, and 5,000 nt for other linear sequences. The completeness was computed as a ratio between the length of our phage sequence and the length of matched reference genomes by CheckV[49] and the threshold was set to 10.0%. Phage contigs passing these two filters were then run through VIBRANT[50] with the "virome" flag to further remove obvious non-viral sequences[50]. Based on MIUViG recommended parameters[51], phages were grouped into "populations" if they shared ≥95% nucleotide identity across ≥85% of the genome using BLASTN and CheckV.

### 3.3.8 Analysis of genotypic associations with disease severity

Two previously sequenced retrospective cohorts from the same hospital were included to increase power[26,52]. In the analyses of toxigenic vs. nontoxigenic isolates from clade 1, pyseer[53] was run using a SNP distance matrix (using snp-dist as above), binary genotypes (presence or absence of *tcdB*), and Panaroo-derived gene presence/absence data. In the analysis of CDI suspicion, all isolates from clade 1 were used that represented one isolate per patient-episode. Isolates recovered from environmental surfaces were excluded. Using these assemblies, a core genome alignment was generated using Prokka[39] and Panaroo[42] as above. SNP distances were inferred from the core-gene alignment using snp-dists[54]. Binary phenotypes were coded for the variable CDI suspicion, whereby isolates associated with a clinically tested stool were associated with symptomatic

colonization (TRUE). Isolates that were associated with a surveillance stool and had no clinical testing associated with that patient timepoint were coded as non-symptomatic colonization (FALSE). Gene candidates filtered based on 'high-bse', and were annotated HMMER on RefSeq databases and using a bacteriophage-specific tool VIBRANT[50]. Selected outputs were visualized in R using the beta coefficient as the x-axis and the $-\log_{10}$(likelihood ratio test p-value) as the y-axis.

3.3.9 Reference assembly collection

We chose 23 reference assemblies from Knight, et al[16] for Figure 3.9.2c because of their MLST-clade associations (Supplementary Table 2). References span Clades 1-5 and cryptic clades C-1, C-2, and C-3, with one reference from each of the three most frequent MLSTs in each clade. Cryptic clade C-3 only had 2 reference assemblies available. References were annotated and included in phylogenetic tree construction as above.

All *Clostridioides difficile* genomes available on the National Institutes of Health (NIH) National Library of Medicine (NLM) were acquired for Figure 3.9.4c construction. References from NCBI (Supplementary Table 4) were included if they had less than 200 contigs. Assemblies that met these quality requirements were annotated and phylogenetically clustered as above.

# 3.4 Results

3.4.1 Surveillance of *C. difficile* reservoirs in hospital wards reveals patient colonization and environmental contamination.

We prospectively collected patient and environmental samples to investigate genomic determinants of *C. difficile* carriage, transmission, and CDI (Figure 3.9.1). Across the study period, we enrolled 384 patients from 647 unique hospital admissions, and collected patient specimens upon admission and weekly thereafter (Figure 3.11.1). We collected at least one specimen (clinical stool collected as part of routine care, study collected stool, or study collected rectal swab) from

364 admissions for a total of 1290 patient specimens (Table 3.10.1). We selectively cultured *C. difficile* from 151 stool specimens or rectal swabs if stool was unavailable or culture-negative. We also collected weekly swabs from the bedrails, sink surfaces, and in-room keyboards, for a total of 3045 swabs from each site. We cultured all environmental swabs collected from rooms in which patients that ever produced culturable *C. difficile* were housed, for a total of 398 swab sets plus one and two additional keyboard and sink handle swabs, respectively. In total, 22/398 (5.5%) of bedrail swabs cultured and 4/399 (1.0%) of keyboard swabs cultured were culture-positive for *C. difficile* (Figure 3.9.2a). *C. difficile* was never recovered from sink surfaces (all sinks on these units are hands-less activated) or other sampled sites. Collapsing multiple positive samples from the same patient admission results in 20 positive bedrails (20/79, 25.3% of all admissions with positive patient specimens) and 4 positive keyboards (4/79, 5.06% of all admissions with positive patient specimens) (Figure 3.9.2b).

Results from selective culture indicated that 21.7% of unique admissions (79/364 admissions with available specimens) were culture-positive for *C. difficile* at some point during their admission (Figure 3.9.2b, Table 3.10.1). Of these, 57 were toxigenic culture positive. 19 (4% of all admissions) patient-admissions were considered “colonized on admission” (i.e., toxigenic culture positive within the first three calendar days of admission), and toxigenic *C. difficile* was acquired in 6 (2%) admissions. For most toxigenic culture positive admissions (32; 9% of all admissions), *C. difficile* acquisition was considered indeterminate, meaning the earliest toxigenic culture positive specimen was collected on calendar day 4 or later during admission. Full admission-level culture results can be found in Table 3.10.1.

3.4.2 *C. difficile* carriers outnumbered patients with CDI

Patients with CDI were identified through routine clinical care, with CDI defined as patients who had stool submitted for *C. difficile* testing, as ordered by the clinical team when suspicious for CDI, and who tested positive for *C. difficile* toxins by enzyme immunoassay (EIA+). Otherwise, if they were culture positive and EIA- or culture positive and not EIA tested, they were considered carriers. Overall, 25 positive EIAs occurred during the study period; of these, 17 occurred during admissions with study specimens available for culture. Among these 17 admissions, 3 (18%) were considered new *C. difficile* acquisition; 6 (35%) had indeterminate timing of *C. difficile* acquisition; 3 (18%) were false positive EIAs, 3 (18%) were colonized on admission/pre-existing colonization, and 2 (12%) were recurrent CDI (Table 3.10.1). The substantial detection of longitudinal patient *C. difficile* colonization prompted us to investigate the genomic correlates of *C. difficile*-associated disease and transmission in these two patient populations.

### 3.4.3 Phylogenetic clustering reveals lack of hypervirulent strains, presence of cryptic clades

We conducted whole-genome sequencing to ascertain phylogenetic distances among isolates and to identify closely related strains of *C. difficile.* We identified 141 isolate genomes as *C. difficile* (using a 75% alignment and 96% average nucleotide identity [ANI] threshold). One isolate was identified as *Clostridium innocuum* and five isolates were classified as *C. difficile* genomospecies (92-93% ANI). To contextualize population structure, we applied a previously established MLST-derived clade definition to our isolate cohort[16]. The majority of *C. difficile* isolates were from Clade 1 (131/146, 89.7% of *C. difficile* and genomospecies, Figure 3.9.2c). Four patient-derived isolates were identified from clade 2, but only one was of the hypervirulent strain ST1 (PCR ribotype 027)[6]. We found that the distribution of STs associated with carriers was significantly different from that of STs associated with CDI patients ($p<0.001$, Fisher's exact test, Figure 3.9.2b) suggesting some strain-specificity to disease outcome.

Interestingly, the five genomospecies isolates clustered with other isolates belonging to a recently discovered *C. difficile* cryptic clade C-1 (Figure 3.11.2). While cryptic clades are genomically divergent from *C. difficile*, these isolates can produce homologs to TcdA/B and cause CDI-like disease in humans[16,55]. In a clinical setting, they are frequently identified by MALDI-TOF MS as *C. difficile* and diagnosed as causative of CDI[55]. These data highlight the novel distribution of circulating *C. difficile* strains in the two study wards. While many patients with multiple isolates had homogeneous signatures of colonization (with closely related isolates), four patients (4/72 patients with positive cultures, 6%) produced isolates from distinct ST types.

### 3.4.4 Carriers and CDI patients contribute to transmission networks and environmental contamination

Given the predominance of clade 1 isolates, we sought to identify clonal populations of *C. difficile* strains, indicative of direct *C. difficile* contamination (patient-environment) or transmission (patient-patient). We compared pairwise, core genome SNP distances within MLST groups to identify networks of transmission connecting isolates <=2 SNPs apart (Figure 3.11.3). We identified a total of 25 strain networks, 2 of which contain patient isolates from different patients (networks 17 and 31, Figure 3a,d). These strain networks were temporally linked, as there were significantly fewer days between same-network isolates than isolates from different networks ($p<2.2e-16$, Wilcoxon, Figure 3b). We also sought to understand if CDI patients were more likely to contaminate bedrails than carriers. While we found slightly higher numbers of total bedrail isolates collected and unique bedrails contaminated by networks with CDI patients, neither comparison reached statistical significance (ns, Student's t-test, Figure 3.11.4a, b).

We compared strain connections among a single patient's isolates from stool or rectal swab ('patient'), and between these isolates and environmental isolates from their immediate

surroundings (‘bedrail’ or ‘keyboard’, Figure 3c). While the majority of bedrail isolates fell within the same network as patient isolates from that room (33/44 comparisons, 75%), 25% (11/44 comparisons) were genomically distinct, suggesting contamination from alternate sources. Keyboards were mostly colonized with distinct strains from the patient (22%, 2/9 comparisons), indicating other routes of contamination ($p<0.05$, Fisher’s exact test, BH corrected. Figure 3c). Among the networks that contain multiple patients, we found no instances of potential transmission from the inhabitant of one room to the subsequent inhabitant. However, in both instances, each potential transmission is associated with a temporal overlap in patient stay in the same ward, providing epidemiological support for putative transmission (Figure 3d). Importantly, we found no networks connecting patients with CDI to *C. difficile* carriers, suggesting successful containment through contact precaution protocols. Two patients (Patients 2026 and 2056) carried a strain of *C. difficile* and later developed CDI with that same strain. These data suggest that direct transmission from CDI patients may no longer be the driving force behind patient CDI in this setting on contact precautions, and prompted us to investigate the relationship between isolate genetic diversity and patient symptomology.

### 3.4.5 Accessory genomic elements are associated with host CDI symptoms

Despite evidence of transmission in this prospective study, a minority of patients were diagnosed with CDI relative to those asymptomatically colonized with *C. difficile* in part due to the presence of nontoxigenic *C. difficile* isolates (Figure 3.9.2b). To power our investigation of virulence determinants across patient-colonizing *C. difficile* strains, we performed whole genome sequencing on 102 additional patient-derived *C. difficile* isolates from a previously described *C. difficile*-colonized/CDI cohort from the same hospital[26], where all patients had clinical suspicion of CDI (CDI suspicion), defined by a clinician ordering an EIA test during patient admission.

Using an MLST-based clade definition as above, we identified that most CDI cases result from isolates within clade 1, though clade 2 isolates were more likely to be associated with CDI status (Figure 3.9.4a). The latter finding supports previous data indicating that clade 2 isolates are hypervirulent, often attributed to the presence of the binary toxin operon or increased expression from the PaLoc[19,56,57]. Meanwhile, some clade 1 isolates contain no toxin genes, indicating a diversity of colonization strategies in this lineage. Pangenomic comparison of nontoxigenic versus toxigenic isolates revealed that in addition to the PaLoc, the majority of our toxigenic isolates from clade 1 (95/131 of our cohort) possess remnants of the binary toxin operon (Figure 3.9.4b, *cdtR and cdtA/B* pseudogenes). Interestingly, we found that nontoxigenic isolates had a higher diversity of phage populations relative to toxigenic isolates (Figure 3.11.5, p=5.7e-8, Wilcoxon). Given the previous report that full-length *cdtAB* was identified only within Clades 2,\ 3, and 5[16], we investigated the conservation of *cdtR* (the transcriptional regulator of the binary toxin locus) across *C. difficile* strains (containing 5 lineages). We additionally examined >1400 *C. difficile* genome assemblies from NCBI (Supplementary Table 4, Figure 3.9.4c). *cdtR* (unlike *cdtAB*) was dispersed across clade 1 and significantly associated with *tcdB* (Figure 3.9.4d, Fisher's exact test, BH corrected), suggesting a selective pressure to maintain some element of both toxin loci in these isolates. Notably, these operons are not syntenic, further underlining the significance of the association. From this association, we sought to further understand why some toxigenic clade 1 isolates cause CDI and some colonize without symptoms. Using 148 toxigenic clade 1 isolates collected from this study and two previous studies from the same hospital[26,52], we utilized a bacterial GWAS approach, *pyseer*[53], that identifies genetic traits associated with strains corresponding to patients with CDI symptoms. Using CDI suspicion (see Methods) as an outcome variable, we found that, multiple amidases (including *cwlD*), putative transcriptional regulators,

and many genes of unknown function were enriched in isolates associated with CDI symptoms (Figure 3.9.4e). These data indicate that the most prevalent, circulating *Cd* strains that cause CDI are not the hypervirulent clade 2 strains, but highlight the possibility that remnant genomic features from epidemic strains and other features may contribute to virulence in this hospital clade of *C. difficile*.

# 3.5 Discussion

Through our prospective genomics study of two hospital wards, we were able to identify connections between contamination of different surfaces and the strains carried by hospitalized patients and quantify some spread between carriers. Our estimates of the prevalence of patients with CDI (3.8%) agree with other estimates of 2-4% CDI in patients with cancer[58-60]. While many studies have quantified surface contamination, few have had the genomic resolution to identify clonality between isolates indicating transmission or patient shedding[61-63].

We observed distinct contamination between a patient's bedrail that differed from the strain the patient themselves carried, indicating that the bedrail may be a point of transmission. Further, we did not identify any instances of CDI that could be genomically linked to an earlier CDI case or *C. difficile* carrier. We identified two possible instances of transmission between carriers, though neither of these occurrences resulted in CDI. As this finding is in the context of contact precautions for CDI patients, it indicates that these strategies are successful at limiting transmission of *C. difficile* that causes CDI, and there is limited risk of CDI due to transmission from carriers. These findings confirm previous suggestions that carriers are not a significant risk for transmission leading to CDI[64,65].

Our data suggests the need to investigate diverse lineages of *C. difficile* beyond previous epidemic strains to clarify mechanisms of disease. Among 79 culture positive admissions, we only isolated the epidemic PCR ribotype 027 strain once, causing just one case of CDI within our cohort. Because the overall burden of Clade 1 isolates was so high, we hypothesize that understanding the mechanisms and genomic factors by which these isolates cause disease may become more important as the burden of PCR ribotype 027 decreases[66]. While Clade 1 isolates associated with CDI symptoms are expectedly toxigenic (containing the toxin genes in the PaLoc), we also found an enrichment in two different amidase genes, that could either contribution to differences in germination rate or possess endolysin function[67,68]. How the function of such a gene contributes to an increase in symptomology remains to be understood. Further, we confirmed a genetic relationship between *cdtR* and *tcdB* across *C. difficile* lineages that indicates some evolutionary pressure for maintaining the regulatory gene of the less prevalent toxin operon (*cdtR*). This phylogenomic analysis supports recent functional data from clade 2 isolates that the presence of *cdtR* increases the expression of *tcdB* disease severity in an animal model of CDI[57]. While this was previously suggested *in vitro*, it is unclear how generalizable this relationship is across lineages[56]. In fact, we predict that clade 1 isolates containing only *cdtR* and the PaLoc may produce more toxin *in vivo* than those without *cdtR*. Future studies are warranted to investigate the role of both classes of genes implicated in this phenotype.

Our study has a number of important limitations. As this study focused on *C. difficile* colonization, disease, and transmission in two wards in the same hospital system, studies with increased sample size or meta-analysis studies are necessary to understand generalizable epidemiological measurements of *C. difficile*-patient dynamics[15]. For example,

we were unable to fully quantify in-unit transmission, as not all patients were able to provide stool specimens and/or consented to rectal swabs within 3 days of admission. Additionally, since we did not culture all environmental swabs or specimens, we likely missed some instances of surface contamination or more transient patient carriage, and thus expect that we underestimated the frequencies of contamination and carriage in these wards. Further, the patients housed in the leukemia and HCT ICUs are unique due to their long hospital stays and high antibiotic exposure[69]. While this population was selected specifically to allow us to increase our sample sizes, these patient characteristics could contribute to extended *C. difficile* colonization time relative to other hospital patient cohorts. Finally, we note the evidence for multi-strain colonization within a single patient (Patient 2330). This patient was diagnosed with CDI, but only nontoxigenic *C. difficile* was isolated (network 10). This could be due to co-colonization, and we never isolated the toxigenic isolate responsible for the CDI, or a false positive toxin EIA. Given our approach of only culturing and sequencing single isolates per patient timepoint, future studies are needed to investigate the extent of within-patient *C. difficile* strain diversity by interrogating additional cultured isolates per samples or via metagenomic methods[70].

Despite these limitations, this work highlights new investigative directions for the prevention of CDI. This work and others find risk for patients carrying *C. difficile* long-term in development of CDI, and we hypothesize that the mechanisms of virulence may be more complex than previous epidemic strains. We also hypothesize that non-CDI carriers contribute to the expansion of *C. difficile* transmission networks and emphasize the need to update infection prevention efforts as this landscape evolves. Indeed, though much human and animal research has focused on epidemic strains that are two decades old, we and others

have identified more disease and colonization, largely from clade 1 lineages. We also investigate gene flux of phage like elements, that may play an important role in colonization, particularly in nontoxigenic isolates. Moreover, within this lineage we found a mosaic representation of genes associated with the PaLoc that highlight the possibility of different mechanisms of colonization and virulence by this population of *C. difficile*. Future studies utilizing other human cohorts or animal models are warranted to investigate disease and pathogenicity caused by Clade 1 *C. difficile* strains.

# 3.6 Conclusions

Our study provides new insight into the nature of prevalent *C. difficile* strains in a hospital setting, transmission between carriers, and pathogen evolution during circulation. Longitudinal sampling of surfaces and patient stool revealed that both toxigenic and nontoxigenic strains of *C. difficile* clade 1 are prevalent in these two wards. Moreover, our estimation of carriage patterns emphasizes the need for further investigation into longitudinal carriage of *C. difficile* and its increased risk for CDI. We also note distinct differences in phage carriage between toxigenic and nontoxigenic *C. difficile*. We identity novel associations of accessory genes with CDI symptomology and toxigenicity (*cdtR* and *cwlD*). Our data highlights the complexities of understanding disease from this pathogen in a hospital setting and the need to investigate mechanisms of *in vivo* persistence and virulence of prevalent lineages in the host gut microbiome.

# 3.7 Supplementary Information

3.7.1 List of Abbreviations

BAP: blood agar plate

CCFA-HT: Cycloserine-Cefoxitin Fructose Agar with Horse Blood and Taurocholate

CCMB-TAL: Cycloserine Cefoxitin Mannitol Broth with Taurocholate and Lysozyme

CDI: *Clostridioides difficile* infection

EIA: enzyme immunoassay

HAI: healthcare-associated infection

HGT: horizontal gene transfer

MALDI-TOF MS: Matrix-assisted laser desorption/ionization-time of flight mass spectrometry

NTCD: non-toxigenic *C. difficile*

PaLoc: pathogenicity locus

TCD: toxigenic *C. difficile*

TSB: tryptic soy broth

### 3.7.2 Supplementary Information

Supplementary information and tables can be found in the full text of this manuscript.

## 3.8 Funding and Declarations

### 3.8.1 Ethics approval and consent to participate

The study protocol was approved by the Washington University Human Research Protection Office (IRB #201810103). All participants provided written informed consent.

### 3.8.2 Consent for publication

Not applicable.

### 3.8.3 Availability of data and materials

The datasets generated and analyzed during the current study are available in NCBI GenBank under BioProject accession no. PRJNA980715.

#### 3.8.4 Competing interests

The authors declare that they have no competing interests.


#### 3.8.5 Funding

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#### 3.8.6 Authors' contributions

SRSF, KAR, ERD, and GD participated in idea formulation and funding for this project. TH, KAR, CC, ZHI, ELS, and ERD conducted participant enrollment, sample collection, and microbiological isolation. EPN, SRSF, KZ, and GD conducted all sequencing analysis and figure generation. EPN and SRSF completed the writing of the manuscript. All authors read and approved the final manuscript.


#### 3.8.7 Acknowledgements

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## 3.9 Figures

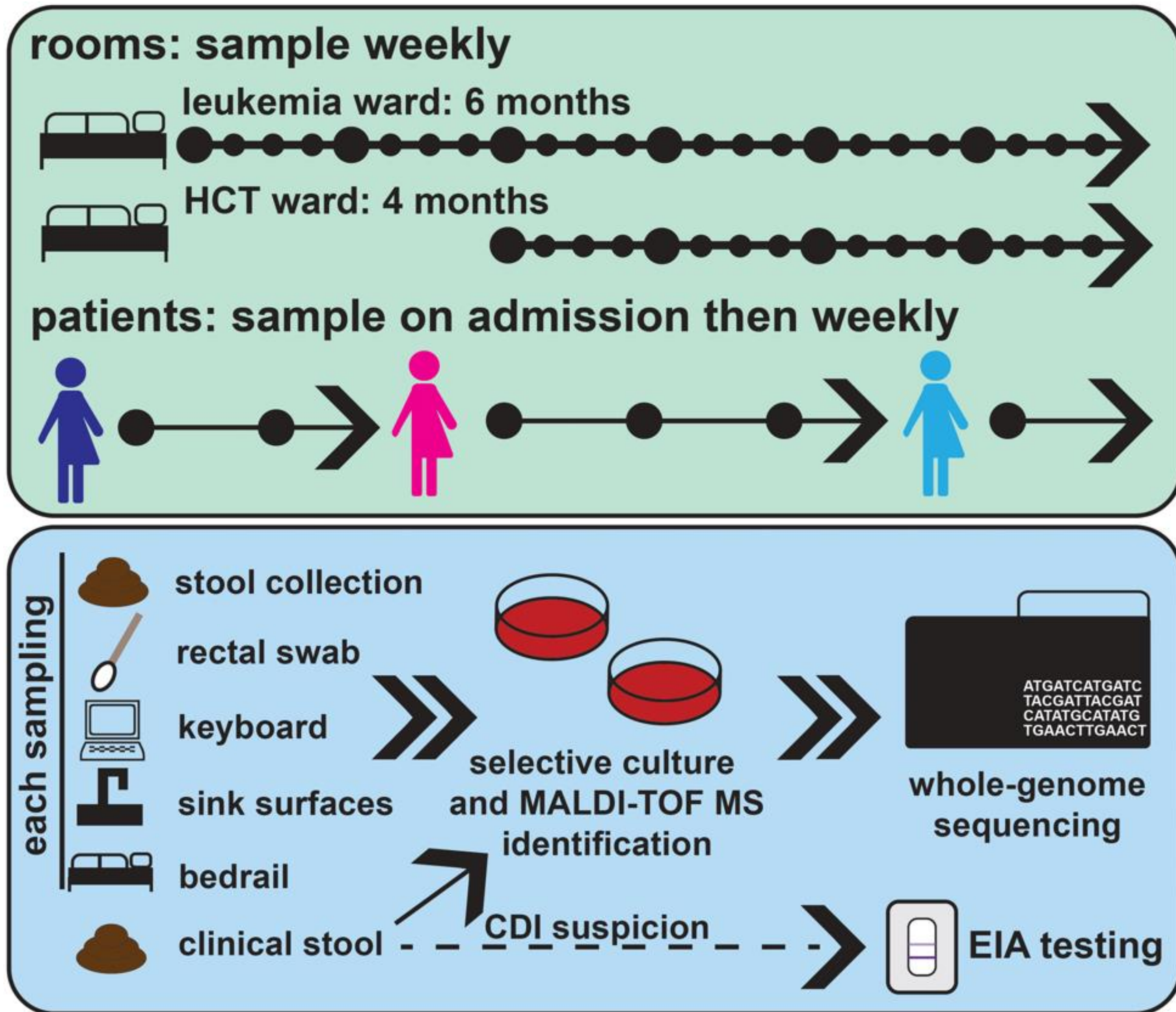


**Figure 3.9.1 Study sampling and testing overview.** We sampled a leukemia and hematopoietic stem cell transplant ward at Barnes-Jewish Hospital in St. Louis, USA for 6 and 4 months respectively. Patients were enrolled and sampled upon admission, and then weekly for their time in the study wards. Surfaces were sampled weekly across the duration of the study. All samples and stool collected as part of routine clinical care were subjected to selective culture and MALDI-TOF MS identification, and isolates were whole-genome sequenced. Results of EIA testing as part of routine care were obtained.

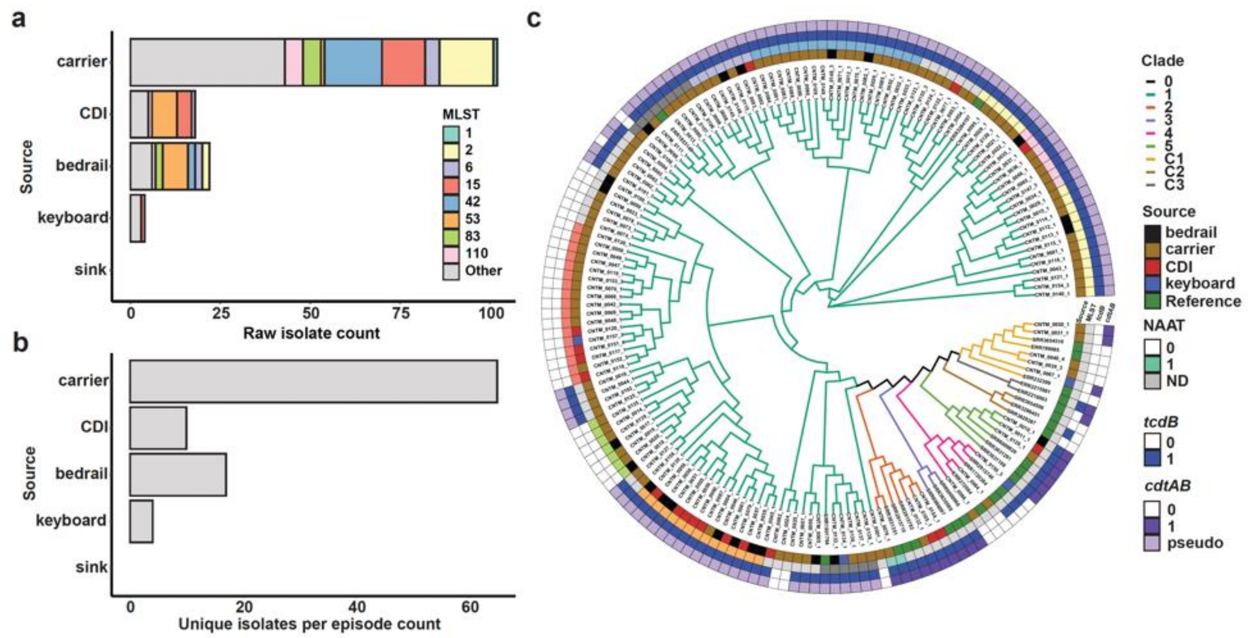


**Figure 3.9.2 Total samples collected and phylogenetic relationships reveal carriers outnumber CDI patients and bedrails are the most commonly contaminated surface.** Total **a)** isolates collected and **b)** culture-positive episodes from each source. We found more carriers than CDI patients, and bedrails yielded the most C. difficile isolates. **c)** Cladogram of all isolates collected during this study plus references.

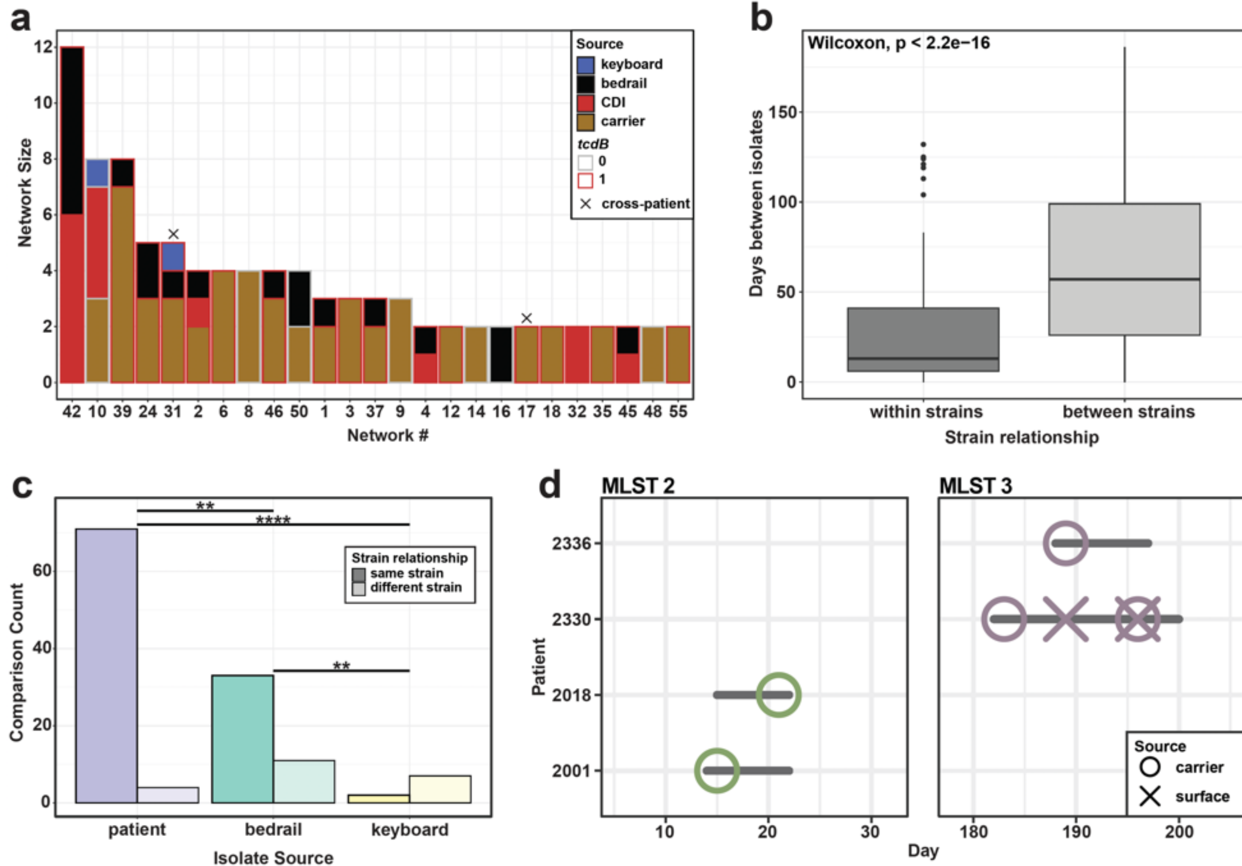


**Figure 3.9.3 Surfaces are a site of environmental contamination and potential for transmission from colonized and CDI patients. a)** Strain networks were defined by <=2 MLST core gene SNP cutoff. Network 10 includes the non-toxigenic isolates from Patient 2245 that are likely not responsible for the CDI. **b)** Absolute value of days between isolates within strains and between strains. Isolates within the same strain were significantly temporally linked ($p<2.2e-16$, Wilcoxon test). **c)** Number of comparisons in each group that fall within strain cutoff. Patient: between two isolates collected from the same patient; bedrail: between a patient isolate and an isolate taken from their bedrail; keyboard: between a patient isolate and an isolate taken from their keyboard. Fisher's exact test, BH corrected. **d)** Strain tracking diagram of transmission networks associated with more than one patient. Colors indicate MLST of network and horizontal lines indicate stay in a room. Patient 2330 sheds C. difficile onto the bedrail and patient 2336 later is identified as a carrier of the same strain.

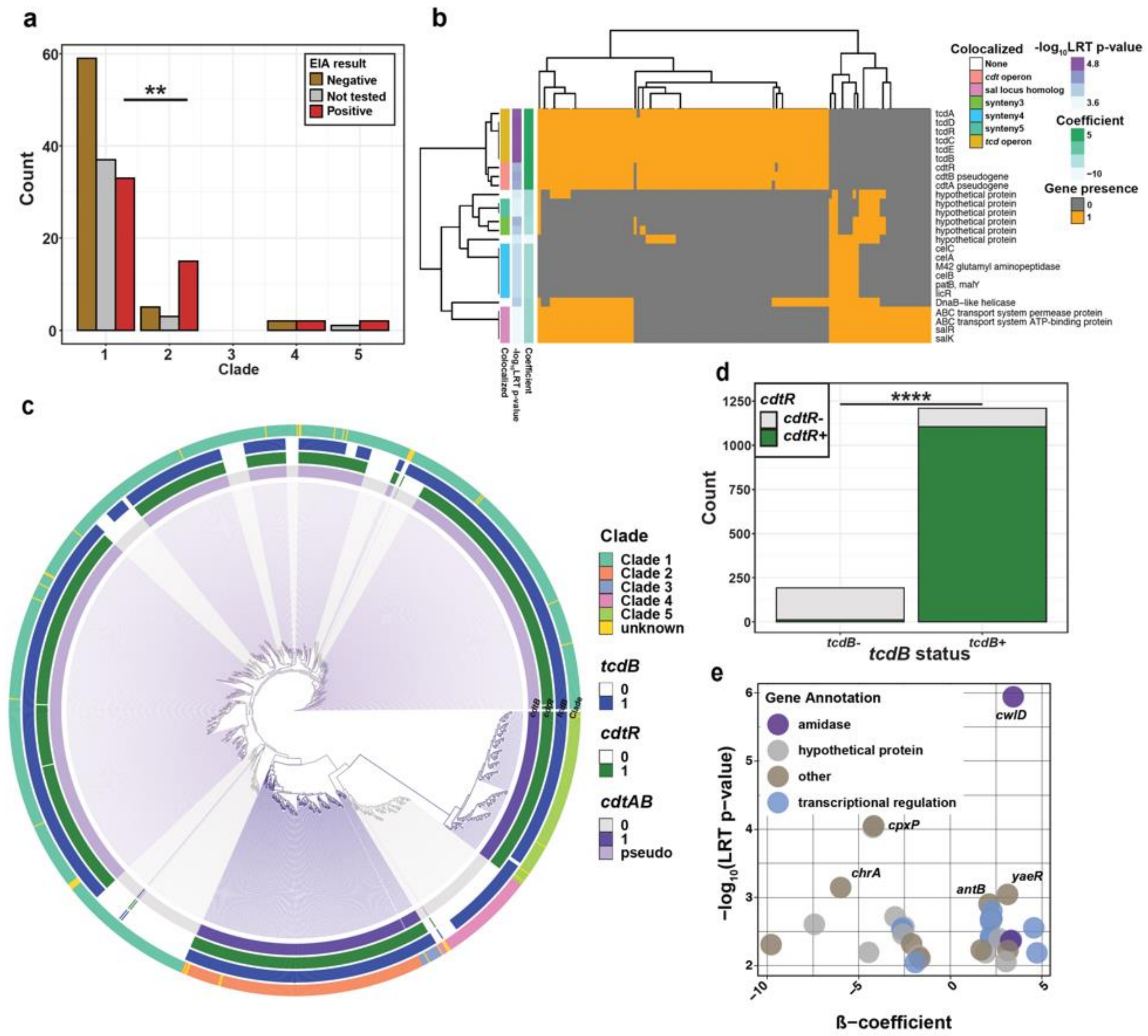


**Figure 3.9.4 Clade 1 is responsible for the majority of CDI cases and carries unique correlates to symptom severity. a)** EIA status by clade across this and a previous study26. Fisher's exact test, p<0.01. **b)** Differentially abundant genes between toxigenic and nontoxigenic isolates in clade 1 from this study. Genes with a population structure adjusted p-value of <0.001 as produced by pyseer. **c)** Phylogenetic tree of >1400 C. difficile isolates from NCBI (Supplementary Table 4) depicting presence of binary toxin and PaLoc operons. **d)** Presence of full-length cdtR and association with tcdB presence. **e)** Filtered results (p-values <0.01) pyseer analysis evaluating gene association with CDI suspicion in Clade 1 isolates using the phylogenetically-corrected p-values (LRT). Purple color indicates p<0.001. Positive beta coefficient indicates gene association with CDI suspicion, while negative beta indicates asymptomatic colonization.

## 3.10 Tables

Table 3.10.1 C. difficile epidemiology on the admission level (N=647 admissions)

| **Variable** | **N (%)** |
|---|---|
| Admissions with ≥1 stool and/or rectal swab specimen collected | 364 / 647 (56) |
| Culture positive any time during admission | 79 / 364 (22) |
| Toxigenic culture positive at any time during admission | 57 / 364 (16) |
| Toxigenic culture positive on admission (**Colonized on admission**) | 19 / 364 (4) |
| Toxigenic culture negative on admission, toxigenic culture positive later in admission (**Acquisition)** | 6 / 364 (2) |
| Unknown colonization status on admission, toxigenic culture positive later in admission (**Indeterminate acquisition**) | 32 / 364 (9) |
| Non-toxigenic culture positive at any time during admission | 26 / 364 (7) |
| Non-toxigenic culture positive on admission (**Non-toxigenic colonized on admission**) | 6 / 364 (2) |
| Non-toxigenic culture negative on admission, toxigenic culture positive later in admission (**Non-toxigenic acquisition**)[a] | 2 / 364 (1) |
| Unknown colonization status on admission, non-toxigenic culture positive later in admission (**Indeterminate acquisition**)[b] | 18 / 364 (5) |
| EIA positive during admission | 25 / 647 (4) |
| EIA positive during admission with specimen(s) collected | 17 / 364 (5) |
| Indeterminate toxigenic *C. difficile* acquisition | 6 / 17 (35) |
| New acquisition | 3 / 17 (18) |
| False positive EIA[c] | 3 /17 (18) |
| Colnoized on admission or pre-existing colonization | 3 / 17 (18) |
| Recurrent CDI | 2 / 17 (12) |

[a]Includes one patient admission with non-toxigenic *C. difficile* acquisition who was co-colonized with toxigenic *C. difficile* on admission. (This patient is also counted in the toxigenic culture positive colonized on admission row.)

[b]Includes two patient admissions co-colonized with toxigenic *C. difficile*; both toxigenic and non-toxigenic were indeterminate acquisition. (These patients are also counted in the toxigenic culture indeterminate acquisition row.)

[c]These results were considered false positive EIAs because both the clinical stool specimen and additional surveillance stool or rectal swab specimens were toxigenic culture negative. One participant had a culture negative clinical specimen and culture negative stool and rectal swab specimens from the subsequent day. The second participant had a culture negative clinical specimen and six additional culture negative specimens (5 stool, 1 rectal swab) collected during admission, ranging from three days prior to clinical stool collection through 26 days after clinical stool collection. The third participant had four non-toxigenic culture positive specimens during the EIA+ admission (including the clinical stool; dates ranged from 8 days prior to EIA+ through 6 days post-EIA+ collection) and three non-toxigenic culture positive specimens during a subsequent admission; all seven non-toxigenic culture positive specimens were the same strain. None of the three participants had a toxigenic culture positive specimen during the EIA+ admission.

## 3.11 Supplementary Figures

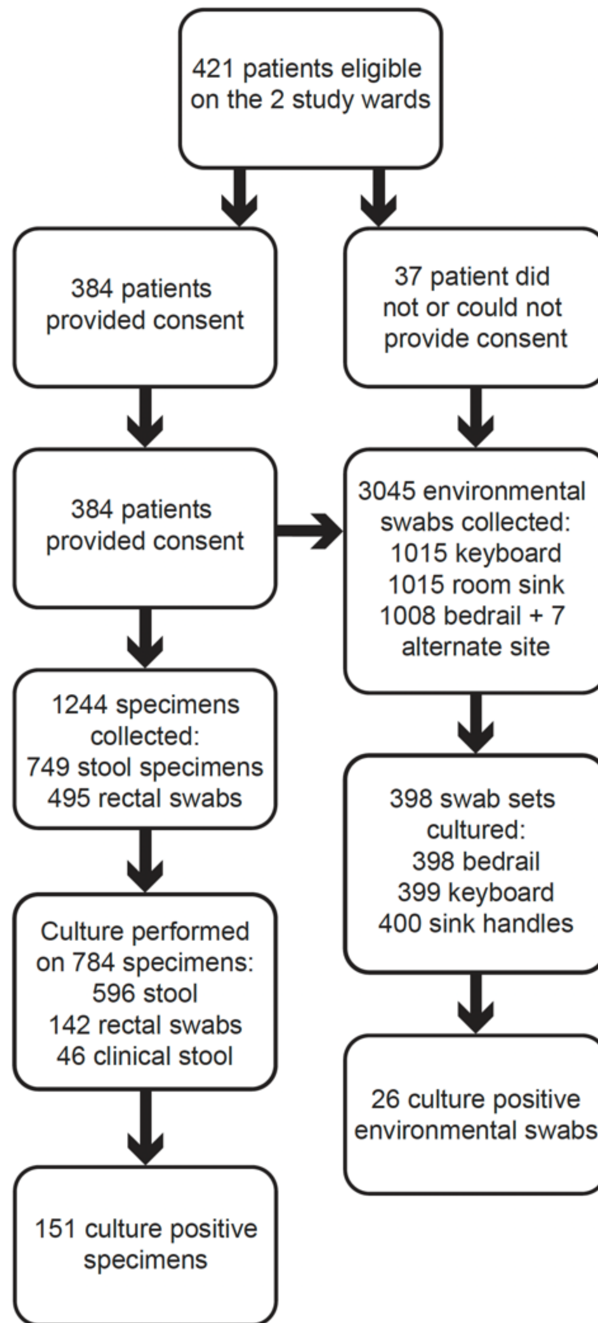


**Figure 3.11.1 Bubble plot of enrollment, collection, and culture numbers.**

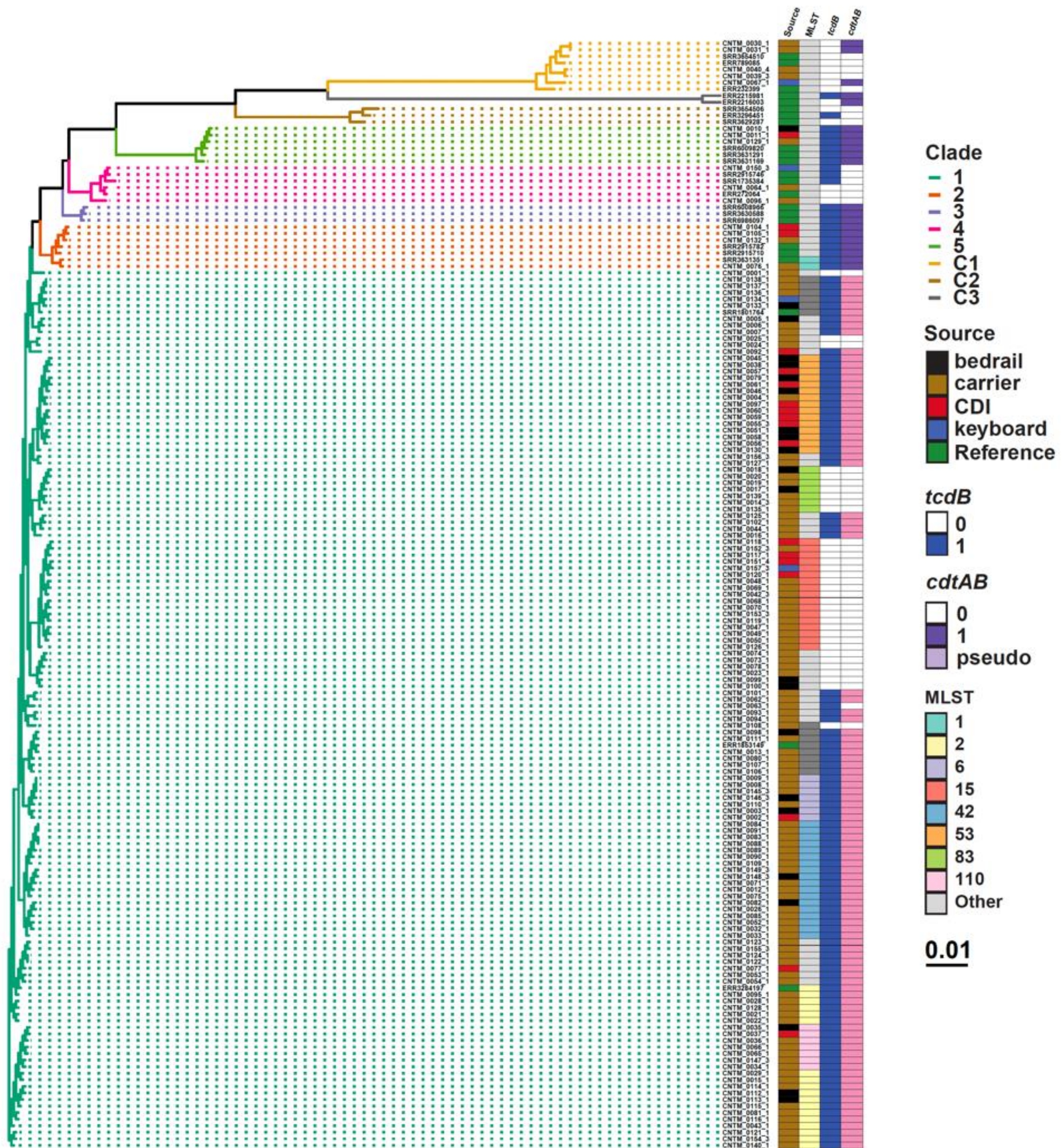


**Figure 3.11.2 Phylogenetic tree of isolates collected in this study and select references (Supplementary Table 2).**

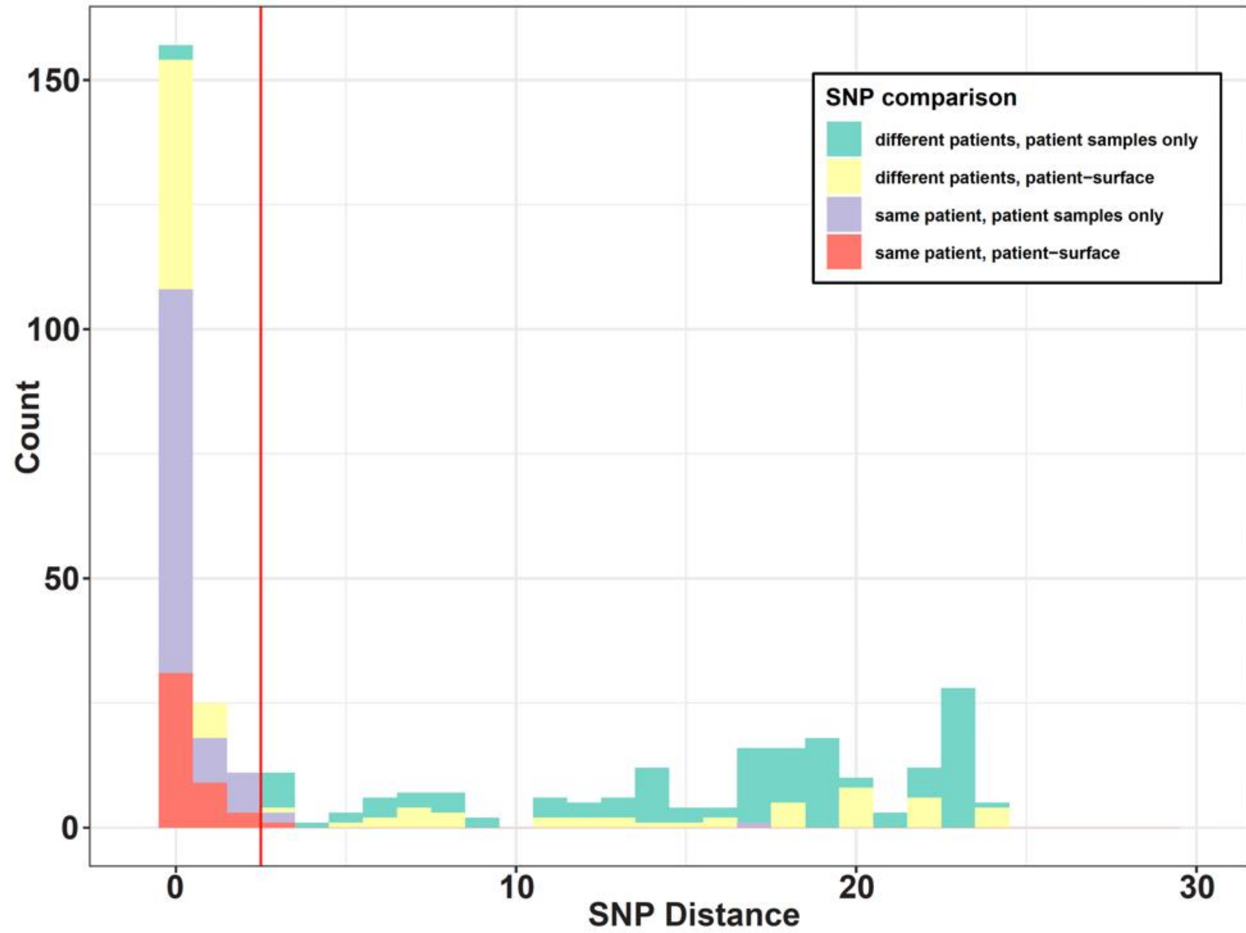


**Figure 3.11.3 Histogram of core genome SNP distances between different within-MLST isolate comparisons, zoomed to show SNP cutoff (red line at 2 SNPs).**

**Figure 3.11.4 Bedrail isolates versus CDI presence in network. A)** Total number of bedrail isolates in networks either containing a CDI case or not containing a CDI case. **B)** Number of unique bedrails contaminated in a network either containing a CDI case or not containing a CDI case. Student's t-test.

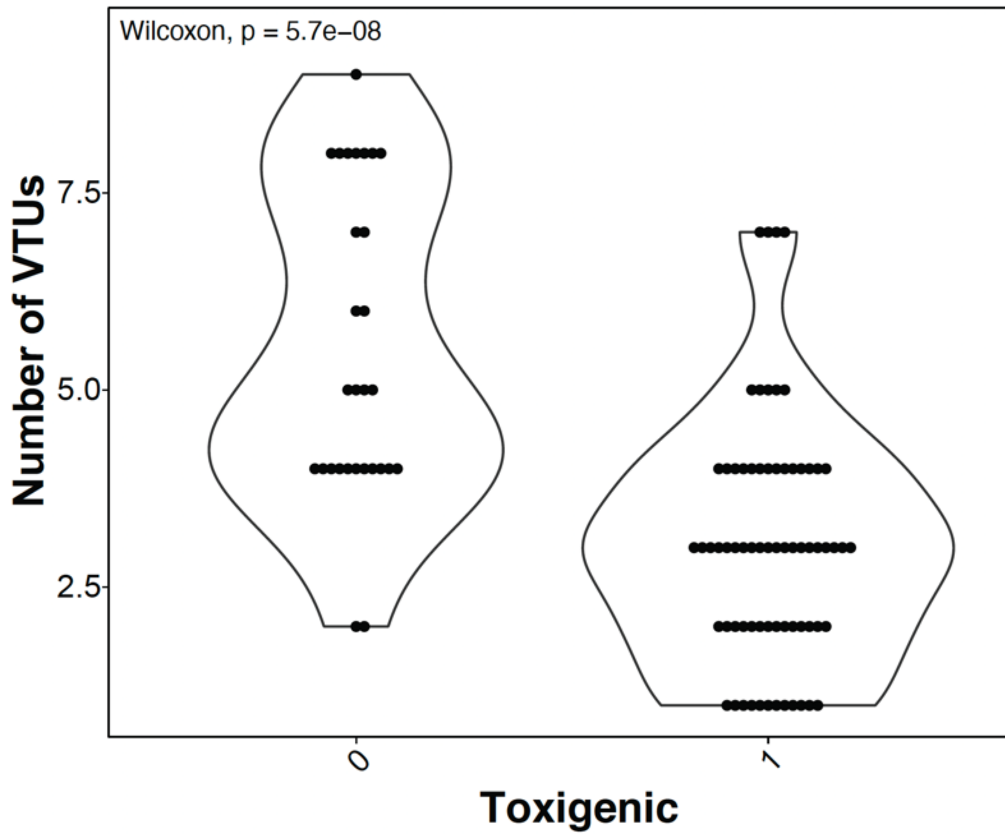


**Figure 3.11.5 Phage population richness across toxigenic and nontoxigenic isolates in our cohort.** Wilcoxon test, p=5.7e-8.

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# Chapter 4

# Environmental hygiene intervention reduces hospital sink drain antibiotic resistant organism burden

The contents of this chapter are adapted from a manuscript that is currently in submission:

**Newcomer EP**, O'Neil C, Vogt L, McDonald D, Cass C, Wallace MA, Hink T, Yerbic F, Meunks C, Gordon R, Stewart H, Arter O, Amor M, Jolani K, Alvarado K, Valencia A, Samuels C, Peacock K, Park D, Struttmann E, Sukhum KV, Burnham CAD, Dantas G, Kwon J. Environmental hygiene intervention reduces hospital sink drain ARO burden. 2023, *In submission.*

# 4.1 Key Points

4.1.1 Question

Does a sink cleaning protocol utilizing bleach wipes and foamed peracid based disinfectant reduce antibiotic resistant organism (ARO) burden in hospital sinks?

4.1.2 Findings

This environmental hygiene intervention was able to reduce Gram-negative bacterial burden at high (5x/week) and low (1x/week) interventional frequencies by up to 80%. It also reduced *Pseudomonas aeruginosa* and *Stenotrophomonas maltophilia* recovery by up to 82% in the low frequency and 100% in the high frequency.

4.1.3 Meaning

This sink cleaning protocol was able to reduce ARO burden in sink drains.

# 4.2 Abstract

Healthcare associated infections (HAIs) associated with hospital sinks have been noted for years, however, there remains no standardized environmental hygiene protocol to combat sink-related HAIs. The goal of this study was to evaluate the impact of an environmental hygiene intervention on antibiotic resistant organism (ARO) recovered from hospital sinks. This study was a longitudinal environmental hygiene intervention. We collected E-swabs (Copan, Murrieta, CA) of sink drains and surrounding surfaces during a pre-intervention, two intervention periods, and two post-intervention periods. Samples were selectively cultured for AROs. We obtained information on HAIs that occurred in study rooms. This study took place in 18 rooms in a stem cell transplant and oncology ICU and a surgical ICU at a large tertiary care academic medical center. All patients housed in the study wards during the study period. At control (0x/week), low frequency (1x/week), and high frequency (5x/week) intervals, we wiped sink surfaces with 10% bleach wipes and

pumped a foamed peracid disinfectant (Virasept, Ecolab, St. Paul, MN) into sink drains, allowing a contact time of 3 minutes before rinsing. We investigated the total and Gram-negative burden of recoverable bacteria from sink surfaces before, during, and after intervention. We quantified the number of unique antibiotic resistant organisms, *Pseudomonas aeruginosa*, and *Stenotrophomonas maltophilia* we recovered across intervention phases. We quantified the number of HAIs identified in rooms in each study arm. The intervention reduced total microbial and Gram-negative burden at both frequencies, with the high frequency reducing the proportion of sink drains yielding Gram-negatives by >80% ($p<0.05$). The low and high frequencies reduced the proportion of sink drains yielding *Pseudomonas aeruginosa* (up to 82% and 100%, respectively) or *Stenotrophomonas maltophilia* (up to 68% and 94%, respectively). No significant differences were found in the number of HAIs identified in each study arm. A sink cleaning protocol utilizing bleach wipes and a peracid based disinfectant delivered via foaming pump effectively reduced ARO burden in sinks drains. The study protocol was approved by the Washington University Human Research Protection Office (IRB #202008081).

## 4.3 Introduction

Healthcare-associated infections (HAIs), particularly those caused by antibiotic resistant organisms (AROs), are on the rise and cause substantial morbidity, mortality, and financial burden for patients[1-7]. Recent HAI outbreak studies have revealed that hospital plumbing, particularly sink drains, can be linked to ARO transmission and outbreaks[6, 8-13]. Patients in intensive care units (ICUs) are at elevated risk for HAIs due to frailty, comorbidities, and frequent antibiotic exposures[14]. Many infection prevention measures implemented in hospitals, such as contact precautions for *Clostridioides difficile* infections and improved hand hygiene have greatly reduced HAIs[15-17]. However, there are currently no standardized environmental hygiene protocols for sink

drains[18]. The growing recognition that sink drains may function as ARO reservoirs obliges the development of protocols to reduce ARO burden to prevent HAIs.

Approaches to disinfecting premise plumbing have varied depending on the source and type of contamination. Outbreaks of water-associated pathogens, such as *Legionella* spp. and *Pseudomonas aeruginosa*, have been linked to contamination of tap water or upstream water infrastructure[19-24]. Therefore, remediation often involves the addition of a water filter or faucet with a built-in filter. However, water filters alone have no impact on secondary contamination from other sources[25]. To reduce the risk of secondary contamination, some have proposed the complete removal of sinks, or 'water-free' ICUs, but this may be impractical and prohibitively expensive for most healthcare facilities[26, 27]. Chemical-based disinfectants, such as bleach, hydrogen peroxide, or acetic acid, may have activity against AROs, but pouring liquid disinfectants into sink drains may lead to insufficient contact time and can produce noxious vapors[28-30]. To overcome this limitation, we tested the ability of an environmental hygiene intervention utilizing foamed peracid based disinfectant to reduce the burden of AROs in sink drains in two ICUs at a large academic medical center[28].

## 4.4 Methods

### 4.4.1 Study design

The study protocol was approved by the Washington University Human Research Protection Office (IRB #202008081).

This study was conducted in the stem cell transplant ICU (SCTO-ICU) and surgical ICU (SICU) at Barnes-Jewish Hospital (BJH), a 1,278-bed tertiary care academic medical center in St. Louis, Missouri, United States[31]. These ICUs are distinct from one another, but are located on the same floor, have the same room and sink design, and share staff. Eighteen patient rooms were included

in the intervention trial: 8 in the SCTO-ICU housing SCTO-ICU patients, 8 in the SICU housing SICU patients, and 2 in the SICU but housing SCTO-ICU patients. Six rooms were randomly assigned to each of 3 intervention arms: a control arm, a low-frequency intervention arm (1x/week), and a high frequency intervention (5x/week) arm. The drains in two shared spaces in each ICU, the housekeeping closet (HC) and soiled utility room (SU), were also included; the shared spaces in the SICU were assigned to the low-frequency intervention arm and the shared spaces in the SCTO-ICU were assigned to the high frequency intervention (Figure 4.9.1).

The study included 5 phases: a 16-week baseline period (Phase 1), a 27-week first intervention period (Phase 2), a 16-week follow-up period (Phase 3), a 23-week second intervention period (Phase 4), and a 16-week follow-up period (Phase 5). During both intervention periods, the low frequency intervention arm received the intervention once per week and the high frequency intervention arm received the intervention 5 times per week; control rooms received only routine cleaning by hospital staff and no intervention (Figure 4.9.1). During the follow-up periods, all rooms only received routine cleaning by hospital staff and no intervention.

4.4.2 Environmental hygiene intervention

For sinks in patient rooms, the intervention included 2 steps. First, Sani-Cloth™ Bleach Germicidal Disposable Wipes (PDI™, Woodcliff Lake, NJ) were used to clean the faucet, sink bowl, lower counter surrounding the sink, and raised counter next to the sink. A separate wipe was used for each surface, and, after a contact time of 10 minutes, the surfaces were rinsed with distilled water and then wiped dry with a paper towel. Next, a Foam-It Pump (FOAMit, Grand Rapids, MI) was used to infuse 10 ounces of a peracid based disinfectant (Virasept, Ecolab, St. Paul, MN), which is EPA-approved for biofilm disinfection, directly into the sink drains[32]. After a dwell time of 3 minutes, the faucet was run for 30 seconds. In the HC and SU, a larger FOAMit pump was

used to infuse 32 ounces of Virasept into the primary sink drain. No bleach wipes were used in these shared spaces due to a lack of sink surfaces. Each room was assigned its own FOAMit pump to reduce potential cross contamination and the pumps were cleaned after each use.

#### 4.4.3 Environmental swabbing and selective culture

Environmental samples were collected in study rooms a total of 31 times during the 2-year study period. During non-intervention phases, samples were collected weekly for 3 weeks and then monthly until the start of the next phase. During intervention phases, samples were collected monthly. At each sampling time point, 3 E-swabs (Copan, Murrieta, CA) were moistened with sterile molecular water and held in tandem to sample each of the following surfaces in each patient room: the sink drain, the faucet, the sink bowl, the lower sink counter, and the raised sink counter (Figure 4.9.1)[6]. Samples were also collected from sink drains in the HC and SU rooms in both ICUs. On dates when the intervention was applied, environmental samples were collected prior to application of the intervention. After collection, all E-swabs were placed into Aimes transport medium (Thermo Fisher Scientific, Lenexa, KS) and stored at room temperature for up to 4 hours before culturing.

Full details on selective culture processing can be found in Supplementary Table 1. Briefly, for each surface sample, 100 μL of eluate was cross-streaked onto each of the selective agars found in Supplementary Table 1. Colony counts up to 200 per morphotype were recorded and general details of growth were described. Unique morphotypes were sub-cultured to blood agar plates (BAP) for isolation and identified using matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry (MS) using the VITEK MS (bioMerieux, Durnham, NC). All culturing, colony selection, and identification was conducted by a trained microbiologist.

#### 4.4.4 Water sampling and selective culture

During alternating weeks during the intervention phases, and week 3 and month 2 of the non-intervention phases, water samples were collected from the sinks in the included patient rooms, HCs, and SUs. Each faucet was run for 10-20 seconds before 1 L of water was collected directly into a sterile plastic container. Water samples were stored at room temperature for up to 24 hours before processing. Samples were then poured through a sterile membrane filter (Pall, Port Washington, NY).[33] The filter was then placed grid side up on a blood agar plate and incubated for up to 48 hours at 35°C. Water samples were considered negative if ≤50 colonies grew on a heterotrophic plate, which is 1 order of magnitude below than the Environmental Protection Agency (EPA) standard[34, 35]. Unique colony morphologies were identified as described above and confirmed AROs (Supplementary Table 1) were used for isolate count analysis.

4.4.5 HAI data collection

Data on HAIs among patients admitted to the SCTO-ICU and SICU during the study period, including central line-associated blood stream infections (CLABSIs), catheter-associated urinary tract infections (CAUTIs), and ventilator-associated events (VAEs), were obtained from BJH Infection Prevention. This data was generated in accordance with standard National Healthcare Safety Network (NHSN) surveillance protocols.

4.4.6 Statistical analysis

All data analysis was conducted using Rstudio and visualized using the ggplot2 package[36]. We used pairwise Wilcoxon tests with BH correction to compare isolate counts between different environmental surfaces (Figure 4.9.2). We used a Kruskal-Wallis test followed by a Dunn's test to identify differences between intervention groups during the baseline phase. We conducted permutation tests with BH correction to compare proportions of rooms to baseline in Figures 4.9.3-4. Full results can be found in Supplementary Tables 2-7 and figure generation code can be found in Supplementary File 1.

## 4.5 Results

4.5.1 Specimen collection and microbial culture

We collected a total of 2,766 environmental swabs and 234 water samples during the study period. From these, we identified a total of 1,187 bacterial isolates (Figure 4.10.1, Supplementary Table 8).

4.5.2 Sink drains and surrounding surfaces provide the most frequent growth on BAPs

During the baseline phase, we recovered viable, cultivatable growth (growth) on BAPs from a relatively high proportion of patient room sink drains (mean=0.87, se=0.3) and shared area drains (mean=1.00, se=0) each week (Figure 4.9.2a). We also recovered growth from high proportions of lower sink counters (mean=0.80, se=0.6) and raised sink counters (mean=0.92, se=0.2). We recovered growth from a moderate proportion of sink bowls (mean=0.58, se=0.8) and faucets (mean=0.50, se=0.6). Although water sampling frequently yielded growth (mean=0.8, se=0.15), growth was rarely above EPA-designated levels (mean=0.06, se=0.06)[35]. All surface and water sample microbiology results are in Supplementary Tables 9 and 10.

4.5.3 Sink drains house the most unique ARO isolates, particularly *Pseudomonas aeruginosa* and *Stenotrophomonas maltophilia*

Patient room sink drains yielded a mean of 2.12 (se=0.11) unique ARO isolates per drain, per week, while we recovered a mean of <0.3 ARO isolates per surface, per week from other patient room surfaces (Figure 4.9.2b). The shared area drains frequently yielded the most ARO isolates, with a mean of 5.13 isolates (se=0.43) per drain, per week.

Across all sample types, the most isolated genera were *Pseudomonas* and *Stenotrophomonas*, which comprised 37.9% (450/1187) and 19.9% (236/1187) of isolates (Figure 4.10.2), respectively. Of these, *P. aeruginosa* and *S. maltophilia* were the most frequently isolated species, comprising 18.4% (219/1187) and 19.8% (235/1187), respectively, of all isolates collected. We recovered *P. aeruginosa* and *S. maltophilia* from a mean of 0.37 (se=0.04) and 0.52 (se=0.06)

patient room sink drains per week, respectively. This was higher than all other patient room surfaces, but similar to the proportion of shared area sink drains yielding these organisms (Figure 4.9.2c,d).

4.5.4 The intervention reduced the proportion of sink drains from which we recovered growth.

During the baseline period, we recovered growth from fewer sink drains that received the high frequency intervention than those that received the low frequency intervention, on BAP ($p<0.05$, Dunn's test), but not on MAC plates (Figure 4.9.3a,b).

When comparing the two intervention periods to the baseline period, the low frequency intervention rooms showed 31% and 36% decreases in the proportion of drains from which we recovered growth on BAP ($p<0.05$, permutation test, BH corrected). This extended into the two follow-up phases, where we recovered growth from 31% and 19% fewer drains per week ($p<0.05$, Figure 4.9.3a). The high frequency intervention also showed significant decreases in the proportion of drains yielding growth on BAP, with 33% and 62% decreases during the intervention phases ($p<0.05$). Control room drains showed insignificant decreases of 4% and 7% relative to baseline during the intervention phases (ns, $p>0.05$). There were no significant decreases in the proportion of surfaces with growth on BAP from non-drain patient room surfaces during either intervention period (ns, $p>0.05$, Figure 4.10.2).

When comparing Gram-negative yield on MAC plates, we recovered growth from 39% and 42% fewer sink drains in the low frequency intervention rooms during intervention phases; however, neither decrease reached statistical significance (ns, $p>0.05$, Figure 4.9.3b). Drains from the high frequency intervention rooms had reductions by 85% and 83% during the two intervention phases followed by decreases of 41% and 62% in the follow-up phases ($p<0.05$). The control rooms, in

contrast, had 2% and 23% reductions in proportion of drains with recovery on MAC when compared to baseline, none of which reached statistical significance (ns, $p>0.05$).

4.5.5 Both interventions significantly reduced recovery of AROs from sink drains

In the low-frequency intervention rooms, the number of unique AROs recovered from sink drain samples decreased by 48% and 66% in the intervention phases relative to baseline ($p<0.05$). The high frequency intervention room drains showed even more pronounced decreases: 69% and 97% less unique AROs were recovered during intervention phases than during baseline ($p<0.05$) (Figure 4.9.4a). For multiple weeks during both intervention phases, we recovered no AROs from the high frequency intervention drains. The high frequency intervention rooms maintained this significant reduction in ARO recovery throughout both post-intervention phases, with 61% and 65% decreases relative to baseline ($p<0.01$). We also recovered fewer unique AROs from drains in the low frequency intervention drains during the follow-up periods (36% and 10% reductions versus baseline), but these decreases were not statistically significant (ns, $p>0.05$). In the control rooms, the number of unique AROs also decreased significantly during the intervention periods; by 38% and 50% relative to baseline, as well as by 56% in the first follow-up phase ($p<0.05$) (Figure 4.9.4a).

The low frequency intervention rooms experienced 62% and 82% decreases in *P. aeruginosa* recovery frequency, and 44% and 68% decreases in *S. maltophilia* recovery during the two intervention periods, relative to baseline. These decreases were variably statistically significant (Figures 4.9.4b,c). In the high frequency intervention rooms, the proportion of drains with recovery of *P. aeruginosa* significantly decreased by 81% and 100% during intervention phases ($p<0.05$) (Figure 4.9.4b). Similarly, recovery of *S. maltophilia*, decreased by 72% (ns, $p>0.05$) and 94% ($p<0.05$) during intervention phases (Figure 4.9.4c). In the control rooms, we observed 3% and

36% statistically insignificant reductions in proportion of drains with recovery of *P. aeruginosa* and 27% and 54% statistically insignificant reductions proportion of drains with recovery of *S. maltophilia* (ns, p>0.05).

There were no significant decreases in unique AROs, *P. aeruginosa*, or *S. maltophilia* from non-drain surfaces in the control rooms or either intervention group (p>0.05) (Figure 4.10.3).

4.5.6 The interventions had no identifiable effects on HAIs in study rooms

During the study period, BJH Infection Prevention identified a total of 69 patient CLABSIs, VAEs, and CAUTIs in the study ICUs that they determined to be healthcare-associated by NHSN standards. There were no significant differences in the rates of HAIs across the 5 phases of the study (ns, p>0.05) (Figure 4.10.4).

## 4.6 Discussion

In this study, we identified sink drains as a common and significant source of AROs. While we found that both sink drains and surrounding sink surfaces yielded viable, cultivable bacteria, sink drains were the dominant source of ARO isolates, including *P. aeruginosa* and *S. maltophilia*. This finding is consistent with previous studies, which have identified sink drains as a potential reservoir for AROs that may cause serious HAIs[6, 8, 11-13, 37, 38]. Sinks and sink drains have also been implicated as the probable source of multiple hospital outbreaks of *P. aeruginosa*[11-13, 37] and *S. maltophilia*[39].

We tested an environmental hygiene intervention consisting of a foaming chemical disinfectant applied to sink drains and bleach wipes applied to sink surfaces, implemented at two different frequencies (once per week and five times per week). The intervention reduced the proportion of sink drains with Gram-negative bacteria recovered by up to 42% for the low frequency and up to 85% for the high frequency when compared to pre-intervention proportions. This finding is

important because outbreaks associated with sink drain contamination are frequently associated with Gram-negative bacteria[6, 8, 10-12, 30, 37, 40]. Crucially, the intervention reduced the proportion of drains from which we recovered *P. aeruginosa* and *S. maltophilia,* which are known to form environmental reservoirs and biofilms and are a common cause of HAIs[6, 11-13, 37, 39, 41]. While both intervention frequencies were associated with significant decreases in ARO isolate recovery, the impact of high frequency intervention on *P. aeruginosa* and *S. maltophilia* recovery resulted in a lasting impact extending through the 2 post-intervention phases. In fact, the high frequency intervention was associated with up to 100% decreases in the proportion of sink drains from which we recovered *P. aeruginosa* and 94% for *S. maltophilia*. The success of this intervention may be due to the use of foamed chemical disinfectant. Disinfectants delivered in foam form, as compared to liquid disinfectants, would have increased contact time with the interior surfaces of sink drain, which may aid in breaking down biofilms[34, 42].

While previous studies have tested a wide variety of potential solutions to reduce ARO burden in hospital sinks, many of these strategies have limited or contradictory evidence for their effectiveness, or are not practical to implement in most real-world hospital environments because they are expensive, time-consuming, and/or complicated to implement[26, 27, 43, 44]. The intervention proposed here utilizes commercially available products but do require training and personnel time to implement. While we found that the intervention was most effective against key AROs known to cause HAIs when applied 5 times a week, further study is necessary to determine feasibility of the intervention in a real-world, hospital setting by hospital personnel.

Despite our success at reducing microbial burden and ARO carriage in sink drains, this study is subject to several limitations. First, this intervention was performed by trained study personnel, not hospital staff. Additional research is therefore needed to evaluate the feasibility and

effectiveness of the intervention when performed by hospital staff. Second, when culturing environmental samples, we tailored our selective culture methods to isolate bacteria specifically associated with HAIs and sink drain-associated outbreaks. These methods may miss other organisms that may cause HAIs, including fungi. Third, this study involved only two ICUs and a relatively short follow-up period. While we observed that the impact of our sink hygiene intervention continued after the intervention was no longer applied, future work is needed to determine the full duration of this impact, and to determine whether the intervention is similarly effective in other ICU and non-ICU healthcare settings. Fourth, while our intervention reduced ARO burden in hospital sink drains, it did not have an observable impact on patient HAIs. This could be due to the small sample size and to the relatively short follow-up period. Because only 69 HAIs were identified among patients admitted to the two study ICUs during the 2-year study period, this left us under- powered to identify potential changes in HAIs during the intervention periods. Future work is needed to directly explore the impact of the intervention on patient infections.

Establishing evidence-based methods to reduce the risk of HAIs caused by AROs is critical as rates of antibiotic resistance continue to rise globally[45-47]. While reducing ARO contamination in the healthcare environment, and particularly sink drains, appears to be a likely way to reduce HAIs, there are currently no standardized, evidence-based practices for cleaning or disinfecting sink drains in healthcare settings in the United States[48]. Our results showcase the potential of an environmental hygiene intervention in reducing microbial growth in sink drains. The study provides critical groundwork for establishing practical and standardized protocols for hospital sink hygiene.

# 4.7 Supplementary Information

Supplementary information and tables can be found in the full text of this manuscript.

# 4.8 Funding and Declarations

4.8.1 Data Sharing Statement

All culture collection and analysis data are available in the Supplementary Tables.

4.8.2 Funding and Declarations

This work was supported in part by an award to J.H.K., G.D., and C.D.B. from the Agency for Healthcare Research and Quality (AHRQ) of the US Department of Health & Human Services (DHHS) (grant number R01HS027621); an award to G.D. by the National Institute of Allergy and Infectious Diseases (NIAID) of the National Institutes of Health (NIH) (grant number U01AI123394); and awards to J.H.K. from NIAID (grant number 1K23AI137321), Barnes-Jewish Hospital Foundation (award number 5102), and the Washington University Institute of Clinical and Translational Sciences grant UL1TR002345 from the National Center for Advancing Translational Sciences (NCATS) of the NIH (Award number 4462). K.V.S. was supported by a Research Scholar Award from the Society for Healthcare Epidemiology of America. The content is solely the responsibility of the authors and does not necessarily represent the official views of the funding agencies. The authors thank the nursing, patient care, and administrative staff of the SCTO-ICU and SICU at Barnes-Jewish Hospital and the Environmental Services staff at Barnes-Jewish Hospital, Lindsay Selner, MBA, MHA, BJC Healthcare Director of Activation Planning, Colleen McEvoy, MD, Director of the Stem Cell Transplant and Oncology Intensive Care Unit (SCTO-ICU) at Barnes-Jewish Hospital (BJH), Courtney McCullough, RN, BSN, Clinical Nurse Manager of the BJH SCTO-ICU, Mana Paolo Vinzon, RN, BSN, MBA, Assistant Clinical Nurse Manager of the BJH SCTO-ICU, William Buol, CEH, Senior Manager of Environmental Services at BJH, and Peter Westervelt, MD, PhD, Section Director of Bone Marrow Transplantation &

Leukemia at Washington University. The authors thank members of the Dantas lab for helpful general discussions and comments on the manuscript. The authors thank the BJH Infection Prevention group for their kind assistance obtaining relevant HAI data.

## 4.9 Figures

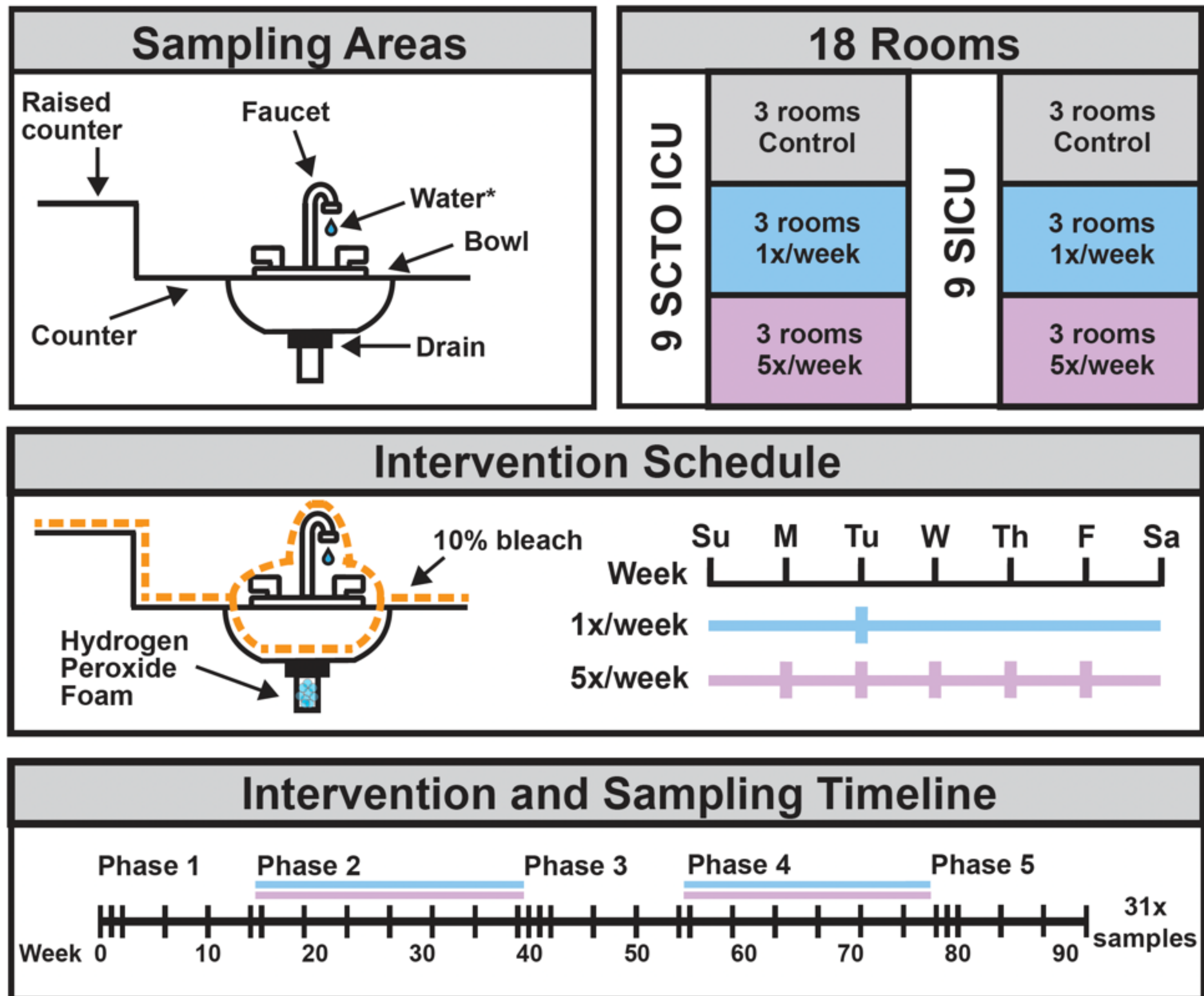


**Figure 4.9.1 Study design overview.** We tested 2 frequencies of the foamed disinfectant and bleach wipe intervention in 18 rooms across a SCTO-ICU and SICU at Barnes Jewish Hospital. We sampled multiple surfaces including sink drains across a baseline phase followed by two rounds of intervention phase and follow-up phase. Water was not sampled every week (see Methods).

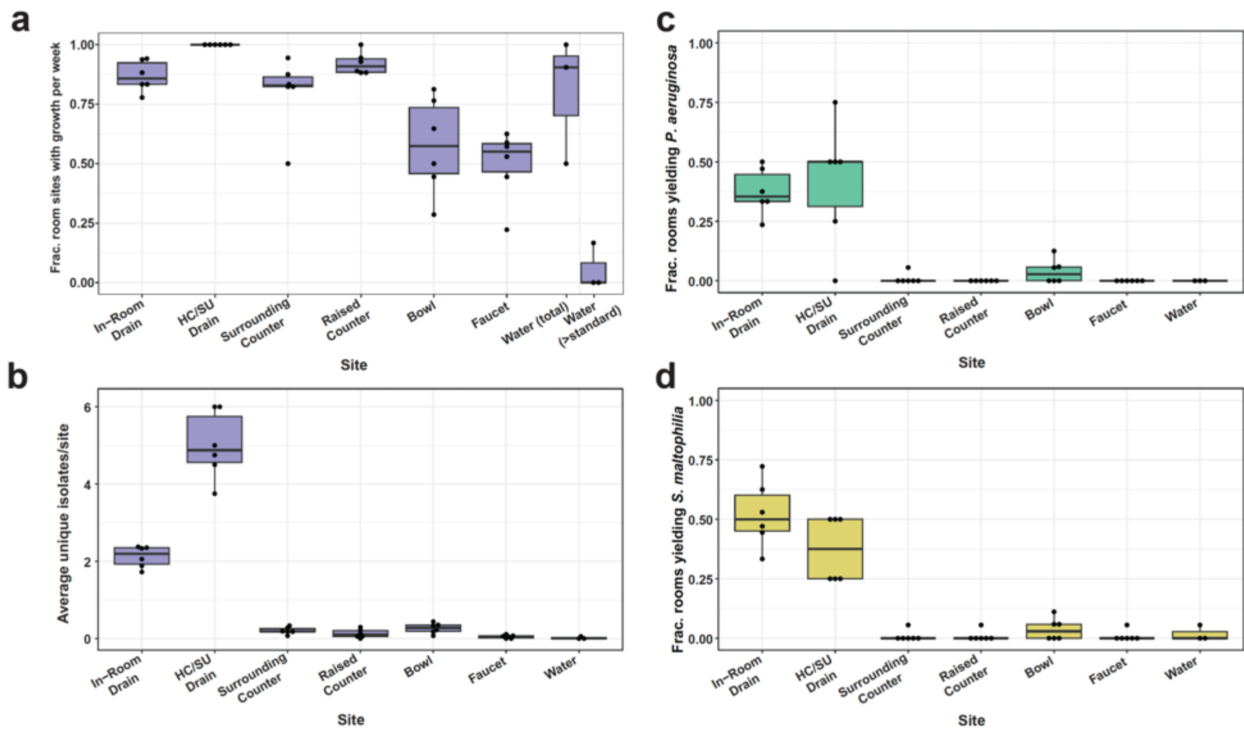


**Figure 4.9.2 Patient room sink drains yielded the most AROs associated with HAIs. a)** Average weekly proportion of rooms with growth on BAP from each site. Average weekly **b)** unique ARO isolates **c)** proportion of rooms yielding *P. aeruginosa* and **d)** proportion of rooms yielding *S. maltophilia*. Differences in proportion of rooms yielding growth or AROs between sites were tested by permutation test and BH corrected. All significance testing results can be found in Supplementary Table 2.

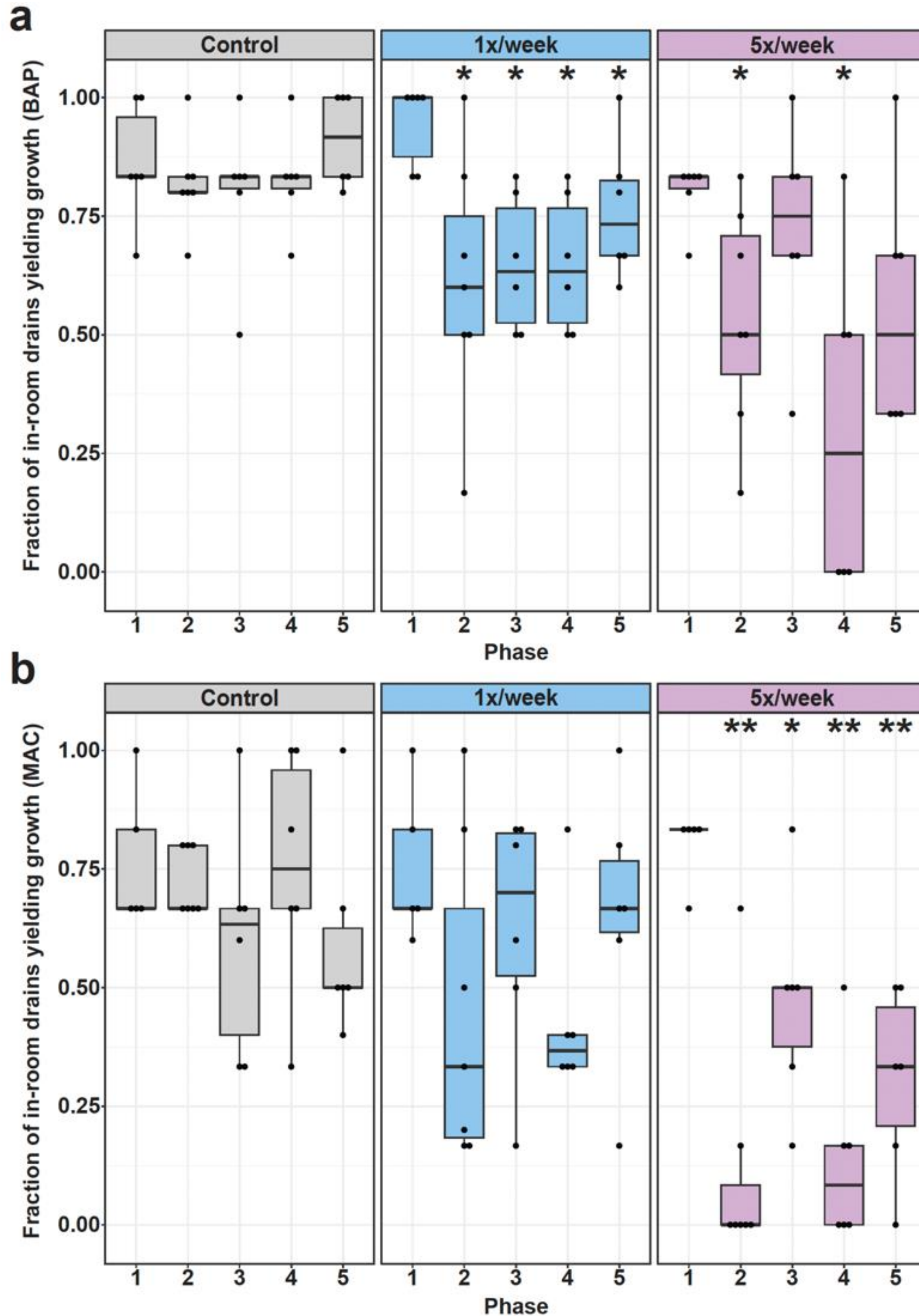


**Figure 4.9.3 Both interventions reduced the proportion of rooms with viable cultivable growth.** Average weekly proportion of rooms in each intervention group that yielded viable cultivable growth on **a)** BAP and **b)** MAC plates. Dunn's test with BH correction was used to evaluate differences between groups in Phase 1 (baseline). Changes within groups versus Phase 1 were tested using permutation tests and BH corrected; significant changes are denoted above the phase that has changed versus Phase 1 as follows: *: $p < 0.05$, **: $p < 0.01$. All significance testing results can be found in Supplementary Table 3.

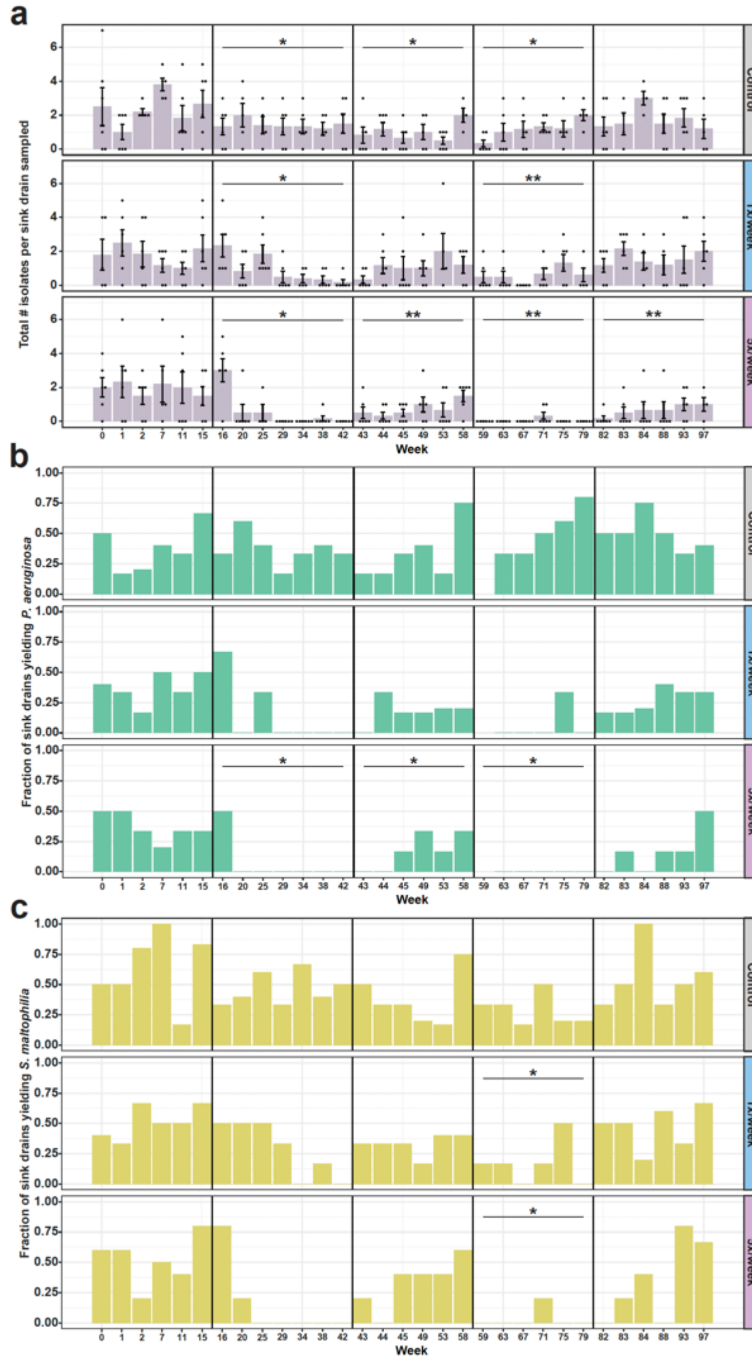


**Figure 4.9.4 Interventions reduced the proportion of patient room sink drains yielding ARO isolates. a)** Weekly counts per sink drain of unique AROs. Proportion of rooms yielding **b)** P. aeruginosa and **c)** S. maltophilia from patient room sink drain. Both interventions significantly reduced viable cultivable ARO isolates, and frequently resulted in no P. aeruginosa or S. maltophilia collected for multiple weeks. Significant changes when compared to Phase 1 (permutation test, BH corrected) are denoted above the phase that has changed versus Phase 1 as follows: *: $p < 0.05$, **: $p < 0.01$; all significance testing results can be found in Supplementary Table 5.

# 4.10 Supplementary Figures

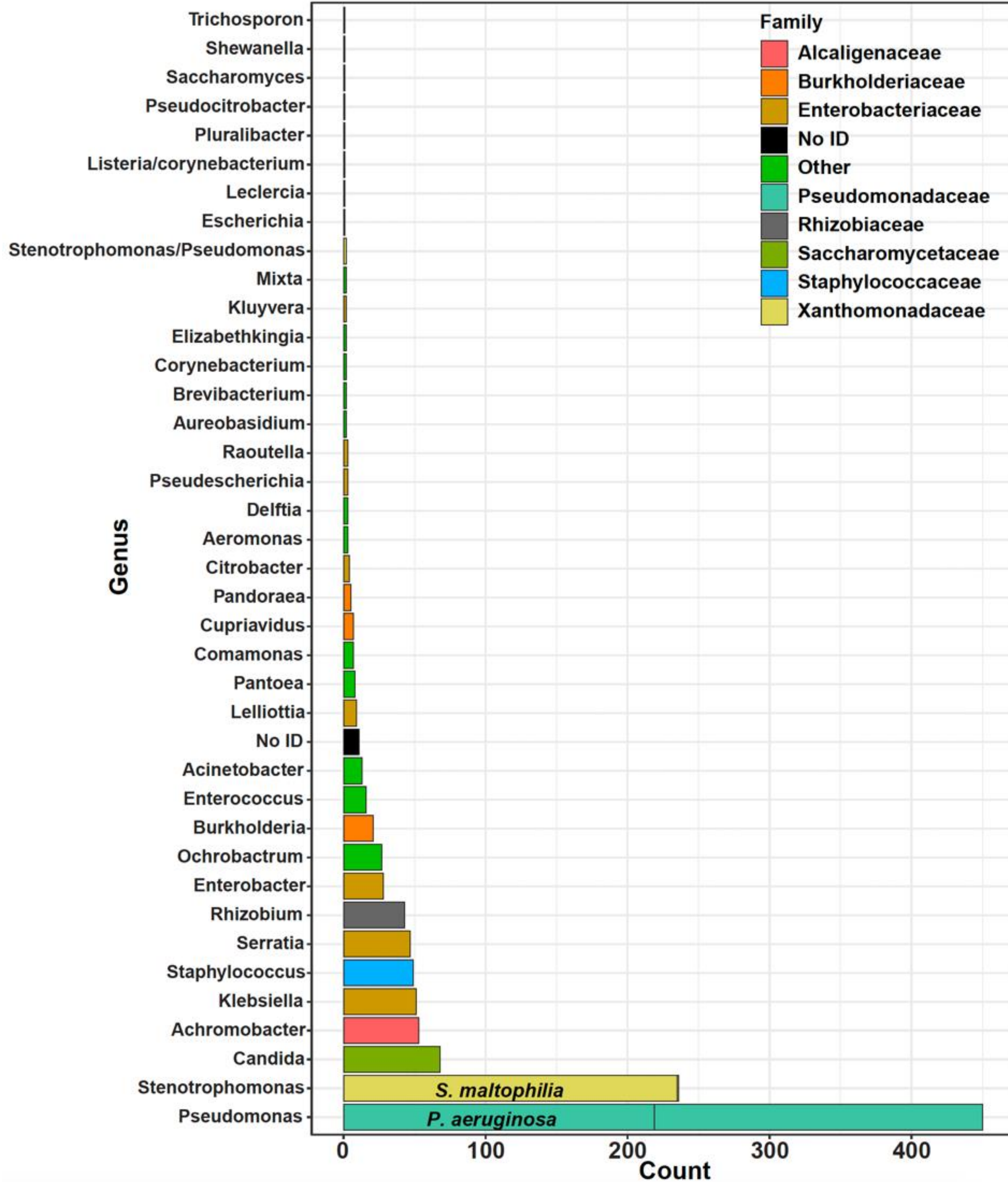


**Figure 4.10.1 All isolates collected during this study.** Histogram of all isolates collected and identified by MALDI-TOF MS divided by genera and colored by family. The 2 most common genera were Pseudomonas and Stenotrophomonas, replicating our previous findings.6, 49 The most frequently isolated species were P. aeruginosa and S. maltophilia (outlined in black), AROs commonly associated with HAIs.

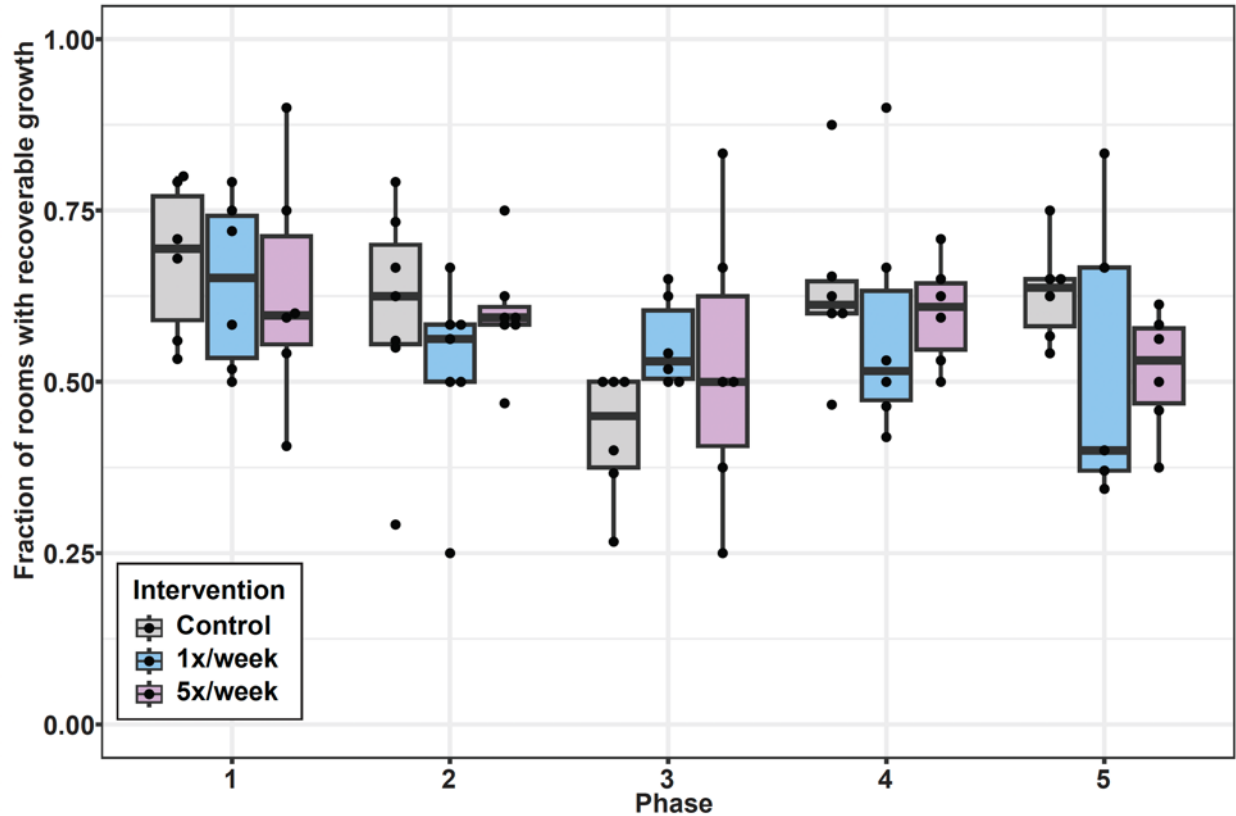


**Figure 4.10.2 Interventions had no significant impact on the proportion of non-drain surfaces with viable cultivable growth on BAP.** Average weekly proportion of non-drain surfaces yielding growth on BAP. Neither intervention had any significant decreases on the proportion of non-drain surfaces yielding growth (permutation test, BH corrected). All significance testing can be found in Supplementary Table 4.

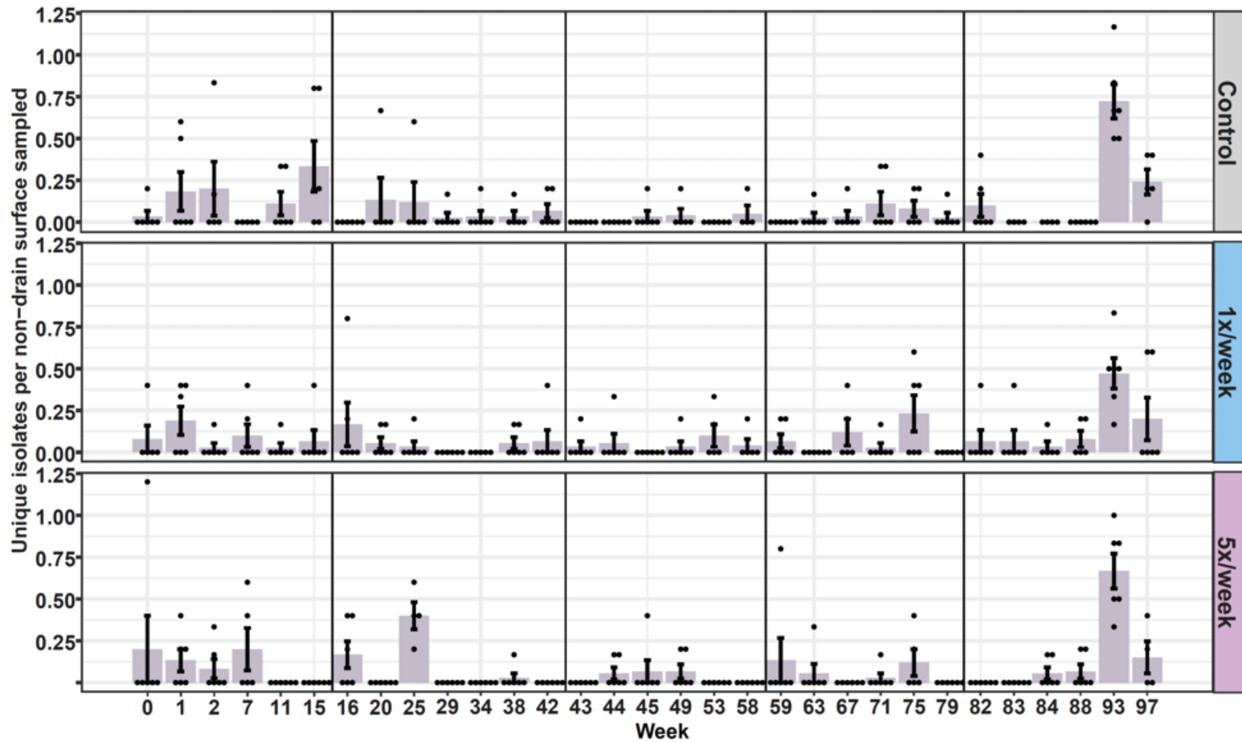


**Figure 4.10.3 Interventions had no significant impact on the number of unique AROs cultured from non-drain surfaces.** Weekly counts per room of unique AROs cultured from non-sink drain surfaces. Neither intervention resulted in a significant change (permutation test, BH corrected). All significance testing can be found in Supplementary Table 6.

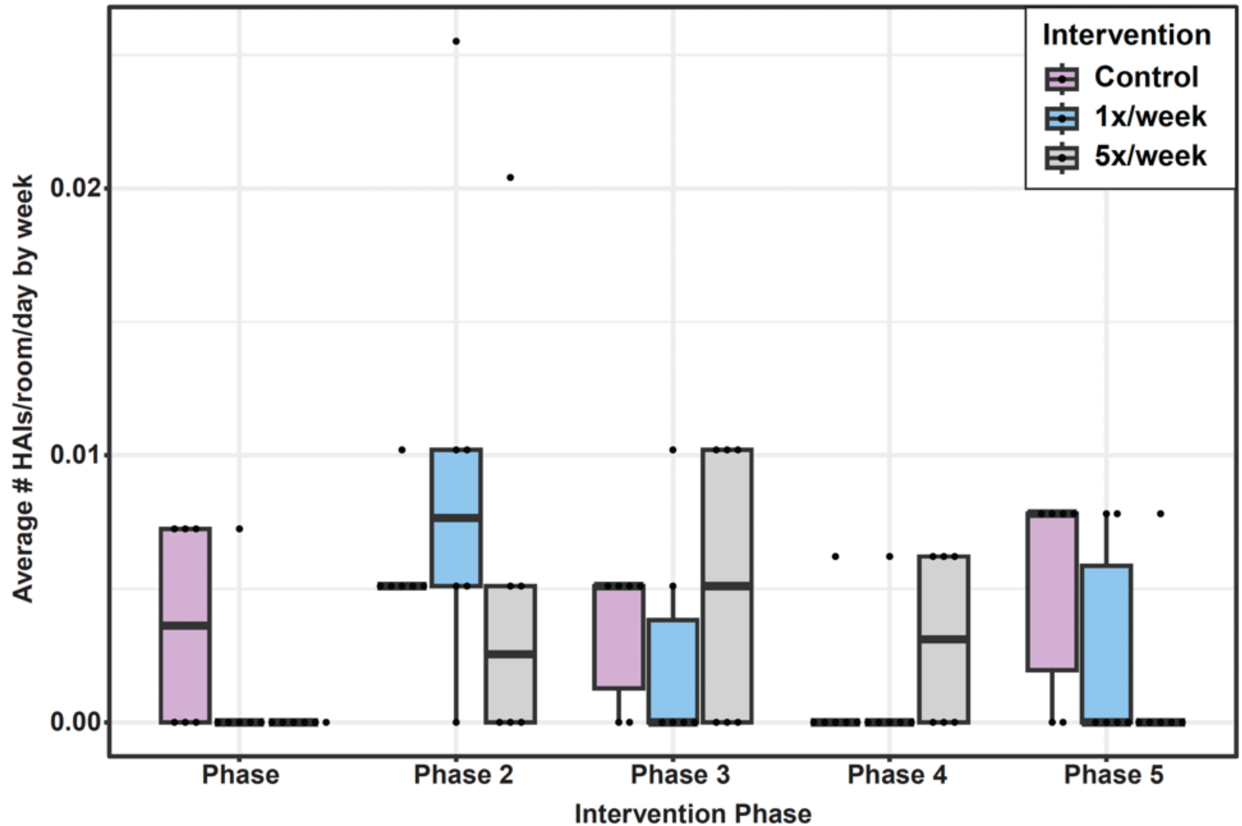


**Figure 4.10.4 Average number of HAIs per room per day by week across sampling periods.** All central line-associated blood stream infections (CLABSIs), catheter-associated urinary tract infections (CAUTIs), and ventilator-associated events (VAEs) that occurred in the study rooms across the course of the study. HAIs per phase were calculated for each sampling period. No significant differences were found between groups (permutation test, BH corrected), likely due to low infection rates (n=70). All significance testing can be found in Supplementary Table 7.

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# Chapter 5

# Sink drain hygiene intervention does not increase antibiotic resistance or disinfectant resistance in opportunistic premise plumbing pathogens

The contents of this chapter are adapted from a manuscript that is currently in preparation:

**Newcomer EP**, O'Neil C, Vogt L, McDonald D, Cass C, Wallace MA, Hink T, Yerbic F, Meunks C, Gordon R, Stewart H, Arter O, Amor M, Jolani K, Alvarado K, Valencia A, Samuels C, Peacock K, Park D, Struttmann E, Sukhum KV, Burnham CAD, Dantas G, Kwon J. Sink drain hygiene intervention does not increase antibiotic resistance or disinfectant resistance in OPPPs. 2023, *In preparation.*

# 5.1 Abstract

Opportunistic premise plumbing pathogens (OPPPs) establish reservoirs in hospital plumbing and can cause healthcare associated infections (HAIs). There is currently no standardized protocol for sink drain disinfection to reduce OPPP burden. We implemented a 10% bleach wipe and foamed disinfectant protocol to reduce OPPP burden in hospital sink drains. We tested two frequencies of intervention and compared to control rooms, and collected surface, water, clinical stool and specimen samples throughout the study. We included environmental and clinical isolate data from a previous study in the same wards. We conducted whole-genome sequencing on all isolates identified as *Pseudomonas* spp. or *Stenotrophomonas* spp., and tracked the intervention's impacts on reservoirs over time. We found several strains of *P. aeruginosa* and *S. maltophilia* that maintained reservoirs in sink drains from the previous study through this study, exhibiting over 3 years of colonization. During and after interventions, *P. aeruginosa* reservoirs were often replaced with new strains ($p<0.05$, Fisher's exact test, BH corrected), while *S. maltophilia* reservoirs returned with the same strains. The intervention had no observable impact on the average number of antibiotic resistance genes carried by strains of either species. Neither species showed any adaptive mutations for disinfection resistance in strains exposed to the intervention. This intervention could reduce OPPP burden in sink drains without increasing antibiotic resistance gene burden or selecting for disinfection resistance. This also reveals differing potential mechanisms for reservoir maintenance by *P. aeruginosa* and *S. maltophilia*.

# 5.2 Introduction

Antibiotic resistant organisms (AROs) can inhabit hospital water and plumbing surfaces and cause healthcare associated infections (HAIs)[1-4]. These opportunistic premise plumbing pathogens (OPPPs) can establish reservoirs in hospital water, sinks, and plumbing surfaces[1-3,5-7]. Many

OPPPs can form thick biofilms in hospital sinks that resist standard disinfection efforts and may increase patient exposure to AROs[8]. Numerous outbreaks of OPPPs in healthcare facilities, including pathogens such as *Pseudomonas aeruginosa*, *Stenotrophomonas maltophilia*, *Legionella spp.,* and *Burkholderia* spp. have derived from hospital sink drains. These outbreaks have been associated with patient morbidity and mortality, as well as increased healthcare costs[6,9-13]. Moreover, recent work has revealed that infections traced to sink drain colonization can also occur in non-outbreak settings, suggesting that the true impact of OPPs may be underestimated[1,2].

Despite growing concern over the risk of sink drain colonization, there is no standardized protocol for sink drain decontamination in healthcare facilities[4]. Some have proposed structural interventions as a potential solution for reducing OPPPs in healthcare settings, but these strategies are often difficult and costly to implement. One proposed intervention targets planktonic OPPPs circulating through the water system by installing filters on faucets, but this strategy doesn't impact OPPPs colonizing pipes or sink drain surfaces[14-17]. Others have implemented fully 'waterless' hospital rooms, but this complicates patient care and requires a large initial investment to restructure rooms[18,19]. As a last resort in an outbreak setting, some facilities have elected to fully replace the sink infrastructure in the affected rooms; however, this option is expensive and impractical[11,20].

Many chemical interventions have also been piloted, but chemical disinfectants are often associated with health and safety risks for both patients and staff. They may also lack efficacy against the biofilms formed by OPPPs[21-23]; however, some work has suggested that using a disinfectant foam versus a liquid disinfectant can increase contact time with sink drain surfaces, increasing the ability of a disinfectant to break down OPPP biofilms[23,24]. If implemented as part

of routine disinfection protocols, this practical and cost-effective strategy could potentially reduce or eliminate OPPP reservoirs in hospital sink drains and protect patients from HAIs.

We sought to investigate the effectiveness of a recently EPA-approved foaming peracid-based disinfectant for the reduction of OPPPs in hospital sink drains[25]. We used pumps to inject the disinfectant foam into sink drains, while also using 10% bleach wipes to reduce ARO contamination on sink surfaces. We tested this intervention protocol when applied at two frequences: once per week and five times per week. We have previously published on the clinical success of this intervention, and found it reduced total ARO burden, as well as the burden of *Pseudomonas aeruginosa* and *Stenotrophomonas maltophilia* in hospital sinks[26]. For this follow-up study, our objective was to evaluate the impact of this intervention strategy on OPPP colonization through a genomics lens. We utilized genomics analyses to track *P. aeruginosa* and *S. maltophilia* isolates from environmental swabs and patient specimens that were collected during this study period and a previous study (HM study) conducted in the same rooms[1]. We found that specific strains of *P. aeruginosa* and *S. maltophilia* strains were identified in the same sink drains during both study periods, indicating reservoirs lasting up to 3 years. We also found that after cessation of the intervention, new *P. aeruginosa* strains quickly recolonized the sink drains, while *S. maltophilia* strains that were previously colonizing the drains returned, suggesting a variety of colonization mechanisms. Finally, we did not identify any increases in antimicrobial resistance (AMR) genes or adaptive mutations to resist the intervention in intervention rooms.

## 5.3 Methods

### 5.3.1 Study design

This study was conducted at Barnes-Jewish Hospital (BJH), a tertiary care academic medical center in St. Louis, Missouri, United States[27]. The study protocol was approved by the Washington

University Human Research Protection Office under IRB #202008081. The analysis of the clinical results from this work has previously been published under Newcomer, et al[26].

The intervention was tested in a stem cell transplant intensive care unit (SCTO-ICU) and a surgical intensive care unit (SICU). These are distinct units, but are located on the same floor, have the same patient room and sink design, and share staff. A total of 18 patient rooms were selected for study inclusion. We randomly assigned six rooms to each of three arms: a control arm that received no intervention, a low-frequency intervention arm that received the intervention 1x/week, and a high-frequency intervention arm that received the intervention 5x/week. All rooms received routine, standard of care, cleaning throughout the study period. Two shared areas in each ward, the housekeeping closet (HC) and soiled utility closet (SU), were also included in the study. The shared areas in the SICU were assigned to the low-frequency intervention arm and the shared areas in the SCTO-ICU were assigned to the high-frequency intervention.

This study included 5 phases, beginning with a 16-week baseline period (Phase 1), during which no rooms received the intervention. This was followed by a 27-week intervention phase (Phase 2), during which the intervention was applied to the low- and high-frequency intervention rooms, then a 16-week non-intervention period (Phase 3), during which no rooms received the intervention. The intervention was then repeated for 23 weeks (Phase 4), followed by another 16-week non-intervention follow-up period (Phase 5) (see Figure 5.8.1).

### 5.3.2 Intervention design

For the sinks in the intervention patient rooms, the intervention included two steps. First, Sani-Cloth™ Bleach Germicidal Disposable Wipes (PDI™, Woodcliff Lake, NJ) were used to clean the faucet, sink bowl, lower counter surrounding the sink, and raised counter next to the sink (Figure 5.8.1). A new wipe was used for each surface. After a 10-minute contact time, the surfaces

were rinsed with distilled water and dried with a paper towel. Second, a 1.5L Foam-It Pump (FOAMit, Grand Rapids, MI) was used to infuse 10 ounces of a foamed peracid-based disinfectant (Virasept, Ecolab, St. Paul, MN) directly into the sink drain. This disinfectant has recently been approved by the EPA for biofilm disinfection[25]. The foam was allowed to dwell for 3 minutes and then rinsed for 30 seconds with water from the faucet. Each room was assigned its own FOAMit pump and pumps were cleaned after each use to reduce cross contamination.

In the shared areas (HC and SU), a 10-gallon, battery-powered FOAMit pump (FOAMit, Grand Rapids, MI) was used to infuse 32 ounces of the disinfectant into the primary sink drain. Because these shared areas do not have other sink surfaces other than a faucet, no bleach wipes were used in these settings.

### 5.3.3 Surface sampling and selective culture

Sink surfaces in each intervention and control room were sampled 31 times over the course of this 2-year study (Figure 5.8.1). During each non-intervention phase (Phases 1, 3, and 5), samples were collected weekly for the first 3 weeks, and then monthly for the remainder of the phase. Samples were collected monthly during both intervention phases. At each sampling time point, surface samples were collected from 5 surfaces in each patient room: the sink drain, the faucet, the sink bowl, the counter immediately surrounding the sink bowl, and the raised counter area next to the sink[1] (Figure 5.8.1). For each sample, 3 E-swabs (Copan, Murrieta, CA) were moistened with molecular water and used to swab the surface. For drains, swabs were inserted through the grate and swiped around the drain pipe. In the shared areas, only drain samples were collected. After sample collection, E-swabs were placed into Aimes transport medium (Thermo Fisher Scientific, Lenexa, KS) and stored at room temperature for up to 4 hours before culturing.

Full details on selective culturing methods can be found in Appendix 1. Briefly, eluate from E-swabs was cross-streaked onto selective agars. One colony of each unique morphotype was selected and subcultured to a blood agar plate (Hardy). After isolation, colonies were identified using matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry (MS) using the VITEK MS (bioMerieux, Durnham, NC), and a stock was frozen at -80°C in tryptic soy broth (TSB) and glycerol. All culturing, colony selection, and identification were conducted by a trained microbiologist.

### 5.3.4 Water sampling and selective culture

Water samples from the faucets in patient rooms and shared area rooms were collected a total of 12 times during the study period: twice during each non-intervention period (Phase 1,3, and 5) and 3 times during each intervention period (Phase 2 and 4). For each sample, the faucet was run for 10-20 seconds before collecting 1 L of water directly into a sterile plastic container. Samples were stored at room temperature for up to 24 hours before processing. All water was poured through a sterile membrane filter (Pall, Port Washington, NY) and the filter was placed grid-side up on a blood agar plate. Plates were incubated for up to 48 hours at 35°C. Unique colony morphologies were isolated and identified as above, and TSB-glycerol stocks of AROs were frozen at -80°C.

### 5.3.5 Clinical isolate collection

During the study period, we identified clinical specimens, collected during routine patient care from patients admitted to the two study wards, that were positive for *Pseudomonas aeruginosa* or *Stenotrophomonas maltophilia.* These isolates were obtained from frozen stocks in the clinical microbiology laboratory, when available. Remnant stool submitted for *C. difficile* testing was collected for ARO selective culture.

### 5.3.6 HM study isolates

The two hospital wards that were included in this intervention study had also undergone sampling during a previous study conducted 2 years earlier (HM study)[1]. Sequenced reads from *P. aeruginosa* and *S. maltophilia* isolates from environmental, water, and clinical samples collected during the HM study were collected from BioProject PRJNA741123 for inclusion in this analysis.

### 5.3.7 Whole genome sequencing and *de novo* genome assembly

Genomic DNA (gDNA) was extracted from frozen stocks of the environmental, water, and clinical *Pseudomonas* and *Stenotrophomonas* isolates collected for this study using the QIAmp BiOstic Bacteremia DNA Kit (Qiagen) and quantified using the PicoGreen dsDNA assay (Thermo Fisher Scientific). gDNA from each isolate was diluted to 0.5 ng/uL for library preparation using a modified Nextera kit (Illumina) protocol[28] and pooled for sequencing. Pooled libraries were sequenced on the NovaSeq 6000 platform (Illumina) to obtain 2 x 150 bp reads. Demultiplexed reads were adapter sequenced trimmed and quality filtered using Trimmomatic v0.38[29] and read quality was assessed using FastQC and MultiQC[30,31]. Trimmed reads from this study and the HM study were assembled into draft genomes with Unicycler v0.4.7 and assessed for quality with BBMap, Quast, and CheckM[32-35]. Assemblies were considered high quality and used for further analysis if they had less than 500 contigs greater than 1000 bp in length, greater than 90% coverage, less than 5% contamination, and N50 greater than 10,000 bp.

### 5.3.8 Isolate species identification and annotation

Isolate species was confirmed first by using a Mash Screen and then average nucleotide identity (ANI) to RefSeq type strains as done previously[1]. Briefly, the top three hits from Mash Screen[36] were identified for each isolate. Then, ANI between the isolate and the top three hits was calculated using dnadiff[37]. Species assignment was dependent on >75% alignment and >96% ANI to a type strain; if no match was found the isolate was classified as genomospecies of the genus level

taxonomy call[38]. Isolates identified as *Pseudomonas aeruginosa* or *Stenotrophomonas maltophilia* were retained for this analysis.

### 5.3.9 Phylogenetic analysis and strain tracking

Multi locus sequencing typing (MLST) type was determined with mlst[39]. Genomes were annotated for coding sequences using Prokka[40] and a species-specific annotated reference (*P. aeruginosa*: GCF_000006765.1; *S. maltophilia*: GCF_900475405.1). Annotated genomes of each species were inputted into Panaroo[41] to construct a core gene alignment, and phylogenetic trees were constructed with FastTree 2[42].

Pairwise core gene single nucleotide polymorphisms (core SNPs) were called within each species using snippy-core[43]. A core SNP strain threshold was empirically determined by plotting a histogram of all pairwise core SNPs; the threshold for *P. aeruginosa* was set to 30 core gene SNPs and the threshold for *S. maltophilia* was set to 39 core gene SNPs. All isolates within that threshold from one another were considered the same strain.

### 5.3.10 Within-strain evolution characterization

Antimicrobial resistance genes were annotated using AMRFinderPlus[44] and total counts were calculated for each isolate.

Tracking of mutations within strains was conducted as done previously[45]. Strain groups with at least one isolate collected in Phases 2-5 were included in this analysis. Briefly, cleaned reads were subsampled from each isolate in a strain to construct a strain specific pseudo-reference assembly. Assembly and coding sequenced annotation was done, as described above. Whole genome SNPs were identified within each strain by aligning reads from each isolate to the pseudo-reference

assembly with snippy[43]. The gene location of each SNP was identified using a custom Python script and the Prokka annotations.

The number of SNPs identified within each strain was randomly permuted across the pseudo-reference assembly 1000 times using a custom Python script. Mutations in genes labeled as 'hypothetical protein', 'intron' or 'unknown node' were ignored, and gene alleles were collapsed into the same gene. Finally, a custom R script was used to identify genes that were mutated more frequently than by chance using an empirical distribution function with BH correction. All custom scripts described here are available on the Dantas Lab Github.

## 5.4 Results

### 5.4.1 *Pseudomonas* spp. and *Stenotrophomonas* spp. isolate collection results.

We identified a total of 448 *Pseudomonas* spp. isolates from surface samples, 4 from water samples, 7 from stool cultures, and 114 from clinical specimens. Of these, 219 surfaces isolates, 0 water isolates, 5 stool isolates, and 114 clinical isolates were identified as *P. aeruginosa* by MALD-TOF MS. After sequencing, we confirmed 180 *P. aeruginosa* isolates from surface samples, 0 from water samples, 5 from stool cultures, and 99 from clinical specimen isolates. Of the surface isolates, 5 were identified as other *Pseudomonas* species, and 34 failed to sequence; of the clinical isolates, 2 were identified as other species and 13 failed to sequence.

In total, we found 236 *Stenotrophomonas* spp. isolates from surface samples, 1 from water samples, 2 from stool cultures, and 2 from clinical specimens. Of these, 235 isolates from surface samples were identified by MALDI-TOF MS as *S. maltophilia*, along with 1 from water samples, 2 from stool samples, and 2 from clinical samples. After sequencing, we confirmed 88 isolates from surface samples as *S. maltophilia*, and none from other sources. The remaining *Stenotrophomonas* spp. isolates were largely *S. geniculata* or *Stenotrophomonas* genomospecies,

and 10 surface isolates failed to sequence. The 2 clinical isolates were identified as *Stenotrophomonas muris* by ANI. Additionally, 3 isolates identified by MALDI-TOF MS as *Pseudomonas* spp. were identified by ANI as *S. maltophilia*.

5.4.2 *Pseudomonas aeruginosa* strains inhabit sink drains across at least 3 years.

We constructed a phylogenetic tree to identify potential clustering among all surface, water, stool, and clinical *P. aeruginosa* isolates collected during this study and the HM study (Figure 5.8.2a). The isolates clustered by MLST type, although many of the clinical and environmental isolates collected did not have a defined MLST type. These undefined allele sets were submitted to PubMLST for ST type definition.

We observed clustering and overlap of environmental *P. aeruginosa* isolates between the two studies within many MLST types. This suggests long-term colonization, as the same *P. aeruginosa* STs from sink drains identified during the HM study were still present in sink drains during this study. However, during both studies, a plurality of environmental isolates were identified as MLST type 1894, with n=62/180 environmental isolates (34.4%) from this study and n=46/78 environmental isolates (59.0%) from the HM study. This suggests that this ST type remains prevalent in these hospital wards, despite being relatively undocumented on PubMLST. While there were 3 bloodstream infections (BSIs) caused by ST 1894 in the previous study, no clinical isolates from ST 1894 were identified in the intervention study.

We calculated pairwise, core gene single nucleotide polymorphisms (SNPs) to define and track strains of *P. aeruginosa*. A core gene SNP threshold of ≤30 SNPs was empirically defined based on a histogram of the pairwise core gene SNPs (Figure 5.9.1a,b). This threshold was used to define 53 strain groups of *P. aeruginosa* (Figure 5.9.2a). Again, the most common of these strain groups

was roup 63, which contains ST 1894 (n=109 isolates). The remaining strain groups ranged in size from 2-16 isolates, and 63 isolates did not fall within a strain group.

Longitudinal strain tracking of these isolates is depicted in Figure 5.8.2b, which depicts environmental isolates from strain groups containing >5 isolates across the HM study and this study. Strain group 63 (containing isolates of ST 1894) was the most prevalent strain identified in environmental samples collected during the HM study and, during the baseline period of the intervention study, it was still present in 4 of the 6 patient rooms that were included in both studies. Strains 69 and 74 were also still found in the same patient rooms where they had been identified in the earlier environmental sampling study. Notably, these strains continued to be isolated from sink drains over the course of this intervention study, particularly in control rooms. This indicates that longer-term colonization of these sink drains for at least 3 total years of observation. Longitudinal strain tracking of all strain groups can be found in Figure 5.9.3a.

### 5.4.3 Interventions may reduce unique *P. aeruginosa* strains colonizing drains, but new strains recolonize once the intervention is no longer applied.

We previously observed significant decreases in the total number of environmental *P. aeruginosa* isolates collected from intervention rooms during the two intervention phases[46]. Here, we sought to investigate the effect of the intervention on the number of unique strain groups present on sink surfaces. We plotted the average number of unique strains isolated from surfaces in each intervention arm at each sampling time point, and calculated a rolling average across every 4 data points to help visualize trends (Figure 5.8.2c). While both intervention groups appeared to have decreases in the number of unique strains during the two intervention periods, this decrease was only statistically significant versus the baseline levels for the 5x/week intervention group during the second intervention phase ($p<0.05$, permutation test, BH corrected). Additionally, during the

non-intervention follow-up phases, the number of unique strain groups identified in rooms assigned to both intervention frequencies increased to once again be at the same level as the control rooms.

To investigate whether this apparent rebound of strains was the old strains returning or new strains colonizing these sink drains, we compared the number of strains that were new (hadn't been isolated in each room in an earlier phase, colored in yellow) or recurring (had been isolated from each room in an earlier phase, colored in grey) in Figure 5.8.2d. Totaled across Phases 2-5 (all intervention and non-intervention follow-up phases), both the 1x/week and 5x/week interventions showed significantly more new strains versus recurring strains when compared with control rooms ($p<0.01$ and $p<0.001$, Fisher's exact test, BH corrected). Proportional visualization of these findings can be found in Figure 5.9.4a. This indicates that after the intervention treatments, new strains of *P. aeruginosa* are colonizing these sinks, rather than the original strains returning.

5.4 4 *S. maltophilia* strains can inhabit sink drains for at least 3 years, but colonization is not ubiquitous.

Phylogenetic clustering revealed associations between *S. maltophilia* isolates collected in the previous study and in this study (Figure 5.8.3a). The isolates clustered primarily by MLST, with the most frequent MLST group being ST 1. Some ST groups were exclusive to either the previous study (like ST 27) or the new study (like ST 365), but others showed overlap between the two studies (ST 1 and ST 84). No clinical isolates in either study were identified as *S. maltophilia* by ANI.

As done above, we defined *S. maltophilia* strain groups using a core SNP cutoff of 39 SNPs (Figure 5.9.1c,d and 5.9.2b). The largest of these strain group was group 2, which contained isolates of ST1 and was isolated in both studies. These strains are depicted longitudinally in Figure 5.8.3b,

which shows all strains that contained >5 isolates (all *S. maltophilia* isolate tracking in Figure 5.9.3b). In contrast to the *P. aeruginosa*, there are several rooms without isolated throughout the study period. Despite this, at least two strain groups (groups 2 and 5) were recovered in the same rooms across both studies, spanning across 2-3 years.

5.4.5 *S. maltophilia* strains return to sinks after intervention is stopped.

Though we previously identified significant decreases in the number of *S. maltophilia* isolates collected both during and after intervention phases[46], we again sought to investigate the different strain dynamics both during and after the interventions. When examining the number of unique *S. maltophilia* strains present in each intervention group over time (Figure 5.8.3c), we found no significant differences in either the 1x/week or 5x/week intervention groups when compared to their pre-intervention counts (ns, $p>0.05$, permutation test, BH corrected). Even when visualized by a 4-timepoint rolling average, there is a less clear impact of the intervention on the number of unique strains present.

This finding paired with the knowledge that total *S. maltophilia* counts still decreased during intervention phases suggested that new strains may not be replacing the old strains after interventions. We examined the number of *S. maltophilia* strains that were new (hadn't been isolated in each room in an earlier phase, colored in yellow) or recurring (had been isolated from each room in an earlier phase, colored in grey) in Figure 5.8.3d. Proportional visualization of these findings can be found in Figure 5.9.4b). Indeed, much like in *P. aeruginosa,* the *S. maltophilia* found in phases 2-5 in the control rooms was frequently a recurring strain. However, the number of new versus recurring *S. maltophilia* strains found in phases 2-5 in both intervention groups were not significantly different than those in the control rooms (ns, $p>0.05$, Fisher's exact test, BH

corrected). Instead of new strains being isolated from sink drains, the same strains that were found prior to the intervention are returning once the intervention is stopped.

5.4.6 Interventions do not appear to select for increased antimicrobial resistance or other adaptations in *P. aeruginosa.*

An intervention could inadvertently select for undesired traits in the microbes we are targeting, such as selecting for increased antibiotic resistance or adaptive mechanisms against the intervention itself. Particularly because many new strains colonized sink drains after interventions, we first sought to ensure that the interventions did not result in increased antimicrobial resistance (AMR) in *P. aeruginosa* on sink surfaces. The overall burden of AMR in this cohort of *P. aeruginosa* is low and correlates with relatively susceptible *P. aeruginosa* when subjected to antimicrobial susceptibility testing (Figure 5.9.5a). We compared the average number of AMR genes per isolate per week across phases in each intervention group in Figure 5.8.4a. We found no significant differences in any phase in either intervention group when compared to the baseline phase for that group (ns, $p>0.05$, permutation test, BH corrected). Indeed, some of the post-intervention phases trend towards fewer AMR genes, potentially indicating that the new *P. aeruginosa* isolates may carry less resistance.

Next, we investigated genes that acquired mutations in strain groups over time (see Methods). We included isolates from all strain groups that contained at least one isolate in phases 2-5, and thus in intervention rooms could have been subjected to the invention. Singleton isolates would provide no potential information into mutations and were excluded. For the control group (n=4 strains) and intervention groups (n=11 strains), we annotated which genes and how many times genes acquired mutations in each strain group. Then, we randomly permuted that number of mutations across the genome, and calculated a frequency at which we expected a given gene to be mutated. We then

filtered out genes that were not mutated at a statistically significant frequency, thus only including genes that were observed to be under selective pressure. We found no significant differences in the proportion of strains that carried mutations in any gene between the control and intervention groups (Figure 5.8.4b, only genes with mutations in m≥2 strains shown). We expected some genes to be under selection even in the control rooms, as sinks are not the ideal environment for *P. aeruginosa*. The lack of other genes under selective pressure in the intervention groups suggests that even strains that do colonize long-term through the interventions are not mutating to adapt to those pressures.

### 5.4.7 *S. maltophilia* don't exhibit increased AMR or other adaptations in response to interventions.

As *S. maltophilia* exhibited long term colonization even after interventions, we sought to confirm that its prolonged presence did not results in acquired AMR genes or adaptations. As above, we annotated AMR genes and found most carried the same 3-4 genes, though one isolate carried an additional 9 AMR genes not found in any other *S. maltophilia* isolates (Figure 5.9.5b). These findings are similarly reflected in the susceptibility shown in the AST results. Once again, we found no significant differences in the average number of AMR genes per isolate per week across any phase in the intervention groups (ns, p>0.05, permutation test, BH corrected). This indicates that even with long-term colonization, these strains are not acquiring additional AMR genes.

Finally, we investigated gene mutations in *S. maltophilia* as was done above. We included n=4 strains from control rooms and n=7 strains from intervention rooms. Like *P. aeruginosa*, we found no significant differences in proportion of strains carrying mutations in any genes (Figure 5.8.5b, only genes with mutations in m≥2 strains shown). These data are particularly important for *S.*

*maltophilia*, as it tended towards the same strains returning after intervention rather than strain replacement.

5.4.8 Few clinical isolates associated with sink colonizing *P. aeruginosa.*

Of the 114 *P. aeruginosa* clinical isolates collected for this study, 9 were associated with the same strain groups as surface *P. aeruginosa* isolates (Figure 5.9.6). These isolates represent 6 incidences of colonization across 6 unique patients. Of these 6 patients, 4 yielded isolates that were of strain groups that had been isolated from sink surfaces several weeks prior to the isolation from the patient. These connections implicate that 6.25% (4/64) of patients with clinical specimens colonized with *P. aeruginosa* are genomically linked with prior sink drain colonization. While fortunately relatively few patients produced clinical *P. aeruginosa* isolates associated with sink colonizing strains, this left us underpowered to evaluate the effect of this intervention on patient carriage or infections.

# 5.5 Discussion

A structured protocol for the removal of AROs from hospital sink drains is urgently needed to reduce the risk of HAIs[3]. In this study, we investigated a novel use of bleach wipes and foamed peracid-based disinfectant and found it effectively reduced total bacterial burden as well as specific AROs, *P. aeruginosa* and *S. maltophilia*[26]. Here, we investigated the genomic effects of this intervention on *P. aeruginosa* and *S. maltophilia* inhabiting hospital sink drains. We identified multiple strains of both *P. aeruginosa* and *S. maltophilia* colonizing the same sink drains for over 3 years, which to date is the longest observed residence of OPPPs, and likely will continue unless remediated. Interestingly, *P. aeruginosa* was more likely to be replaced with new strains after treatment, while *S. maltophilia* tended to rebound or be recolonized with the same strains. We also found no indication of an increase in AMR genes or adaptations against the intervention in strains

of either species. These findings support the use of this intervention as it does not appear to increase the risk or virulence of sink drain colonizers.

One question that remains is the mechanism by which these organisms maintain colonization. We hypothesize two different mechanisms for this problem: 1) OPPPs grow deep into sink drains, so after any perturbations higher in the system, they can regrow back into the accessible drain, or 2) these organisms are constantly being seeding from a common source and may only maintain temporary residence if that source were eliminated. One study found *P. aeruginosa* growth at least as deep as the pipes going into the wall[22]. This suggests a deeper reservoir that supports mechanism 1 but does not rule out the potential combined effect of these two hypotheses. Indeed, we found that after the intervention, the same *S. maltophilia* strains that were in sink drains prior to the intervention returned, rather than new strains colonizing; this was mirrored in the control rooms. This could be due to either mechanism, as the same strain could grow back or be reseeded. However, the *P. aeruginosa* result in this study differed in that after the intervention; nearly half of the strains identified were novel strains to the sink drains, suggesting seeding from diverse outside sources. Despite this, the *P. aeruginosa* strains in the control rooms largely remained the same. This contrast may indicate that *P. aeruginosa* provides a sort of 'colonization resistance' against new *P. aeruginosa* strains colonizing, and if that growth is removed or reduced, new strains are able to establish growth. Further work is needed to understand how these dynamics play into long-term colonization and risk to patients.

As this is a chemical intervention on hospital environment bacteria, we sought to ensure that this intervention does not inadvertently cultivate additional AMR or resistance to the disinfectants utilized. In the context of *P. aeruginosa*, our main concern was that after eliminating or removing the strains that have inhabited sink drains long-term, the new strains that colonize could carry

additional antibiotic resistance, therefore posing a more significant threat to patients. In parallel, both the *P. aeruginosa* and *S. maltophilia* colonized sink surfaces long-term throughout the intervention, and this longitudinal exposure may provide opportunities for these OPPPs to acquire additional AMR genes through horizontal gene transfer. Fortunately, we found no significant increases in antibiotic resistance in strains of either species identified during or after the intervention, validating its safety with respect to AMR gene burden.

Outside of AMR gene acquisition or new carriage, we also sought to explore any other adaptive mutations that may provide resistance to disinfectants. *P. aeruginosa* and *S. maltophilia* can both carry a variety of mechanisms to resist or develop resistance to disinfectants such as the peracidbased disinfectant used in this study. First, both can form biofilms, or conglomerations of bacterial cells and secreted extracellular matrix, in sink drains[47-49]. One study found that biofilms formed by some strains of *P. aeruginosa* were able to tolerate up to 0.02% peracetic acid on endoscopes[47]. However, for many weeks during the intervention periods we isolated little to no *P. aeruginosa*, so we do not currently believe that biofilms alone are sufficient to resist this intervention[26]. However, both organisms can employ a variety of other mechanisms such as porins or enzymatic degradation, which have proven effective in resistance against other types of disinfectants[50,51]. We investigated the frequency of gene mutations in strains of *P. aeruginosa* and *S. maltophilia* from control rooms and intervention rooms to ensure that the intervention does not encourage new mutational adaptations to resist the disinfectants utilized. We identified several genes under mutational pressures in both the control and intervention groups, such as *oprD* in *P. aeruginosa*, an outer membrane protein associated with carbapenem resistance[52], and *smf-1* in *S. maltophilia*, an important participant in early-stage biofilm formation[53]. However, we found no significant differences in the frequency of mutations in these genes between the two groups,

suggesting these pressures may be due to the stressors of colonizing sink drains, rather than something unique to the intervention. These results provide a promising foundation suggesting that this intervention is not increasing the risk of AMR gene carriage or disinfectant resistance.

Finally, we found too few clinical isolates associated with sink surface isolates to power an investigation into the effect of this intervention on patient infections and carriage. However, even these small numbers provide us with a minimum estimation of patient clinical specimens that may be attributable to sink drains; this key finding is constantly in debate as it is critical for acquiring investment and resources to combat OPPPs in hospitals. The 6.25% reported here is likely underestimating the impact of these organisms, as we don't have samples from patients who resided in these rooms and then later developed infections in other rooms.

While our preliminary results are promising, this study does have several limitations. First, the sample sizes are small, and limited our ability to observe differences in mutations over time. A larger study across more rooms or wards will likely capture more strains and help elucidate adaptive mutations that we were underpowered to identify. Further, it's possible that a longer application of this intervention could result in additional mutations of AMR gene acquisitions. Next, while our sampling methods allow us to regularly isolate AROs such as *P. aeruginosa* and *S. maltophilia*, we hypothesize that we are missing isolates even if the organism is still growing in the drain. Even for strains that are present throughout the course of the study, there are weeks where it was not isolated, and we suspect this is due to sampling efficacy rather than actual lack of colonization. This study also took place in one SICU and one SCTO-ICU, two wards that house patients with a relatively very high risk of opportunistic infections by the OPPPs studied here. Our findings on the percentage of patients colonized by these organisms that were associated with sink colonizing organisms may not translate to different wards to hospital systems. These wards also

have different cleaning practices than other healthcare settings and may result in different pressures and efficacy of the intervention.

In this study, we evaluated the impact of bleach wipes and a foamed peracid-based disinfectant on OPPPs on hospital sink surfaces. We found that strains of *P. aeruginosa* and *S. maltophilia* can inhabit sink drains for at least 3 years without outside intervention, and could be responsible for at least 6.25% of *P, aeruginosa* in patient clinical specimens. This intervention does not result in an increase in AMR gene burden or promote disinfectant resistance in these OPPPs. Though this work took place in only 18 rooms, it is an important step in validating this intervention as a standardized intervention for HAI prevention. Outbreaks due to these organisms and others from plumbing colonization have occurred globally, and urgent remediation is necessary to protect patients. Future work in a study across more wards and hospital facilities is needed to continue to evaluate the impact of this innovation.

## 5.6 Supplementary Information

Supplementary information and tables can be found in the full text of this manuscript.

## 5.7 Funding and Declarations

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## 5.8 Figures

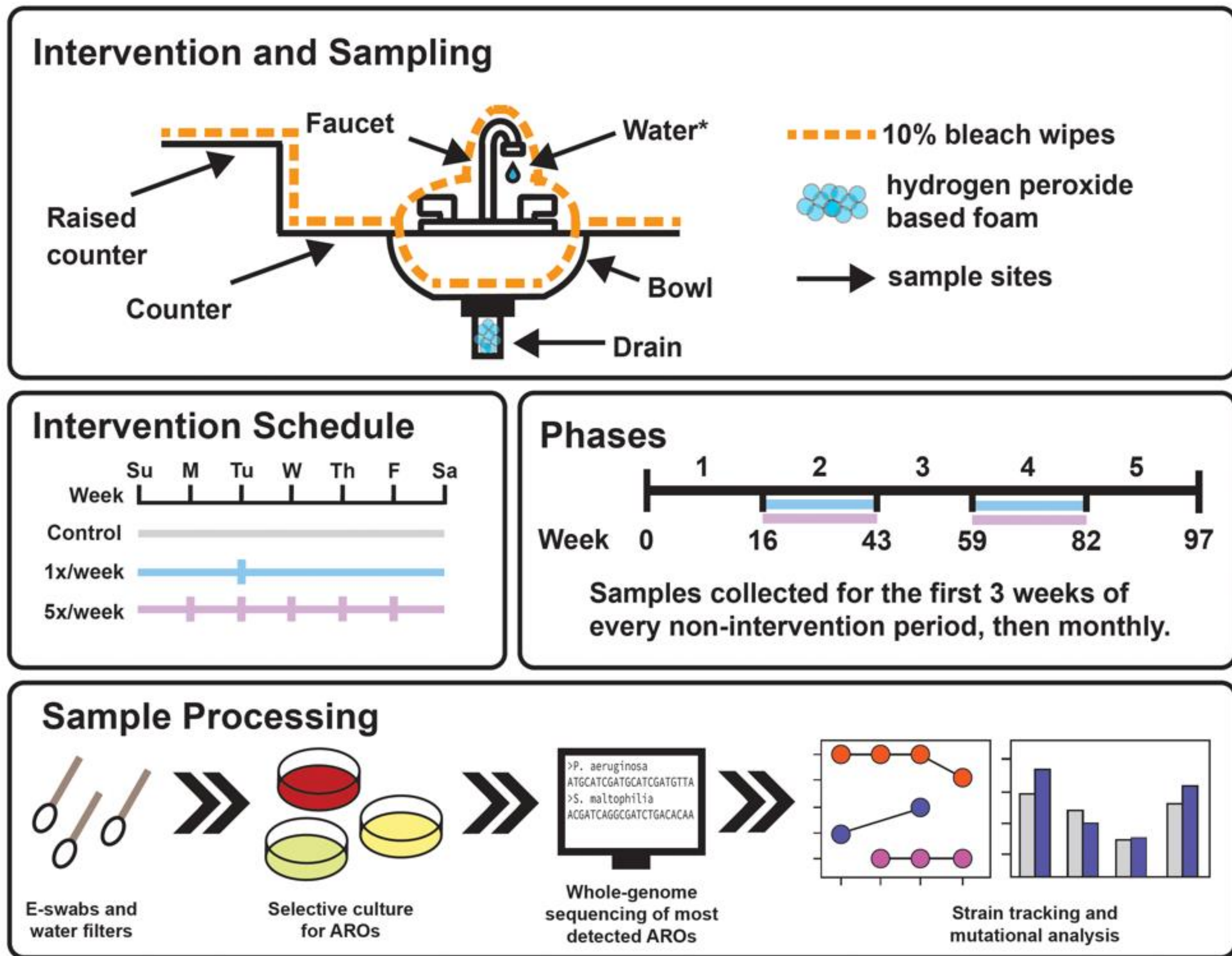



**Figure 5.8.1 Study design and sample processing overview.** The objective of this study was to evaluate the impact of an environmental hygiene intervention consisting of 10% bleach wiped down on all sink surfaces and a hydrogen peroxide-based foam pumped into the sink drains for a contact time of 3 minutes. This intervention was utilized at both 1x/week and 5x/week frequencies during two intervention phases across the study. We collected E-swab samples monthly and for the first 3 weeks of non-intervention phases, and at each timepoint collected samples from the sink drain, sink bowl, faucet, surrounding counter, and raised counter. Water samples were collected regularly throughout the study. Eluate from swabs and water filters were selectively cultured to isolate AROs, and the most frequent antibiotic resistant organisms (AROs) recovered were Pseudomonas spp. and Stenotrophomonas spp. We conducted whole genome sequencing on all Pseudomonas and Stenotrophomonas isolates for this genomics analysis.

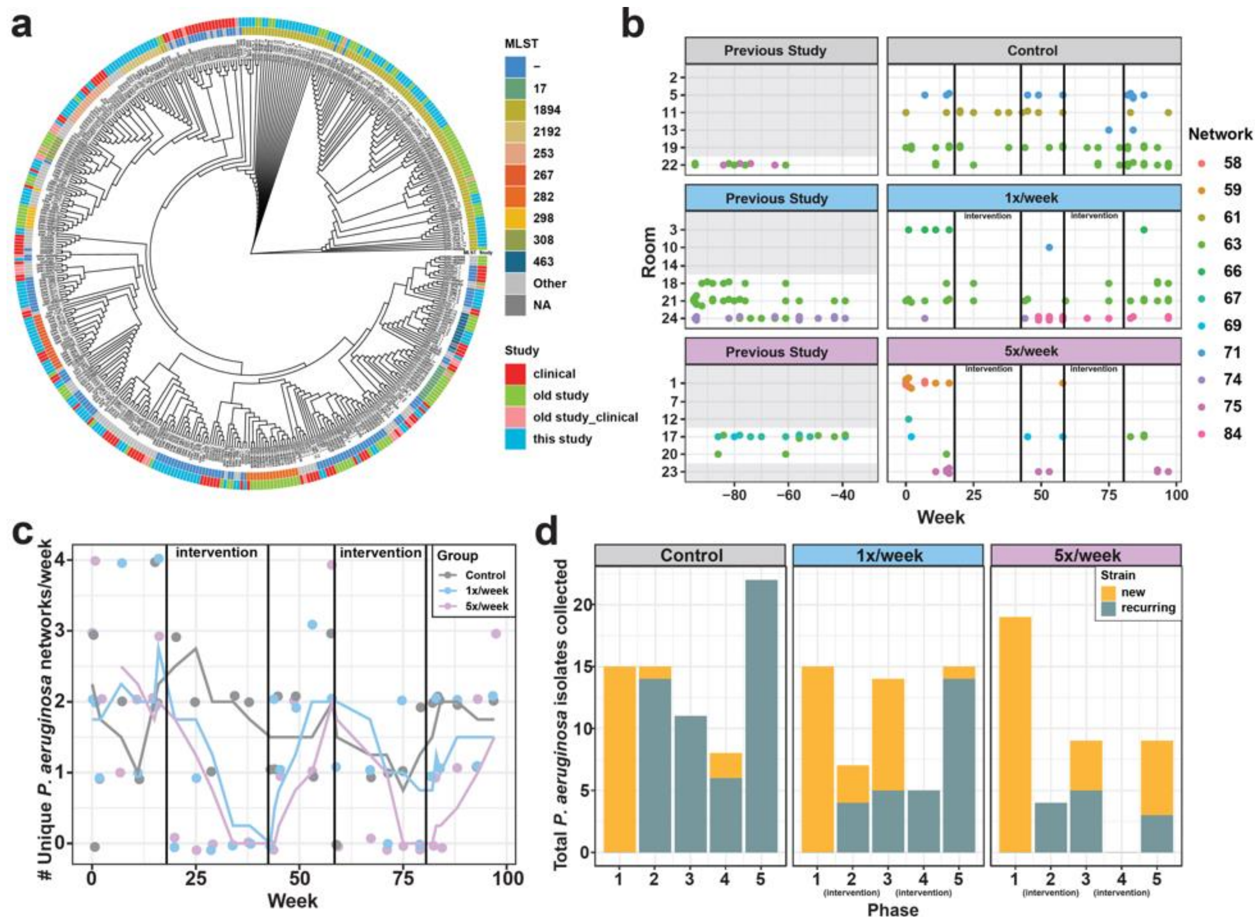



**Figure 5.8.2 Pseudomonas aeruginosa strain tracking, removal, and replacement. a)** Cladogram of all P. aeruginosa isolates collected in this study. Inner ring is colored by MLST group, only most common MLST assignments (>5 isolates collected) shown. MLST '-' indicates unidentified MLST. Outer ring is colored by study and source of isolate. **b)** Tracking of most common strain groups (groups with >5 isolates) from this study and a previous study in the same ward 1. Grey boxes mark rooms where samples were not collected in the previous study. Interventions are labeled in the second and fourth phases of this study. **c)** Number of unique strain groups found each week in each intervention group. Lines represent rolling mean across 4 collection timepoints to highlight general trends. 5x/week intervention had significantly less unique P. aeruginosa strains in the second intervention phase than pre-intervention ($p<0.05$, permutation test, BH corrected). **d)** Count of P. aeruginosa isolates that were strains collected from the same room an earlier phase (recurring, grey) or not seen yet in that room (new, yellow). Intervention rooms had significantly higher proportions of new strains versus recurring strains after interventions begun (totaled across phases 2-5) than controls rooms in both the 1x/week ($p<0.001$, Fisher's exact test, BH corrected) and 5x/week ($p<0.0001$, Fisher's exact test, BH corrected) interventions.

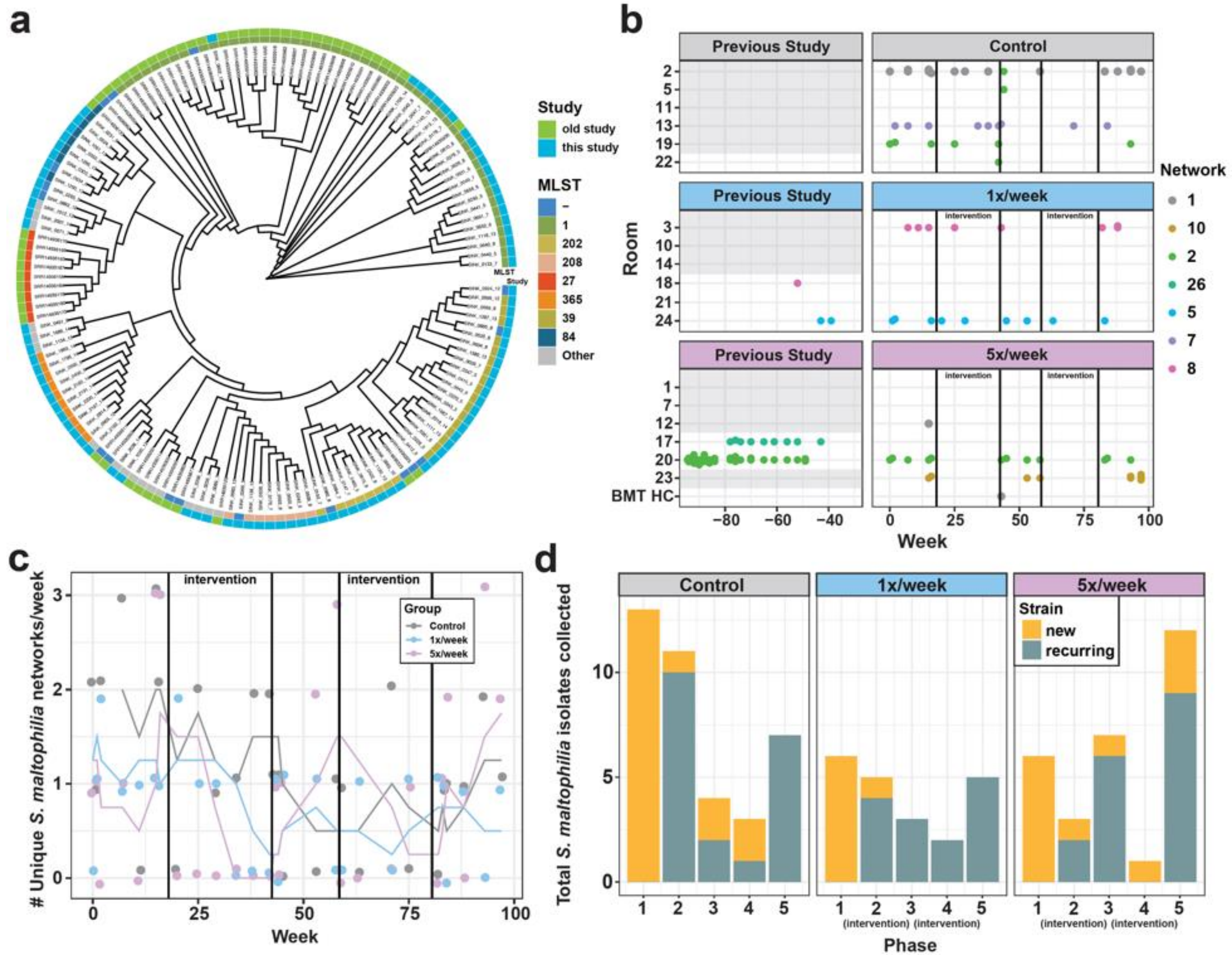



**Figure 5.8.3 Stenotrophomonas maltophilia strain tracking, knockback, and return. a)** Cladogram of all S. maltophilia isolates collected in this study. Inner ring is colored by MLST group, only most common MLST assignments (>5 isolates collected) shown. Outer ring is colored by study and source of isolate. **b)** Tracking of most common strain groups (groups with >5 isolates) from this study and a previous study in the same ward1. Grey boxes mark rooms where samples were not collected in the previous study. Interventions are labeled in the second and fourth phases of this study. **c)** Number of unique S. maltophilia strain groups found each week in each intervention group. Lines represent rolling mean across 4 collected timepoints to highlight general trends. No intervention groups had significantly different numbers of unique isolates when compared with their respective pre-intervention values (ns, p>0.05, permutation test, BH corrected). **d)** Count of S. maltophilia isolates that were strains collected from the same room in an earlier phase (recurring, grey) or not yet seen in that room (new, yellow). Rooms in either intervention group did not have significantly different proportions of new strains versus recurring strains after interventions began (totaled across phases 2-5) than control rooms (ns, p>0.05, Fisher's exact test, BH corrected).

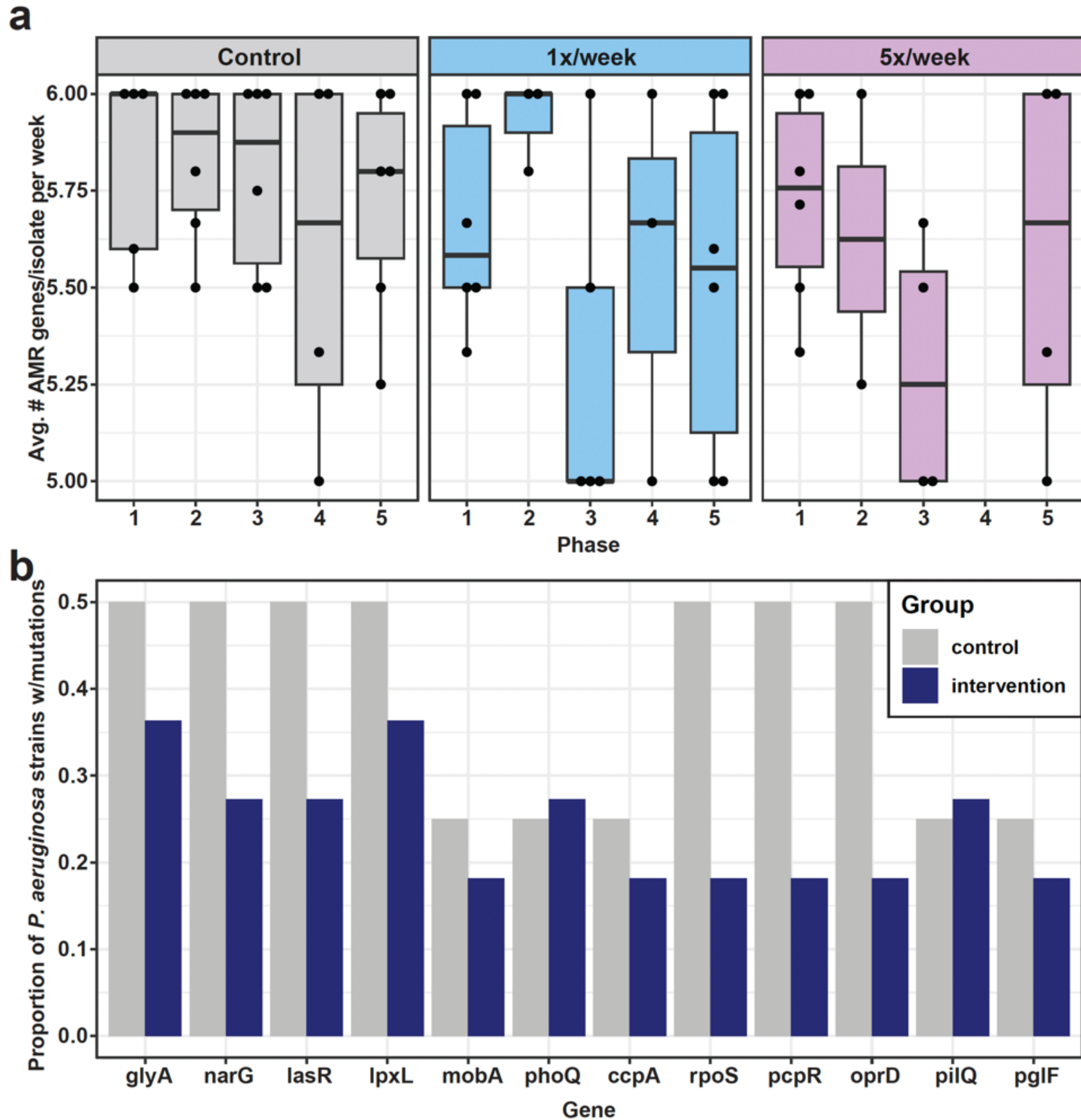



**Figure 5.8.4 Intervention does not significantly impact presence of AMR genes or frequency of gene mutations in Pseudomonas aeruginosa strains in sink drains. a)** Average number of AMR genes per P. aeruginosa isolate collected per week. No significant differences in AMR gene presence between any intervention or post-intervention phases and the pre-intervention phase (ns, p>0.05, permutation test, BH corrected). **b)** Proportion of strains in either control (n=4 strains) or intervention (n=11 strains, 1x/week and 5x/week) groups with within-strain mutations in genes. Strains from intervention rooms were required to contain at least one isolate from Phases 2-5. Only genes mutated in  2 strains in either group shown. The proportion of strains in the intervention group carrying mutations in any gene were never significantly higher than in the control group (ns, p>0.05, Fisher's exact test, BH corrected).

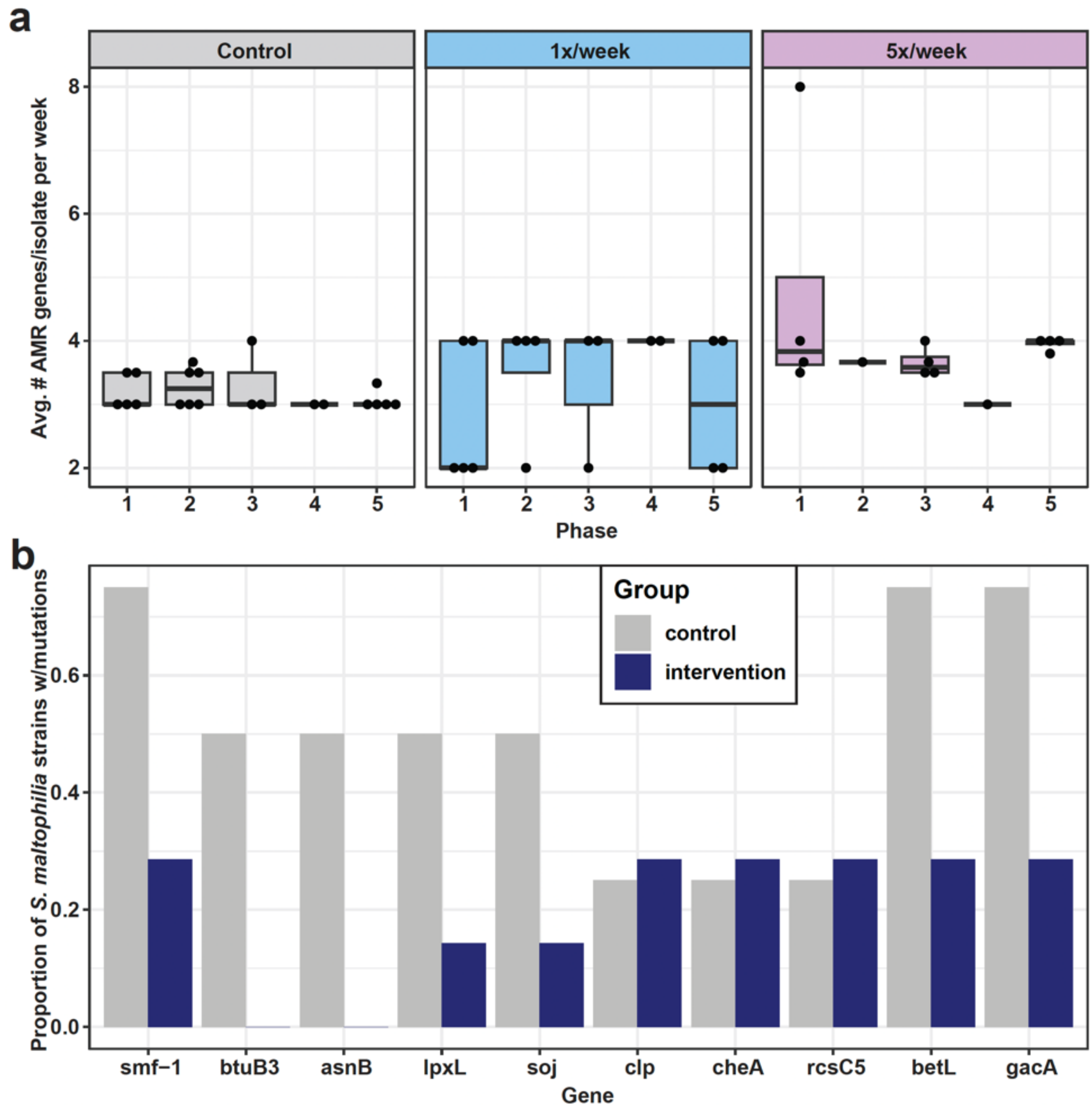



**Figure 5.8.5 Intervention does not significantly impact presence of AMR genes or evolutionary pressures in Stenotrophomonas maltophilia strains in sink drains. a)** Average number of AMR genes per S. maltophilia isolate collected per week. No significant differences in AMR gene presence between any intervention or post-intervention phases and the pre-intervention phase (ns, p>0.05, permutation test, BH corrected). **b)** Proportion of strains in either control (n=4 strains) or intervention (n=7 strains, 1x/week and 5x/week) groups with within-strain mutations in genes. Strains from intervention rooms were required to contain at least one isolates from Phases 2-5. The proportion of strains in the intervention group carrying mutations in any gene were never significantly higher than in the control group (ns, p>0.05, Fisher's exact test, BH corrected).

# 5.9 Supplementary Figures

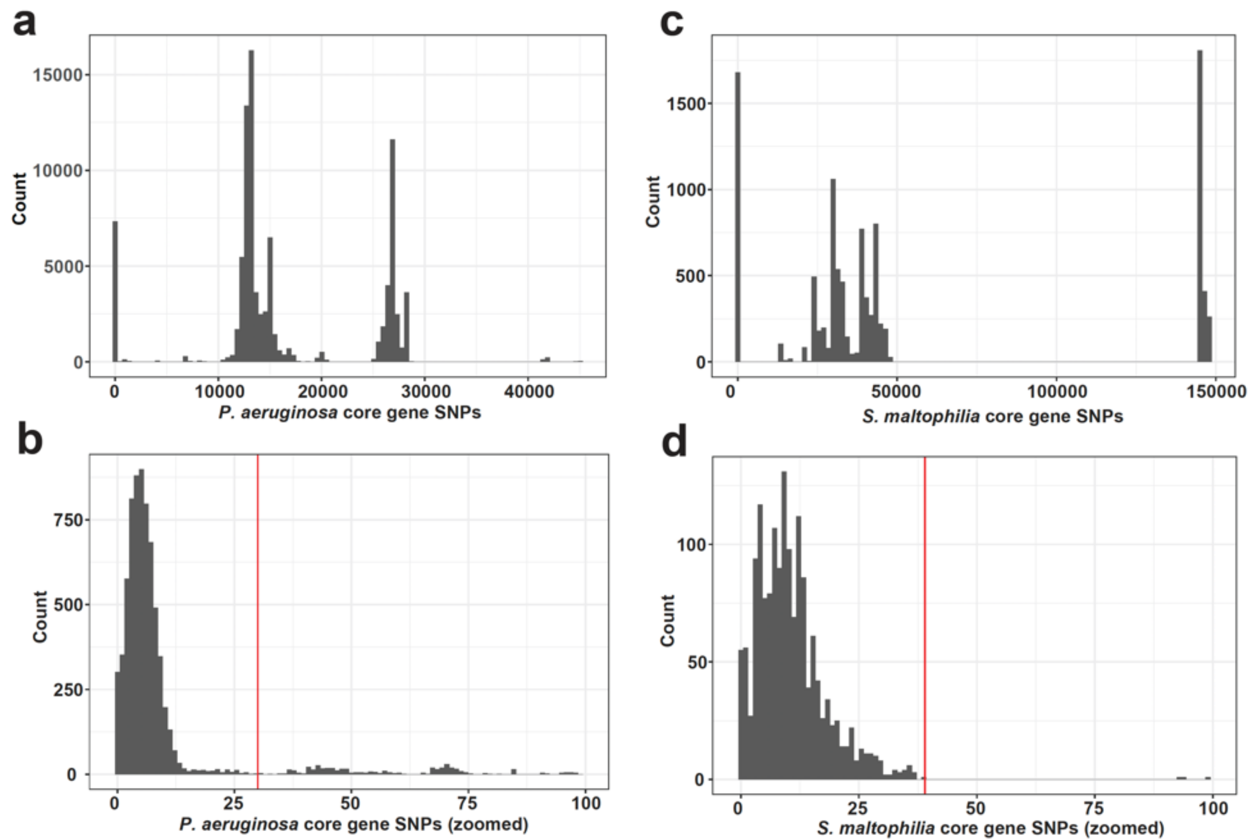



**Figure 5.9.1 Definition of strain groups by pairwise core gene SNP analysis. A)** Histogram and **b)** zoomed histogram of all pairwise, core gene SNP distances between P. aeruginosa isolates. Core gene SNP cutoff for strain groups was set to 30 core gene SNPs. **c)** Histogram and **d)** zoomed histogram of all pairwise, core gene SNP distances between S. maltophilia isolates. Core gene SNP cutoff for strain groups was set to 39 core gene SNPs.

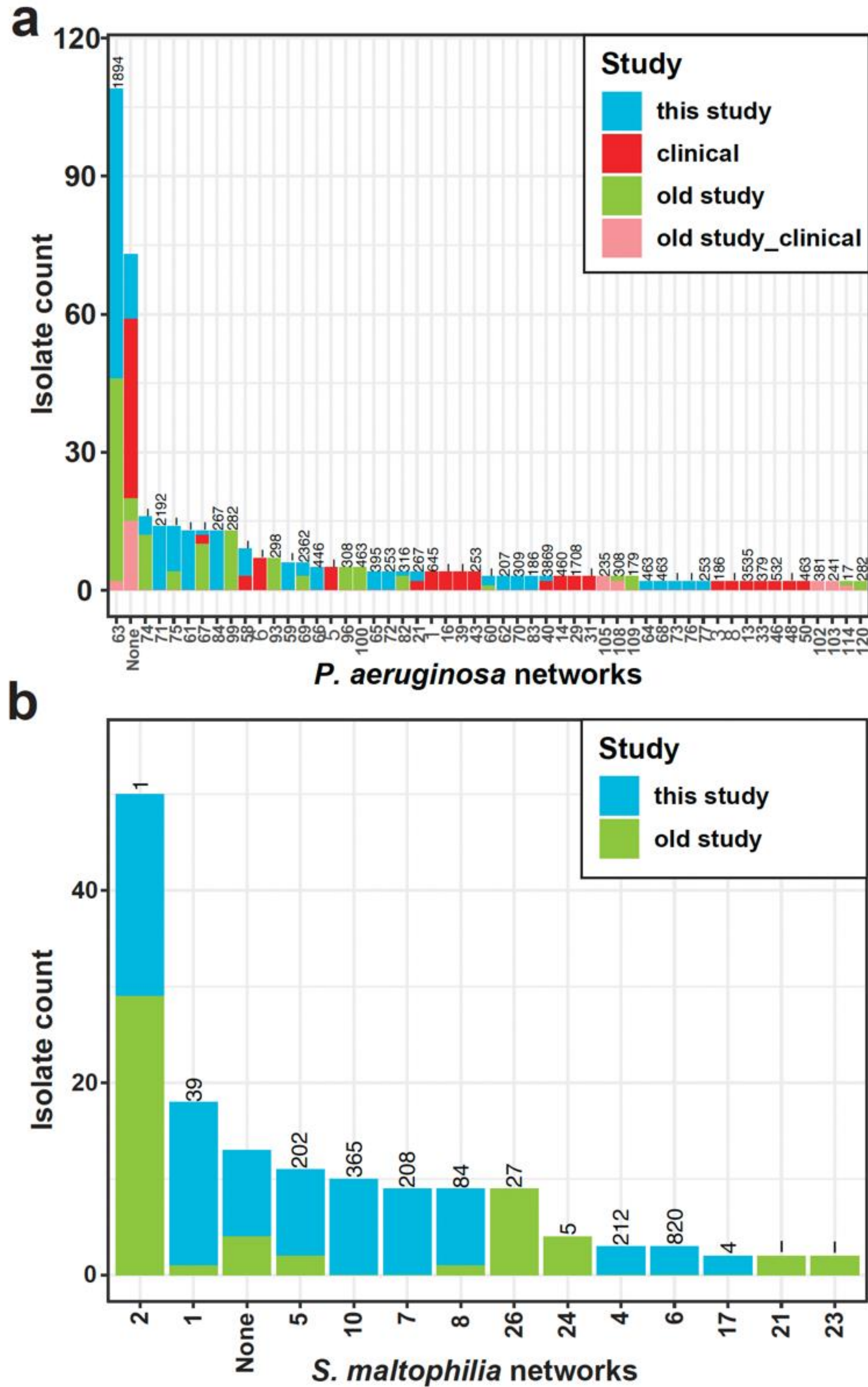



**Figure 5.9.2 Strain groups as determined by core gene SNP cutoff.** Strain groups of **a)** *P. aeruginosa* and **b)** *S. maltophilia* as defined by the core gene SNP cutoffs. Stacked bar plots colored by source of isolate, and each bar is labeled with the MLST type contained within the group.

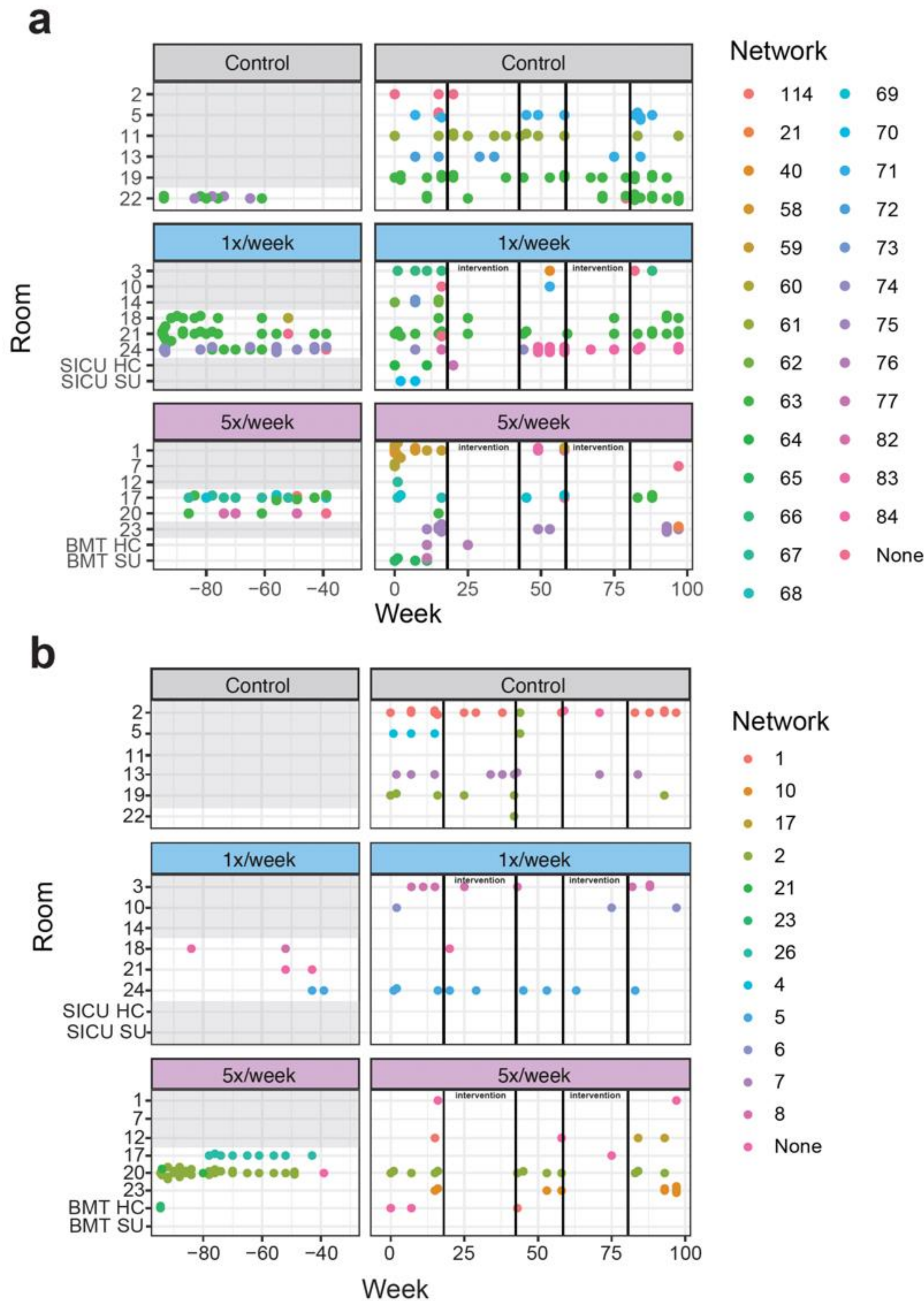



**Figure 5.9.3 Strain tracking of all strain groups and non-group isolates.** Longitudinal tracking of all **a)** *P. aeruginosa* and **b)** *S. maltophilia* isolates. from this study and a previous study in the same ward[1]. Grey boxes mark rooms where samples were not collected in the previous study. Interventions are labeled in the second and fourth phases of this study. Isolates collected from the same strain group depicted in the same color, isolates not within a strain group are all colored the same salmon pink.

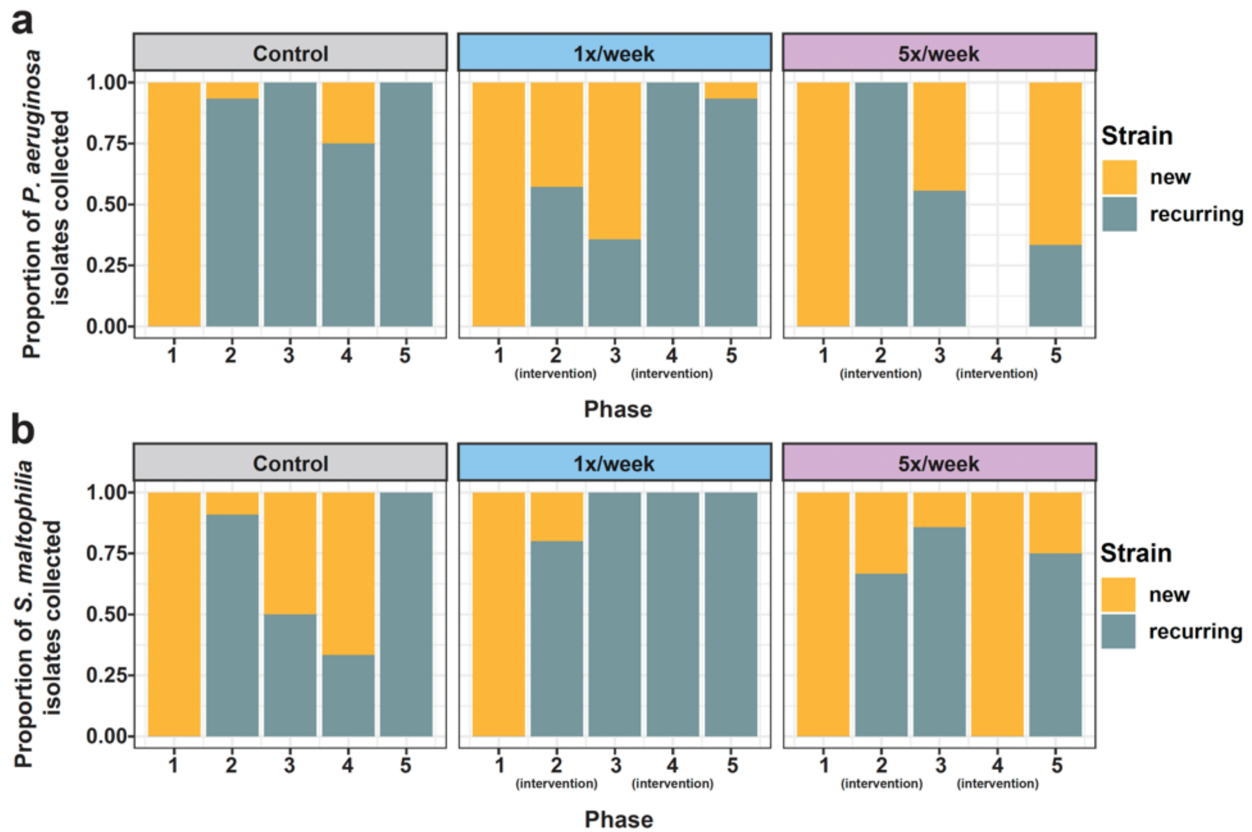
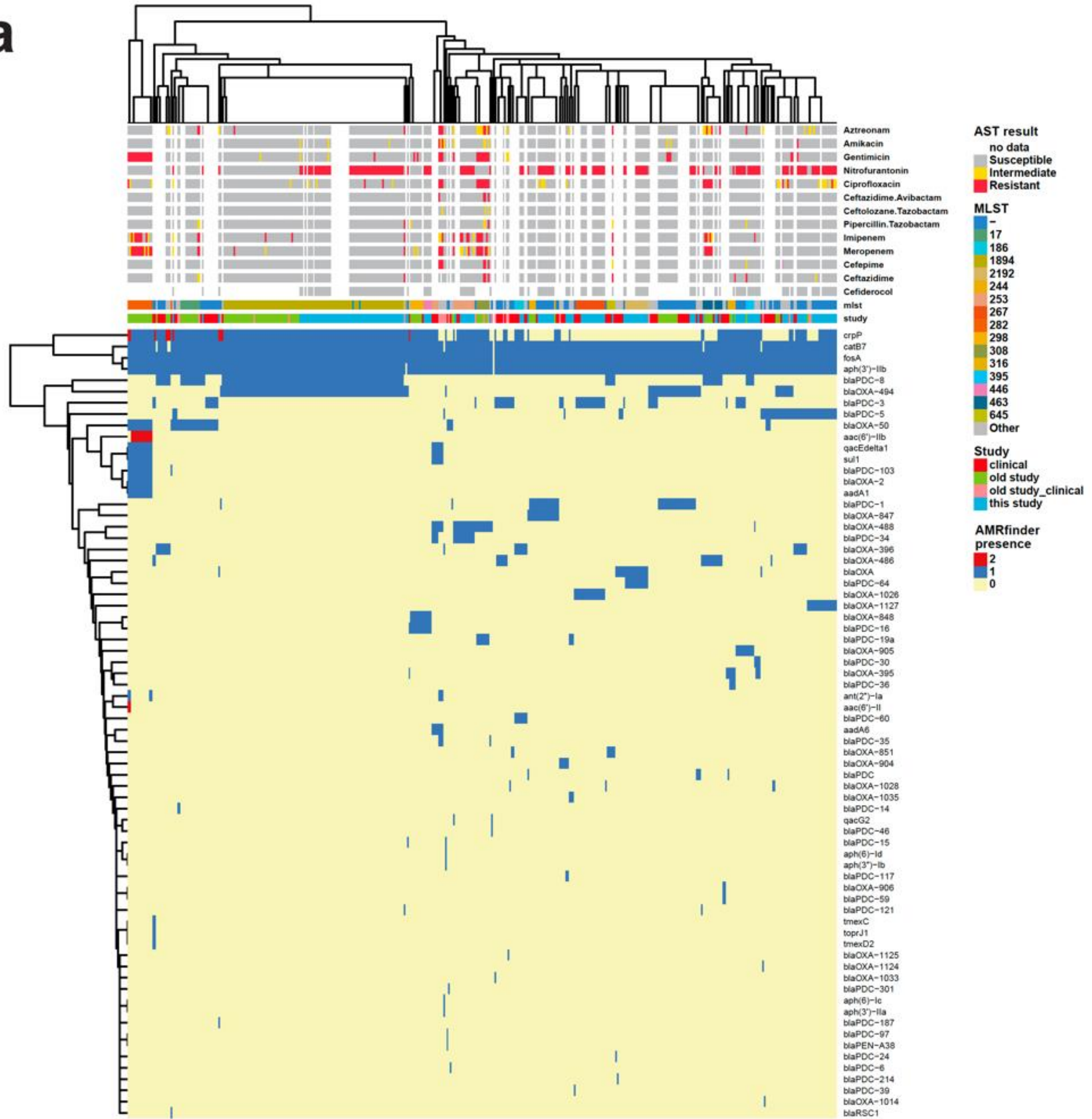



**Figure 5.9.4 Proportional return of recurring strains.** Proportional new versus recurring strains of **a)** *P. aeruginosa* and **b)** *S. maltophilia* collected in each phase. Intervention rooms in **a)** had significantly higher proportions of new strains versus recurring strains after interventions began (totaled across phases 2-5) than controls rooms in both the 1x/week ($p<0.001$, Fisher's exact test, BH corrected) and 5x/week ($p<0.0001$, Fisher's exact test, BH corrected) interventions. Interventions rooms in **b)** had no significant differences in proportion of new strains versus recurring strains after interventions began (ns, $p>0.05$, Fisher's Exact test, BH corrected).


a
Aztreonam
Amikacin
Gentimicin
Nitrofurantonin
Ciprofloxacin
Ceftazidime.Avibactam
Ceftolozane.Tazobactam
Pipercillin.Tazobactam
Imipenem
Meropenem
Cefepime
Ceftazidime
Cefiderocol
mlst
study
crpP
catB7
fosA
aph(3')-IIb
blaPDC-8
blaOXA-494
blaPDC-3
blaPDC-5
blaOXA-50
aac(6')-IIb
qacEdelta1
sul1
blaPDC-103
blaOXA-2
aadA1
blaPDC-1
blaOXA-847
blaOXA-488
blaPDC-34
blaOXA-396
blaOXA-486
blaOXA
blaPDC-64
blaOXA-1026
blaOXA-1127
blaOXA-848
blaPDC-16
blaPDC-19a
blaOXA-905
blaPDC-30
blaOXA-395
blaPDC-36
ant(2")-Ia
aac(6')-II
blaPDC-60
aadA6
blaPDC-35
blaOXA-851
blaOXA-904
blaPDC
blaOXA-1028
blaOXA-1035
blaPDC-14
qacG2
blaPDC-46
blaPDC-15
aph(6)-Id
aph(3")-Ib
blaPDC-117
blaOXA-906
blaPDC-59
blaPDC-121
tmexC
toprJ1
tmexD2
blaOXA-1125
blaOXA-1124
blaOXA-1033
blaPDC-301
aph(6)-Ic
aph(3')-IIa
blaPDC-187
blaPDC-97
blaPEN-A38
blaPDC-24
blaPDC-6
blaPDC-214
blaPDC-39
blaOXA-1014
blaRSC1
AST result
no data
Susceptible
Intermediate
Resistant
MLST
-
17
186
1894
2192
244
253
267
282
298
308
316
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446
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Other
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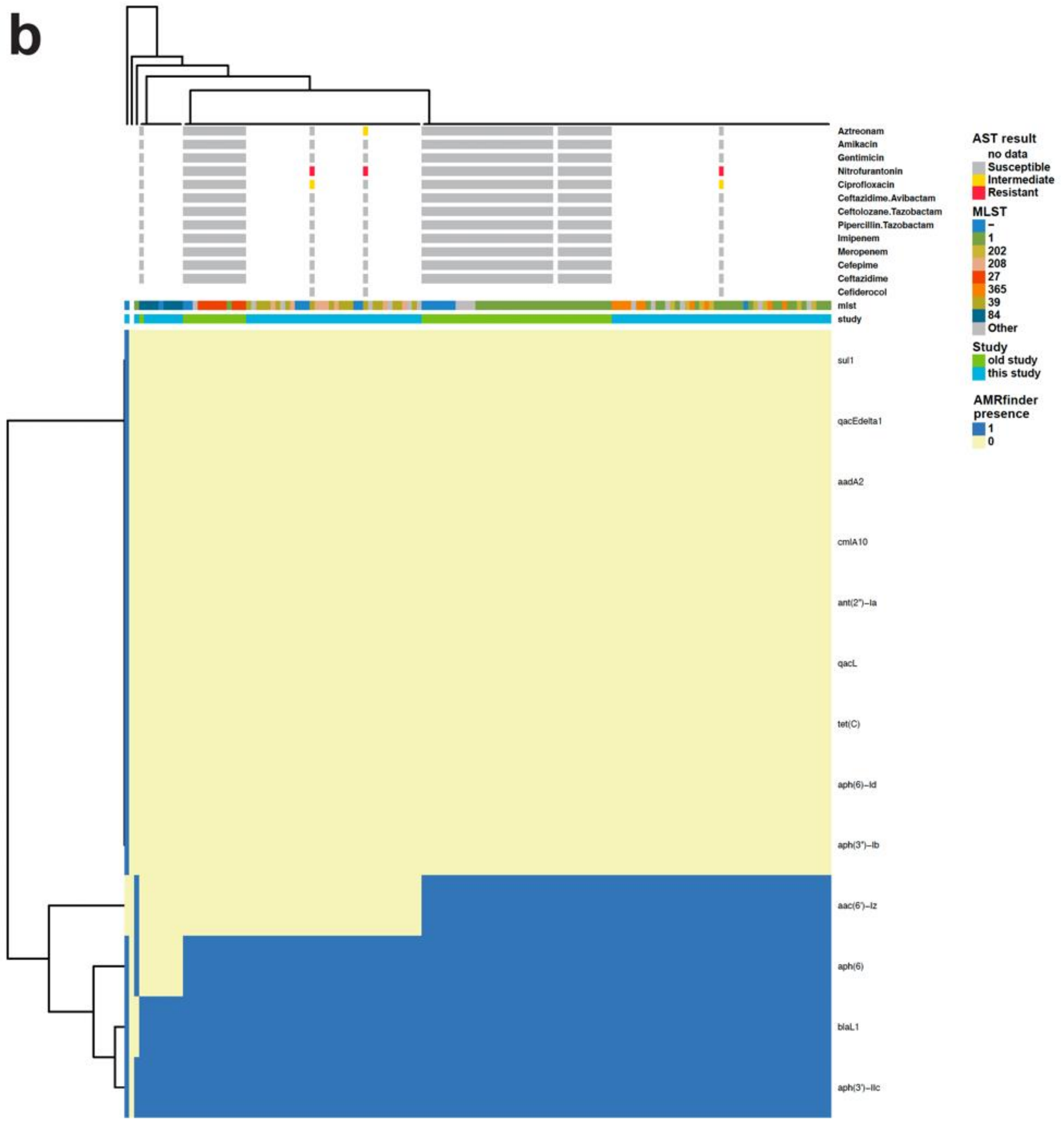


**Figure 5.9.5 AMRFinder and AST results.** Antibiotic susceptibility testing results and AMRFinder genes identified in **a)** *P. aeruginosa* and **b)** *S. maltophilia* isolates. Only most frequent (≥5 isolates collected) MLST groups shown. ASTs will be updated once clinical lab work is completed.

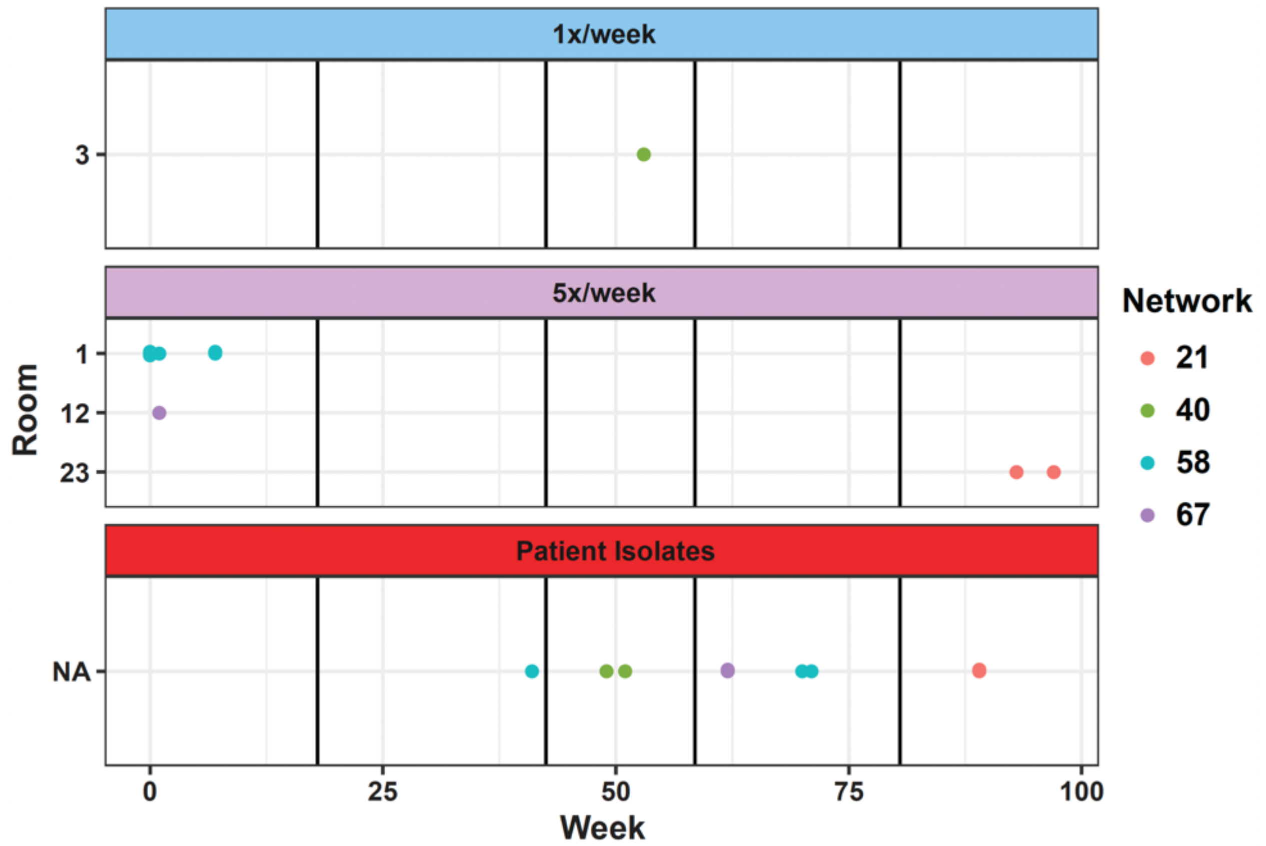


**Figure 5.9.6 Strain groups associated with clinical infections.** Strain group tracking of all groups that contain both clinical and environmental isolates collected in this study. Only relevant rooms shown.

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# Chapter 6. Conclusion

The work described in this thesis encompasses several aspects of transmission and contamination by bacteria in the hospital environment. First, we addressed the two mechanisms of transmission, which many organisms use in parallel: 1) transmission to patients from an environmental reservoir, and 2) transmission between patients using the hospital environment as an intermediary. In Chapter 2, we delved into this first mechanism with the goal of understanding which surfaces play a critical role in housing antibiotic resistant organisms (AROs) that can cause patient infections. In this work, we learned about the critical importance of opportunistic premise plumbing pathogens (OPPPs) in a hospital setting, and how their transmission from sink drains can cause healthcare-associated infections (HAIs) even in non-outbreak settings. In Chapter 3, we turned our focus to the second mechanism, and studied how patients contaminate their surroundings with *Clostridioides difficile* spores which are then transmit to other patients. With the knowledge that *C. difficile* transmits through this mechanism, hospitals implement contact precautions that have drastically decreased transmission from patients with active *C. difficile* infection (CDI). In this context, we described a new landscape of prevalent *C. difficile* strains in a hospital and characterized genomic factors that may explain why these strains are predominant. Finally, we aimed to challenge the AROs identified in Chapter 2 by implementing an intervention to reduce bacterial growth in hospital sink drains in Chapters 4 and 5. In Chapter 4, we examined the clinical results of this intervention, and found exciting significance demonstrating the success of this intervention in reducing both total bacterial load as well as that of specific AROs. We then utilized whole-genome sequencing of ARO isolates in Chapter 5 to characterize the genomic effects of this intervention and found crucial distinctions between the mechanisms by which these organisms persist in sink drains. This work provided me with a strong foundation in many tenants of

healthcare epidemiology, but there are several new directions that each of these projects could take.

A key limitation to sink-related studies described here is the size and power of analysis, as well as translatability to other settings. In Chapter 2, we identified 4 *P. aeruginosa* blood stream infection (BSI) isolates that were tied to previous colonization of sink drains in that ward across 1 year. If these findings were translatable to all other rooms at Barnes-Jewish Hospital, a 1,278-bed facility, that could easily scale up to approximately 852 *P. aeruginosa* BSIs per year that are associated with sink drain colonization. *P. aeruginosa* can additionally cause several other kinds of infections not studied in this work, like urinary tract infections and ventilator-associated pneumonias, which could further drive up those numbers. With that in mind, not all patients are as high-risk for HAIs as the patients housed in the stem cell transplant and oncology intensive care unit (SCTO-ICU), and we expect the attributable number of BSIs and other infections to be significantly lower in other wards. The looming unknown fraction of HAIs that are due to transmission from sink drains is an investigation that many in this field are anxious to pursue. Others and I on the 'hospital microbiome' team have made plans to address these questions. We have recently received notice of funding from the Washington University Institute of Clinical and Translational Sciences to begin a broader cross-sectional study collecting sink and sink drain samples from a variety of wards, clinical, and non-clinical spaces across Barnes-Jewish Hospital. With this work, we aim to characterize a diverse landscape of OPPPs living in different sink drain settings. In Chapter 2, we identified mostly *Pseudomonas aeruginosa* and *Stenotrophomonas maltophilia*, but others have characterized other OPPPs such as *Legionella* spp., *Burkholderia* spp, and *Mycobacterium avium* as frequent colonizers of hospital plumbing, so we expect to find more diversity as our range expands. Though the study will not be longitudinal and may lack the ability to identify HAIs

following sink drain colonization, the team will use these results to acquire additional NIH funding for that purpose. Ideally, this future work will have a longitudinal aspect to identify attributable infections across several wards of varying patient risk levels. Eventually, this work should be expanded outside the hospital, and cover other healthcare facilities such as long-term care facilities and retirement homes.

In addition to physically larger studies, there is much room for growth in the optimization of the sink studies' protocols. Currently, isolates are collected through a swabbing and selective culture for ARO isolation protocol, which is both expensive and time intensive. Even with trained clinical microbiologists, we still frequently observe weeks of culture where no AROs are isolated from sink drains, even though it seems unlikely that long-term inhabitants of sink drains would just skip weeks. Additionally, I had the opportunity to meet with the CDC Epicenters Environmental team, and a large concern of this group was the financial and personnel feasibility of this intensive study. The use of metagenomic sequencing methods could benefit these studies by reducing both costs and effort and help to detect those strains with missing weeks. I helped to conceptualize a project in which we plate swabs from sink drains on a selective agar (largely selective for *Pseudomonas* and *Stenotrophomonas* species, given our previous findings in these rooms), and then metagenomically sequence a sweep of the entire plate. This project has been passed on to another member of the Dantas Lab, but I believe it could streamline the process of strain detection to reduce financial and time costs. As an added benefit, this technique could potentially help identify strains from weeks that we were not able to isolate them using culture-based methods, or diverse strains that were not selected from the plates.

The *C. difficile* work described in Chapter 3 suffers from the difficulties of small sample sizes much like the work in Chapter 2, though the solution is not nearly as straightforward. In the sink-

related work, expanding the study requires additional environmental sampling and collection of remnant clinical specimens. In the *C. difficile* study, most of our samples came from patient sampling, and the culture positive proportion of patients is much lower despite the wards chosen for the study in Chapter 3 being selected specifically due to the high incidence of CDI. Expanding outside of those wards will lead to lower returns on the efforts put into collection of patient specimens. Though transmission from CDI patients to others can still be identified in hospital systems, we know that contact precautions for CDI patients implemented in the early-mid 2000s were largely successful in reducing transmission. In addition, there is debatable benefit to implementing contact precautions to all patients or just carriers, and this carries a significant financial burden as well.

Rather than focusing on transmission in the future, my interest in the *C. difficile* field lies more in understanding the dynamics and virulence of *C. difficile* in the gut, through the lens of next generation sequencing. The use of both metagenomic and long-read sequencing could help supplement our findings on how this pathogen causes disease, and how the host resists disease. First, we frequently encountered patients who seemed to longitudinally carry a strain of *C. difficile*, but would seem to temporarily have weeks where no *C. difficile* was isolated. Rather than this being intermittent colonization, we could metagenomically sequence the stool and rectal swabs, and align reads to a known assembly from a different timepoint. This would allow us to be less reliant on culturing methods to confirm presence of *C. difficile*. In addition, rather than picking one single isolate per specimen, we could detect co-colonization of multiple *C. difficile* strains. This phenomenon was largely thought to be uncommon until Seekatz, et al identified up to 52.8% co-colonization rates. Identifying co-colonizing strains could first supplement our transmission findings by revealing strains that we hadn't previously observed, but also provide deeper insight

into which strains and virulence factors are truly responsible for CDI. Obtaining metagenomic information from these specimens could also help educate other analyses concerning the impact of the gut microbiome on disease progression. The addition of long-read sequencing could also provide insight into the mechanisms behind *C. difficile* infection and colonization. *C. difficile* has an incredibly plastic genome and is highly subject to horizontal gene transfer (HGT) from other bacteria. However, identification of those acquired genes is difficult without a contiguous genome. In our experience, the AT rich nature of the *C. difficile* genome makes *de novo* assembly such as the methods used in Chapter 3 relatively difficult, and result in highly fragmented draft genomes. Long-read sequencing of these genomes can help bridge gaps created by repetitive sequences and reveal acquisition or loss of genes over time. Understanding the functions that are selected for during colonization is critical in understanding how this organism establishes itself, and what factors may influence the progression towards CDI. Indeed, this knowledge will be key in the development of microbial treatments such as fecal microbiota transplants.

The knowledge gained from Chapters 4 and 5 showcases just the beginning of the potential of sink drain hygiene interventions. There are both methodological and technological advancements that could benefit the structure of this intervention in future work. First, as we observed in Chapter 4, the 5x/week intervention reduced recoverable AROs by a larger fraction than the 1x/week intervention, but both reached no *P. aeruginosa* or *S. maltophilia* recovery by late in the intervention phase. Future work could investigate the potential of a variable frequency of intervention: starting off with several weeks of 5x/week intervention, to ‘knock back’ the growth, and then maintenance intervention at a lower frequency like 1-2x/week. This could exploit the benefit of the high frequency intervention initially but reduce the financial and time costs necessary for maintenance. Though in this study research personnel implemented the intervention, these

methods may be more attainable for integrating into routine cleaning protocols. Another aspect of this intervention that could be improved is the apparatus by which the disinfectant is foamed and pumped into the drain. The study in Chapters 4 and 5 utilized two sizes of jug with a foaming pump, connecting tube, and nozzle to infuse the foamed disinfectant into the drains. This equipment is bulky and was not easy to work with and would require extra training for staff. There are already products on the market specifically for foaming cleaning products into sink drains, though they are largely for removing hair or oil clogs. The apparatus is a large bottle with a suction cup attached to the top, and when the suction cup is pressed down over the drain, the foam is pumped out (like the function of a whipped cream cannister or spray paint). A collaboration with one of these companies to produce a similar device that foams the disinfectant used in this study could greatly increase the feasibility of implementing such an intervention.

Finally, though this intervention largely seems successful in reducing ARO burden in hospital sink drains, much more work is needed before this type of intervention could be implemented in regular practice. Because this intervention only took place in 18 rooms, we were not able to identify any reduction in HAIs, or truly quantify the attributable fraction of colonized or infected patients that is due to sink drain colonization. While our findings were promising, we are not yet able to quantify any additional safety that this intervention lends patients, and thus cannot communicate the financial costs associated with these infections to hospitals and other healthcare facilities. The burden of proof is still on researchers to comprehensively analyze this intervention, as well as other proposed interventions, in their ability to benefit patients and hospitals in a way that is feasible and economical. As part of my involvement with the CDC Epicenters Environmental group, I participated in a discussion about the potential for funding a large multi-institutional study investigating exactly these aspects of sink drain interventions. A study of this scale will involve

much planning and organization of responsibilities but would likely provide sufficient evidence for healthcare facilities to begin addressing the problem of sink drain colonization.

The work described here is just the tip of the iceberg when it comes to infectious disease prevention and epidemiology. My work has been narrowly focused on HAIs, and I have not addressed other transmission routes, infection mechanisms, or organisms outside the ones chosen here. I was incredibly fortunate to gain exposure to countless other aspects of infectious disease epidemiology throughout this experience through collaborations, classes, conferences, mentorship, and other educational opportunities. These exposures have inspired me to pursue a career in public health and epidemiology, where I will use my knowledge and skills as a Genomic Epidemiologist at Rush University Medical Center. There, I will work in part with the Chicago Department of Public Health as they expand their genomic surveillance to cover multi-drug resistant organisms of concern and continue to build upon my experiences to contribute to the field of public health. I am so incredibly grateful for the amazing peers, mentors, friends, and family that have motivated and supported me through this process and look forward to continuing my education throughout my career.