Whole-genome sequencing identifies highly related *Pseudomonas aeruginosa* strains in multiple washbasin U-bends at several locations in one hospital: evidence for trafficking of potential pathogens via wastewater pipes

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Running title: Trafficking of bacteria in wastewater pipes

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Summary

Background: Hand washbasin U-bends have increasingly been associated with nosocomial outbreaks by Gram-negative bacteria, including *Pseudomonas aeruginosa* which is virtually ubiquitous in U-bends. Wastewater networks servicing U-bends are potential highways for trafficking pathogenic bacteria.

Aim: To use *P. aeruginosa* to investigate trafficking of bacteria between hospital washbasin U-bends.

Methods: Twenty-five washbasin U-bends in five locations in the Dublin Dental University Hospital (DDUH) were investigated for trafficking of *P. aeruginosa*; 10 in Clinic 2 (C2), 10 in the Accident & Emergency Department (A&E) and five in three other locations. In addition washbasin faucet samples (n=80) and mains and faucet water samples (n=72) were cultured for *P. aeruginosa*. Selected *P. aeruginosa* isolates recovered over 29 months underwent whole-genome sequencing and relatedness was interpreted using whole-genome multilocus typing (wgMLST) and pairwise single nucleotide polymorphism (SNP) analysis.

Findings: *P. aeruginosa* was recovered from all U-bends but not from faucets or water. Eighty-three isolates yielded 10 sequence types (STs), with ST560 and ST179 from A&E, C2, and two other locations predominating (70%). ST560 was also recovered from a common downstream pipe. Isolates within ST560 and ST179 were highly related regardless of source. ST560 divided into Cluster I (n=25) and Cluster II (n=2) with average allelic differences and SNPs of 3 and 0, and 2 and 5, respectively. The 31 ST179 isolates exhibited an average allelic difference and SNPs of 3 and 12, respectively.

Conclusion: Highly related *P. aeruginosa* strains were identified in multiple U-bends in several DDUH locations, indicating trafficking via the wastewater network.

Keywords: Washbasin U-bends, *Pseudomonas aeruginosa*, whole-genome sequencing, wastewater pipes, strain trafficking, biofilm.

Introduction

Hand washing is vital to reduce the spread of infection. Ironically, while the presence of hand washbasins in hospitals promotes hand washing, it also provides increased associated infection risks[1].

In hospitals, the wastewater pipe network is a complex and lengthy system servicing sanitary fixtures throughout the facilities. Wastewater pipes are constantly damp which encourages biofilm growth[2]. Wastewater networks open to the environment throughout hospital buildings in areas occupied by patients and staff at washbasin, sink and shower drains. Wastewater traps are a fundamental part of sanitary fixtures including washbasins, sinks, baths, showers and toilets and prevent sewer gas entering buildings from wastewater pipes. Traps are situated below the drain outlet and consist of shaped pipework (e.g. U-bends) that retain ~~a~~ water forming a seal against the ingress of sewer gas[3]. This water stagnates when the fixtures are idle and biofilms form readily within the retained water section of the trap and can extend to the fixture drain outlet[3]. Microorganisms present in these biofilms can contaminate the washbasin and the surrounding environment, particularly if tap water directly impacts the drain causing splashing and aerosol formation[3–5].

Many studies have described nosocomial outbreaks caused predominantly by Gram-negative bacteria, associated directly or indirectly with contaminated washbasin and sink drains[1,4,6–9]. Furthermore, many recent reports have highlighted the importance of washbasin and sink drains in the nosocomial transmission of carbapenemase-producing Enterobacteriaceae, an emerging health threat globally[9].

Previous studies demonstrated that bacteria present in washbasin and sink drains can be aerosolised by the impact of tap water flow and can contaminate the washbasin, taps and local environmental surfaces[1,10,11]. An *in vitro* study using a monoculture of a laboratory strain of *Escherichia coli* expressing green fluorescent protein (GFP) showed that biofilm in a sink U-bend model system grows upwards towards the sink drain outlet and that subsequent splatter contaminates the bowl and surrounding area[5]. This study also showed trafficking of *E. coli* to adjacent sinks via common wastewater pipes. Deasy *et al.*[3] reported the growth of biofilm between washbasin U-bends and drain outlets in a hospital setting. It is not surprising that bacteria should spread from the U-bends of adjoining sanitary fixtures via common pipework, and over time perhaps via the wastewater pipe network to the U-bends of distantly located fixtures.

This study investigated whether individual strains of bacteria are distributed throughout a

washbasin wastewater pipe network in order to provide evidence for strain trafficking in a hospital setting. For this purpose, *Pseudomonas aeruginosa* isolates from washbasin U-bends were used as a marker organism because it is among the most frequently encountered bacteria identified from hospital washbasin U-bends. The genetic relatedness of *P. aeruginosa* isolates from multiple washbasin U-bends at adjacent and distant sites in one hospital was investigated using whole-genome sequencing (WGS). The study also investigated whether regular decontamination of washbasin U-bends affects the population structure of *P. aeruginosa*.

Methods

Hand washbasins

Twenty-five ceramic hand washbasins in the Dublin Dental University Hospital (DDUH) were investigated. Twenty-four were hospital-pattern (HP) washbasins with offset drain outlets[3,12]. One domestic pattern (DP) washbasin had the drain located directly below the tap water flow. Each HP washbasin faucet had a thermostatic mixing valve set to provide water at 38ºC. The DP washbasin had a manual mixer tap. All washbasins were used for hand washing only with Tork Extra Mild Liquid Soap (SCA Hygiene Products Ltd., Bedfordshire, United Kingdom), all were in frequent use daily on weekdays and fitted with identical polypropylene U-bends with two integrated sampling ports as described in detail previously[3]. Unscrewing a cap from each port permitted access to the U-bend interior for sampling.

Washbasins were selected to represent the diversity of large clinics and other areas in different DDUH locations. Ten HP washbasins were located in the ground floor Accident & Emergency Department (A&E; equipped with 11 washbasins in total), and 10 in Clinic 2 (C2; equipped with 15 washbasins in total) on the second floor. C2 and A&E were refurbished in August 2017 and 2016, respectively, with identical new washbasins, faucets, U-bends and wastewater pipes[3]. Cold water to washbasin faucets was provided from a 15,000-L tank supplied with mains water, which also supplied a calorifier providing faucet hot water. Hot and cold water supplied to DDUH washbasins is treated with residual anolyte (2.5 ppm), an electrochemically activated disinfectant solution composed predominately of hypochlorous acid[13]. Both clinics operate as outpatient facilities Monday-Friday. Additional HP washbasins from different locations in DDUH included one in the first floor Central Sterile Services Department (CSSD; equipped with one washbasin) and three in West Clinic (WC; equipped with six washbasins) on the ground floor. The DP washbasin was in a third floor staff bathroom, distant from clinics (Supplemental Figure S1).

The 10 washbasins investigated in Clinic 2 (C2) are located in five bays, each with three washbasins; two washbasins in each bay were included in the study. The U-bend of each C2 washbasin was connected via a 1-m vertical pipe to one of a series of five horizontal wastewater pipes, each of which serviced individual bays. Each pipe discharged water into an individual vertical pipe, which passed through the building into the basement (Supplemental Figure S1). Three of the five vertical pipes connected to a larger common horizontal wastewater pipe connected to the municipal sewer at the building perimeter. The other two vertical pipes connected to a separate common horizontal wastewater pipe that discharged wastewater to the

municipal sewer at a separate outlet. The U-bends of each A&E washbasin were connected via 1-m vertical pipes to a common horizontal wastewater pipe that discharged water into a vertical pipe connected to the same large common wastewater pipe servicing C2. The CSSD washbasin discharged water into one of the vertical wastewater pipes servicing C2. The washbasin U-bends in WC and the DP washbasin discharge wastewater to the sewer system at different outlets to C2 and A&E (Supplemental Figure S1).

Since their installation in August 2016, A&E washbasin U-bends underwent automated decontamination three times weekly, involving sequential treatments with two electrochemically-activated (ECA) solutions generated from brine; catholyte (80 ppm NaOH) with detergent properties and anolyte (632 ppm HOCl) with disinfectant properties[3]. This involves completely filling U-bends with ECA solutions sequentially for 10 min each, facilitated by closing an electronic valve on the common wastewater outflow pipe[3]. Other DDUH washbasin U-bends were not decontaminated.

Testing water and faucets for P. aeruginosa

Seventy-two 1-L water samples, eight from the washbasin cold water supply, eight from the mains supply, and 56 from washbasin faucets (16 each from A&E and C2) were tested for *P. aeruginosa*. Samples were taken in sterile bottles, neutralised with 0.5% sodium thiosulphate[14] and vacuum filtered through 0.45 µm filters (Sartorius, Göttingen, Germany), followed by incubation on *Pseudomonas aeruginosa* selective agar (PSCN). Swab samples of 20 representative DDUH washbasin faucets (including 5 each from A&E and C2) were sampled four times at six-month intervals with swabs dipped in sodium thiosulphate (0.5%) and cultured on PSCN.

Recovery of P. aeruginosa *from U-bends*

U-bends were flushed with water prior to sampling. *Pseudomonas aeruginosa* was recovered by swab sampling U-bend interiors via sampling ports using sterile cotton swabs (Venturi Transystem, Copan, Italy) dipped in 0.5% (w/v) sodium thiosulphate solution[3,13]. C2 U-bends were sampled once weekly for 52 weeks (n=520), whereas A&E U-bends were sampled immediately following decontamination, 24 h- and 48 h-post decontamination for 52 weeks (n=1560). Average bacterial densities were calculated from these samples.

Swab tips were suspended in 1 ml of sterile phosphate buffered saline, vortexed, serially diluted and 100 µl aliquots spread in duplicate on Colombia Blood agar (CBA), Reasoner's 2A (R2A) agar and PSCN as described[3]. Presumptive *P. aeruginosa* isolates were recovered on

PSCN, purified and identified using Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry (MALDI-TOF-MS)[3]. Isolates were stored at -80°C in Microbank cryovials (Prolab Diagnostics, Cheshire, United Kingdom). Unless otherwise stated, a single isolate from each sample was stored (see study design and isolate selection section below).

Study design and isolate selection

C2 was selected as a model clinic to investigate the population of *P. aeruginosa* in washbasins U-bends (n=10) by WGS. A six-month time frame was established for the selection of *P. aeruginosa* isolates to be sequenced (February–July 2018) to reduce WGS costs. Overall 55 isolates were sequenced (Supplemental Table S1). These included five *P. aeruginosa* isolates from at least three independent U-bends recovered monthly for the six month period (n=30). An additional 17 *P. aeruginosa* from one U-bend (B2D3) consisted of isolates recovered at intervals of at least a week over the six-months. The remaining eight isolates consisted of separate *P. aeruginosa* isolates recovered in February 2019 from one B2D3 sample following completion of the sampling period.

Twenty-one *P. aeruginosa* isolates from ECA-treated A&E U-bends were investigated (Supplemental Table S1). Isolates recovered over a longer sampling period (January 2017–March 2019) were selected for WGS because the majority of ECA-treated U-bend samples failed to yield *P. aeruginosa*[3]. Isolates from three time points were investigated: immediately after ECA treatment(n=7), 24-h post-ECA treatment(n=7), and 48-h post-ECA treatment(n=7).

Additional isolates from other location in DDUH were included in this study: three from separate WC U-bends (June and July 2017), two from a CSSD U-bend (May and June 2017) and two from the DP washbasin U-bend (May and August 2017). Three additional isolates recovered in May 2019 from the main common wastewater collection pipe servicing C2 and A&E at the point of discharge into the municipal sewer were also investigated. A total 83 U-bend isolates and three additional wastewater pipe isolates were selected for sequencing from DDUH.

A selection of *P. aeruginosa* comparator isolates from separate washbasin U-bends from two other Irish hospitals (n=9), from a dental chair water reservoir from a clinic outside of DDUH (n=2) and isolates previously recovered from dental suction systems (n=7)[15] were investigated as comparator isolates. The *P. aeruginosa* reference strains PA01[16] and ATCC15442[17] were also included. In total, 104 environmnetal isolates and two reference isolates were sequenced.

Whole-Genome Sequencing

Genomic DNA was extracted from *P. aeruginosa* using the Qiagen DNeasy Blood and Tissue kit (Qiagen, West Sussex, United Kingdom). Sequencing libraries were prepared using the Nextera DNA Flex kit (Illumina, Eindhoven, The Netherlands) and paired-end reads generated using the MiSeq Reagent kit v2 (500 cycles) (Illumina) using the MiSeq sequencing platform. All isolate sequences passed quality metrics of average Q30 >30 with an average read coverage of ≥ 50X. Read qualities were checked using Galaxy software tools[18] and where necessary, reads with a Phred score of <30 were trimmed using Trimmomatic software[18].

Genome assembly and analysis

The BioNumerics v7.6 (Applied Maths, Sint-Martens-Latem, Belgium) suite of software applications were used to analyse WGS data. FASTQ files were uploaded to BioNumerics and the raw reads *de novo* assembled and contigs generated utilising SPAdes v6.4[19]. The BioNumerics *P. aeruginosa* whole genome (wg) multi-locus typing (MLST) scheme consisting of 15,136 loci was utilised for assembly-free and assembly-based allelic detection. The MLST profile of each isolate was determined using PubMLST (BioNumerics). Pairwise single nucleotide polymorphism (SNP) analysis was utilised. SNP filter exclusion parameters were set to remove potential indel-related SNPs (SNPs occurring within 12 bp), positions with ambiguous base calls, and SNPs in repeat regions. Minimal spanning trees (MSTs) were generated using BioNumerics, based on Kruskal's algorithm.

Statistical Analysis

Statistical significance was determined using an unpaired, two-tailed Student's *t*-test with 95% confidence interval using GraphPad Prism v.5 (GraphPad, San Diego, USA).

Results

P. aeruginosa *from C2 and A&E U-bends*

Pseudomonas aeruginosa was recovered from all U-bends in C2 and A&E during the study. C2 U-bends were not decontaminated, whereas A&E U-bends were decontaminated three-times weekly with ECA solutions. The average bacterial densities from the 10 A&E U-bends showed a >4.4 log reduction on all media (CBA, R2A and PSCN) immediately after disinfection relative to the corresponding average bacterial densities ~~compared with~~ from the 10 C2 U-bends over the 12-month study. Reductions were highly significant (P <0.0001) on all media. The average bacterial densities in C2 U-bends (520 samples, all *P. aeruginosa*-positive) on CBA and PSCN was 1,862,000 (±678,076) CFU/swab and 1,547,000 (±807,633) CFU/swab, respectively. The corresponding average bacterial densities in A&E U-bends immediately following ECA treatment (520 samples, 6% *P. aeruginosa*-positive) was 28.6 (±57.13) and 13.54 (±77.63) CFU/swab, respectively.

The approximate abundance of *P. aeruginosa* relative to other bacteria in U-bends from each clinic was determined. Over a period of five weeks, representatives of the different colony types recovered on CBA from A&E U-bends were identified using MALDI-TOF-MS. *Pseudomonas aeruginosa* accounted for 58% of all colony types identifiable. Similarly, over a period of two weeks *P. aeruginosa* accounted for 32% of all colony types identifiable from C2 U-bends.

P. aeruginosa *STs in DDUH U-bends*

Sequencing of 55 *P. aeruginosa* isolates selected from C2 U-bends yielded four STs (ST179, ST252, ST298, ST560). ST179 and ST560 accounted for 49% (27/55) and 35% (19/55) of isolates, respectively (Supplemental Table S1). C2 U-bend B2D3 was sampled weekly during the same period and 17 isolates from separate samples belonged to ST179 (9 isolates, average allelic difference of 1 [range 0–2]) and ST560 (8 isolates, average allelic difference of 1 [range 0–2]). Eight isolates from one B2D3 sample belonged to ST179 (average allelic difference of 2 [range 0–7]). Analysis of all C2 ST560 (n=19) and ST179 isolates (n=27) showed that isolates within each ST were very closely related (average allelic difference of 1 [range 0–4] and 2 [range 0–14], respectively).

Sequencing of 21 *P. aeruginosa* isolates selected from seven A&E U-bends yielded six STs, including ST308 (n=7), ST560 (n=4), ST773 (n=4), ST296 (n=3), ST179 (n=2), and ST27

(n=1). The four ST560 and two ST179 isolates exhibited an average allelic difference of 14 (range 0–35) and 0–7 allelic differences, respectively. Overall, the allelic difference range for the two most abundant STs recovered from C2 and A&E, ST179 and ST560, exhibited an average allelic difference of 3 (range 0–17) and 10 (range 0–64), respectively.

Seven *P. aeruginosa* isolates from three other washbasin U-bends in CSSD (n=2), WC (n=3) and the staff bathroom (n=2) yielded four STs, (ST27, ST179, ST253, and ST560) (Supplemental Table S1). Three isolates belonging to ST253 and ST560 were recovered from the main common wastewater pipe receiving wastewater from A&E, C2 and CSSD at the point of discharge to the municipal sewer.

P. aeruginosa *STs among comparator isolates*

The 11 *P. aeruginosa* isolates investigated from three other healthcare facilities (including 9 isolates from washbasin U-bends in two acute hospitals) yielded eight STs (ST17, ST253, ST282, ST298, ST313, ST348, ST390 and ST395) (Supplemental Table S1). Only two of these (ST253 and ST298) were identified in DDUH. The ST298 isolate from Hospital 2 exhibited 135 allelic differences to the ST298 isolates (n=6) from C2 (ST298 was not identified in A&E U-bends). Furthermore, the two ST253 isolates identified in the common wastewater pipe servicing C2 and A&E, exhibited 53 allelic differences to the single ST253 isolate from Hospital 2. *P. aeruginosa* recovered from dental suction systems (n=7) yielded two STs including ST1320 (n=6; average of allelic differences 4 [range 0–11]) and ST2865 (n=1).

Population structure of DDUH and comparator P. aeruginosa

A MST based on wgMLST profiles was generated showing the STs of all isolates investigated (Figure 1a). Overall eight *P. aeruginosa* STs were identified among 83 isolates from DDUH U-bends and three from the common wastewater pipe (ST27, ST179, ST252, ST253, ST296, ST298, ST308, ST560, ST773). One of the predominant DDUH STs, ST179 (n=31), exhibited an average allelic difference of 3 (range 0–17), indicating these isolates were very closely related (Figure 1a). Isolates of the second predominant ST, ST560 (n=27), exhibited an average allelic difference of 7 (range 0–64), suggesting these isolates were more diverse. However, two isolate clusters were evident within ST560; Cluster I (n=25; average allelic difference of 3 [range 0–21]) and Cluster II (n=2; no allelic differences) (Figure 1a).

ST560 and ST179 isolates were also investigated by pairwise SNP analysis; these isolates exhibited an average of 9 SNPs (range 0–66) and 12 SNPs (range 0–38), respectively (Figure 1b & 1c). ST560 Cluster I isolates exhibited an average of 2 SNPs (range 0–8) (including isolates

from C2, A&E, CSSD and the common wastewater pipe), whereas the two ST560 Cluster II isolates (from A&E) exhibited five SNPs. Cluster II was differentiated from Cluster I by 59 SNPs (Figure 1b). ST179 isolates (including isolates from C2, A&E, and the staff bathroom) exhibited an average of 12 SNPs (range 0–38) (Figure 1c). Two isolates from U-bends in A&E and the staff bathroom exhibited no SNPs (Figure 1c).

Effects of A&E U-bend decontamination on the P. aeruginosa *population structure*

Of the 21 isolates sequenced from A&E, four STs were identified immediately after U-bend decontamination (ST296, ST308, ST560 and ST773), four 24 h-post disinfection (ST27, ST179, ST308 and ST560) and three 48 h-post disinfection (ST296, ST308, ST773). Isolates from STs ST308, ST296, ST773, and ST560 were identified between two or more of the sampling time points. ST308 was the only ST recovered at all three time points, and the seven isolates exhibited an average allelic difference of 4 (range 0–8).

Testing DDUH water for P. aeruginosa

Pseudomonas aeruginosa was not detected in the potable mains water supply (n=8), the anolyte-treated water supply to washbasin faucets (n=8), and washbasin faucet water (n=56). Swab sampling of 20 DDUH washbasin faucets including five each from C2 and A&E on four occasions each also failed to detect *P. aeruginosa*.

Discussion

Washbasin U-bends and drains have increasingly been identified as reservoirs for nosocomial infections[5,6,8,20]. Here, *P. aeruginosa* was used as a marker organism for washbasin U-bend contamination, where it is virtually ubiquitous, and many reports have linked nosocomial transmission of *P. aeruginosa* to contaminated U-bends[20,21]. WGS was used to investigate the distribution of *P. aeruginosa* STs in washbasin U-bends in DDUH focusing on two separate clinics on different floors, each with a common water supply, similar usage and equipped with identical washbasins, U-bends and wastewater pipes. The wastewater pipes from each clinic discharged into common outflow pipes connected to the municipal sewer (Supplemental Figure S1). C2 U-bends were not decontaminated during the study, whereas A&E U-bends were decontaminated three times weekly. Consequently, the burden of *P. aeruginosa* in A&E U-bends immediately after decontamination was significantly reduced with a >4.4 log reduction in overall bacterial counts over 12 months relative to the corresponding bacterial counts in C2 U-bends. Nonetheless, *P. aeruginosa* was recovered from every washbasin investigated during the study. A previous study investigated the *P. aeruginosa* population diversity at two wastewater sampling sites in a French hospital using MLST and identified 15 different STs from 30 samples[23]. Here, the diversity of *P. aeruginosa* in C2 U-bends yielded only four STs among 55 isolates, of which ST179 and ST560 predominated (83.6%). Six STs were identified among 21 *P. aeruginosa* isolates from A&E U-bends, with ST179 and ST560 accounting for 28.6%. All STs identified were previously recovered from the environment, and all except ST296, have been associated with clinical infections[24–28].

In this study the allelic and SNP thresholds of relatedness for *P. aeruginosa* isolates were set at <14 allelic differences and <37 SNPs, as previously suggested[29–31]. Isolates within ST179 and ST560 were very closely related based on wgMLST and SNP analyses regardless of the location of recovery (Figure 1 and Supplemental Figure S1). ST560 represented 32.5% of all isolates investigated and were recovered only from DDUH (C2, A&E, CSSD U-bends, and the main wastewater pipe common to all three). The average allelic differences and SNPs within ST560 isolates was 7 (range 0–64) and 9 (range 0–66), respectively (Figure 1). However, on closer inspection the 27 ST560 isolates group into two clusters, Cluster I (n=25) and Cluster II (n=2). Cluster I isolates exhibited average allelic and SNP differences of 3 (range 0–21) and 2 (range 0–8), respectively, whereas Cluster II isolates exhibited no allelic differences and five SNPs (Figure 1a and 1b). Similarly, ST179 accounted for 37.3% of all isolates investigated and were recovered only from DDUH U-bends (C2, A&E and the staff bathroom). The average

391 allelic differences and SNPs within ST179 isolates was 3 (range 0–17) and 12 (range 0–38),
392 respectively (Figure 1a and 1c). Interestingly, an ST179 isolate (E24Aug) from an A&E U-bend
393 and an ST179 isolate (LP3F2) from the staff bathroom U-bend exhibited zero SNPs (Figure 1
394 and Supplemental Table S1). These U-bends are located at opposite ends of DDUH, separated by
395 a distance of approximately 132 m. These results revealed the presence of very closely related *P.*
396 *aeruginosa* isolates in washbasin U-bends in several different areas of DDUH (i.e. C2, A&E,
397 CSSD, staff bathroom) and in one of the main wastewater outflow pipes. C2 washbasin U-bend
398 B2D3 was selected to investigate the diversity of isolates in an individual U-bend. Seventeen
399 isolates recovered at intervals of at least a week belonged to ST179 (n=9) and ST560 (n=8) and
400 isolates within each ST were very closely related (both with an average allelic difference of 1
401 [range 0–2]). These findings reveal the persistence and stability of isolates in an individual U-
402 bend; at least during the six-month period isolates were sequenced. At the end of the study, eight
403 isolates from one sample from B2D3 belonged to ST179 and exhibited an average allelic
404 difference of 2 (range 0–7). Isolates from A&E U-bends were included to investigate the effects
405 of regular U-bend decontamination on *P. aeruginosa* diversity. The abundance and prevalence of
406 *P. aeruginosa* in A&E U-bends was significantly lower than non-decontaminated U-bends
407 elsewhere in DDUH and the range of STs was slightly higher (6 vs. 4); however, the majority of
408 ST560 (25/27) and all ST179 (n=31) isolates from the former were very closely related to
409 isolates from the latter (Figure 1b and 1c).

410 *Pseudomonas aeruginosa* isolates in U-bends could have originated from the supply
411 water, faucets, water discharged down U-bends and wastewater pipes. Hot and cold water
412 supplying washbasin faucets in DDUH has been treated continuously with residual anolyte (2.5
413 ppm) since 2012[13]. During the present study *P. aeruginosa* was not isolated from washbasin
414 faucets, mains water, the anolyte-treated washbasin water supply, or from faucet output water. A
415 previous year-long study from DDUH also failed to detect *P. aeruginosa* in anolyte-treated
416 washbasin faucet water or faucets from the same sites[13]. Anolyte readily penetrates biofilms in
417 water systems and very likely was a significant factor contributing to the failure to detect *P.*
418 *aeruginosa* in washbasin water and faucets in the present and previous studies[3,13,14]. These
419 findings suggest that supply water was unlikely to be a significant source of *P. aeruginosa* in U-
420 bends; otherwise a wider range of STs would be expected. It is highly unlikely that the residual
421 anolyte (2.5 ppm) used to treat washbasin tap water in DDUH had any significant effect on
422 bioburden in DDUH U-bends due to high densities of bacteria recovered from non-
423 decontaminated U-bends. A previous study demonstrated that small amounts of organic matter
424 (i.e. 1 mg/ml of bovine serum albumin) completely neutralises the free available chlorine present

in 100 ppm anolyte, a concentration 40-times higher than that used to treat washbasin water in DDUH[32]. *Pseudomonas aeruginosa* can be carried transiently on the hands[33]. However, if hand washing were a frequent contributor of *P. aeruginosa* to U-bends, a far wider range of STs would be anticipated. The detection of highly related strains in U-bends in four separate DDUH locations (C2, A&E, CSSD, and the staff bathroom) and the main wastewater outflow pipe indicates that the wastewater pipe network is a more likely contributor to U-bend contamination. Interestingly, ST560 and ST179 isolates were recovered from U-bends in adjacent clinical bays in C2. Washbasins in individual bays in C2 do not share common proximal wastewater pipes; common pipework occurs more distally in the network, suggesting trafficking of isolates occurs from more downstream regions (Supplemental Figure S1). The low diversity identified in *P. aeruginosa* from U-bends in this study may be associated with ECA decontamination of A&E U-bends. Large volumes of spent ECA solutions are discharged following decontamination, which likely reduces the burden of *P. aeruginosa* in downstream pipework and this may reduce trafficking[3].

Trafficking of bacteria in wastewater pipes could occur by wastewater flow, bacterial motility and air currents. Water discharged down washbasin drains can traffic bacteria in U-bends and pipes to distal sites in the network. As mentioned above, the model U-bend and wastewater system supplied with nutrients in the absence of faucet water flow demonstrated that an *E. coli* strain expressing GFP exhibited an average growth of one inch per day along the pipework[5]. Flagellar motility has been shown to be an essential element in the ability of *P. aeruginosa* to form biofilms on surfaces and tissues[34]. Air currents occur in wastewater pipes both by airflow down into the sewer, and inversely, within wastewater networks[35]. The flow of water in pipes results in a partial vacuum that draws air behind the flow of water. All three methods likely contribute to the dissemination of related strains in wastewater networks.

Conclusions

Previous studies suggested that washbasin U-bends and associated fittings are potential highways for trafficking potentially pathogenic bacteria[5,11]. This study confirmed this using high-resolution WGS typing for the first time and demonstrated the distribution of highly related strains of *P. aeruginosa* in multiple washbasin U-bends in different locations in a hospital setting. Consideration should be given to effective decontamination of wastewater pipes in hospitals, at least in critical areas. The use of ECA solutions for this purpose has already yielded encouraging results[3,36].

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Conflict of interest statement

None declared.

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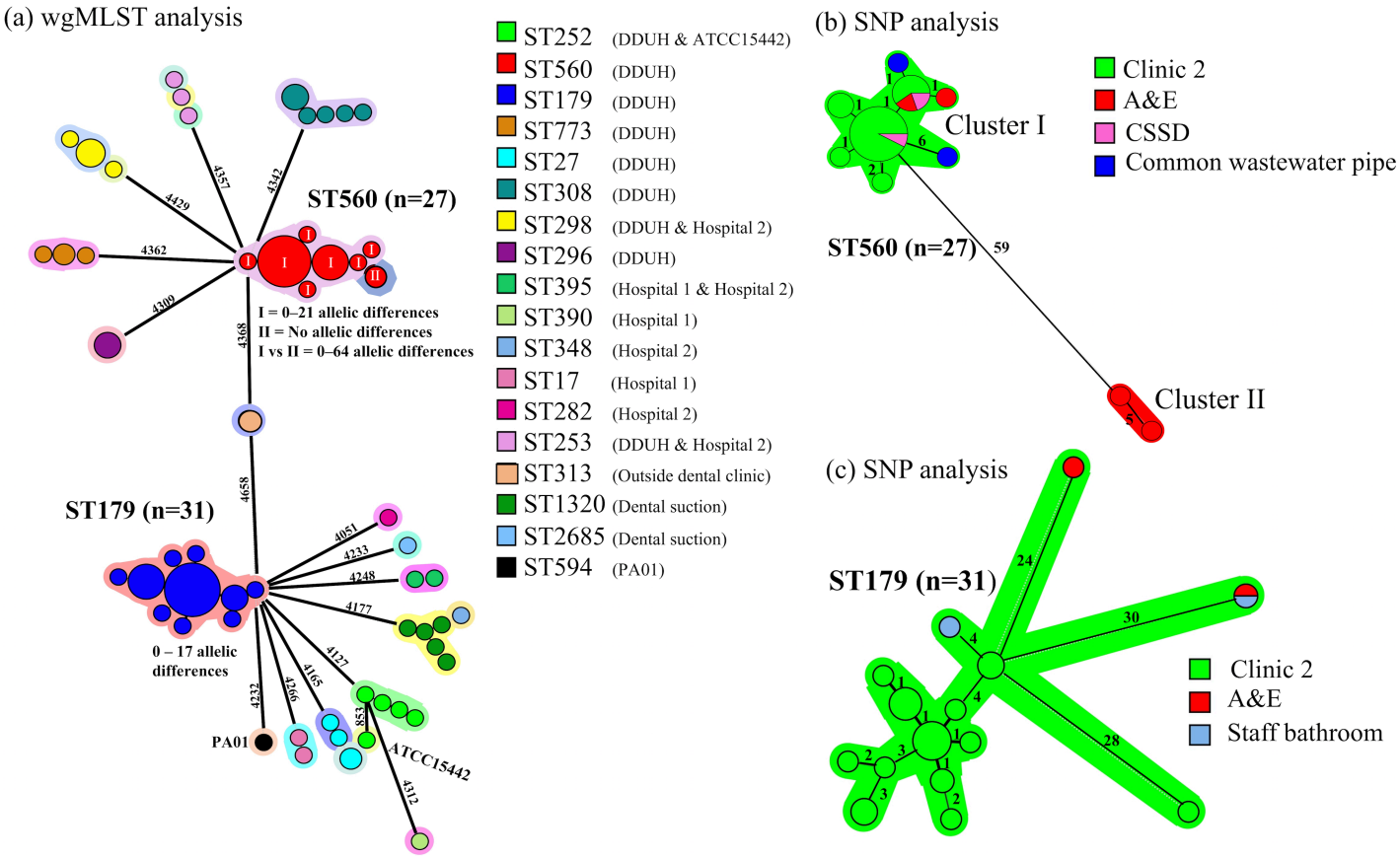
Figure 1 legend. Minimum spanning trees (MSTs) based on whole-genome multi-locus sequence typing (wgMLST) and single nucleotide polymorphism (SNP) data of *P. aeruginosa* isolates. (a) An MST based on wgMLST data of all 106 isolates investigated showing the relationships between the sequence types (STs) identified including the PA01 and ATCC15442 reference strains. Numbers on the branches show allelic differences between ST isolate groupings. The colour-coded key to the right of the figure identifies STs and the origin of isolates within each ST. Eighty-three isolates were recovered from DDUH U-bends (ST179, ST252, ST298, ST560, ST308, ST773, ST296 and ST27) and three (ST253 and ST560) from the main common wastewater pipe servicing washbasins in Clinic 2 (C2), A&E and CSSD. ST179 and ST560 accounted for 37.3% (31/83) and 32.5% (27/83) of the total DDUH isolates sequenced. ST560 isolates had two distinct clusters; Cluster I consisted of 25 isolates, whereas Cluster II consisted of two isolates. ST179 and ST560 were not identified among comparator isolates investigated. The allelic threshold of relatedness for *P. aeruginosa* isolates were set at <14 allelic differences as previously suggested[29].

(b) An MST based on the SNP analysis of the 27 ST560 DDUH isolates recovered from C2, A&E, CSSD and the main common wastewater pipe. The threshold of isolate relatedness was set as <37 SNP differences as previously suggested[31]. The isolates formed two distinct groups, Clusters I and II, differentiated by 59 SNPs. The average SNPs within the 25 isolates of Clusters I was 2 (range 0–8), whereas the two Cluster II isolates differed by five SNPs. Isolates within each of the two clusters revealed by SNP analysis corresponded to the same isolates identified within the two ST560 clusters identified by wgMLST analysis. These findings confirmed that isolates within each cluster were very closely related

(c) An MST based on the SNP profiles of the 31 ST179 DDUH isolates recovered from C2, A&E and the staff bathroom. Isolates differed by an average of 12 (range 0–38) SNPs.

Figure 1


(a) wgMLST analysis
ST560 (n=27)
ST179 (n=31)
I = 0–21 allelic differences
II = No allelic differences
I vs II = 0–64 allelic differences
0 – 17 allelic differences
PA01
ATCC15442
4357
4342
4429
4362
4309
4368
4658
4051
4233
4248
4177
4127
4165
4366
4232
4312
ST252 (DDUH & ATCC15442)
ST560 (DDUH)
ST179 (DDUH)
ST773 (DDUH)
ST27 (DDUH)
ST308 (DDUH)
ST298 (DDUH & Hospital 2)
ST296 (DDUH)
ST395 (Hospital 1 & Hospital 2)
ST390 (Hospital 1)
ST348 (Hospital 2)
ST17 (Hospital 1)
ST282 (Hospital 2)
ST253 (DDUH & Hospital 2)
ST313 (Outside dental clinic)
ST1320 (Dental suction)
ST2685 (Dental suction)
ST594 (PA01)
(b) SNP analysis
Cluster I
ST560 (n=27)
59
Cluster II
Clinic 2
A&E
CSSD
Common wastewater pipe
(c) SNP analysis
ST179 (n=31)
24
30
28
Clinic 2
A&E
Staff bathroom

Supplemental Table S1. Recovery timeline and sequence types of the 106 *P. aeruginosa* isolates investigated by whole-genome sequencing in this study.

Sequence Type	**Isolate name**	**Date of isolation**	**Location**[a]
ST179	AE24Jan	January 2017	A&E - washbasin 5 (24 h post disinfection)
	LP3F1	May 2017	DP washbasin in staff bathroom
	AE24Aug	August 2017	A&E - washbasin n 9 (24 h post disinfection)
	LP3F2	August 2017	DP washbasin in staff bathroom
	B2D3bFeb	February 2018	C2 - washbasin B2D3
	B2D3cMar	March 2018	C2 - washbasin B2D3
	B3D4Apr	April 2018	C2 - washbasin B3D4
	B2D3aMay	May 2018	C2 - washbasin B2D3
	B2D3bMay	May 2018	C2 - washbasin B2D3
	B2D3cMay	May 2018	C2 - washbasin B2D3
	B3D2May	May 2018	C2 - washbasin B3D2
	B2D3dMay	May 2018	C2 - washbasin B2D3
	B3D4May	May 2018	C2 - washbasin B3D4
	B1D2Jun	June 2018	C2 - washbasin B1D2
	B2D3aJun	June 2018	C2 - washbasin B2D3
	B3D2Jun	June 2018	C2 - washbasin B3D2
	B3D4Jun	June 2018	C2 - washbasin B3D4
	B4D3Jun	June 2018	C2 - washbasin B4D3
	B2D3cJun	June 2018	C2 - washbasin B2D3
	B1D4bJul	July 2018	C2 - washbasin B1D4
	B1D4cJul	July 2018	C2 - washbasin B1D4
	B2D3aJul	July 2018	C2 - washbasin B2D3
	B2D3bJul	July 2018	C2 - washbasin B2D3
	B2D3SNAP1	February 2019	C2 - washbasin B2D3
	B2D3SNAP2	February 2019	C2 - washbasin B2D3
	B2D3SNAP3	February 2019	C2 - washbasin B2D3
	B2D3SNAP4	February 2019	C2 - washbasin B2D3
	B2D3SNAP5	February 2019	C2 - washbasin B2D3
	B2D3SNAP6	February 2019	C2 - washbasin B2D3
	B2D3SNAP7	February 2019	C2 - washbasin B2D3
	B2D3SNAP8	February 2019	C2 - washbasin B2D3
ST560	AE24aMay	May 2017	A&E - washbasin 4 (24 h post disinfection)

	AE24cMay	May 2017	A&E - washbasin 3 (24 h post disinfection)
	CSSD1	May 2017	Central Sterile Services Department
	CSSD2	June 2017	Central Sterile Services Department
	AEDaJul	July 2017	A&E - washbasin 4 (immediately after disinfection)
	B1D4Feb	February 2018	C2 - washbasin B1D4
	B2D3aFeb	February 2018	C2 - washbasin B2D3
	B2D3cFeb	February 2018	C2 - washbasin B2D3
	B3D4Feb	February 2018	C2 - washbasin B3D4
	B1D4Mar	March 2018	C2 - washbasin B1D4
	B2D2Mar	March 2018	C2 - washbasin B2D2
	B2D3aMar	March 2018	C2 - washbasin B2D3
	B2D3bMar	March 2018	C2 - washbasin B2D3
	B3D4Mar	March 2018	C2 - washbasin B3D4
	B4D3Mar	March 2018	C2 - washbasin B4D3
	B2D3aApr	April 2018	C2 - washbasin B2D3
	B2D3bApr	April 2018	C2 - washbasin B2D3
	B2D3cApr	April 2018	C2 - washbasin B2D3
	B3D2Apr	April 2018	C2 - washbasin B3D2
	B2D3bJun	June 2018	C2 - washbasin B2D3
	B1D4aJul	July 2018	C2 - washbasin B1D4
	B2D3cJul	July 2018	C2 - washbasin B2D3
	B5D4Jul	July 2018	C2 - washbasin B5D4
	B5D4aJul	July 2018	C2 - washbasin B5D4
	AEDaOct	October 2018	A&E - washbasin 3 (immediately after disinfection)
	CWP3	May 2019	Common wastewater pipe[b]
	CWP6	May 2019	Common wastewater pipe[b]
ST308	AEDbJul	July 2017	A&E - washbasin 7 (immediately after disinfection)
	AEDApr	April 2018	A&E - washbasin 1 (immediately after disinfection)
	AE48aJun	June 2018	A&E - washbasin 1 (48 h post disinfection)
	AE48bJun	June 2018	A&E - washbasin 7 (48 h post disinfection)
	AE24Feb	February 2019	A&E - washbasin 1 (24 h post disinfection)
	AE24Mar	March 2019	A&E - washbasin 1 (24 h post disinfection)
	AE48Mar	March 2019	A&E - washbasin 1 (48 h post disinfection)
ST298	B5D2Feb	February 2018	C2 - washbasin B5D2
	B5D2Apr	April 2018	C2 - washbasin B5D2
	B5D4Apr	April 2018	C2 - washbasin B5D4

	B5D2Jun	June 2018	C2 - washbasin B5D2
	B5D4bJul	July 2018	C2 - washbasin B5D4
	DH11	November 2018	Hospital 2[c]
ST1320	DenS1	2005[1]	Recovered from dental suction systems
	DenS2	2005[1]	Recovered from dental suction systems
	DenS3	2005[1]	Recovered from dental suction systems
	DenS4	2005[1]	Recovered from dental suction systems
	DenS5	2005[1]	Recovered from dental suction systems
	DenS6	2005[1]	Recovered from dental suction systems
ST252	B5D4Feb	February 2018	C2 - washbasin B5D4
	B5D4Mar	March 2018	C2 - washbasin B5D4
	B1D2aMay	May 2018	C2 - washbasin B1D2
	B1D2bMay	May 2018	C2 - washbasin B1D2
	ATCC15442	N/A	American Type Culture Collection 15442
ST773	AE48Aug	August 2018	A&E - washbasin 5 (48 h post disinfection)
	AEDJan	January 2019	A&E - washbasin 7 (immediately after disinfection)
	AE48aJan	January 2019	A&E - washbasin 5 (48 h post disinfection)
	AE48bJan	January 2019	A&E - washbasin 1 (48 h post disinfection)
ST27	AE24bMay	May 2017	A&E - washbasin 2 (24 h post disinfection)
	West1	June 2017	West Clinic
	West2	June 2017	West Clinic
	West3	July 2017	West Clinic
ST296	AEDJun	June 2018	A&E - washbasin 7 (immediately after disinfection)
	AE48Sep	September 2018	A&E - washbasin 2 (48 h post disinfection)
	AEDbOct	October 2018	A&E - washbasin 7 (immediately after disinfection)
ST253	DH6	November 2018	Hospital 2[c]
	CWP2	May 2019	Common wastewater pipe[b]
ST17	LH1	March 2019	Hospital 1[c]
	LH4	March 2019	Hospital 1[c]
ST313	LPDP1	February 2019	Water sample from dental chair unit water reservoir outside DDUH
	LPDP2	February 2019	Water sample from dental chair unit water reservoir outside DDUH
ST395	DH13	November 2018	Hospital 2[c]
	LH3	March 2019	Hospital 1[c]
ST282	DH1	November 2018	Hospital 2[c]
ST348	DH10	November 2018	Hospital 2[c]

ST390	LH2	March 2019	Hospital 1[c]
ST549	PA01	N/A	Genbank (AE004091.2) [2]
ST2685	DenS7	2005[1]	Recovered from dental suction systems

Abbreviations: DDUH, Dublin Dental University Hospital; A&E, Accident and Emergency Department; C2, Clinic 2; DP, domestic pattern washbasin; CSSD, Central Sterile Services Department. [a]All isolates were recovered from swab sampled U-bends unless otherwise stated. [b]Common wastewater pipe collecting wastewater from Clinic 2, CSSD, A&E. [c]Hospitals 1 and 2 are located 121 km and 8 km from DDUH, respectively.

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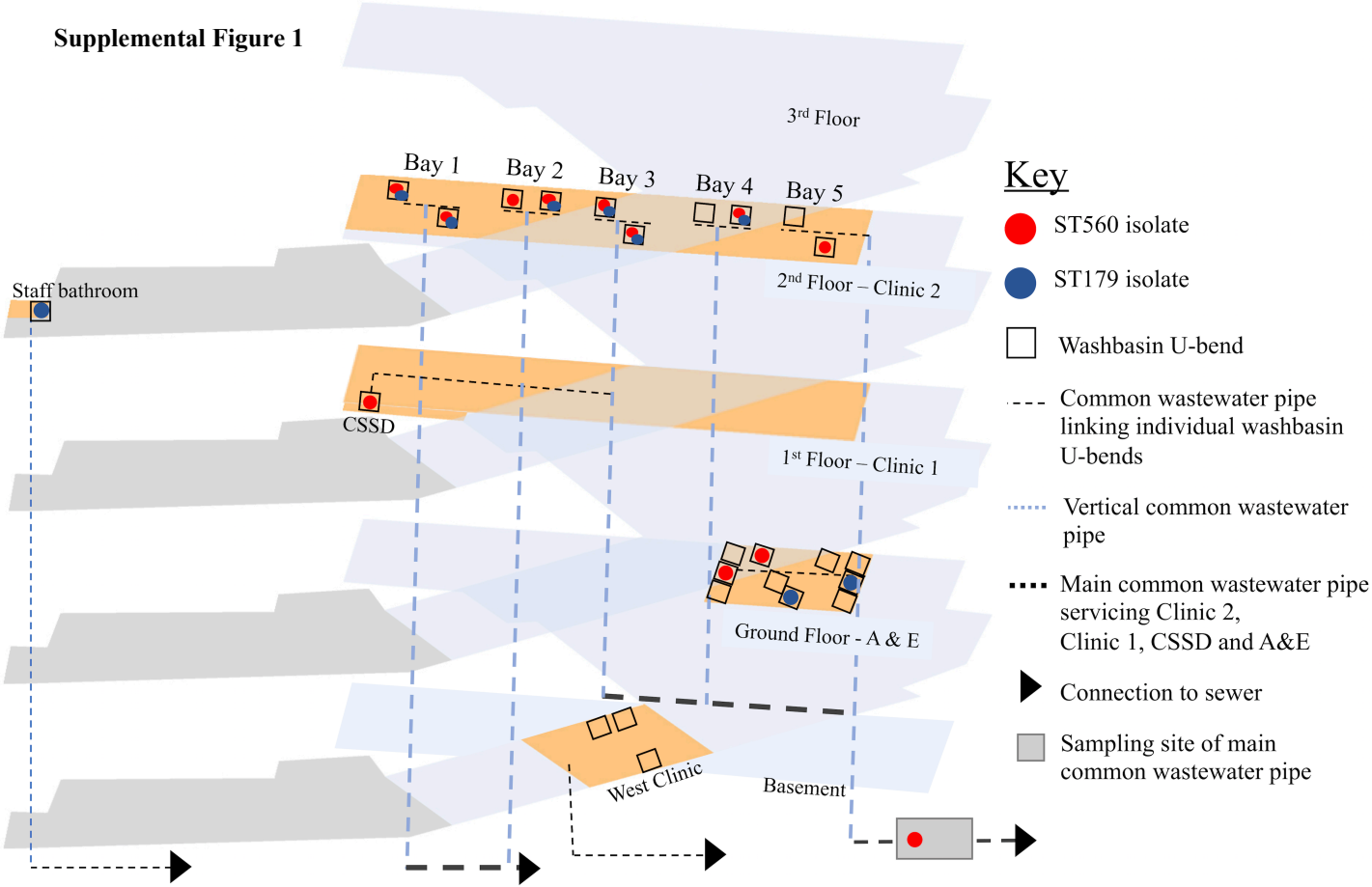
Supplemental Figure 1



Supplemental Figure S1 legend. Schematic showing the relative locations of Dublin Dental University Hospital (DDUH) washbasins investigated showing the distribution of the two predominant *Pseudomonas aeruginosa* sequence types (STs) identified (ST560 and ST179) in U-bends. The pale blue (clinical) and grey (administrative) sections represent different areas of DDUH. The 10 washbasins investigated in Clinic 2 (C2) are located in five bays, each with three washbasins (two washbasins from each bay were included in the study). Wastewater from each bay is discharged into a separate vertical wastewater pipe that passes down through the building into the basement. The vertical pipes servicing bays 3–5 from each clinic connect to a large common horizontal wastewater pipe in the DDUH basement where it discharges to the municipal sewer at the building perimeter. This common wastewater pipe also receives washbasin wastewater from the Accident and Emergency Department (A&E) and the Central Sterile Services Department (CSSD). Wastewater from clinical bays 1 and 2 connect to a separate horizontal wastewater pipe in the basement and discharge wastewater to the municipal sewer at a different exit point. Washbasin wastewater from bathrooms in the administration area and West Clinic (WC) discharge wastewater to the municipal sewer at separate connections. Ten washbasins each from C2 and A&E, one from CSSD, three from WC and one from a staff bathroom were included in the study.

C2, A&E, WC and CSSD were equipped with identical ceramic hospital-pattern washbasins (Armitage Shanks, Staffordshire, United Kingdom)[1]. Washbasins were equipped with identical polypropylene U-bends (McAlpine Plumbing Products, Glasgow, Scotland) with two access ports for sampling purposes [1]. Wastewater pipes and fittings were made of polyvinylchloride or acrylonitritebutadiene- styrene and all pipe connections apart from U-bends were chemically welded to minimize potential for leaks[1]. Bay 3 in C2, CSSD and A&E were approximately 38 m, 30 m and 12 m distant from the common wastewater collection pipe sampling point. The staff bathroom was located approximately 120 m from the sampling point common wastewater collection pipe.

ST560 was recovered from 12 washbasin U-bends in C2, A&E, CSSD, and the common wastewater collection pipe servicing clinical bays 3–5 just prior to discharge into the municipal sewer. ST179 was recovered from 9 washbasin U-bends in C2, A&E, and the staff bathroom. SNP analysis of the 27 ST560 isolates grouped the isolates into two clusters. In the present study the allelic and SNP thresholds of relatedness for *P. aeruginosa* isolates were set at <14 allelic differences and <37 SNPs, as previously suggested [2–4]. Cluster I isolates (*N*=25) exhibit an average SNP difference of 2 (range 0–8) and were recovered in C2, A&E, CSSD and the common wastewater pipe. Cluster II exhibits five SNP differences between two isolates recovered in A&E. SNP analysis of the 31 ST179 isolates recovered exhibit an average SNP difference of 12 (range 0–38). However, interestingly, there was no SNP differences between isolates recovered from the A&E and the staff bathroom.

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