Pseudomonas aeruginosa infection in augmented care: the molecular ecology and transmission dynamics in four large UK hospitals

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Running title: Transmission P. aeruginosa from water
Summary

Background: *Pseudomonas aeruginosa* is a common opportunistic pathogen and molecular typing in outbreaks has linked patient acquisition to contaminated hospital water systems.

Aim: To elucidate the role of *P. aeruginosa* transmission rates in non-outbreak augmented care setting in the UK.

Methods: Over a 16-week period, all water outlets in augmented care units of four hospitals were sampled for *P. aeruginosa* and clinical isolates were collected. Outlet and clinical *P. aeruginosa* isolates underwent whole genome sequencing (WGS), which with epidemiological data identified acquisition from water as definite (level 1), probable (level 2), possible (level 3), and no evidence (level 4).

Findings: Outlets were positive in each hospital on all three occasions, W (16%), X (2.5%), Y (0.9%) and Z (2%), and there were 51 persistently positive outlets in total. WGS identified likely transmission (at levels 1, 2 and 3) from outlets to patients in three hospitals for *P. aeruginosa* positive patients: W (63%), X (54.5%) and Z (26%). According to the criteria (intimate epidemiological link and no phylogenetic distance), approximately 5% of patients in the study ‘definitely’ acquired their *P. aeruginosa* from their water outlets in ICU. This study found extensive evidence of transmission from the outlet to the patients particularly in the newest hospital (W), which had the highest rate of positive outlets.

Conclusions: The overall findings suggest that water outlets are the most likely source of *P. aeruginosa* nosocomial infections in some settings, and that widespread introduction of control measures would have a substantial impact on infections.

Key Words: 

*P. aeruginosa*, water-outlets, transmission, molecular-ecology, infections, augmented-care
Introduction

_Pseudomonas aeruginosa_ is an important nosocomial pathogen in immunocompromised and critically ill patients and it is the fourth most common health care acquired infection (HCAI) pathogen in the USA [1] and the third most common cause of Gram-negative bacteraemia in hospitals in England, Wales and Northern Ireland [2].

Transmission of _P. aeruginosa_ in the healthcare environment has been due to cross-contamination from a variety of environmental reservoirs [3-5]. Contaminated hospital water outlets have been implicated in outbreaks in neonatal and adult critical care units [3-9], including the high profile neonatal unit incident in Northern Ireland [8], where water outlets (last two metres) and associated fittings were identified as the source of infections that led to fatalities. Consequently, UK national guidance was published by the Department of Health for water sampling and control measures [10]. Whilst outbreaks of _P. aeruginosa_ cross-infection in critical care units have been attributed to environmental sources, the role of such sources in sporadic (non-outbreak) pseudomonal infection situations has generally been poorly characterised in the UK [3].

Whole genome sequence typing (WGST) delivers a very high level of specificity and resolution compared to previously used typing methods such as pulsed-field gel electrophoresis (PFGE), and variable number tandem repeats (VNTR) which have been applied to _P. aeruginosa_ outbreaks in critical care [4, 6, 8, 11]. WGST has been used to investigate transmission in outbreaks of _Staphylococcus aureus_ [12] _Acinetobacter baumannii_ [13] as well as _P. aeruginosa_ [9, 14, 15] and previously used to demonstrate that transmission of waterborne isolates to burns patients most likely occurs during hydrotherapy, which is used in the management of burns patient’s wounds [16].

To date there has been no comprehensive longitudinal multi-centre studies of the transmission dynamics of _P. aeruginosa_ in UK critical care settings employing WGST. This study undertook a defined snapshot survey using WGST, simultaneously across four large UK hospitals, to investigate the burden
of *P. aeruginosa* infection or colonisation in adults (caused by transmission from water outlets) in the non-outbreak augmented care setting.

**Methods**

**Study design and patients**

Four NHS hospitals (anonymised to W, X, Y and Z) with diverse water sources, buildings, and plumbing infrastructures from a wide geographical area in England participated in this study (Table I). A total of 23 augmented care units (ACUs) across the 4 hospitals were studied, from which all 881 water outlets (774 taps and 107 showers) were sampled.

During a 16-week period (30th August - 6th December 2014) water sampling of outlets was undertaken at all sites, on three occasions (designated ‘beginning’, ‘middle’ and ‘end’) within a 10-day window. The ACUs of the hospitals were described as ‘non-outbreak’ with respect to *P. aeruginosa* as there was no observed increase in incidence of *P. aeruginosa* infections above the average number (from both clinical and environmental observations), and hence these units were not under enhanced surveillance.

All clinical isolates of *P. aeruginosa*, which were retained as part of routine care from patients satisfied the inclusion criteria as follows: an inpatient in one of the included wards for more than 48 hours, with no pre-existing *P. aeruginosa* colonisation/infection, and without an isolate with the same antibiogram and colony morphology having already been saved. The bed, ward number and nearest water outlet (make and model) when sample(s) were retained. No clinical details or patient identifiable data were recorded and we were unable to obtain ethical approval to screen all patients for *P. aeruginosa* carriage upon ICU admission.
Microbiological methods

Pre-flush samples were taken at each hospital as described previously [10]. Water (100 mL) samples were filtered (0.45µM membrane filter) and cultured on cysteine lactose electrolyte deficient agar (CLED) agar (Biomerieux) and on cetyl trimethylammonium bromide and nalidixic acid (CN) agar plate (Biomerieux) (48 hours incubation at 37°C). Oxidase positive colonies were identified using MALDI-TOF (Biomerieux).

Molecular methods and bioinformatics analysis

DNA extraction of P. aeruginosa isolates was performed by emulsifying (5µl) each bacterial colony in molecular grade water (100µl) and then heated (10 minutes at 95°C). DNA was isolated and cleaned by adding the supernatant to an equal volume of Ampure XP beads (Beckmann Coulter, High Wycombe, UK).

Whole genome sequencing was performed as described previously [16]. Reads were demultiplexed and then mapped against the P. aeruginosa PAO1 reference genome (NC_002516.2). Clusters were identified using a rapid genotyping method described previously [17]. To call variants, representative isolates from each cluster were mapped against P. aeruginosa reference genomes from RefSeq [18] and the NCTC 3000 project using bwa-mem (v0.7.15) [19]. The best reference was selected for each cluster based on greatest proportion of mapped reads. Variants were called using FreeBayes Variant calls and filtered for allele frequency and recombination. Phylogenetic trees were constructed for each cluster using RAxML (v8.2.10) [20]. In addition all isolates were MLST typed in silico using StringMLST (v0.5.1.1) [21, 22].
**Definition of transmission events**

The likelihood of transmission was assessed through a multifactorial approach taking into consideration i) the cluster defined dendrograms of environmental water and clinical isolates, ii) a measure of phylogenetic distance, and iii) the metadata relating to geographical locations when samples were taken.

- All clinical isolates within the same cluster as environmental isolates were analysed for potential transmissions by both phylogenetic distance and epidemiological link.
- Pairwise phylogenetic distances between clinical and environmental isolates were measured for each cluster with distances used to identify similarity to an environmental source.
- Metadata for clinical isolates defining ward and bed locations was analysed for potential epidemiological links to the sampling location of environmental isolates.

Four categories of transmission were defined; Level 1 (definite): ‘Patient isolate genome indistinguishable from water outlet *P. aeruginosa* genome (phylogenetic distance of 0) with epidemiological link to at least ward level’ (patient and outlet linked in time and place), Level 2 (probable): ‘Patient isolate genome highly similar to an outlet genome (but with a phylogenetic distance of >0), and with an epidemiological link to outlet’, Level 3 (possible) ‘Patient isolate genome present in the same phylogenetic cluster as a *P. aeruginosa* water outlet genome but without an epidemiological link to outlet’, and Level 4 (no evidence): clearly not a hospital water transmission (no hospital water isolates present in the same cluster as the patient isolates and no epidemiological link). The likely direction of transmission (and temporality) was then inferred based on the dates that the water and clinical isolates were collected.
Results

The overall proportion of outlets positive for *P. aeruginosa* (as a percentage of the total outlets sampled per hospital site) was 26.3%, 10.4%, 6.5% and 8.2% for hospitals W, X, Y & Z respectively. Some outlets (16% in hospital W) remained positive throughout the study (Table 1). The hospital built most recently (2010; with the newest plumbing system (hospital W)) had the highest rate of *P. aeruginosa* positive outlets, and the greatest number of clinical isolates. Hospital Y (the only hospital with a Copper Silver Ionisation (CSI) water treatment system for the prevention of Legionella infections), had the lowest positivity rates from the water outlets, and the fewest number of clinical isolates.

There were 120 clinical *P. aeruginosa* isolates collected from 78 eligible patients during the study (Supplementary Table II). The majority were from respiratory samples (50%), with the remainder from swabs (25.4%), urine (11.2%), blood cultures (6.7%), tissues (4.2%) and fluids (2.5%). Only one patient isolate was not included in the analysis due to inadequate sequencing depth.

Sequence diversity of *P. aeruginosa* isolates

A total of 552 (120 clinical and 432 water) *P. aeruginosa* isolates were analysed. One clinical isolate failed sequencing, and <10% of the water isolates could not be sequenced. We assigned the isolates to 17 clusters which were defined as clades containing at least one patient and one water outlet isolate i.e. a potential transmission. A total of 460 (83%) isolates belonged to a designated cluster, and clusters were consistent with the results of multi-locus sequence typing data reported by StringMLST (Supplementary Table III).

Analysis of the isolates for sequence diversity (Figure 1) demonstrated different clades associated with different hospitals. Hospital W had the largest number of positive water outlet isolates (181 from 231 sampled assets), but also the most defined clusters with the majority of the isolates belonging to one
of three large clusters (ST395, ST17 and ST179). The environmental water outlet isolates from the other hospitals were more diverse, with a smaller proportion of isolates contained in three small clusters (hospital X), one cluster (hospital Z), or with limited clustering (hospital Y).

**Detection of linked environmental and clinical isolates**

Although all clusters were analysed, further diversity analysis was performed on ST395, as it was present in each hospital (Supplementary Figure 2). Phylogenetic distance was lower within the same hospital when compared to different hospitals, suggesting each hospital was colonised by a local clone or clones. Phylogenetic distance of isolates from the same ward was similar to that of different wards, suggesting that transmission of *P. aeruginosa* to other wards only accounted for a minority of transmissions. Phylogenetic distance of isolates from the same outlet was markedly reduced compared to those from different outlets, suggesting the persistence of a single genotype within an outlet. We have chosen to represent phylogenetic distance data as a violin plot as it allows visualisation of the density distribution for the large number of datapoints from the pairwise comparison of isolates [23].

Clinical isolates (ranging from one to seven per patient) were collected from 78 patients across the four hospitals. The transmission category was determined for every clinical isolate, using the epidemiological information alongside phylogenetic distance. The highest transmission category was then recorded per patient (Supplementary Table IIII) and the positive water and patient sampling results for hospital W are presented (Supplementary Figure 3).

**Direction of transmission inference**

A summary of the patient acquisition/transmission events for each classification level, and each hospital, is shown in Supplementary Table II. Where multiple clinical isolates existed for a patient, all were investigated (to determine the particular classification levels of all the clinical isolates), and the highest classification level recorded in Supplementary Table III for that patient.
Using this approach, 5.1% of the patients were classified as level 1 (‘definitely’ acquired their *P. aeruginosa* isolate from hospital water outlets), 18% were level 2 (‘probably’...), 24.3% are level 3 (‘possibly’...), and 51.3% were level 4 (there is ‘no evidence’ that they have acquired their *P. aeruginosa* isolate from the hospital water outlets). A single clinical isolate from one of the patients could not be sequenced (1.3%). Consequently, considering the study as a whole, approximately 50% of patient’s most likely acquired their *P. aeruginosa* isolate from the hospital water outlets.

**Intra-hospital transmission events**

The transmission rates varied across the hospitals involved in the study (Supplementary Table II) and were calculated per hospital by dividing those transmission events classified as level 1-3 by the total number of events and multiplying by 100. Hospital W had the highest transmission rate (63.3%), suggesting that nearly two-thirds of patients acquired their *P. aeruginosa* from the hospital water outlets whilst being nursed in augmented care. There were no patient acquisitions at hospital Y, and only a small number of clinical *P. aeruginosa* isolates were collected during the study period, despite a similar rate of sample collection.

Hospital W had four patients with a clinical *P. aeruginosa* isolate classified as level 1 (‘definite’). For example, patient 28, was nursed close to an outlet which was positive on all three sampling events (30th August, 23rd October and 6th December 2014). Three clinical *P. aeruginosa* isolates were recovered from this patient (from sputum samples collected in early December) during the course of their treatment, and two of these clinical isolates were indistinguishable from the water isolates recovered from this outlet that was positive on all three sampling events. Interestingly, although classified as level 2 (owing to the phylogenetic distance being 0.002 and therefore greater than 0), this particular outlet had resulted in a previous patient (patient 3) also acquiring infection, with a swab and sputum sample positive on 8th September (water outlet first positive on 30th August). It is therefore assumed that both patients most likely acquired their *P. aeruginosa* from the identified hospital water outlet.
Although we only have evidence of ‘definite’ transmission from hospital W, there is evidence of probable (level 2), and possible (level 3) transmission for both hospitals X and Z. A probable transmission event in hospital X involved a patient who was nursed in a room with en-suite facilities including a handwash basin, and shower. *P. aeruginosa* was isolated from a blood culture collected from the patient on 21\textsuperscript{st} October 2014 and is closely genetically related (phylogenetic distance of 0.003) to those collected from the handwash basin, and shower on both 3\textsuperscript{rd} September and 27\textsuperscript{th} October.

Hospital Z showed four probable transmission events, the temporality of which fit with the affected patients acquiring their *P. aeruginosa* from their nearest hospital water outlet. There was no evidence of water outlet to patient transmission at hospital Y.

**Discussion**

The majority of studies focusing on hospital water delivery and disposal have investigated distinct outbreaks of infection caused by *P. aeruginosa* often involving strains with an easily detectable phenotypic marker e.g. antibiotic resistance [9]. Nevertheless, such studies have established clear links between hospital water *P. aeruginosa* strains, and patient strains.

Many previous studies have been hampered by non-molecular typing methods with low discriminatory power, which overestimates transmission rates. Kerr et al. [3] concluded that because *P. aeruginosa* occurs in a wide range of hospital environments that investigations restricted to outbreaks are not sufficient to define directionality of transmission. This difficulty of interpretation was confirmed in a systematic review [24] which concluded that although water systems can act as a source of *P. aeruginosa* infection, the route of transmission was unclear, as was the directionality.
Strain typing studies using WGS are relatively uncommon and concentrate on outbreaks and did not examine water outlets as a source [7, 9, 15] with two others showing transmission from water outlets to a total of 5 patients [14, 16].

In order to understand the extent and dynamics of the colonisation of ICU tap water outlets a prospective longitudinal study is required. Only one such study has been published of 10 ICU wards in 8 separate hospitals in France performed in 2010 [25]. Over a 5-month period 233 taps were cultured for *P. aeruginosa* and patient colonisation was determined, as 17 patients were found to be colonised with a PFGE typed strain that matched a water isolate. The authors commented on “discrepancies” between units (e.g. 41/81 positive taps came from just 2 units) but did not investigate the reasons for the variation recommending that further studies on routes of transmission and control were needed.

Our study was a longitudinal, non-outbreak epidemiological study in four large hospitals housing 22 ICU wards with 881 tap outlets which were monitored. We looked for correlation between clinical disease associated isolates and water outlet isolates rather than asymptomatic colonisation, also using WGS in which we were able to assign directionality of transmission in some cases. We have shown ‘definite’ and ‘probable’ acquisition of *P. aeruginosa* in three of the four participating hospitals, affecting 19 of 78 (24.3%) patients. These definitions have been based on a measure of phylogenetic distance, epidemiological linkages and are further strengthened owing to the low diversity of water clones encountered (especially in hospital W), making patient contamination of the outlet unlikely. This is a highly conservative estimate, demonstrating that this is a significant potential problem in any ACU.

There is evidence of hospital-specific water associated clones of *P. aeruginosa*. MLST analysis of the WGS data shows that ST179 was found in hospital W only, and ST395 was predominantly associated with hospital W (although was also found in hospitals X, Y and Z) (Figure 1). We hypothesise that the low diversity of *P. aeruginosa* sequences found in hospital W was related to the age of the plumbing infrastructure (newest of the four). The majority (86%) of positive taps in hospital W were of one
model (compared to 38.5%, 64.2%, and 45% in hospitals X, Y and Z, respectively). A possible explanation for the presence of the dominant clone was that they were contaminated during manufacturer’s wet integrity testing of the plumbing components prior to distribution [26]. ST395 has been widely reported in water systems and has been noted to carry a copper resistance gene which might aid survival in copper containing water distribution systems [27]. Interestingly the three large clusters from hospital W (ST395, ST17 and ST179) were also found in the other three hospital groups, suggesting that these represent common aquatic clones, despite considerable geographic separation (200 miles between hospital X and Z).

This is the first study to use patristic distances to investigate genetic relatedness of water and patient P. aeruginosa in the non-outbreak setting. The question of defining strain relatedness by differences in SNP carriage in WGS data is a vexed one. Attempts have been made to both define isolates as indistinguishable using a range of allowed SNP differences as well as proposing directions of transmission [28]. However, genome rearrangements can lead to overestimation of strain differences if SNP counts are considered in isolation. In the case of Mycobacterium tuberculosis (which has no horizontal gene transfer and a relatively slowly evolving genome) such numerical parameters would appear to be ideal and were proposed after sequencing sequential isolates from individuals and family clusters [28]. Their proposed 0-5 SNPs for indistinguishable isolates and >12 SNPs for distinct isolates was applied to a large 14 year outbreak of isoniazid resistant strains in North London [29]. They found that the majority of the 344 isolates differed by only 2 SNPs, but sequencing of 27 individual colonies cultured from a single patient specimen revealed up to 10 SNP differences between colonies. When applying the detailed epidemiological information to the data set it was clear that multiple transmission events had occurred without detectable SNP acquisition. This meant that even identical isolate pairs could not be deduced to have resulted from a direct transmission event without a supporting epidemiological link [29]. This experience has informed our criteria for assigning isolate
relatedness by including epidemiological information and using patristic distance rather than absolute SNP number differences.

There are some limitations of the study. Since the study was a short defined “snapshot” investigation of events over a defined time period, there was no follow up of the patient’s journey during their hospital admission. Consequently, the conservative definitions used to define the transmission events may have underestimated the true transmission rate. Patients were not screened for endogenous (asymptomatic) carriage on admission (owing to ethical considerations) which could be the route for colonisation and/or infection. Additionally, water sampling was performed on three occasions; therefore, it is possible that positives may have been missed in the gaps between samplings. Other wet sources (e.g. sink traps) or patient-to-patient transmissions were not investigated. As hospital Y had a CSI treatment system and copper ions are known to induce a persistor state inducing a viable but non-culturable state thus rendering previously culturable bacteria unculturable on the same media [30, 31]. Pre-treatment with a copper chelator may have enabled more accurate viable counts of *P. aeruginosa* in Hospital Y.

The effect of CSI on bacteria other than Legionella in water distribution systems has been reported [32]. The CSI hot water samples showed no increase in species richness (defined by 16S rDNA sequencing) compared to the cold-water samples, whereas the non-CSI showed high diversity. The low rate of recovery of *P. aeruginosa* at hospital Y may be attributable to the CSI treatment, although other factors such as water temperature, plastic pipes and maintenance activity could also have contributed to this significant finding.

This was the largest prospective study that has demonstrated the importance of *P. aeruginosa* in hospital water, and evidence of onward transmission to patients. In hospital W (where outlets were identified as a likely source[33]), positive water samples have been drastically reduced across critical care in hospital W [34]. A series of holistic interventions (fitting new taps, filters on existing taps, improved tap cleaning, and appropriate disposal of patient waste water), resulted in a 50% reduction
in the number of *P. aeruginosa* clinical patient isolates over a year [34], and a 72% decrease in of *P. aeruginosa* acquisition when positive taps were removed and new ones fitted [35].

This study has shown that colonisation of ICU outlets acts as a persistent source of infection with *P. aeruginosa* and this varies in ACU depending on the level of contamination. There is substantial variation between different facilities and an urgent need to introduce control measures to reduce *P. aeruginosa* colonisation in ICU tap water.

**Conclusion**

In this longitudinal, non-outbreak study of four hospitals, very different levels and patterns of colonisation of water outlets was observed across all three sampling timeframes. Application of WGS to water and clinical isolates demonstrated convincing transmission (level 1) from water to patients for 5% of patients, and probable transmission (level 2) in a further 19% of the studied patients. Strains from water outlets caused a range of clinical infections, including a bacteraemia.

Hospital W had a strikingly high incidence of colonised outlets with a corresponding high incidence of patient isolates, supporting the transmission of *P. aeruginosa* from water outlets causing colonisation/infection in patients. This hospital also had a dominant clone (ST179) which exhibited low intra-isolate diversity, suggesting a common source. Hospital Y had the lowest incidence of colonised outlets which may have been due to the CSI treatment.

There was variability in the frequency of *P. aeruginosa* detected at different hospitals which correlated with the frequency of predicted transmissions. This suggests that in some settings water outlets are the most likely source of *P. aeruginosa* nosocomial infections for the patients in the study with widespread implications for control measures nationally and internationally.
Conflict of Interest

None

Funding and acknowledgments

This study was funded by the Department of Health & Social Care (DHSC) Policy Research Programme (grant number PR-ST_1213-00007), and the NIHR Surgical Reconstruction and Microbiology Research Centre. The NIHR SRMRC is a partnership between The National Institute for Health Research, University Hospitals Birmingham NHS Foundation Trust, the University of Birmingham, and the Royal Centre for Defence Medicine. The views expressed are those of the author(s) and not necessarily those of the NHS, the NIHR or the Department of Health & Social Care. This manuscript is dedicated to Sophia, Rex, Seth and Florence, all of whom have brought great joy into our lives.
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Table

Table I: The dates of construction, number of beds, and percentage of positive outlets per hospital

<table>
<thead>
<tr>
<th>Hospital group</th>
<th>Details of the augmented care ward, including dates of construction</th>
<th>Number of beds</th>
<th>Number of outlets sampled during the study</th>
<th>Number of positive outlets (as a % of all outlets sampled) per sampling period</th>
</tr>
</thead>
<tbody>
<tr>
<td>W</td>
<td>4 wards. 1 hospital site. All 2010</td>
<td>100</td>
<td>231</td>
<td>57 (25%) 63 (28%)</td>
</tr>
<tr>
<td>X</td>
<td>10 wards. 1 hospital site. Built 1974 (but revised 2002-2013)</td>
<td>165</td>
<td>400</td>
<td>47 (12%) 50 (12.5%)</td>
</tr>
<tr>
<td>Y</td>
<td>5 wards across 2 hospital sites. All 1975</td>
<td>72</td>
<td>105</td>
<td>9 (9%) 5 (4.8%)</td>
</tr>
<tr>
<td>Z</td>
<td>3 wards across 2 hospital sites. Two built 1978 (and revised 2009), the other built 2005</td>
<td>64</td>
<td>145</td>
<td>37 (25%) 19 (13%)</td>
</tr>
</tbody>
</table>
Figures

Please see separate image files

Legends

**Figure 1.** Phylogenetic tree for all isolates generated using the rapid genotyping method. The five predominant clades are coloured according to their MLST clonal complex to aid interpretation (a clonal complex is defined as alleles exactly matching a sequence type or a single-locus variant of that sequence type). The annotation bars below indicate the hospital where that isolate was found and the type either water (blue) or patient (orange).

**Supplementary Table II: The transmission events per Trust including the transmission events classified per patient, transmission rate, and clinical specimen types involved**

**Supplementary Table III: Table showing the multi-locus sequence typing data reported by StringMLST for all isolates for seven highly conserved housekeeping genes.**


**Supplementary Table IIII: Showing the categorisation of the transmission events per clinical isolate.**

The table shows the clinical isolates, the ward and outlet that relate to each clinical isolate and the closest waters isolates (in terms of phylogenetic distances). This was then used to assign a category to that clinical/water isolate pair according to the definitions used in the paper:
Level 1 (definite): ‘clinical *P. aeruginosa* genome indistinguishable from water *P. aeruginosa* genome (phylogenetic distance of 0) with epidemiological link to at least ward level’, Level 2 (probable): ‘Patient genome most closely similar to an outlet genome (but with a phylogenetic distance of >0), and with an epidemiological link to outlet’, Level 3 (possible) ‘Patient genome present in the same phylogenetic cluster as a *P. aeruginosa* water genome but without an epidemiological link to outlet’, and Level 4 (no evidence): not clearly a hospital water transmission (no hospital waters present in the same cluster and no epidemiological link). ‘none’ refers to clinical isolates for which there were no related water isolates present in our sampling set.

**Supplementary Figure 2.** Violin plots showing phylogenetic distance between all pairs of isolates from the same hospital (left), the same ward (middle) and same outlet (right) for the ST395 cluster. Phylogenetic distance was calculated by the R package ‘ape’ [36] which are derived from the RAxML branch length themselves based on mutations per site.

**Supplementary Figure 3.** Map of four wards in hospital W, demonstrating association between water outlets and *P. aeruginosa* genetic clusters. The three water sampling periods (beginning, middle and end) are shown separately from top to bottom. *P. aeruginosa* isolates from water isolates (diamonds) and patients (circles) are coloured by their cluster type.