

# BMJ Open Seeking the source of *Pseudomonas aeruginosa* infections in a recently opened hospital: an observational study using whole-genome sequencing

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## ABSTRACT

**Objectives:** *Pseudomonas aeruginosa* is a common nosocomial pathogen responsible for significant morbidity and mortality internationally. Patients may become colonised or infected with *P. aeruginosa* after exposure to contaminated sources within the hospital environment. The aim of this study was to determine whether whole-genome sequencing (WGS) can be used to determine the source in a cohort of burns patients at high risk of *P. aeruginosa* acquisition.

**Study design:** An observational prospective cohort study.

**Setting:** Burns care ward and critical care ward in the UK.

**Participants:** Patients with >7% total burns by surface area were recruited into the study.

**Methods:** All patients were screened for *P. aeruginosa* on admission and samples taken from their immediate environment, including water. Screening patients who subsequently developed a positive *P. aeruginosa* microbiology result were subject to enhanced environmental surveillance. All isolates of *P. aeruginosa* were genome sequenced. Sequence analysis looked at similarity and relatedness between isolates.

**Results:** WGS for 141 *P. aeruginosa* isolates were obtained from patients, hospital water and the ward environment. Phylogenetic analysis revealed eight distinct clades, with a single clade representing the majority of environmental isolates in the burns unit. Isolates from three patients had identical genotypes compared with water isolates from the same room. There was clear clustering of water isolates by room and outlet, allowing the source of acquisitions to be unambiguously identified. Whole-genome shotgun sequencing of biofilm DNA extracted from a thermostatic mixer valve revealed this was the source of a *P. aeruginosa* subpopulation previously detected in water. In the remaining two cases there was no clear link to the hospital environment.

**Conclusions:** This study reveals that WGS can be used for source tracking of *P. aeruginosa* in a hospital setting, and that acquisitions can be traced to a specific source within a hospital ward.

## Strengths and limitations of this study

- We have demonstrated that whole-genome sequencing can be used for source tracking of *Pseudomonas aeruginosa* in a hospital setting.
- We show convincing evidence that transmission has occurred directly from water to patients, but other routes are as likely.
- The main limitation of the study was the sample size, which could be attributable to interventions being carried out during the study.
- Our study focused on a burns unit and critical care unit in a newly built hospital. Modes of *P. aeruginosa* transmission may be different in hospitals with different styles of plumbing and on other augmented care units.

## INTRODUCTION

*Pseudomonas aeruginosa* is a ubiquitous Gram-negative bacterium and an important opportunistic pathogen in the healthcare setting. *P. aeruginosa* particularly affects those with impaired host or mucosal immunity and has a broad range of presentations including respiratory infections in cystic fibrosis and mechanically ventilated patients, bloodstream infections in premature neonates and wounds in burns injuries. Nosocomial *P. aeruginosa* outbreaks are frequently reported and associated with water sources such as taps, showers, mixer valves and flow straighteners, sink traps and drains.<sup>1–10</sup> Other potential routes of transmission include cross-infection, for example, carriage on the hands of healthcare workers, and through contaminated medical equipment such as endoscopic devices.<sup>3,5</sup>

In the UK, the role of water in the transmission of *P. aeruginosa* in healthcare settings has become a matter of urgent concern in response to a recent high-profile outbreak

affecting a neonatal critical care unit in Belfast in 2012.<sup>11</sup> This source was eventually determined to be sink taps.<sup>11–13</sup> National guidance is now in place detailing enhanced procedures for routine water sampling on augmented care units, with directed interventions such as disinfection and replacement of high-risk plumbing parts required.<sup>14</sup>

Historical phenotypic typing methods for *P. aeruginosa* such as O-antigen serotyping have more recently been replaced by molecular typing methods such as pulsed-field gel electrophoresis (PFGE), variable number tandem repeat analysis, random amplification of polymorphic DNA and multilocus sequencing typing (MLST).<sup>15</sup> These methods have been used to investigate outbreaks of *P. aeruginosa* within hospitals.<sup>4 16–18</sup> However, such techniques have important limitations for source tracking of infections in hospitals as they sample limited numbers of sites in the genome which may result in false clustering of unrelated strains.<sup>19</sup> In the past 5 years, whole-genome sequencing (WGS) has started to be used to investigate outbreaks in hospitals. WGS is attractive because of its digital, sharable format and ultra-high resolution, which is able to discriminate two isolates differing by just a single mutation. WGS has been successfully used to determine likely transmission chains during outbreaks of *Staphylococcus aureus*, *Acinetobacter baumannii* and *Klebsiella pneumoniae*.<sup>19–21</sup> Benchtop sequencing instruments now offer a cost-effective approach for bringing bacterial WGS to the clinical environment.<sup>22</sup>

In this study, we explore the utility of WGS to determine the likely sources of *P. aeruginosa* in an at-risk population of burns patients. In the UK and US burns patients receive shower cart hydrotherapy as a mainstay of burns treatment.<sup>23–26</sup> A previous hospital audit suggested that up to one-third of such patients became colonised with *P. aeruginosa*. We hypothesised that this high rate of acquisition may relate to transmission from hospital shower water during therapy. We therefore wished to understand the importance of transmission from water compared with alternative routes such as cross-infection and endogenous carriage.

## MATERIALS AND METHODS

### Hospital setting

An observational, prospective study design was employed in a burns centre serving approximately 13.7 million people across the Midlands region of England with 300 admissions annually. Opened in June 2010, the burns centre comprises a purpose built 15-bed ward with 11 side-rooms and 2 dual-bedded rooms. Patients requiring mechanical ventilation and organ support are usually treated in two self-contained burns cubicles located within the trauma critical care unit. Despite the observational nature of the study, sampling was carried out during implementation of interim national guidance on control of *P. aeruginosa* issued by the Department of

Health. These guidelines were issued in draft form March 2012, and subsequently revised in March 2013. This meant that parallel water sampling and engineering interventions were being undertaken during the period of study. In addition, some enhanced infection prevention measures were also introduced in response to an outbreak of a multidrug-resistant *A. baumannii*.

### Study design and patient selection

Patients admitted to the burns unit were eligible for the screening phase of the study if they had burns injuries covering greater than 7% total body surface area (TBSA). Patients were screened as soon as possible after admission after they had given written informed consent. When appropriate, legal consultee advice was sought for patients lacking capacity due to emergency treatment. On admission, recruited patients were screened for carriage of *P. aeruginosa* (wounds, urine and stool) using standard microbiology techniques. Samples were then taken as part of routine microbiology service during the patients stay. Environmental and water samples were taken after the patient was admitted to the burns centre. If during the period of stay *P. aeruginosa* was isolated from a patient sample the patient was recruited into the second phase of the study. In this phase, patients had wound swabs taken at each dressing change as well as twice-weekly urine samples. The patient's environment and water from outlets in their bed space were sampled weekly for the duration of their stay, and after discharge (post-cleaning). Termination of the study was planned after 30 screening patient admissions, or a year, whichever came soonest, after which 10 patients were expected to acquire *P. aeruginosa*. This prediction was based on a previous local audit which suggested about one-thirds of burns patients became colonised with *P. aeruginosa*.

### Microbiological and molecular methods

*P. aeruginosa* isolates were obtained from wound swab, urine, stool, environmental and water samples. *P. aeruginosa* was isolated from wound swabs, urine and stool by inoculation onto cysteine lactose electrolyte deficient agar (CLED) and cetrimide agar and incubation for 24 h at 37°C. Stool samples were cultured overnight in a cetrimide enrichment broth before subculture onto CLED. Identification was confirmed by resistance to C-390 and the VITEK 2 GN identification card. Antibiotic sensitivity assays were performed using the VITEK 2 AST N-210 card (bioMérieux, Basingstoke, UK).

The patient's environment (shower head rosette, drain, shower chair or trolley, bedside table, patient chair, instruments in contact with the patient) was sampled over a 10 cm<sup>2</sup> area by a Polywipe sponge. The sponge was placed in tryptic soy broth incubated for 24 h at 37°C then subcultured onto CLED and cetrimide agar. During water sampling, water was taken from the patient's shower, or tap if a shower was not present. Shower heads were not removed for water sampling. At

least 200 mL of water was collected into a vessel containing sodium thiosulfate as a neutraliser. In duplicate, 100 mL of water was filtered through a 0.45 µ filter and the filters placed onto CLED plates and cetrimide agar. Plates were incubated at 37°C for 48 h and the number of organisms per 100 mL quantified.

For storage and DNA extraction a single colony was purified from the primary culture plate. When different colony morphologies were observed, a single colony from each type was purified. Additionally, for a randomly selected water sample, 24 colonies were individually picked from one water-filter primary microbiological plate for sequencing. Isolates were stored on Biobank beads at -20°C prior to DNA extraction. Organisms were resuscitated on CLED agar plates and genome DNA either extracted directly using the MOBIO UltraClean Microbial DNA Kit, or from overnight LB broth culture using a Qiagen Genomic-Tip 100G.

### DNA extraction and sequencing

Genomic DNA was prepared from single colony picks using the MOBIO Ultraclean microbial kit (MOBIO, Carlsbad, USA). 1 ng input DNA, as quantified by Qubit (Life Technologies, Carlsbad, USA) was used to prepare genomic libraries for sequencing using the Illumina Nextera XT DNA sample kit as per manufacturer's protocol (Illumina, San Diego, USA). Libraries were sequenced on the Illumina MiSeq using a paired-end protocol resulting in read lengths between 150 and 300 bases. A single additional sample, isolate 910, was chosen as a representative member of Clade 5 for long-read sequencing. DNA from this sample was fragmented using a Hydroshear (Digilab, Marlborough, Massachusetts, USA) using the recommended protocol for 10 kb fragments and further size-selected on a BluePippin instrument (Sage Science, Massachusetts, USA) with a 7 kb minimum size cut-off. The library was sequenced on two SMRT Cells using the Pacific Biosciences RS II instrument at the Norwegian Sequencing Centre, Oslo. C4-P2 chemistry was chosen because it favours long, more accurate reads for *de novo* assembly.

### Stool PCR

For simple presence/absence detection of *P. aeruginosa* in stool samples using PCR, a stool sample was collected into a stool collection tube containing stool DNA stabiliser. Total DNA was extracted using the PSP Spin Stool DNA Plus kit (Stratec Molecular). PCR amplification of species specific regions of the 16S rDNA gene was carried out using primers PA-SS-F: GGGGGATCTTCG GACCTCA and PA-SS-R: TCCTTAGAGTGCCCCACCCG<sup>12</sup> in the following conditions: 0.5 µM of each primer, 1.5 mM MgCl<sub>2</sub>, 0.2 mM dNTP's using BIOTAQ DNA Polymerase and buffer set. After initial denaturation at 96°C for 2 min, 30 cycles of 96°C for 30 s, 62°C for 30 s and 72°C for 30 s were completed with a final extension of 72°C for 5 min. Products were visualised for size on an 1.5% agarose gel.

### Bioinformatics methods

Illumina MiSeq reads from each isolate were adapter and quality trimmed before use with Trimmomatic. Phylogenetic reconstruction of isolates sequenced in this study were combined with data from a global collection of 55 *P. aeruginosa* strains collected world-wide which have been previously analysed by Stewart *et al.*<sup>28</sup> For each of the published strains, 600 000 paired-end reads of length 250 bases were simulated using wgsim (<https://github.com/lh3/wgsim>) from the complete or draft genome assembly deposited in Genbank. Read sets were mapped against the *P. aeruginosa* PAO1 reference genome using BWA-MEM 0.7.5a-r405 using default settings.<sup>29</sup> Single nucleotide polymorphisms were called using VarScan 2.3.6 and filtered for regions with an excessive number of variants. These may represent regions of recombination, misalignments or strong Darwinian selection.<sup>30</sup> FastTree (V2.1.7) was used for phylogenetic reconstruction. This software estimates an approximate maximum-likelihood tree under the Jukes-Cantor model of nucleotide evolution with a single rate for each site (CAT).<sup>31</sup> Trees were drawn in FigTree (<http://tree.bio.ed.ac.uk/software/figtree/>).

For *in silico* MLST prediction, trimmed reads were assembled *de novo* using Velvet<sup>32</sup> with a k-mer size of 81 and searched using nucleotide BLAST against the multi-locus sequence database downloaded from the pubMLST website on 5 August 2013 (<http://pubmlst.org/paeruginosa/>).<sup>33</sup> For Clade E isolates, in order to exhaustively search for discriminatory mutations, a nearly complete reference genome was generated by *de novo* assembly using Pacific Biosciences sequencing data. Reads were assembled using the 'RS\_HGAP\_Assembly.3' pipeline within SMRT Portal V2.2.0. Illumina reads from the same sample were mapped to this draft genome assembly in order to correct remaining indel errors in the assembly using Pilon (<http://www.broadinstitute.org/software/pilon/>). Isolates belonging to each clade were mapped individually against either the PacBio reference (Clade E) or *P. aeruginosa* PAO1 (NC\_002516; Clades C, D and G).

Variants (single nucleotide polymorphisms and short insertion-deletions) were called using SAMtools mpileup and VarScan with an allele frequency threshold of 80%.<sup>30</sup> Non-informative positions and regions of putative recombination were removed, the later with a variant density filter of more than 3 SNPs every 1000 nucleotides. Analysing samples in each clade individually maximised the number of variants detected by reducing the likelihood of the position being uncovered by a subset of samples. From these variants fine-grained phylogenetic trees were reconstructed for each clade using FastTree. The scripts used to perform this analysis are available at [http://www.github.com/joshquick/snp\\_calling\\_scripts](http://www.github.com/joshquick/snp_calling_scripts). Approximate-maximum-likelihood phylogenetic trees were generated using FastTree and visualised in FigTree. For whole-genome shotgun metagenomics analysis, reads were analysed using the Kraken taxonomic classifier

software with the supplied *minikraken* database.<sup>34</sup> Reads from the metagenomics data set were aligned to *P. aeruginosa* Clade E as in the previous section and phylogenetic placement was carried out using pplacer in conjunction with FastTree.<sup>35</sup> Sequence data is available from the European Nucleotide Archive for the Illumina data (ERP006056) and the corrected Pacific Biosciences assembly (ERP006058).

## RESULTS

### Study results

Recruitment lasted a period of 300 days, ending according to protocol after the enrolment of 30 screening patients. In total, we detected *P. aeruginosa* in five patients. Of these patients, three had *P. aeruginosa* detected only in burns wound swabs, one had *P. aeruginosa* detected in their burns wound and in their urine, and one had *P. aeruginosa* in their sputum. One additional eligible patient did not consent to enter the study and was excluded. The average age in the study group was 41 years. Males predominated with a male-to-female ration of 2.3:1. Flame burns were the most common mechanism of injury, followed by scalds and mixed flame/flash injuries. The average burn size of the study group was 12.5% of the TBSA and 27% of patients sustained an inhalation injury. Eight patients required admission to intensive trauma unit (ITU) and the majority required surgical treatment of their burns with excision and skin grafting (80%). A large majority of the study group (83%) received shower cart hydrotherapy as a routine part of their wound management to encourage healing through wound debridement and decontamination. The average length of hospital stay (LOS) was 17 days and taking into account burn size, the average was 1.4 days per % TBSA.

### The water and environment in burns and critical care units are frequently colonised by *P. aeruginosa*

A total of 282 water and environmental samples were screened for *P. aeruginosa* of which 39/78 (50%) were positive in water samples, 25/96 (26%) were positive from the wet environment and 7/108 (6%) were positive from the dry environment. A total of 86 genome sequences were generated from the 71 positives, as in some cases multiple colony picks were sequenced. Seventy-eight patient samples were screened for *P. aeruginosa* of which 39 (50%) were positive. A total of 55 genome sequences were generated, as in some cases multiple colony picks were sequenced. In total, 141 genomes were sequenced; water and environmental (n=86) and patient (n=55). Genomes were sequenced to a mean coverage of 24.4x, with the minimum coverage of a sample being 14x and highest 64.7x.

When placed in the context of a global collection of *P. aeruginosa* strains, phylogenetic reconstruction demonstrated isolates in our study fell into eight clades (figure 1A). As has been reported previously, there was no

strong association between ecological context and position in the phylogenetic tree.<sup>28</sup> Isolates in this study are most closely related to strains from a variety of settings. The majority of isolates (52%) belong to Clade E (figure 1B), whose nearest sequenced relative is the Liverpool Epidemic Strain, a clone often isolated from patients in the UK and Canada with cystic fibrosis.<sup>36 37</sup> Isolates from Clade E were found in the burns unit's water and the ward environment, as well as from two patient's wounds. However it was never detected in the critical care unit. Clade E was detected throughout the study in a total of 10 different rooms (figure 2).

### Inferred potential transmission events by WGS

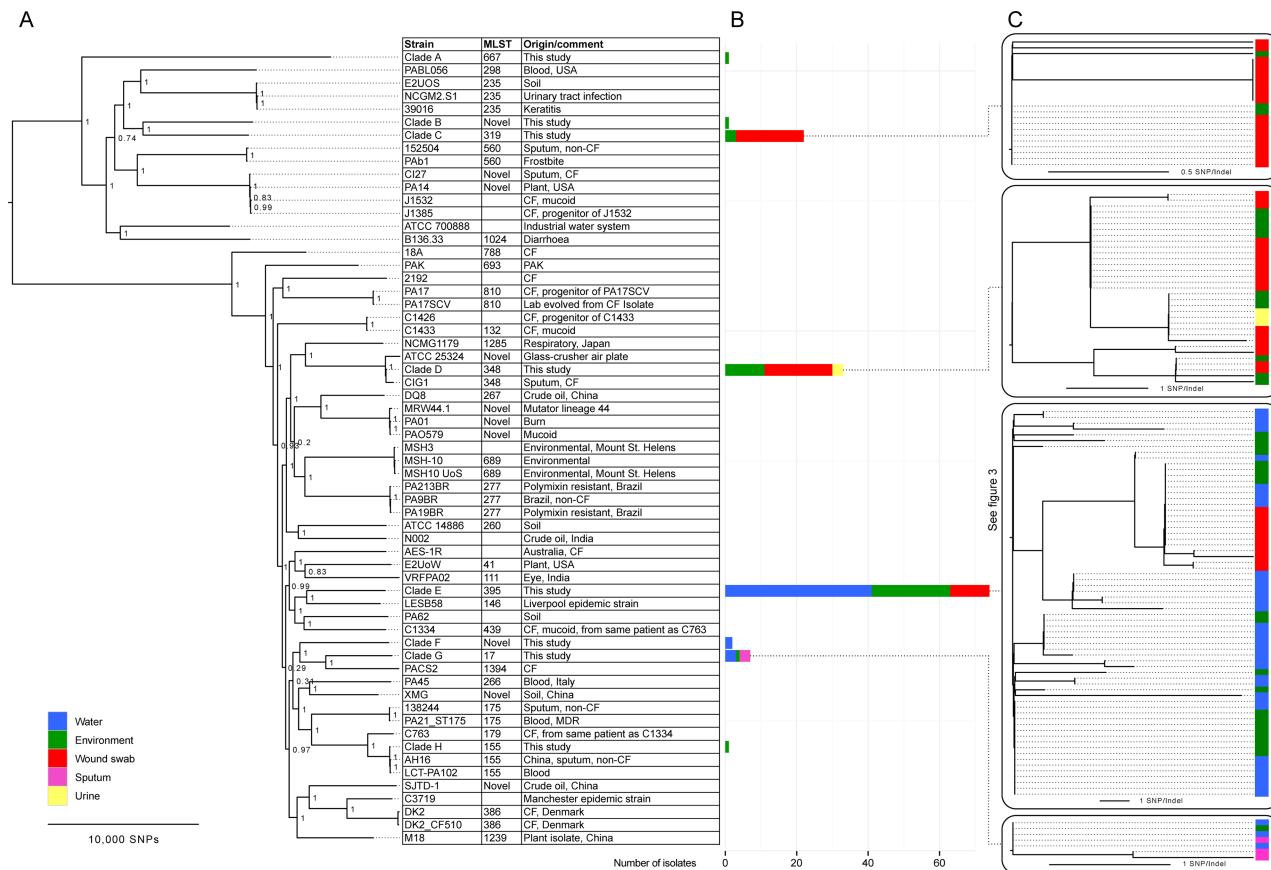
Microevolutionary changes occurring over rapid time-scales (ie, days to months) have been used to detect potential chains of transmission in hospital and community outbreaks.<sup>19–21 38 39</sup> The number of distinct mutations between given isolates has been used to infer whether transmission events are likely to have occurred. Such inferences are aided by prior knowledge of mutation rates in similar populations. Two patients (1 and 4) in our study both had *P. aeruginosa* from Clade E isolated from their wounds. These isolates had an indistinguishable genotype from those present in water and the environment of the room they were nursed within (figures 1C and 3). This genotype was detected in the patient's shower water after initial patient screening, during screening of the second patient admission, twice during the second patient's stay and then 127 days later (days 27, 65, 89 and 216, respectively). When water isolates were positive, the genotype was also detected in wet environment sites (shower drain, shower rosette and patient's trolley) on the same days.

Patient 5 was nursed on the critical care unit due to concomitant medical problems. *P. aeruginosa* belonging to Clade G was isolated from sputum during this time. Identical genotypes were detected contemporaneously in the water from the associated sink and sink tap handle (see online supplementary appendix 4).

Two further patients (patients 2 and 3) were positive for *P. aeruginosa*. Isolates from these patients belonged to Clade C and D, respectively. Neither clade was ever isolated from hospital water. In both cases, identical genotypes were detectable in the environment associated with the patient but these were not detected before or after the patients' stay, indicating that the environment was not persistently contaminated. During the course of patient 3's stay, the dry environment such as the bedside table was contaminated, as was the patient's door handle and shower chair. However, after patient discharge, the strain associated with this patient was never seen again during the course of the study in any location.

### WGS permits source tracking of *P. aeruginosa* to individual water outlets

WGS has been reported previously for source tracking, but never for the detection of transmission events from



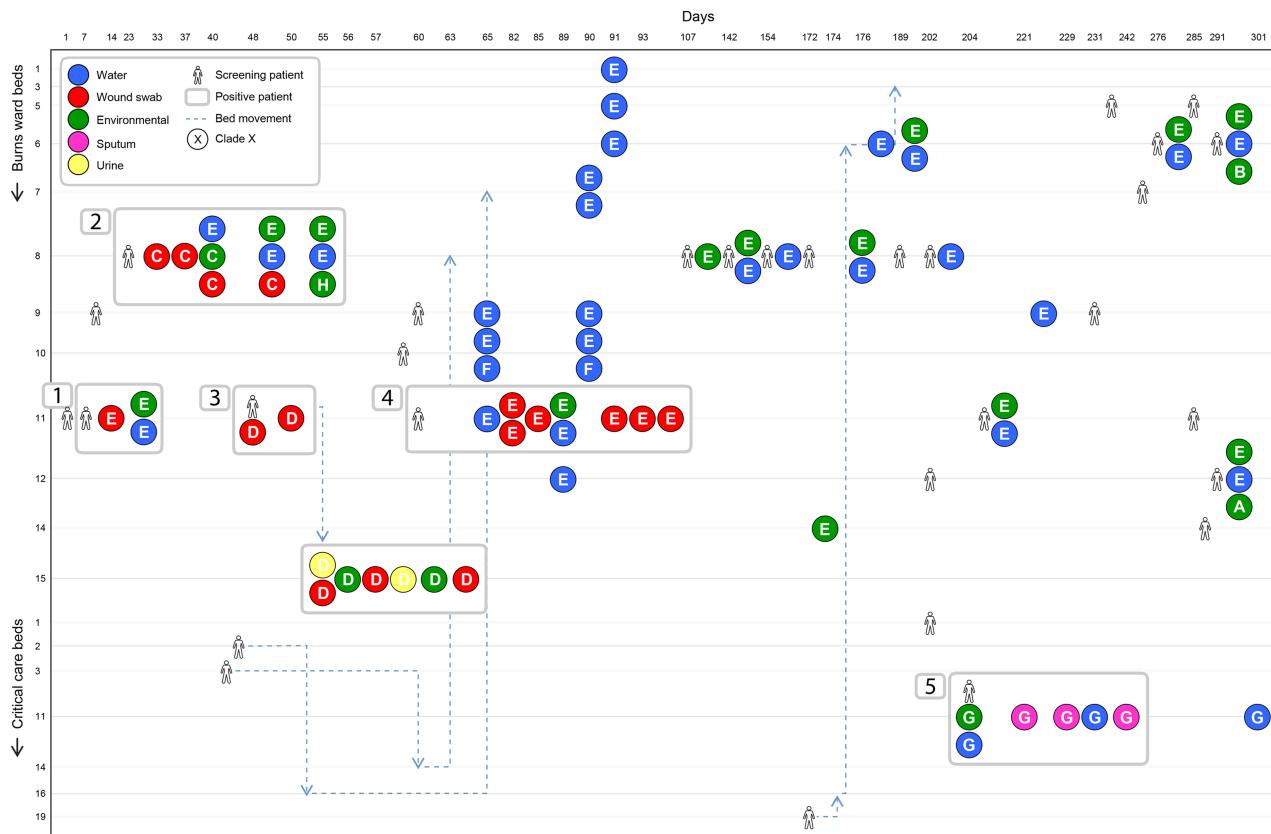
**Figure 1** An overview of all samples collected during the study in global phylogenetic context with other sequenced strains of *Pseudomonas aeruginosa* from the set of Stewart *et al.*<sup>28</sup> Samples collected in this study are widely dispersed in the tree, which contains isolates from different environments (A). Bar plots indicate the numbers of each type of sample collected (B). Microdiversity within each clade is shown, with the colour bar indicating the source of each sample (C).

hospital water.<sup>40</sup> Phylogenetic reconstruction within Clade E, the most commonly detected water clone demonstrated additional diversity within this clone, with a total of 46 mutations detected an average genetic distance between isolates of 4.1 mutations (figure 3). The reconstruction demonstrated clear evidence of clustering of genotypes both by room and outlet (figure 3). When *P. aeruginosa* was detected in the wet environment (eg, shower rosettes and drains) these genotypes were most often identical to those found in water, indicating that the water was likely the ultimate source of that clone. Genotypic variation was seen between outlets within the same room. For example, tap water sampled from room 11 had a distinct genotype from that sampled from shower water in the same room and this was consistently found over multiple samplings. Notably, isolates from two patients fell within the cluster originating from shower water, indicating that shower hydrotherapy was the most likely source of infection. Two plasmids (designated pBURNS1 and pBURNS2) were detected in this study set, which both demonstrated geographical clustering, with pBURNS1 only being detectable in isolates from room 8 and pBURNS2 only being detectable in isolates from the shower water in room 9.

### Rapid evolution of antibiotic resistance associated with treatment

*P. aeruginosa* is commonly associated with antibiotic resistance due to a number of predisposing features including intrinsic resistance, a repertoire of efflux pumps and antibiotic-inactivating enzymes including  $\beta$ -lactamases.<sup>41</sup> Three infected patients (2, 3 and 5) received antibiotic therapy, and in each case this was associated with the development of resistance to at least one therapeutic agent. Associated mutations were detected that were either partially or fully explanatory of the phenotype (online supplementary appendix 12).

Patient 2 was treated with ciprofloxacin, nitrofurantoin and vancomycin (see online supplementary appendix 11 for full details). Eight of 21 (38%) tested isolates from this patient were ciprofloxacin resistant. Seven of eight isolates (88%) of the ciprofloxacin-resistant strains were distinguishable from the other isolates by a single SNP in *mexS* (annotated as PA2491 in *P. aeruginosa* PAO1; see online supplementary appendix 1 and 7). This SNP was predicted to result in a non-synonymous amino acid substitution. Disruption of this gene has been shown to cause increased expression of the *mexEF-oprN* multidrug efflux pump, associated with resistance to quinolones.<sup>42</sup>



**Figure 2** A schematic view of the 300-day study of *Pseudomonas aeruginosa* in a burns centre and critical care unit. Time in days is shown along the x axis with bed numbers in the critical care unit and burns unit along the y axis. Each circular icon indicates a positive isolate of *P. aeruginosa*. The icon's logotype indicates which environment it originated from (wound, urine, sputum, environmental or water). The filled colour of the icon indicates the clade it belongs to. Patient icons represent the enrolment of a screening patient into the study and their location. Patient movements around the hospital are noted by dotted lines. The five patients infected with *P. aeruginosa* are denoted by rounded boxes. Boxes are coloured according to the patient number. In the event two or more isolates of the same source and clade were collected on the same day, these have been collapsed into a single circular icon.

Patient 3 was not treated with antibiotics, but isolates associated with this patient demonstrated differences in resistance to timentin and piperacillin-tazobactam. These changes were associated with non-synonymous mutations in *gacA*, the response regulator of the GacA/GacS two-component system and in *lasR*, a transcriptional activator required for transcription of elastase and LasA protease (online supplementary appendices 2 and 8).

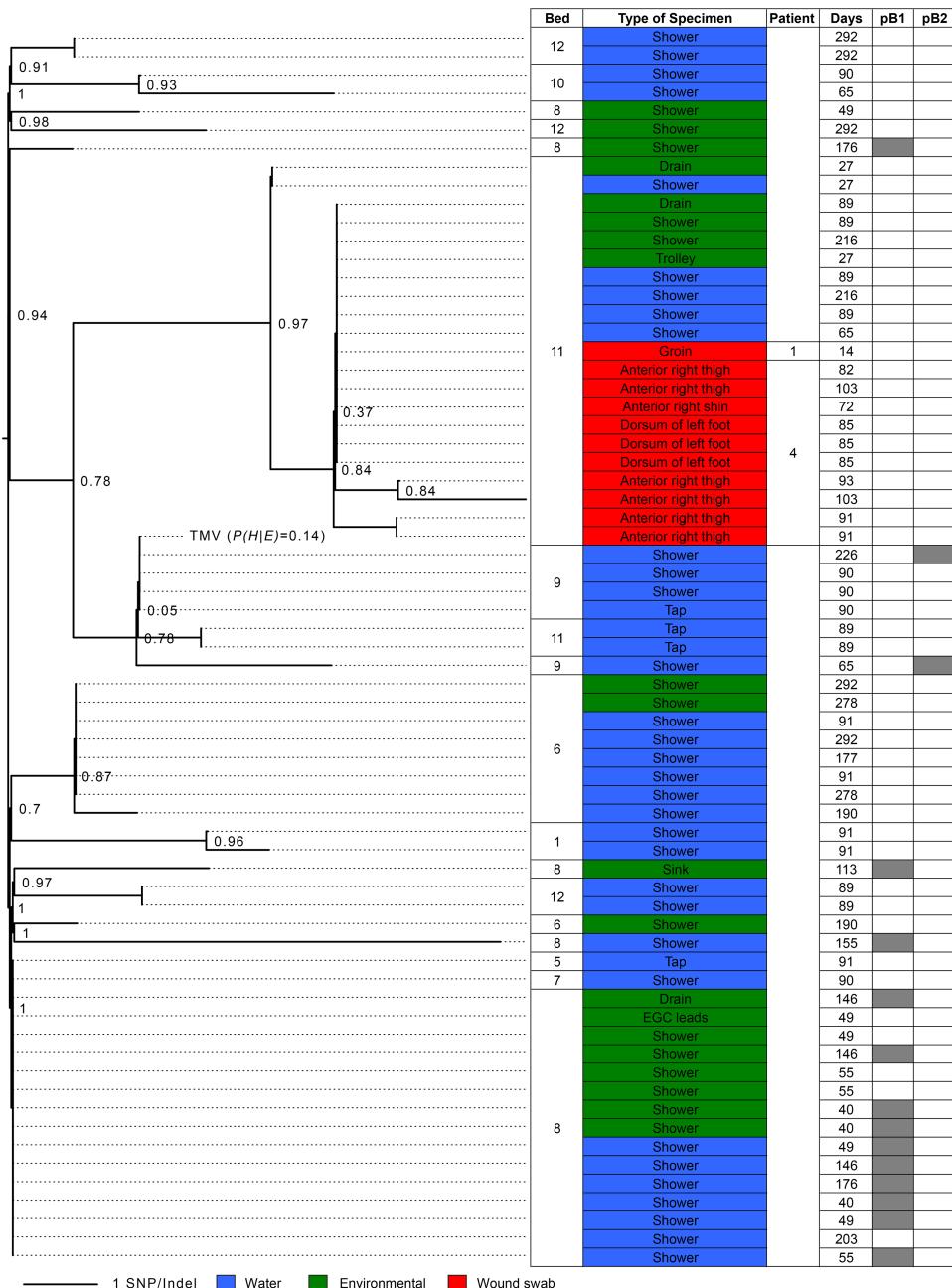
Patient 4 was treated with meropenem, piperacillin/tazobactam, flucloxacillin and colistin. Five isolates collected 10–18 days after initiation of meropenem showed resistance to imipenem and intermediate resistance to meropenem (see online supplementary appendix 3 and 9). The most likely mutation responsible for this phenotype was detectable in two isolates, both of which had a frame-shift mutation in the gene coding for the membrane porin OprD.<sup>43</sup>

Patient 5 had a prolonged stay in ITU and had multiple medical problems including *A. baumannii* infection and was treated with nine antibiotic agents including ciprofloxacin, meropenem and piperacillin-tazobactam. Serial isolates from this patient demonstrated the

stepwise acquisition of two mutations (online supplementary appendix 4). The first was in *nalC*, a probable repressor of the TetR/AcrR family (online supplementary appendix 10).<sup>44</sup> On inspection of the sequence alignment in this region, a large deletion of 196 nucleotide bases was seen compared to the reference PAO1 strain. This mutation was seen in association with full resistance to piperacillin-tazobactam, ceftazidime, aztreonam, meropenem and intermediate resistance to ciprofloxacin. This deletion is likely to result in over-expression of efflux pumps involving the *mexAB-oprM* operon.<sup>44 45</sup> Ciprofloxacin resistance in a later isolate corresponded to the stepwise acquisition of a second mutation. This mutation is predicted to affect the well-studied DNA gyrase subunit A gene (*gyrA*) which is strongly associated with ciprofloxacin resistance.<sup>46</sup>

#### Confirmation of *P. aeruginosa* genotypes in biofilms by whole-genome metagenomic shotgun sequencing

*P. aeruginosa* is able to produce and survive in biofilms. Plumbing parts such as flow straighteners, shower rosettes,



**Figure 3** The high-resolution phylogenetic reconstruction of Clade E isolates. This demonstrates the clustering of genotypes by bed space. Patient associated samples are contained within a room 11 clade. This clade contains water samples from the shower and environmental samples from the shower, drain and trolley. The water samples from the room 11 tap are in a distinct clade, indicating the biofilm within the tap has a distinct genotype to the shower. This suggests environmental contamination was more likely to arise from contaminated shower water than tap water. Details of sampling site, days since start of study and presence of pBURNS plasmids are also shown. The likely phylogenetic position of *Pseudomonas aeruginosa* detected in a biofilm from a thermostatic mixer valve is shown in the clade associated with room 9 and indicated 'TMV'.

flexible hoses, solenoid valves and thermostatic mixer valves (TMV) are particularly at risk of biofilm formation due to factors including surface areas, convoluted designs and inadequate pasteurisation.<sup>47</sup> To confirm the presence of *P. aeruginosa* in water fittings associated with rooms on the burns unit, we obtained a TMV removed by the hospital estates team from the shower in room nine as part of compliance with UK guidelines for managing *P. aeruginosa*

in hospitals. On visual inspection, a biofilm was present which was scraped from the surface with a sterile scalpel. DNA from this biofilm was extracted for whole-genome shotgun sequencing. The majority of reads did not map to any known bacterial taxa. The most abundant taxon identified was *P. aeruginosa* (3%). Subsequent alignment to the *P. aeruginosa* Clade E reference covered 94% of the 6.3 million base reference genome at a median coverage of

5×, confirming that reads were correctly classified to this species and not other environmental *Pseudomonas* species. Alignment to the *P. aeruginosa* Clade E reference genome followed by phylogenetic placement of reads demonstrated that it fell into the same clade as previously recovered isolates from the shower or tap in room 9 (indicated on figure 3, and in online supplementary appendix 6).

## DISCUSSION

The hospital environment has been intimately linked with *P. aeruginosa* infection for over 50 years yet hospital acquisitions, clusters and outbreaks remain a common occurrence and understanding precise routes of transmission can be difficult.<sup>47 48</sup> Our results demonstrate that, even in a new hospital, *P. aeruginosa* can become rapidly endemic in hospital plumbing. Furthermore, by linking *P. aeruginosa* genotypes recovered from patients to specific individual water outlets, we offer compelling evidence of unidirectional transmission from water to patients. Further, by sequencing of a biofilm identified in a TMV from a hospital water system, we can identify the likely common source of genotypes found in water and in the hospital environment.

Our results suggest that use of WGS can reduce ambiguity about potential transmission events in hospitals and consequently inform infection prevention efforts about the direction and sequence of transmission. Typing schemes such as MLST and PFGE are much lower resolution methods and would not be able to provide sufficient information to permit such inferences to be made. It is notable that the burns unit was colonised by a single clone, meaning that it was very unlikely that water outlets at each bed space were colonised as a result of transmissions from the patient or environment. For this to happen would require multiple transmission events from separate patients with the same clone, for which there is no evidence. Instead we speculate that this clone was introduced to the hospital associated with its commissioning. One hypothesis is that particular plumbing fittings, that is, the TMV may have been colonised simultaneously by a clone circulating in water. Clade E (ST395) has been frequently reported associated with water, so this remains a possibility.<sup>49 50</sup> However, it is possible that plumbing fittings are installed 'pre-seeded' with *P. aeruginosa* as has already been proposed by Kelsey.<sup>3 5 47</sup> Investigation of an outbreak in Wales implicated new plumbing parts as a potential source of *P. aeruginosa*. New plumbing components are often tested by companies prior to their supply and it is possible they were contaminated prior to distribution. The limited amount of diversity (average 4 SNPs) seen within this clade is consistent with a single founding genotype coinciding with the opening of the burns unit, based on estimates from a previous study using WGS which reported that mutations accumulate at a rate of approximately one every 3–4 months in a hospital-associated clone.<sup>51</sup> However, our results suggest

that our isolates accumulate mutations even more slowly. This may be due to reduced growth rates in nutritionally-poor biofilms.<sup>52</sup>

It is notable that antibiotic resistance to multiple first-line agents developed rapidly in response to therapy. These results underline the importance of selecting appropriate antibiotic therapy in *P. aeruginosa* infections. It is reassuring however that antibiotic resistance genotypes selected *in vivo* did not show evidence of persistence in the ward environment or transmission to other patients.

Our study has certain limitations. Based on a previous audit, we expected around one-third of patients screened for *P. aeruginosa* would develop colonisation or clinical infection. In fact, only 5 out of 30 of patients were colonised. This may have been related to guidance and engineering interventions being put in place during the study as detailed in national guidance issued while this study was on-going. In addition, infection control policies were revised to address control of an outbreak of a multidrug resistant *A. baumannii* in this same burns unit. Following these interventions, only 1 of the last 20 patients recruited was infected with *P. aeruginosa* which may demonstrate the importance of national guidance in reducing transmissions.

By focusing on burns patients who receive hydrotherapy, our study population were at extremely high risk of waterborne infection. In other patient groups it may be that alternative routes of transmission including cross-infection or endogenous carriage play a more important role. Our results suggest that our burns unit is endemically colonised with a distinct clone of *P. aeruginosa* that may have been imported coinciding with the opening of the hospital. Other intensive care units, particularly those which have been open for longer may harbour a greater diversity of *P. aeruginosa* as a result of increased opportunities for clones to be imported.

One potential application for WGS in infection control would be to determine whether cases are as a result of water transmission, or represent sporadic clones originating from the wider environment. Despite improved guidance concerning improved engineering infection control practices and the introduction of the water safety group in the UK, it may not be realistic to eliminate *P. aeruginosa* from hospitals entirely. In augmented care units such as ITUs, burns units and neonatal wards where *P. aeruginosa* poses a significant risk to vulnerable patients, the increased resolution offered by WGS will justify its use, particularly as the costs continue to fall.

In conclusion, we have identified through WGS clear evidence for transmission of *P. aeruginosa* from specific water outlets to burns patients and offer a forensic-level framework for dealing with outbreaks linked to hospital water. We expect WGS will continue to make inroads into clinical microbiology and become a vital tool for tracking *P. aeruginosa* in the hospital environment, helping inform targeted control measures to help protect patients at risk of infection.

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**Contributors** MJP, NSM and BO conceived the study. CMW and AB enrolled patients into study and collected samples. NC collected environmental and water samples. NC, CC and MN processed samples and performed microbiology. NC, CC and JQ did sequencing. JQ, NC, CMT and NJL analysed the data. NJL, NC, JQ, MJP and BO wrote the paper. All authors commented on the manuscript draft.

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**Data sharing statement** Pacific Biosciences raw data files are available from the corresponding author (Nicholas J Loman, n.j.loman@bham.ac.uk).

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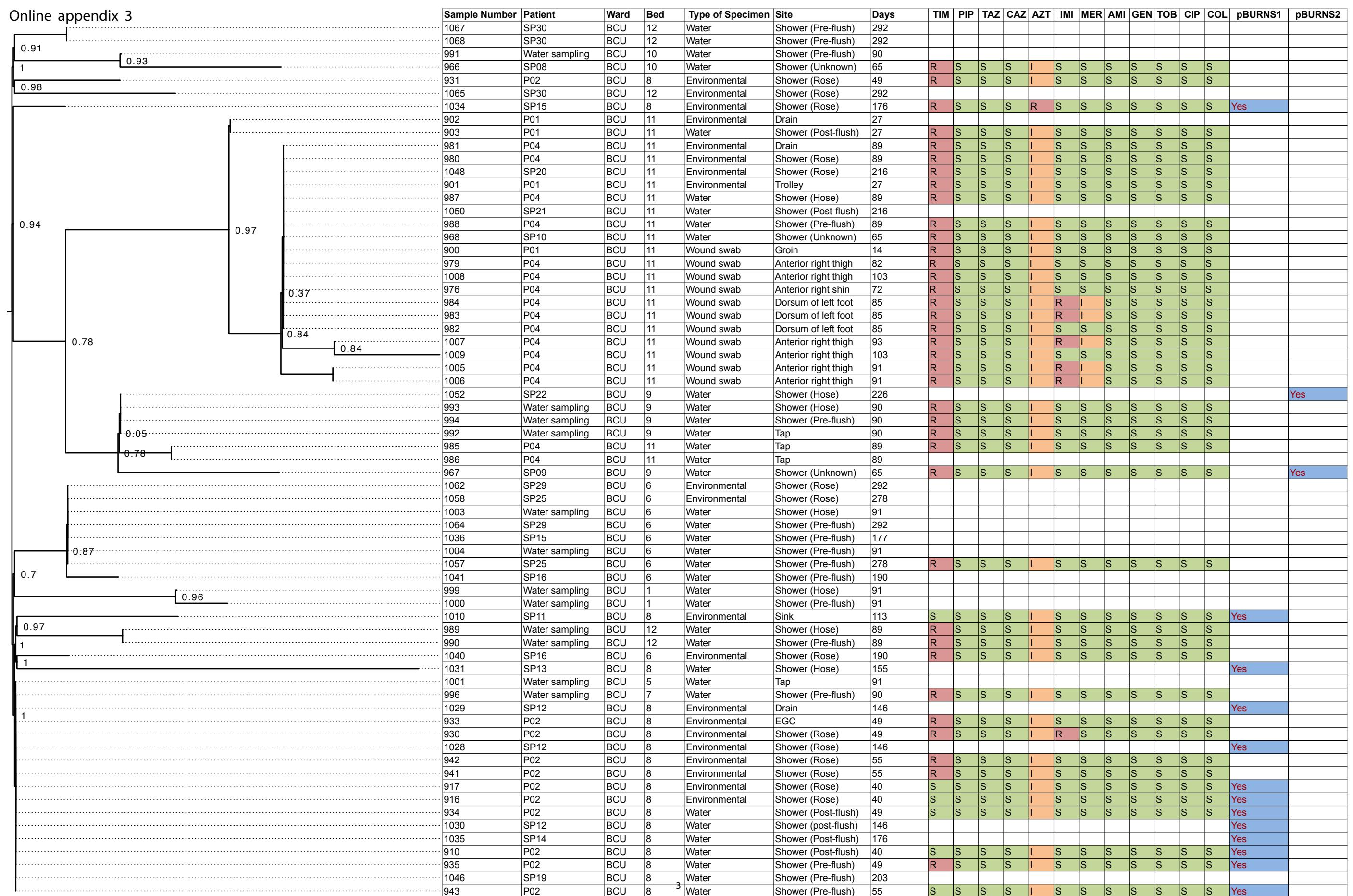
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Online appendix 1	Sample Number	Patient	Ward	Bed	Type of Specimen	Site	Days	TIM	PIP	TAZ	CAZ	AZT	IMI	MER	AMI	GEN	TOB	CIP	COL
0.94	922	P02	BCU	8	Wound swab	Upper back	40	R	I	I	S	I	S	S	S	S	S	S	
	921	P02	BCU	8	Wound swab	Upper back	40												
	913	P02	BCU	8	Environmental	Shower trolley	40	R	S	S	S	I	S	S	S	S	S	S	
	919	P02	BCU	8	Wound swab	Chest	40	R	I	I	S	I	I	S	S	S	R	S	
0.93	926	P02	BCU	8	Wound swab	Right palm	44	R	I	I	S	I	I	S	S	S	R	S	
	909	P02	BCU	8	Wound swab	Anterior left upper-arm	37	R	I	I	S	I	R	S	S	S	R	S	
	908	P02	BCU	8	Wound swab	Anterior left upper-arm	37	R	I	I	S	I	S	S	S	S	S	S	
	925	P02	BCU	8	Wound swab	Anterior left upper-arm	44	R	I	I	S	I	I	S	S	S	R	S	
	932	P02	BCU	8	Wound swab	Back of head	49	R	I	I	S	I	R	S	S	S	R	S	
	928	P02	BCU	8	Wound swab	Upper back	44	R	I	I	S	I	I	I	S	S	R	S	
	927	P02	BCU	8	Wound swab	Upper back	44	R	I	I	S	I	I	I	S	S	R	S	
	915	P02	BCU	8	Environmental	Chair	40	R	I	I	S	I	S	S	S	S	S	S	
	914	P02	BCU	8	Environmental	Chair	40	R	S	S	S	I	S	S	S	S	S	S	
	904	P02	BCU	8	Tissue	Anterior right upper-arm	33	R	S	S	S	I	S	S	S	S	S	S	
	905	P02	BCU	8	Wound swab	Chest	37	R	I	I	S	I	I	S	S	S	S	S	
	906	P02	BCU	8	Wound swab	Chest	37	R	I	I	S	I	I	S	S	S	S	S	
	920	P02	BCU	8	Wound swab	Chest	40	R	I	I	S	I	R	I	S	S	R	S	
	918	P02	BCU	8	Wound swab	Chest	40	R	I	I	S	I	S	S	S	S	S	S	
	907	P02	BCU	8	Wound swab	Abdomen	37	R	I	I	S	I	S	S	S	S	S	S	
	911	P02	BCU	8	Wound swab	Anterior left forearm	37	R	I	I	S	I	I	S	S	S	S	S	
	912	P02	BCU	8	Wound swab	Anterior left forearm	37	R	I	I	S	I	I	S	S	S	S	S	
	923	P02	BCU	8	Wound swab	Posterior left upper-arm	40	R	I	I	S	I	S	S	S	S	S	S	

## Online appendix 2

Study Number	Patient	Ward	Bed	Type of Specimen	Site	Days	TIM	PIP	TAZ	CAZ	AZT	IMI	MER	AMI	GEN	TOB	CIP	COL
937	P03	BCU	11	Wound swab	Anterior left thigh	50												
936	P03	BCU	11	Wound swab	Anterior left thigh	50												
929	P03	BCU	11	Wound swab	Posterior left thigh	48	R	S	S	S	I	R	I	S	S	S	S	
951	P03	BCU	15	Environmental	Bedside table	56	R	I	I	S	I	R	I	S	S	S	S	
960	P03	BCU	15	Environmental	Bedside table	62	R	S	S	S	I	R	I	S	S	S	S	
961	P03	BCU	15	Environmental	Door handle	62	R	I	I	S	I	R	I	S	S	S	S	
958	P03	BCU	15	Environmental	Shower chair	62	R	I	I	S	I	R	I	S	S	S	S	
957	P03	BCU	15	Environmental	Shower chair	62	R	S	S	S	I	R	I	S	S	S	S	
0.86																		
940	P03	BCU	15	Wound swab	Anterior right thigh	55	S	S	S	S	I	I	I	I	S	S	S	S
970	P03	BCU	15	Wound swab	Anterior right thigh	64	R	I	I	S	I	R	I	S	S	S	S	
944	P03	BCU	15	Wound swab	Anterior right shin	55	R	I	I	S	I	R	I	S	S	S	S	
945	P03	BCU	15	Wound swab	Anterior right shin	55	R	S	S	S	I	R	I	S	S	S	S	
946	P03	BCU	15	Wound swab	Anterior left thigh	55	R	S	S	S	I	R	I	S	S	S	S	
954	P03	BCU	15	Wound swab	Anterior left thigh	57	R	I	I	S	I	R	I	S	S	S	S	
947	P03	BCU	15	Wound swab	Anterior left shin	55	R	S	S	S	I	R	I	S	S	S	S	
972	P03	BCU	15	Wound swab	Posterior left shin	64	S	S	S	S	I	R	I	S	S	S	S	
0.17																		
949	P03	BCU	15	Wound swab	Posterior right thigh	55	R	I	I	S	I	R	I	S	S	S	S	
950	P03	BCU	15	Environmental	Bedside table	56	R	S	S	S	I	R	I	S	S	S	S	
959	P03	BCU	15	Environmental	Bedside table	62	R	I	I	S	I	R	I	S	S	S	S	
953	P03	BCU	15	Environmental	Toilet flush	56	R	S	S	S	I	I	I	S	S	S	S	
938	P03	BCU	15	Urine	Urine	55	R	S	S	S	I	R	I	S	S	S	S	
939	P03	BCU	15	Urine	Urine	55	R	S	S	S	I	R	I	S	S	S	S	
956	P03	BCU	15	Urine	Urine	58	R	S	S	S	I	R	I	S	S	S	S	
969	P03	BCU	15	Wound swab	Anterior right thigh	64	R	S	S	S	I	R	I	S	S	S	S	
971	P03	BCU	15	Wound swab	Anterior left thigh	64	R	I	I	S	I	R	I	S	S	S	S	
973	P03	BCU	15	Wound swab	Posterior right thigh	64	R	S	S	S	I	R	I	S	S	S	S	
0.86																		
955	P03	BCU	15	Wound swab	Anterior left thigh	57	S	S	S	S	I	R	I	S	S	S	S	
975	P03	BCU	15	Wound swab	Posterior right shin	64	S	S	S	S	I	R	I	S	S	S	S	
0.77																		
962	P03	BCU	15	Environmental	Shower chair	62	S	S	S	S	I	R	I	S	S	S	S	
948	P03	BCU	15	Wound swab	Posterior right thigh	55	S	S	S	S	I	R	I	S	S	S	S	
974	P03	BCU	15	Wound swab	Posterior right thigh	64	S	S	S	S	I	I	I	S	S	S	S	
0.79																		
963	P03	BCU	15	Environmental	Bedside table	62	S	S	S	S	I	I	I	S	S	S	S	
964	P03	BCU	15	Environmental	Bedside table	62	S	S	S	S	I	R	I	S	S	S	S	

1 SNP/Indel

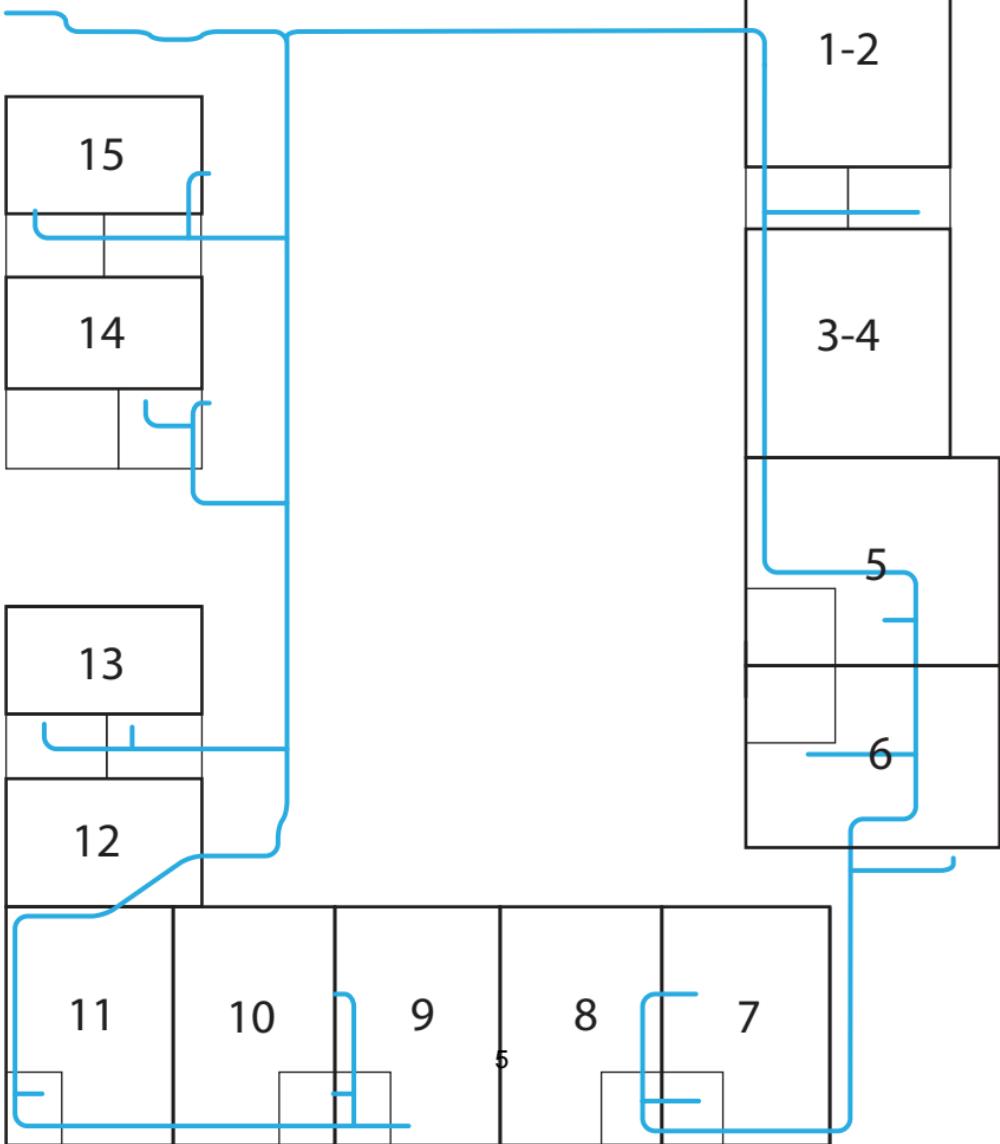


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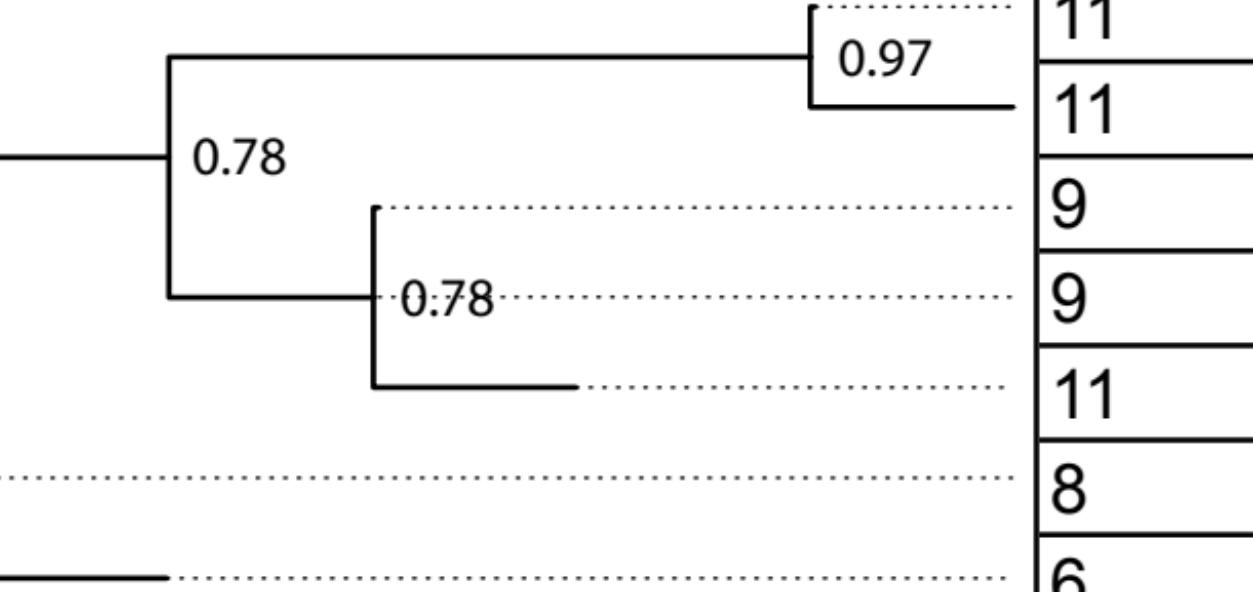
Online appendix 4	Sample Number	Patient	Ward	Bed	Type of Specimen	Site	Days	TIM	PIP	TAZ	CAZ	AZT	IMI	MER	AMI	GEN	TOB	CIP	COL
.....	1069	Water sampling	WCCB	11	Water	Tap	301	R	S	S	S	I	R	I	S	S	S	S	S
	1045	SP20	WCCB	11	Environmental	Tap handle	204												
	1047	SP20	WCCB	11	Water	Tap	204	R	S	S	S	I	R	I	I	R	S	S	S
	1049	P05	WCCB	11	Sputum	Sputum	221		I	I	S	I	I	I	I	R	S	S	S
	1053	P05	WCCB	11	Water	Tap	231	R	I	I	S	I	R	S	S	S	S	S	S
	1054	P05	WCCB <sub>4</sub>	11	Sputum	Sputum	229	R	R	R	R	R	R	I	R	S	I	S	S
	1056	P05	WCCB	11	Sputum	Sputum	242		R	R	R	S	R	R		S	S	R	S

1 SNP/Indel

## Online appendix 5



## Online appendix 6



<b>Bed</b>	<b>Type of Specimen</b>	<b>Alignment</b>
11	Shower	T <del>CCT</del> ACTCC-CACAG <del>A</del> CCTAACCT-----G--ACCGAAATCCTTTCCG-C <del>G</del> GGCG
11	Shower	T <del>CCT</del> ACTCC-TACAG <del>A</del> CCTAACCT-----G--ACCGAAATCCTTTCCG-C <del>G</del> GGCG
9	Tap	T <del>GCA</del> ACTCC-CACAG <del>A</del> CCTAACGT-----G--ACCGAAATCCTTTCCG-C <del>G</del> GGCG
9	TMV	T <del>GCA</del> -CT-C-CACAG <del>A</del> ---AACGT-----G--ACCG--A-CCTTTTC-G-C--GGCG
11	Tap	T <del>GTA</del> ACTCC-CACAG <del>A</del> CCTAACGT-----G--ACCGAAATCCTTTCCG-C <del>G</del> GGCG
8	Shower	T <del>CCA</del> ACTCC-CACAG <del>A</del> CCTAACGT-----G--ACCGAAATCCTTTCCG-C <del>T</del> GGCG
6	Shower	T <del>CCA</del> ACTCC-CACAG <del>C</del> CCTAACGT-----G--ACCGAAATCCTTTCCG-C <del>T</del> GGCG

## Online appendix 7

Chromosome	Position	Ref	Alt	Mean depth	No calls	Hom calls	Het calls	Effect	Effect impact	Functional class	Codon change	Amino acid change	Gene name	904	905	906	907	908	909	911	912	913	914	915	918	919	920	921	922	923	925	926	927	928	932
NC_002516	1558800	CCATATG	C	40	0	1	0	CODON_DELETION	MODERATE		cata/g-	HM211-	lasR														C								
NC_002516	2806409	G	A	23	0	8	0	NON_SYNONYMOUS_CODING	MODERATE	MISSENSE	Cac/Tac	H321Y	PA2491							A	A							A	A	A	A	A			
NC_002516	3804666	GCTTGC	G	19	0	1	0	FRAME_SHIFT	HIGH		-	-72	PA3399																			G			
NC_002516	4148397	A	T	21	0	1	0	NON_SYNONYMOUS_CODING	MODERATE	MISSENSE	aTt/aAt	I181N	wspD															T							

# Online appendix 8

Chromosome	Position	Ref	Alt	Mean depth	No calls	Hom calls	Het calls	Effect	Effect Impact	Functional class	Codon change	Amino acid change	Gene name	929	936	937	938	939	940	944	945	946	947	948	949	950	951	953	954	955	956	957	958	959	960	961	962	963	964	969	970	971	972	973	974	975
NC_002516	5748989	CGGGAGTGG	C	30	0	0	0	FRAME SHIFT	HIGH	-33	P40512																																			
NC_002516	856001	TGGCCTGG	T	31	0	1	0	FRAME SHIFT	HIGH	-	putP																																			
NC_002516	1558520	C	T	41	0	5	0	NON_SYNONYMOUS_CODING	Moderate	Missense	cCc/tG	P117L	lasR																																	
NC_002516	1558668	C	CTT	36	0	2	0	FRAME SHIFT	HIGH	-1/T	lasR																																			
NC_002516	2840713	A	C	19	0	0	26	0	NON_SYNONYMOUS_CODING	Moderate	Missense	t/G/tCc	D119A	PA2523	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C							
NC_002516	2936290	G	A	20	0	0	7	0	NON_SYNONYMOUS_CODING	Moderate	Missense	cCc/tG	P40512																																	
NC_002516	3486673	A	T	25	0	0	1	0	NON_SYNONYMOUS_CODING	Moderate	Missense	cTg/cAg	L171Q	PA3106																																
NC_002516	3584085	G	A	23	0	2	0	NON_SYNONYMOUS_CODING	Moderate	Missense	gCc/gTc	A87V	glfR		A	A																														
NC_002516	5796903	C	T	14	0	0	1	0	NON_SYNONYMOUS_CODING	Moderate	Missense	gCc/gTc	A317V	mutY																																
NC_002516	5853846	A	C	26	0	1	0	NON_SYNONYMOUS_CODING	Moderate	Missense	Acc/cCc	T88P	PA5201																																	

Online appendix 9

# Online appendix 10

Chromosome	Position	Ref	Alt	Mean depth	No calls	Hom calls	Het calls	Effect	Effect impact	Functional class	Codon change	Amino acid change	Gene name	1045	1047	1049	1053	1054	1056	1069
NC_002516	3558951	G	A	35	0	1	0	NON_SYNONYMOUS_CODING	MODERATE	MISSENSE	aCc/aTc	T83I	gyrA				A			
NC_002516	4166773	A	G	22	0	2	0	NON_SYNONYMOUS_CODING	MODERATE	MISSENSE	Acg/Gcg	T86A	nalC				G	G		

Patient	Antibiotic/antifungal	Start	End	Start	End
2	Ciprofloxacin	16/10/2012	06/11/2012		
	Nitrofurantoin	16/10/2012	11/11/2012		
	Vancomycin	29/10/2012	03/11/2012		
4	Flucloxacillin	23/11/2012	30/11/2012		
	Piperacillin/tazobactam	30/11/2012	03/12/2012		
	Meropenem	03/12/2012	08/12/2012		
5	Colistin	15/12/2012	21/12/2012		
	Gentamycin	12/04/2013	12/04/2013		
	Co-amoxiclav	13/04/2013	14/04/2013		
5	Erthromycin	14/04/2013	21/04/2013	11/05/2013	19/05/2013
	Piperacillin/tazobactam	14/04/2013	18/04/2013		
	Meropenem	20/04/2013	08/05/2013	19/05/2013	20/05/2013
5	Caspofungin	26/04/2013	14/05/2013	20/05/2013	21/05/2013
	Linezolid	01/05/2013	12/05/2013		
	Ciprofloxacin	06/05/2013	16/05/2013	20/05/2013	21/05/2013
5	Colistin	20/05/2013	23/05/2013		

<b>Gene</b>	<b>Mutation type</b>	<b>Effect</b>	<b>AA substitution</b>	<b>Resistance phenotype</b>	<b>Samples</b>
gyrA	SNP	non-synonymous	T83I	Ciprofloxacin	1056
nalC	indel	-	-	Meropenem	1054, 1056
mexS	SNP	non-synonymous	H321Y	Ciprofloxacin	908, 909, 919, 925-928, 932
oprD	indel	frame shift	-400?	Imipenem/meropenem	1005, 1006