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Complete List of Authors:	Quick, Josh; Institute of Microbiology and Infection, University of Birmingham; Queen Elizabeth Hospital, NIHR Surgical Reconstruction and Microbiology Research Centre Cumley, Nicola; Queen Elizabeth Hospital, NIHR Surgical Reconstruction and Microbiology Research Centre Wearn, Chris; University Hospital Birmingham Foundation Trust, Healing Foundation Centre for Burns Research; Queen Elizabeth Hospital, NIHR Surgical Reconstruction and Microbiology Research Centre Niebel, Marc; Queen Elizabeth Hospital, NIHR Surgical Reconstruction and Microbiology Research Centre Constantinidou, Chrystala; University of Warwick, Division of Microbiology and Immunology Pallen, Mark; University of Warwick, Division of Microbiology and Immunology Moiemen, Naiem; Queen Elizabeth Hospital, NIHR Surgical Reconstruction and Microbiology Research Centre; University Hospital Birmingham Foundation Trust, Healing Foundation Centre for Burns Research Bamford, Amy; Queen Elizabeth Hospital, NIHR Surgical Reconstruction and Microbiology Research Centre; University Hospital Birmingham Foundation Trust, Healing Foundation Centre for Burns Research Oppenheim, Beryl; Queen Elizabeth Hospital, NIHR Surgical Reconstruction and Microbiology Research Centre Loman, Nicholas; University of Birmingham, Institute of Microbiology and Infection
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For peer review only

Title

Seeking the source of *Pseudomonas aeruginosa* infections in a recently opened hospital: a role for whole-genome sequencing

Authors

Joshua Quick^{1,2*}, Nicola Cumley^{2*}, Chris M. Wearn^{2,3}, Marc Niebel², Chrystala Constantinidou⁴, Mark J. Pallen⁴, Naiem S. Moiemmen^{2,3}, Amy Bamford^{2,3}, Beryl Oppenheim^{2#}, Nicholas J. Loman^{1#}

Affiliations

¹Institute of Microbiology and Infection, University of Birmingham, Birmingham, United Kingdom

²NIHR Surgical Reconstruction and Microbiology Research Centre, Queen Elizabeth Hospital, Birmingham, United Kingdom

³Healing Foundation Centre for Burns Research, University Hospital Birmingham Foundation Trust, Birmingham, United Kingdom

⁴Division of Microbiology and Immunology, University of Warwick, Warwick, United Kingdom

*Contributed equally

Correspondence:

Dr Nicholas James Loman

Institute of Microbiology and Infection, University of Birmingham, Birmingham, B15 2TT United Kingdom

1
2
3 Telephone: +44 (0) 121 414 8849
4
5

6 Email: n.j.loman@bham.ac.uk
7
8

9 Dr Beryl Oppenheim
10

11
12 Clinical Microbiology, University Hospitals Birmingham NHS Foundation Trust
13

14 Queen Elizabeth Hospital Birmingham, Mindelsohn Way, Edgbaston
15

16 Birmingham, B15 2WB
17

18
19
20 Telephone: +44 (0) 121 371 6523
21

22 Email: Beryl.Oppenheim@uhb.nhs.uk
23
24

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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 United Kingdom.

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 whole-genome sequenced. Sequence analysis looked at similarity and relatedness between isolates.

Results

1
2
3 Whole-genome sequences for 141 *P. aeruginosa* isolates were obtained from
4 patients, hospital water and the ward environment. Phylogenetic analysis revealed
5 eight distinct clades, with a single clade representing the majority of environmental
6 isolates in the burns unit. Isolates from three patients had identical genotypes
7 compared with water isolates from the same room. There was clear clustering of
8 water isolates by room and outlet, allowing the source of acquisitions to be
9 unambiguously identified. Whole-genome shotgun sequencing of biofilm DNA
10 extracted from a thermostatic mixer valve revealed this was the source of a *P.*
11 *aeruginosa* subpopulation previously detected in water. In the remaining two cases
12 there was no clear link to the hospital environment.
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25 **Conclusions**

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

Strengths and limitations of the study

- We have demonstrated that whole-genome sequencing can be used for source tracking of *P. 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.[1-10] Other potential routes of transmission include cross-infection, for example carriage on the hands of health care workers, and through contaminated medical equipment such as endoscopic devices.[3,5]

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.[11] This source was eventually determined to be sink taps.[11-13] 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.[14]

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 (VNTR) and random amplification of polymorphic DNA (RAPD) and multi-locus sequencing typing (MLST). These methods have been used to investigate outbreaks of *P. aeruginosa* within hospitals.[4,15-17] However, such techniques have important limitations for

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2
3 source tracking of infections in hospitals as they sample limited numbers of sites in
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5 the genome which may result in false clustering of unrelated strains.[18] In the past
6
7 five years, whole-genome sequencing (WGS) has started to be used to investigate
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9 outbreaks in hospitals. WGS is attractive because of its digital, sharable format and
10
11 ultra-high resolution which is able to discriminate two isolates differing by just a
12
13 single mutation. WGS has been successfully used to determine likely transmission
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15 chains during outbreaks of *Staphylococcus aureus*, *Acinetobacter baumannii* and
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17 *Klebsiella pneumoniae*. [18-20] Benchtop sequencing instruments now offer a cost-
18
19 effective approach for bringing bacterial WGS to the clinical environment. [1,21]
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24 In this study, we explore the utility of WGS to determine the likely sources of *P.*
25
26 *aeruginosa* in an at-risk population of burns patients. In the UK and US burns
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28 patients receive shower cart hydrotherapy as a mainstay of burns treatment.[22-25]
29
30 A previous hospital audit suggested that up to one-third of such patients became
31
32 colonised with *P. aeruginosa*. We hypothesised that this high rate of acquisition may
33
34 relate to transmission from hospital shower water during therapy. We therefore
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36 wished to understand the importance of transmission from water compared with
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38 alternative routes such as cross-infection and endogenous carriage.
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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 (CCU). 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 multi-drug resistant *A. baumannii*.

Study design and patient selection

Ethical approval was sought and received from the National Research Ethics Service committee in the West Midlands (reference number 12/WM/0181). 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

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3 microbiology techniques. Samples were then taken as part of routine microbiology
4 service during the patients stay. Environmental and water samples were taken after
5 the patient was admitted to the burns centre. If during the period of stay *P.*
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10 *aeruginosa* was isolated from a patient sample the patient was recruited into the
11 second phase of the study. In this phase, patients had wound swabs taken at each
12 dressing change as well as twice-weekly urine samples. The patient's environment
13 and water from outlets in their bed space were sampled weekly for the duration of
14 their stay, and after discharge (post-cleaning). Termination of the study was planned
15 after 30 screening patient admissions, or a year, whichever came soonest, after
16 which 10 patients were expected to acquire *P. aeruginosa*.
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25 26 **Microbiological and molecular methods**

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29 *P. aeruginosa* isolates were obtained from wound swab, urine, stool, environmental
30 and water samples. *P. aeruginosa* was isolated from wound swabs, urine and stool
31 by inoculation onto cysteine lactose electrolyte deficient agar (CLED) and cetrimide
32 agar and incubation for 24 hours at 37°C. Stool samples were cultured overnight in a
33 cetrimide enrichment broth before subculture onto CLED. Identification was
34 confirmed by resistance to C-390 and the VITEK® 2 GN identification card. Antibiotic
35 sensitivity assays were performed using the VITEK® 2 AST N-210 card (bioMérieux,
36 Basingstoke, UK).
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47 The patient's environment (shower head rosette, drain, shower chair or trolley,
48 bedside table, patient chair, instruments in contact with the patient) was sampled
49 over a 10cm² area by a Polywipe™ sponge. The sponge was placed in tryptic soy
50 broth incubated for 24 hours at 37°C then sub-cultured onto CLED and cetrimide
51 agar. During water sampling, water was taken from the patient's shower, or tap if a
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3 shower was not present. In duplicate, 100ml of water was filtered through a 0.45
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5 micron filter and the filters placed onto CLED plates and cetrimide agar. Plates were
6
7 incubated at 37°C for 48 hours and the number of organisms per 100ml quantified.
8
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10 For storage and DNA extraction a single colony was purified from the primary culture
11
12 plate. When different colony morphologies were observed, a single colony from each
13
14 type was purified. Additionally, for a randomly selected water sample, 24 colonies
15
16 were individually picked from one water-filter primary microbiological plate for
17
18 sequencing. Isolates were stored on Biobank beads at -20°C prior to DNA extraction.
19
20 Organisms were resuscitated on CLED agar plates and genome DNA either
21
22 extracted directly using the MOBIO UltraClean Microbial DNA Kit, or from overnight
23
24 LB broth culture using a Qiagen Genomic-Tip 100G.
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29 **DNA extraction and sequencing**

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32 Genomic DNA was prepared from single colony picks using the MIOBIO Ultraclean
33
34 microbial kit (MOBIO, Carlsbad, USA). 1ng input DNA, as quantified by Qubit (Life
35
36 Technologies, Carlsbad, USA) was used to prepare genomic libraries for sequencing
37
38 using the Illumina Nextera XT™ DNA sample kit as per manufacturer's protocol
39
40 (Illumina, San Diego, USA). Libraries were sequenced on the Illumina MiSeq using a
41
42 paired-end protocol resulting in read lengths between 150 and 300 bases. A single
43
44 additional sample, isolate 910, was chosen as a representative member of Clade 5
45
46 for long-read sequencing. DNA from this sample was fragmented using a
47
48 Hydroshear (Digilab, Marlborough, MA) using the recommended protocol for 10kb
49
50 fragments and further size-selected on a Blue Pippin instrument (Sage Science,
51
52 MA) with a 7kb minimum size cut-off. The library was sequenced on two zero-mode
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54 waveguides (ZMWs) using the Pacific Biosciences RS II instrument at the Norwegian
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3 Sequencing Centre, Oslo. C4-P2 chemistry was chosen because it favours long,
4
5 more accurate reads for *de novo* assembly.
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8 **Stool PCR**

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10 For detection of *P. aeruginosa* in stool samples using PCR, a stool sample was
11
12 collected into a stool collection tube containing stool DNA stabilizer. Total DNA was
13
14 extracted using the PSP Spin Stool DNA Plus kit (Strattec Molecular). PCR
15
16 amplification of species specific regions of the 16S rDNA gene was carried out using
17
18 primers PA-SS-F: GGGGGATCTTCGGACCTCA and PA-SS-R:
19
20 TCCTTAGAGTGCCACCCG [12] in the following conditions: 0.5µM of each primer,
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22 1.5mM MgCl₂, 0.2mM dNTP's using BIOTAQ™ DNA Polymerase and buffer set.
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24 After initial denaturation at 96°C for 2 minutes, 30 cycles of 96°C for 30 seconds, 62°
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26 C for 30 seconds and 72°C for 30 seconds were completed with a final extension of
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28 72°C for 5 minutes. Products were visualised for size on an 1.5% agarose gel.
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34 **Bioinformatics methods**

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37 Illumina MiSeq reads from each isolate were adapter and quality trimmed before use
38
39 with Trimmomatic.[26] Phylogenetic reconstruction of isolates sequenced in this
40
41 study were combined with data from a global collection of 55 *P. aeruginosa* strains
42
43 collected world-wide which have been previously analysed by Stewart et al. [27] For
44
45 each of the published strains, 600,000 paired-end reads were simulated from the
46
47 complete or draft genome assembly deposited in Genbank. Read sets were mapped
48
49 against the *P. aeruginosa* PAO1 reference genome using BWA-MEM 0.7.5a-r405
50
51 using default settings.[28] Single nucleotide polymorphisms were called using
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53 VarScan 2.3.6 and filtered for regions with an excessive number of variants, which
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55 may represent regions of recombination or strong Darwinian selection.[29] FastTree
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3 (version 2.1.7) was used for phylogenetic reconstruction. This software estimates an
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5 approximate maximum-likelihood tree under the Jukes-Cantor model of nucleotide
6
7 evolution with a single rate for each site (CAT).[30] Trees were drawn in FigTree
8
9 (<http://tree.bio.ed.ac.uk/software/figtree/>).

10
11
12 For *in silico* MLST prediction, trimmed reads were assembled *de novo* using Velvet
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14 [31] with a k-mer size of 81 and searched using nucleotide BLAST against the multi-
15
16 locus sequence database downloaded from the pubMLST website on 2013-08-05
17
18 (<http://pubmlst.org/paeruginosa/>). For Clade 5 isolates, in order to exhaustively
19
20 search for discriminatory mutations, a nearly complete reference genome was
21
22 generated by *de novo* assembly using Pacific Biosciences sequencing data. Reads
23
24 were assembled using the 'RS_HGAP_Assembly.3' pipeline within SMRT Portal
25
26 v2.2.0. Illumina reads from the same sample were mapped to this draft genome
27
28 assembly in order to correct remaining indel errors in the assembly using Pilon
29
30 (<http://www.broadinstitute.org/software/pilon/>). Isolates belonging to each clade were
31
32 mapped individually against either the PacBio reference (Clade 5) or *P. aeruginosa*
33
34 PAO1 (NC_002516) (Clades 3, 4 and 7).

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37 Variants (single nucleotide polymorphisms and short insertion-deletions) were called
38
39 using SAMtools mpileup and VarScan with an allele frequency threshold of 80%.[29]

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42 Non-informative positions and regions of putative recombination were removed, the
43
44 later with a variant density filter of more than 3 SNPs every 1000 nucleotides.

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46
47 Analysing samples in each clade individually maximised the number of variants
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49 detected by reducing the likelihood of the position being uncovered by a subset of
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51 samples. From these variants fine-grained phylogenetic trees were reconstructed for
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53 each clade using FastTree. The scripts used to perform this analysis are available at
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3 http://www.github.com/joshquicksnp_calling_scripts. Approximate-maximum-
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5 likelihood phylogenetic trees were generated using FastTree and FigTree. For
6
7 whole-genome shotgun metagenomics analysis, reads were analysed using the
8
9 Kraken taxonomic classifier software with the supplied *minikraken* database.[32]
10
11 Reads from the metagenomics dataset were aligned to *P. aeruginosa* PAO1 as in
12
13 the previous section and phylogenetic placement was carried out using pplacer in
14
15 conjunction with FastTree.[33] Sequence data is available from the European
16
17 Nucleotide Archive for the Illumina data (ERP006056) and the corrected Pacific
18
19 Biosciences assembly (ERP006058).
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21

22 23 **RESULTS**

24 25 **Study results**

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27 Recruitment lasted a period of 300 days, ending according to protocol after the
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29 enrolment of 30 screening patients. One additional eligible patient did not consent to
30
31 enter the study and was excluded. The average age in the study group was 41
32
33 years. Males predominated with a male-to-female ration of 2.3:1. Flame burns were
34
35 the most common mechanism of injury, followed by scalds and mixed flame/flash
36
37 injuries. The average burn size of the study group was 12.5% of the total body
38
39 surface area (TBSA) and 27% of patients sustained an inhalation injury. Eight
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41 patients required admission to ITU and the majority required surgical treatment of
42
43 their burns with excision and skin grafting (80%). A large majority of the study group
44
45 (83%) received shower cart hydrotherapy as a routine part of their wound
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47 management to encourage healing through wound debridement and
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49 decontamination. The average length of hospital stay (LOS) was 17 days and taking
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51 into account burn size, the average was 1.4 days per % TBSA.
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3 **The water and environment in burns and critical care units are frequently**
4 **colonised by *P. aeruginosa***
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8 A total of 282 water and environmental samples were screened for *P. aeruginosa* of
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10 which 39/78 (50%) were positive in water samples, 25/96 (26%) were positive from
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12 the wet environment and 7/108 (6%) were positive from the dry environment. A total
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14 of 83 genome sequences were generated from the 71 positive, as in some cases
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16 multiple colony picks were sequenced. When placed in the context of a global
17
18 collection of *P. aeruginosa* strains, phylogenetic reconstruction demonstrated
19
20 isolates in our study fell into eight clades (figure 1, Panel A). As has been reported
21
22 previously, there was no strong association between ecological context and position
23
24 in the phylogenetic tree.[27] Isolates in this study are most closely related to strains
25
26 from a variety of settings. The majority of isolates (52%) belong to Clade E (figure 1
27
28 Panel B), whose nearest sequenced relative is the Liverpool Epidemic Strain, a
29
30 clone often isolated from patients in the UK and Canada with cystic fibrosis. [34,35]
31
32 Isolates from clade E were found in burns unit water and the ward environment, as
33
34 well as from two patient's wounds. However it was never detected in the critical care
35
36 unit. Clade E was detected throughout the study in a total of 10 different rooms
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38 (figure 2).
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44 **Detection of potential transmission events by whole-genome sequencing**
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47 Microevolutionary changes occurring over rapid time-scales (i.e. days to months)
48
49 have been used to detect potential chains of transmission in hospital and community
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51 outbreaks.[18-20,36,37] The number of distinct mutations between given isolates
52
53 has been used to infer whether transmission events are likely to have occurred.
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55 Such inferences are aided by prior knowledge of mutation rates in similar
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3 populations. Two patients (1 and 4) in our study both had *P. aeruginosa* from clade E
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5 isolated from their wounds. These isolates had an indistinguishable genotype from
6
7 those present in water and the environment of the room they were nursed within
8
9 (figure 1 Panel C and figure 3). This genotype was detected in the patient's shower
10
11 water after initial patient screening, during screening of the second patient
12
13 admission, twice during the second patient's stay and then 127 days later (days 27,
14
15 65, 89 and 216 respectively). When water isolates were positive, the genotype was
16
17 also detected in wet environment sites (shower drain, shower rosette and patient's
18
19 trolley) on the same days.
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24 Patient E was nursed on the critical care unit due to concomitant medical problems.
25
26 *P. aeruginosa* belonging to clade G was isolated from sputum during this time..
27

28 Identical genotypes were detected contemporaneously in the water from the
29
30 associated sink and sink tap handle (see online supplementary appendix 4).
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34 Two further patients (patients 2 and 3) were positive for *P. aeruginosa*. Isolates from
35
36 these patients belonged to clades C and D respectively. Neither clade was ever
37
38 isolated from hospital water. In both cases, identical genotypes were detectable in
39
40 the environment associated with the patient but these were not detected before or
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42 after the patients' stay, indicating that the environment was not persistently
43
44 contaminated. During the course of patient 3's stay, the dry environment such as the
45
46 bedside table was contaminated, as was the patient's door handle and shower chair.
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48 However, after patient discharge, the strain associated with this patient was never
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50 seen again during the course of the study in any location.
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55 **Whole-genome sequencing permits source tracking of *P. aeruginosa* to**
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57 **individual water outlets**
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3 Whole-genome sequencing has been reported previously for source tracking, but
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5 never for the detection of transmission events from hospital water.[38] Phylogenetic
6
7 reconstruction within Clade E, the most commonly detected water clone
8
9 demonstrated additional diversity within this clone, with a total of 46 mutations
10
11 detected an average genetic distance between isolates of 4.1 mutations (figure 3).
12
13 The reconstruction demonstrated clear evidence of clustering of genotypes both by
14
15 room and outlet (figure 3). When *P. aeruginosa* was detected in the wet environment
16
17 (e.g. shower rosettes and drains) these genotypes were most often identical to those
18
19 found in water, indicating that the water was likely the ultimate source of that clone.
20
21 Genotypic variation was seen between outlets within the same room. For example,
22
23 tap water sampled from room 11 had a distinct genotype from that sampled from
24
25 shower water in the same room and this was consistently found over multiple
26
27 samplings. Notably, isolates from two patients fell within the cluster originating from
28
29 shower water, indicating that shower hydrotherapy was the most likely source of
30
31 infection. Two plasmids (designated pBURNS1 and pBURNS2) were detected in this
32
33 study set, which both demonstrated geographical clustering, with pBURNS1 only
34
35 being detectable in isolates from room 8 and pBURNS2 only being detectable in
36
37 isolates from the shower water in room 9.
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44 **Rapid evolution of antibiotic resistance associated with treatment**

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46 *P. aeruginosa* is commonly associated with antibiotic resistance due to a number of
47
48 predisposing features including intrinsic resistance, a repertoire of efflux pumps,
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50 antibiotic-inactivating enzymes including beta-lactamases and natural
51
52 transformability. [39] Three infected patients (2, 3 and 5) received antibiotic therapy,
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54 and in each case this was associated with the development of resistance to at least
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3 one therapeutic agent. Associated mutations were detected that were either partially
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5 or fully explanatory of the phenotype.
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8 Patient 2 was treated with ciprofloxacin, nitrofurantoin and vancomycin (see online
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10 supplementary appendix 11 for full details). 8/21 (38%) tested isolates from this
11
12 patient were ciprofloxacin resistant. 7/8 isolates (88%) of the ciprofloxacin-resistant
13
14 strains were distinguishable from the other isolates by a single SNP in *mexS*
15
16 (annotated as PA2491 in *P. aeruginosa* PAO1) (see online supplementary appendix
17
18 1 and 7). This SNP was predicted to result in a non-synonymous amino acid
19
20 substitution. Disruption of this gene has been shown to cause increased expression
21
22 of the mexEF-oprN multidrug efflux pump, associated with resistance to
23
24 quinolones.[40]
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29 Patient 4 was treated with meropenem, piperacillin/tazobactam, flucloxacillin and
30
31 colistin. Five isolates collected 10 to 18 days after initiation of meropenem showed
32
33 resistance to imipenem and intermediate resistance to meropenem (see online
34
35 supplementary appendix 3 and 9). The most likely mutation responsible for this
36
37 phenotype was detectable in two isolates, both of which had a frame-shift mutation in
38
39 the gene coding for the membrane porin *oprD*. [41]
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44 Patient 5 had a prolonged stay in ITU and had multiple medical problems including
45
46 *A. baumannii* infection and was treated with nine antibiotic agents including
47
48 ciprofloxacin, meropenem and piperacillin-tazobactam. Serial isolates from this
49
50 patient demonstrated the stepwise acquisition of two mutations. The first was in
51
52 *nalC*, a probable repressor of the TetR/AcrR family (see online supplementary
53
54 appendix 10). [42] On inspection of the sequence alignment in this region, a large
55
56 deletion of 196 nucleotide bases was seen compared to the reference PAO1 strain.
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3 This mutation was seen in association with full resistance to piperacillin-tazobactam,
4
5 ceftazidime, aztreonam, meropenem and intermediate resistance to ciprofloxacin.
6

7 This deletion is likely to result in over-expression of efflux pumps involving the
8
9 *mexAB-oprM* operon. [42,43] Ciprofloxacin resistance in a later isolate corresponded
10
11 to the stepwise acquisition of a second mutation. This mutation is predicted to affect
12
13 the well-studied DNA gyrase subunit A gene (*gyrA*) which is strongly associated with
14
15 ciprofloxacin resistance.[44]
16
17

18 19 **Confirmation of *P. aeruginosa* genotypes in biofilms by whole-genome** 20 21 **metagenomic shotgun sequencing** 22 23

24 *P. aeruginosa* is able to produce and survive in biofilms. Plumbing parts such as flow
25
26 straighteners, shower rosettes, flexible hoses, solenoid valves and thermostatic
27
28 mixer valves (TMV) are particularly at risk of biofilm formation due to factors
29
30 including surface areas, convoluted designs and inadequate pasteurisation. [45] To
31
32 confirm the presence of *P. aeruginosa* in water fittings associated with rooms on the
33
34 burns unit, we obtained a TMV removed by the hospital estates team from the
35
36 shower in room nine as part of compliance with UK guidelines for managing *P.*
37
38 *aeruginosa* in hospitals. On visual inspection, a biofilm was present which was
39
40 scraped from the surface with a sterile scalpel. DNA from this biofilm was extracted
41
42 for whole-genome shotgun sequencing. The majority of reads did not map to any
43
44 known bacterial taxa. The most abundant taxon identified was *Pseudomonas*
45
46 *aeruginosa* (3%). Subsequent alignment to the *P. aeruginosa* PAO1 reference
47
48 genome covered 94% of the 6.3 million base reference genome at a median coverage of 5x,
49
50 confirming that reads were correctly classified to this species and not other
51
52 environmental *Pseudomonas* species. Alignment to the *P. aeruginosa* clade E
53
54 reference genome followed by phylogenetic placement of reads demonstrated that it
55
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1
2
3 fell into the same clade as previously recovered isolates from the shower or tap in
4
5 room 9 (indicated on figure 3, and in online supplementary appendix 6).
6
7

8 **DISCUSSION**

9
10 The hospital environment has been intimately linked with *P. aeruginosa* infection for
11
12 over 50 years yet hospital acquisitions, clusters and outbreaks remain a common
13
14 occurrence and understanding precise routes of transmission can be difficult. [45,46]
15

16
17 Our results demonstrate that, even in a new hospital, *P. aeruginosa* can become
18
19 rapidly endemic in hospital plumbing. Furthermore, by linking *P. aeruginosa*
20
21 genotypes recovered from patients to specific individual water outlets, we offer
22
23 compelling evidence of unidirectional transmission from water to patients. Further, by
24
25 sequencing of a biofilm identified in a TMV from a hospital water system, we can
26
27 identify the likely common source of genotypes found in water and in the hospital
28
29 environment.
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32

33
34 Our results suggest that use of whole-genome sequencing can reduce ambiguity
35
36 about potential transmission events in hospitals and consequently inform infection
37
38 prevention efforts about the direction and sequence of transmission. It is notable that
39
40 the burns unit was colonised by a single clone, meaning that it was very unlikely that
41
42 water outlets at each bed space were colonised as a result of transmissions from the
43
44 patient or environment. For this to happen would require multiple transmission
45
46 events from separate patients with the same clone, for which there is not evidence.
47
48 Instead we speculate that this clone was introduced to the hospital associated with
49
50 its commissioning. One hypothesis is that particular plumbing fittings, i.e. the TMV
51
52 may have been colonised simultaneously by a clone circulating in water. Clone E
53
54 (ST395) has been frequently reported associated with water, so this remains a
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1
2
3 possibility. [47,48] However, it is possible that plumbing fittings are installed 'pre-
4
5 seeded' with *P. aeruginosa* as has already been proposed by Kelsey.[3,5,45]

6
7 Investigation of an outbreak in Wales implicated new plumbing parts as a potential
8
9 source of *P. aeruginosa*. New plumbing components are often tested by companies
10
11 prior to their supply and it is possible they were contaminated prior to distribution.

12
13 The limited amount of diversity (average 4 SNPs) seen within this clade is consistent
14
15 with a single founding genotype coinciding with the opening of the burns unit, based
16
17 on estimates from a previous study using WGS which reported that mutations
18
19 accumulate at a rate of approximately one every 3-4 months in a hospital-associated
20
21 clone.[49] However our results suggest that our isolates accumulate mutations even
22
23 more slowly. This may be due to reduced growth rates in nutritionally-poor
24
25 biofilms.[50]
26
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28

29
30 It is notable that antibiotic resistance to multiple first-line agents developed rapidly in
31
32 response to therapy. These results underline the importance of selecting appropriate
33
34 antibiotic therapy in *P. aeruginosa* infections. It is reassuring however that antibiotic
35
36 resistance genotypes selected *in vivo* did not show evidence of persistence in the
37
38 ward environment or transmission to other patients.
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42
43 Our study has certain limitations. Based on a previous audit, we expected around
44
45 one-third of patients screened for *P. aeruginosa* would develop colonisation or
46
47 clinical infection. In fact, only 5 out of 30 of patients were colonised. This may have
48
49 been related to guidance and engineering interventions being put in place during the
50
51 study as detailed in national guidance issued whilst this study was on-going. In
52
53 addition, infection control policies were revised to address control of an outbreak of a
54
55 multi-drug resistant *A. baumannii* in this same burns unit. Following these
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1
2
3 interventions, only 1 of the last 20 patients recruited was infected with *P. aeruginosa*
4
5 which may demonstrate the importance of national guidance in reducing
6
7 transmissions.
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10
11 By focusing on burns patients who receive hydrotherapy, our study population were
12
13 at extremely high risk of waterborne infection. In other patient groups it may be that
14
15 alternative routes of transmission including cross-infection or endogenous carriage
16
17 play a more important role. Our results suggest that our burns unit is endemically
18
19 colonised with a distinct clone of *P. aeruginosa* that may have been imported
20
21 coinciding with the opening of the hospital. Other intensive care units, particularly
22
23 those which have been open for longer may have harbour a greater diversity of *P.*
24
25 *aeruginosa* as a result of increased opportunities for clones to be imported.
26
27

28
29 One potential application for WGS in infection control would be to determine whether
30
31 cases are as a result of water transmission, or represent sporadic clones originating
32
33 from the wider environment. Despite improved guidance concerning improved
34
35 engineering infection control practices and the introduction of the water safety group
36
37 in the UK, it may not be realistic to eliminate *P. aeruginosa* from hospitals entirely. In
38
39 augmented care units such as ITUs, burns units and neonatal wards where *P.*
40
41 *aeruginosa* poses a significant risk to vulnerable patients, the increased resolution
42
43 offered by WGS will justify its use, particularly as the costs continue to fall.
44
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48
49 In conclusion, we have identified through WGS clear evidence for transmission of *P.*
50
51 *aeruginosa* from specific water outlets to burns patients and offer a forensic-level
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53 framework for dealing with outbreaks linked to hospital water. We expect WGS will
54
55 continue to make inroads into clinical microbiology and become a vital tool for
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tracking *P. aeruginosa* in the hospital environment, helping inform targeted control measures to help protect patients at risk of infection.

For peer review only

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Contributors

MP NM BO conceived the study. CW and AB enrolled patients into study and collected samples. NC collected environmental and water samples. NC CC MN processed samples and performed microbiology. NC CC JQ did sequencing. JQ NC NL analysed the data. NL NC JQ MP BO wrote the paper. All authors commented on the manuscript draft.

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Competing interests

None

Data Sharing Statement

Pacific Biosciences raw data files are available from the corresponding author (Nicholas J Loman, n.j.loman@bham.ac.uk) and will be made available via Data Dryad.

Ethics approval

The study protocol received approval from National Research Ethics Service committee in the West Midlands (reference number 12/WM/0181).

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Figure and Table Legends

Figure 1 Legend

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48 An overview of all samples collected during the study in global phylogenetic context
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50 with other sequenced strains of *P. aeruginosa*. Samples collected in this study are
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52 widely dispersed in the tree, which contains isolates from different environments
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54 (Panel A). Bar plots indicate the numbers of each type of sample collected (Panel C).
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3 Microdiversity within each clade is shown, with the colour bar indicating the source of
4 each sample (Panel C).
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8 9 **Figure 2 Legend**

10
11 A schematic view of the 300-day study of *P. aeruginosa* in a burns centre and critical
12 care unit. Time in days is shown along the X-axis with location along the Y-axis.
13

14 Each circular icon indicates a positive isolate of *P. aeruginosa*. The icon's logotype
15 indicates which environment it originated from (wound, urine/sputum, environment or
16 water). The filled colour of the icon indicates the clade it belongs to. Patient icons
17 represent the enrolment of a screening patient into the study and their location.
18 Patient movements around the hospital are noted by dotted lines. The five patients
19 infected with *P. aeruginosa* are denoted by rounded boxes. Boxes are coloured
20 according to the patient number.
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32 33 **Figure 3 Legend**

34
35 The high-resolution phylogenetic reconstruction of clade E isolates. This
36 demonstrates the clustering of genotypes by bed space. Patient associated samples
37 are contained within the room 11 clade. This clade contains water samples from the
38 shower and environmental samples from the shower, drain and trolley. The water
39 samples from the room 11 tap are in a distinct clade, indicating the biofilm within the
40 tap has a distinct genotype to the shower. This also indicated environmental
41 contamination was more likely to arise from contaminated shower water than tap
42 water. Details of sampling site, days since start of study and presence of pBURNS
43 plasmids are also shown. The likely phylogenetic position of *P. aeruginosa* detected
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3 in a biofilm from a thermostatic mixer valve is shown in the clade associated with
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5 room 9 and indicated 'TMV'.
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8 **Appendix 1**

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11 Phylogenetic tree, sample origin and antibiotic-resistance phenotype for clade C
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13 isolates.
14

15 **Appendix 2**

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18 Phylogenetic tree, sample origin and antibiotic-resistance phenotype for clade D
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20 isolates.
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22

23 **Appendix 3**

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26 Phylogenetic tree, sample origin and antibiotic-resistance phenotype for clade E
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28 isolates.
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31 **Appendix 4**

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34 Phylogenetic tree, sample origin and antibiotic-resistance phenotype for clade G
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36 isolates.
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39 **Appendix 5**

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42 A physical map of the burns unit, indicating individual patient bed areas, shower
43
44 areas. The water supply is indicated by a blue line.
45

46 **Appendix 6**

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49 Clade E isolates in phylogenetic context with the metagenomics sample from a tap
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51 mixer valve.
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54 **Appendix 7**

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57 Single nucleotide and small indel variants detected within clade C isolates.
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Appendix 8

Single nucleotide and small indel variants detected within clade D isolates.

Appendix 9

Single nucleotide and small indel variants detected within clade E isolates.

Appendix 10

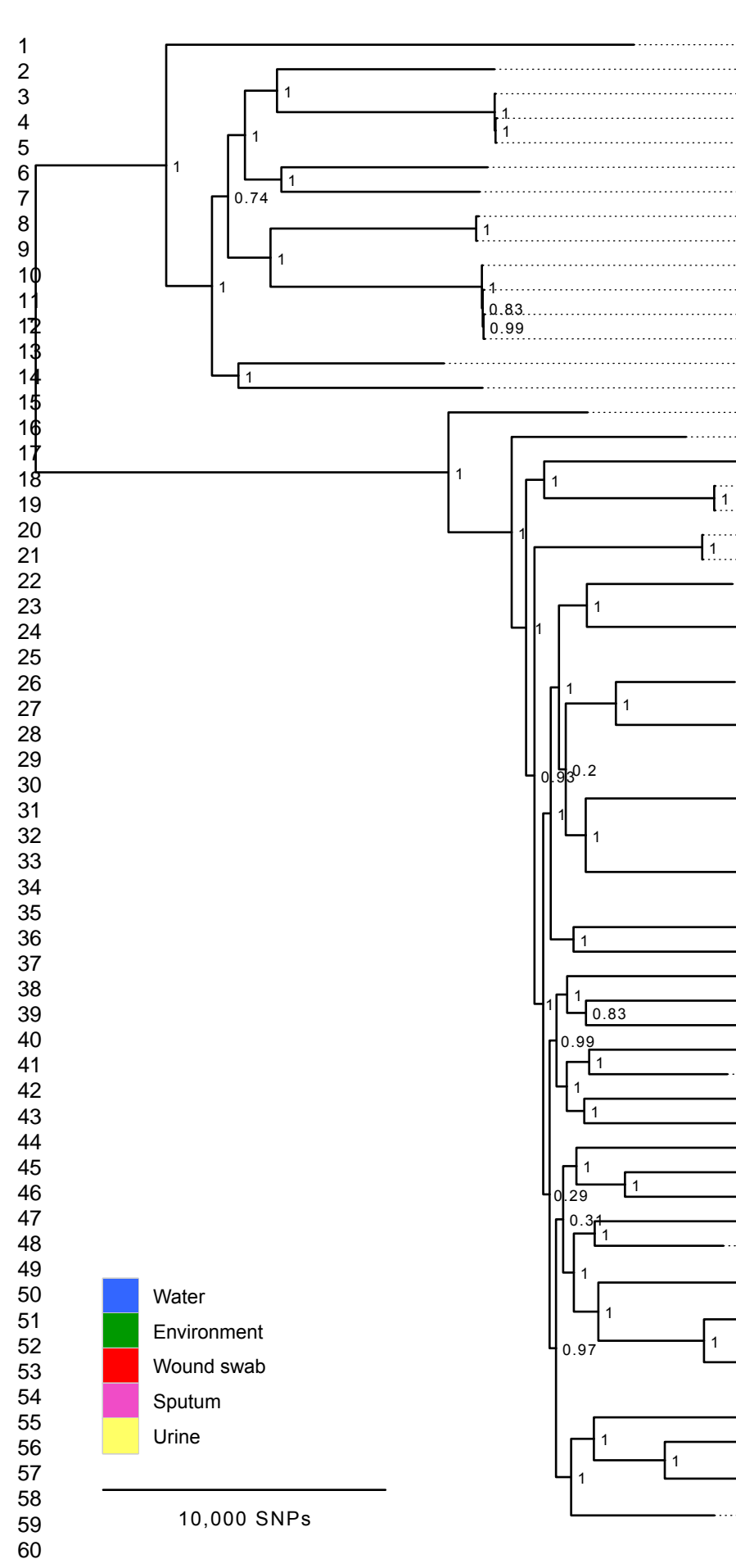
Single nucleotide and small indel variants detected within clade G isolates.

Appendix 11

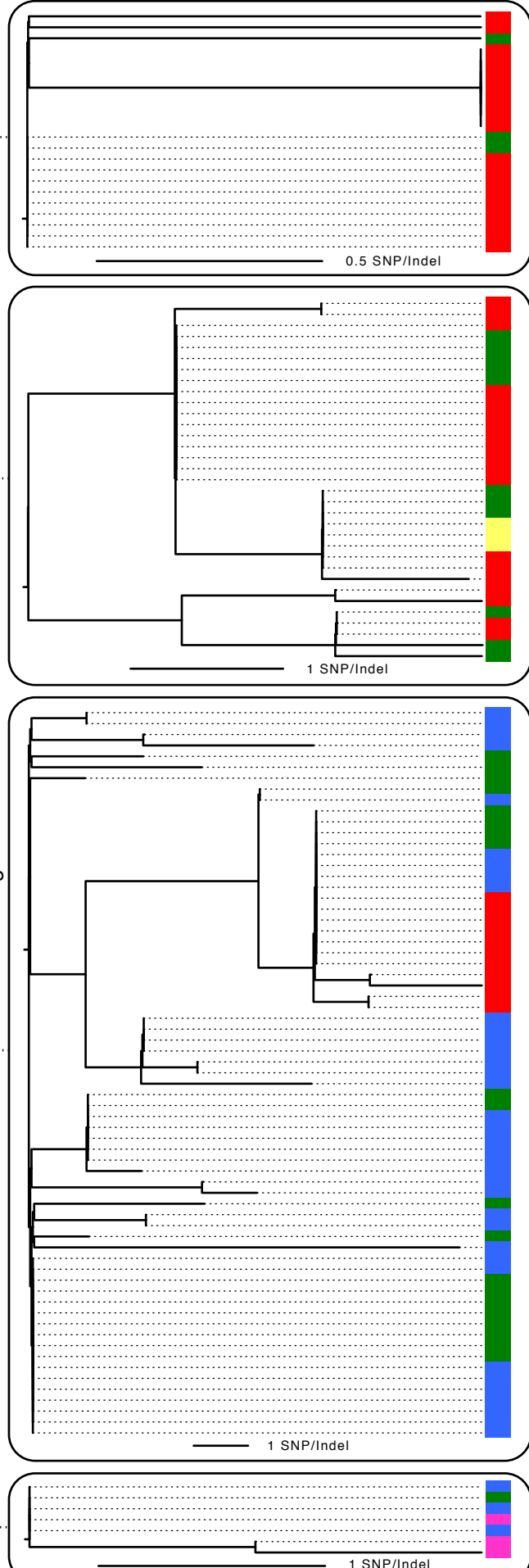
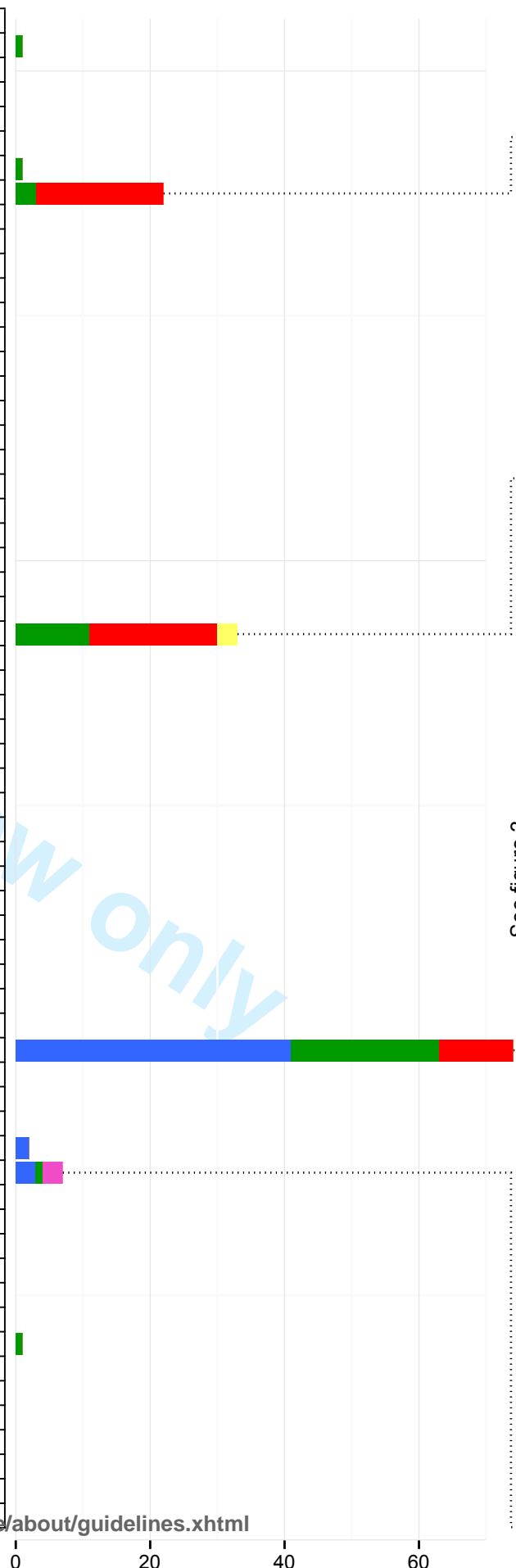
The type of antibiotics administered to the five patients in this study.

Appendix 12

Mutations predicted to be associated with antibiotic-resistance.



Strain	MLST	Origin/comment
Clade A	667	This study
PABL056	298	Blood, USA
E2UOS	235	Soil
NCGM2.S1	235	Urinary tract infection
39016	235	Keratitis
Clade B	Novel	This study
Clade C	319	This study
152504	560	Sputum, non-CF
PAb1	560	Frostbite
CI27	Novel	Sputum, CF
PA14	Novel	Plant, USA
J1532		CF, mucoid
J1385		CF, progenitor of J1532
ATCC 700888		Industrial water system
B136.33	1024	Diarrhoea
18A	788	CF
PAK	693	PAK
2192		CF
PA17	810	CF, progenitor of PA17SCV
PA17SCV	810	Lab evolved from CF Isolate
C1426		CF, progenitor of C1433
C1433	132	CF, mucoid
NCMG1179	1285	Respiratory, Japan
ATCC 25324	Novel	Glass-crusher air plate
Clade D	348	This study
CIG1	348	Sputum, CF
DQ8	267	Crude oil, China
MRW44.1	Novel	Mutator lineage 44
PA01	Novel	Burn
PA0579	Novel	Mucoid
MSH3		Environmental, Mount St. Helens
MSH-10	689	Environmental
MSH10 UoS	689	Environmental, Mount St. Helens
PA213BR	277	Polymixin resistant, Brazil
PA9BR	277	Brazil, non-CF
PA19BR	277	Polymixin resistant, Brazil
ATCC 14886	260	Soil
N002		Crude oil, India
AES-1R		Australia, CF
E2UoW	41	Plant, USA
VRFPA02	111	Eye, India
Clade E	395	This study
LESB58	146	Liverpool epidemic strain
PA62		Soil
C1334	439	CF, mucoid, from same patient as C763
Clade F	Novel	This study
Clade G	17	This study
PACS2	1394	CF
PA45	266	Blood, Italy
XMG	Novel	Soil, China
138244	175	Sputum, non-CF
PA21_ST175	175	Blood, MDR
C763	179	CF, from same patient as C1334
Clade H	155	This study
AH16	155	China, sputum, non-CF
LCT-PA102	155	Blood
SJTD-1	Novel	Crude oil, China
C3719		Manchester epidemic strain
DK2	386	CF, Denmark
DK2_CF510	386	CF, Denmark
M18	1239	Plant isolate, China



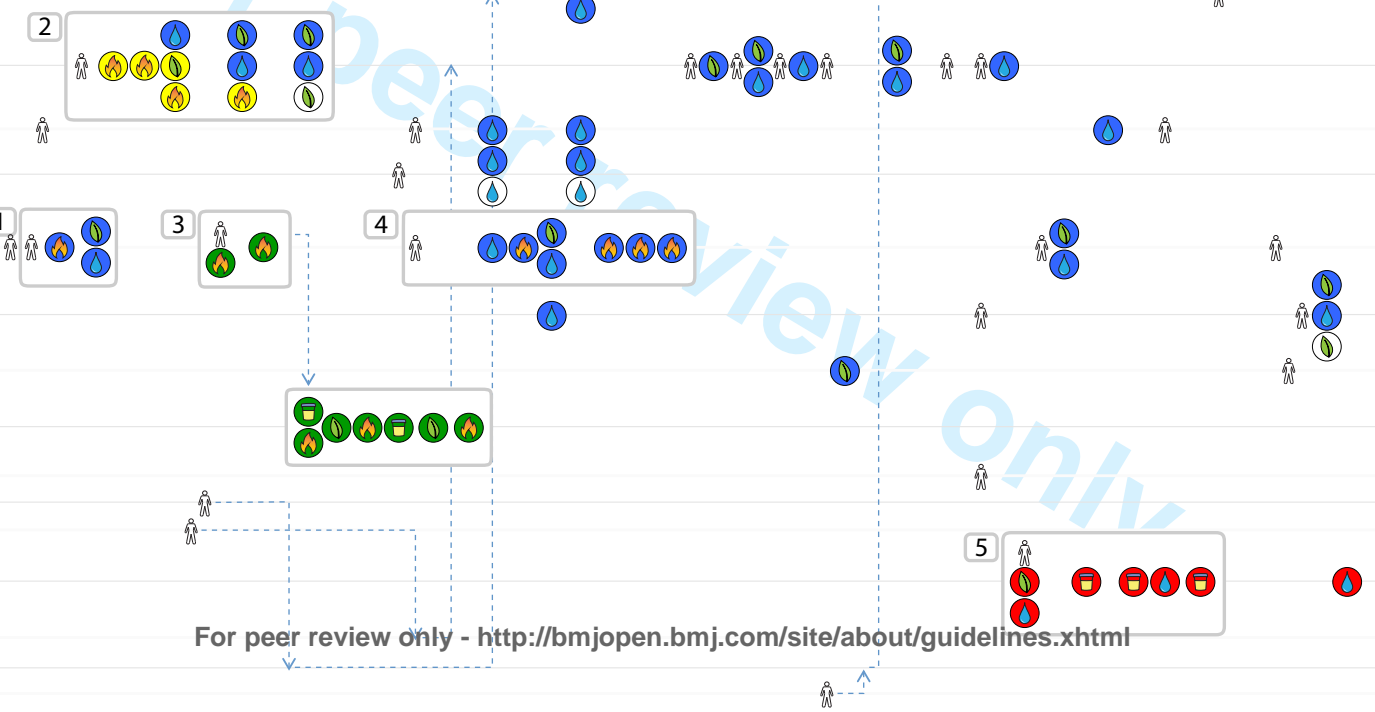
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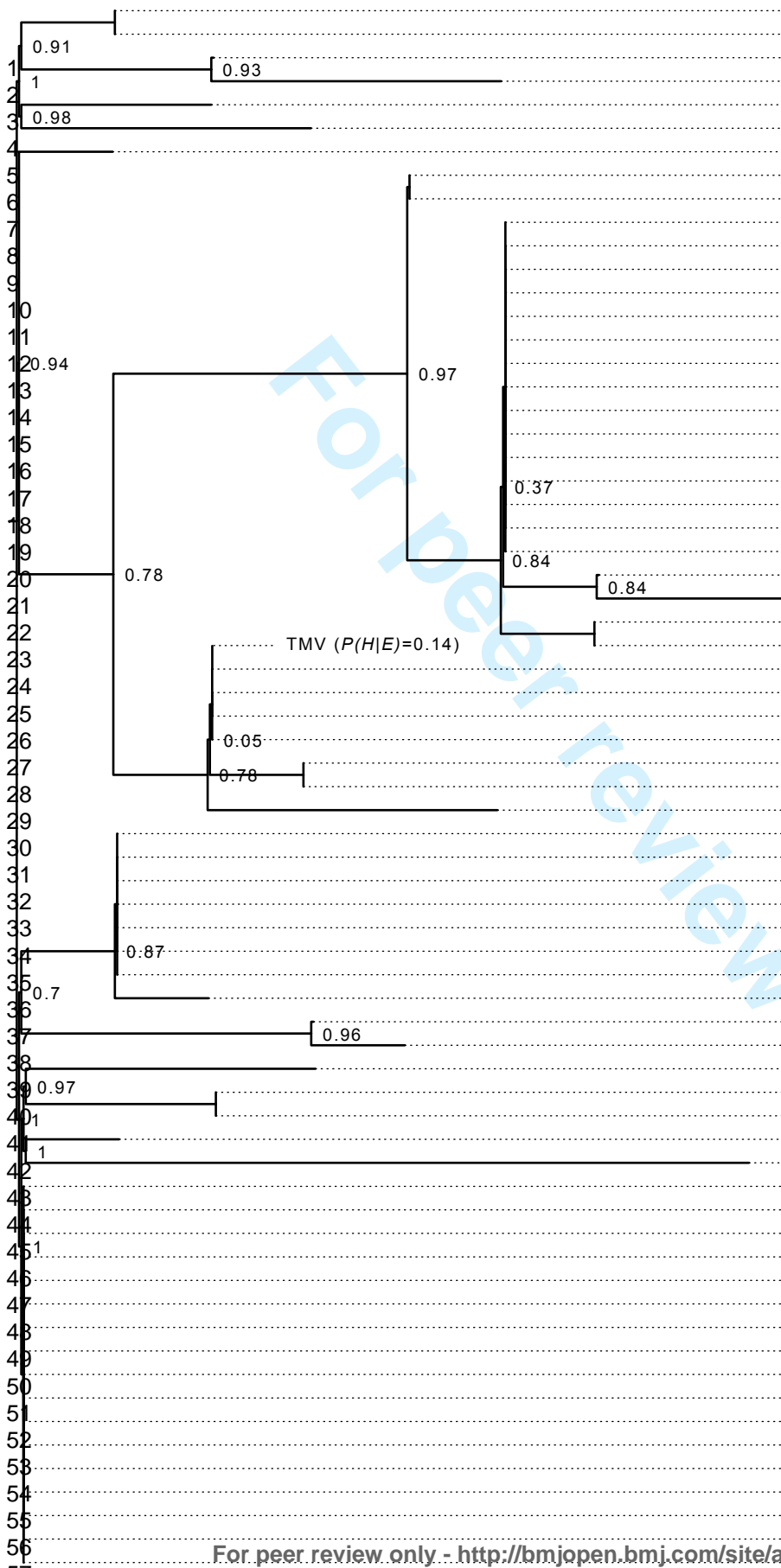
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Legend:

- Yellow circle: Clade C
- Green circle: Clade D
- Blue circle: Clade E
- Red circle: Clade G
- White circle: Other clade
- Water drop icon: Water
- Burn icon: Burn
- Leaf icon: Environmental
- Urine/sputum icon: Urine/sputum
- Person icon: Screening patient
- White box icon: Positive patient
- Dashed arrow icon: Bed movement



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Bed	Type of Specimen	Days	pB1	pB2
12	Shower	292		
	Shower	292		
10	Shower	90		
	Shower	65		
8	Shower	49		
12	Shower	292		
8	Shower	176		
	Drain	27		
	Shower	27		
	Drain	89		
	Shower	89		
	Shower	216		
	Trolley	27		
	Shower	89		
	Shower	216		
	Shower	89		
	Shower	65		
11	Groin	14		
	Anterior right thigh	53		
	Anterior right thigh	103		
	Anterior right shin	53		
	Dorsum of left foot	85		
	Dorsum of left foot	85		
	Dorsum of left foot	85		
	Anterior right thigh	93		
	Anterior right thigh	103		
	Anterior right thigh	91		
	Anterior right thigh	91		
	Shower	226		
9	Shower	90		
	Shower	90		
	Tap	90		
11	Tap	89		
	Tap	89		
9	Shower	65		
	Shower	292		
	Shower	278		
	Shower	91		
6	Shower	292		
	Shower	177		
	Shower	91		
	Shower	278		
	Shower	190		
	Shower	91		
1	Shower	91		
8	Sink	113		
12	Shower	89		
	Shower	89		
6	Shower	190		
8	Shower	155		
5	Tap	91		
7	Shower	90		
	Drain	146		
	ECG leads	49		
	Shower	49		
	Shower	146		
	Shower	55		
	Shower	55		
	Shower	40		
8	Shower	40		
	Shower	49		
	Shower	146		
	Shower	176		
	Shower	40		
	Shower	49		
	Shower	203		
	Shower	55		

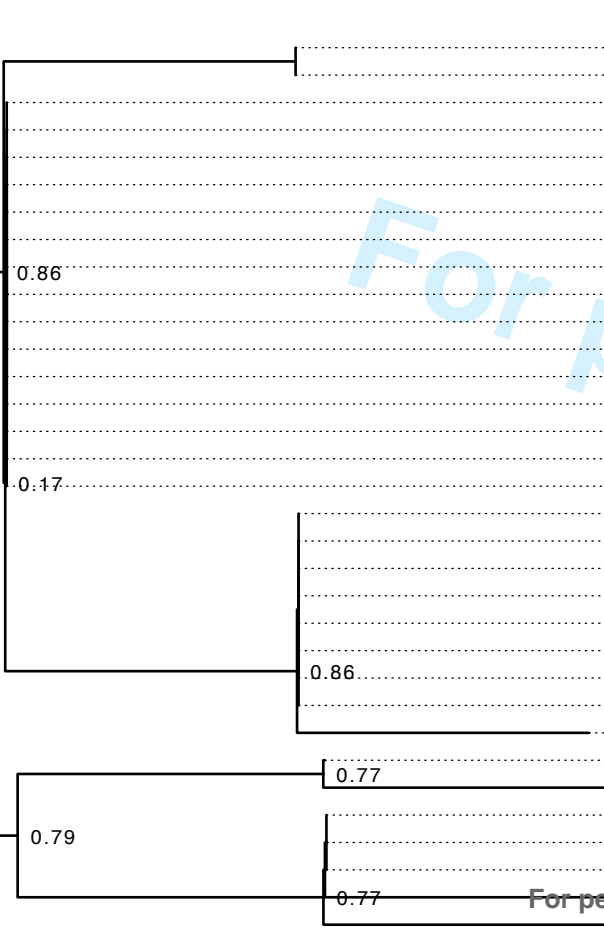
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Sample Number	Patient	Ward	Bed	Type of Specimen	Site	Date	TIM	PIP	TAZ	CAZ	AZT	IMI	MER	AMI	GEN	TOP	GIP	GOL
922	P02	BCU	8	Wound swab	Upper back	29/10/12	R	I	I	S	I	S	S	S	S	S	S	S
921	P02	BCU	8	Wound swab	Upper back	29/10/12												
913	P02	BCU	8	Environmental	Shower trolley	29/10/12	R	S	S	S	I	S	S	S	S	S	S	S
919	P02	BCU	8	Wound swab	Chest	29/10/12	R	I	I	S	I	I	S	S	S	S	R	S
926	P02	BCU	8	Wound swab	Right palm	02/11/12	R	I	I	S	I	I	S	S	S	S	R	S
909	P02	BCU	8	Wound swab	Anterior left upper-arm	26/10/12	R	I	I	S	I	R	S	S	S	S	R	S
908	P02	BCU	8	Wound swab	Anterior left upper-arm	26/10/12	R	I	I	S	I	S	S	S	S	S	S	S
925	P02	BCU	8	Wound swab	Anterior left upper-arm	02/11/12	R	I	I	S	I	I	S	S	S	S	R	S
932	P02	BCU	8	Wound swab	Back of head	07/11/12	R	I	I	S	I	R	S	S	S	S	R	S
928	P02	BCU	8	Wound swab	Upper back	02/11/12	R	I	I	S	I	I	I	S	S	S	R	S
927	P02	BCU	8	Wound swab	Upper back	02/11/12	R	I	I	S	I	I	I	S	S	S	R	S
915	P02	BCU	8	Environmental	Chair	29/10/12	R	I	I	S	I	S	S	S	S	S	S	S
914	P02	BCU	8	Environmental	Chair	29/10/12	R	S	S	S	I	S	S	S	S	S	S	S
904	P02	BCU	8	Tissue	Anterior right upper-arm	22/10/12	R	S	S	S	I	S	S	S	S	S	S	S
905	P02	BCU	8	Wound swab	Chest	26/10/12	R	I	I	S	I	I	S	S	S	S	S	S
906	P02	BCU	8	Wound swab	Chest	26/10/12	R	I	I	S	I	I	S	S	S	S	S	S
920	P02	BCU	8	Wound swab	Chest	29/10/12	R	I	I	S	I	R	I	S	S	S	R	S
918	P02	BCU	8	Wound swab	Chest	29/10/12	R	I	I	S	I	S	S	S	S	S	S	S
907	P02	BCU	8	Wound swab	Abdomen	26/10/12	R	I	I	S	I	S	S	S	S	S	S	S
911	P02	BCU	8	Wound swab	Anterior left forearm	26/10/12	R	I	I	S	I	I	S	S	S	S	S	S
912	P02	BCU	8	Wound swab	Anterior left forearm	26/10/12	R	I	I	S	I	I	S	S	S	S	S	S
923	P02	BCU	8	Wound swab	Posterior left upper-arm	29/10/12	R	I	I	S	I	S	S	S	S	S	S	S

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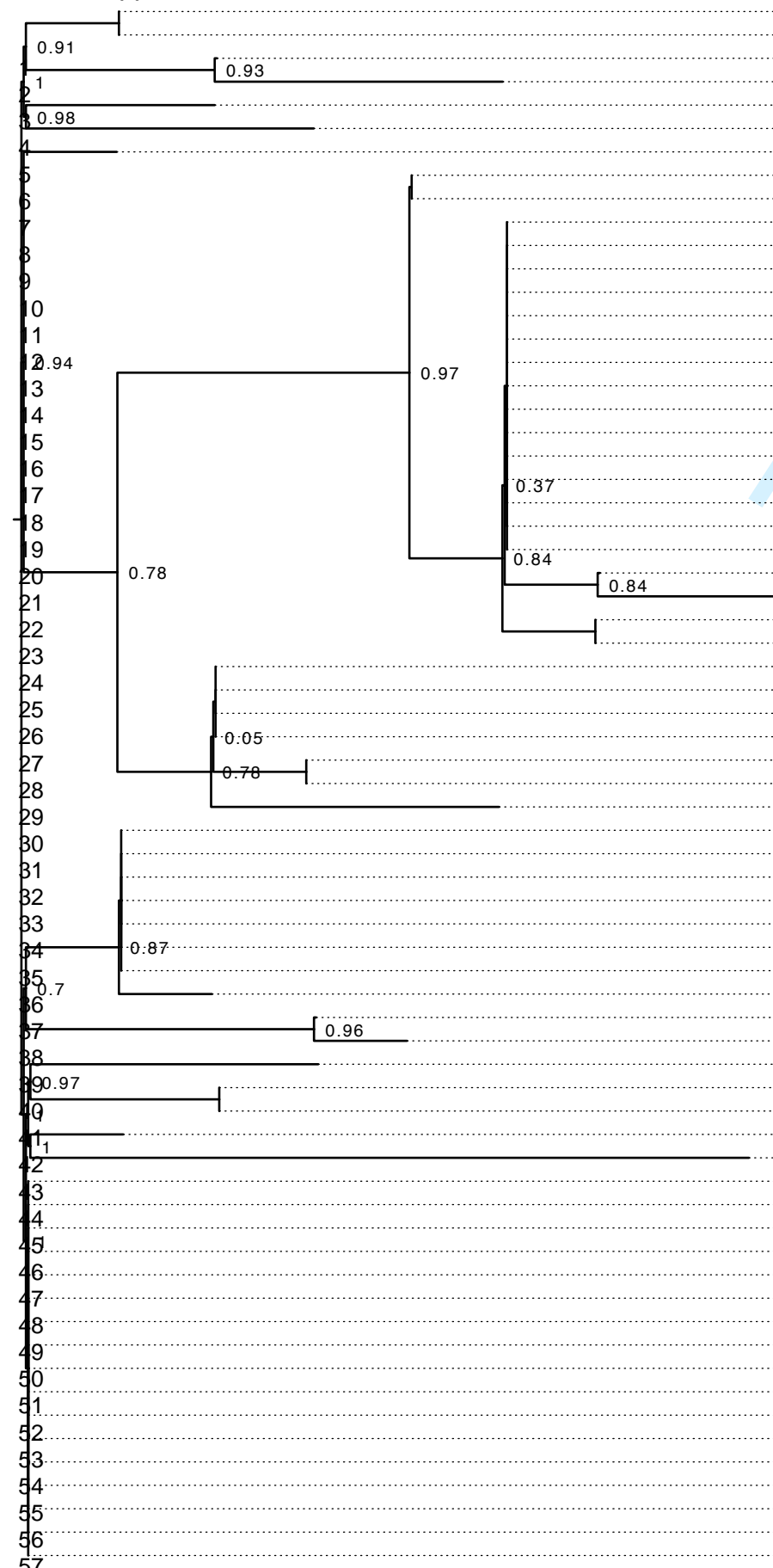
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Study Number	Patient	BMW	Open Bed	Type of Specimen	Site	Date	TIM	PIP	TAZ	CAZ	AZT	IMI	MER	AMI	GEN	TOB	CIP	COL
937	P03	BCU	11	Wound swab	Anterior left thigh	08/11/12												
936	P03	BCU	11	Wound swab	Anterior left thigh	08/11/12												
929	P03	BCU	11	Wound swab	Posterior left thigh	06/11/12	R	S	S	S	I	R	I	S	S	S	S	S
951	P03	BCU	15	Environmental	Bedside table	14/11/12	R	I	I	S	I	R	I	S	S	S	S	S
960	P03	BCU	15	Environmental	Bedside table	20/11/12	R	S	S	S	I	R	I	S	S	S	S	S
961	P03	BCU	15	Environmental	Door handle	20/11/12	R	I	I	S	I	R	I	S	S	S	S	S
958	P03	BCU	15	Environmental	Shower chair	20/11/12	R	I	I	S	I	R	I	S	S	S	S	S
957	P03	BCU	15	Environmental	Shower chair	20/11/12	R	S	S	S	I	R	I	S	S	S	S	S
940	P03	BCU	15	Wound swab	Anterior right thigh	13/11/12	S	S	S	S	I	I	I	S	S	S	S	S
970	P03	BCU	15	Wound swab	Anterior right thigh	22/11/12	R	I	I	S	I	R	I	S	S	S	S	S
944	P03	BCU	15	Wound swab	Anterior right shin	13/11/12	R	I	I	S	I	R	I	S	S	S	S	S
945	P03	BCU	15	Wound swab	Anterior right shin	13/11/12	R	S	S	S	I	R	I	S	S	S	S	S
946	P03	BCU	15	Wound swab	Anterior left thigh	13/11/12	R	S	S	S	I	R	I	S	S	S	S	S
954	P03	BCU	15	Wound swab	Anterior left thigh	15/11/12	R	I	I	S	I	R	I	S	S	S	S	S
947	P03	BCU	15	Wound swab	Anterior left shin	13/11/12	R	S	S	S	I	R	I	S	S	S	S	S
972	P03	BCU	15	Wound swab	Posterior left shin	22/11/12	S	S	S	S	I	R	I	S	S	S	S	S
949	P03	BCU	15	Wound swab	Posterior right thigh	13/11/12	R	I	I	S	I	R	I	S	S	S	S	S
950	P03	BCU	15	Environmental	Bedside table	14/11/12	R	S	S	S	I	R	I	S	S	S	S	S
959	P03	BCU	15	Environmental	Bedside table	20/11/12	R	I	I	S	I	R	I	S	S	S	S	S
953	P03	BCU	15	Environmental	Toilet flush	14/11/12	R	S	S	S	I	I	I	S	S	S	S	S
938	P03	BCU	15	Urine	Urine	13/11/12	R	S	S	S	I	R	I	S	S	S	S	S
939	P03	BCU	15	Urine	Urine	13/11/12	R	S	S	S	I	R	I	S	S	S	S	S
956	P03	BCU	15	Urine	Urine	16/11/12	R	S	S	S	I	R	I	S	S	S	S	S
969	P03	BCU	15	Wound swab	Anterior right thigh	22/11/12	R	S	S	S	I	R	I	S	S	S	S	S
971	P03	BCU	15	Wound swab	Anterior left thigh	22/11/12	R	I	I	S	I	R	I	S	S	S	S	S
973	P03	BCU	15	Wound swab	Posterior right thigh	22/11/12	R	S	S	S	I	R	I	S	S	S	S	S
955	P03	BCU	15	Wound swab	Anterior left thigh	15/11/12	S	S	S	S	I	R	I	S	S	S	S	S
975	P03	BCU	15	Wound swab	Posterior right shin	22/11/12	S	S	S	S	I	R	I	S	S	S	S	S
962	P03	BCU	15	Environmental	Shower chair	20/11/12	S	S	S	S	I	R	I	S	S	S	S	S
948	P03	BCU	15	Wound swab	Posterior right thigh	13/11/12	S	S	S	S	I	R	I	S	S	S	S	S
974	P03	BCU	15	Wound swab	Posterior right thigh	22/11/12	S	S	S	S	I	I	I	S	S	S	S	S
963	P03	BCU	15	Environmental	Bedside table	20/11/12	S	S	S	S	I	I	I	S	S	S	S	S
964	P03	BCU	15	Environmental	Bedside table	20/11/12	S	S	S	S	I	R	I	S	S	S	S	S

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Sample Number	Patient	Ward	BMJ	Open of Specimen	Site	Date	TIM	PIP	TAZ	CAZ	AZT	IMI	MER	AMI	GEN	TOB	CIP	COL	pBURNS	Page 28 of 48
1067	SP30	BCU	12	Water	Shower (Pre-flush)	08/07/13														
1068	SP30	BCU	12	Water	Shower (Pre-flush)	08/07/13														
991	Water sampling	BCU	10	Water	Shower (Pre-flush)	18/12/12														
966	SP08	BCU	10	Water	Shower (Unknown)	23/11/12	R	S	S	S	I	S	S	S	S	S	S	S		
931	P02	BCU	8	Environmental	Shower (Rose)	07/11/12	R	S	S	S	I	S	S	S	S	S	S	S		
1065	SP30	BCU	12	Environmental	Shower (Rose)	08/07/13														
1034	SP15	BCU	8	Environmental	Shower (Rose)	14/03/13	R	S	S	S	R	S	S	S	S	S	S	S	Yes	
902	P01	BCU	11	Environmental	Drain	16/10/12														
903	P01	BCU	11	Water	Shower (Post-flush)	16/10/12	R	S	S	S	I	S	S	S	S	S	S	S		
981	P04	BCU	11	Environmental	Drain	17/12/12	R	S	S	S	I	S	S	S	S	S	S	S		
980	P04	BCU	11	Environmental	Shower (Rose)	17/12/12	R	S	S	S	I	S	S	S	S	S	S	S		
1048	SP20	BCU	11	Environmental	Shower (Rose)	23/04/13	R	S	S	S	I	S	S	S	S	S	S	S		
901	P01	BCU	11	Environmental	Trolley	16/10/12	R	S	S	S	I	S	S	S	S	S	S	S		
987	P04	BCU	11	Water	Shower (Hose)	17/12/12	R	S	S	S	I	S	S	S	S	S	S	S		
1050	SP21	BCU	11	Water	Shower (Post-flush)	23/04/13														
988	P04	BCU	11	Water	Shower (Pre-flush)	17/12/12	R	S	S	S	I	S	S	S	S	S	S	S		
968	SP10	BCU	11	Water	Shower (Unknown)	23/11/12	R	S	S	S	I	S	S	S	S	S	S	S		
900	P01	BCU	11	Wound swab	Groin	03/10/12	R	S	S	S	I	S	S	S	S	S	S	S		
979	P04	BCU	11	Wound swab	Anterior right thigh	11/11/12	R	S	S	S	I	S	S	S	S	S	S	S		
1008	P04	BCU	11	Wound swab	Anterior right thigh	31/12/12	R	S	S	S	I	S	S	S	S	S	S	S		
976	P04	BCU	11	Wound swab	Anterior right shin	11/11/12	R	S	S	S	I	S	S	S	S	S	S	S		
984	P04	BCU	11	Wound swab	Dorsum of left foot	13/12/12	R	S	S	S	I	R	I	S	S	S	S	S		
983	P04	BCU	11	Wound swab	Dorsum of left foot	13/12/12	R	S	S	S	I	R	I	S	S	S	S	S		
982	P04	BCU	11	Wound swab	Dorsum of left foot	13/12/12	R	S	S	S	I	S	S	S	S	S	S	S		
1007	P04	BCU	11	Wound swab	Anterior right thigh	21/12/12	R	S	S	S	I	R	I	S	S	S	S	S		
1009	P04	BCU	11	Wound swab	Anterior right thigh	31/12/12	R	S	S	S	I	S	S	S	S	S	S	S		
1005	P04	BCU	11	Wound swab	Anterior right thigh	19/12/12	R	S	S	S	I	R	I	S	S	S	S	S		
1006	P04	BCU	11	Wound swab	Anterior right thigh	19/12/12	R	S	S	S	I	R	I	S	S	S	S	S		
1052	SP22	BCU	9	Water	Shower (Hose)	03/05/13													Yes	
993	Water sampling	BCU	9	Water	Shower (Hose)	18/12/12	R	S	S	S	I	S	S	S	S	S	S	S		
994	Water sampling	BCU	9	Water	Shower (Pre-flush)	18/12/12	R	S	S	S	I	S	S	S	S	S	S	S		
992	Water sampling	BCU	9	Water	Tap	18/12/12	R	S	S	S	I	S	S	S	S	S	S	S		
985	P04	BCU	11	Water	Tap	17/12/12	R	S	S	S	I	S	S	S	S	S	S	S		
986	P04	BCU	11	Water	Tap	17/12/12														
967	SP09	BCU	9	Water	Shower (Unknown)	23/11/12	R	S	S	S	I	S	S	S	S	S	S	S	Yes	
1062	SP29	BCU	6	Environmental	Shower (Rose)	08/07/13														
1058	SP25	BCU	6	Environmental	Shower (Rose)	24/06/13														
1003	Water sampling	BCU	6	Water	Shower (Hose)	19/12/12														
1064	SP29	BCU	6	Water	Shower (Pre-flush)	08/07/13														
1036	SP15	BCU	6	Water	Shower (Pre-flush)	15/03/13														
1004	Water sampling	BCU	6	Water	Shower (Pre-flush)	19/12/12														
1057	SP25	BCU	6	Water	Shower (Pre-flush)	24/06/13	R	S	S	S	I	S	S	S	S	S	S	S		
1041	SP16	BCU	6	Water	Shower (Pre-flush)	28/03/13														
999	Water sampling	BCU	1	Water	Shower (Hose)	19/12/12														
1000	Water sampling	BCU	1	Water	Shower (Pre-flush)	19/12/12														
1010	SP11	BCU	8	Environmental	Sink	10/01/13													Yes	
989	Water sampling	BCU	12	Water	Shower (Hose)	17/12/12	R	S	S	S	I	S	S	S	S	S	S	S		
990	Water sampling	BCU	12	Water	Shower (Pre-flush)	17/12/12	R	S	S	S	I	S	S	S	S	S	S	S		
1040	SP16	BCU	6	Environmental	Shower (Rose)	28/03/13	R	S	S	S	I	S	S	S	S	S	S	S		
1031	SP13	BCU	8	Water	Shower (Hose)	21/02/13													Yes	
1001	Water sampling	BCU	5	Water	Tap	19/12/12														
996	Water sampling	BCU	7	Water	Shower (Pre-flush)	18/12/12	R	S	S	S	I	S	S	S	S	S	S	S		
1029	SP12	BCU	8	Environmental	Drain	12/02/13													Yes	
933	P02	BCU	8	Environmental	EGC	07/11/12	R	S	S	S	I	S	S	S	S	S	S	S		
930	P02	BCU	8	Environmental	Shower (Rose)	07/11/12	R	S	S	S	I	R	S	S	S	S	S	S		
1028	SP12	BCU	8	Environmental	Shower (Rose)	12/02/13													Yes	
942	P02	BCU	8	Environmental	Shower (Rose)	13/11/12	R	S	S	S	I	S	S	S	S	S	S	S		
941	P02	BCU	8	Environmental	Shower (Rose)	13/11/12	R	S	S	S	I	S	S	S	S	S	S	S		
917	P02	BCU	8	Environmental	Shower (Rose)	29/10/12	S	S	S	S	I	S	S	S	S	S	S	S	Yes	
916	P02	BCU	8	Environmental	Shower (Rose)	29/10/12	S	S	S	S	I	S	S	S	S	S	S	S	Yes	
934	P02	BCU	8	Water	Shower (Post-flush)	07/11/12	S	S	S	S	I	S	S	S	S	S	S	S	Yes	
1030	SP12	BCU	8	Water	Shower (post-flush)	12/02/13													Yes	
1035	SP14	BCU	8	Water	Shower (Post-flush)	14/03/13													Yes	
910	P02	BCU	8	Water	Shower (Post-flush)	29/10/12	S	S	S	S	I	S	S	S	S	S	S	S	Yes	
935	P02	BCU	8	Water	Shower (Pre-flush)	07/11/12	R	S	S	S	I	S	S	S	S	S	S	S	Yes	
1046	SP19	BCU	8	Water	Shower (Pre-flush)	10/04/13														
943	P02	BCU	8	Water	Shower (Pre-flush)	13/11/12	S	S	S	S	I	S	S	S	S	S	S	S	Yes	

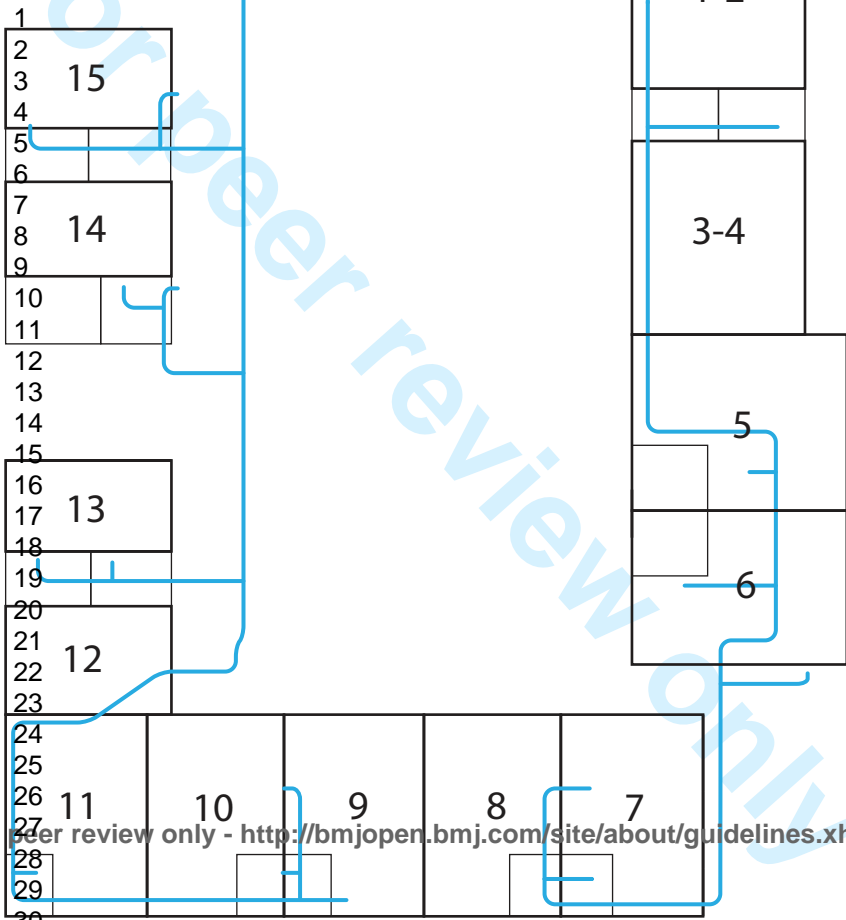
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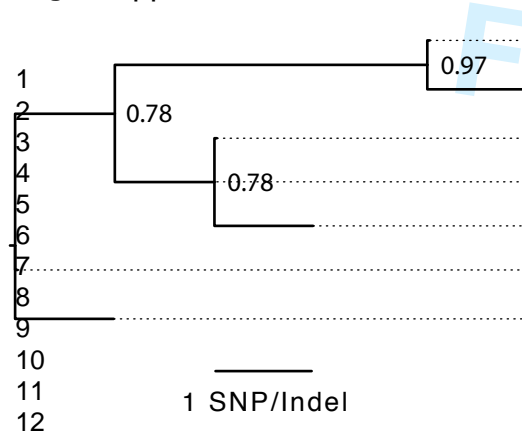
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Study Number	Patient	WCCB	WU Open	Type of Specimen	Site	Date	TIM	PIP	TAZ	CAZ	AZT	IMI	MER	AMI	GEN	TOB	CIP	COL
1069	Water sampling	WCCB	11	Water	Tap	17/07/13	R	S	S	S	I	R	I	S	S	S	S	S
1045	SP20	WCCB	11	Environmental	Tap handle	11/04/13												
1047	SP20	WCCB	11	Water	Tap	11/04/13	R	S	S	S	I	R	I	I	R	S	S	S
1049	P05	WCCB	11	Sputum	Sputum	28/04/13		I	I	S	I	I	I	I	R	S	S	S
1053	P05	WCCB	11	Water	Tap	08/05/13	R	I	I	S	I	R	S	S	S	S	S	S
1054	P05	WCCB	11	Sputum	Sputum	06/05/13	R	R	R	R	R	R	R	I	R	S	I	S
1056	P05	WCCB	11	Sputum	Sputum	19/05/13		R	R	R	S	R	R		S	S	R	S

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Bed	Type of Specimen	Alignment
11	Shower	T CCTACTCC - CACAGACCTAACCT - - - - - G - - ACCGAAATCCTTTTCCG - C GAGGCG
11	Shower	T CCTACTCC - TACAGACCTAACCT - - - - - G - - ACCGAAATCCTTTTCCG - C GAGGCG
9	Tap	T GCAACTCC - CACAGACCTAACGT - - - - - G - - ACCGAAATCCTTTTCCG - C GC GGCG
9	TMV	T GCA - CT - C - CACAGA - - - AACGT - - - - - G - - ACCG - - A - CCTTTTC - G - C - - GGCG
11	Tap	T GTA ACTCC - CACAGACCTAACGT - - - - - G - - ACCGAAATCCTTTTCCG - C GC GGCG
8	Shower	T CCAACTCC - CACAGACCTAACGT - - - - - G - - ACCGAAATCCTTTTCCG - C TC GGCG
6	Shower	T CCAACTCC - CACAGCCTAACGT - - - - - G - - ACCGAAATCCTTTTCCG - C TC GGCG

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Study	Year	Age	Sex	Site	Study design	Outcome	Effect size	95% CI	Quality score	Notes
1	2008	18-24	M	USA	Case-control	MI	1.5	1.2-1.8	10	
2	2009	18-24	M	USA	Case-control	MI	1.2	1.0-1.4	10	
3	2010	18-24	M	USA	Case-control	MI	1.8	1.5-2.1	10	
4	2011	18-24	M	USA	Case-control	MI	1.4	1.1-1.7	10	
5	2012	18-24	M	USA	Case-control	MI	1.6	1.3-1.9	10	
6	2013	18-24	M	USA	Case-control	MI	1.3	1.0-1.6	10	
7	2014	18-24	M	USA	Case-control	MI	1.7	1.4-2.0	10	
8	2015	18-24	M	USA	Case-control	MI	1.5	1.2-1.8	10	
9	2016	18-24	M	USA	Case-control	MI	1.4	1.1-1.7	10	
10	2017	18-24	M	USA	Case-control	MI	1.6	1.3-1.9	10	
11	2018	18-24	M	USA	Case-control	MI	1.3	1.0-1.6	10	
12	2019	18-24	M	USA	Case-control	MI	1.7	1.4-2.0	10	
13	2020	18-24	M	USA	Case-control	MI	1.5	1.2-1.8	10	
14	2021	18-24	M	USA	Case-control	MI	1.4	1.1-1.7	10	
15	2022	18-24	M	USA	Case-control	MI	1.6	1.3-1.9	10	
16	2023	18-24	M	USA	Case-control	MI	1.3	1.0-1.6	10	
17	2024	18-24	M	USA	Case-control	MI	1.7	1.4-2.0	10	
18	2025	18-24	M	USA	Case-control	MI	1.5	1.2-1.8	10	
19	2026	18-24	M	USA	Case-control	MI	1.4	1.1-1.7	10	
20	2027	18-24	M	USA	Case-control	MI	1.6	1.3-1.9	10	
21	2028	18-24	M	USA	Case-control	MI	1.3	1.0-1.6	10	
22	2029	18-24	M	USA	Case-control	MI	1.7	1.4-2.0	10	
23	2030	18-24	M	USA	Case-control	MI	1.5	1.2-1.8	10	
24	2031	18-24	M	USA	Case-control	MI	1.4	1.1-1.7	10	
25	2032	18-24	M	USA	Case-control	MI	1.6	1.3-1.9	10	
26	2033	18-24	M	USA	Case-control	MI	1.3	1.0-1.6	10	
27	2034	18-24	M	USA	Case-control	MI	1.7	1.4-2.0	10	
28	2035	18-24	M	USA	Case-control	MI	1.5	1.2-1.8	10	
29	2036	18-24	M	USA	Case-control	MI	1.4	1.1-1.7	10	
30	2037	18-24	M	USA	Case-control	MI	1.6	1.3-1.9	10	
31	2038	18-24	M	USA	Case-control	MI	1.3	1.0-1.6	10	
32	2039	18-24	M	USA	Case-control	MI	1.7	1.4-2.0	10	
33	2040	18-24	M	USA	Case-control	MI	1.5	1.2-1.8	10	
34	2041	18-24	M	USA	Case-control	MI	1.4	1.1-1.7	10	
35	2042	18-24	M	USA	Case-control	MI	1.6	1.3-1.9	10	
36	2043	18-24	M	USA	Case-control	MI	1.3	1.0-1.6	10	
37	2044	18-24	M	USA	Case-control	MI	1.7	1.4-2.0	10	
38	2045	18-24	M	USA	Case-control	MI	1.5	1.2-1.8	10	
39	2046	18-24	M	USA	Case-control	MI	1.4	1.1-1.7	10	
40	2047	18-24	M	USA	Case-control	MI	1.6	1.3-1.9	10	
41	2048	18-24	M	USA	Case-control	MI	1.3	1.0-1.6	10	
42	2049	18-24	M	USA	Case-control	MI	1.7	1.4-2.0	10	
43	2050	18-24	M	USA	Case-control	MI	1.5	1.2-1.8	10	
44	2051	18-24	M	USA	Case-control	MI	1.4	1.1-1.7	10	
45	2052	18-24	M	USA	Case-control	MI	1.6	1.3-1.9	10	
46	2053	18-24	M	USA	Case-control	MI	1.3	1.0-1.6	10	
47	2054	18-24	M	USA	Case-control	MI	1.7	1.4-2.0	10	

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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	

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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		
	Colistin	15/12/2012	21/12/2012		
5	Gentamycin	12/04/2013	12/04/2013		
	Co-amoxiclav	13/04/2013	14/04/2013		
	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
	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
	Colistin	20/05/2013	23/05/2013		

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Gene	Mutation type	Effect	AA substitution	Resistance phenotype	Samples
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

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STROBE 2007 (v4) Statement—Checklist of items that should be included in reports of cohort studies

Section/Topic	Item #	Recommendation	Reported on page #
Title and abstract	1	(a) Indicate the study's design with a commonly used term in the title or the abstract	3
		(b) Provide in the abstract an informative and balanced summary of what was done and what was found	3-4
Introduction			
Background/rationale	2	Explain the scientific background and rationale for the investigation being reported	6
Objectives	3	State specific objectives, including any prespecified hypotheses	7
Methods			
Study design	4	Present key elements of study design early in the paper	8-9
Setting	5	Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection	8
Participants	6	(a) Give the eligibility criteria, and the sources and methods of selection of participants. Describe methods of follow-up	8
		(b) For matched studies, give matching criteria and number of exposed and unexposed	n/a
Variables	7	Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable	8-9
Data sources/ measurement	8*	For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group	8-9
Bias	9	Describe any efforts to address potential sources of bias	n/a
Study size	10	Explain how the study size was arrived at	9
Quantitative variables	11	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen and why	n/a
Statistical methods	12	(a) Describe all statistical methods, including those used to control for confounding	??
		(b) Describe any methods used to examine subgroups and interactions	n/a
		(c) Explain how missing data were addressed	n/a
		(d) If applicable, explain how loss to follow-up was addressed	n/a
		(e) Describe any sensitivity analyses	n/a
Results			

Comment [MN1]: For JAMA it was in eMethods, however not sure where it is going to be now...

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Participants	13*	(a) Report numbers of individuals at each stage of study—eg numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed (b) Give reasons for non-participation at each stage (c) Consider use of a flow diagram	10 10 -
Descriptive data	14*	(a) Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders (b) Indicate number of participants with missing data for each variable of interest (c) Summarise follow-up time (eg, average and total amount)	10-11 10 n/a
Outcome data	15*	Report numbers of outcome events or summary measures over time	10-11
Main results	16	(a) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (eg, 95% confidence interval). Make clear which confounders were adjusted for and why they were included (b) Report category boundaries when continuous variables were categorized (c) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period	n/a n/a n/a
Other analyses	17	Report other analyses done—eg analyses of subgroups and interactions, and sensitivity analyses	n/a
Discussion			
Key results	18	Summarise key results with reference to study objectives	16
Limitations			
Interpretation	20	Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence	16-18
Generalisability	21	Discuss the generalisability (external validity) of the study results	18
Other information			
Funding	22	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based	19

*Give information separately for cases and controls in case-control studies and, if applicable, for exposed and unexposed groups in cohort and cross-sectional studies.

Note: An Explanation and Elaboration article discusses each checklist item and gives methodological background and published examples of transparent reporting. The STROBE checklist is best used in conjunction with this article (freely available on the Web sites of PLoS Medicine at <http://www.plosmedicine.org/>, Annals of Internal Medicine at <http://www.annals.org/>, and Epidemiology at <http://www.epidem.com/>). Information on the STROBE Initiative is available at www.strobe-statement.org.

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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Manuscript ID:	bmjopen-2014-006278.R1
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Manuscripts

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Title

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

Authors

Joshua Quick ^{1,2*}, Nicola Cumley ^{2*}, Christopher M. Wearn ^{2,3}, Marc Niebel ²,
Chrystala Constantinidou ⁴, Chris M Thomas ¹, Mark J. Pallen ⁴, Naiem S. Moiemem
^{2,3}, Amy Bamford ^{2,3}, Beryl Oppenheim ^{2#}, Nicholas J. Loman ^{1#}

Affiliations

¹Institute of Microbiology and Infection, University of Birmingham, Birmingham,
United Kingdom

²NIHR Surgical Reconstruction and Microbiology Research Centre, Queen Elizabeth
Hospital, Birmingham, United Kingdom

³Healing Foundation Centre for Burns Research, University Hospital Birmingham
Foundation Trust, Birmingham, United Kingdom

⁴Division of Microbiology and Immunology, University of Warwick, Warwick, United
Kingdom

*Contributed equally

Correspondence:

Dr Nicholas James Loman

Institute of Microbiology and Infection, University of Birmingham, Birmingham, B15
2TT United Kingdom

1
2
3 Telephone: +44 (0) 121 414 8849
4
5

6 Email: n.j.loman@bham.ac.uk
7
8

9 Dr Beryl Oppenheim
10

11
12 Clinical Microbiology, University Hospitals Birmingham NHS Foundation Trust
13

14 Queen Elizabeth Hospital Birmingham, Mindelsohn Way, Edgbaston
15

16 Birmingham, B15 2WB
17
18

19
20 Telephone: +44 (0) 121 371 6523
21

22 Email: Beryl.Oppenheim@uhb.nhs.uk
23
24

25 **Keywords**
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28 High-Throughput DNA sequencing; Pseudomonas aeruginosa; Burns, Hydrotherapy
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31 **Word Count**
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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 United Kingdom.

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 whole-genome sequenced. Sequence analysis looked at similarity and relatedness between isolates.

Results

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

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

Strengths and limitations of the study

- We have demonstrated that whole-genome sequencing can be used for source tracking of *P. 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.[1-10] Other potential routes of transmission include cross-infection, for example carriage on the hands of health care workers, and through contaminated medical equipment such as endoscopic devices.[3,5]

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.[11] This source was eventually determined to be sink taps.[11-13] 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.[14]

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 (VNTR) and random amplification of polymorphic DNA (RAPD) and multi-locus sequencing typing (MLST).[15] These methods have been used to investigate outbreaks of *P. aeruginosa* within hospitals.[4,16-18] However, such techniques have important

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3 limitations for source tracking of infections in hospitals as they sample limited
4 numbers of sites in the genome which may result in false clustering of unrelated
5 strains.[19] In the past five years, whole-genome sequencing (WGS) has started to
6 be used to investigate outbreaks in hospitals. WGS is attractive because of its digital,
7 sharable format and ultra-high resolution, which is able to discriminate two isolates
8 differing by just a single mutation. WGS has been successfully used to determine
9 likely transmission chains during outbreaks of *Staphylococcus aureus*, *Acinetobacter*
10 *baumannii* and *Klebsiella pneumoniae*. [19-21] Benchtop sequencing instruments
11 now offer a cost-effective approach for bringing bacterial WGS to the clinical
12 environment. [1,22]
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26 In this study, we explore the utility of WGS to determine the likely sources of *P.*
27 *aeruginosa* in an at-risk population of burns patients. In the UK and US burns
28 patients receive shower cart hydrotherapy as a mainstay of burns treatment. [23-26]
29 A previous hospital audit suggested that up to one-third of such patients became
30 colonised with *P. aeruginosa*. We hypothesised that this high rate of acquisition may
31 relate to transmission from hospital shower water during therapy. We therefore
32 wished to understand the importance of transmission from water compared with
33 alternative routes such as cross-infection and endogenous carriage.
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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 (CCU). 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 multi-drug resistant *A. baumannii*.

Study design and patient selection

Ethical approval was sought and received from the National Research Ethics Service committee in the West Midlands (reference number 12/WM/0181). 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

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3 microbiology techniques. Samples were then taken as part of routine microbiology
4 service during the patients stay. Environmental and water samples were taken after
5 the patient was admitted to the burns centre. If during the period of stay *P.*
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10 *aeruginosa* was isolated from a patient sample the patient was recruited into the
11 second phase of the study. In this phase, patients had wound swabs taken at each
12 dressing change as well as twice-weekly urine samples. The patient's environment
13 and water from outlets in their bed space were sampled weekly for the duration of
14 their stay, and after discharge (post-cleaning). Termination of the study was planned
15 after 30 screening patient admissions, or a year, whichever came soonest, after
16 which 10 patients were expected to acquire *P. aeruginosa*. This prediction was
17 based on a previous local audit which suggested about one-thirds of burns patients
18 became colonised with *P. aeruginosa*..
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30 **Microbiological and molecular methods**

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33 *P. aeruginosa* isolates were obtained from wound swab, urine, stool, environmental
34 and water samples. *P. aeruginosa* was isolated from wound swabs, urine and stool
35 by inoculation onto cysteine lactose electrolyte deficient agar (CLED) and cetrimide
36 agar and incubation for 24 hours at 37°C. Stool samples were cultured overnight in a
37 cetrimide enrichment broth before subculture onto CLED. Identification was
38 confirmed by resistance to C-390 and the VITEK® 2 GN identification card. Antibiotic
39 sensitivity assays were performed using the VITEK® 2 AST N-210 card (bioMérieux,
40 Basingstoke, UK).
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52 The patient's environment (shower head rosette, drain, shower chair or trolley,
53 bedside table, patient chair, instruments in contact with the patient) was sampled
54 over a 10cm² area by a Polywipes™ sponge. The sponge was placed in tryptic soy
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3 broth incubated for 24 hours at 37°C then sub-cultured onto CLED and cetrimide
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5 agar. During water sampling, water was taken from the patient's shower, or tap if a
6
7 shower was not present. Shower heads were not removed for water sampling. At
8
9 least 200ml of water was collected into a vessel containing sodium thiosulphate as a
10
11 neutraliser. In duplicate, 100ml of water was filtered through a 0.45 micron filter and
12
13 the filters placed onto CLED plates and cetrimide agar. Plates were incubated at
14
15 37°C for 48 hours and the number of organisms per 100ml quantified.
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19 For storage and DNA extraction a single colony was purified from the primary culture
20
21 plate. When different colony morphologies were observed, a single colony from each
22
23 type was purified. Additionally, for a randomly selected water sample, 24 colonies
24
25 were individually picked from one water-filter primary microbiological plate for
26
27 sequencing. Isolates were stored on Biobank beads at -20°C prior to DNA extraction.
28
29 Organisms were resuscitated on CLED agar plates and genome DNA either
30
31 extracted directly using the MOBIO UltraClean Microbial DNA Kit, or from overnight
32
33 LB broth culture using a Qiagen Genomic-Tip 100G.
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38 **DNA extraction and sequencing**

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40 Genomic DNA was prepared from single colony picks using the MIOBIO Ultraclean
41
42 microbial kit (MOBIO, Carlsbad, USA). 1ng input DNA, as quantified by Qubit (Life
43
44 Technologies, Carlsbad, USA) was used to prepare genomic libraries for sequencing
45
46 using the Illumina Nextera XT™ DNA sample kit as per manufacturer's protocol
47
48 (Illumina, San Diego, USA). Libraries were sequenced on the Illumina MiSeq using a
49
50 paired-end protocol resulting in read lengths between 150 and 300 bases. A single
51
52 additional sample, isolate 910, was chosen as a representative member of Clade 5
53
54 for long-read sequencing. DNA from this sample was fragmented using a
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3 Hydroshear (Digilab, Marlborough, MA) using the recommended protocol for 10kb
4 fragments and further size-selected on a Blue Pippin instrument (Sage Science,
5 MA) with a 7kb minimum size cut-off. The library was sequenced on two SMRT Cells
6 using the Pacific Biosciences RS II instrument at the Norwegian Sequencing Centre,
7 Oslo. C4-P2 chemistry was chosen because it favours long, more accurate reads for
8 *de novo* assembly.
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16 17 **Stool PCR**

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19 For simple presence/absence detection of *P. aeruginosa* in stool samples using
20 PCR, a stool sample was collected into a stool collection tube containing stool DNA
21 stabilizer. Total DNA was extracted using the PSP Spin Stool DNA Plus kit (Strattec
22 Molecular). PCR amplification of species specific regions of the 16S rDNA gene was
23 carried out using primers PA-SS-F: GGGGGATCTTCGGACCTCA and PA-SS-R:
24 TCCTTAGAGTGCCACCCG [12] in the following conditions: 0.5µM of each primer,
25 1.5mM MgCl₂, 0.2mM dNTP's using BIOTAQ™ DNA Polymerase and buffer set.
26 After initial denaturation at 96°C for 2 minutes, 30 cycles of 96°C for 30 seconds, 62°
27 C for 30 seconds and 72°C for 30 seconds were completed with a final extension of
28 72°C for 5 minutes. Products were visualised for size on an 1.5% agarose gel.
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43 **Bioinformatics methods**

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46 Illumina MiSeq reads from each isolate were adapter and quality trimmed before use
47 with Trimmomatic.[27] Phylogenetic reconstruction of isolates sequenced in this
48 study were combined with data from a global collection of 55 *P. aeruginosa* strains
49 collected world-wide which have been previously analysed by Stewart et al. [28] For
50 each of the published strains, 600,000 paired-end reads of length 250 bases were
51 simulated using wgsim (<https://github.com/lh3/wgsim>) from the complete or draft
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3 genome assembly deposited in Genbank. Read sets were mapped against the *P.*
4 *aeruginosa* PAO1 reference genome using BWA-MEM 0.7.5a-r405 using default
5 settings.[29] Single nucleotide polymorphisms were called using VarScan 2.3.6 and
6 filtered for regions with an excessive number of variants. These may represent
7 regions of recombination, misalignments or strong Darwinian selection.[30] FastTree
8 (version 2.1.7) was used for phylogenetic reconstruction. This software estimates an
9 approximate maximum-likelihood tree under the Jukes-Cantor model of nucleotide
10 evolution with a single rate for each site (CAT).[31] Trees were drawn in FigTree
11 (<http://tree.bio.ed.ac.uk/software/figtree/>).

12
13
14 For *in silico* MLST prediction, trimmed reads were assembled *de novo* using Velvet
15 [32] with a k-mer size of 81 and searched using nucleotide BLAST against the multi-
16 locus sequence database downloaded from the pubMLST website on 2013-08-05
17 (<http://pubmlst.org/paeruginosa/>) [33]. For Clade E isolates, in order to exhaustively
18 search for discriminatory mutations, a nearly complete reference genome was
19 generated by *de novo* assembly using Pacific Biosciences sequencing data. Reads
20 were assembled using the 'RS_HGAP_Assembly.3' pipeline within SMRT Portal
21 v2.2.0. Illumina reads from the same sample were mapped to this draft genome
22 assembly in order to correct remaining indel errors in the assembly using Pilon
23 (<http://www.broadinstitute.org/software/pilon/>). Isolates belonging to each clade were
24 mapped individually against either the PacBio reference (Clade E) or *P. aeruginosa*
25 PAO1 (NC_002516) (Clades C, D and G).

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28 Variants (single nucleotide polymorphisms and short insertion-deletions) were called
29 using SAMtools mpileup and VarScan with an allele frequency threshold of 80%.**[30]**
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31 Non-informative positions and regions of putative recombination were removed, the
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3 later with a variant density filter of more than 3 SNPs every 1000 nucleotides.
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5 Analysing samples in each clade individually maximised the number of variants
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7 detected by reducing the likelihood of the position being uncovered by a subset of
8
9 samples. From these variants fine-grained phylogenetic trees were reconstructed for
10
11 each clade using FastTree. The scripts used to perform this analysis are available at
12
13 http://www.github.com/joshquick/snp_calling_scripts. Approximate-maximum-
14
15 likelihood phylogenetic trees were generated using FastTree and visualised in
16
17 FigTree. For whole-genome shotgun metagenomics analysis, reads were analysed
18
19 using the Kraken taxonomic classifier software with the supplied *minikraken*
20
21 database.^[34] Reads from the metagenomics dataset were aligned to *P. aeruginosa*
22
23 PAO1 as in the previous section and phylogenetic placement was carried out using
24
25 pplacer in conjunction with FastTree.^[35] Sequence data is available from the
26
27 European Nucleotide Archive for the Illumina data (ERP006056) and the corrected
28
29 Pacific Biosciences assembly (ERP006058).
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34 RESULTS

35 Study results

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37 Recruitment lasted a period of 300 days, ending according to protocol after the
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39 enrolment of 30 screening patients. In total, we detected *P. aeruginosa* in five
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41 patients. Of these patients, three had *P. aeruginosa* detected only in burns wound
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43 swabs, one had *P. aeruginosa* detected in both their burns wound and in their urine,
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45 and one had *P. aeruginosa* in their sputum. One additional eligible patient did not
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47 consent to enter the study and was excluded. The average age in the study group
48
49 was 41 years. Males predominated with a male-to-female ration of 2.3:1. Flame
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51 burns were the most common mechanism of injury, followed by scalds and mixed
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53 flame/flash injuries. The average burn size of the study group was 12.5% of the total
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3 body surface area (TBSA) and 27% of patients sustained an inhalation injury. Eight
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5 patients required admission to ITU and the majority required surgical treatment of
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7 their burns with excision and skin grafting (80%). A large majority of the study group
8
9 (83%) received shower cart hydrotherapy as a routine part of their wound
10
11 management to encourage healing through wound debridement and
12
13 decontamination. The average length of hospital stay (LOS) was 17 days and taking
14
15 into account burn size, the average was 1.4 days per % TBSA.
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20 **The water and environment in burns and critical care units are frequently**
21
22 **colonised by *P. aeruginosa***
23

24 A total of 282 water and environmental samples were screened for *P. aeruginosa* of
25
26 which 39/78 (50%) were positive in water samples, 25/96 (26%) were positive from
27
28 the wet environment and 7/108 (6%) were positive from the dry environment. A total
29
30 of 86 genome sequences were generated from the 71 positives, as in some cases
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32 multiple colony picks were sequenced. 78 patient samples were screened for *P.*
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34 *aeruginosa* of which 39 (50%) were positive. A total of 55 genome sequences were
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36 generated, as in some cases multiple colony picks were sequenced. In total, 141
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38 genomes were sequenced; water and environmental (n=86) and patient (n=55).
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40 Genomes were sequenced to a mean coverage of 24.4x, with the minimum
41
42 coverage of a sample being 14x and highest 64.7x.
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47 When placed in the context of a global collection of *P. aeruginosa* strains,
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49 phylogenetic reconstruction demonstrated isolates in our study fell into eight clades
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51 (figure 1, Panel A). As has been reported previously, there was no strong association
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53 between ecological context and position in the phylogenetic tree.[28] Isolates in this
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55 study are most closely related to strains from a variety of settings. The majority of
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3 isolates (52%) belong to Clade E (figure 1 Panel B), whose nearest sequenced
4 relative is the Liverpool Epidemic Strain, a clone often isolated from patients in the
5 UK and Canada with cystic fibrosis. [36,37] Isolates from Clade E were found in the
6 burns unit's water and the ward environment, as well as from two patient's wounds.
7
8 However it was never detected in the critical care unit. Clade E was detected
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10 throughout the study in a total of 10 different rooms (figure 2).
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17 **Inferring potential transmission events by whole-genome sequencing**

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19 Microevolutionary changes occurring over rapid time-scales (i.e. days to months)
20 have been used to detect potential chains of transmission in hospital and community
21 outbreaks.[19-21,38,39] The number of distinct mutations between given isolates
22 has been used to infer whether transmission events are likely to have occurred.
23
24 Such inferences are aided by prior knowledge of mutation rates in similar
25 populations. Two patients (1 and 4) in our study both had *P. aeruginosa* from Clade
26 E isolated from their wounds. These isolates had an indistinguishable genotype from
27 those present in water and the environment of the room they were nursed within
28 (figure 1 Panel C and figure 3). This genotype was detected in the patient's shower
29 water after initial patient screening, during screening of the second patient
30 admission, twice during the second patient's stay and then 127 days later (days 27,
31 65, 89 and 216 respectively). When water isolates were positive, the genotype was
32 also detected in wet environment sites (shower drain, shower rosette and patient's
33 trolley) on the same days.
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52 Patient 5 was nursed on the critical care unit due to concomitant medical problems.
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54 *P. aeruginosa* belonging to Clade G was isolated from sputum during this time.
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3 Identical genotypes were detected contemporaneously in the water from the
4 associated sink and sink tap handle (see online supplementary appendix 4).
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8 Two further patients (patients 2 and 3) were positive for *P. aeruginosa*. Isolates from
9 these patients belonged to Clade C and D respectively. Neither clade was ever
10 isolated from hospital water. In both cases, identical genotypes were detectable in
11 the environment associated with the patient but these were not detected before or
12 after the patients' stay, indicating that the environment was not persistently
13 contaminated. During the course of patient 3's stay, the dry environment such as the
14 bedside table was contaminated, as was the patient's door handle and shower chair.
15 However, after patient discharge, the strain associated with this patient was never
16 seen again during the course of the study in any location.
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29 **Whole-genome sequencing permits source tracking of *P. aeruginosa* to** 30 **individual water outlets** 31 32

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34 Whole-genome sequencing has been reported previously for source tracking, but
35 never for the detection of transmission events from hospital water.[40] Phylogenetic
36 reconstruction within Clade E, the most commonly detected water clone
37 demonstrated additional diversity within this clone, with a total of 46 mutations
38 detected an average genetic distance between isolates of 4.1 mutations (figure 3).
39 The reconstruction demonstrated clear evidence of clustering of genotypes both by
40 room and outlet (figure 3). When *P. aeruginosa* was detected in the wet environment
41 (e.g. shower rosettes and drains) these genotypes were most often identical to those
42 found in water, indicating that the water was likely the ultimate source of that clone.
43 Genotypic variation was seen between outlets within the same room. For example,
44 tap water sampled from room 11 had a distinct genotype from that sampled from
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3 shower water in the same room and this was consistently found over multiple
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5 samplings. Notably, isolates from two patients fell within the cluster originating from
6
7 shower water, indicating that shower hydrotherapy was the most likely source of
8
9 infection. Two plasmids (designated pBURNS1 and pBURNS2) were detected in this
10
11 study set, which both demonstrated geographical clustering, with pBURNS1 only
12
13 being detectable in isolates from room 8 and pBURNS2 only being detectable in
14
15 isolates from the shower water in room 9.
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19 **Rapid evolution of antibiotic resistance associated with treatment**

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21
22 *P. aeruginosa* is commonly associated with antibiotic resistance due to a number of
23
24 predisposing features including intrinsic resistance, a repertoire of efflux pumps and
25
26 antibiotic-inactivating enzymes including beta-lactamases. [41] Three infected
27
28 patients (2, 3 and 5) received antibiotic therapy, and in each case this was
29
30 associated with the development of resistance to at least one therapeutic agent.
31
32 Associated mutations were detected that were either partially or fully explanatory of
33
34 the phenotype (online supplementary appendix 12).
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39 Patient 2 was treated with ciprofloxacin, nitrofurantoin and vancomycin (see online
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41 supplementary appendix 11 for full details). 8/21 (38%) tested isolates from this
42
43 patient were ciprofloxacin resistant. 7/8 isolates (88%) of the ciprofloxacin-resistant
44
45 strains were distinguishable from the other isolates by a single SNP in *mexS*
46
47 (annotated as PA2491 in *P. aeruginosa* PAO1) (see online supplementary appendix
48
49 1 and 7). This SNP was predicted to result in a non-synonymous amino acid
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51 substitution. Disruption of this gene has been shown to cause increased expression
52
53 of the *mexEF-oprN* multidrug efflux pump, associated with resistance to
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55 quinolones.[42]
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3 Patient 3 was not treated with antibiotics, but isolates associated with this patient
4
5 demonstrated differences in resistance to timentin and piperacillin-tazobactam.
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7 These changes were associated with non-synonymous mutations in *gacA*, the
8 response regulator of the GacA/GacS two-component system and in *lasR*, a
9 transcriptional activator required for transcription of elastase and LasA protease
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11 (online supplementary appendices 2 and 8).
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17 Patient 4 was treated with meropenem, piperacillin/tazobactam, flucloxacillin and
18 colistin. Five isolates collected 10 to 18 days after initiation of meropenem showed
19 resistance to imipenem and intermediate resistance to meropenem (see online
20 supplementary appendix 3 and 9). The most likely mutation responsible for this
21 phenotype was detectable in two isolates, both of which had a frame-shift mutation in
22 the gene coding for the membrane porin *oprD*. [43]
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31 Patient 5 had a prolonged stay in ITU and had multiple medical problems including
32 *A. baumannii* infection and was treated with nine antibiotic agents including
33 ciprofloxacin, meropenem and piperacillin-tazobactam. Serial isolates from this
34 patient demonstrated the stepwise acquisition of two mutations (online
35 supplementary appendix 4). The first was in *nalC*, a probable repressor of the
36 TetR/AcrR family (online supplementary appendix 10). [44] On inspection of the
37 sequence alignment in this region, a large deletion of 196 nucleotide bases was
38 seen compared to the reference PAO1 strain. This mutation was seen in association
39 with full resistance to piperacillin-tazobactam, ceftazidime, aztreonam, meropenem
40 and intermediate resistance to ciprofloxacin. This deletion is likely to result in over-
41 expression of efflux pumps involving the *mexAB-oprM* operon. [44,45] Ciprofloxacin
42 resistance in a later isolate corresponded to the stepwise acquisition of a second
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3 mutation. This mutation is predicted to affect the well-studied DNA gyrase subunit A
4 gene (*gyrA*) which is strongly associated with ciprofloxacin resistance.[46]
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8 **Confirmation of *P. aeruginosa* genotypes in biofilms by whole-genome** 9 **metagenomic shotgun sequencing** 10

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12
13 *P. aeruginosa* is able to produce and survive in biofilms. Plumbing parts such as flow
14 straighteners, shower rosettes, flexible hoses, solenoid valves and thermostatic
15 mixer valves (TMV) are particularly at risk of biofilm formation due to factors
16 including surface areas, convoluted designs and inadequate pasteurisation. [47] To
17 confirm the presence of *P. aeruginosa* in water fittings associated with rooms on the
18 burns unit, we obtained a TMV removed by the hospital estates team from the
19 shower in room nine as part of compliance with UK guidelines for managing *P.*
20 *aeruginosa* in hospitals. On visual inspection, a biofilm was present which was
21 scraped from the surface with a sterile scalpel. DNA from this biofilm was extracted
22 for whole-genome shotgun sequencing. The majority of reads did not map to any
23 known bacterial taxa. The most abundant taxon identified was *Pseudomonas*
24 *aeruginosa* (3%). Subsequent alignment to the *P. aeruginosa* PAO1 reference
25 covered 94% of the 6.3 million base reference genome at a median coverage of 5x,
26 confirming that reads were correctly classified to this species and not other
27 environmental *Pseudomonas* species. Alignment to the *P. aeruginosa* Clade E
28 reference genome followed by phylogenetic placement of reads demonstrated that it
29 fell into the same clade as previously recovered isolates from the shower or tap in
30 room 9 (indicated on figure 3, and in online supplementary appendix 6).
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53 **DISCUSSION** 54 55 56 57 58 59 60

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3 The hospital environment has been intimately linked with *P. aeruginosa* infection for
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5 over 50 years yet hospital acquisitions, clusters and outbreaks remain a common
6
7 occurrence and understanding precise routes of transmission can be difficult. [47,48]
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10 Our results demonstrate that, even in a new hospital, *P. aeruginosa* can become
11
12 rapidly endemic in hospital plumbing. Furthermore, by linking *P. aeruginosa*
13
14 genotypes recovered from patients to specific individual water outlets, we offer
15
16 compelling evidence of unidirectional transmission from water to patients. Further, by
17
18 sequencing of a biofilm identified in a TMV from a hospital water system, we can
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20 identify the likely common source of genotypes found in water and in the hospital
21
22 environment.
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26 Our results suggest that use of whole-genome sequencing can reduce ambiguity
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28 about potential transmission events in hospitals and consequently inform infection
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30 prevention efforts about the direction and sequence of transmission. Typing
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32 schemes such as MLST and PFGE are much lower resolution methods and would
33
34 not be able to provide sufficient information to permit such inferences to be made. It
35
36 is notable that the burns unit was colonised by a single clone, meaning that it was
37
38 very unlikely that water outlets at each bed space were colonised as a result of
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40 transmissions from the patient or environment. For this to happen would require
41
42 multiple transmission events from separate patients with the same clone, for which
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44 there is not evidence. Instead we speculate that this clone was introduced to the
45
46 hospital associated with its commissioning. One hypothesis is that particular
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48 plumbing fittings, i.e. the TMV may have been colonised simultaneously by a clone
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50 circulating in water. Clade E (ST395) has been frequently reported associated with
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52 water, so this remains a possibility. [49,50] However, it is possible that plumbing
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54 fittings are installed 'pre-seeded' with *P. aeruginosa* as has already been proposed
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3 by Kelsey.[3,5,47] Investigation of an outbreak in Wales implicated new plumbing
4 parts as a potential source of *P. aeruginosa*. New plumbing components are often
5 tested by companies prior to their supply and it is possible they were contaminated
6 prior to distribution. The limited amount of diversity (average 4 SNPs) seen within
7 this clade is consistent with a single founding genotype coinciding with the opening
8 of the burns unit, based on estimates from a previous study using WGS which
9 reported that mutations accumulate at a rate of approximately one every 3-4 months
10 in a hospital-associated clone.[51] However our results suggest that our isolates
11 accumulate mutations even more slowly. This may be due to reduced growth rates in
12 nutritionally-poor biofilms.[52]
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26 It is notable that antibiotic resistance to multiple first-line agents developed rapidly in
27 response to therapy. These results underline the importance of selecting appropriate
28 antibiotic therapy in *P. aeruginosa* infections. It is reassuring however that antibiotic
29 resistance genotypes selected *in vivo* did not show evidence of persistence in the
30 ward environment or transmission to other patients.
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38 Our study has certain limitations. Based on a previous audit, we expected around
39 one-third of patients screened for *P. aeruginosa* would develop colonisation or
40 clinical infection. In fact, only 5 out of 30 of patients were colonised. This may have
41 been related to guidance and engineering interventions being put in place during the
42 study as detailed in national guidance issued whilst this study was on-going. In
43 addition, infection control policies were revised to address control of an outbreak of a
44 multi-drug resistant *A. baumannii* in this same burns unit. Following these
45 interventions, only 1 of the last 20 patients recruited was infected with *P. aeruginosa*
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3 which may demonstrate the importance of national guidance in reducing
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5 transmissions.
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9 By focusing on burns patients who receive hydrotherapy, our study population were
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11 at extremely high risk of waterborne infection. In other patient groups it may be that
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13 alternative routes of transmission including cross-infection or endogenous carriage
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15 play a more important role. Our results suggest that our burns unit is endemically
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17 colonised with a distinct clone of *P. aeruginosa* that may have been imported
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19 coinciding with the opening of the hospital. Other intensive care units, particularly
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21 those which have been open for longer may have harbour a greater diversity of *P.*
22
23 *aeruginosa* as a result of increased opportunities for clones to be imported.
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27 One potential application for WGS in infection control would be to determine whether
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29 cases are as a result of water transmission, or represent sporadic clones originating
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31 from the wider environment. Despite improved guidance concerning improved
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33 engineering infection control practices and the introduction of the water safety group
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35 in the UK, it may not be realistic to eliminate *P. aeruginosa* from hospitals entirely. In
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37 augmented care units such as ITUs, burns units and neonatal wards where *P.*
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39 *aeruginosa* poses a significant risk to vulnerable patients, the increased resolution
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41 offered by WGS will justify its use, particularly as the costs continue to fall.
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46 In conclusion, we have identified through WGS clear evidence for transmission of *P.*
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48 *aeruginosa* from specific water outlets to burns patients and offer a forensic-level
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50 framework for dealing with outbreaks linked to hospital water. We expect WGS will
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52 continue to make inroads into clinical microbiology and become a vital tool for
53
54 tracking *P. aeruginosa* in the hospital environment, helping inform targeted control
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56 measures to help protect patients at risk of infection.
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Contributors

MP NM BO conceived the study. CW and AB enrolled patients into study and collected samples. NC collected environmental and water samples. NC CC MN processed samples and performed microbiology. NC CC JQ did sequencing. JQ NC CT NL analysed the data. NL NC JQ MP BO wrote the paper. All authors commented on the manuscript draft.

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Competing interests

None

Data Sharing Statement

1
2
3 Pacific Biosciences raw data files are available from the corresponding author
4
5 (Nicholas J Loman, n.j.loman@bham.ac.uk) and will be made available via Data
6
7 Dryad.
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10 **Ethics approval**

11
12 The study protocol received approval from National Research Ethics Service
13
14 committee in the West Midlands (reference number 12/WM/0181).
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Figure and Table Legends

Figure 1 Legend

An overview of all samples collected during the study in global phylogenetic context with other sequenced strains of *P. aeruginosa* from the set of Stewart *et al.*[28]

Samples collected in this study are widely dispersed in the tree, which contains isolates from different environments (Panel A). Bar plots indicate the numbers of each type of sample collected (Panel B). Microdiversity within each clade is shown, with the colour bar indicating the source of each sample (Panel C).

Figure 2 Legend

A schematic view of the 300-day study of *P. 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, environment 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.

Figure 3 Legend

1
2
3 The high-resolution phylogenetic reconstruction of Clade E isolates. This
4
5 demonstrates the clustering of genotypes by bed space. Patient associated samples
6
7 are contained within the room 11 clade. This clade contains water samples from the
8
9 shower and environmental samples from the shower, drain and trolley. The water
10
11 samples from the room 11 tap are in a distinct clade, indicating the biofilm within the
12
13 tap has a distinct genotype to the shower. This suggests environmental
14
15 contamination was more likely to arise from contaminated shower water than tap
16
17 water. Details of sampling site, days since start of study and presence of pBURNS
18
19 plasmids are also shown. The likely phylogenetic position of *P. aeruginosa* detected
20
21 in a biofilm from a thermostatic mixer valve is shown in the clade associated with
22
23 room 9 and indicated 'TMV'.
24
25
26
27

28 **Appendix 1**

29
30
31 Phylogenetic tree, sample origin and antibiotic-resistance phenotype for Clade C
32
33 isolates. The patient column refers to the sampling event. If a sample was collected
34
35 from a patient colonised with *P. aeruginosa* or that patient's environment the patient
36
37 number if marked. Patient numbers starting with SP relate to screening patients who
38
39 were not colonised by *P. aeruginosa*.
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42

43 **Appendix 2**

44
45 Phylogenetic tree, sample origin and antibiotic-resistance phenotype for Clade D
46
47 isolates.
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49

50 **Appendix 3**

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52
53 Phylogenetic tree, sample origin and antibiotic-resistance phenotype for Clade E
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55 isolates.
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57

58 **Appendix 4**

1
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3 Phylogenetic tree, sample origin and antibiotic-resistance phenotype for Clade G
4 isolates.
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7 8 **Appendix 5** 9

10 A physical map of the burns unit, indicating individual patient bed areas, shower
11 areas. The water supply is indicated by a blue line.
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14 15 **Appendix 6** 16

17 Clade E isolates in phylogenetic context with the metagenomics sample from a tap
18 mixer valve.
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21 22 **Appendix 7** 23

24 Single nucleotide and small indel variants detected within Clade C isolates.
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27 28 **Appendix 8** 29

30 Single nucleotide and small indel variants detected within Clade D isolates.
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33 34 **Appendix 9** 35

36 Single nucleotide and small indel variants detected within Clade E isolates.
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39 40 **Appendix 10** 41

42 Single nucleotide and small indel variants detected within Clade G isolates.
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45 46 **Appendix 11** 47

48 The type of antibiotics administered to the five patients in this study.
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50 51 **Appendix 12** 52

53 Mutations predicted to be associated with antibiotic-resistance.
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Title

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

Authors

Joshua Quick ^{1,2*}, Nicola Cumley ^{2*}, Christopher M. Wearn ^{2,3}, Marc Niebel ²,
Chrystala Constantinidou ⁴, [Chris M Thomas](#) ¹, Mark J. Pallen ⁴, Naiem S. Moiemem ^{2,3}, Amy Bamford ^{2,3}, Beryl Oppenheim ^{2#}, Nicholas J. Loman ^{1#}

Affiliations

¹Institute of Microbiology and Infection, University of Birmingham, Birmingham, United Kingdom

²NIHR Surgical Reconstruction and Microbiology Research Centre, Queen Elizabeth Hospital, Birmingham, United Kingdom

³Healing Foundation Centre for Burns Research, University Hospital Birmingham Foundation Trust, Birmingham, United Kingdom

⁴Division of Microbiology and Immunology, University of Warwick, Warwick, United Kingdom

*Contributed equally

Correspondence:

Dr Nicholas James Loman

Institute of Microbiology and Infection, University of Birmingham, Birmingham, B15 2TT United Kingdom

1
2
3 Telephone: +44 (0) 121 414 8849
4
5

6 Email: n.j.loman@bham.ac.uk
7
8

9 Dr Beryl Oppenheim
10

11
12 Clinical Microbiology, University Hospitals Birmingham NHS Foundation Trust
13

14 Queen Elizabeth Hospital Birmingham, Mindelsohn Way, Edgbaston
15

16 Birmingham, B15 2WB
17

18
19
20 Telephone: +44 (0) 121 371 6523
21

22 Email: Beryl.Oppenheim@uhb.nhs.uk
23
24

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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 United Kingdom.

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 whole-genome sequenced. Sequence analysis looked at similarity and relatedness between isolates.

Results

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

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

Strengths and limitations of the study

- We have demonstrated that whole-genome sequencing can be used for source tracking of *P. 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.[1-10] Other potential routes of transmission include cross-infection, for example carriage on the hands of health care workers, and through contaminated medical equipment such as endoscopic devices.[3,5]

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.[11] This source was eventually determined to be sink taps.[11-13] 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.[14]

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 (VNTR) and random amplification of polymorphic DNA (RAPD) and multi-locus sequencing typing (MLST).[15] These methods have been used to investigate outbreaks of *P. aeruginosa* within hospitals.[4,16-18] However, such techniques have important

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3 limitations for source tracking of infections in hospitals as they sample limited
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5 numbers of sites in the genome which may result in false clustering of unrelated
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7 strains.[19] In the past five years, whole-genome sequencing (WGS) has started to
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9 be used to investigate outbreaks in hospitals. WGS is attractive because of its digital,
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11 sharable format and ultra-high resolution, which is able to discriminate two isolates
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13 differing by just a single mutation. WGS has been successfully used to determine
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15 likely transmission chains during outbreaks of *Staphylococcus aureus*, *Acinetobacter*
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17 *baumannii* and *Klebsiella pneumoniae*. [19-21] Benchtop sequencing instruments
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19 now offer a cost-effective approach for bringing bacterial WGS to the clinical
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21 environment. [1,22]
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26 In this study, we explore the utility of WGS to determine the likely sources of *P.*
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28 *aeruginosa* in an at-risk population of burns patients. In the UK and US burns
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30 patients receive shower cart hydrotherapy as a mainstay of burns treatment. [23-26]
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32 A previous hospital audit suggested that up to one-third of such patients became
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34 colonised with *P. aeruginosa*. We hypothesised that this high rate of acquisition may
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36 relate to transmission from hospital shower water during therapy. We therefore
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38 wished to understand the importance of transmission from water compared with
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40 alternative routes such as cross-infection and endogenous carriage.
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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 (CCU). 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 multi-drug resistant *A. baumannii*.

Study design and patient selection

Ethical approval was sought and received from the National Research Ethics Service committee in the West Midlands (reference number 12/WM/0181). 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

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3 microbiology techniques. Samples were then taken as part of routine microbiology
4 service during the patients stay. Environmental and water samples were taken after
5 the patient was admitted to the burns centre. If during the period of stay *P.*
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10 *aeruginosa* was isolated from a patient sample the patient was recruited into the
11 second phase of the study. In this phase, patients had wound swabs taken at each
12 dressing change as well as twice-weekly urine samples. The patient's environment
13 and water from outlets in their bed space were sampled weekly for the duration of
14 their stay, and after discharge (post-cleaning). Termination of the study was planned
15 after 30 screening patient admissions, or a year, whichever came soonest, after
16 which 10 patients were expected to acquire *P. aeruginosa*. This prediction was
17 based on a previous local audit which suggested about one-thirds of burns patients
18 became colonised with *P. aeruginosa*..
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30 **Microbiological and molecular methods**

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33 *P. aeruginosa* isolates were obtained from wound swab, urine, stool, environmental
34 and water samples. *P. aeruginosa* was isolated from wound swabs, urine and stool
35 by inoculation onto cysteine lactose electrolyte deficient agar (CLED) and cetrimide
36 agar and incubation for 24 hours at 37°C. Stool samples were cultured overnight in a
37 cetrimide enrichment broth before subculture onto CLED. Identification was
38 confirmed by resistance to C-390 and the VITEK® 2 GN identification card. Antibiotic
39 sensitivity assays were performed using the VITEK® 2 AST N-210 card (bioMérieux,
40 Basingstoke, UK).
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52 The patient's environment (shower head rosette, drain, shower chair or trolley,
53 bedside table, patient chair, instruments in contact with the patient) was sampled
54 over a 10cm² area by a Polywipe™ sponge. The sponge was placed in tryptic soy
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3 broth incubated for 24 hours at 37°C then sub-cultured onto CLED and cetrimide
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5 agar. During water sampling, water was taken from the patient's shower, or tap if a
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7 shower was not present. Shower heads were not removed for water sampling. At
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9 least 200ml of water was collected into a vessel containing sodium thiosulphate as a
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11 neutraliser. In duplicate, 100ml of water was filtered through a 0.45 micron filter and
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13 the filters placed onto CLED plates and cetrimide agar. Plates were incubated at
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15 37°C for 48 hours and the number of organisms per 100ml quantified.
16
17

18
19 For storage and DNA extraction a single colony was purified from the primary culture
20
21 plate. When different colony morphologies were observed, a single colony from each
22
23 type was purified. Additionally, for a randomly selected water sample, 24 colonies
24
25 were individually picked from one water-filter primary microbiological plate for
26
27 sequencing. Isolates were stored on Biobank beads at -20°C prior to DNA extraction.
28
29 Organisms were resuscitated on CLED agar plates and genome DNA either
30
31 extracted directly using the MOBIO UltraClean Microbial DNA Kit, or from overnight
32
33 LB broth culture using a Qiagen Genomic-Tip 100G.
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38 **DNA extraction and sequencing**

39
40 Genomic DNA was prepared from single colony picks using the MIOBIO Ultraclean
41
42 microbial kit (MOBIO, Carlsbad, USA). 1ng input DNA, as quantified by Qubit (Life
43
44 Technologies, Carlsbad, USA) was used to prepare genomic libraries for sequencing
45
46 using the Illumina Nextera XT™ DNA sample kit as per manufacturer's protocol
47
48 (Illumina, San Diego, USA). Libraries were sequenced on the Illumina MiSeq using a
49
50 paired-end protocol resulting in read lengths between 150 and 300 bases. A single
51
52 additional sample, isolate 910, was chosen as a representative member of Clade 5
53
54 for long-read sequencing. DNA from this sample was fragmented using a
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3 Hydroshear (Digilab, Marlborough, MA) using the recommended protocol for 10kb
4 fragments and further size-selected on a Blue Pippin instrument (Sage Science,
5 MA) with a 7kb minimum size cut-off. The library was sequenced on two SMRT Cells
6 using the Pacific Biosciences RS II instrument at the Norwegian Sequencing Centre,
7 Oslo. C4-P2 chemistry was chosen because it favours long, more accurate reads for
8 *de novo* assembly.
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16 17 **Stool PCR**

18
19 For simple presence/absence detection of *P. aeruginosa* in stool samples using
20 PCR, a stool sample was collected into a stool collection tube containing stool DNA
21 stabilizer. Total DNA was extracted using the PSP Spin Stool DNA Plus kit (Strattec
22 Molecular). PCR amplification of species specific regions of the 16S rDNA gene was
23 carried out using primers PA-SS-F: GGGGGATCTTCGGACCTCA and PA-SS-R:
24 TCCTTAGAGTGCCACCCG [12] in the following conditions: 0.5µM of each primer,
25 1.5mM MgCl₂, 0.2mM dNTP's using BIOTAQ™ DNA Polymerase and buffer set.
26
27 After initial denaturation at 96°C for 2 minutes, 30 cycles of 96°C for 30 seconds, 62°
28 C for 30 seconds and 72°C for 30 seconds were completed with a final extension of
29 72°C for 5 minutes. Products were visualised for size on an 1.5% agarose gel.
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43 **Bioinformatics methods**

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46 Illumina MiSeq reads from each isolate were adapter and quality trimmed before use
47 with Trimmomatic.[27] Phylogenetic reconstruction of isolates sequenced in this
48 study were combined with data from a global collection of 55 *P. aeruginosa* strains
49 collected world-wide which have been previously analysed by Stewart et al. [28] For
50 each of the published strains, 600,000 paired-end reads of length 250 bases were
51 simulated using wgsim (<https://github.com/lh3/wgsim>) from the complete or draft
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3 genome assembly deposited in Genbank. Read sets were mapped against the *P.*
4 *aeruginosa* PAO1 reference genome using BWA-MEM 0.7.5a-r405 using default
5 settings.[29] Single nucleotide polymorphisms were called using VarScan 2.3.6 and
6 filtered for regions with an excessive number of variants. These may represent
7 regions of recombination, misalignments or strong Darwinian selection.[30] FastTree
8 (version 2.1.7) was used for phylogenetic reconstruction. This software estimates an
9 approximate maximum-likelihood tree under the Jukes-Cantor model of nucleotide
10 evolution with a single rate for each site (CAT).[31] Trees were drawn in FigTree
11 (<http://tree.bio.ed.ac.uk/software/figtree/>).

12
13
14 For *in silico* MLST prediction, trimmed reads were assembled *de novo* using Velvet
15 [32] with a k-mer size of 81 and searched using nucleotide BLAST against the multi-
16 locus sequence database downloaded from the pubMLST website on 2013-08-05
17 (<http://pubmlst.org/paeruginosa/>) [33]. For Clade E isolates, in order to exhaustively
18 search for discriminatory mutations, a nearly complete reference genome was
19 generated by *de novo* assembly using Pacific Biosciences sequencing data. Reads
20 were assembled using the 'RS_HGAP_Assembly.3' pipeline within SMRT Portal
21 v2.2.0. Illumina reads from the same sample were mapped to this draft genome
22 assembly in order to correct remaining indel errors in the assembly using Pilon
23 (<http://www.broadinstitute.org/software/pilon/>). Isolates belonging to each clade were
24 mapped individually against either the PacBio reference (Clade E) or *P. aeruginosa*
25 PAO1 (NC_002516) (Clades C, D and G).

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28 Variants (single nucleotide polymorphisms and short insertion-deletions) were called
29 using SAMtools mpileup and VarScan with an allele frequency threshold of 80%.**[30]**
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31 Non-informative positions and regions of putative recombination were removed, the
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3 later with a variant density filter of more than 3 SNPs every 1000 nucleotides.
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5 Analysing samples in each clade individually maximised the number of variants
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7 detected by reducing the likelihood of the position being uncovered by a subset of
8
9 samples. From these variants fine-grained phylogenetic trees were reconstructed for
10
11 each clade using FastTree. The scripts used to perform this analysis are available at
12
13 http://www.github.com/joshquick/snp_calling_scripts. Approximate-maximum-
14
15 likelihood phylogenetic trees were generated using FastTree and visualised in
16
17 FigTree. For whole-genome shotgun metagenomics analysis, reads were analysed
18
19 using the Kraken taxonomic classifier software with the supplied *minikraken*
20
21 database.^[34] Reads from the metagenomics dataset were aligned to *P. aeruginosa*
22
23 PAO1 as in the previous section and phylogenetic placement was carried out using
24
25 pplacer in conjunction with FastTree.^[35] Sequence data is available from the
26
27 European Nucleotide Archive for the Illumina data (ERP006056) and the corrected
28
29 Pacific Biosciences assembly (ERP006058).
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34 RESULTS

35 Study results

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37 Recruitment lasted a period of 300 days, ending according to protocol after the
38
39 enrolment of 30 screening patients. In total, we detected *P. aeruginosa* in five
40
41 patients. Of these patients, three had *P. aeruginosa* detected only in burns wound
42
43 swabs, one had *P. aeruginosa* detected in both their burns wound and in their urine,
44
45 and one had *P. aeruginosa* in their sputum. One additional eligible patient did not
46
47 consent to enter the study and was excluded. The average age in the study group
48
49 was 41 years. Males predominated with a male-to-female ration of 2.3:1. Flame
50
51 burns were the most common mechanism of injury, followed by scalds and mixed
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53 flame/flash injuries. The average burn size of the study group was 12.5% of the total
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3 body surface area (TBSA) and 27% of patients sustained an inhalation injury. Eight
4
5 patients required admission to ITU and the majority required surgical treatment of
6
7 their burns with excision and skin grafting (80%). A large majority of the study group
8
9 (83%) received shower cart hydrotherapy as a routine part of their wound
10
11 management to encourage healing through wound debridement and
12
13 decontamination. The average length of hospital stay (LOS) was 17 days and taking
14
15 into account burn size, the average was 1.4 days per % TBSA.
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19
20 **The water and environment in burns and critical care units are frequently**
21
22 **colonised by *P. aeruginosa***
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24
25 A total of 282 water and environmental samples were screened for *P. aeruginosa* of
26
27 which 39/78 (50%) were positive in water samples, 25/96 (26%) were positive from
28
29 the wet environment and 7/108 (6%) were positive from the dry environment. A total
30
31 of 86 genome sequences were generated from the 71 positives, as in some cases
32
33 multiple colony picks were sequenced. 78 patient samples were screened for *P.*
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35 *aeruginosa* of which 39 (50%) were positive. A total of 55 genome sequences were
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37 generated, as in some cases multiple colony picks were sequenced. In total, 141
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39 genomes were sequenced; water and environmental (n=86) and patient (n=55).
40
41 Genomes were sequenced to a mean coverage of 24.4x, with the minimum
42
43 coverage of a sample being 14x and highest 64.7x.
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48 When placed in the context of a global collection of *P. aeruginosa* strains,
49
50 phylogenetic reconstruction demonstrated isolates in our study fell into eight clades
51
52 (figure 1, Panel A). As has been reported previously, there was no strong association
53
54 between ecological context and position in the phylogenetic tree.[28] Isolates in this
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56 study are most closely related to strains from a variety of settings. The majority of
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3 isolates (52%) belong to Clade E (figure 1 Panel B), whose nearest sequenced
4
5 relative is the Liverpool Epidemic Strain, a clone often isolated from patients in the
6
7 UK and Canada with cystic fibrosis. [36,37] Isolates from Clade E were found in the
8
9 burns unit's water and the ward environment, as well as from two patient's wounds.
10
11 However it was never detected in the critical care unit. Clade E was detected
12
13 throughout the study in a total of 10 different rooms (figure 2).
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15

16 17 **Inferring potential transmission events by whole-genome sequencing**

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19 Microevolutionary changes occurring over rapid time-scales (i.e. days to months)
20
21 have been used to detect potential chains of transmission in hospital and community
22
23 outbreaks.[19-21,38,39] The number of distinct mutations between given isolates
24
25 has been used to infer whether transmission events are likely to have occurred.
26
27 Such inferences are aided by prior knowledge of mutation rates in similar
28
29 populations. Two patients (1 and 4) in our study both had *P. aeruginosa* from Clade
30
31 E isolated from their wounds. These isolates had an indistinguishable genotype from
32
33 those present in water and the environment of the room they were nursed within
34
35 (figure 1 Panel C and figure 3). This genotype was detected in the patient's shower
36
37 water after initial patient screening, during screening of the second patient
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39 admission, twice during the second patient's stay and then 127 days later (days 27,
40
41 65, 89 and 216 respectively). When water isolates were positive, the genotype was
42
43 also detected in wet environment sites (shower drain, shower rosette and patient's
44
45 trolley) on the same days.
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52 Patient 5 was nursed on the critical care unit due to concomitant medical problems.
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54 *P. aeruginosa* belonging to Clade G was isolated from sputum during this time.
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3 Identical genotypes were detected contemporaneously in the water from the
4 associated sink and sink tap handle (see online supplementary appendix 4).
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8 Two further patients (patients 2 and 3) were positive for *P. aeruginosa*. Isolates from
9 these patients belonged to Clade C and D respectively. Neither clade was ever
10 isolated from hospital water. In both cases, identical genotypes were detectable in
11 the environment associated with the patient but these were not detected before or
12 after the patients' stay, indicating that the environment was not persistently
13 contaminated. During the course of patient 3's stay, the dry environment such as the
14 bedside table was contaminated, as was the patient's door handle and shower chair.
15 However, after patient discharge, the strain associated with this patient was never
16 seen again during the course of the study in any location.
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29 **Whole-genome sequencing permits source tracking of *P. aeruginosa* to** 30 **individual water outlets** 31 32

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34 Whole-genome sequencing has been reported previously for source tracking, but
35 never for the detection of transmission events from hospital water.[40] Phylogenetic
36 reconstruction within Clade E, the most commonly detected water clone
37 demonstrated additional diversity within this clone, with a total of 46 mutations
38 detected an average genetic distance between isolates of 4.1 mutations (figure 3).
39 The reconstruction demonstrated clear evidence of clustering of genotypes both by
40 room and outlet (figure 3). When *P. aeruginosa* was detected in the wet environment
41 (e.g. shower rosettes and drains) these genotypes were most often identical to those
42 found in water, indicating that the water was likely the ultimate source of that clone.
43 Genotypic variation was seen between outlets within the same room. For example,
44 tap water sampled from room 11 had a distinct genotype from that sampled from
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3 shower water in the same room and this was consistently found over multiple
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5 samplings. Notably, isolates from two patients fell within the cluster originating from
6
7 shower water, indicating that shower hydrotherapy was the most likely source of
8
9 infection. Two plasmids (designated pBURNS1 and pBURNS2) were detected in this
10
11 study set, which both demonstrated geographical clustering, with pBURNS1 only
12
13 being detectable in isolates from room 8 and pBURNS2 only being detectable in
14
15 isolates from the shower water in room 9.
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19 **Rapid evolution of antibiotic resistance associated with treatment**

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21
22 *P. aeruginosa* is commonly associated with antibiotic resistance due to a number of
23
24 predisposing features including intrinsic resistance, a repertoire of efflux pumps and
25
26 antibiotic-inactivating enzymes including beta-lactamases. [41] Three infected
27
28 patients (2, 3 and 5) received antibiotic therapy, and in each case this was
29
30 associated with the development of resistance to at least one therapeutic agent.
31
32 Associated mutations were detected that were either partially or fully explanatory of
33
34 the phenotype (online supplementary appendix 12).
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39 Patient 2 was treated with ciprofloxacin, nitrofurantoin and vancomycin (see online
40
41 supplementary appendix 11 for full details). 8/21 (38%) tested isolates from this
42
43 patient were ciprofloxacin resistant. 7/8 isolates (88%) of the ciprofloxacin-resistant
44
45 strains were distinguishable from the other isolates by a single SNP in *mexS*
46
47 (annotated as PA2491 in *P. aeruginosa* PAO1) (see online supplementary appendix
48
49 1 and 7). This SNP was predicted to result in a non-synonymous amino acid
50
51 substitution. Disruption of this gene has been shown to cause increased expression
52
53 of the *mexEF-oprN* multidrug efflux pump, associated with resistance to
54
55 quinolones.[42]
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3 Patient 3 was not treated with antibiotics, but isolates associated with this patient
4
5 demonstrated differences in resistance to timentin and piperacillin-tazobactam.
6

7 These changes were associated with non-synonymous mutations in *gacA*, the
8 response regulator of the GacA/GacS two-component system and in *lasR*, a
9 transcriptional activator required for transcription of elastase and LasA protease
10
11 (online supplementary appendices 2 and 8).
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17 Patient 4 was treated with meropenem, piperacillin/tazobactam, flucloxacillin and
18 colistin. Five isolates collected 10 to 18 days after initiation of meropenem showed
19 resistance to imipenem and intermediate resistance to meropenem (see online
20 supplementary appendix 3 and 9). The most likely mutation responsible for this
21 phenotype was detectable in two isolates, both of which had a frame-shift mutation in
22 the gene coding for the membrane porin *oprD*. [43]
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31 Patient 5 had a prolonged stay in ITU and had multiple medical problems including
32 *A. baumannii* infection and was treated with nine antibiotic agents including
33 ciprofloxacin, meropenem and piperacillin-tazobactam. Serial isolates from this
34 patient demonstrated the stepwise acquisition of two mutations (online
35 supplementary appendix 4). The first was in *nalC*, a probable repressor of the
36 TetR/AcrR family (online supplementary appendix 10). [44] On inspection of the
37 sequence alignment in this region, a large deletion of 196 nucleotide bases was
38 seen compared to the reference PAO1 strain. This mutation was seen in association
39 with full resistance to piperacillin-tazobactam, ceftazidime, aztreonam, meropenem
40 and intermediate resistance to ciprofloxacin. This deletion is likely to result in over-
41 expression of efflux pumps involving the *mexAB-oprM* operon. [44,45] Ciprofloxacin
42 resistance in a later isolate corresponded to the stepwise acquisition of a second
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3 mutation. This mutation is predicted to affect the well-studied DNA gyrase subunit A
4 gene (*gyrA*) which is strongly associated with ciprofloxacin resistance.[46]
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8 **Confirmation of *P. aeruginosa* genotypes in biofilms by whole-genome** 9 **metagenomic shotgun sequencing** 10

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12
13 *P. aeruginosa* is able to produce and survive in biofilms. Plumbing parts such as flow
14 straighteners, shower rosettes, flexible hoses, solenoid valves and thermostatic
15 mixer valves (TMV) are particularly at risk of biofilm formation due to factors
16 including surface areas, convoluted designs and inadequate pasteurisation. [47] To
17 confirm the presence of *P. aeruginosa* in water fittings associated with rooms on the
18 burns unit, we obtained a TMV removed by the hospital estates team from the
19 shower in room nine as part of compliance with UK guidelines for managing *P.*
20 *aeruginosa* in hospitals. On visual inspection, a biofilm was present which was
21 scraped from the surface with a sterile scalpel. DNA from this biofilm was extracted
22 for whole-genome shotgun sequencing. The majority of reads did not map to any
23 known bacterial taxa. The most abundant taxon identified was *Pseudomonas*
24 *aeruginosa* (3%). Subsequent alignment to the *P. aeruginosa* PAO1 reference
25 covered 94% of the 6.3 million base reference genome at a median coverage of 5x,
26 confirming that reads were correctly classified to this species and not other
27 environmental *Pseudomonas* species. Alignment to the *P. aeruginosa* Clade E
28 reference genome followed by phylogenetic placement of reads demonstrated that it
29 fell into the same clade as previously recovered isolates from the shower or tap in
30 room 9 (indicated on figure 3, and in online supplementary appendix 6).
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53 **DISCUSSION** 54 55 56 57 58 59 60

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3 The hospital environment has been intimately linked with *P. aeruginosa* infection for
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5 over 50 years yet hospital acquisitions, clusters and outbreaks remain a common
6
7 occurrence and understanding precise routes of transmission can be difficult. [47,48]
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10 Our results demonstrate that, even in a new hospital, *P. aeruginosa* can become
11
12 rapidly endemic in hospital plumbing. Furthermore, by linking *P. aeruginosa*
13
14 genotypes recovered from patients to specific individual water outlets, we offer
15
16 compelling evidence of unidirectional transmission from water to patients. Further, by
17
18 sequencing of a biofilm identified in a TMV from a hospital water system, we can
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20 identify the likely common source of genotypes found in water and in the hospital
21
22 environment.
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26 Our results suggest that use of whole-genome sequencing can reduce ambiguity
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28 about potential transmission events in hospitals and consequently inform infection
29
30 prevention efforts about the direction and sequence of transmission. Typing
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32 schemes such as MLST and PFGE are much lower resolution methods and would
33
34 not be able to provide sufficient information to permit such inferences to be made. It
35
36 is notable that the burns unit was colonised by a single clone, meaning that it was
37
38 very unlikely that water outlets at each bed space were colonised as a result of
39
40 transmissions from the patient or environment. For this to happen would require
41
42 multiple transmission events from separate patients with the same clone, for which
43
44 there is not evidence. Instead we speculate that this clone was introduced to the
45
46 hospital associated with its commissioning. One hypothesis is that particular
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48 plumbing fittings, i.e. the TMV may have been colonised simultaneously by a clone
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50 circulating in water. Clade E (ST395) has been frequently reported associated with
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52 water, so this remains a possibility. [49,50] However, it is possible that plumbing
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54 fittings are installed 'pre-seeded' with *P. aeruginosa* as has already been proposed
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3 by Kelsey.[3,5,47] Investigation of an outbreak in Wales implicated new plumbing
4 parts as a potential source of *P. aeruginosa*. New plumbing components are often
5 tested by companies prior to their supply and it is possible they were contaminated
6 prior to distribution. The limited amount of diversity (average 4 SNPs) seen within
7 this clade is consistent with a single founding genotype coinciding with the opening
8 of the burns unit, based on estimates from a previous study using WGS which
9 reported that mutations accumulate at a rate of approximately one every 3-4 months
10 in a hospital-associated clone.[51] However our results suggest that our isolates
11 accumulate mutations even more slowly. This may be due to reduced growth rates in
12 nutritionally-poor biofilms.[52]
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26 It is notable that antibiotic resistance to multiple first-line agents developed rapidly in
27 response to therapy. These results underline the importance of selecting appropriate
28 antibiotic therapy in *P. aeruginosa* infections. It is reassuring however that antibiotic
29 resistance genotypes selected *in vivo* did not show evidence of persistence in the
30 ward environment or transmission to other patients.
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38 Our study has certain limitations. Based on a previous audit, we expected around
39 one-third of patients screened for *P. aeruginosa* would develop colonisation or
40 clinical infection. In fact, only 5 out of 30 of patients were colonised. This may have
41 been related to guidance and engineering interventions being put in place during the
42 study as detailed in national guidance issued whilst this study was on-going. In
43 addition, infection control policies were revised to address control of an outbreak of a
44 multi-drug resistant *A. baumannii* in this same burns unit. Following these
45 interventions, only 1 of the last 20 patients recruited was infected with *P. aeruginosa*
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3 which may demonstrate the importance of national guidance in reducing
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5 transmissions.
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9 By focusing on burns patients who receive hydrotherapy, our study population were
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11 at extremely high risk of waterborne infection. In other patient groups it may be that
12
13 alternative routes of transmission including cross-infection or endogenous carriage
14
15 play a more important role. Our results suggest that our burns unit is endemically
16
17 colonised with a distinct clone of *P. aeruginosa* that may have been imported
18
19 coinciding with the opening of the hospital. Other intensive care units, particularly
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21 those which have been open for longer may have harbour a greater diversity of *P.*
22
23 *aeruginosa* as a result of increased opportunities for clones to be imported.
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26
27 One potential application for WGS in infection control would be to determine whether
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29 cases are as a result of water transmission, or represent sporadic clones originating
30
31 from the wider environment. Despite improved guidance concerning improved
32
33 engineering infection control practices and the introduction of the water safety group
34
35 in the UK, it may not be realistic to eliminate *P. aeruginosa* from hospitals entirely. In
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37 augmented care units such as ITUs, burns units and neonatal wards where *P.*
38
39 *aeruginosa* poses a significant risk to vulnerable patients, the increased resolution
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41 offered by WGS will justify its use, particularly as the costs continue to fall.
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46 In conclusion, we have identified through WGS clear evidence for transmission of *P.*
47
48 *aeruginosa* from specific water outlets to burns patients and offer a forensic-level
49
50 framework for dealing with outbreaks linked to hospital water. We expect WGS will
51
52 continue to make inroads into clinical microbiology and become a vital tool for
53
54 tracking *P. aeruginosa* in the hospital environment, helping inform targeted control
55
56 measures to help protect patients at risk of infection.
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Contributors

MP NM BO conceived the study. CW and AB enrolled patients into study and collected samples. NC collected environmental and water samples. NC CC MN processed samples and performed microbiology. NC CC JQ did sequencing. JQ NC CI NL analysed the data. NL NC JQ MP BO wrote the paper. All authors commented on the manuscript draft.

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Competing interests

None

Data Sharing Statement

Pacific Biosciences raw data files are available from the corresponding author (Nicholas J Loman, n.j.loman@bham.ac.uk) and will be made available via Data Dryad.

Ethics approval

The study protocol received approval from National Research Ethics Service committee in the West Midlands (reference number 12/WM/0181).

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Figure and Table Legends

Figure 1 Legend

An overview of all samples collected during the study in global phylogenetic context with other sequenced strains of *P. aeruginosa* from the set of Stewart *et al.*[28]

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3 Samples collected in this study are widely dispersed in the tree, which contains
4 isolates from different environments (Panel A). Bar plots indicate the numbers of
5 each type of sample collected (Panel B). Microdiversity within each clade is shown,
6 with the colour bar indicating the source of each sample (Panel C).
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12 **Figure 2 Legend**

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16 A schematic view of the 300-day study of *P. aeruginosa* in a burns centre and critical
17 care unit. Time in days is shown along the X-axis with bed numbers in the critical
18 care unit and burns unit along the Y-axis. Each circular icon indicates a positive
19 isolate of *P. aeruginosa*. The icon's logotype indicates which environment it
20 originated from (wound, urine/sputum, environment or water). The filled colour of the
21 icon indicates the clade it belongs to. Patient icons represent the enrolment of a
22 screening patient into the study and their location. Patient movements around the
23 hospital are noted by dotted lines. The five patients infected with *P. aeruginosa* are
24 denoted by rounded boxes. Boxes are coloured according to the patient number. In
25 the event two or more isolates of the same source and clade were collected on the
26 same day, these have been collapsed into a single circular icon.
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42 **Figure 3 Legend**

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45 The high-resolution phylogenetic reconstruction of Clade E isolates. This
46 demonstrates the clustering of genotypes by bed space. Patient associated samples
47 are contained within the room 11 clade. This clade contains water samples from the
48 shower and environmental samples from the shower, drain and trolley. The water
49 samples from the room 11 tap are in a distinct clade, indicating the biofilm within the
50 tap has a distinct genotype to the shower. This suggests environmental
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3 contamination was more likely to arise from contaminated shower water than tap
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5 water. Details of sampling site, days since start of study and presence of pBURNS
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7 plasmids are also shown. The likely phylogenetic position of *P. aeruginosa* detected
8
9 in a biofilm from a thermostatic mixer valve is shown in the clade associated with
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11 room 9 and indicated 'TMV'.
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14 15 **Appendix 1**

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18 Phylogenetic tree, sample origin and antibiotic-resistance phenotype for Clade C
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20 isolates. The patient column refers to the sampling event. If a sample was collected
21
22 from a patient colonised with *P. aeruginosa* or that patient's environment the patient
23
24 number if marked. Patient numbers starting with SP relate to screening patients who
25
26 were not colonised by *P. aeruginosa*.
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29 30 **Appendix 2**

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32 Phylogenetic tree, sample origin and antibiotic-resistance phenotype for Clade D
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34 isolates.
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37 38 **Appendix 3**

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40 Phylogenetic tree, sample origin and antibiotic-resistance phenotype for Clade E
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42 isolates.
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45 46 **Appendix 4**

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48 Phylogenetic tree, sample origin and antibiotic-resistance phenotype for Clade G
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50 isolates.
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53 54 **Appendix 5**

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56 A physical map of the burns unit, indicating individual patient bed areas, shower
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58 areas. The water supply is indicated by a blue line.
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Appendix 6

Clade E isolates in phylogenetic context with the metagenomics sample from a tap mixer valve.

Appendix 7

Single nucleotide and small indel variants detected within Clade C isolates.

Appendix 8

Single nucleotide and small indel variants detected within Clade D isolates.

Appendix 9

Single nucleotide and small indel variants detected within Clade E isolates.

Appendix 10

Single nucleotide and small indel variants detected within Clade G isolates.

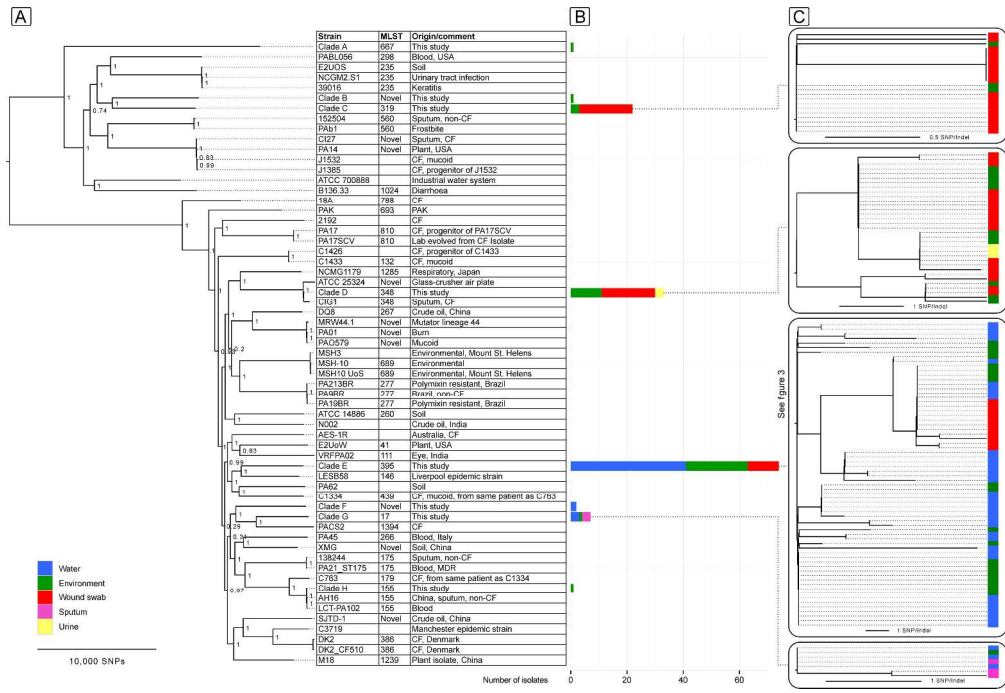
Appendix 11

The type of antibiotics administered to the five patients in this study.

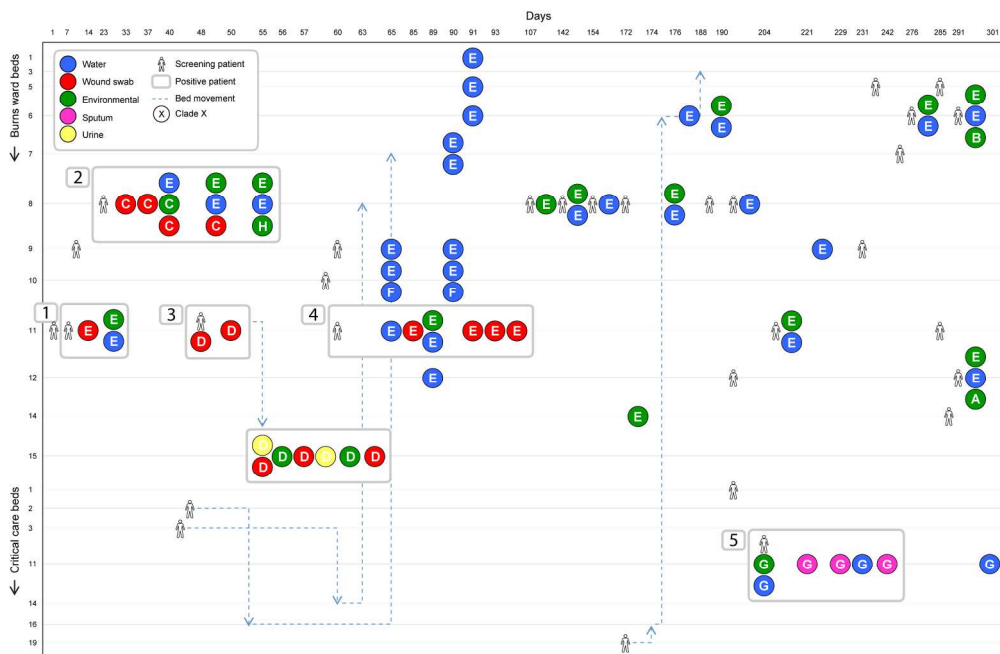
Appendix 12

Mutations predicted to be associated with antibiotic-resistance.

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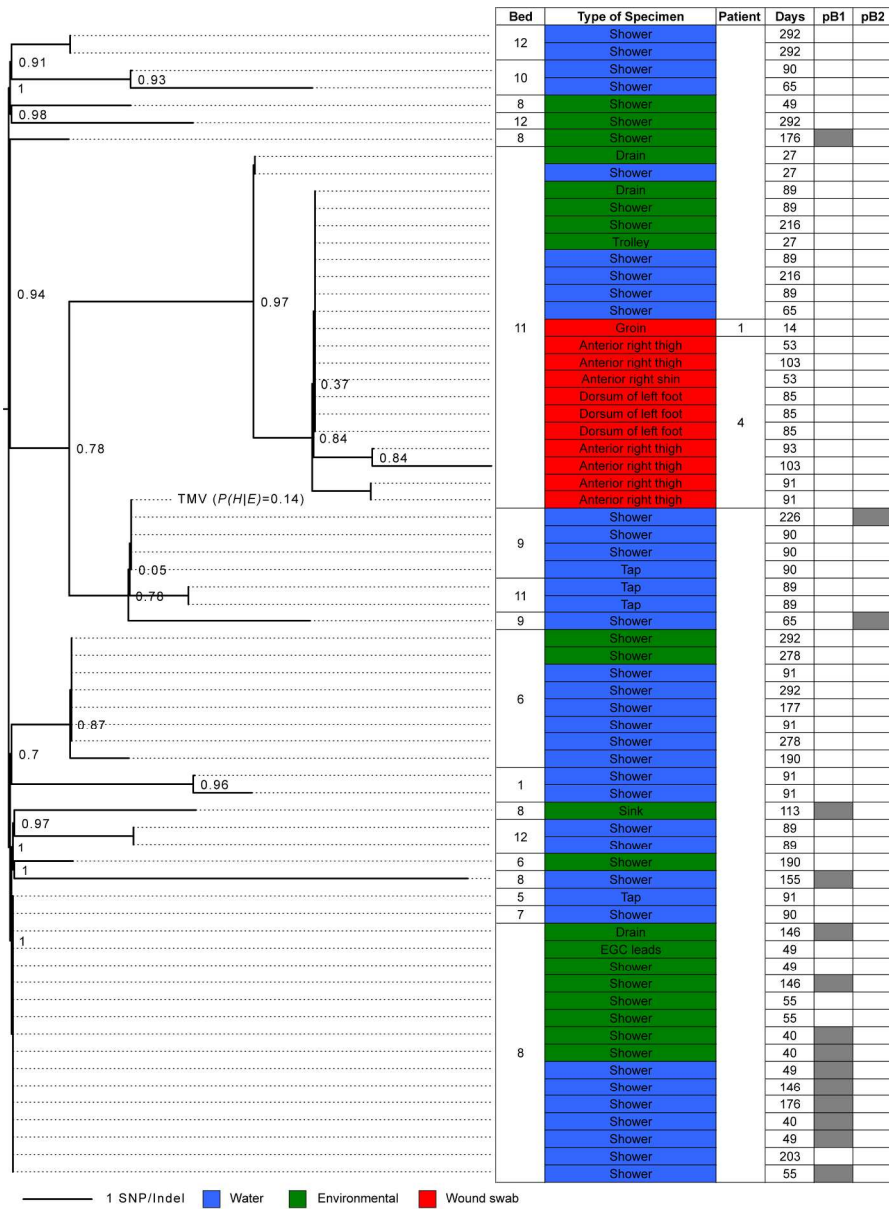


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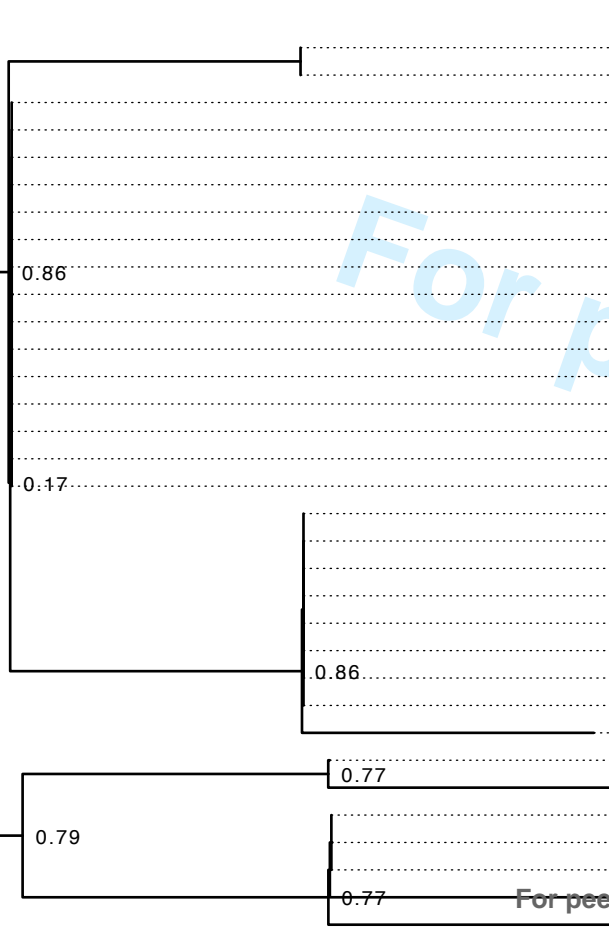


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Sample Number	Patient	Ward	Bed	Type of Specimen	Site	Days	TIM	PIP	TAZ	CAZ	AZT	IMI	MER	AMI	GEN	TOB	CIP	COL
922	P02	BCU	8	Wound swab	Upper back	40	R	I	I	S	I	S	S	S	S	S	S	S
921	P02	BCU	8	Wound swab	Upper back	40												
913	P02	BCU	8	Environmental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

1 SNP/Indel

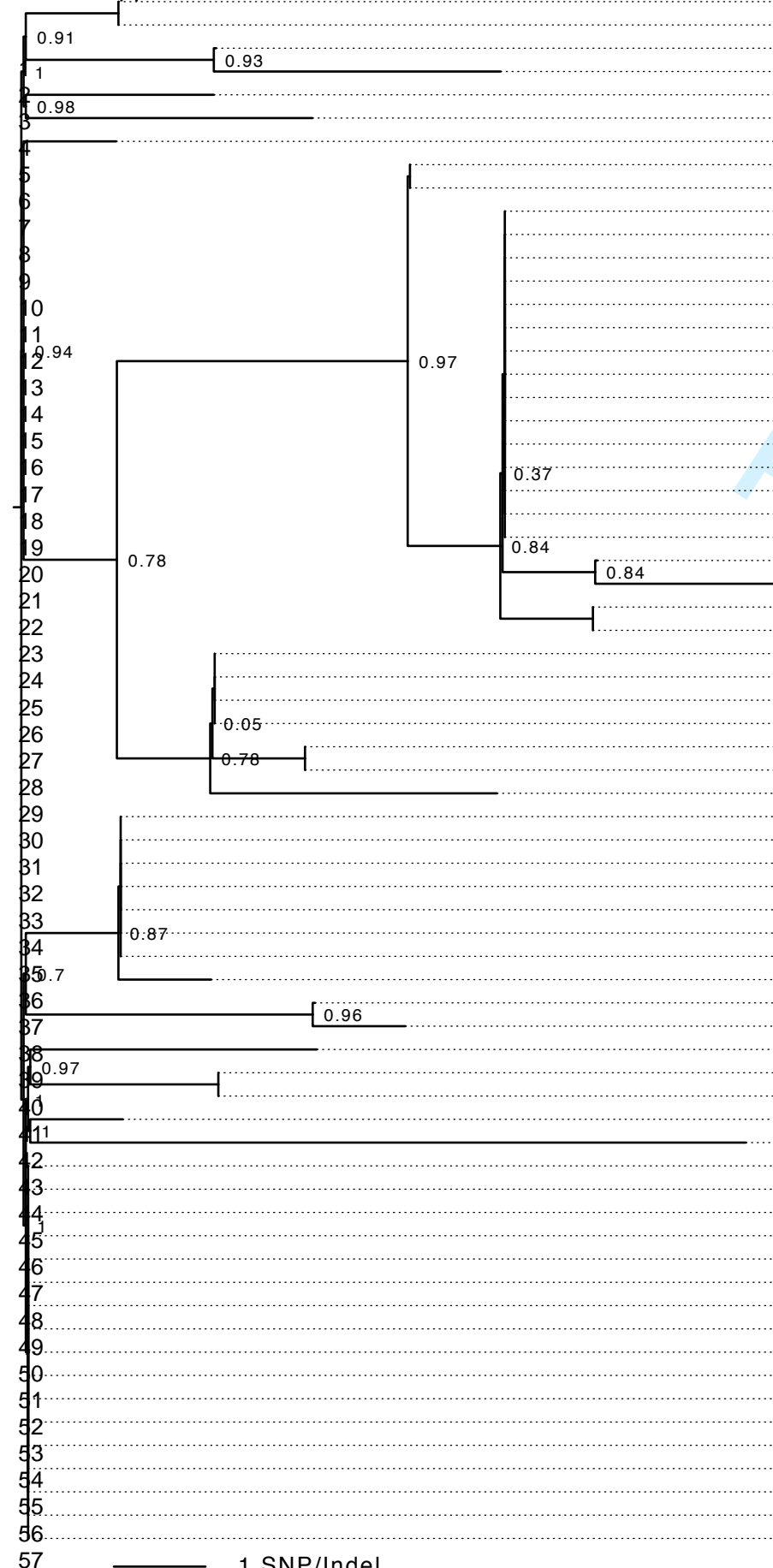
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Study Number	Patient	BMJ	Open	Bed	Type of Specimen	Site	Days	TIM	PIP	TAZ	CAZ	AZT	IMI	MER	AMI	GEN	TOB	CIP	COL
937	P03	BCU		11	Wound swab	Anterior left thigh	50												
936	P03	BCU		11	Wound swab	Anterior left thigh	50												
929	P03	BCU		11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

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Sample Number	Patient	Ward	Bed	Type of Specimen	Site	Days	TIM	PIP	TAZ	CAZ	AZT	IMI	MER	AMI	GEN	TOB	CIP	COL	pBURNS1	pBURNS2	
1067	SP30	BCU	12	Water	Shower (Pre-flush)	292															
1068	SP30	BCU	12	Water	Shower (Pre-flush)	292															
991	Water sampling	BCU	10	Water	Shower (Pre-flush)	90															
966	SP08	BCU	10	Water	Shower (Unknown)	65	R	S	S	S	I	S	S	S	S	S	S	S			
931	P02	BCU	8	Environmental	Shower (Rose)	49	R	S	S	S	I	S	S	S	S	S	S	S			
1065	SP30	BCU	12	Environmental	Shower (Rose)	292															
1034	SP15	BCU	8	Environmental	Shower (Rose)	176	R	S	S	S	R	S	S	S	S	S	S	S	Yes		
902	P01	BCU	11	Environmental	Drain	27															
903	P01	BCU	11	Water	Shower (Post-flush)	27	R	S	S	S	I	S	S	S	S	S	S	S			
981	P04	BCU	11	Environmental	Drain	89	R	S	S	S	I	S	S	S	S	S	S	S			
980	P04	BCU	11	Environmental	Shower (Rose)	89	R	S	S	S	I	S	S	S	S	S	S	S			
1048	SP20	BCU	11	Environmental	Shower (Rose)	216	R	S	S	S	I	S	S	S	S	S	S	S			
901	P01	BCU	11	Environmental	Trolley	27	R	S	S	S	I	S	S	S	S	S	S	S			
987	P04	BCU	11	Water	Shower (Hose)	89	R	S	S	S	I	S	S	S	S	S	S	S			
1050	SP21	BCU	11	Water	Shower (Post-flush)	216															
988	P04	BCU	11	Water	Shower (Pre-flush)	89	R	S	S	S	I	S	S	S	S	S	S	S			
968	SP10	BCU	11	Water	Shower (Unknown)	65	R	S	S	S	I	S	S	S	S	S	S	S			
900	P01	BCU	11	Wound swab	Groin	14	R	S	S	S	I	S	S	S	S	S	S	S			
979	P04	BCU	11	Wound swab	Anterior right thigh	53	R	S	S	S	I	S	S	S	S	S	S	S			
1008	P04	BCU	11	Wound swab	Anterior right thigh	103	R	S	S	S	I	S	S	S	S	S	S	S			
976	P04	BCU	11	Wound swab	Anterior right shin	53	R	S	S	S	I	S	S	S	S	S	S	S			
984	P04	BCU	11	Wound swab	Dorsum of left foot	85	R	S	S	S	I	R	I	S	S	S	S	S			
983	P04	BCU	11	Wound swab	Dorsum of left foot	85	R	S	S	S	I	R	I	S	S	S	S	S			
982	P04	BCU	11	Wound swab	Dorsum of left foot	85	R	S	S	S	I	S	S	S	S	S	S	S			
1007	P04	BCU	11	Wound swab	Anterior right thigh	93	R	S	S	S	I	R	I	S	S	S	S	S			
1009	P04	BCU	11	Wound swab	Anterior right thigh	103	R	S	S	S	I	S	S	S	S	S	S	S			
1005	P04	BCU	11	Wound swab	Anterior right thigh	91	R	S	S	S	I	R	I	S	S	S	S	S			
1006	P04	BCU	11	Wound swab	Anterior right thigh	91	R	S	S	S	I	R	I	S	S	S	S	S			
1052	SP22	BCU	9	Water	Shower (Hose)	226														Yes	
993	Water sampling	BCU	9	Water	Shower (Hose)	90	R	S	S	S	I	S	S	S	S	S	S	S			
994	Water sampling	BCU	9	Water	Shower (Pre-flush)	90	R	S	S	S	I	S	S	S	S	S	S	S			
992	Water sampling	BCU	9	Water	Tap	90	R	S	S	S	I	S	S	S	S	S	S	S			
985	P04	BCU	11	Water	Tap	89	R	S	S	S	I	S	S	S	S	S	S	S			
986	P04	BCU	11	Water	Tap	89															
967	SP09	BCU	9	Water	Shower (Unknown)	65	R	S	S	S	I	S	S	S	S	S	S	S		Yes	
1062	SP29	BCU	6	Environmental	Shower (Rose)	292															
1058	SP25	BCU	6	Environmental	Shower (Rose)	278															
1003	Water sampling	BCU	6	Water	Shower (Hose)	91															
1064	SP29	BCU	6	Water	Shower (Pre-flush)	292															
1036	SP15	BCU	6	Water	Shower (Pre-flush)	177															
1004	Water sampling	BCU	6	Water	Shower (Pre-flush)	91															
1057	SP25	BCU	6	Water	Shower (Pre-flush)	278	R	S	S	S	I	S	S	S	S	S	S	S			
1041	SP16	BCU	6	Water	Shower (Pre-flush)	190															
999	Water sampling	BCU	1	Water	Shower (Hose)	91															
1000	Water sampling	BCU	1	Water	Shower (Pre-flush)	91															
1010	SP11	BCU	8	Environmental	Sink	113	S	S	S	S	I	S	S	S	S	S	S	S	Yes		
989	Water sampling	BCU	12	Water	Shower (Hose)	89	R	S	S	S	I	S	S	S	S	S	S	S			
990	Water sampling	BCU	12	Water	Shower (Pre-flush)	89	R	S	S	S	I	S	S	S	S	S	S	S			
1040	SP16	BCU	6	Environmental	Shower (Rose)	190	R	S	S	S	I	S	S	S	S	S	S	S			
1031	SP13	BCU	8	Water	Shower (Hose)	155														Yes	
1001	Water sampling	BCU	5	Water	Tap	91															
996	Water sampling	BCU	7	Water	Shower (Pre-flush)	90	R	S	S	S	I	S	S	S	S	S	S	S			
1029	SP12	BCU	8	Environmental	Drain	146														Yes	
933	P02	BCU	8	Environmental	EGC	49	R	S	S	S	I	S	S	S	S	S	S	S			
930	P02	BCU	8	Environmental	Shower (Rose)	49	R	S	S	S	I	R	S	S	S	S	S	S			
1028	SP12	BCU	8	Environmental	Shower (Rose)	146														Yes	
942	P02	BCU	8	Environmental	Shower (Rose)	55	R	S	S	S	I	S	S	S	S	S	S	S			
941	P02	BCU	8	Environmental	Shower (Rose)	55	R	S	S	S	I	S	S	S	S	S	S	S			
917	P02	BCU	8	Environmental	Shower (Rose)	40	S	S	S	S	I	S	S	S	S	S	S	S	Yes		
916	P02	BCU	8	Environmental	Shower (Rose)	40	S	S	S	S	I	S	S	S	S	S	S	S	Yes		
934	P02	BCU	8	Water	Shower (Post-flush)	49	S	S	S	S	I	S	S	S	S	S	S	S	Yes		
1030	SP12	BCU	8	Water	Shower (post-flush)	146														Yes	
1035	SP14	BCU	8	Water	Shower (Post-flush)	176														Yes	
910	P02	BCU	8	Water	Shower (Post-flush)	40	S	S	S	S	I	S	S	S	S	S	S	S	Yes		
935	P02	BCU	8	Water	Shower (Pre-flush)	49	R	S	S	S	I	S	S	S	S	S	S	S	Yes		
1046	SP19	BCU	8	Water	Shower (Pre-flush)	203															
943	P02	BCU	8	Water	Shower (Pre-flush)	55	S	S	S	S	I	S	S	S	S	S	S	S	Yes		

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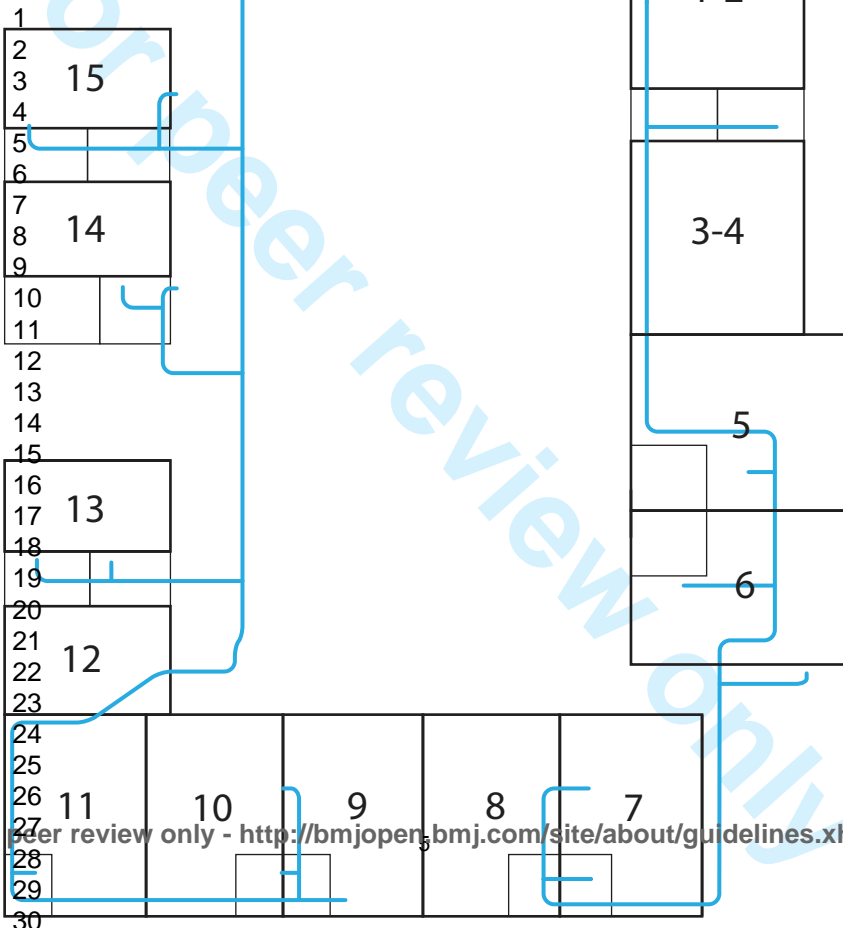
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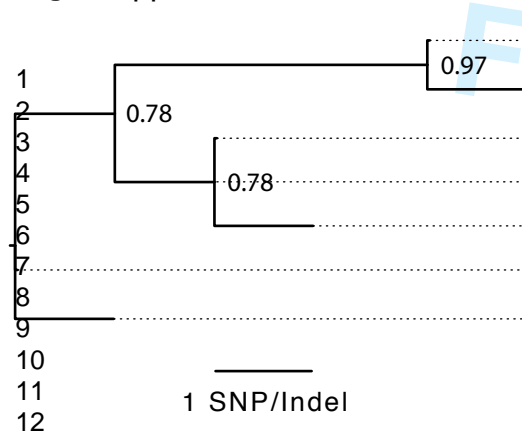
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Sample Number	Patient	WCCB	Open	Bed	Type of Specimen	Site	Days	TIM	PIP	TAZ	CAZ	AZT	IMI	MER	AMI	GEN	TOB	CIP	COL
1069	Water sampling	WCCB	11	11	Water	Tap	301	R	S	S	S	I	R	I	S	S	S	S	S
1045	SP20	WCCB	11	11	Environmental	Tap handle	204												
1047	SP20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

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Bed	Type of Specimen	Alignment
11	Shower	T CCTACTCC - CACAGACCTAACCT - - - - - G - - ACCGAAATCCTTTTCCG - C GAGGCG
11	Shower	T CCTACTCC - TACAGACCTAACCT - - - - - G - - ACCGAAATCCTTTTCCG - C GAGGCG
9	Tap	T GCAACTCC - CACAGACCTAACGT - - - - - G - - ACCGAAATCCTTTTCCG - C GC GGCG
9	TMV	T GCA - CT - C - CACAGA - - - AACGT - - - - - G - - ACCG - - A - CCTTTTC - G - C - - GGCG
11	Tap	T GTA ACTCC - CACAGACCTAACGT - - - - - G - - ACCGAAATCCTTTTCCG - C GC GGCG
8	Shower	T CCAACTCC - CACAGACCTAACGT - - - - - G - - ACCGAAATCCTTTTCCG - C TC GGCG
6	Shower	T CCAACTCC - CACAGCCTAACGT - - - - - G - - ACCGAAATCCTTTTCCG - C TC GGCG

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Study	Year	Age	Sex	Site	Study design	Outcome	Effect size	95% CI	Quality score	Notes
1	2008	18-24	M	USA	Case-control	MI	1.5	1.2-1.8	10	
2	2009	18-24	M	USA	Case-control	MI	1.2	1.0-1.4	10	
3	2010	18-24	M	USA	Case-control	MI	1.8	1.5-2.1	10	
4	2011	18-24	M	USA	Case-control	MI	1.4	1.1-1.7	10	
5	2012	18-24	M	USA	Case-control	MI	1.6	1.3-1.9	10	
6	2013	18-24	M	USA	Case-control	MI	1.3	1.0-1.6	10	
7	2014	18-24	M	USA	Case-control	MI	1.7	1.4-2.0	10	
8	2015	18-24	M	USA	Case-control	MI	1.5	1.2-1.8	10	
9	2016	18-24	M	USA	Case-control	MI	1.4	1.1-1.7	10	
10	2017	18-24	M	USA	Case-control	MI	1.6	1.3-1.9	10	
11	2018	18-24	M	USA	Case-control	MI	1.3	1.0-1.6	10	
12	2019	18-24	M	USA	Case-control	MI	1.7	1.4-2.0	10	
13	2020	18-24	M	USA	Case-control	MI	1.5	1.2-1.8	10	
14	2021	18-24	M	USA	Case-control	MI	1.4	1.1-1.7	10	
15	2022	18-24	M	USA	Case-control	MI	1.6	1.3-1.9	10	
16	2023	18-24	M	USA	Case-control	MI	1.3	1.0-1.6	10	
17	2024	18-24	M	USA	Case-control	MI	1.7	1.4-2.0	10	
18	2025	18-24	M	USA	Case-control	MI	1.5	1.2-1.8	10	
19	2026	18-24	M	USA	Case-control	MI	1.4	1.1-1.7	10	
20	2027	18-24	M	USA	Case-control	MI	1.6	1.3-1.9	10	
21	2028	18-24	M	USA	Case-control	MI	1.3	1.0-1.6	10	
22	2029	18-24	M	USA	Case-control	MI	1.7	1.4-2.0	10	
23	2030	18-24	M	USA	Case-control	MI	1.5	1.2-1.8	10	
24	2031	18-24	M	USA	Case-control	MI	1.4	1.1-1.7	10	
25	2032	18-24	M	USA	Case-control	MI	1.6	1.3-1.9	10	
26	2033	18-24	M	USA	Case-control	MI	1.3	1.0-1.6	10	
27	2034	18-24	M	USA	Case-control	MI	1.7	1.4-2.0	10	
28	2035	18-24	M	USA	Case-control	MI	1.5	1.2-1.8	10	
29	2036	18-24	M	USA	Case-control	MI	1.4	1.1-1.7	10	
30	2037	18-24	M	USA	Case-control	MI	1.6	1.3-1.9	10	
31	2038	18-24	M	USA	Case-control	MI	1.3	1.0-1.6	10	
32	2039	18-24	M	USA	Case-control	MI	1.7	1.4-2.0	10	
33	2040	18-24	M	USA	Case-control	MI	1.5	1.2-1.8	10	
34	2041	18-24	M	USA	Case-control	MI	1.4	1.1-1.7	10	
35	2042	18-24	M	USA	Case-control	MI	1.6	1.3-1.9	10	
36	2043	18-24	M	USA	Case-control	MI	1.3	1.0-1.6	10	
37	2044	18-24	M	USA	Case-control	MI	1.7	1.4-2.0	10	
38	2045	18-24	M	USA	Case-control	MI	1.5	1.2-1.8	10	
39	2046	18-24	M	USA	Case-control	MI	1.4	1.1-1.7	10	
40	2047	18-24	M	USA	Case-control	MI	1.6	1.3-1.9	10	
41	2048	18-24	M	USA	Case-control	MI	1.3	1.0-1.6	10	
42	2049	18-24	M	USA	Case-control	MI	1.7	1.4-2.0	10	
43	2050	18-24	M	USA	Case-control	MI	1.5	1.2-1.8	10	
44	2051	18-24	M	USA	Case-control	MI	1.4	1.1-1.7	10	
45	2052	18-24	M	USA	Case-control	MI	1.6	1.3-1.9	10	
46	2053	18-24	M	USA	Case-control	MI	1.3	1.0-1.6	10	
47	2054	18-24	M	USA	Case-control	MI	1.7	1.4-2.0	10	

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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	

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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		
	Colistin	15/12/2012	21/12/2012		
5	Gentamycin	12/04/2013	12/04/2013		
	Co-amoxiclav	13/04/2013	14/04/2013		
	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
	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
	Colistin	20/05/2013	23/05/2013		

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Gene	Mutation type	Effect	AA substitution	Resistance phenotype	Samples
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

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STROBE 2007 (v4) Statement—Checklist of items that should be included in reports of *cohort studies*

Section/Topic	Item #	Recommendation	Reported on page #
Title and abstract	1	(a) Indicate the study's design with a commonly used term in the title or the abstract	3
		(b) Provide in the abstract an informative and balanced summary of what was done and what was found	3-4
Introduction			
Background/rationale	2	Explain the scientific background and rationale for the investigation being reported	6
Objectives	3	State specific objectives, including any prespecified hypotheses	7
Methods			
Study design	4	Present key elements of study design early in the paper	8-9
Setting	5	Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection	8
Participants	6	(a) Give the eligibility criteria, and the sources and methods of selection of participants. Describe methods of follow-up	8
		(b) For matched studies, give matching criteria and number of exposed and unexposed	n/a
Variables	7	Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable	8-9
Data sources/ measurement	8*	For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group	8-9
Bias	9	Describe any efforts to address potential sources of bias	n/a
Study size	10	Explain how the study size was arrived at	9
Quantitative variables	11	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen and why	n/a
Statistical methods	12	(a) Describe all statistical methods, including those used to control for confounding	11-13
		(b) Describe any methods used to examine subgroups and interactions	n/a
		(c) Explain how missing data were addressed	n/a
		(d) If applicable, explain how loss to follow-up was addressed	n/a
		(e) Describe any sensitivity analyses	n/a
Results			

Participants	13*	(a) Report numbers of individuals at each stage of study—eg numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed	10
		(b) Give reasons for non-participation at each stage	10
		(c) Consider use of a flow diagram	-
Descriptive data	14*	(a) Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders	10-11
		(b) Indicate number of participants with missing data for each variable of interest	10
		(c) Summarise follow-up time (eg, average and total amount)	n/a
Outcome data	15*	Report numbers of outcome events or summary measures over time	10-11
Main results	16	(a) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (eg, 95% confidence interval). Make clear which confounders were adjusted for and why they were included	n/a
		(b) Report category boundaries when continuous variables were categorized	n/a
		(c) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period	n/a
Other analyses	17	Report other analyses done—eg analyses of subgroups and interactions, and sensitivity analyses	n/a
Discussion			
Key results	18	Summarise key results with reference to study objectives	16
Limitations			
Interpretation	20	Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence	16-18
Generalisability	21	Discuss the generalisability (external validity) of the study results	18
Other information			
Funding	22	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based	19

*Give information separately for cases and controls in case-control studies and, if applicable, for exposed and unexposed groups in cohort and cross-sectional studies.

Note: An Explanation and Elaboration article discusses each checklist item and gives methodological background and published examples of transparent reporting. The STROBE checklist is best used in conjunction with this article (freely available on the Web sites of PLoS Medicine at <http://www.plosmedicine.org/>, Annals of Internal Medicine at <http://www.annals.org/>, and Epidemiology at <http://www.epidem.com/>). Information on the STROBE Initiative is available at www.strobe-statement.org.