A citywide, clonal outbreak of *Pseudomonas aeruginosa* during a drought.

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Declaration

DECLARATION

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Dedication and Acknowledgments

Dedication

Firstly, I want to dedicate this dissertation to my wife Mariet, and daughter Cornelia for their unconditional support, love and always understanding time spent away from home working on the project. I will forever be grateful.

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Part A: Abstract

Background

Outbreaks of community-acquired *Pseudomonas aeruginosa* are typically small and localized. We investigated an increase in *P. aeruginosa* clinical isolates in Cape Town, South Africa during a severe drought.

Methods

Cases were defined as *P. aeruginosa* isolated from any clinical sample, and "wild-type" as susceptibility to all antibiotics tested. Residential addresses of community-acquired wild-type cases were mapped. Whole genome sequencing and multi-locus sequence typing were used to determine clonality and identify virulence genes. A modified case-control study in a subset of patients with bloodstream infection compared demographic and clinical characteristics between sequence types.

Results

The outbreak lasted 10 months from December, 2016 to September, 2017 with 3,321 documented cases. At the peak, cases reached 2.3-fold baseline and the city's dams reached a nadir of 19% capacity. Cases were distributed widely across the city. Multi-locus sequence type (ST) 303 was found in 27 of 42 (64%) blood culture isolates of *P. aeruginosa* during the outbreak, one of 19 (5%) before, and none of 11 after. ST303 infection was independently associated with younger age, but not with co-morbidities nor increased mortality. Fifty-one virulence genes were differentially present in ST303 compared with other sequence types, including genes involved in biofilm formation, iron uptake, and gut penetration.

Conclusion

The investigation confirmed a citywide outbreak of *P. aeruginosa* coinciding with and potentially related to a severe drought. We identified a predominant outbreak-associated clone, ST303, which harboured genes that could contribute to virulence and survival in drought-related conditions. Enhanced surveillance for *P. aeruginosa* during periods of drought is recommended.

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Part D: Abbreviations

AMR	Antimicrobial resistance
BSI	Bloodstream infection
CFU	Colony forming units
DNA	Deoxyribonucleic acid
ESRI	Environmental Systems Research Institute
GIS	Geographic information systems
HAI	Health care-associated infection
HIV	Human immunodeficiency virus
ICU	Intensive care unit
ISO	International Organization for Standardization
LPS	Lipopolysaccharide layer
SANS	South African National Standards
SNP	Single nucleotide polymorphisms
VAP	Ventilator associated pneumonia
VF	Virulence factor
WGS	Whole genome sequencing
WHO	World Health Organization

Part E: Introduction, Literature review and Context

Introduction, literature review and context

Pseudomonas aeruginosa and water-associated outbreaks

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Introduction

Pseudomonas aeruginosa is a Gram-negative, aerobic, oxidase positive, non-sporulating, motile, non-lactose fermenting rod that typically causes opportunistic infections.[1] It occurs naturally in soil and water. These bacteria are notoriously difficult to eradicate and treat, displaying biocide and multidrug resistance (MDR).[2] This organism is an important cause of health care associated infections.[3] Hospital outbreaks of *P. aeruginosa*, usually MDR(i.e. resistance to at least three classes of antibiotics), have been described in neonatal units, intensive care surroundings and in the wards.[4,5] Numerous hospital acquired outbreaks of *P. aeruginosa* have been associated with showers, faucets, sinks, and sink drains;[6-8] however, the ubiquitous nature of this organism in the environment makes the source of an outbreak difficult to identify.[1]

Pseudomonas can form biofilms, survive in most environments, and may colonize water systems.[9] Sloughing of the biofilm may increase the prevalence of planktonic bacteria in drinking water. A study performed in Mafikeng, South Africa identified *Pseudomonas* in biofilms from raw untreated water entering the Mondimola dam and treated drinking water from the Mondimola dam treatment plant.[9] However, data still remains limited on *Pseudomonas* distribution in community water infrastructure systems. To date, no widespread community outbreaks of *P. aeruginosa* has been identified in the literature, with only small common source community-acquired outbreaks reported. This review will focus on *P. aeruginosa* and outbreaks, its association with water shortages and climate change, and identify knowledge gaps and the importance to identify community-acquired *P. aeruginosa* infections in the clinical setting.

Clinical features and risk factors for *P. aeruginosa* infections

The World Health Organization (WHO) defines a nosocomial or health care-associated infection (HAI) as an infection acquired during hospital care, which was not incubating or present at the time of admission.[10] These infections may appear after discharge from the health care facility and also include occupational infections among health care workers at the facility.[10] Nosocomial infections related to *P. aeruginosa* include serious conditions such as urosepsis, bacteraemia, pneumonia, infective endocarditis, osteomyelitis, and secondary wound infections associated with burns.[11] Vulnerable patients at higher risk for pseudomonal infections include those that are immunocompromised, especially if neutropenic secondary to chemotherapy or malignancies.[12] Patients in high care or intensive care units are predisposed due to multiple invasive interventions, such as vascular and urinary catheters, endotracheal tubes, and underlying comorbidities including cystic fibrosis and bronchiectasis.[13] A large retrospective study that looked at more than 28 000 hospital acquired infections from 463 hospitals across the United States, found *P. aeruginosa* to be the seventh commonest cause of catheter-related bloodstream infections and second most frequent cause for ventilator associated pneumonia (VAP).[14] Mortality (ranging between 18% to 61%) and morbidity attributable to *P. aeruginosa* remains considerable among bacteraemia and VAP cases treated with incorrect empiric antibiotic therapy.[15,16]

Folliculitis and hot foot syndrome following recreational water exposure (swimming pools, jacuzzies, hot tubs) are infections usually encountered from the community setting in healthy individuals.[17] Keratitis from *Pseudomonas* in contact lens users is well documented.[18] *P. aeruginosa* endophthalmitis is a feared complication of eye surgery, and may result in visual impairment or the loss of an eye if not treated promptly.[19]

Antibiotic-resistant *P. aeruginosa*, a priority pathogen in hospitals and antibiotic-susceptible *P. aeruginosa* acquired in the community

Carbapenem-resistant *P. aeruginosa* isolates are on the WHO list of priority antibiotic-resistant pathogens that pose a major threat to public and human health.[20] They are responsible for hospital outbreaks of HAIs globally.[21] Several mechanisms of carbapenem-resistance have been identified.[22] Carbapenemase enzymes that hydrolyses beta-lactam antibiotics are the most critical mechanism for carbapenem-resistance in *P. aeruginosa*.[23] A recent systematic review and meta-analysis of carbapenem-resistant *P. aeruginosa* (24 studies) from the African continent, showed a total of almost 3 000 *P. aeruginosa* isolates collected between 2007 and 2018 across Africa.[20] The carbapenem-resistant *P. aeruginosa* pooled prevalence was 21.4% with VIM (Verona integrin-encoded metallo-beta-lactamases) isolates representing the most prominent carbapenemase gene (Class B according to Amber classification).[20] Most of these studies was from Northern Africa (Egypt and Algeria), with the remainder carried out in the Southern African countries (South Africa, Nigeria, Tanzania, Uganda, Ghana) and no reports from central Africa as yet. [20]

In addition, *P. aeruginosa* displays resistance to various other antibiotics, including the quinolones, aminoglycosides and polymyxins. Broadly, the major mechanisms of resistance are classified into adaptive (biofilm formation), intrinsic (expression of efflux pumps [example.g., MexCD-OprJ], low outer membrane permeability [e.g., OprD] and the production of antibiotic-inactivating enzymes), and acquired (spontaneous mutational changes or horizontal resistance genes transfer).[24] Frequently, resistance to many antibiotics is conferred by these different mechanisms simultaneously. Thus, the antibiotic treatment options against MDR *P. aeruginosa* are limited and of clinical concern.[25]

Community-acquired isolates of *P. aeruginosa* are usually susceptible to carbapenems and other anti-pseudomonal antibiotics.[26] Prediction of *P. aeruginosa* in patients presenting from the community setting is challenging. A point-prevalence study that recruited 3 193 community-acquired pneumonia patients from 222 hospitals and 52 countries around the world, found *P. aeruginosa* and antibiotic-resistant *P. aeruginosa* in 4.2% and 2.0% of clinical samples, respectively.[27] Risk factors identified in their multivariate analysis for the acquisition of *P. aeruginosa* community-acquired pneumonia included: invasive respiratory and/or vasopressor support during the initial 24 hours of hospitalisation, bronchiectasis, tracheostomy, prior *Pseudomonas* infection/colonisation, and very severe chronic obstructive pulmonary disease. [27] Rojas *et al*., 2019 reports in their systematic review and meta-analysis of risk factors associated with community-acquired *P. aeruginosa* bloodstream infection (BSI) solid tumour, previous antibiotic use, hematologic malignancy, and neutropenia as the most prevalent predisposing factors. An increased 30-day all-cause mortality was associated with inappropriate empiric antibiotic use.[28] Therefore, predisposing factors should be borne in mind when selecting antibiotics for the treatment of community-acquired sepsis where *P. aeruginosa* is a potential aetiological agent. However, overuse of anti-pseudomonal agents should be avoided to reduce selective pressure for this microorganism. Future research should focus on validated predictor scores for community-acquired pseudomonal infections to reduce mortality and/or overuse of anti-pseudomonal antibiotics.

P. aeruginosa **virulence factors, biofilm formation, and water system colonisation**

The wide spectrum of infections associated with *P. aeruginosa* is partially explained by the bacterium's battery of virulence factors. Virulence factors include enzymes such as elastases, proteases, cytotoxins, phospholipases, and secretion export systems.[29] The virulence genes

expressed by *P. aeruginosa* are cell density-dependent, and these differ between the planktonic (free living) and biofilm stages. The biofilm stage can precede the planktonic stage and vice versa, especially if the bacteria is nutrient deprived.[30]

A biofilm is an assembly of surface-associated mono- or poly-microbial aggregated cells that are enclosed in an extracellular polymeric substance matrix (Figure 1).[31] It prevents water loss from the bacterium's micro-environment through its direct hygroscopic properties and ability to ensure an architecture that reduces water evaporation.[32] Clinically, this capsule-like exopolysaccharide produced by mucoid *P. aeruginosa* strains is important to avoid phagocytic engulfment by macrophages, increase resistance to antimicrobial agents, and provides an adherence mechanism. This is prominent in patients with chronic pulmonary lung conditions and cystic fibrosis.[33]

The lipopolysaccharide layer (LPS) present on the cell surface of Gram-negative bacteria provides the microorganism with unique properties. The LPS is an essential component of the outer membrane, transforming it into a permeability barrier for hydrophobic molecules such as antibiotic compounds. [34] In addition, it plays an important role in host-bacteria interactions and modulating the host immune system response. The LPS consists of lipid A, core oligosaccharide, and the O-antigen region. [34] *P. aeruginosa* is capable of synthesizing two types of LPS, the A and B-band patterns. The A-band contains a homopolymer O-antigen chain length lipopolysaccharide, while 20 different O-serotype structures exist among the B-band O-antigen heteropolymer. The genes for the B-band chain are clustered in an operon (*wzz*, *wzy*, *wzx*, *wbpL*, *wbpK*, *wbpJ*, *wbpI*, *wbpH*, *wbpG*, *wbpE*, *wbpD*, *wbpB*, and *wbpA*) and expressed concurrently.[35] These proteins may potentially alter the surface charge and electrostatic

potential of the outer layer of the bacterium, increasing host cell adhesion ability compared to the A-band, and enhancing its pathogenic capability.[36]

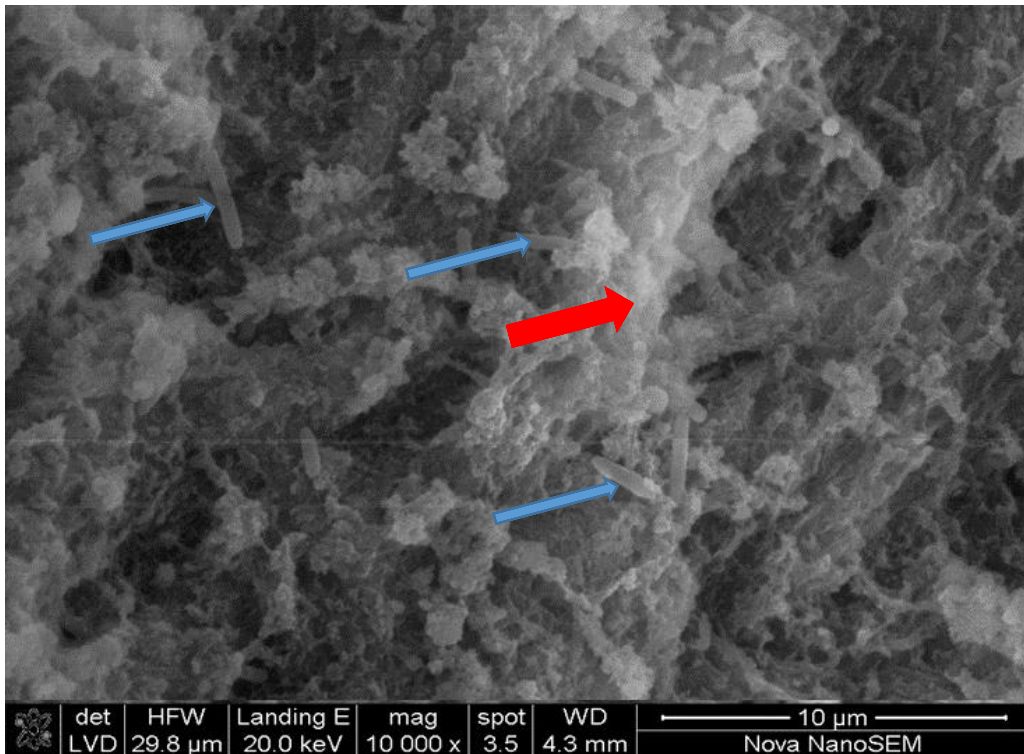

Figure 1. Scanning electron microscopy image of *P. aeruginosa* (10 000 X magnification). Note the abundant exopolysaccharide matrix (red arrow) surrounding bacilli (blue arrows), that support the notion of biofilm formation to the surface. (Picture taken by Dr. CJ. Opperman under the supervision of Dr. M. Waldron from the Aaron Klug, Centre of Imaging and Analysis, Electron Microscope Unit, University of Cape Town).

Iron uptake from the environment is essential for bacterial growth and mature biofilm formation in *P. aeruginosa*.[37] The two best known iron uptake systems in *Pseudomonas* are pyoverdine (high affinity) and pyochelin (low affinity). These iron scavenger and transport molecules are fluorescent, water soluble pigments that are evident when the organism is

isolated on culture media (Figure 2).[38] Extracellular iron is transported into the cell and bound to these siderophores.[39] The ferric-siderophore complexes are transported back into the cell with the aid of an outer membrane protein (FpvA). FpvR is a cytoplasmic membrane-associated anti-sigma factor that controls the activities of sigma factor FpvI responsible for the transcription of the ferric-pyoverdine receptor gene, fpvA.[40] Furthermore, *pvdD* is a pyoverdine biosynthesis gene involved in the synthesis of the siderophore.[41]

The type III secretion system that injects effector proteins into the cytoplasm of the invaded cell are important in the disruption of host cell cytoskeleton (ExoS and ExoY). [42]Not only do these virulence factors interfere with epithelium barriers, but also play an important role in evading innate immune response components, such as macrophages and neutrophils.[40] Flagella hook-associated protein 1 (FlgK), type IV pilus assembly protein (PilC) and pilV required for type 4 fimbriae biogenesis, has been shown to facilitate *P. aeruginosa* translocation through Caco-2 cell-monolayers *in vitro*. Theoretically these virulence factors may increase the risk of invasive disease after oral ingestion.[43] The bacterium's flagella may be important during stagnant water conditions to reach a surface, mediate attachment, facilitate development, and spread along a biofilm.[44]

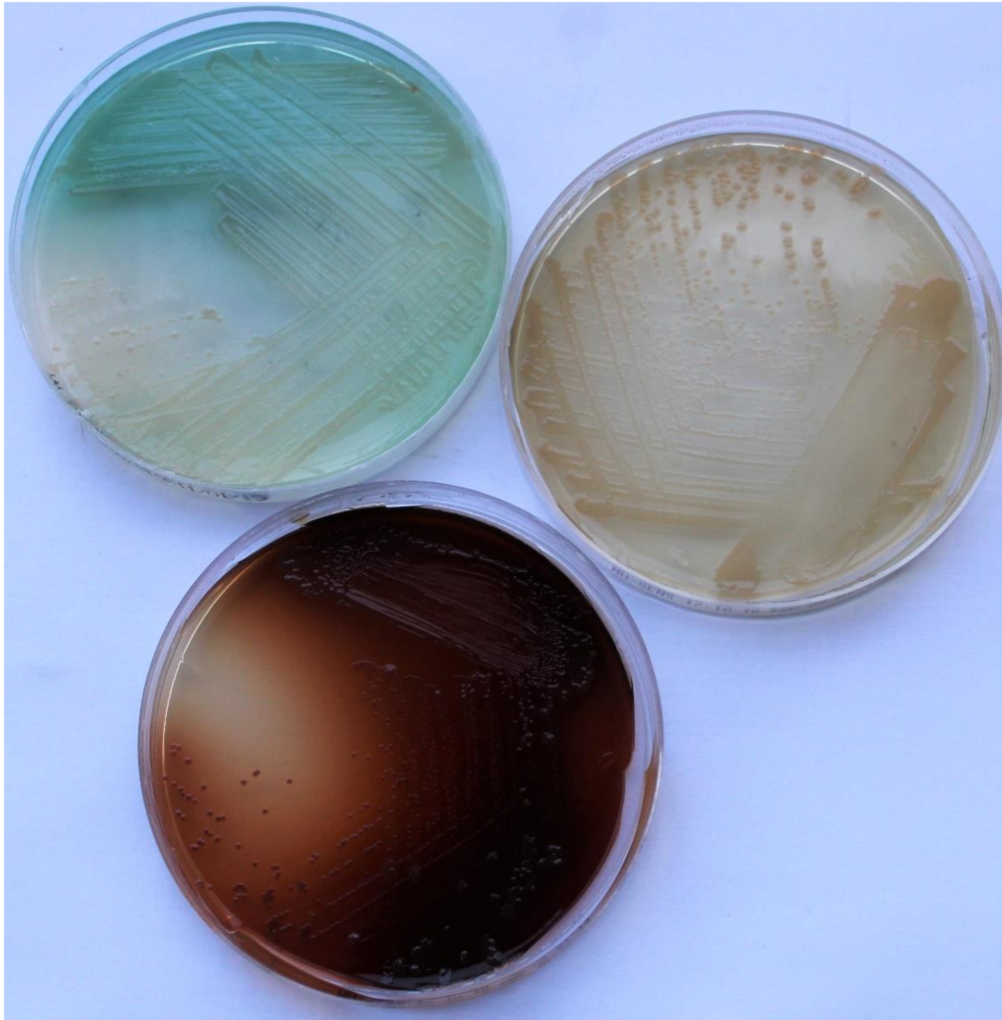

Figure 2. Different pigmentation colours of *P. aeruginosa* strains grown on Mueller-Hinton agar, incubated aerobically at 42°C for 24 hours in the microbiology laboratory, National Health Laboratory Service (NHLS), Groote Schuur Hospital (GSH). *P. aeruginosa* colonies can develop a blue-green colour (pyocyanin), a yellow-green colour (pyoverdine), a brown-to-black colour (pyomelanin), or a red-brown colour (pyorubin), among others (Pictures taken by Dr. CJ. Opperman).

Evidence for *P. aeruginosa* water-associated transmission and pathogenesis in community-acquired infections

Epidemiological evidence of hot tubs, spas, and whirlpools as a source of *P. aeruginosa* infection is well established. The majority of outbreaks include dermatitis, folliculitis, followed by otitis externa, keratitis, and urinary tract infections.[45-47] Some strains are well adapted to survive in high temperature and chlorine environments.[48] It is postulated that they contain a consortium of virulence factors that include proteases and lechithinases which provoke a skin inflammatory reaction in the presence of hydration and heat.[49] Other factors that may contribute to the pathogenesis of *P. aeruginosa* in these conditions, include skin pore dilatation during increased water temperatures, longer exposure times to water, and the potential of aerosols produced by hydrojet circulation systems.[50] *P. aeruginosa* has the capability to adhere and form biofilms to polyvinyl chloride piping, such as those used in water distribution systems, making it extremely difficult to remove.[48]

Swimming pools are associated with recreational disease outbreaks involving dermatitis and otitis externa *P. aeruginosa* infections. [51] This was evident in an outbreak among a competitive swimming team that trained twice daily, from whom 18 out of 25 developed ear infections.[51] In recent years, play equipment and water slides have become known to be colonised with *P. aeruginosa*. In one such an event, 151/593 children developed folliculitis on their lower back and buttocks. The organism was not only isolated from the water slides, but also the fire truck that supplied the water.[52]

Tap water has been described as a source of multiple *P. aeruginosa* nosocomial outbreaks.[53-56] Colonization of tap hardware and plumbing fixtures are the probable cause for exposure.

Patients can be exposed by drinking water, showering, bathing, or medical equipment rinsed with tap water. An association of *P. aeruginosa* pneumonia and aerosols generated by tap usage and splashing in sinks has been found.[57] Although multiple studies have investigated such outbreaks, most fail to find the organism in the distribution system, so the source remains unclear. One of the earliest links of a contaminated water supply causing an outbreak occurred in 1971 when 10 neonates in a hospital nursery acquired *P. aeruginosa* umbilical cord infection from groundwater contaminated by sewage.[50] A few small outbreaks of community-acquired *P. aeruginosa* have been associated with drinking water sources.[45,58] In one study, contaminated well water use resulted in shower and bath colonisation with subsequent infection. The author suggested that folliculitis secondary to *P. aeruginosa* could be much higher than reported due to its clinical confusion with scabies, insect bites, and other events. [1]

P. aeruginosa thrives best in the distal elements of plumbing systems. [59] Similar colonization of city water systems with *P. aeruginosa* persister cells (viable but not cultural on routine laboratory media) during nutrient poor conditions and in the presence of inhibitors, such as chlorine and copper has been documented.[59] Stagnation with biofilm formation on the inner surface of plastic-based and stainless steel containers used in rural households for the storing of drinking water during water restrictions is concerning, as it may function as a potential contaminated drinking water source during outbreaks.[60] In addition, portable home drinking water treatment technologies are important to improve the quality of drinking water, but little attention is given to prevent biofilm formation during storage.[61,62]

Water quality standards, monitoring, and risk assessment

In 1995, the European Union published a Drinking Water Directive draft, stating a permissible *P. aeruginosa* count of zero per 250 ml of bottled water. Broadbent, 1996 suggested a standard of no *P. aeruginosa* in a 100ml water sample collected from recreational hot tubs, spas and whirlpools, with ongoing monthly tests to ensure the water is safe.[50] Although these recommendations was made, the risk for *P. aeruginosa* gastrointestinal colonization from ingesting drinking water remains low, and they are usually found in 2% or less of water samples or at concentrations of 3-4 colony forming units (CFU)/ml, including bottled water.[62] Currently, there is no mandatory monitoring of municipality drinking water in South African for *P. aeruginosa*, which is in keeping with WHO guidelines.[64] Countries such as France recommend that *P. aeruginosa* values be below 1 CFU/100ml in the water systems of health care facilities with at-risk populations.[59]

It is unclear if enhanced water sampling and clinical specimen surveillance for *P. aeruginosa* during water restricted conditions should be implemented. Heterotrophic cell counts, coliforms, chlorine levels, and other parameters at water distribution points were uniformly within South African National Standards (SANS 241:2015) during the outbreak presented in this manuscript. Moreover, South Africa is currently drafting new drinking water standards which include specific monitoring for *P. aeruginosa* (unpublished correspondence: Water Quality and Source Protection, Bulk Water, Bulk Services Water and Sanitation, Water and Waste, City of Cape Town, South Africa, 12 December 2020).

Climate change and *P. aeruginosa* infections

Climate change is defined as an extreme weather event pattern or long-term shift in weather conditions. It has resulted in the emergence, redistribution, and resurgence of global infectious diseases.[65] Climate can impact the distribution, survival, and reproduction of disease pathogens, including parasites, bacteria, fungi, and viruses.[66] Vector (ticks, misquotes, snails) borne diseases, including, dengue, African trypanosomiasis, tick borne encephalitis, yellow-fever, malaria, lyme disease, and plague to mention a few is emerging in some non-endemic geographical areas due to a change in the weather conditions.[67] Diseases such as giardiasis, cholera, and salmonellosis have shown increased outbreaks during flooding or elevated temperatures.[68] Wind and dust storms can act as a transport vehicle for airborne viruses and fungal spores. In fact, human influenza virus can cross the Pacific with prevailing winds to spread from Asia to the Americas.[69]

The relationship between infectious diseases and climate change is well documented with many diseases having a seasonal peak in incidence, including *P. aeruginosa*.[70,71] *P. aeruginosa* infections are notoriously associated with warmer peak months of the year. Eber *et al.*, 2011 conducted a retrospective study evaluating BSIs over an eight-year period in 132 hospitals located across the United States. Interestingly, they found that a mean monthly temperature increase of 5.6°C, correlated with an 8.0% (95% CI 6.0-10.1) increase in *P. aeruginosa* BSIs. Furthermore, an increase in humidity independently resulted in a higher frequency of *P. aeruginosa* BSIs (5.8% increase for every 10% elevation in humidity, 95%CI 3.1-8.6).[70] After stratifying infections by site in a study done at a tertiary hospital in Brazil looking at 844 cases of *P. aeruginosa*, a significant association between urinary tract infections and ambient temperature ($R = 0.29$, $p = 0.021$) as well as precipitation ($R= 0.27$, $p = 0.036$) was found.[70]

In addition, chronic lung diseases, such as cystic fibrosis have shown a higher prevalence for *P. aeruginosa* acquisition in areas with higher ambient temperatures, during summer seasons, and among countries with a close proximity to the equator.[73,74] However, to date no published literature is available showing a direct link between drought and *P. aeruginosa* clinical infections.

An association between infectious diseases and drier environments has been postulated. Some authors suggest bacterial charge modifications during temperature alterations,[75] while others hypothesize that high temperatures interfere with human behavior, including recreational water activities resulting in seasonal variability.[76] This school of thought have been supported by a South-East England study looking at over 7 000 otitis externa patients between 2008 and 2016 from which *P. aeruginosa* was isolated at a proportional incidence of 36%. Isolates were mainly from children between 5 and 15 years of age and peaked during the summer months with increased water contact time during the holidays.[77] To conclude, seasonal patterns may vary between different subpopulations, geographic locations, microorganisms, and their clinical sites. Therefore, understanding seasonal fluctuations are essential to develop efficient prevention and control strategies.

Geographical distribution of community-acquired *P. aeruginosa*

Geographic information systems (GIS) can be used to map trends in disease transmission, geographical prevalence of disease, and the spatial-temporal aspects of disease occurrence.[78] ArcGIS is an ESRI (Environmental Systems Research Institute) developed platform that contains location services and tools for mapping and location-based analytics. ArcGIS software has been utilised in studies of the geographical distribution of cystic fibrosis *P. aeruginosa*

genotypes from patients in Australia.[79] From the 983 patients attending 18 cystic fibrosis centers in the study, some genotypes were found to be almost exclusive to specific areas, such as AUST-06 to Queensland or AUST-01 to South-Eastern Australia. In another study, spatial maps were implemented to indicate the proportion of children with *P. aeruginosa* per state and median days to acquisition. In this cohort of 3 623 young children with cystic fibrosis across the United States, 47% acquired *P. aeruginosa* between 2003 to 2009.[80] No literature has been published on the use of this mapping tool to showcase the distribution of community-acquired outbreaks of *P. aeruginosa*. Challenges that prevent the widespread use of GIS include the lack of technical and analytical skills, incomplete sets of data, and limited access to GIS infrastructure.[81]

Molecular typing and epidemiological investigations

Molecular typing is an important instrument to identify the source of an outbreak, detect identical or closely related strains, and recognize transmission patterns of nosocomial infections.[82] Outbreaks are usually caused by a single etiological agent and are genetically or clonally related because of point mutations (micro-evolutions) from an ancestor strain.[83] The large and complex genome (5-7 MB) of *P. aeruginosa* is a major reason for its capability to cause a broad spectrum of infections and to adapt to a variety of environmental niches.[30] While the core genome genes are essential for survival and is relatively preserved among species with high sequence conservation, it is the accessory genes that equip the bacteria with its strain specific peculiarities. In addition, recombination and horizontal gene transfer are established mechanisms to discard or acquire genomic segments between species.[84] Therefore, molecular typing allows the investigation of this genetic diversity, but may also give insight to important

phenotypic features, such as antibiotic resistance patterns, pathogenicity, host specificity, and virulence factors.[85]

Several molecular typing techniques have been developed for discriminating *P. aeruginosa* strains. Methods are based on DNA (deoxyribonucleic acid) sequencing [e.g. whole genome sequencing and multilocus sequence typing (MLST)], DNA banding patterns [e.g. pulsed-field gel electrophoresis (PFGE), variable-number tandem repeat (VNTR), and restriction fragment length polymorphism (RLFP)] or DNA hybridization (DNA micro and macro-arrays).[86] Pulsed-field gel electrophoresis is a labor-intensive method with high discriminative capability but lacks reproducibility between laboratories. Standardized protocols are still lacking with this technique for *P. aeruginosa* and a PFGE pulsotype database is not available.[87] MLST is a sequencing method that identifies single nucleotide polymorphisms (SNPs) and genomic rearrangements in six to seven housekeeping genes. The advantage of this method over DNA banding pattern-based techniques are high reproducibility and the potential to be subjected to high-throughput systems. The reliable data output is useful to establish a global database. [86] However, MLST still remains an expensive method compared to non-sequence based methods.[88] Species-specific oligonucleotide microarray typing using SNPs at various conserved loci have shown to be both cost-effective and reliable to identify genetic informative traits between strains.[89] A constraining element of the above fingerprinting techniques is their limit for discrimination when performing phylogenetic or relatedness analysis, since typing is based on only a few alleles or loci. [89]

Whole genome sequencing (WGS) can provide high level resolution to differentiate strains and investigate outbreaks.[83] The volume of sequence data from WGS makes *de novo* assembly

plausible in a single multiplex run for small bacterial genomes, without sacrificing coverage depth.[90] Although, WGS has higher error rates compared to Sanger sequencing, its repeated sequencing of DNA fragments mitigates this concern to obtain sequence consensus.[91] Core-genome MLST (cgMLST), whole-genome MLST (wgMLST) and core-genome single nucleotide polymorphism (cgSNP) analysis have become well-known genotypic typing methods to investigate outbreaks.[92] Isolates with mutations that do not affect restriction sites in the genome can have the same PFGE patterns, but different cgMLST/ wgMLST profiles that discriminate them. One of the challenges with the above mentioned WGS techniques remains the difficulty to determine the threshold SNP cut-off value that defines a single clone during an outbreak. Core-genome genes are not lost or gained as frequently as horizontal transfer genes and for this reason may be a more suitable area to investigate clonal relatedness.[93]

Knowledge gaps related to *Pseudomonas aeruginosa* and community-acquired outbreaks

Although various small, localised outbreaks of community-acquired *P. aeruginosa* have been described as mentioned above, to date no previous work has been published on a large-scale community-acquired outbreak. Defining the origin of *Pseudomonas* are lacking in most outbreak studies. Little is known about what species or quantities of *Pseudomonas* occur in water distribution or undisinfected groundwater systems. More studies are needed to investigate if colonisation of the gastrointestinal tract among healthy individuals result in disseminated infection. In general, the inoculation dose and clinical response data are lacking, not only for skin infections (bathing), but also oral ingestion and risk of colonisation. It is unclear if opportunistic and pathogenic bacteria, such as *Pseudomonas* with enhanced biofilm capability factors can be selected from the community during water-stress conditions.

Conclusion

P. aeruginosa colonisation and distribution via water is a plausible vector of community-acquired outbreaks, given the prolific amount of data linking *Pseudomonas* to hospital water infrastructure systems. New tools such as WGS are improving our understanding of virulence factors, biofilm formation, and antibiotic resistance analysis, and therefore our understanding of pathogenesis and treatment modalities. As our knowledge of outbreaks during changing climactic conditions expands so will surveillance practices and antibiotic policy. We are only just starting to unravel these dynamic social and environmental determinants of health in the 21st century.

Cape Town drought coinciding with the outbreak

During 2016, the Western Cape was experiencing its worst drought in 113 years (Figure 3). The combined average dam levels, providing water to the Cape Metropolitan reached a nadir of 19% capacity in May 2017.[94] This immense concern at the time grabbed the attention of the world as Cape Town could have been the first city to run out of water. Over the same period, we noted an increase in isolates of pan-susceptible (“wild type”- susceptible to all antibiotics tested against in the laboratory) *P. aeruginosa* from the diagnostic laboratory at the NHLS, GSH, Cape Town, South Africa. Initial investigation suggested that infections were acquired in the community; some were severe and required ICU (intensive care unit) admission. It was postulated that the increase in *P. aeruginosa* laboratory isolates may have been related to the drought conditions, given the capability of *Pseudomonas* to form biofilms and the possibility of low-pressure water pipes acting as a potential reservoir. There were no simultaneous increases in isolation of *Escherichia coli* from clinical samples, a well-known proxy for water quality nor did a change occur in water disinfection practices during the outbreak.[95] Thus, water

quality in Cape Town was not in question during the drought. Stringent water restrictions were escalated to stage 5 (70 litres/person/day) in Cape Town at the time, and the rainy season was coming to an end with water storage in some areas a possibility.

Figure 3. The Theewaterkloof dam is one of six major dams providing drinking water to the Cape Town area. These images taken by the once South African meteorologist, Derek van Dam, and published in the media, show the recession of dam levels between 2013 and 2017.

Statement reflecting the aim and objectives of the research study

The study was a collaboration between the University of Cape Town, Division of Medical Microbiology, GSH, NHLS and the Department of Water and Sanitation, City of Cape Town.

The aim of this study was to describe a community-acquired *P. aeruginosa* outbreak with descriptive and molecular epidemiology during a period of severe drought in Cape Town, South Africa.

The objectives were to:

1. Develop a case definition for an outbreak-related case.
2. Describe the outbreak area with geographic mapping and illustrate the distribution of cases with epidemic curves.
3. Explore a link between the *P. aeruginosa* outbreak and the water system infrastructure of the Cape Metropolitan area in a time of high-water stress. Analyse water samples around the Cape Metropolitan area to identify a potential point source in the community.
4. Perform WGS of *P. aeruginosa* isolated from clinical cultures before, during and after the suspected outbreak to identify genotypic clonal variation and virulence factors.

Study site

The NHLS is a laboratory situated within GSH, Cape Town, South Africa. It services approximately 80% of the population in the hospital's drainage area, providing services to numerous facilities including False Bay hospital, Mitchells Plain hospital, New Somerset Hospital, GSH, Red Cross Hospital, Mowbray Maternity hospital, Victoria Hospital, and various midwife obstetric units and clinics around Cape Town. The microbiology laboratory at GSH performs approximately 450000 tests annually. GSH is a tertiary hospital with 893 beds and several outpatient departments.

Objective 1

A case definition must be established to identify and count cases to determine who may be affected in the outbreak. Components of the case definition will include laboratory findings and/or clinical presentation. A broader definition will be considered initially before refining the definition to be more specific and exclude unrelated cases and nosocomial infections. A time frame will be incorporated into the definition to select patients within the outbreak period.

Objective 2

Descriptive epidemiology will be implemented to display the outbreak. Case distribution with epidemic curves will show *P. aeruginosa* numbers in different specimen types, antibiotic sensitivity profiles, distribution among wards and variation between different years. The residential addresses of patients identified as cases will be obtained from the Western Cape Government of Health, data centre and will be used to illustrate potential clustering when geographic mapping is applied with the ArcGIS software system (The software is licensed within the department of Pathology, division of Medical Microbiology, University of Cape Town).

Objective 3

Common water points and reservoirs will be identified, and environmental sampling done for *P. aeruginosa* as discussed with the Department of Water and Sanitation, and Biological Scientific Laboratory serves, City of Cape Town.

Objective 4

P. aeruginosa isolated from clinical cultures will be used to evaluate clonality and/or presence of new virulence factors expressed during a time of high-water stress. WGS will be done on isolates of selected cases and non-outbreak strains (controls), stored in -70°C freezers within the NHLS, department of Pathology, division of Medical Microbiology, GSH. Deoxyribonucleic acid extracting will be done with commercial kits. WGS with the Illumina sequencer will be performed within the division of Medical Microbiology, University of Cape Town. The results will be reviewed with bioinformatics software. The genotype and patient profiles of isolates before during and after the exposure will be compared.

Permission from provincial and local authorities and ethical review

Written permission to conduct the investigations will be sought from the Western Cape provincial government, local municipality, Department of Water and Sanitation and NHLS. The protocol will be submitted to the Human Research Ethics Committee of the University of Cape Town, and the Provincial Research Committee of the Western Cape Province.

Data analysis, interpretation, management, and confidentiality

Data will be imported into Stata version 16.1 (StataCorp, College Station, TX, USA) for descriptive statistical analyses of cases and controls. Geographic mapping will be done according to patient residence with data obtained from the Western Cape Department of Health. Nosocomial infections may be excluded with admission dates used as a proxy. Nosocomial or HAI is defined as a case of *P. aeruginosa* from a clinical sample collected after 48 hours of admission to the healthcare facility.

Information gained from the study will primarily be used to inform potential public health and water quality interventions. Secondarily, results will be used for research purposes and may be presented at congresses or published in peer reviewed journals. Completed results will be stored securely on a computer, password protected and only be available to investigators. Samples will be connected to a numbered code which will be delinked from patient identifiers, once relevant data for the study is entered. The research will conform to the Helsinki ethical guidelines for research in developing countries.

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Part F: Journal Manuscript

A citywide, clonal outbreak of *Pseudomonas aeruginosa* during a drought

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Key words: *Pseudomonas aeruginosa*; outbreak; clone; drought; Cape Town

A citywide, clonal outbreak of *Pseudomonas aeruginosa* during a drought; *March* 2021

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Abstract

Background

Outbreaks of community-acquired *Pseudomonas aeruginosa* are typically small and localized. We investigated an increase in *P. aeruginosa* clinical isolates in Cape Town, South Africa during a severe drought.

Methods

Cases were defined as *P. aeruginosa* isolated from any clinical sample, and "wild-type" as susceptibility to all antibiotics tested. Residential addresses of community-acquired wild-type cases were mapped. Whole genome sequencing and multi-locus sequence typing were used to determine clonality and identify virulence genes. A modified case-control study in a subset of patients with bloodstream infection compared demographic and clinical characteristics between sequence types.

Results

The outbreak lasted 10 months from December, 2016 to September, 2017 with 3,321 documented cases. At the peak, cases reached 2.3-fold baseline and the city's dams reached a nadir of 19% capacity. Cases were distributed widely across the city. Multi-locus sequence type (ST) 303 was found in 27 of 42 (64%) blood culture isolates of *P. aeruginosa* during the outbreak, one of 19 (5%) before, and none of 11 after. ST303 infection was independently associated with younger age, but not with co-morbidities nor increased mortality. Fifty-one virulence genes were differentially present in ST303 compared with other sequence types, including genes involved in biofilm formation, iron uptake, and gut penetration.

Conclusion

The investigation confirmed a citywide outbreak of *P. aeruginosa* coinciding with and potentially related to a severe drought. We identified a predominant outbreak-associated clone, ST303, which harboured genes that could contribute to virulence and survival in drought-related conditions. Enhanced surveillance for *P. aeruginosa* during periods of drought is recommended.

Introduction

The Gram-negative bacterium *Pseudomonas aeruginosa* is ubiquitous in the environment and water sources.[1] It is an important opportunistic pathogen of compromised and hospitalised patients, in whom it causes a range of infections. Outbreaks of *P. aeruginosa*, frequently multi-drug resistant, occur in neonatal units, ICUs, and hospital wards,[2-4] and are associated with hospital water distribution systems.[5,6] In the community setting, small localized common-source outbreaks of *P. aeruginosa* associated with recreational water (e.g. whirlpool baths) have resulted in superficial infection, although invasive disease has been described.[1,7,8]

We noted an increase of *P. aeruginosa* isolates at our large, public-sector laboratory in Cape Town, Western Cape, South Africa, beginning December 2016. Many of the isolates were pan-susceptible (“wild-type”) and appeared to be community-acquired. Some infections, as reported by a neighbouring tertiary hospital in Cape Town, were severe and required ICU admission.[9] At the time, the city was experiencing its worst drought in 113 years.[10] We investigated the suspected outbreak using descriptive epidemiology, spatial mapping, water sampling, genomic analyses, and a modified case-control study.

Methods

Laboratory setting and microbiological testing

The investigating laboratory, one of two public-sector National Health Laboratory Service laboratories in Cape Town, serves 65 government healthcare facilities (58 primary healthcare facilities, three district, two regional, and two tertiary hospitals) with a referral area of 755 km^2 and estimated population of 1.85 million.

Bacterial identification and antibiotic minimal inhibitory concentrations were determined using the Vitek 2 system (bioMérieux, Marcy-l’Étoile, France). Clinical & Laboratory Standards Institute breakpoints were applied.[11] Automated aerobic blood cultures were performed using the BacT/ALERT system (bioMérieux). Isolates of *P. aeruginosa* were stored in TSB-glycerol at -70º C.

Definitions

A case was defined as a patient with *P. aeruginosa* isolated from a clinical sample, irrespective of sample type or anatomical site. Only the first isolate from each patient was counted. A wild-type case was defined as *P. aeruginosa* susceptible to all antipseudomonal antibiotics tested

(ciprofloxacin, meropenem, imipenem, cefepime, ceftazidime, piperacillin-tazobactam, gentamicin, and amikacin); a drug-resistant case as *P. aeruginosa* non-susceptible to one or more antibiotics. A community-acquired case had *P. aeruginosa* from a sample collected before or within 48 hours of admission to hospital, and a nosocomial case, after. A bloodstream infection (BSI) was defined as one or more blood cultures yielding *P. aeruginosa*. The outbreak period (December 1, 2016 to September 31, 2017) began when cases/month exceeded 1.25 times the monthly average (calculated over 6 months) and ended when cases/month dropped below that threshold. The “outbreak-associated clone” was defined as *P. aeruginosa* multi-locus sequence type (ST) 303.

Descriptive epidemiology, municipal water indices, and spatial mapping

Data for the broader Western Cape province were obtained from the National Health Laboratory Service centralized data repository from January 1, 2013 to October 31, 2020. Detailed epidemiological, demographic, clinical, and laboratory data (including antibiotic-susceptibility testing results) were analysed only for cases identified at the investigating laboratory, between October 1, 2015 and December 31, 2018. Data were imported into Stata version 16.1 (StataCorp, College Station, TX) and Microsoft Excel (Microsoft Corporation, Redmond, WA). Epidemic curves were used to illustrate cases over time, by sample type, and healthcare facility type.

Residential addresses of community-acquired wild-type cases, where available on the laboratory information system, were plotted onto population-density maps using ArcGIS®Pro version 2.0 (Environmental Systems Research Institute, Redlands, CA). Cases per 100,000 population, based on 2011 Census estimates, were presented by sub-place. Spatial mapping

was performed for two periods: the 10-month outbreak and the same 10 calendar months 1 year before the outbreak.

Key indices reflecting Cape Town's municipal water supply were obtained from the Department of Water and Sanitation, City of Cape Town and included annual water inflow, combined storage dam levels, and municipal water usage restrictions imposed (Table S1).

Water sampling

The Department of Water and Sanitation, City of Cape Town collected weekly or bi-weekly samples from 230 designated drinking water sampling sites across the distribution system, excluding residents' homes, between February 5, 2018 and April 23, 2018 (after the outbreak period). Samples were collected according to ISO 5667-5:2006,[12]subjected to membrane filtration, and cultured on cetrimide agar (supplementary methods). *P. aeruginosa* was expressed as colony forming units (CFU)/100 ml.

Whole genome sequencing and analysis

Isolates selected for whole genome sequencing (WGS) included all community-acquired wild-type *P. aeruginosa* blood cultures from October 10, 2015 to October 28, 2018; a collection of community-acquired wild-type isolates from other sample types randomly stored during the outbreak; five randomly selected nosocomial wild-type blood cultures obtained during the outbreak; three randomly selected nosocomial drug-resistant isolates obtained during the outbreak; and five randomly selected water sample isolates. DNA was sequenced by MiSeq (Illumina, Inc., San Diego, CA) 150 bp paired-end sequencing. Sequenced data were aligned with reference PA01. Multi-locus sequence typing, virulence factor, and antimicrobial resistance gene detection were performed with SRST2.[13] For antimicrobial resistance

detection, the ARG-ANNOT database[14] was employed. A genetic relatedness dendrogram was drawn using core genome SNPs. Fisher's exact tests with multiple testing correction identified genes that were differentially present/absent in ST303 isolates compared to other sequence types, with adjusted P values ≤0.05 deemed significant.

Modified case-control study

A modified case-control study compared ST303 BSI ("cases") to all other wild-type non-ST303 BSI ("controls"), irrespective of sample timing relative to the outbreak. Clinical and demographic data, including underlying illness and outcome, were obtained from case notes or provided by the Provincial Health Data Centre, Western Cape Department of Health. Descriptive statistics, Mann-Whitney U test, Chi-squared test, and logistic regression were used in Stata to compare demographics, risk factors, and outcomes. Variables with a P value ≤0.05 were entered into the final model.

The study was approved by the Human Research Ethics Committee, University of Cape Town (HREC REF: 068/2018) and the Department of Health, Western Cape Government.

Results

Context

Annual water inflow reached an 89-year low in 2017,[15] and in May, 2017, combined dam storage levels reached a nadir of 19% capacity (Figure 1A). The City of Cape Town imposed increasingly stringent water restrictions from November 1, 2016 onwards (Table S1).

Description of the increase in cases

The outbreak commenced in December, 2016 when cases/month at the investigating laboratory rose from the preceding 6-month average of 66 to 87 (1.3-fold increase), peaked between March (154 cases; 2.3-fold) and June, 2017 (147 cases; 2.2-fold), and ended after 10 months in September, 2017 (Figure 1A). The increase was associated with a broader outbreak (3,321 cases) localized to the City of Cape Town and adjacent Cape Winelands over the same period (Figure 1B).

Of 1,152 cases identified at the investigating laboratory during the outbreak (53% male; median age, 38 years; interquartile range, 23 to 59), 866 (75%) were wild-type (compared to 64% in the 10-month calendar period 1 year before the outbreak; $P<0.001$), and of those, 451 (52%) were community-acquired. The 451 community-acquired wild-type cases were identified from 180 (40%) urine samples, 98 (22%) pus swabs, 92 (20%) respiratory samples, 38 (8%) blood cultures, 22 (5%) tissue samples, and 21 (5%) fluid aspirates or other samples (Figure S1). The community-acquired wild-type cases were from general wards (33%), emergency departments (20%), ICUs (20%), and outpatient departments or clinics (12%); 15% had no ward indicated (Figure S2).

Whole genome sequencing and analysis

Thirty-five different *P. aeruginosa* sequence types from 95 sequenced isolates, three of which were novel (ST3465, ST3473, and ST3474), were identified (Figure 2). The most frequent was ST303, occurring in one of 19 (5%) blood culture isolates obtained before the outbreak, 27 of 42 (64%) during, and none of 11 after. All sequenced *P. aeruginosa* isolates from 10 urine samples, two of three respiratory samples, three of four pus swabs, and both fluid aspirates obtained during the outbreak were ST303 (Table S3). Four of five nosocomial wild-type BSI

isolates were ST303, but none of three drug-resistant isolates were ST303. Core SNP distance analysis showed clustering of ST303 isolates (Figures 2, S3, and S4). Individual case summaries of the 95 isolates which underwent WGS can be found in Table S3.

Fifty-one virulence factors were identified in a significantly greater proportion of ST303 isolates compared to other sequence types (Figure 2, Table S4). These virulence factors included clusters of genes involved in B-band O-antigen chain lipopolysaccharide biosynthesis, iron uptake and potential gastrointestinal penetration.

Spatial mapping

Community-acquired wild-type cases resided in a wide geographic distribution across the laboratory's referral area before (152 cases in 74 sub-places) and during (324 cases in 89 sub-places) the outbreak (Figure 3). ST303 cases were evenly geographically distributed among overall cases.

Modified case-control study

There were 28 patients with confirmed ST303 BSI (cases) and 40 patients with BSI due to non-ST303 (controls) (Table 1). Patients with ST303 were significantly younger (median, 29 vs. 54 years; P=0.004). There were eight neonates and one child (aged 12) in the ST303 group, while all controls were adults. Fewer patients with ST303 had any comorbidity on univariate analysis (23 vs. 46%; P=0.04); after adjusting for age, the difference was not significant (adjusted odds ratio, 1.0; 95% confidence interval, 0.2 to 4.2). Thirty-day mortality was no different between the two groups (50%).

Water sampling

709 municipal water samples were collected from 230 sites (Table S2). Each site was sampled one to nine times over the 76-day sampling period. Ninety-four of 230 (41%) sites had 1-100 CFU/100 ml *P. aeruginosa* on one or more occasions, and 12 of 230 (5%) sites had >100 CFU/100 ml on one or more occasions. Four of five randomly chosen *P. aeruginosa* isolates from municipal water samples were wild-type (one was resistant to gentamicin) and none was ST303.

Discussion

We confirmed a 10-month citywide increase of *P. aeruginosa* cases, representing more than double the usual monthly case burden. A single, clonal multi-locus sequence type, ST303, previously isolated from the surface of a river in Queensland, Australia,[16] predominated among sequenced isolates during the outbreak, and was rare outside the outbreak period, suggesting a clonal, point-source outbreak.

The outbreak coincided with Cape Town's worst drought in 113 years and peaked at the nadir of the water shortage.[10] Proliferation and dissemination of the outbreak-associated clone might have been promoted by a range of drought-related factors, including lowering of water pressure in the municipal distribution system,[1,17] enforcement of daily water usage restrictions, and implementation of water-saving practices (e.g. use of rainwater storage tanks, re-use of grey water, less frequent bathing, and less flushing of toilets).[18] *Pseudomonas* has a predilection for warm, stagnant water[19,20] and readily forms biofilm, a consortium of bacteria attached to a surface and enclosed in an extracellular polysaccharide matrix which protects it from environmental stressors.[21] Our virulence factor analysis suggested the outbreak-associated clone had enhanced capacity for biofilm production, through the presence of genes involved in

lipopolysaccharide biosynthesis and iron uptake, which may have allowed it to colonize a particular water source or distribution system,[22] and hold a survival advantage in drought-related conditions.[23-26] During unsteady water flow, biofilm can dislodge, and planktonic cells carried to distal parts of the distribution system and termination points.[27] We hypothesize that dislodging of biofilm containing the clonal strain, with widespread seeding of municipal water during the drought, may have led to high levels of gastrointestinal colonisation among residents, predisposing those with risk factors to development of clinical infection.[1]

We showed that the outbreak-associated clone possessed genes which could theoretically increase gastrointestinal penetration, with risk of invasive disease after ingestion.[28] The risk for *P. aeruginosa* colonization from ingesting drinking water is low at concentrations <1.5 CFU/100 ml[1], but water provided in healthcare facilities with at-risk populations should have *P. aeruginosa* ≤1 CFU/100 ml.[27] While *P. aeruginosa* has previously been found in 2% or less of drinking water samples,[1,29] we found *P. aeruginosa* at >100 CFU/100 ml in 5% of sampling sites, albeit in a study conducted immediately after the outbreak.

Municipal water in Cape Town is supplied by the Western Cape Water Supply System, a network of rivers, dams, underground water sources, reservoirs, pumping stations, and pipelines. There was no obvious geographic clustering of cases to suggest one of these sources was contaminated; however, admixture along the distribution system[30,31] may have obscured a proximal source. The citywide extent of the outbreak-associated clone likely excludes a source in distal parts of the distribution system or in residential plumbing. Water treatment in Cape Town includes chlorine disinfection, but *Pseudomonas* can be resistant to this process,[32] and the city does not monitor drinking water for *P. aeruginosa* routinely, in line with WHO guidelines.[33] Nonetheless, heterotrophic cell counts and chlorine levels at water distribution

points were uniformly within national standards (SANS 241:2015) during the outbreak.[34-36] Moreover, we cannot rule out other potential sources, such as contamination of processed foods[1] or beverages (e.g. soft drinks). South Africa recently experienced the largest recorded outbreak of listeriosis, linked to a single meat-processing plant.[37] The outbreak resolved as water storage dams were replenished by winter rains and has not recurred as of November 30, 2020; however, a causal association with the drought remains hypothetical. Many diseases have a seasonal peak;[19,20] *P. aeruginosa* sepsis in previously well Taiwanese children was more common during warm weather.[7]

The outbreak-associated clone possessed genes for the B-band O-antigen chain length lipopolysaccharide, potentially conferring enhanced virulence. This protein may alter the surface charge and electrostatic potential of the bacterial outer layer, resulting in increased cell adhesion compared to the A-band.[38] Patients with the outbreak-associated clone had predisposing risk factors for *P. aeruginosa* infection, such as solid organ tumours, hematologic malignancy, and neutropenia.[39] However, the modified case-control study failed to identify differences in co-morbidities or mortality when comparing patients with the outbreak-associated clone to patients with other sequence types. Overall, 30-day mortality in BSI cases (50%) was greater than that previously reported (34%).[39]

Patients with the outbreak-associated clone were significantly younger than patients with other sequence types. We identified eight cases of community-acquired BSI in neonates (seven during the outbreak, all the outbreak-associated clone). In contrast, no cases of wild-type *P. aeruginosa* in neonates were identified in the same 10 calendar months 1 year before the outbreak (data not shown). A previous outbreak of neonatal sepsis was attributed to umbilical cord infection from drinking water contaminated with *P. aeruginosa*.[1] Another potential source

of neonatal infection is maternal urogenital tract colonization.[40] Indeed, urinary cases increased prominently during the outbreak, and all 10 sequenced urinary isolates were the outbreak-associated clone. Urogenital tract colonisation might have occurred while bathing in contaminated water[1] or from toilet splashback. Aerosolization of contaminated water was hypothesized to be the route of acquisition of *P. aeruginosa* respiratory infection at a neighbouring institution during the outbreak.[9]

We focused our investigation on community-acquired wild-type *P. aeruginosa*, as the increase was primarily attributable to wild-type cases and community strains are typically drug-susceptible.[32] However, we did identify the outbreak clone in four of five nosocomial wild-type cases, suggesting either a common source shared between the community and healthcare facilities, undetected colonisation prior to hospitalisation, or spread of the clone within hospitals. *P. aeruginosa* infection had a substantial background incidence, which may have obscured risk factors associated with the outbreak. Further study limitations include that detailed data and isolates were obtained from a subset of patients at one of two public-sector laboratories in Cape Town, and data were also not obtained from private laboratories. Water sampling was conducted after the outbreak, and only five isolates were available for WGS. We did not investigate socio-economic or other population-level factors, nor risk factors related to food or beverage exposure. Future research will focus on the virulence and biofilm potential of the outbreak-associated clone.

In summary, we identified a citywide, clonal outbreak of *P. aeruginosa* during a severe drought. The outbreak-associated clone, ST303 may have enhanced capacity to persist and produce biofilms in drought-related conditions, and colonize water distribution systems or food and beverage production facilities. The strain possessed numerous virulence determinants

facilitating invasion across the gastrointestinal tract and subsequent systemic illness. As climate change increases the frequency of drought, it may pose yet unrecognized threats to public health.[41] Consideration should be given to disease surveillance and testing of water distribution systems for pathogens such as *P. aeruginosa* which may proliferate under drought conditions.

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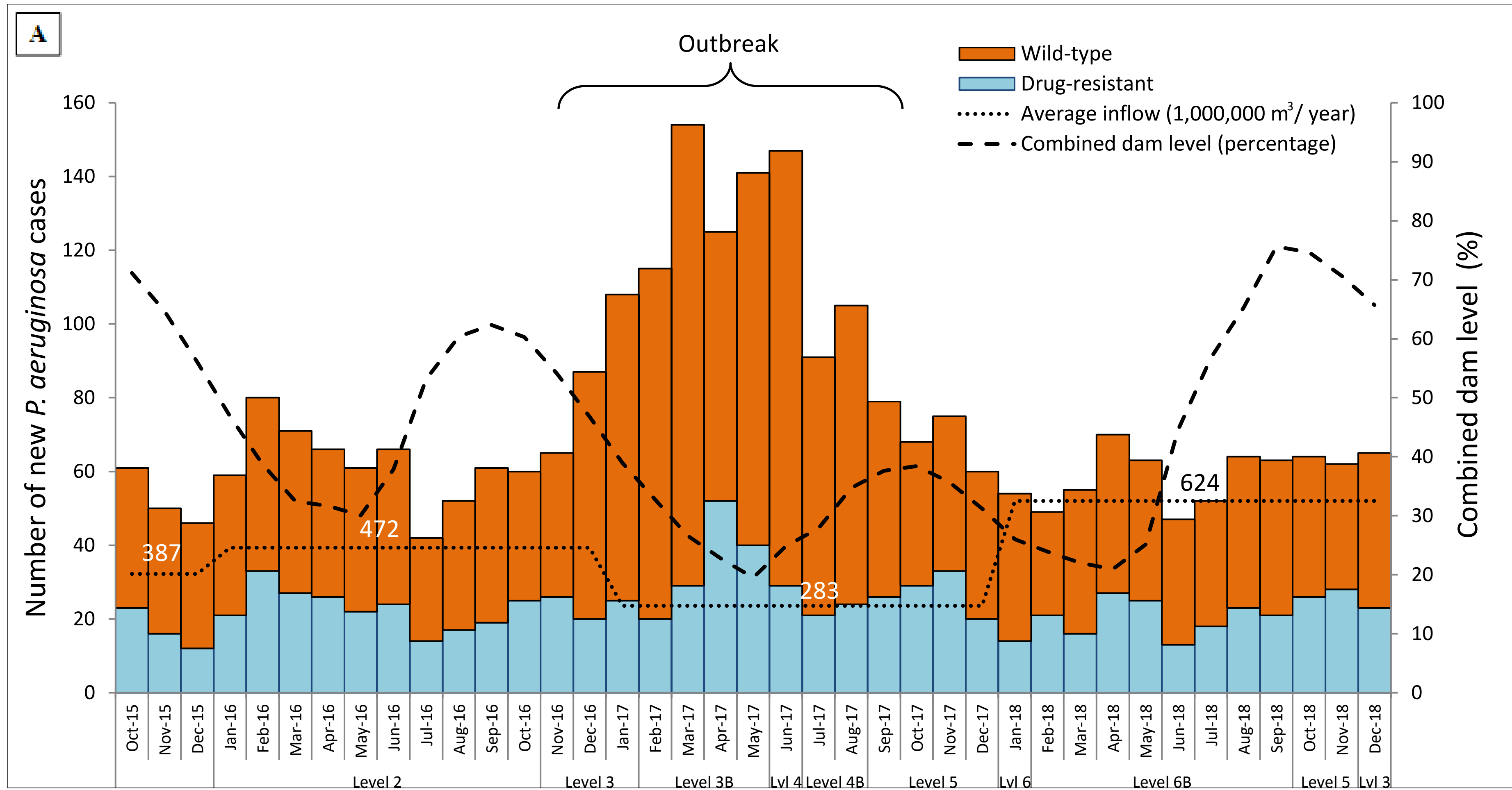
A
Outbreak
Wild-type
Drug-resistant
Average inflow (1,000,000 m³/ year)
Combined dam level (percentage)
Number of new P. aeruginosa cases
Combined dam level (%)
387
472
283
624
Oct-15
Nov-15
Dec-15
Jan-16
Feb-16
Mar-16
Apr-16
May-16
Jun-16
Jul-16
Aug-16
Sep-16
Oct-16
Nov-16
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Level 5
Lvl 3

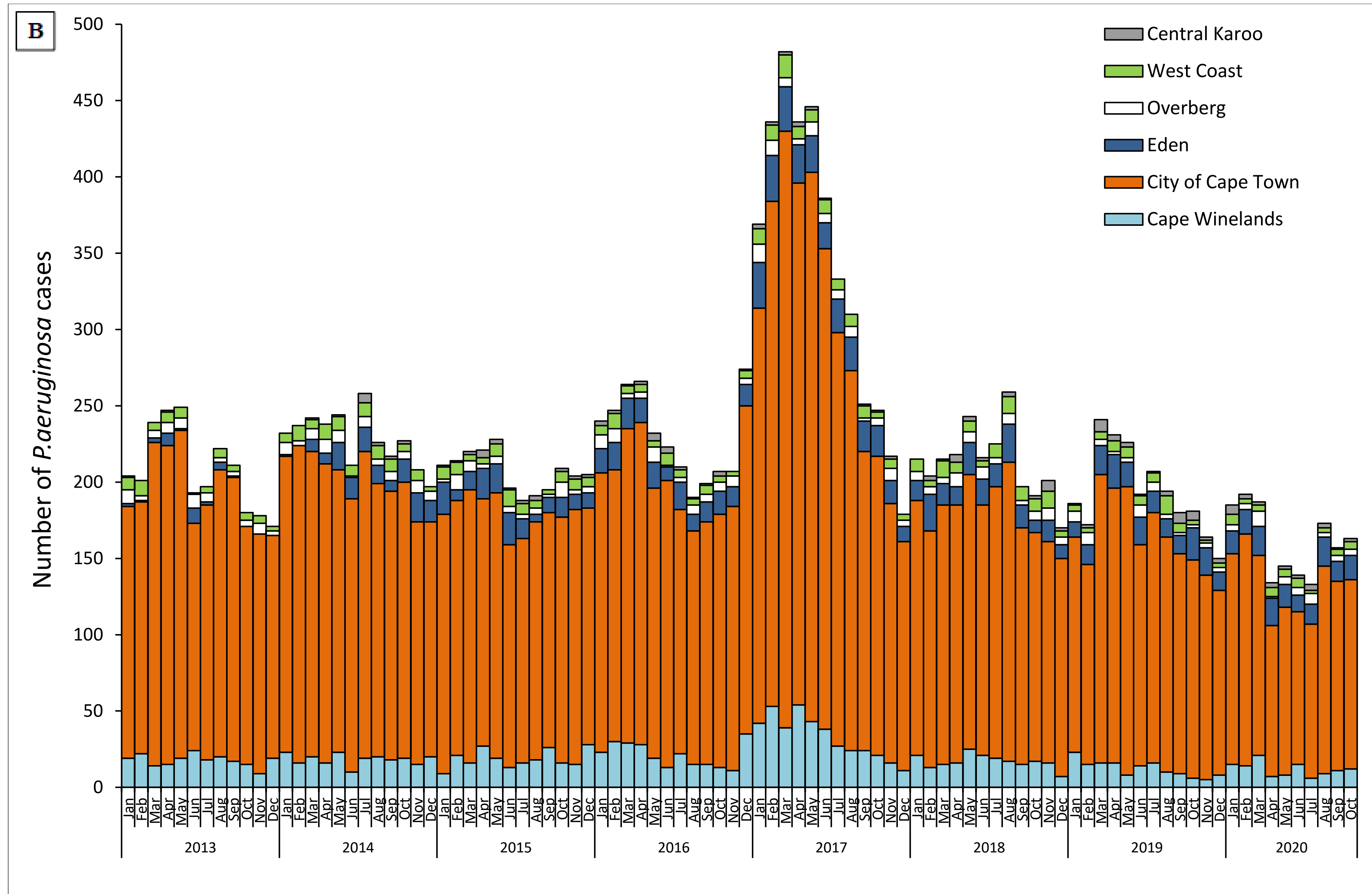
B
Number of *P.aeruginosa* cases
0
50
100
150
200
250
300
350
400
450
500
Central Karoo
West Coast
Overberg
Eden
City of Cape Town
Cape Winelands
Jan
Feb
Mar
Apr
May
Jun
Jul
Aug
Sep
Oct
Nov
Dec
2013
2014
2015
2016
2017
2018
2019
2020

Figure 1. Number of new wild-type and drug-resistant *P. aeruginosa* monthly cases isolated from all clinical sample types submitted to the investigating laboratory from October 1, 2015 to December 31, 2018. Wild-type is defined as *P. aeruginosa* susceptible to ciprofloxacin, meropenem, imipenem, cefepime, ceftazidime, piperacillin-tazobactam, gentamicin, and amikacin and drug-resistant as non-susceptibility to one or more antibiotics. The outbreak period is indicated between December 1, 2016 and September 30, 2017. Regional annual water inflow (total volume of incoming water from rivers, streams, rainfall, and underground water sources) is displayed as 1,000,000 m^3 water / year and reached an 89-year low in 2017. Cape Town's combined storage dam levels (from six major and eight minor storage dams) are shown as percentage of total capacity (data provided by the Department of Water and Sanitation). To reduce water use, the City of Cape Town imposed water restriction level 2 on January 1, 2016, incrementally increasing to level 6B on February 1, 2018 (Table S1) (Panel A). Number of new monthly *P. aeruginosa* cases isolated from all clinical sample types submitted to public-sector laboratories across districts in the Western Cape province from January 1, 2013 to October 31, 2020 (Panel B).

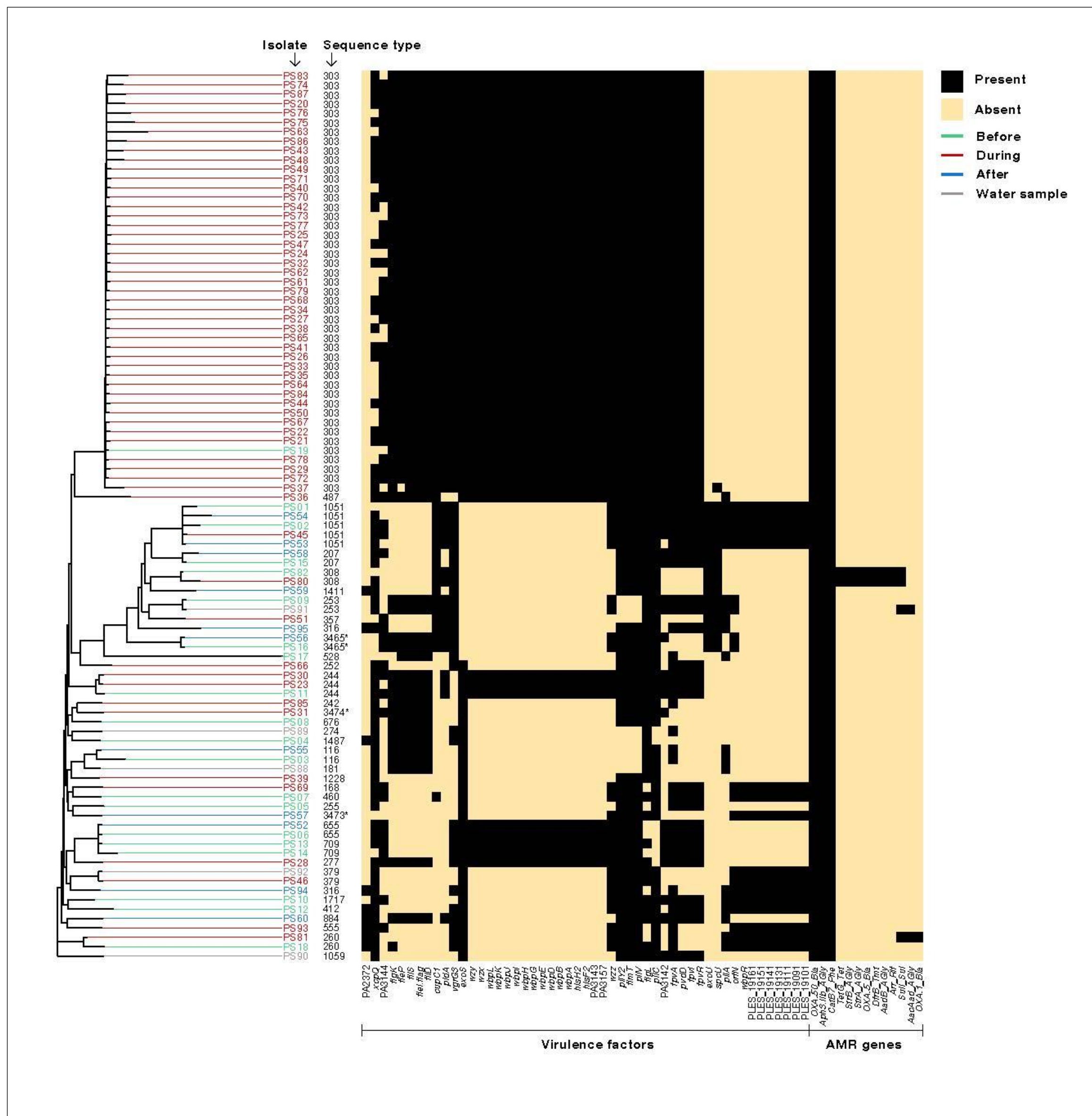

Figure 2. Genetic relatedness dendrogram of 95 whole genome sequence isolates. Red- during the outbreak, green- before the outbreak, blue- after the outbreak, orange- environmental isolate. Based on multi-locus sequence typing, 35 different sequence types were identified, including three novel sequence types (*). Drug-resistant isolates were included in the analysis (PS 80-82), and five water samples (PS 88-92). All remaining clinical isolates were wild-type *P. aeruginosa*. Virulence factors and antimicrobial resistance (AMR) genes present are shown in black. Only virulence factors that were significantly different by Fisher's exact test (adjusted P value ≤0.05) between the ST303 and non-ST303 isolates are shown.

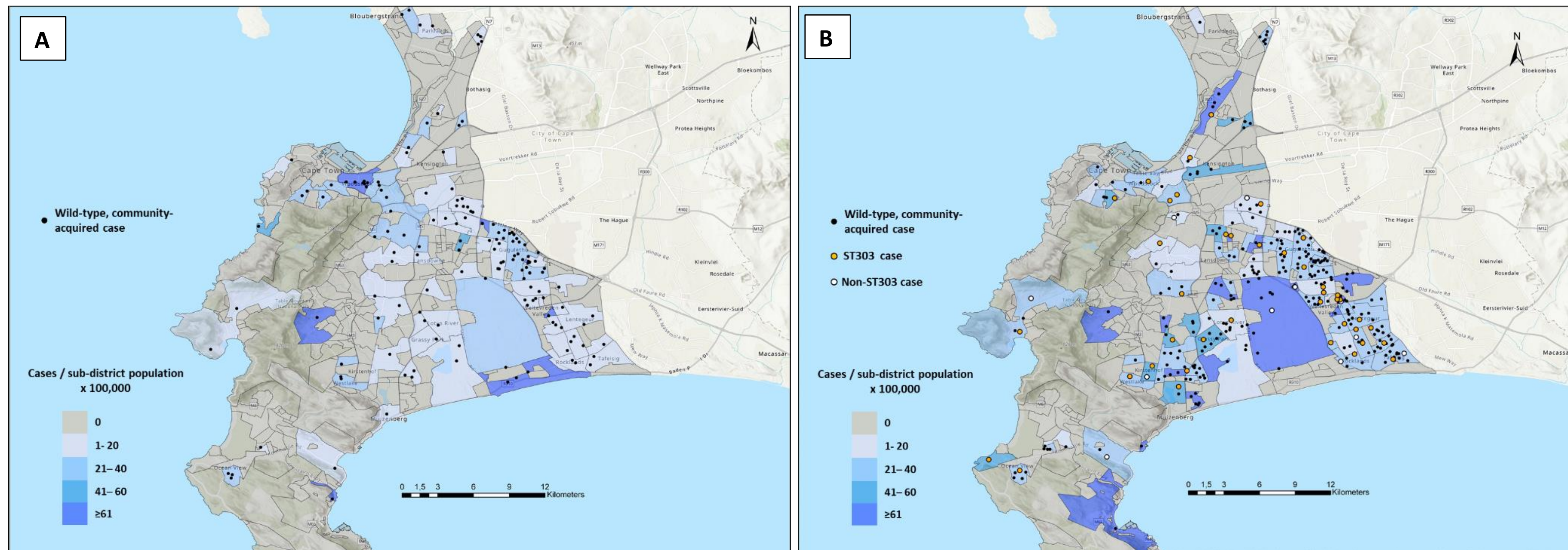

Figure 3. A total of 152 community-acquired wild-type *P. aeruginosa* cases (from 74 sub-places) were detected in a 10-month period 1 year before the outbreak (December 1, 2015 to September 30, 2016) (Panel A), and 324 (from 89 sub-places) during the outbreak (December 1, 2016 to September 30, 2017) (Panel B). The distribution of the outbreak clone (ST303) (yellow circles) and non-ST303 cases (white circles) are shown during the outbreak (B). Shading of sub-places was based on population density statistics from 2011 census data and categories were expressed as cases / sub-district population x 100,000. Cases residing outside the investigating laboratory's drainage area were excluded.

Table 1. Demographic and clinical characteristics of patients with *P. aeruginosa* during the outbreak, comparing patients with ST303 and non-ST303 multi-locus sequence type bloodstream infection								
	ST303 bloodstream infection cases	**Non-ST303 blood stream infection cases**	**P value***	**Unadjusted odds ratio (95% confidence interval)***	**Adjusted odds ratio (95% confidence interval)***	**Total cases during the outbreak**	**Wild-type cases during the outbreak**	**Wild-type community-acquired cases during the outbreak**
Explanatory variable	**N=28 (%)**	**N=40 (%)**				**N=1152 (%)**	**N=866 (%)**	**N=452 (%)**
Demographic characteristic								
Sex								
Female	11 539	20 (50)		1.0		539 (47)	435 (50)	230 (51)
Male	17 613	20 (50)	0.38	1.54 (0.6-4.1)		613 (53)	431 (50)	222 (49)
Age (median [IQR])	29.0 (0.0-56.5)	40.1 (43.5-65)	0.003	0.96† (0.94-0.98)	0.96 (0.93-0.99)	38 (23-59)	38 (23-58)	39 (24-60)
Age category								
< 1 month	8 8	0 (0)		ND		8 (1)	8 (1)	8 (2)
1 month to 16 years	1 188	0 (0)		ND		188 (16)	137 (16)	65 (14)
> 16 years	19 954	40 (100)	0.001	ND		954 (83)	721 (83)	379 (84)
Comorbidities								
Malignancy								
Absent	26 (93)	33 (83)		1.0				
Present	2 (7)	7 (18)	0.22	0.4 (0.1-1.9)				
HIV								
Negative	11 (39)	17 (43)		1.0				
Positive	7 (25)	8 (20)	0.71	1.3 (0.4-4.5)				
Unknown	10 (36)	15 (38)		-				
Chronic kidney disease								
Absent	25 (89)	35 (88)		1.0				
Present	2 (7)	4 (10)	0.90	1.0 (0.2-6.2)				
Unknown	1 (4)	(3)		-				
Diabetes mellitus								
Absent	23 (82)	29 (73)		1.0				
Present	5 (18)	11 (28)	0.36	0.6 (0.2-1.9)				
Chronic obstructive pulmonary disease								
Absent	24 (86)	33 (85)		1.0				
Present	3 (11)	6 (15)	0.86	0.6 (0.2-1.7)				
Unknown	1 (4)	(3)						
Epilepsy								

Absent	27 (96)	36 (90)		ND ND	
Present	0 (0)	3 (8)	0.36	ND ND	
Unknown	1 (4)	(4)		-	
Hypertension					
Absent	22 (79)	27 (68)		1.0	
Present	6 (21)	13 (33)	0.32	0.6 (0.2-1.7)	
Previous tuberculosis					
No	27 (96)	34 (85)		1.0	
Yes	1 (4)	6 (15)	0.13	0.2 (0.02-1.9)	
Any comorbidity					
No	15 (54)	31 (78)		1.0	
Yes	13 (46)	9 (23)	0.04	0.3 (0.1-0.96)	1.0 (0.2-4.2)
Source of infection					
Intra-abdominal	7 (25)	11 (28)	-	-	
Urogenital	5 (18)	7 (18)	-	-	
Neutropenic sepsis	3 (11)	8 (20)	-	-	
Osteomyelitis	2 (7)	2 (5)	-	-	
Skin and Soft tissue	2 (7)	3 (8)	-	-	
Lower respiratory tract	1 (4)	8 (20)	-	-	
Septic arthritis	0 (0)	1 (3)	-	-	
Neonatal sepsis	8 (29)	0 (0)	-	-	
Antibiotic exposure in the previous 30 days‡					
No	22 (79)	28 (70)		1.0	
Yes	6 (21)	12 (30)	0.43	0.6 (0.0-2.0)	
Outcome					
30-Day mortality					
Alive	14 (50)	20 (50)		-	
Deceased	14 (50)	20 (50)	1.00	-	
Received antipseudomonal antibiotics within 2 days of culture result§					
No	16 (57)	22 (55)		-	
Yes	12 (43)	18 (45)	0.86	-	

* The P value, unadjusted and adjusted odds ratios only apply to ST303 versus non-ST303

† Per 1-year increase

‡ Antibiotic exposure of any class and duration

§ Antipseudomonal antibiotic (ciprofloxacin, meropenem, imipenem, cefepime, ceftazidime, piperacillin-tazobactam, gentamicin, and/or amikacin) administered within 2 days of the culture result

ND = not determined (unable to determine odds ratio due to a zero value), IQR = interquartile range

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Supplementary water sampling methods

Samples were collected according to ISO 5667-5 2006. Water Quality – sampling – part 5: Guidance on sampling of drinking water from treatment works and piped distribution systems. Water taps were cleaned, disinfected, and flushed for 3 minutes before adjusting the stream into a steady flow. Samples were collected in plastic sterilized bottles containing sodium thiosulphate for neutralizing the activity of free residual chlorine and transported within 6 hours of their collection to the Biologic Sciences, Scientific Services, Department of Water and Sanitation. Collected water samples were not exposed to direct sunlight and kept on ice during transport. 100 ml of the water samples was analyzed by the membrane filtration method. Briefly, water samples were filtered on 0.45µm mixed cellulose ester based membrane of 47mm diameter and cultured on a selective *Pseudomonas* agar, cetrimide, for the isolation and enumeration of *Pseudomonas aeruginosa*. Using a sterile forceps, membranes were transferred carefully onto a Petri dish ensuring that no air bubble was trapped between the agar and membrane. Petri dishes were inverted and incubated at 37° C aerobically, and examined after 48 hours. *P. aeruginosa* colonies developed a blue-green colour (pyocyanin), a yellow-green colour (pyoverdine), a brown-to-black colour (pyomelanin), or a red-brown colour (pyorubin), among others. *P. aeruginosa* colonies fluoresce under ultraviolet light illumination. All the fluorescent colonies were counted and expressed as colony forming units (CFU)/100 ml.

Supplementary whole genome sequencing methods

Sequencing data quality control

Illumina MiSeq 150 bp raw, paired reads were filtered using Trimmomatic 0.36[1],removing any adapter sequences (listed in the 'adapters.fa' file of the bbmap-37.10 installation) initially looking for seed matches (16 bases) with a maximum of 2 mismatches; seeds were extended and removed if (in the case of paired end reads) a score of 30 was reached (about 50 bases). Leading and trailing low-quality bases (below phred quality of 3) were removed. Reads were scanned with a 4-base wide sliding window, cutting when the average quality per base dropped below 15. Reads shorter than 36 bases long after these steps were removed.

Sequence data quality control (Trimmomatic, FastQC[2], MultiQC[3]) was performed as part of the Nextflow-based[4] Tychus pipeline[5] (https://github.com/kviljoen/Tychus/tree/ilifu), which was customized as needed. Singularity images (one for the Tychus alignment module and one for the Tychus assembly module) were built based on the original Dockerfiles for Tychus [https://github.com/Abdo-Lab/Tychus] using docker2singularity [https://github.com/singularityhub/docker2singularity].

SNP-based genetic relatedness dendrogram

A genetic relatedness dendrogram was drawn using core genome SNPs to avoid variation obtained through horizontal gene transfer. Trimmed reads were aligned to the *P. aeruginosa* reference genome (ASM676v1) using Bowtie2[6] 2.3.4.3 and SNP's were called with Freebayes[7] version v1.2.0-4-gd15209e with default settings; to prevent inclusion of spurious variation caused by sequencing artefacts, positions were skipped when no more than 2 (-min-alternate-count) or 0.05 (min-alternate-fraction) non-clonal observations of an alternate were found in

one sample. SNP calls were integrated into the *P. aeruginosa* ASM676v1 reference genome using BCFtools[8] 1.3.1. SNP-integrated genomes were used as input to kSNP3[9] 3.1 to extract the core SNPs fasta and construct a dendrogram based on these core SNPs, where core SNPs refer to SNPs that were present across all genomes. The resulting core SNPs dendrogram (.tre file) was imported into R to create publication quality graphics alongside virulence factor and antimicrobial resistance gene detection data. SNP-based genetic relatedness profiling was executed via the alignment module of the Tychus pipeline described in the previous section (https://github.com/kviljoen/Tychus/tree/ilifu).

The SNP-based dendrogram was constructed alongside a heatmap of virulence factors (VFs) and antimicrobial resistance genes (AMRs) using the R packages ggtree[10], ggimage [https://github.com/GuangchuangYu/ggimage] and GeneMates (heatMapPAM function).[11] Fisher's exact tests with multiple testing correction was conducted with metagenomeSeq's fitPA() function to identify VFs that were differentially present/absent in ST303 and non-ST303 samples, with adjusted P values ≤0.05 deemed significant.[12]

A heatmap of pairwise similarity in core SNPs was constructed in R using the heatmapColorDistance function from the R package colordistance.[13] The input distance matrix was computed with snp-dists (https://github.com/tseemann/snp-dists) based on the core SNPs fasta file from kSNP3 as input. Values were clustered by complete-linkage hierarchical clustering.

De novo genome assembly

Short reads were assembled into contigs using the assembly module of the Nextflow-based pipeline Tychus. The Tychus assembly module uses the results from multiple assemblers

(ABySS[14], Velvet[15], SPAdes[16], IDBA-UD[17]) to construct a consensus assembly using CISA[18] with fewer and longer contigs that can be used as a draft genome for further downstream processes. Assemblies are evaluated with QUAST[19] based on common scoring metrics, such as number of contigs, contig size, and N50.

Multi-locus sequence typing, virulence factor and antimicrobial resistance gene profiling

Multi-locus sequence typing (MLST) and detection of virulence factors and antimicrobial resistance genes were performed with SRST2[20], using a gene length coverage of 80%, and 90% identity cut-off, implemented as a Nextflow pipeline [https://github.com/kviljoen/uct-srst2]. The MLST database and reference table for *P. aeruginosa* was obtained with the getmlst.py script from SRST2 (https://github.com/katholt/srst2/tree/master/scripts), with command: `getmlst.py --species "*Pseudomonas aeruginosa*"`. Alleles included acsA, aroE, guaA, mutL, nuoD, ppsA and trpE. Alignments for MLST results generated with SRST2 that were classified as 'uncertain' were manually inspected. Most uncertain hits were classified as such since they had one or two low coverage bases at the first or last 2bp of the read. By doing multiple sequence alignments for all alleles for each of the 7 markers (acsA, aroE, guaA, mutL, nuoD, ppsA, trpE) we could establish whether the first 2bp and last 2bp were necessary to distinguish from all the other alleles. In most cases these bases were not discriminatory, and the 'uncertain' assignment could be passed. Alignment was done with MAFFT[21] and viewed in Jalview[22]. Alignments are available on reasonable request. In cases where SRST2 reported SNPs, the short-read alignment results were compared to the *P. aeruginosa* assembled contigs (from the Tychus assembly module) and those with 100% match between allele sequence and contig were accepted.

For AMR detection the ARGannot database was downloaded from the SRST2 github page https://github.com/katholt/srst2/tree/master/data/ARGannot_r3.fasta. For VF detection the VFDB database (http://www.mgc.ac.cn/VFs/download.htm) was downloaded. Databases were converted to single line FASTAs for use with SRST2. AMR and VF genes were considered present if reads covered at least 80% of the gene length with an identity of at least 90%.

Table S1. City of Cape Town water restriction categories, combined dam storage levels and daily per capita water limits from 2016 to 2018*			
Date	**City water restriction level**	**Combined dam level† (%)**	**Daily water limit‡**
January 1, 2016	2	55.4	-
November 1, 2016	3	60.3	-
February 1, 2017	3B	38.7	-
June 1, 2017	4	19.4	100
July 1, 2017	4B	25,0	87
September 3, 2017	5	36.1	87
January 1, 2018	6	31,0	87
February 1, 2018	6B	25.9	50
October 1, 2018	5	75.9	70
December 1, 2018	3	70.3	105

* Table was modified from the Water outlook report, December, 2018, published by the Department of Water and Sanitation, City of Cape Town.

† Major dams included are Theewaterskloof, Voëlvlei, Berg Rivier, Wemmershoek, and Streenbras Upper and Lower, with eight smaller dams draining into Cape Town.

‡ Daily water limit is indicated as litres / person / day

Table S2. Municipal water sampling in Cape Town between February 5, 2018 and April 23, 2018					
Sub-district*	**No of sites**	**No of samples†**	**Highest growth category (number of sites [%])**		
			<1 CFU/100 ml	**1-100 CFU/100 ml**	**>100 CFU/100 ml**
Eastern	24	72	18 (75)	5 (21)	1 (4)
Khayelitsha	12	37	2 (17)	9 (75)	1 (8)
Klipfontein	16	61	5 (31)	10 (63)	1 (6)
Mitchells Plain	28	68	20 (71)	7 (25)	1 (4)
Northern	29	100	12 (41)	13 (45)	4 (14)
Southern	44	90	23 (52)	20 (46)	1 (2)
Tygerberg	31	89	17 (55)	12 (39)	2 (6)
Western	46	192	27 (59)	18 (39)	1 (2)
Total	**230**	**709**	**124 (54)**	**94 (41)**	**12 (5)**

* Sub-districts are according to the Cape Metro District Health Plan 2018/19 – 2020/21, Western Cape Government, Department of Health, South Africa.

† Each site was sampled on 1-9 occasions

Table S3. Profile of 95 isolates submitted for whole genome sequencing

Isolate	Age (years)	Wild-type* or MRO†	Clinical sample type	Source‡	Relation to outbreak§	Sequence type ǁ	Outbreak-associated clone¶	HIV status**	Clinical detail	30-day mortality
PS1	65	Wild-type	Blood culture	Community	Before	1051	No	N	Lymphoma with neutropenic sepsis and a bone marrow transplant	Alive
PS2	64	Wild-type	Blood culture	Community	Before	1051	No	U	Pneumonia with sepsis	Deceased
PS3	57	Wild-type	Blood culture	Community	Before	116	No	U	Open tibia fracture with a septic wound	Alive
PS4	52	Wild-type	Blood culture	Community	Before	1487	No	U	Hepatic encephalopathy with cholangitis	Alive
PS5	60	Wild-type	Blood culture	Community	Before	255	No	U	Multiple myeloma with neutropenic sepsis	Deceased
PS6	31	Wild-type	Blood culture	Community	Before	655	No	N	Polytrauma, motor vehicle accident, septic humerus	Alive
PS7	47	Wild-type	Blood culture	Community	Before	460	No	P	Disseminated tuberculosis with abdominal sepsis	Deceased
PS8	59	Wild-type	Blood culture	Community	Before	676	No	N	Polytrauma, pulmonary embolus, spinal injury	Alive
PS9	44	Wild-type	Blood culture	Community	Before	253	No	U	Cholangitis with a stent placed	Deceased
PS10	86	Wild-type	Blood culture	Community	Before	1717	No	U	Uncomplicated urinary tract infection	Alive
PS11	63	Wild-type	Blood culture	Community	Before	244	No	U	Acute kidney injury secondary to a urinary tract infection	Alive
PS12	87	Wild-type	Blood culture	Community	Before	412	No	U	Pneumonia with sepsis	Deceased
PS13	37	Wild-type	Blood culture	Community	Before	709	No	P	Acute kidney injury secondary to a urinary tract infection	Alive
PS14	76	Wild-type	Blood culture	Community	Before	709	No	N	Congestive cardiac failure with pneumonia	Deceased
PS15	45	Wild-type	Blood culture	Community	Before	207	No	U	Cholecystitis with a stent placed	Alive
PS16	24	Wild-type	Blood culture	Community	Before	3465	No	U	Gunshot to abdomen, colon, liver, and stomach injury	Deceased
PS17	32	Wild-type	Blood culture	Community	Before	528	No	N	Cervical cancer with infection around left nephrostomy	Alive
PS18	54	Wild-type	Blood culture	Community	Before	260	No	U	Follicular lymphoma, neutropenia, bullous impetigo	Deceased
PS19	1 day	Wild-type	Blood culture	Community	Before	303	Yes	U	Neonatal sepsis of unknown aetiology	Deceased
PS20	58	Wild-type	Blood culture	Community	During	303	Yes	U	Pneumonia without septic shock	Deceased
PS21	76	Wild-type	Blood culture	Community	During	303	Yes	P	Barrett's oesophagus with gastritis	Deceased
PS22	60	Wild-type	Blood culture	Community	During	303	Yes	N	Oesophagitis with a peptic ulcer	Alive
PS23	45	Wild-type	Blood culture	Community	During	244	No	P	Chronic productive cough, deceased before workup	Deceased
PS24	50	Wild-type	Blood culture	Community	During	303	Yes	P	Necrotizing fasciitis of the perineum and left groin	Alive
PS25	20	Wild-type	Blood culture	Community	During	303	Yes	N	Gunshot, multiple areas of necrotic bowel resection	Alive
PS26	80	Wild-type	Blood culture	Community	During	303	Yes	P	Scrotal abscess with benign prostatic hypertrophy	Alive

PS27	1 days	Wild-type	Blood culture	Community	During	303	Yes	U	Neonatal sepsis of unknown aetiology	Alive
PS28	74	Wild-type	Blood culture	Community	During	277	No	U	Right necrotic testis with sepsis	Diseased
PS29	2 days	Wild-type	Blood culture	Community	During	303	Yes	U	Premature neonate with sepsis of unknown aetiology	Alive
PS30	75	Wild-type	Blood culture	Community	During	244	No	U	Muciunous adenocarcinoma, neutropenic	Deceased
PS31	65	Wild-type	Blood culture	Community	During	3474	No	N	Squamous cell penile cancer, neutropenic	Alive
PS32	19	Wild-type	Blood culture	Community	During	303	Yes	U	Gunshot abdomen, intra-abdominal infection	Diseased
PS33	1 day	Wild-type	Blood culture	Community	During	303	Yes	N	Neonatal Sepsis with unknown aetiology	Deceased
PS34	1 day	Wild-type	Blood culture	Community	During	303	Yes	U	Neonatal Sepsis with unknown aetiology	Deceased
PS35	1 day	Wild-type	Blood culture	Community	During	303	Yes	U	Neonatal Sepsis with unknown aetiology	Deceased
PS36	40	Wild-type	Blood culture	Community	During	487	No	P	Chronic productive cough, deceased before workup	Deceased
PS37	1 day	Wild-type	Blood culture	Community	During	303	Yes	U	Neonatal Sepsis with unknown aetiology	Alive
PS38	63	Wild-type	Blood culture	Community	During	303	Yes	N	Right hemi- lobectomy of the prostate	Deceased
PS39	67	Wild-type	Blood culture	Community	During	1228	No	N	Acute kidney injury secondary to urinary tract infection	Alive
PS40	55	Wild-type	Blood culture	Community	During	303	Yes	N	Prostatic cancer with neutropenia	Deceased
PS41	39	Wild-type	Blood culture	Community	During	303	Yes	P	Diffuse large cell lymphoma with neutropenia	Diseased
PS42	1 day	Wild-type	Blood culture	Community	During	303	Yes	N	Neonatal sepsis with unknown aetiology	Deceased
PS43	51	Wild-type	Blood culture	Community	During	303	Yes	P	Upper gastrointestinal bleed	Alive
PS44	72	Wild-type	Blood culture	Community	During	303	Yes	U	Acute kidney injury secondary to a urinary tract infection	Alive
PS45	44	Wild-type	Blood culture	Community	During	1051	No	N	Pneumonia with pulmonary oedema	Alive
PS46	39	Wild-type	Blood culture	Community	During	379	No	N	Acute abdomen, perforated peptic ulcer	Deceased
PS47	18	Wild-type	Blood culture	Community	During	303	Yes	N	Multiple gunshot wounds, abdominal infection	Alive
PS48	44	Wild-type	Blood culture	Community	During	303	Yes	P	Cervix carcinoma with bleeding and urinary retention	Deceased
PS49	12	Wild-type	Blood culture	Community	During	303	Yes	N	Acute myeloid leukaemia, neutropenic	Deceased
PS50	81	Wild-type	Blood culture	Community	During	303	Yes	P	Femur fracture with osteomyelitis	Alive
PS51	43	Wild-type	Blood culture	Community	During	357	No	N	Multiple myeloma with neutropenia	Alive
PS52	48	Wild-type	Blood culture	Community	During	655	No	N	Large cell lymphoma, neutropenia	Alive
PS53	43	Wild-type	Blood culture	Community	After	1051	No	P	Poly trauma, septic shock, liver injury, vertebral fracture	Deceased
PS54	48	Wild-type	Blood culture	Community	After	1051	No	P	Chronic diarrhoea, no dysentery	Alive
PS55	46	Wild-type	Blood culture	Community	After	116	No	N	Malignancy (Bone, abdominal)? neutropenic	Deceased

PS56	38	Wild-type	Blood culture	Community	After	3465	No	N	Chronic productive cough, deceased before workup	Deceased
PS57	40	Wild-type	Blood culture	Community	After	3473	No	P	Uncomplicated urinary tract infection	Deceased
PS58	63	Wild-type	Blood culture	Community	After	207	No	U	Upper limb cellulitis with septic shock	Deceased
PS59	74	Wild-type	Blood culture	Community	After	1411	No	P	Diabetic foot with critical limb ischaemia	Deceased
PS60	55	Wild-type	Blood culture	Community	After	884	No	N	Gangrenous appendix dissected with abdominal infection	Alive
PS61	22	Wild-type	Urine	Community	During	303	Yes	U	Pregnant with a complicated urinary tract infection	Alive
PS62	28	Wild-type	Tracheal aspirate	Community	During	303	Yes	N	Acute liver failure with hepatitis B	Alive
PS63	76	Wild-type	Urine	Community	During	303	Yes	U	Urinary retention of unknown aetiology	Deceased
PS64	58	Wild-type	Urine	Community	During	303	Yes	U	Gastritis, chronic cardiac and renal failure	Alive
PS65	6	Wild-type	Urine	Community	During	303	Yes	U	Uncomplicated urinary tract infection	Alive
PS66	52	Wild-type	Abscess deep swab	Community	During	252	No	U	Perforated appendix with intra-abdominal infection	Deceased
PS67	77	Wild-type	Urine	Community	During	303	Yes	U	Benign prostatic hypertrophy with urethral catheter	Alive
PS68	8	Wild-type	Swab superficial	Community	During	303	Yes	U	Ear discharge without any known co-morbidities	Alive
PS69	26	Wild-type	Tracheal aspirate	Community	During	168	No	U	Intubated, spinal cord injury due to gunshot	Alive
PS70	40	Wild-type	Fluid-aspirate	Community	During	303	Yes	U	Intubated, gunshot trauma with abdominal infection	Alive
PS71	72	Wild-type	Fluid-aspirate	Community	During	303	Yes	N	Acute kidney injury secondary to lower leg cellulitis	Deceased
PS72	34	Wild-type	Wound swab	Community	During	303	Yes	U	Sepsis of abdominal wound post caesarean section	Alive
PS73	32	Wild-type	Urine	Community	During	303	Yes	U	Infective endocarditis	Alive
PS74	62	Wild-type	Swab superficial	Community	During	303	Yes	N	Infective endocarditis	Alive
PS75	72	Wild-type	Urine	Community	During	303	Yes	U	Acute kidney injury secondary to urosepsis	Deceased
PS76	46	Wild-type	Urine	Community	During	303	Yes	N	Obstructive uropathy with a bladder ulcer	Alive
PS77	76	Wild-type	Urine	Community	During	303	Yes	N	Delirium secondary to a urinary tract infection	Alive
PS78	27	Wild-type	Urine	Community	During	303	Yes	N	Acute on chronic renal failure	Alive
PS79	57	Wild-type	Sputum	Community	During	303	Yes	U	Acute onset pneumonia	Alive
PS80	32	MRO	Blood culture	Nosocomial	During	308	No	P	Acute kidney injury with pancreatitis	Alive
PS81	57	MRO	Blood culture	Nosocomial	During	260	No	P	Urinary tract infection with a stent placed	Alive
PS82	25	MRO	Fluid - aspirate	Nosocomial	During	308	No	N	Liver transplant with abdominal sepsis	Alive
PS83	30	Wild-type	Blood culture	Nosocomial	During	303	Yes	N	Spermatic cord injury with left necrotic testicle	Alive

PS84	26	Wild-type	Blood culture	Nosocomial	During	303	Yes	N	Multiple gunshot wounds with abdominal sepsis	Deceased
PS85	61	Wild-type	Blood culture	Nosocomial	During	242	No	N	Post heart transplant with sternotomy sepsis	Deceased
PS86	47	Wild-type	Blood culture	Nosocomial	During	303	Yes	U	Paravertebral abscess with collapse	Alive
PS87	27	Wild-type	Blood culture	Nosocomial	During	303	yes	N	Ruptured left ovary mass in septic shock	Alive
PS88	Not applicable	Wild-type	Water	Environmental	After	181	No	Not applicable	Not applicable	Water
PS89	Not applicable	Wild-type	Water	Environmental	After	274	No	Not applicable	Not applicable	Water
PS90	Not applicable	Wild-type	Water	Environmental	After	1059	No	Not applicable	Not applicable	Water
PS91	Not applicable	Wild-type	Water	Environmental	After	253	No	Not applicable	Not applicable	Water
PS92	Not applicable	Wild-type	Water	Environmental	After	379	No	Not applicable	Not applicable	Water
PS93	54	Wild-type	Blood culture	Community	After	555	No	U	Strangulated hernia with necrotic ileum resection	Deceased
PS94	68	Wild-type	Blood culture	Community	After	316	No	N	Perforated diverticulitis with sigmoid resection	Deceased
PS95	17	Wild-type	Blood culture	Community	After	316	No	N	Paraplegia with hip septic arthritis	Alive

* Wild-type: susceptibility to ciprofloxacin, meropenem, imipenem, cefepime, ceftazidime, piperacillin-tazobactam, gentamicin, and amikacin

† MRO- multidrug resistant organism

‡ Community-acquired clinical samples were collected before or within 48 hours of admission, and nosocomial samples, after 48 hours. Water samples were collected from February 5, 2018 to April 23, 2018.

§ Outbreak period was from December 1, 2016 to September 31, 2017

¶ Outbreak-associated clone was defined as ST303

‖ Sequence type confirmed with multi-locus sequence typing

** Human immunodeficiency virus is reported as P-positive, N-negative, U-untested

Table S4. Fifty-one virulence factors differentially present or absent in ST303 and non-ST303 isolates*					
Virulence Factor	Odds-Ratio	Lower†	Upper†	P value	Adjusted P value
PA2372	0	0	0,502119602	0,002886484	0,009685758
PA3142	Infinity	12,57130015	Infinity	1,89E-10	1,19E-09
PA3143	Infinity	40,21692115	Infinity	1,92E-18	1,81E-17
PA3144	5,416643647	2,027077527	15,68172728	0,000173421	0,00059515
PA3157	Infinity	40,21692115	Infinity	1,92E-18	1,81E-17
PLES_19091	0	0	0,217293996	1,01E-05	3,72E-05
PLES_19101	0	0	0,217293996	1,01E-05	3,72E-05
PLES_19111	0	0	0,217293996	1,01E-05	3,72E-05
PLES_19131	0	0	0,217293996	1,01E-05	3,72E-05
PLES_19141	0	0	0,217293996	1,01E-05	3,72E-05
PLES_19151	0	0	0,217293996	1,01E-05	3,72E-05
PLES_19161	0	0	0,217293996	1,01E-05	3,72E-05
cupC1	Infinity	17,3188938	Infinity	1,34E-12	1,12E-11
exoS	Infinity	5,05952592	Infinity	4,27E-06	2,01E-05
exoU	0	0	0,217293996	1,01E-05	3,72E-05
fimT	Infinity	1,686973197	Infinity	0,00619026	0,018694585
fleI.flag	Infinity	15,95858234	Infinity	4,83E-12	3,31E-11
fleP	68,56033866	9,990135475	2937,992176	9,18E-11	6,03E-10
flgK	Infinity	15,95858234	Infinity	4,83E-12	3,31E-11
flgL	Infinity	1,686973197	Infinity	0,00619026	0,018694585
fliD	Infinity	15,95858234	Infinity	4,83E-12	3,31E-11
fliS	Infinity	15,95858234	Infinity	4,83E-12	3,31E-11
fpvA	Infinity	3,758367893	Infinity	5,40E-05	0,000194312
fpvI	Infinity	8,46109927	Infinity	1,72E-08	9,61E-08
fpvR	Infinity	8,46109927	Infinity	1,72E-08	9,61E-08
hisF2	Infinity	40,21692115	Infinity	1,92E-18	1,81E-17
hisH2	Infinity	40,21692115	Infinity	1,92E-18	1,81E-17
orfN	0	0	0,151453085	2,94E-07	1,48E-06
pilA	0	0	0,151453085	2,94E-07	1,48E-06
pilC	Infinity	1,396631265	Infinity	0,013145964	0,038922365
pilV	Infinity	1,686973197	Infinity	0,00619026	0,018694585
pilY2	Infinity	1,686973197	Infinity	0,00619026	0,018694585
pldA	Infinity	18,80226956	Infinity	3,58E-13	3,18E-12
pvdD	Infinity	8,46109927	Infinity	1,72E-08	9,61E-08
spcU	0,049511156	0,001131733	0,349022405	0,00010327	0,000362647
vgrG3	Infinity	6,05781407	Infinity	7,29E-07	3,55E-06
wbpA	Infinity	40,21692115	Infinity	1,92E-18	1,81E-17
wbpB	Infinity	40,21692115	Infinity	1,92E-18	1,81E-17
wbpD	Infinity	40,21692115	Infinity	1,92E-18	1,81E-17
wbpE	Infinity	40,21692115	Infinity	1,92E-18	1,81E-17
wbpG	Infinity	40,21692115	Infinity	1,92E-18	1,81E-17
wbpH	Infinity	40,21692115	Infinity	1,92E-18	1,81E-17
wbpI	Infinity	40,21692115	Infinity	1,92E-18	1,81E-17
wbpJ	Infinity	40,21692115	Infinity	1,92E-18	1,81E-17
wbpK	Infinity	40,21692115	Infinity	1,92E-18	1,81E-17
wbpL	Infinity	40,21692115	Infinity	1,92E-18	1,81E-17
wbpR	0	0	0,217293996	1,01E-05	3,72E-05
wzx	Infinity	40,21692115	Infinity	1,92E-18	1,81E-17
wzy	Infinity	40,21692115	Infinity	1,92E-18	1,81E-17
wzz	Infinity	7,184052601	Infinity	1,16E-07	6,27E-07
xcpQ	0,241921352	0,07949041	0,676148723	0,003260265	0,010702176

* Fisher's exact tests with multiple testing correction were used to identify virulence factors that were differentially present/absent in ST303 and non-ST303 isolates, with adjusted P values ≤0.05 deemed significant

† Upper and Lower: Boundaries for the 95% confidence interval

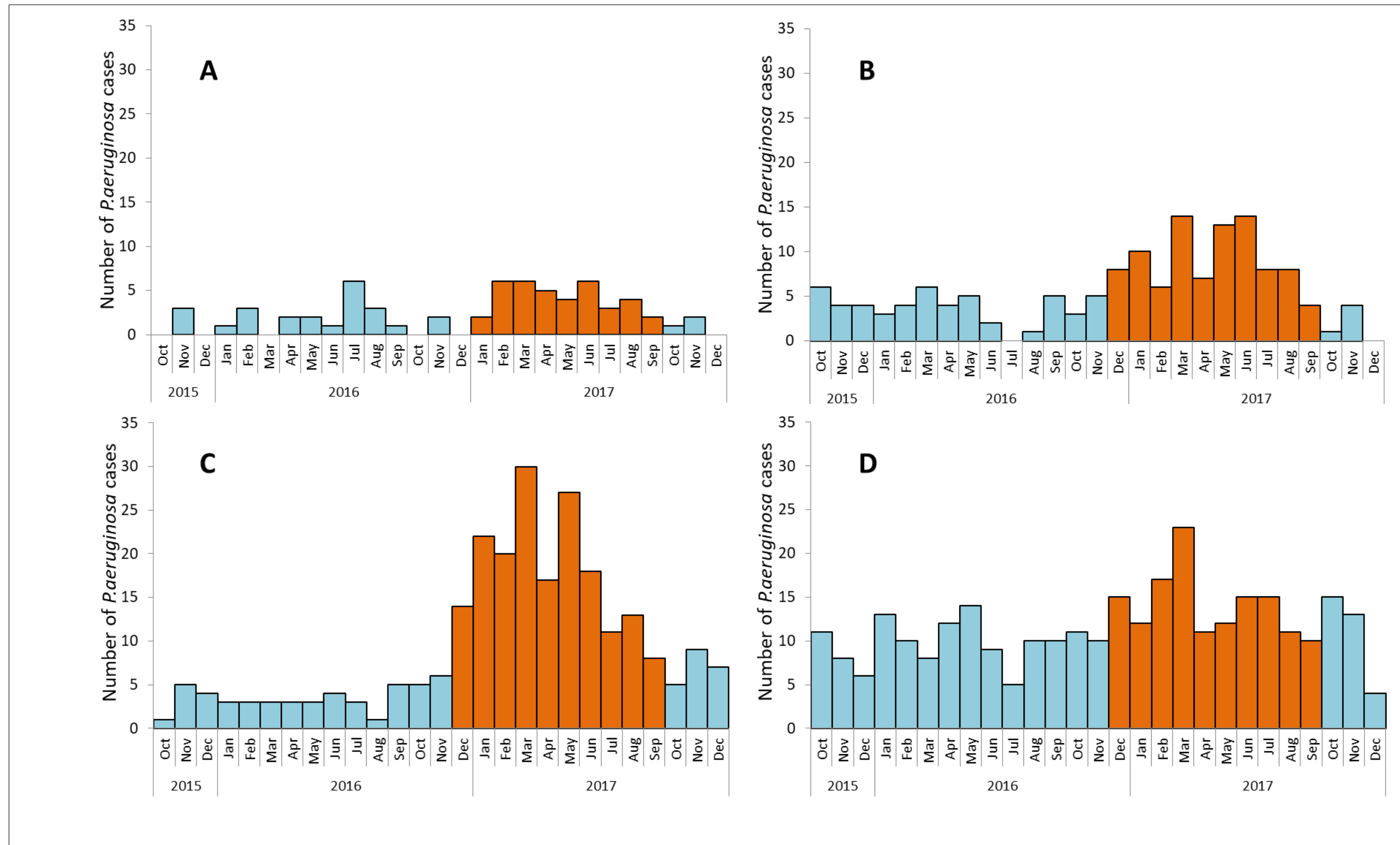

Figure S1. Number of community-acquired wild-type *P. aeruginosa* cultured from clinical samples between October 1, 2015 and December 31, 2017. Orange areas indicate the outbreak period (December 1, 2016 to September 31, 2017). A- Blood culture, B- Respiratory samples, C- Urine D- Tissue, pus swabs, fluid aspirates and other.

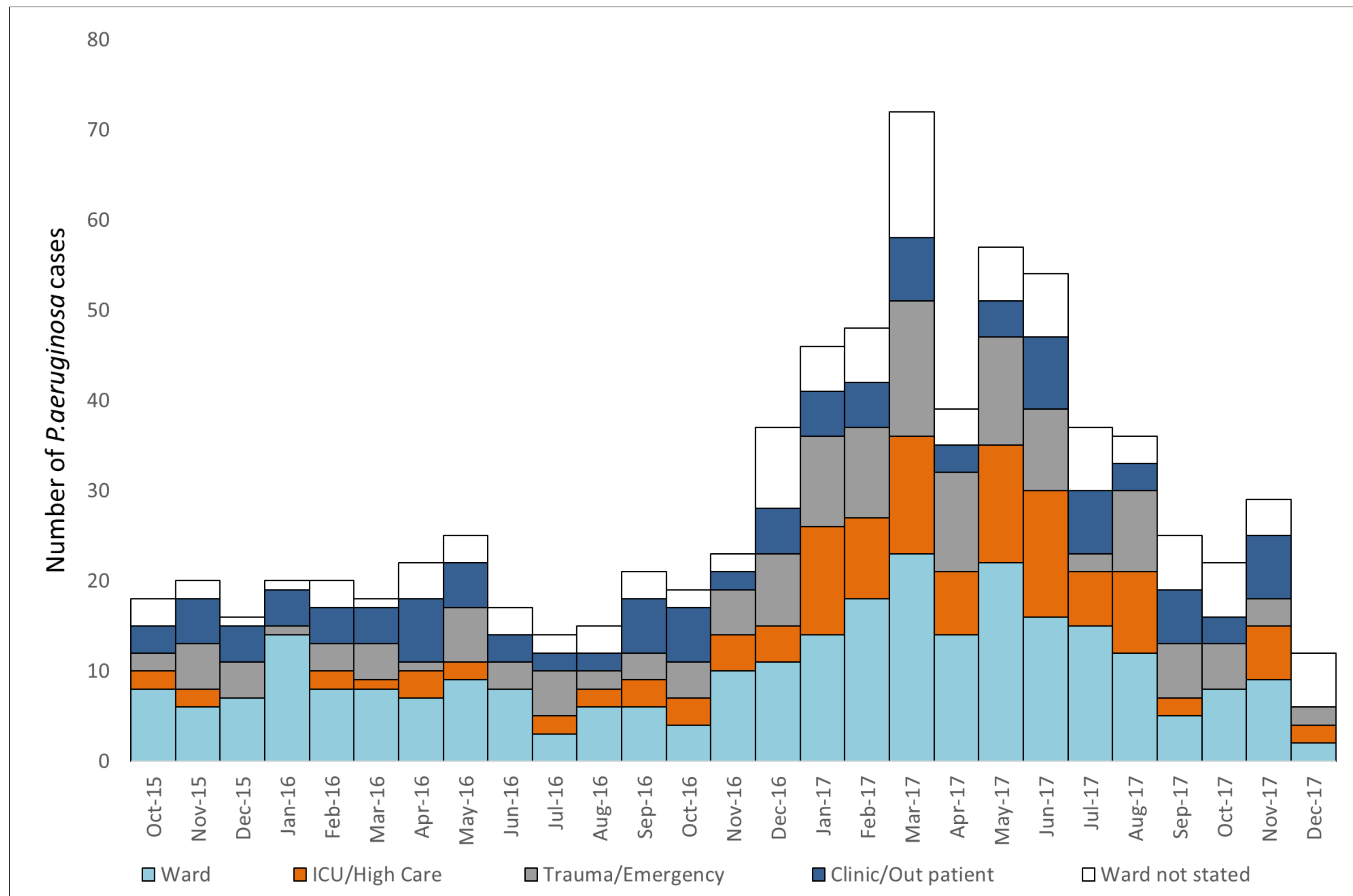

Figure S2. Submitting ward type of clinical samples in the hospital where a wild-type, community-acquired *P. aeruginosa* was cultured from between October 1, 2015 and December 31, 2017. The outbreak period was from December 1, 2016 to September 30, 2017. Only the site of the first sample taken from a patient is indicated. ICU-Intensive care unit.

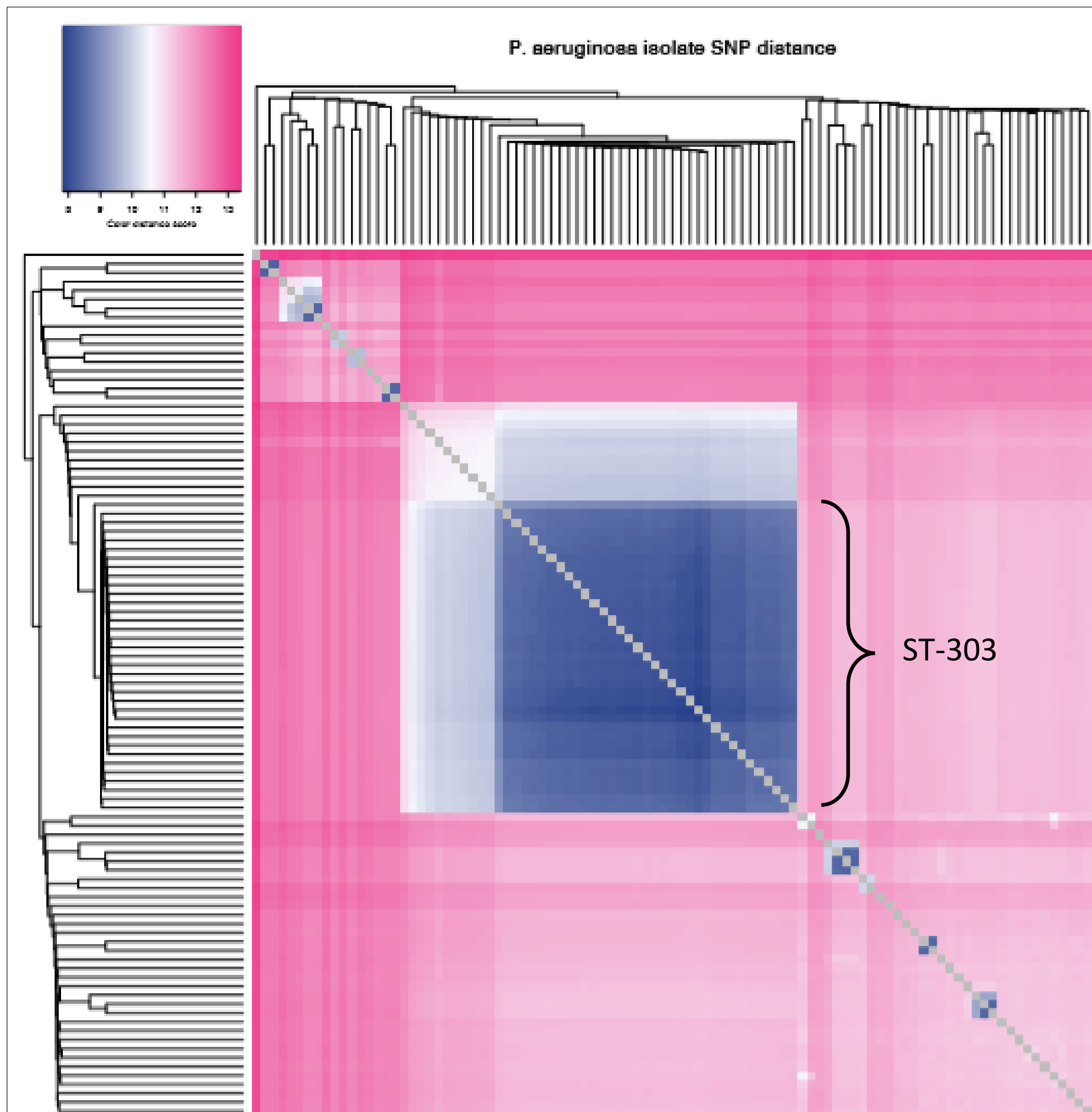

Figure S3. Core genome-SNP distance heat map illustrating clustering of ST303 isolates. A heatmap of pairwise similarity in core-SNPs was constructed using the heatmapColorDistance function from the R package, colordistance. The input distance matrix was computed with SNP-dists. Raw, paired reads were filtered using Trimmomatic 0.36. Trimmed reads were aligned to the *P. aeruginosa* reference genome using Bowtie2 2.3.4.3 and SNPs were called with Freebayes version v1.2.0-4-gd15209e. Short reads were assembled into contigs using the assembly module of the Nextflow-based pipeline Tychus.

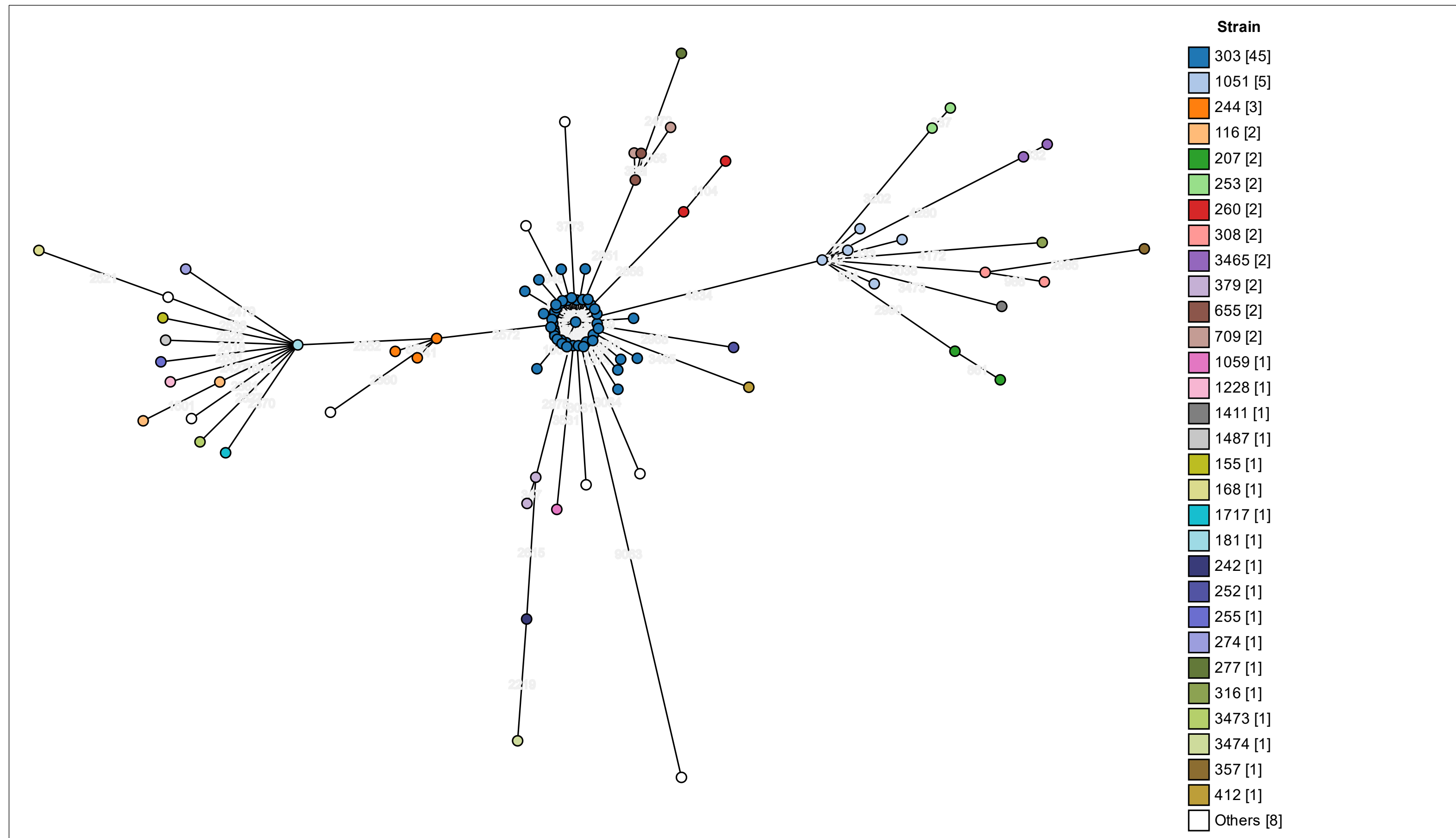

Figure S4. Core genome SNP difference minimum spanning tree generated from 95 sequenced *P. aeruginosa* isolates (Table S3) constructed in GrapeTree software (Version 1.5.0)[23]. The core genome SNP differences between isolates are depicted on the branches with the nodes color coded according to the sequence type (ST). Thirty-five different STs were identified, three novel (ST3474, ST3473, and ST3465). All ST303 isolates clustered around a single node. PAO1: *P. aeruginosa* reference strain.

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Part G: Instructions to authors from the journal

Manuscript is prepared in publication-ready format for the *New England Journal of Medicine*.

General information for original clinical research reports:

Maximum words: 2,700 (excluding the abstract)

Elements:

• Abstract

• Maximum of five (5) tables and figures

• Up to 40 references

Authorship

All individuals meeting International Committee of Medical Journal Editors (ICMJE) criteria for article authorship must be named as article authors. There are no limits to the number of authors that can be submitted with manuscripts.

Word-Count Limits

Manuscript word-count limits cited below include all information from introduction through conclusion or discussion. They exclude abstracts, figure legends, and table notes. Manuscripts far exceeding word-count limits will be returned unread.

Statistical reporting guidelines:

Our Statistical Consultants recommend the following best statistical practices in manuscripts submitted to the *Journal*. We recommend that you follow them in the design and reporting of research studies.

For all studies:

- The Methods section of all manuscripts should contain a brief description of sample size and power considerations for the study, as well as a brief description of the methods for primary and secondary analyses.
- The Methods section of all manuscripts should include a description of how missing data have been handled. Unless missingness is rare, a complete case analysis is generally not acceptable as the primary analysis and should be replaced by methods that are appropriate, given the missingness mechanism. Multiple imputation or inverse probability case weights can be used when data are missing at random; model-based methods may be more appropriate when missingness may be informative.
- Significance tests should be accompanied by confidence intervals for estimated effect sizes, measures of association, or other parameters of interest. The confidence intervals should be adjusted to match any adjustment made to significance levels in the corresponding test.
- Unless one-sided tests are required by study design, such as in non-inferiority clinical trials, all reported P values should be two-sided. In general, P values larger than 0.01 should be reported to two decimal places, and those between 0.01 and 0.001 to three decimal places; P values smaller than 0.001 should be reported as $P<0.001$. Notable exceptions to this policy include P values arising from tests associated with stopping rules in clinical trials or from genome-wide association studies.

- Results should be presented with no more precision than is of scientific value and is meaningful given the available sample size. For example, measures of association, such as odds ratios, should ordinarily be reported to two significant digits. Results derived from models should be limited to the appropriate number of significant digits.

Key Journal Style elements:

Units of Measurement

Authors should express all measurements in conventional units, with Système International (SI) units given in parentheses throughout the text. Figures and tables should use conventional units, with conversion factors given in legends or footnotes. In accordance with the Uniform Requirements, however, manuscripts containing only SI units will not be returned for that reason.

Abbreviations

Except for units of measurement, abbreviations are strongly discouraged; the first time an abbreviation appears, it should be preceded by the words for which it stands.

Drug Names

Generic names should be used. When proprietary brands are used in research, include the brand name and the name of the manufacturer in parentheses after the first mention of the generic name in the Methods section.

Prepare Materials for submission:

Manuscript Text File

Compile all text, references, figure legends, and tables into a single double-spaced digital file (preferably an MS Word document). NEJM will also accept text (.txt), or Rich Text Format (.rtf) files.

Title Page

Create a title page that includes:

- Manuscript title
- Each author's name, highest degree, and affiliation/institution
- Contact information for one corresponding author

Abstract

Provide an abstract of not more than 250 words with four labeled paragraphs containing the following:

- Background: Problem being addressed in the study
- Methods: How the study was performed
- Results: Salient results
- Conclusions: What the authors conclude from study results

References

References must be double-spaced and numbered consecutively as they are cited. References first cited in a table or figure legend should be numbered so they will be in sequence with references cited in the text at the point where the table or figure is first mentioned. List all

citation authors when there are six or fewer; when there are seven or more, list the first three, followed by et al.

The following are sample references:

1. Shapiro AMJ, Lakey JRT, Ryan EA, et al. Islet transplantation in seven patients with type 1 diabetes mellitus using a glucocorticoid-free immunosuppressive regimen. N Engl J Med 2000;343:230-8.
2. Goadsby PJ. Pathophysiology of headache. In: Silberstein SD, Lipton RB, Dalessio DJ, eds. Wolff's headache and other head pain. 7th ed. Oxford, England: Oxford University Press, 2001:57-72.
3. Kuczmarski RJ, Ogden CL, Grammer-Strawn LM, et al. CDC growth charts: United States. Advance data from vital and health statistics. No. 314. Hyattsville, Md.: National Center for Health Statistics, 2000. (DHHS publication no. (PHS) 2000-1250 0-0431.)
4. Medicare: trends in fees, utilization, and expenditures for imaging services before and after implementation of the Deficit Reduction Act of 2005. Washington, DC: Government Accountability Office, September 2008. (http://www.gao.gov/new.items/d081102r.pdf. opens in new tab.)

Numbered references to personal communications, unpublished data, or manuscripts either “in preparation” or “submitted for publication” are unacceptable. If essential, such materials can be incorporated at appropriate places in the text.

Tables

All tables should be included at the end of the manuscript text file. Double-space tables (including footnotes) and provide a title for each table. For Original Articles, there is normally

a limit of five figures and tables (total) per manuscript. Extensive tables or supplementary materials will be published as supplemental materials with the digital version of the article.

Figures and Illustrations:

Authors can either insert figures into text files (preferred) or upload figure files separately. Low-resolution images may be submitted for peer review, but be aware that NEJM may, at a later stage, request high-resolution versions that comply fully with detailed Technical Guidelines for Figures. opens in new tab.

Supplementary Appendix:

A manuscript's Supplementary Appendix should be paginated, with a table of contents, followed by a list of investigators (if there is one), text (such as methods), figures, tables, and then references. Reference citations in the Appendix and the corresponding list of references should be self-contained with respect to the Appendix. The Appendix must be submitted in two formats: PDF and MS Word (or another editable text format). The Appendix will not be edited for style and will be presented online as additional information provided by the authors.

Supplementary Figures and Tables:

For outcome scales, provide in the figure legend or table footnotes the range, sign, and minimally important difference (if known). There must be an informative reference citation for the scale. Each figure should include a title and a legend, which should appear on the same page as the figure itself. Tables in the Supplementary Appendix should be labelled Table S1, Table S2, etc. Each table should be accompanied by a title and, if necessary, footnotes.

Part H: Institutional Approvals

GROOTE SCHUUR HOSPITAL
Enquiries: Dr Bernadette Eick
E-mail : Bernadette.Eick@westerncape.gov.za

Professor M. Nicol
MEDICAL MICROBIOLOGY

E-mail: Stefanopperman1@gmail.com

Dear Professor Nicol

RESEARCH PROJECT: An Investigation Of A Community-Acquired Pseudomonas Aeruginosa Outbreak In The Cape Metropolitan Area During A Time Of High Water Stress (Mmed. Dr C. Opperman)

Your recent letter to the hospital refers.

You are granted permission to proceed with your research, which is valid until **28 February 2019.**

Please note the following:

a) Your research may not interfere with normal patient care.
b) Hospital staff may not be asked to assist with the research.
c) No additional costs to the hospital should be incurred i.e. Lab, consumables or stationary.
d) **No patient folders may be removed from the premises or be inaccessible.**
e) Please provide the research assistant/field worker with a copy of this letter as verification of approval.
f) Confidentiality must be maintained at all times.
g) Should you at any time require photographs of your subjects, please obtain the necessary indemnity forms from our Public Relations Office (E45 OMB or ext. 2187/2188).
h) Should you require additional research time beyond the stipulated expiry date, please apply for an extension.
i) Please discuss the study with the HOD before commencing.
j) Please introduce yourself to the person in charge of an area before commencing.
k) On completion of your research, please forward any recommendations/findings that can be beneficial to use to take further action that may inform redevelopment of future policy / review guidelines.
l) **Kindly submit a copy of the publication or report to this office on completion of the research.**

I would like to wish you every success with the project.

Yours sincerely

DR BERNADETTE EICK
CHIEF OPERATIONAL OFFICER
Date: 7 March 2018

C.C. Mr L. Naidoo
Dr B. Jacobs

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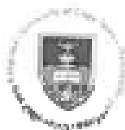
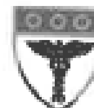


UNIVERSITY OF CAPE TOWN
Faculty of Health Sciences
Human Research Ethics Committee



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Groote Schuur Hospital
Observatory 7925
Telephone [021] 404 7682
Email: nosi.tsama@uct.ac.za
Website: www.health.uct.ac.za/fhs/research/humanethics/forms

05 February 2018

HREC REF: 068/2018

Prof M Nicol
Medical Microbiology
NHLS
GSH

Dear Prof Nicol

PROJECT TITLE: AN INVESTIGATION OF A COMMUNITY-ACQUIRED PSEUDOMONAS AERUGINOSA OUTBREAK IN THE CAPE METROPOLITAN AREA DURING A TIME OF HIGH WATER STRESS (MMeD-CANDIDATE-Dr C Opperman)

Thank you for submitting your study to the Faculty of Health Sciences Human Research Ethics Committee for review.

It is a pleasure to inform you that the HREC has **formally approved** the above-mentioned study.

Approval is granted for one year until the 28th February 2019.

Please submit a progress form, using the standardised Annual Report Form if the study continues beyond the approval period. Please submit a Standard Closure form if the study is completed within the approval period.
(Forms can be found on our website: www.health.uct.ac.za/fhs/research/humanethics/forms)

We acknowledge that the following student will be involved in this study: Dr CJ Opperman.

Please note that for all studies approved by the HREC, the principal investigator **must** obtain appropriate institutional approval before the research may occur.

Please quote the HREC REF in all your correspondence.

Please note that the ongoing ethical conduct of the study remains the responsibility of the principal investigator.

Yours sincerely

PROFESSOR M BLOCKMAN
CHAIRPERSON, FHS HUMAN RESEARCH ETHICS COMMITTEE
Federal Wide Assurance Number: FWA00001637.

UNIVERSITY OF CAPE TOWN

FACULTY OF HEALTH SCIENCES
Human Research Ethics Committee

FHS016: Annual Progress Report / Renewal

HREC office use only (FWA00001637; IRB00001938) **This serves as notification of annual approval, including any documentation described below.**			
☑ Approved	Annual progress report	Approved until/next renewal date	28.02.2020
☐ Not approved	See attached comments		
Signature Chairperson of the HREC	[signature]	Date Signed	30/1/2019

Comments to PI from the HREC

Principal Investigator to complete the following:

1. Protocol information

Date form submitted	29 January 2019		
HREC REF Number	068/2018	Current Ethics Approval was granted until	28.02.2019
Protocol title	An investigation of a community-acquired *Pseudomonas aeruginosa* outbreak in the Cape Metropolitan area during a time of high water stress		
Protocol number (if applicable)	None applicable		
Are there any sub-studies linked to this study?	☐ Yes **X** No		
If yes, could you please provide the HREC Ref's for all sub-studies? Note: A separate FHS016 must be submitted for each sub-study. **None applicable**			
Principal Investigator	Chad Centner		
Department / Office Internal Mail Address	Department of Microbiology, NHLS, C18 Laboratory, Groote Schuur Hospital, Anzio Road Observatory, 7925		

1.1 Does this protocol receive US Federal funding?	☐ Yes	**X** No
1.2 If the study receives US Federal Funding, does the annual report require full committee approval?	☐ Yes	**X** No

11 February 2014 Page 1 of 5 FHS016

(Note: Please complete the Closure form (FHS010) if the study is completed within the approval period)