Klebsiella pneumoniae ST307 with bla$_{OXA-181}$, South Africa, 2014–2016

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Klebsiella pneumoniae sequence type (ST) 307 is an emerging global antimicrobial drug–resistant clone. We used whole-genome sequencing and PCR to characterize *K. pneumoniae* ST307 with oxacillinase (OXA) 181 carbapenemase across several private hospitals in South Africa during 2014–2016. The South Africa ST307 belonged to a different clade (clade VI) with unique genomic characteristics when compared with global ST307 (clades I–V). Bayesian evolution analysis showed that clade VI emerged around March 2013 in Gauteng Province, South Africa, and then evolved during 2014 into 2 distinct lineages. *K. pneumoniae* ST307 clade VI with OXA-181 disseminated over a 15-month period within 42 hospitals in 23 cities across 6 northeastern provinces, affecting 350 patients. The rapid expansion of ST307 was most likely due to intrahospital, interhospital, intercity, and interprovince movements of patients. This study highlights the importance of molecular surveillance for tracking emerging antimicrobial clones.

The World Health Organization recently identified global spread of antimicrobial resistance (AMR) as one of the most serious recent threats to human health (*1*). The emergence and spread of carbapenem resistance is a substantial public health concern, because these agents are regarded as one of the last effective therapies available for treating serious infections caused by gram-negative bacteria. Carbapenemases, the predominant cause of carbapenem resistance, are commonly harbored on plasmids that are able to transfer between members of the family *Enterobacteriaceae* (*2*). The most common carbapenemases among clinical *Enterobacteriaceae* are the *Klebsiella pneumoniae* carbapenemases (KPCs; Ambler class A); the metallo-β-lactamases (IMPs, VIMs, NDMs; Ambler class B); and oxacillinase 48 (OXA-48)–like (Ambler class D) enzymes.

The OXA-48–like carbapenemases are identified mostly in *K. pneumoniae* and *Escherichia coli* and include the following enzymes: OXA-48, OXA-162, OXA-181, OXA-204, OXA-232, OXA-244, OXA-245, and OXA-247 (*3*). They are active against penicillins and weakly hydrolyze carbapenems, with limited activities against broad-spectrum cephalosporins and most β-lactam inhibitors.

The earliest reported case in South Africa of a *K. pneumoniae* that contained bla$_{OXA-48}$ occurred in Johannesburg in 2011; the case-patient had previously been hospitalized in Egypt (*4*). The report also described OXA-181–producing *K. pneumoniae* from different Johannesburg and Cape Town private hospitals. Laboratory surveillance reports showed that OXA-48–like enzymes are the second most common carbapenemase (after NDMs) in various healthcare centers across South Africa (*5*).

The molecular diagnostic reference center at Ampath Laboratories (Ampath-MDRC) in Pretoria, Gauteng Province, South Africa, experienced a substantial increase of *K. pneumoniae* with bla$_{OXA-48-like}$ during 2014–2016 from different private hospitals across northeastern South Africa. We designed a study to investigate the underlying molecular mechanisms associated with the increase of *K. pneumoniae* with OXA-48–like enzymes. We obtained ethics approval from the Conjoint Health Research Ethics Board at the University of Calgary (REB17-1010) and from the Research Ethics Committee (REC), Faculty of Health Sciences, University of Pretoria (protocol nos. 240/2016 and 104/2017).

Materials and Methods

Setting and Workflow

We described South Africa's health system and the role of Ampath-MDRC (Appendix, https://wwwnc.cdc.gov/EID/

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article/25/4/18-1482-App1.pdf). We also summarized the study workflow (Appendix Figure 1).

We screened all carbapenem-nonsusceptible *K. pneumoniae* at Ampath-MDRC using a PCR for bla_{NDM}, bla_{KPC}, bla_{OXA-48}–like, bla_{IMP}, bla_{VIM}, and bla_{GES}. We performed pulsed-field gel electrophoresis (PFGE) on 471 *K. pneumoniae* isolates positive for OXA-48–like enzyme to determine if some of the isolates were genetically related. We identified 1 dominant pulsotype (pulsotype A) and a related pulsotype (AR) and selected isolates representing them from different geographic sites over various time periods. We used these isolates for initial Illumina short-read sequencing (n = 28) (Illumina, http://www.illumina.com) and PacBio long-read sequencing (n = 1) (Pacific Biosciences, http://www.pacb.com) to determine if they belong to the same sequence type and to identify the OXA-48–like enzyme.

We identified the pulsotypes as sequence type (ST) 307 containing OXA-181 on IncX3 plasmids. We then compared this whole-genome sequencing (WGS) data with the sequences from the US National Center for Biotechnology Information (NCBI) genome database (ftp://ftp.ncbi.nih.gov/genomes) to design PCR primers for the detection of ST307, IncX3, and OXA-181-IS3000 mobile genetic element (MGE). We used previously characterized *K. pneumoniae* STs with different plasmid replicons to verify these primers (Appendix Table 1). We screened *K. pneumoniae* producing OXA-48–like enzyme (n = 471) with PCR primers to identify ST307, IncX3 plasmids, and OXA-181-IS3000 MGE. We selected additional *K. pneumoniae* ST307 isolates (n = 60) from different geographic locations, time points, and specimens to undergo Illumina short-read WGS to elucidate the evolution of ST307 in South Africa.

Bacterial Isolates

During January 2014–December 2016, various Ampath regional clinical laboratories referred 1,247 unique clinical, carbapenem (i.e., ertapenem, meropenem, or imipenem) nonsusceptible *K. pneumoniae* isolates to Ampath-MDRC for PCR confirmation of carbapenemases (Appendix Figure 1). We performed PCR screening for bla_{NDM}, bla_{KPC}, bla_{OXA-48}–like, bla_{IMP}, bla_{VIM}, and bla_{GES} using LightMix modular carbapenemase kits (TIB Molbiol, https://www.tib-molbiol.com) on a LightCycler 480 II instrument (Roche Diagnostics, https://www.roche.com). Details on the identification and susceptibility testing of the bacterial isolates are provided in the Appendix.

PFGE

We performed PFGE on the *K. pneumoniae* isolates with OXA-48–like enzymes (n = 471) to determine if there was a dominant pulsotype among them. The Appendix describes the methodology used and results obtained with PFGE.

PCR

We designed 3 sets of PCR primers for the detection of ST307, IncX3 plasmid, and the IS3000-OXA MGE. We screened *K. pneumoniae* isolates with OXA-48–like enzymes (n = 471) using different primer sequences (Appendix Table 1).

WGS and Data Analysis

We sequenced the *K. pneumoniae* isolates that tested positive by PCR for ST307 (n = 88) using the Illumina NextSeq platform (Appendix). We prepared libraries with the Illumina Nextera XT kit to produce paired end reads of 150 bp for a predicted coverage of ≥75×. We chose *K. pneumoniae* I72 (isolated from urine obtained in Benoni, Gauteng Province, during December 2014) that was positive for $bla_{OXA-181}$ and bla_{OXA-48} for long-read WGS using the RSII platform (Pacific Biosciences) to characterize plasmids.

We compared the South Africa genomes sequenced in this study (deposited in the NCBI Bioproject database [https://www.ncbi.nlm.nih.gov/bioproject] under accession no. PRJNA488070) with 620 ST307 genomes previously deposited in the NCBI Sequence Read Archive (https://www.ncbi.nlm.nih.gov/sra) and the genome database. The global ST307 genomes were from the United States (n = 488), the United Kingdom (n = 45), Norway (n = 30), Italy (n = 10), Thailand (n = 9), Australia (n = 6), Brazil (n = 3), Colombia (n = 3), China (n = 3), Nepal (n = 3), Cambodia (n = 2), France (n = 2), Nigeria (n = 2), Cameroon (n = 1), Guinea (n = 1), Iran (n = 1), Netherlands (n = 1), Pakistan (n = 1), and other unspecified regions (n = 9).

We mapped all genomes to the ST307 reference genome NR5632 (GenBank accession no. CP025143) using Snippy (https://github.com/tseemann/snippy). We predicted prophages using PHASTER (*6*), examined repeated regions using MUMmer (*7*), and predicted putative regions of recombination with Gubbins (*8*), followed by filtering using vcftools. We generated a recombination-free single-nucleotide polymorphism phylogenetic tree, using a general time-reversible model of nucleotide substitution with a gamma model of rate heterogeneity and 4 rate categories, by using RAxML version 8.2.4 (*9*). We conducted hierarchical Bayesian analysis of population structure with 3 nested levels and 10 independent runs of the stochastic optimization algorithm with the a priori upper bound of 10–30 clusters varying across the runs to identify phylogenetic clades. We defined clades using the first level of clustering (*10*) and annotated the phylogenetic tree in iTOL (*11*).

We used BEAST version 2.4.7 (*12*) to estimate a timed phylogeny with concatenated recombination-free core single-nucleotide polymorphism alignment. To increase the accuracy of the time to most recent common ancestor, we included 17 additional ST307 isolates from a Pretoria academic hospital in this analysis. We identified the bla_{OXA181} harboring IncX3 plasmids by de novo assembly using plasmidSPAdes (*13*) and manually investigated the findings

using Bandage assembly graph viewer (*14*) and blastn (https://blast.ncbi.nlm.nih.gov/Blast.cgi), as well as S1-PFGE in combination with Southern blotting (Appendix).

Results

Overview

Ampath-MDRC experienced an increase in OXA-48–like *K. pneumoniae* isolates during July 2015–June 2016, especially from private hospitals in Gauteng Province. We identified by PFGE a dominant pulsotype named A (and a related pulsotype AR) among the OXA-48–like collection. Initial WGS on 28 isolates that belong to pulsotypes A and AR identified them as ST307 with OXA-181 on IncX3 plasmid that was shown using PacBio long-read sequencing. Using WGS, we used PCR primers specific to ST307, IncX3, and OXA-181-IS3000 MGE to screen the OXA-48–like *K. pneumoniae* and showed that 74% belonged to ST307 containing IncX3 plasmids.

WGS of an additional 60 PCR-positive ST307 illustrated, when compared with ST307 from other countries, that the South Africa ST307 belonged to a different clade. The South Africa clade emerged around March 2013 in Gauteng Province, then evolved during 2014 into 2 distinct lineages that spread across northeastern South Africa, affecting 350 patients.

Increase of OXA-48–Like Positive *K. pneumoniae* across Northeastern South Africa

Overall, 574 carbapenemase gene PCR-positive *K. pneumoniae* were detected at Ampath-MDRC during January 2014–December 2016; the total included NDM (n = 58), KPC (n = 10), VIM (n = 35), and OXA-48–like (n = 471) carbapenemases (Appendix Table 2). The OXA-48–like isolates from 2014 came mainly from different private hospitals in Johannesburg and Alberton, Gauteng Province. The numbers of OXA-48–like *K. pneumoniae* increased exponentially toward the end of 2015 and peaked during the first 6 months of 2016 (e.g., we detected 349/471 [74%] of OXA-48–like *K. pneumoniae* during July 2015–June 2016). This increase was especially evident in various hospitals across the province. Private hospitals from other areas in northern and eastern South Africa also experienced increases during 2015–2016 (Appendix Table 2). Throughout the study period, the numbers of *K. pneumoniae* with KPC, NDM, and VIM remained relatively low and stable compared with OXA-48–like *K. pneumoniae*. PCR testing did not detect IMP or GES during this period.

We obtained OXA-48–like *K. pneumoniae* from intraabdominal specimens (n = 78), urine (n = 196), skin and soft tissues (n = 11), blood (n = 76), central venous catheter tips (n = 8), respiratory specimens (n = 99), and rectal specimens (n = 3). The isolates tested nonsusceptible

(intermediate or full resistance) to ampicillin, amoxicillin/clavulanic acid, piperacillin/tazobactam, cefotaxime, ceftazidime, cefepime, and ertapenem. Most isolates were also nonsusceptible to trimethoprim/sulfamethoxazole (98%), gentamicin (98%), ciprofloxacin (92%), and meropenem (52%), whereas 44% of isolates were nonsusceptible to imipenem and 11% to amikacin. The tigecycline (TGC) and colistin MICs were each ≤1 µg/mL, except for 4 isolates that had TGC MICs of 2, 4, 4, and 8 µg/mL.

Dissemination of ST307 across Private Hospitals

PCR screening of OXA-48–like *K. pneumoniae* showed that 350/471 (74%) isolates were positive for ST307, IncX3, and IS3000-OXA-181 (cited as ST307_X3-OXA-181). ST307_X3-OXA-181 appeared in different private hospitals in Johannesburg and Alberton during January–June 2014 (Figures 1, 2). During July 2014–June 2016, ST307_X3-OXA-181 subsequently spread to other private hospitals in Gauteng Province, especially in Pretoria, and to Mpumalanga, North West, Limpopo, Free State, and Eastern Cape Provinces (Figures 1, 2). Gauteng Province was the epicenter of the dissemination of ST307_X3-OXA-181, showing a substantial increase in numbers from 2014 (n = 18) to 2015 (n = 138) (Figure 1).

We isolated ST307_X3-OXA-181 mostly from urine (41%), respiratory (20%), intraabdominal (18%), and blood (16%) specimens; 96% of specimens were submitted from hospital settings. Ampath-MDRC receives ≈80% of community, nursing home, and hospital specimens from the private sector in Gauteng, Mpumalanga, North West, Limpopo, Free State, and Eastern Cape Provinces. We isolated ST307_X3-OXA-181 exclusively from hospital
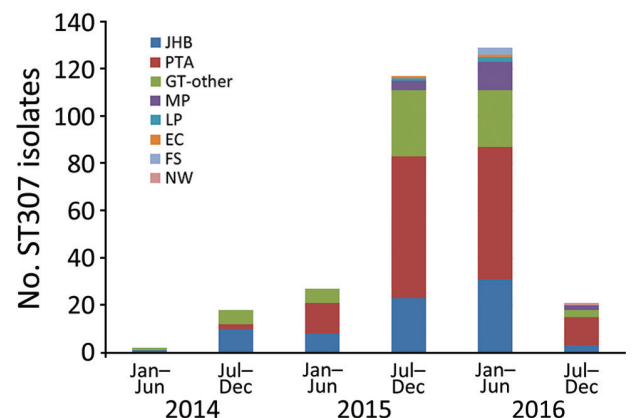
Figure 1. Locations of *Klebsiella pneumoniae* with ST307 oxacillinase 48–like in South Africa during January 2014–December 2016, as identified at Ampath Molecular Diagnostic Reference Center (PTA). EC, Eastern Cape Province; FS, Free State Province; GT-other, other cities in Gauteng Province; JHB, Johannesburg, Gauteng Province; LP, Limpopo Province; MP, Mpumalanga Province; NW, North West Province; PTA, Pretoria, Gauteng Province; ST, sequence type.
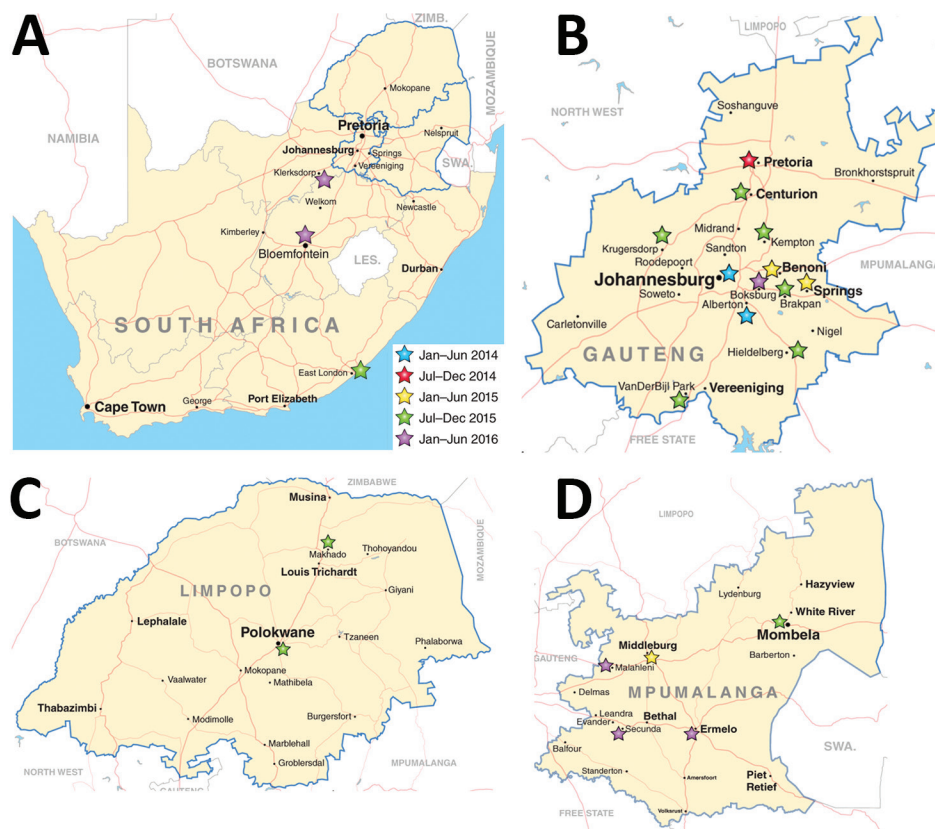
Figure 2. Geographic distribution of *Klebsiella pneumoniae* sequence type 307 with oxacillinase 181 in northeastern South Africa, January 2014–December 2016. A) South Africa; B) Gauteng Province; C) Limpopo Province; D) Mpumalanga Province. Map source: http://d-maps.com.

specimens; therefore, it is unlikely that the community or nursing home sectors were important reservoirs of ST307.

South Africa *K. pneumoniae* ST307 Clade

The overall characteristics of the ST307 sequences from this study (n = 88) were similar to ST307 with bla_{KPC} and $bla_{CTX-M-15}$ sequences obtained previously in Colombia, Italy, and the United Kingdom, and those in this study contained the capsular loci *wzi*-173, capsule 2, π-fimbrial cluster, Type IV secretion system (*15*). The hierarchical Bayesian clustering analysis of 88 South Africa ST307 and additional 620 genomes from publicly available databases divided ST307 into 6 distinct clades: clades I–IV, from the United States (mainly Texas) (*16*); clade V, from various countries (Australia, Brazil, Cambodia, Cameroon, China, Colombia, France, Guinea, Iran, Italy, Nepal, Netherlands, Nigeria, Norway, Pakistan, Thailand, the United Kingdom, and the United States); and clade VI, which consisted of the isolates from South Africa (Figure 3). Clade V also includes a subset of isolates from Texas. Sequence analysis of the mutations in the quinolone resistance–determining regions in chromosomal *gyrA* and *parC* indicated that most of the isolates from clades V and VI contained the ParC-80I and GyrA-83I mutations, whereas isolates from clades I–IV harbored an additional GyrA-87N mutation (Figure 3).

Genomic analysis revealed that clade VI isolates contained some unique characteristics when compared with clades I–V. All of clade VI harbored the plasmid p72_X3_OXA181 with $bla_{OXA-181}$ that contained the IncX3 and truncated ColKp3 replicons (Appendix Figure 2). Approximately 30% of isolates in clades I–V contained various carbapenemase genes, namely bla_{KPC-2} (n = 184), bla_{KPC-3} (n = 4), bla_{NDM-1} (n = 3), bla_{NDM-5} (n = 2), and bla_{OXA-48} (n = 2). KPC-2 was distributed within clades I–V, whereas KPC-3, NDM-1, NDM-5, and OXA-48 were restricted to clade V (Figure 3). Most ST307 isolates in clades I–VI contained the extended-spectrum β-lactamase gene $bla_{CTX-M-15}$ (684/708, 96.6%) that was adjacent to IS*Ecp1* (682/684, 99.7%) situated on a FIB-like plasmid and showed high similarities to the previously described pKPN3–307_type A plasmid (*15*).

We identified 5 prophages (72_phage 1–5) and 1 novel integrative conjugative element (72_ICE) within the 88 sequenced ST307 that belonged to clade VI (Figure 3); the 72_phages 2, 3, and 4 correlated with the previously described ST307 phages 1, 3, and 4 (*15*). The 72_phage1, 72_phage5, and 72_ICE were mainly restricted in clade VI; 72_ICE was unique to clade VI, whereas 72_phage1 was also present in 5 isolates and 72_phage5 in 23 isolates from clade V. The 72_phage3 and 72_phage4 were found in clade VI and a subset of clade V, and the 72_phage 2 was part of ST307 isolates from clusters I–VI (Figure 3).
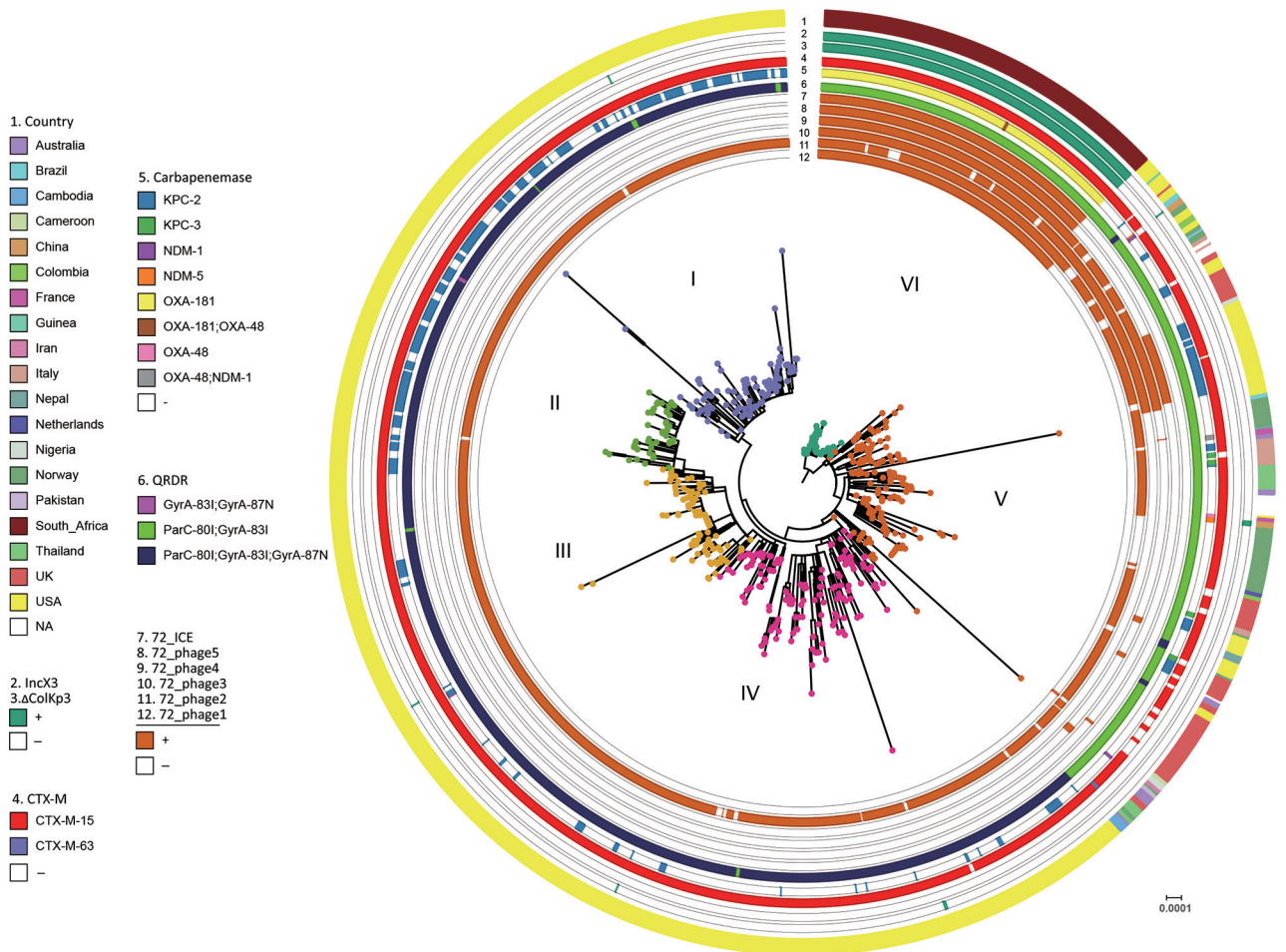
Figure 3. Bayesian phylogenetic analysis of global *Klebsiella pneumoniae* sequence type (ST) 307 isolates. The ST307 genomes included 88 from South Africa (this study) and 620 international isolates from 19 countries (downloaded from the US National Center for Biotechnology Information whole genome shotgun database). ST307 has 6 distinct clades, as indicated on branches. CTX-M, active on cefotaxime first isolated in Munich; KPC, *Klebsiella pneumoniae* carbapenemase; NDM, New Delhi metallo-β-lactamases; OXA, active on oxacillin; QRDR, quinolone resistance determinants.

Origin of ST307 Clade VI

Bayesian evolution analysis conducted with BEAST estimated the time to most recent common ancestor of clade VI around March 2013, ≈2 months before the first isolate (C6) that was collected in May 2013 from a Johannesburg hospital, J1 (Figure 4). We estimated the mean evolutionary rate as 1.16×10^{-6} substitutions/site/year (95% highest posterior density 7.7×10^{-7} to 1.6×10^{-6} substitutions/site/year), corresponding to 5.7 substitutions/genome/year among clade VI. This analysis placed the outbreak node, from which all outbreak isolates were derived, at April 2014 (95% highest posterior density September 2013–September 2014).

Two distinct lineages evolved within Gauteng Province from the outbreak node. Lineage A originated in Johannesburg and lineage B in Alberton, and they spread to different hospitals across Gauteng (both lineages), Mpumalanga (both lineages), Free State (lineage A), Eastern Cape (lineage A), Limpopo (lineage A), and North West Provinces (lineage B) (Figure 4).

Dissemination by Movement of Patients

Genomics, combined with admission, discharge, and transfer data, showed evidence of intrahospital spread. For example, patient I51 in hospital P5 was transferred during December 2015 from the medical intensive care unit to the high-risk unit. Subsequently a highly related ST307 was isolated during January 2016 from patient I68 in the high-risk unit (Figure 4). We also observed interhospital spread when patient I14 was transferred from the surgical intensive care unit at hospital P1 to hospital P4 during September 2016. In December 2016, a highly related ST307 was isolated at hospital P4 from patient K43 (Figure 4). Intercity and interprovince spread were illustrated by patient I2 from the city of Springs, patient K61 from the town of Heidelberg, and patient K22

from the North West Province, all with highly related ST307 isolates, having previously received medical care at hospital J2 in Johannesburg (Figure 4).

Discussion

K. pneumoniae producing OXA-181 was first described as isolated in hospitals in India during 2006–2007 (*17*),
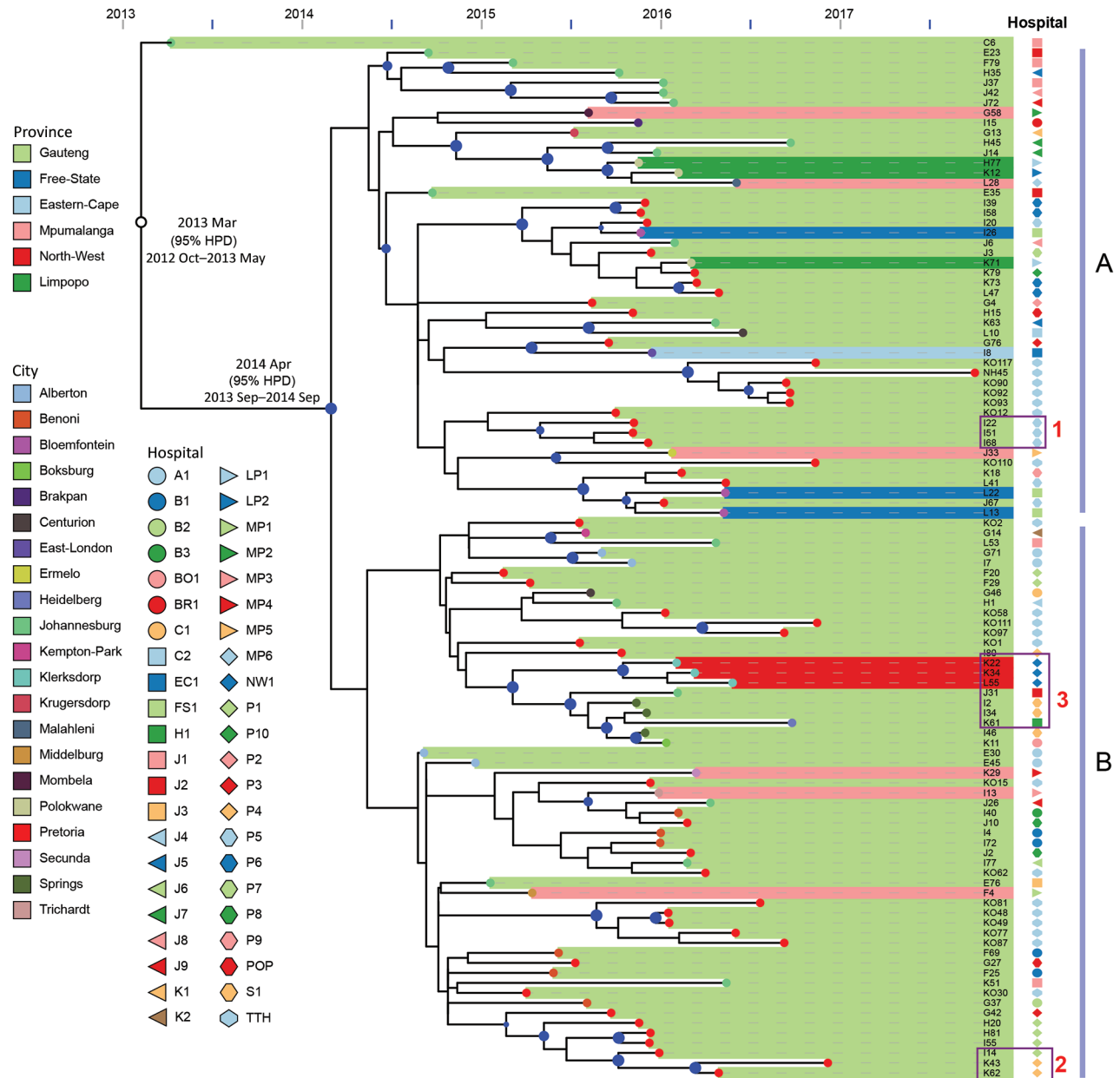
Figure 4. Bayesian evolution analysis of *Klebsiella pneumoniae* sequence type (ST) 307 in South Africa hospitals, January 2014–December 2016. A and B indicate the 2 distinct lineages that evolved within Gauteng Province. Highlighted areas depict the provinces from which isolates were obtained, and colored dots at the tips of areas represent the cities from which the isolates were obtained (e.g., for Gauteng Province, Pretoria is red, Johannesburg is green). Dark blue dots at branch points indicate posterior probability >70%, and dot size is proportional to posterior probability values. Hospital locations are indicated as follows; all are private except for Tshwane tertiary hospital: A1, Alberton; J1–J10, Johannesburg; P1–P8, Pretoria; B1–3, Benoni; BO1, Boksburg; BR1, Brakpan; C1–C2, Centurion; EC1, Eastern Cape Province; FS1, Free State Province; K1, Krugersdorp; K2, Kempton Park; LP1–2, Limpopo Province; MP1–6, Mpumalanga Province; NW1, North West Province; S1, Springs; TTH, Tshwane (tertiary hospital). Box 1 shows intrahospital spread: highly related ST307 isolates were obtained from patient I51 and patient I68 in hospital P5. Box 2 shows interhospital spread: patient I14 was transferred from hospital P1 to hospital P4; 3 months later, highly related ST307 was isolated from patient K43 at hospital P4. Box 3 shows intercity and interprovince spread: patient I2 from Springs, patient K61 from Heidelberg, and patient K22 from North West Province, all with highly related ST307 isolates, had previously received medical care at hospital J2 in Johannesburg. HPD, highest posterior density interval; POP, outpatient.

with a subsequent report from Oman (*18*). OXA-181 has a global distribution, is the second most common OXA-48 derivative, and differs from OXA-48 by 4 amino acid substitutions, while retaining the same spectrum of activity (*3*). Various *K. pneumoniae* clones with OXA-48–like carbapenemases have been described from several localized hospital outbreaks in Africa, Europe, and the Middle East (*3*). Previous studies have shown that *bla*$_{OXA-181}$ are present on ColE2-type (*18*), IncT (*19*), IncN (*20*), and IncX3 (*21*) plasmids.

We describe the dissemination of a carbapenemase-producing clone, *K. pneumoniae* ST307 containing an IncX3 plasmid with *bla*$_{OXA-181}$, across 23 cities and towns within 6 provinces in South Africa. The plasmid (p72_X3_OXA181) was identical to other IncX3 plasmids with *bla*$_{OXA-181}$ previously reported from China (*21*) and Angola (*22*). We tracked (with PCR) and confirmed (with WGS) the presence of ST307 within Gauteng, Mpumalanga, North West, Limpopo, Free State, and Eastern Cape provinces. Genomics combined with admission, discharge, and transfer data showed intrahospital, interhospital, intercity, and interprovince spread to involve 350 patients in 42 private hospitals.

K. pneumoniae ST307 is a superclone that emerged during the mid-1990s and was responsible for several worldwide nosocomial outbreaks (*23*). The earliest published report was in 2013 from hospitals in Texas, USA (*24*), and it has since been reported globally (*23*). ST307 is associated with several antimicrobial resistance determinants, including CTX-M-15 (*25*), KPC (*26*), OXA-48 (*27*), NDM-1 (*28*), and *mcr-1* (*29*). Recent reports from Texas (*16*), Colombia (*26*), and Italy (*30*) have shown that ST307 is replacing ST258 as the most prevalent clone associated with multidrug resistance. Certain characteristics of ST307 may lead to increased fitness, persistence, and adaptation to the hospital environment and the human host (*15*).

K. pneumoniae ST307 belongs to 6 distinct clades: US clades I–IV; an international clade, V; and South Africa clade VI. The presence of 72_ICE, p72_X3_OXA181 is unique to clade VI and, with other genetic changes, may have played a role in the success of this clade. Bayesian evolution analysis showed that clade VI emerged around March 2013 and evolved during 2014 into 2 distinct lineages that spread across northeastern South Africa over a 15-month period.

In 2014, the World Health Organization reported that key tools to tackle AMR, such as basic surveillance systems to track and monitor the problem, do not exist in many countries (*1*). This study highlighted the public health and clinical utility of using WGS data to develop rapid, reliable, and user-friendly molecular surveillance methods such as PCR for tracking emerging AMR clones and plasmids during outbreaks. We designed PCR primers for tracking ST307 and p72_X3_OXA181 across northeastern South

Africa. From a global perspective, this study is an example of a productive collaboration between resource-limited and industrialized countries that rapidly generated cost-effective PCR methodologies to track an emerging AMR clone.

K. pneumoniae ST307 clade VI spread rapidly between various private hospitals across South Africa. The reasons for this are unclear, but the medical community needs to know how and why this happened. The increase in OXA-48–like bacteria occurred during a period of high carbapenem usage in the private sector (J. Coetzee, unpub. data). Patients often visit various private hospitals during their treatment. Our results suggest that the intrahospital, interhospital, intercity, and interprovince movements of patients were responsible for the dissemination of ST307.

It is imperative that the medical community continues to explore the reasons for the spread of ST307. Studies investigating the pathogenicity, fitness, adaptiveness, and evolution of ST307 clade VI are currently in progress. The South Africa clade VI has the potential to be introduced to other countries and the ability to cause devastating countrywide outbreaks associated with substantial healthcare costs. A recent report from the United Kingdom highlighted the economic implications associated with a 10-month outbreak of carbapenemase-producing *K. pneumoniae* affecting 40 patients from 5 hospitals across London, costing around €1.1 million (*31*). Clinical studies are also urgently required to investigate the reasons for the high transmission rates of *K. pneumoniae* ST307. Such projects will serve as models to predict what could happen with the continuing emergence of successful clones among clinically relevant bacteria (*32*).

About the Author

Ms. Lowe is a PhD student at the University of Pretoria. Her research interests include the molecular epidemiology of antimicrobial-resistant organisms.

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etymologia

Anaplasma phagocytophilum [anʺə-plazʹmə faʹgo-sītʺo-fī-lum]

Ronnie Henry

A species of tickborne bacteria that causes human granulocytic anaplasmosis, *Anaplasma* (from the Greek *an-* ["without"] + *plasma* ["shape"]) *phagocytophilum* (named for its affinity for growing in neutrophils: phagocyte + Latin *phile* ["loving"]) has gone by many names. First it was named *Rickettsia* (for Howard Taylor Ricketts) *phagocytophilum*, then *Cytoecetes* (for its similarity to *Cytoecetes microti*) *phagocytophilum*, and then *Ehrlichia* (for Paul Ehrlich) *phagocytophilum*. More recently, *E. equi* and the agent of human granulocytic ehrlichiosis (now anaplasmosis) were combined with *E. phagocytophilum* as *A. phagocytophilum*.
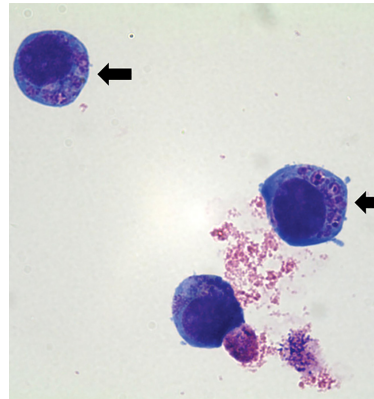
Anaplasma phagocytophilum cultured in human promyelocytic cells, showing morulae as basophilic and intracytoplasmic inclusions (arrows). Wright-Giemsa stain. Original magnification x1,000. Image: Emerg Infect Dis. 2014;20:1708–11.

Source

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Klebsiella pneumoniae ST307 with *bla*_{OXA-181,} South Africa, 2014–2016

Appendix

Healthcare Systems in South Africa

The South Africa healthcare system consists of a large public sector, a smaller private sector, and a nongovernmental organizations sector (which focuses mainly on HIV and tuberculosis). The public health system consists of primary healthcare clinics, district hospitals, and academic hospitals (at the tertiary level) where advanced diagnostic procedures and treatments are provided. There are 376 public hospitals in South Africa. The private healthcare system is made up of healthcare professionals who provide their services on a private basis, usually funded by the subscriptions of persons to medical aid schemes. Private healthcare practitioners provide services through private hospitals. There are 238 private hospitals in South Africa.

Ampath Laboratories

Ampath is a large private clinical laboratory that provides pathology-related services to private healthcare practitioners and their patients, especially to those in Gauteng Province (the northern part of the country). Ampath serves ≈50% of the private healthcare market in South Africa. They have an extensive national network of more than 300 facilities that includes reference laboratories, regional laboratories, satellite laboratories and service depots.

The Ampath Molecular Diagnostic Reference Center (Ampath-MDRC) is situated in Pretoria, Gauteng Province, and routinely screens all carbapenem-nonsusceptible *Enterobacteriaceae* (referred from regional laboratories) for carbapenemases using commercial PCR methodology. Due to the nature of the private healthcare system (various hospitals with different healthcare professionals), it was impossible to collect clinical data from patients in that sector.

Selection of Isolates

We selected *K. pneumoniae* isolates that tested positive by PCR for ST307 (n = 88) to represent different geographic locations, time periods, and specimens. The selection criteria were as follows: n = 5 from a private Alberton hospital A1 (July 2014–December 2015); n = 19 from private Johannesburg hospitals J1–J10 (July 2014– December 2016); n = 31 from 10 private Pretoria hospitals P1–P10 (October 2014 – December 2016); n = 6 from 3 private Benoni hospitals B1–B3 (May 2015–January 2016); n = 2 from 1 private Boksburg hospital BO1 (February 2016, July 2016); n = 1 from private Brakpan hospital BR1 (December 2015); n = 2 from 2 private Centurion hospitals C1–C2 (September 2015, June 2016); n = 1 from a private Eastern Cape hospital EC1 (December 2015), n = 3 from a private Free State hospital FS1 (January– July 2016), n = 1 from a private Krugersdorp hospital K1 (November 2015); n = 1 from a private Kempton Park hospital K2 (September 2015), n = 3 from 2 private Limpopo hospitals LP1–2 (December 2015–April 2016), n = 6 from 6 private Mpumalanga hospitals MP1–MP6 (April 2014– July 2016), n = 3 from a private North-West hospital NW1 (May 2016– July 2016), n = 3 from a private Springs hospital S1 (December 2015–March 2016). The sequenced isolates were obtained from the following specimens: urine (42%), respiratory tract (21%), intra-abdominal (17%), blood (16%), and others (4%).

Validation of Primers and Methodologies

We used the following isolates of *K. pneumoniae* with IncF, IncX3, IncN, IncL/M, IncC types of plasmids for validation of the primers for sensitivity and specificity: ST307 (n = 35), ST11 (n = 9), ST14 (n = 5), ST15 (n = 9), ST17 (n = 1), ST29 (n = 1), ST37 (n = 1), ST39 (n = 3), ST47 (n = 1), ST101 (n = 11), ST147 (n = 12), ST218 (n = 1), ST258 (n = 15), ST278 (n = 8), ST336 (n = 2), ST383 (n = 5), ST391 (n = 2), ST392 (n = 2), ST393 (n = 6), ST405 (n = 1), ST423 (n = 2), ST437 (n = 1), ST485 (n = 2), ST525 (n = 2), ST831 (n = 2), ST972 (n = 1), ST1373 (n = 1), and ST1805 (n = 1).

We tested the performance of the PCR methodologies first as a pilot study and then during implementation. When comparing WGS results with PCR results, the ST307, IncX3 and

IS3000-OXA-181 PCR screening displayed 97% correlation with WGS results, furthering the evidence that the PCR approach was highly reliable.

We performed each amplification with 1X JumpStart Taq ReadyMix (Sigma-Aldrich, https://www.sigmaaldrich.com), 0.1 – 0.2 µM for each primer, and 2 µL of DNA in a total volume of 25 µL. Thermocycling was done using Veriti Thermal Cycler (Applied Biosystems, http://www.thermofisher.com/applied/biosystems) under the following conditions:: initial denaturation 95°C for 5 min; 30 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s, and a final extension of 72°C for 5 min.

We also screened the isolates for OXA-48-IS1999 using the following primers: OXA-R (Appendix Table 1) and IS1999 (3′-CTTAGAGGCCAGCATCAAGC-5′). Only 1 isolate, I72, was positive for both OXA-181 (amplicon of 724 bp) and OXA-48 (amplicon of 307bp).

Identification and Susceptibility Testing of Bacterial Isolates

We used matrix-assisted laser desorption ionization–time of flight mass spectrometry for identification testing (Vitek AMS; bioMérieux Vitek Systems Inc., https://www.biomerieux-usa.com) and VITEK 2 instrument (bioMérieux Vitek Systems) for susceptibility testing. Susceptibilities to the following drugs were determined: ampicillin (AMP), amoxicillin-clavulanic acid (AMC), piperacillin-tazobactam (TZP), cefotaxime (CTX), ceftazidime (CAZ), imipenem (IPM), meropenem (MER), ertapenem (ERT), amikacin (AMK), gentamicin (GEN), ciprofloxacin (CIP), and trimethoprim-sulfamethoxazole (SXT). Throughout this study, results were interpreted using Clinical Laboratory Standards Institute (CLSI) criteria for broth dilution. Carbapenem susceptibilities (ERT, IPM, and MER), colistin (COL), and tigecycline (TGC) MICs were determined using E-tests (bioMérieux Inc.) according to the manufacturer's instructions. The European Committee for Antimicrobial Susceptibility Testing (EUCAST) breakpoint was used for COL and the FDA breakpoint was used for TGC.

Pulsed-Field Gel Electrophoresis (PFGE)

Genetic relatedness of *K. pneumoniae* isolates was examined by comparing *Xba*I digested profiles generated by PFGE analysis using the standardized *E. coli* (O157:H7) protocol from the Centers for Disease Control and Prevention (CDC), Atlanta, GA, USA (*6*). The subsequent

PFGE analyses were performed on a CHEF-MAPPER XA apparatus (Bio-Rad Laboratories, http://www.Bio-Rad.com). Gel images in tiff format were exported to BioNumerics software version 3.0 (Applied Maths, http://www.applied-maths.com) for analysis. Comparisons were made using the band-based Dice coefficient, which is a binary coefficient measuring similarity based upon common and different bands. Dendrograms were generated using Unweighted Pair Group Method using Arithmetic averages (UPGMA) method with 1.5% position tolerance. DNA relatedness was calculated based on the Dice coefficient and isolates were considered to be genetically related if the Dice coefficient correlation was $\geq$80%, which corresponds to the "possibly related (4–6 bands difference)" criteria of Tenover et al. (*7*). The *bla*$_{OXA-181}$ plasmid detection and size estimation was performed using S1-nuclease linearization, followed by PFGE and Southern blot hybridization (*8*).

A dominant pulsotype with >80% similar PFGE profiles (named pulsotype A), was detected among 310/471 (66%) of OXA-48–like isolates (data not shown). Forty isolates exhibited >70% similarity of PFGE profiles to pulsotype A, suggesting that they were related to pulsotype A and were named AR. The remaining isolates were not clonally related, i.e., exhibited <60% similar PFGE profiles and did not show patterns similar to those of pulsotypes A or AR.

Whole-Genome Sequencing and Analysis

The SMRT analysis portal v2.3.0 (Pacific Biosciences, http://www.pacb.com) was used for I72 genome, with raw sequence reads assembled de novo using the HGAP3 protocol, and error correction and polishing with Quiver v1, and closure with Circlator (*1*). Further error correction was performed by mapping Illumina short reads from the same isolate to the PacBio assembly with Pilon v1.22 (*2*). Illumina raw sequencing reads were trimmed using Trimmomatic v0.36 (*3*), followed by de novo assembly, using SPAdes v3.11.1 (*4*) and annotation by Prokka v1.12 (*5*). The multilocus sequence type (MLST) was determined *in silico* using MLST (https://github.com/tseemann/mlst), while the acquired resistance gene and plasmid replicons were determined using Abricate (https://github.com/tseemann/abricate) and ARIBA (*6*) based on ResFinder (*7*) and PlasmidFinder (*8*) databases.

Root-to-tip correlations to test for a temporal signal in the data was performed using TempEST (*9*), and a significant positive slope for the alignment was present (p<0.001, R = 0.527). BEAST v2.4.7 (*10*) was used to estimate a timed phylogeny with concatenated recombination-free core SNP alignment. The GTR substitution model was selected based on evaluation of substitution models in bModelTest (*11*). BEAST analyses were performed using a coalescent constant population model and a coalescent Bayesian skyline model. In addition, a strict clock, with a lognormal prior, and a relaxed clock (both lognormal and exponential) were tested. Markov chain Monte Carlo (MCMC) was run for 100 million iterations sampling every 10 thousand steps and convergence was checked by inspecting the effective sample sizes and parameter value traces in the software Tracer v1.6.0. The model fit across analyses was compared using Tracer v1.6 (*12*). A relax exponential clock with the Bayesian skyline was selected as the most appropriate model.

PacBio Whole-Genome Sequencing of I72

I72 was identified as *K. pneumoniae* subsp. *pneumoniae* ST307and the chromosomal length was 5.430 Mb. It harbored 3 plasmids, namely p72_FIBkpn containing *bla*$_{CTX-M-15}$, p72_L/M_OXA48 containing *bla*$_{OXA-48,}$ and p72_X3_OXA181 containing *bla*$_{OXA-181}$. p72_X3_OXA181 was 51kb in size, harbored the IncX3 and truncated ColKp3 replicons with *bla*$_{OXA-181}$ and *qnrS1* (Appendix Figure 2). The p72_FIBkpn plasmid was 193 kb in size, contained FIIK and FIBK replicons and harbored an array of antimicrobial resistance genes, namely *bla*$_{CTX-M-15}$, *bla*$_{OXA-1}$, *bla*$_{TEM-1}$, *aac(6')-Ib-cr, aph(3'')-Ib, aph (6)-Id*, *qnrB1*, *catB4*, *strAB*, *tetA*, *sul2*, and *dfrA14*. p72_L/M_OXA48 was 64kb in size and contained only 1 antimicrobial resistance gene namely *bla*$_{OXA-48}$ (Appendix Figure 2).

Identification of the *bla*$_{OXA181}$ Harboring IncX3 Plasmids

The genome sequences were de novo assembled using plasmidSPAdes (*13*), followed by extraction of the *bla*$_{OXA181}$-harboring contigs, using blastdbcmd. The *bla*$_{OXA-181}$ contigs were "BLAST" against PlasmidFinder (*8*) database. The results showed that *bla*$_{OXA-181}$ co-existed on the same contig as a truncated ColKp3 replicon gene, similar to that of p72_OXA181_X3.The identification of p72_OXA181_X3 were investigated using Bandage assembly graph viewer (*14*)

and BLASTn. The plasmid region was identified by BLASTn searching for the p72_OXA181_X3 "core" genes, excluding insertion or repeated sequences.

Enterobacteriaceae with Carbapenemases other than *K. pneumoniae* from 2014–16

Eighteen *Enterobacter cloacae* complex and 7 *E. coli* with carbapenemases were isolated at Ampath laboratories during 2014–2016. The majority of *E. cloacae* complex was positive for NDMs (n = 15) followed by OXA-48-like (n = 2) and KPC (n = 1); 2 of the *E. coli* was positive for NDM and 2 for OXA-48-like. WGS plasmid mining, S1-PFGE and Southern blotting showed that p72_X3_OXA181 was present among OXA-48-like *Enterobacter cloacae* complex and *E. coli*.

Infection Prevention and Control Measures

Routine auditing of hospital-acquired infections is part of continuous surveillance at the private hospitals. All carbapenem resistant microorganisms (CRE) detected at Ampath and satellite laboratories were communicated to the treating physician and to the infection prevention control (IP&C) practitioners on a daily basis. When an increase of CRE was noted, the affected hospital implemented CDC guidelines for CRE infection prevention and control (*15*). Outbreak response teams were identified within each hospital management during information sharing meetings with management and heads of departments. Infection prevention practitioners held weekly information sessions with all the wards and increased hand hygiene practices. Contact precautions training and education on donning and removal of gloves and aprons were monitored. Environmental cleaning was systematically actively pursued.

All patients within the same wards with newly laboratory confirmed CREs were screened using rectal swabs inoculated on chromID Carba (bioMérieux). All high-risk patients were screened on admission and cohorted immediately if identified. Active surveillance cultures consisted of stool, rectal swabs, ostomy output, wound swabs and endotracheal suctions as requested by the IPC team. Isolation wards were identified at each hospital and patients were cohorted. Strict access control to wards for staff and visitors was implemented. All patients were bathed with 2% chlorohexidine daily and when transferred in or out of the facility.

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Appendix Table 1. PCR primers and amplification conditions

Primers	Sequence	Target	Amplicon size, bp	Sensitivity	Specificity
307-F2	3′-AGGAAAGTCGCCGCAGGAGGAT-5′	ST307*	628	97%	94%
307-R2	3′-TGCTGCTGCCATAAAACGCACCT-5′				
X3-F1	3′-TGCAGCTCTGAAAGAGGGAGCTAA-5′	IncX3	556	99%	96%
X3-R1	3′-TCTAAGCATGTGAGCCATTCTCCCT-5′				
IS3000-F	3′-TTTACCCGTCAGCGAAAAGT-5′	IS3000-OXA	724	98%	95%
OXA-R	3′-TTTCTTGCCATTCCTTTGCT-5′				

*target; 72_phage 2.

Appendix Table 2. Carbapenemase-producing *Klebsiella pneumoniae* isolates identified in South Africa, January 2014–December 2016

| Strain	Province	Jan–Jun 2014	Jul–Dec 2014	Jan–Jun 2015	Jul–Dec 2015	Jan–Jun 2016	Jul–Dec 2016
KPC (n = 10)		1	0	3	1	5	0
Alberton	Gauteng						
Pretoria	Gauteng	1		1			
East London	East-Cape			2			
Johannesburg	Gauteng				1	3	
Roodepoort	Gauteng					1	
Krugersdorp	Gauteng					1	
NDM (n = 58)		7	19	8	11	11	2
Pretoria	Gauteng		6	2	4	2	1
Johannesburg	Gauteng	6	10	5	4	5	
Alberton	Gauteng				2		
Richards Bay	KwaZulu	1					
Bloemfontein	Free State		3	1	1	2	1
Trichardt	Mpumalanga					1	
Mbombela	Mpumalanga					1	
VIM (n = 35)		7	12	6	8	1	1
Emalahleni	Mpumalanga	1	2		1		
Pretoria	Gauteng	2	6	2	5		1
Johannesburg	Gauteng	4	3	3	2	1	
Bloemfontein	Free State		1				
Springs	Gauteng			1			
OXA-48–like (n = 471)		21	33	41	162	187	27
Johannesburg	Gauteng	15	18	16	54	63	7
Cape Town	Cape		1		7	2	
Alberton	Gauteng	2	8	3	11	7	1
Heidelberg	Gauteng	1				2	1
Krugersdorp	Gauteng	1			2	1	
Pretoria	Gauteng	1	3	16	63	69	14
Somerset	Cape	1					
Springs	Gauteng		2	1	2	3	
Tzaneen	Limpopo		1				
Benoni	Gauteng			4	13	9	1
Middelburg	Mpumalanga			1	2	1	
Brakpan	Gauteng				1	2	
Centurion	Gauteng				1	2	
East London	East Cape				1	1	
Kempton	Gauteng				1	2	
Mbombela	Mpumalanga				1	4	
Polokwane	Limpopo				1	3	
Trichardt	Mpumalanga				1	1	
VDBijlPark	Gauteng				1		
Bloemfontein	Free State					3	
Boksburg	Gauteng					4	
Ermelo	Mpumalanga					1	
Klerksdorp	North West					5	1
Secunda	Mpumalanga					2	1
Emalahleni	Mpumalanga					3	1

Carbapenem non-susceptible *K. pneumoniae*
Ampath-MDRC
n = 1,247

↓

PCR confirmation for carbapenemases
n = 574

↓

PFGE on OXA-48-like isolates
n = 471

↓

Illumina WGS on dominant pulsotype
n = 28

↓

PacBio WGS
n = 28

↓

PCR screening for ST307, IncX3, IS3000
n = 471

↓

Additional Illumina WGS
n = 60
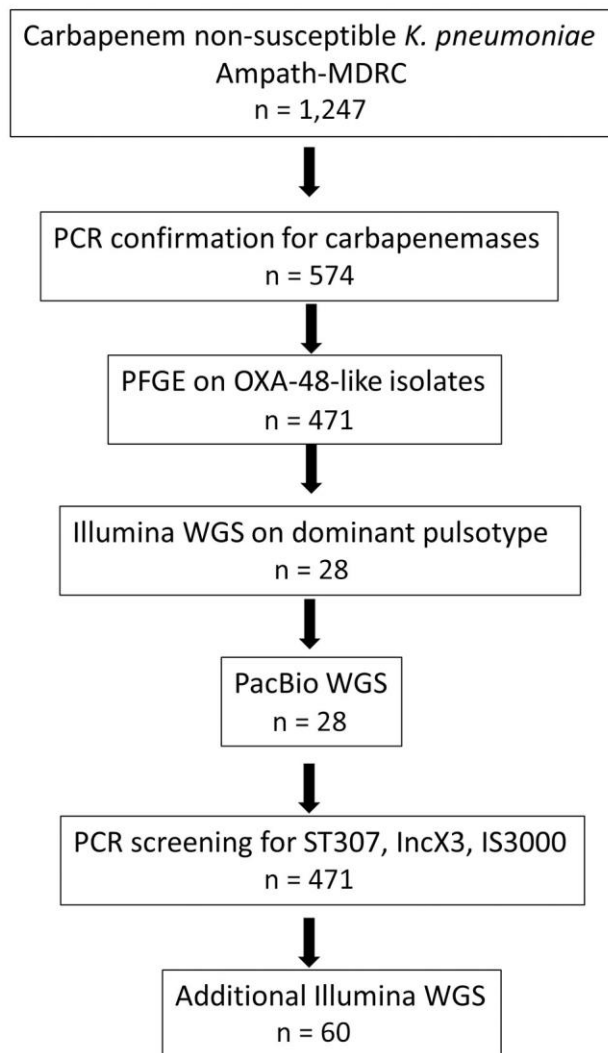
Appendix Figure 1. Workflow for study of *Klebsiella pneumoniae* ST307 with *bla*$_{OXA-181,}$ South Africa, 2014–2016.
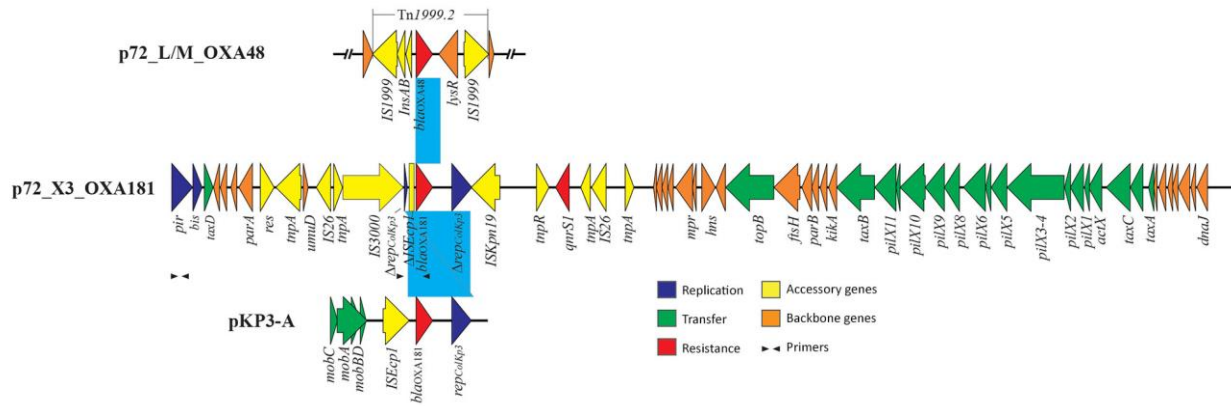
Appendix Figure 2. Sequence homology of p72_X3_OXA181 containing *bla*_{OXA-181} from study of *Klebsiella pneumoniae* ST307, South Africa, 2014–2016.