The dissemination of multidrug resistant *Enterobacter cloacae* throughout the UK and Ireland

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Letter

Enterobacter cloacae is a clinically important gram-negative member of the Enterobacteriaceae*, which has increasingly been recognised as a major pathogen in nosocomial infections. Despite this, knowledge about the population structure and the distribution of virulence factors and antibiotic resistance determinants of this species is scarce. In this study, we analysed a systematic collection of multidrug resistant *E. cloacae* isolated between 2001 and 2011 from bloodstream infections across hospitals in the UK and Ireland. We found that the population is characterized by the presence of multiple clones formed at widely different time periods in the past. The clones exhibit a high degree of geographical heterogeneity, which indicates extensive dissemination of these *E. cloacae* clones across the UK and Ireland. These findings suggest that a diverse, community-based, commensal population underlies multidrug resistant *E. cloacae* infections within hospitals.

Enterobacter cloacae is a non-motile bacterium that belongs to the Enterobacteriaceae family. *E. cloacae* have been recovered from natural environments [1-3]. In addition, *E. cloacae* is an opportunistic pathogen that can cause a wide range of infections, in particular in immune-compromised individuals. *E. cloacae* has been increasingly recognized as a cause of hospital-acquired infections and outbreaks in the past few decades [4-6], as reported in hospitals [7,8], especially in the neonatal settings [9]. The reservoirs for *E. cloacae* in hospitals are thought to include equipment, cleaning solutions and healthcare workers [9-11]. Furthermore, endogenous infection from an established clone carried by individuals prior to admission to hospital has also been described. Multi-locus sequencing typing (MLST) has shown that the global *E. cloacae* population is highly diverse, and includes a limited number of clonal complexes that have spread internationally and independently acquired antibiotic resistance elements [12,13].

A number of antibiotic resistance genes, and to a lesser extent pathogenicity mechanisms, of *E. cloacae* have been elucidated to date [5,14-17]. Multiple virulence factors and extended spectrum beta-lactamases (ESBLs) or the hyper-production of constitutive AmpC beta-lactamase have been shown to be involved in the development of infection and antimicrobial resistance, respectively [6]. Furthermore previous genomic studies have shown that the *E. cloacae* genome harbours a large repertoire of accessory genetic elements that enable colonization of diverse environments and efficient metabolic adaptation to different niches and environmental stresses [6,18-20].

There is a growing recognition that *E. cloacae* is now the third major Enterobacteriaceae species involved in nosocomial infections after *Escherichia coli* and *K. pneumoniae.* To understand the spread of multidrug-resistant pathogens within the population it is necessary to determine their epidemiology and population structure. However, unlike other important pathogens, our knowledge about the population genomics of *E. cloacae* is limited. To fill this gap, we utilized whole genome sequencing of a large systematic collection of multidrug resistant (MDR) *E. cloacae* collected from various hospitals across the UK between 2001 and 2011.

Our collection contained 316 genomes composed of MDR *E. cloacae* collected from 37 hospitals in the UK and Ireland between 2001 and 2011. We first calculated the set of core genes (genes shared by every member of the population) and accessory genes (genes that were variably present between isolates). *De-novo* assembly and annotation of core and accessory genomes showed that the pan-genome of *E. cloacae* contained 44,235 genes, 1,567 and 490 of which were in the hard core (genes present in >99% of samples) and the soft core genome (genes present in >95% and <99% of samples), respectively. This large number of accessory genes indicates a very high degree of flexibility of the *E. cloacae* genome, which reflects the diverse environmental habitats that this bacterium occupies. The core genome was ~1.5 Mbp. We found a high degree of congruence between the pattern of the presence and absence of accessory genes and the genetic distance between the core genomes in our dataset (p < 0.001, Mantel test) suggesting that the MDR clones of which the data set comprises were each formed with a distinct subset of genes which have undergone little change since the clone emerged (Supplementary Figure 1 A).

The distribution of pairwise SNP distances between isolates showed that the population of MDR isolates was highly structured and composed of closely-related clades together with strains that were far more distantly related to other isolates in the collection (Figure 1A and Figure 1B). In line with this, the phylogenetic tree constructed from the core genome variant sites showed that the population consisted of one large primary group with a number of closely related sub-populations (clones) nested within it, and a divergent clade that mainly contained individual isolates that, according to *rpoB* and *hsp60* typing [21], represent separate sub-species in the *E. cloacae* complex including *Enterobacter asburiae* and *Enterobacter kobei* (Supplementary Figure 1 B and 1 C in Supplementary Material). We observed a low degree of geographic clustering in the tree, and the high level of diversity of the species was

also reflected in the number of different MLST sequence types (STs) that made up the population, some of which are the most widespread STs in a global collection of *E. cloacae* [13]. This suggests that isolates in our collection are part of a global circulation of these clones (Supplementary Figure 2 in Supplementary Material).

In order to investigate the recent evolution of *E. cloacae* in greater detail, we analysed the major clones in our collection. Utilizing the clustering method detailed in the methods section, we identified 11 distinct groups that contained more than 9 isolates (Figure 2A). We found that isolates in these groups could be adequately identified by MLST since most clusters contained a single sequence type (Figure 2B, Supplementary Notes 2 in Supplementary Material). As with the whole tree, the clones exhibited a high level of geographical diversity in that the majority of the clusters contain isolates originating from multiple hospitals (Figure 2C). Furthermore, and in line with this, the distribution of the pairwise geographical distances between isolates within each clone were as widespread as those within the whole collection for the majority of clones, and only for clones 14, 2 and 13 was the geographical clustering significant ($p < 0.01$, one-sided t-test < 0.01) (Figure 2D). This implies that, prior to the time of the study, the dissemination of all the different *E. cloacae* MDR clones appeared to have already taken place across the country.

Utilizing the variation within the time of isolation within each cluster (Figure 2E), we estimated the substitution rate for the genome for those clusters where there was a detectable signal to be between 0.5 and 3 SNPs per genome per year (an average of 1.5×10^{-7} SNPs per site per year across the genome) (Supplementary Figures 3 A and S 4 in Supplementary Material). These calculations indicate that these clusters were established between ~10 and ~150 years ago (Supplementary Figure 3 B). Using the average substitution rate, and applying it to the other major clusters for which we could not detect a temporal signal, we calculated the root age for these clusters to be between 50 and 250 years ago (Supplementary Figure 3 B and 4 in Supplementary Material). We conclude that drug resistant *E. cloacae* clones have been continuously arising and expanding over the past several decades. Subsequently, we used these substitution rates to estimate the dates and numbers of putative recent relationships of isolates in different hospitals. To this end we extracted all the pairs of isolates that were less than 40 SNPs apart (which corresponds to ~20 years). We found that these isolates originated from different hospitals across the country, and when we compared the geographical distances between these linked hospitals

with the distribution of pairwise geographical distance for the whole collection we found no significant difference (p > 0.05, one-sided t-test) (Supplementary Figure 3 C in Supplementary Material). The results demonstrate that inter-hospital connectivity between genetically similar isolates exists, but that this is not necessarily between geographically close hospitals (Supplementary Figure 3 C in Supplementary Material). To further examine this finding, we reconstructed the networks for all SNP cutoffs since the most recent clone (clone 7) emerged in the population. Our results show that although the proportion of inter-hospital relationships decreased with stricter cut-off values, the number of isolates in the network also declined rapidly (Supplementary Figure 5 in Supplementary Material). The findings show that even with 316 samples the population is still so diverse that only a very few recent close relationships can be detected.

Within the population, we were able to identify the differential presence of multiple plasmids and putative virulence factors, some of which were clade specific, implying that the population of *E. cloacae* has developed both conserved and variable virulence mechanisms (Supplementary Notes 4 and 5 in Supplementary Material). Furthermore our antimicrobial resistance analysis demonstrated various patterns of emergence of antimicrobial resistance determinants in the collection, where resistance determinants were acquired independently throughout the population (Supplementary Notes S6 in Supplementary Material). This implies a potential for rapid adaptability in *E. cloacae*. The observations of the antimicrobial resistance analysis are particularly important since the emergence of antibiotic resistance for the currently effective antibiotics such as imipenem, tigecycline and ciprofloxacin raises concerns that the resistance profile may extend in the future, in particular through mobile genetic elements. Moreover, the commensal nature of the bacterial population documented here will allow rapid spread of resistant *E. cloacae* throughout the human population, outside of hospital-based infection control, from which it can readily be imported into hospitals.

The dissemination of *E. cloacae* raises questions about the underlying reservoirs and routes of the spread of pathogens between hospitals. A diverse range of sources, including healthcare staff and equipment, waste waster and food have been shown to serve as reservoirs of *E. cloacae* [2,3,22]. Despite being the largest genomic collection of *E. cloacae* studied to date, our collection only contained clinical isolates so we are not able to draw a definitive conclusion about the sources and potential reservoirs of *E. cloacae*. However the

relationship between our isolates and previously published *E. cloacae* genomes indicates that clinical and non-clinical strains originating from the USA, India and China are scattered throughout our phylogeny, both within and outside the clones identified here (Supplementary Figure 17). Clinical isolates recovered from a systematic screen of infections in a single hospital in the USA [23] were also spread across the tree, although mainly in the rarer sub-species. An environmental isolate recovered from oil-contaminated soil in India [24], a food borne isolate from pepper [19] in Hong Kong and an isolate of plant infection origin [20] were also mainly in the rarer sub-species. Together, this suggests that the broad diversity of *E. cloacae* causing infections in the UK may be part of a global population transmitting via different routes involving both clinical and non-clinical sources. To fully elucidate this, a more comprehensive collection, containing isolates of different non-clinical and clinical origins is required, which would serve as the basis for future genomic studies on *E. cloacae*.

Enterobacter infections can be acquired within a hospital or originate from a pathogen within an already colonized patient. Although there are reports tracing infections to the contamination of hospital devices, most of the infections reported previously do not appear to have a common nosocomial source and may be accounted for by previously established clones within patients [25,26]. Our findings are consistent with diverse sources of *E. cloacae* in any individual hospital. The close genetic relationship between isolates from different geographic regions that was frequently observed suggest that individuals that have already been colonised before entering hospital introduce multiple strains into hospitals, some of which may be involved in subsequent intra-hospital transmissions. Isolates from hospital infections therefore represent samplings from the diversity of commensal *E. cloacae* in the community. In this sense the apparent spread of *E. cloacae* between the hospitals found here may only mirror the dissemination of this species throughout the UK and Ireland. The presence of some isolates from the known major globally circulating STs, as well as the close genetic distance between isolates of different source and country of origins, indicate that the geographical distribution of *E. cloacae* lineages goes beyond UK and Ireland and belongs to a global population [7]. From this, it is apparent that controlling *E. cloacae* infections may be more difficult than other nosocomial pathogens, where within-hospital transmission is often the primary source of new infections.

Acknowledgements

Contributions

S.J.P., D.M. and J.P. designed the study. D.M. analysed the data. S.R. performed the *in silico rpoB* and *hsp60* typing. V.M., S.J.P. and J.P. contributed materials and data. S.J.P and J.P. completed ethical approvals. J.P. and S.J.P. were responsible for management of the study.

METHODS

Isolates and antimicrobial susceptibility testing

The study was approved by the National Research Ethics Service (ref: 12/EE/0439) and the CUH Research and Development (R&D) Department. We studied a national collection of 316 *E. cloacae* isolates that were collected as part of the British Society for Antimicrobial Chemotherapy (BSAC) Resistance Surveillance project (www.bsacsurv.org). The collection was part of a bacteraemia surveillance programme (all isolates were from blood of patients with blood infection) that ran between 2001-2011 in 37 hospitals across the United Kingdom and Ireland (UK&I). The strains were identified to species level using API20E strips and the species were further confirmed using Kraken [27]. Selection for the collection was based on the presence of phenotypic resistance to at least 4 antimicrobial groups. We provide a list of isolates in Supplementary Table 1.

Antimicrobials that were tested in this study were beta-lactams (penicillin: amoxicillin, cephalosporins: cefuroxime, cefoxitin, cefotaxime, ceftazidime, and piperacillin-tazobactam and amoxicilin-clavulanic acid), tetracyclines (minocycline, tetracycline and tigecycline), aminoglycosides (gentamicin) and fluoroquinolones (ciprofloxacin). We defined Multidrug

resistance (MDR) as acquired non-susceptibility to at least one agent in three or more antimicrobial categories, as it is proposed in [28]. We then selected Isolates based on the presence of phenotypic resistance to at least one antimicrobial in three of the antimicrobial groups: penicillins, carbapenems, cephalospsorins, tetracyclines, aminoglycoside and fluoroquinolones.

The minimum inhibitory concentration (MIC) for each antimicrobial agent was determined using the BSAC agar dilution method. To obtain an insight into the distribution of MIC values, we compared the distribution of MIC values of our samples with those from the European Committee on Antimicrobial Susceptibility Testing (EUCAST). We obtained these distributions and also the clinical susceptibility breakpoints to determine resistance from the EUCAST website (www.eucast.org) on 02/02/2016.

Sequencing, pan-genome and phylogenetic analysis

DNA was extracted using a QIAxtractor (QIAgen), according to the manufacturer's instructions. Sequencing libraries were constructed as described by the manufacturer (http://www.illumina.com/techniques/sequencing/ngs-library-prep). The sequencing was conducted on an Illumina HiSeq2000 with a read length of 2X100 bp. 96 samples were multiplexed per lane to give an average depth of coverage of 71-fold. The assembled data and raw sequences were deposited in the ENA under the accession numbers provided in Supplementary Table 1. We assembled paired end sequence reads using an in-house assembly and improvement pipeline [29], based on Velvet [30]. Subsequently we annotated the assemblies with Prokka, which takes fragmented *de novo* assemblies [31]. We then used the annotated assemblies produced by Prokka as input to Roary, a tool that rapidly builds large-scale pan-genomes, to construct the core and accessory genome [32] (One isolate was excluded from pan-genome analysis, as it fell outside Roary's similarity cut-offs). To estimate a Maximum Likelihood tree, we employed FastTree version 2.1.3 with generalized time-reversible model to reconstruct the phylogenetic tree from the core genome alignment [33]. We assessed the confidence values through SH-like support. To visualize trees and associated metadata, we used FigTree (http://tree.bio.ed.ac.uk/software/*figtree/*), *Dendroscope 3* [34] and in-house tools.

Divergence analysis and cluster analysis

In addition to phylogenetic analysis we reconstructed the network of relatedness between the isolates using the SeqTrack algorithm function in R package adegenet [35,36]. The algorithm reconstructs ancestries between the sequences, by considering the genetic distances and isolation dates, to obtain a tree with maximum parsimony. In Supplementary Figure 3, we included the network in which the edges that correspond to >40 SNPs, i.e. the number of variant sites in the core genome alignment, were excluded. Given the estimated substitution rate of *E. cloacae*, 40 SNPs is equivalent to SNPs accumulated over approximately ~20 years (see main text). To identify the clusters in the phylogenetic tree, we used the clustering algorithm detailed in the adegenet package. The distance-based clustering algorithm takes the pairwise SNP distance matrix and a SNP cut-off to identify the clones. We tested various SNP cut-off values from 0, producing 315 clusters, to very large values, which collapses the entire sample set into one cluster. To find the best cut-off for clustering we first excluded 14 outliers, i.e. the isolates that were very distantly related to other isolates in the collection, and then varied the SNP cutoffs while measuring the number of clusters identified. The longest plateau where the clustering did not change extended for 3356 SNPs, and this yielded 88 clusters We then used the 11 clusters with more than 9 members, including 194 isolates in total, for downstream analysis.

To assess the output of the clustering method, we compared the result with the output of HierBAPS, which performs Bayesian analysis of population structure to cluster similar genomic sequences, using 2 clustering iterations and 10, 30 and 50 expected number of clusters (k) as input parameters. Our clusters are strongly in agreement with the clusters of the second iteration of BAPS clustering (Supplementary Figure 18).

Phylogenetic analysis and substitution rate calculation

After identifying major clusters on the tree we attempted to estimate the recent nucleotide substitution rate. For the nucleotide substitution rate estimation, we first employed Gubbins [37] to remove high SNP density regions, which signified putative recombination events, from the multiple alignment for each cluster. We then reconstructed the tree for each cluster and plotted root-to-tip distance versus year of isolation for each sample. Subsequently we conducted 10,000 bootstraps with re-sampled years to obtain a distribution for R-squared values. We then compared the real R-squared values with the distribution to check the significance of the temporal signal in each cluster. We found a strong temporal signal for

cluster 8 and weaker signals for clusters 7, 18 and 31. Subsequently we used the BEAST v1.7 package to obtain the substitution rate and the age of the root node for the clusters with a signal [38].

We tested various models including a strict molecular clock (uniform prior distribution for the clock rate model) and a log-normal model with constant population model. For each parameter set we performed 100 million generations with sampling every 10 generations for three times. Besides checking that different runs had converged on similar values, we also assessed the convergence for each model by ensuring that ESS values were greater than 200 for key parameters. A burn-in of 10 million states was discarded from each of the three runs. Since the log-normal clock model always converged, we included the results of this model in the manuscript. We took the age of the root as the height of the root of the Maximum Clade Credibility (MCC) tree reconstructed by combining trees using tree annotator program from the BEAST package.

Molecular Typing of E. cloacae complex, in silico MLST analysis and identification of antimicrobial resistance determinants, virulence factors and plasmids

Using the srst2 package [39], we mapped the paired-end reads to resistance, and plasmid replicon genes in the databases detailed in the srst2 package (coverage cut-off 90%). We also used srst2 to find virulence genes in the Virulence Factors Database (VFDB) (http://www.mgc.ac.cn/VFs/main.htm) in the isolates. We then visualized the genes across the phylogenetic tree using an in-house tool. We determined the STs of the samples using an *in silico* MLST in-house script that uses the assemblies as input. Furthermore, we conducted the *rpoB* and *hsp60* (*groL*) typing of *E. cloacae complex* to detect sub-species of *E. cloacae* within our dataset [21]. To this end, gene sequences for *rpoB* and *hsp60* (*groL*) were extracted from the assembled genomes. These were then trimmed and compared to the reference set [21]. After phylogenetic estimation with RAxML using a general time reversible (GTR) evolutionary model and a gamma correction for among site rate variation [40], isolates were allocated to the different clusters based on the reference dataset. This grouping was then compared back to the whole-genome phylogeny and clustering.

Regression analysis to identify antimicrobial resistance determinants

We developed three linear regression models to identify genetic determinants that are strongly associated with resistance. To this end we used MIC values as a continuous dependent variable in the linear regression models, instead of the categorical resistant/susceptible status. This is because MIC values enabled us to identify the effect of different genetic elements even when all samples are classed as resistant. Moreover, the MIC values have a higher variance than the categorical resistance status and therefore provide more information and allow greater statistical confidence in the results.

In the first linear regression model, we took the MIC values for different antimicrobials as a continuous dependent variable and the presence/absence of individual accessory genes as a categorical predictor variable. By doing so we aimed to identify genes in the accessory genome that are strongly associated (p-value<10^{-5}) with resistance level.

We performed two types of SNP based regression analysis. We first identified genes in which the number of non-synonymous SNPs strongly correlates with resistance levels and identified individual SNPs, the presence of which are strongly associated with MIC levels. To this end, we mapped the short reads against the reference genome *E. cloacae* ATCC 13047 (accession number: CP001918) using SMALT v0.7.4 (https://www.sanger.ac.uk/resources/software/smalt/). The annotation of SNPs was done with an in-house script that combines SAMtools mplieup [41] and BCFtools as detailed in [42] (7 isolates were excluded from the SNP analysis). The SNP association regression model identified known/putative resistance SNPs that were strongly correlated with MIC values (p-value<10^{-5}) primarily for ciprofloxacin. Samples in which SNPs were absent or unknown were excluded from the analysis for each site. We also excluded the sites in which more than 10% of nucleotides across the population were unknown. In the linear regression model we considered MIC values as continuous dependent variable and the presence/absence of individual SNPs as a categorical predictor variable. In our second SNP-based analysis, we aimed to find genes with a strong correlation between their non-synonymous variation pattern and MIC. To this end we developed a linear regression model in which the MIC values for different antimicrobials were considered as the continuous dependent variable and the number of non-synonymous SNPs in individual genes was used as the continuous predictor variable. In all of the above regression models, we accounted for the population structure by including the MLST information as the categorical predictor variable into the

regression models. We set a stringent p-value of 10^{-5} to define strong associations and then screened the hit list of strongly associated genes/SNPs for putative resistance genes/SNPs.

We have provided the output files of pan-genome analysis, the input and R code for the three regression models in a public repository (https://data.mendeley.com/datasets/vg6cnk9bc5/1). We have also included the hit lists for the three regression models as Supplementary Tables 2, 3 and 4.
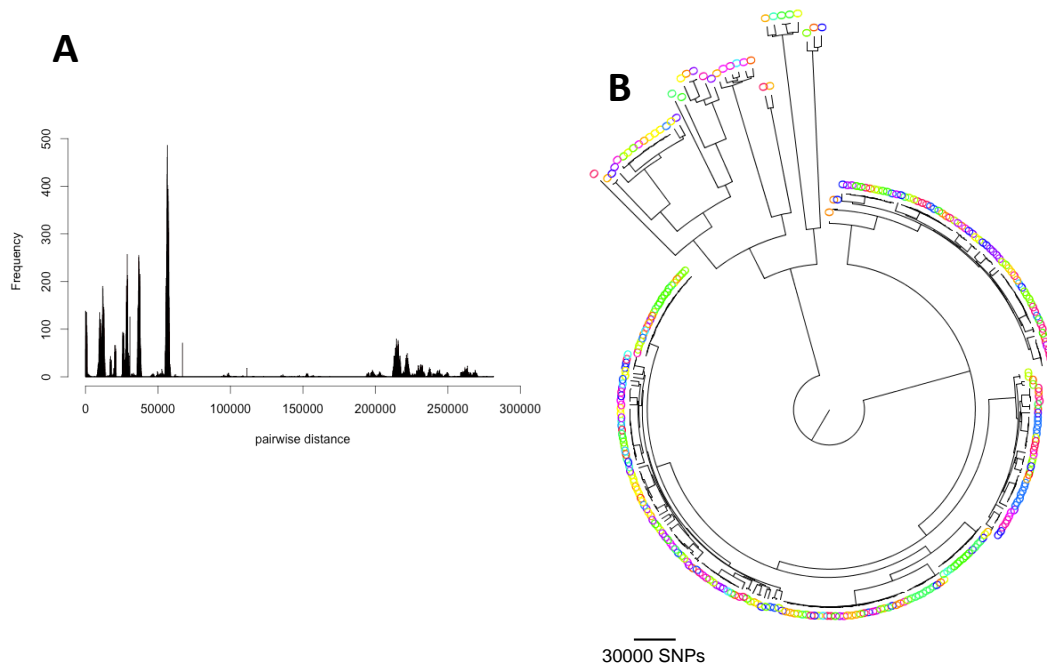
Figures

Figure 1 A) Histogram of pair-wise SNP distance between the core genome sequences of isolates. **B)** Maximum Likelihood tree for the isolates. The colours of the isolates correspond to hospitals of isolation.
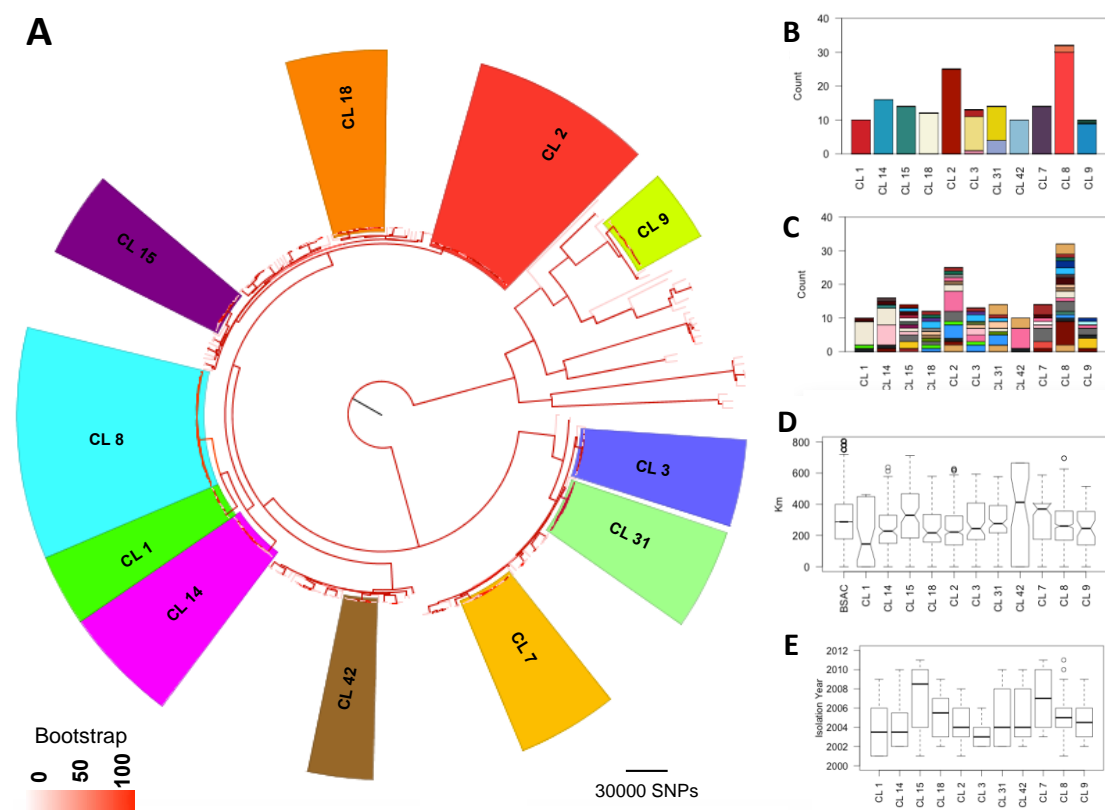
Figure 2 A) Detected clusters (CLs) represented on the phylogenetic tree. The intensity of branch colour corresponds to the bootstrap support (SH support). B) MLST sequence type (ST) composition of each of the clusters. Each colour signifies one ST. C) Hospitals from which isolates in each cluster were identified. D) Distribution of pairwise geographical distance for each cluster compared with the distribution of the whole population. The notched area shows the 95% confidence interval around the median. E) Distribution of the time of isolation for isolates in each cluster. The boxes in D) and E) give the interquartile range, the whiskers indicate the boundary of 1.5 times the interquartile range, and the points beyond that are outliers.

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