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Short Communication

Emerging challenges of whole-genome-sequencing–powered epidemiological surveillance of globally distributed clonal groups of bacterial infections, giving *Acinetobacter baumannii* ST195 as an example

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ABSTRACT

Whole-genome sequencing (WGS) has revolutionized the genotyping of bacterial pathogens and is expected to become the new gold standard for tracing the transmissions of bacterial infectious diseases for public health purposes. However, it is still unexpectedly demanding to employ WGS for global epidemiological surveillance because of the high degree of similarity between the genomes of intercontinental isolates. The aim of this study was to utilize genomically derived bioinformatics analysis to identify globally distributed *A. baumannii* ST195 lineage and differentiation outbreaks to address this issue. The genomic sequences and their related epidemiological metadata of 2850 *A. baumannii* isolates were recruited from NCBI Genbank database. Assignment into sequence type (Oxford scheme) and lineage (global clone 2/CC92) were performed. A total of 91 ST195 *A. baumannii* isolates were subsequently classified to perform the bacterial source tracking analysis by implementing both core genome MLST (cgMLST) and core genome SNP (cgSNP) strategy that were integrated in our recently updated BacWGSTdb 2.0 server. Antibiotic resistance genes were identified using the ResFinder database. The ST195 *A. baumannii* isolates distributed widely in eight countries and harboured multiple antimicrobial resistance genes simultaneously. In most cases, the bacterial isolates recovered from geographically distant sources may present less genomic sequence similarity, i.e., the phylogenetic relationship between these ST195 isolates worldwide was roughly congruent with their country of isolation. However, a few isolates collected from distant geographic regions were revealed to possess smaller genetic distances (less than 8 loci or 20 SNPs) than the threshold without an observable epidemiological link. Our study highlights the emerging challenges entailed in the WGS-powered epidemiological surveillance of globally distributed clonal groups. Standardization is urgently required before WGS can be routinely applied to infectious diseases outbreak investigations.

1. Introduction

Acinetobacter baumannii has emerged worldwide as a predominant opportunistic pathogen responsible for nosocomial infections (Wong et al., 2017). With a strong capacity for clonal transmission and acquirement of antimicrobial resistance determinants, in 2017 the World Health Organization (WHO) announced carbapenem-resistant *A. baumannii* as the top priority pathogen critically requiring research and development of new antibiotics (WHO, 2017). The *A. baumannii* population primarily responsible for nosocomial infections and multiple-drug resistance largely consists of two globally distributed clones, GC1 and GC2 (Zarrilli et al., 2013). The accurate knowledge of an epidemic *A. baumannii* clone commonly relies on molecular epidemiological analysis, such as unrevealing the genetic relatedness of bacterial isolates and their antimicrobial resistance genes, which boosting our insights into the global transmissions and developing optimized *A. baumannii* infection control strategies when associated with a region-specific outbreak (Karah et al., 2012; Maragakis and Perl, 2008; Ruan et al., 2013).

Several molecular typing methods have been established during the last decades, particularly pulsed-field gel electrophoresis (PFGE) and multilocus sequence typing (MLST), which are the gold standard and complementary extend to each other in global epidemiological investigations of bacterial pathogens (Maiden et al., 1998; Tenover et al.,

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1995). However, they lack sufficient resolution for providing strain-specific diagnostics and thus cannot meet contemporary requirements (Maiden et al., 2013). Take *A. baumannii* GC2, the most important clone made up mainly of multiple-drug resistant isolates, as an example: its predominant lineage ST195 disseminates worldwide, rendering MLST unable to delineate on a global scale more delicate transmission routes (Endo et al., 2012; Kao et al., 2014; Zhou et al., 2015). Recently, many successful cases have demonstrated the power of whole-genome sequencing (WGS) in the epidemiological surveillance of infectious disease outbreaks at various geographic scales and in different temporal scenarios (Gardy and Loman, 2018). Because of its single-base resolution, WGS is expected to become the new authoritative norm for genotyping bacterial pathogens for public health purposes (Croucher and Didelot, 2015; Loman and Pallen, 2015).

The WGS-based approaches rely on either the characterization of core-genome single nucleotide polymorphisms (cgSNP) or core-genome gene-by-gene multilocus sequence typing (cgMLST) approach encompassing a stable set of core genome genes (Mellmann et al., 2016). The cgMLST approach allows immediate comparisons of newly determined genotypes with historical data, enabling continuous surveillance, in contrast to SNP-based approaches that call for recalculation once the data set changes unless a preliminarily defined reference genome is given (Ruan et al., 2019). More importantly, cgMLST can hardly be affected by such genetic events as homologous recombination and the lateral transfer of mobile genetic elements (MGEs), which can result in high density SNPs within a short segment and, thereby, distort the true phylogenetic relationships (Awadalla, 2003). The whole-genome comparisons yielded insights into the evolution of pathogenicity in *A. baumannii*, uncovering wide diversity in gene content, including variation in antimicrobial resistance determinants (Fitzpatrick et al., 2016; Sahl et al., 2015; Wright et al., 2014). However, genomic studies of intra-clonal variation in *A. baumannii* have been limited to small or highly localized isolates.

To increase our understanding of the genomic epidemiology of *A. baumannii*, a comparative genomic analysis of 91 *A. baumannii* ST195 clinical isolates recovered from eight countries was performed to determine the phylogenetic relationship between a large collections of publicly available global *A. baumannii* isolates. A few of the isolates collected from very distant geographic regions were revealed to possess smaller genetic distances but without an observable epidemiological link. Our study highlights the emerging challenges entailed in the WGS-powered epidemiological surveillance of globally distributed clonal groups.

2. Material and methods

2.1. Bacterial isolates

A total of 2850 *A. baumannii* genomes currently available from NCBI GenBank non-redundant database and whole-genome shotgun sequence databases (including both complete and draft genomes) were obtained. The relevant clinical metadata were also retrieved from NCBI BioSample database. The sequence type (ST) of the study isolates was determined by Oxford MLST scheme using the whole genome assemblies and a total of 91 ST195 *A. baumannii* isolates were classified to share this feature. The detailed epidemiological characteristics of these isolates implemented in the comparative genomic analysis were described in **Table S1**. The *A. baumannii* ST195 isolates were selected in this study to represent diverse geographical locations, i.e. China (n = 59), Saudi Arabia (n = 13), Malaysia (n = 5), Morocco (n = 5), Lebanon (n = 4), India (n = 2), Thailand (n = 2) and the USA (n = 1) (Fig. 1).

2.2. Genomic epidemiology analysis

The genome annotation was performed using NCBI Prokaryotic Genome Annotation Pipeline (PGAP) (Tatusova et al., 2016). Antimicrobial resistance genes were identified using the ABRicate v0.8.7 screening tool with the ResFinder database at the Center for Genomic Epidemiology (http://www.genomicepidemiology.org/) (Zankari et al., 2012). The bacterial source tracking for implementing both cgSNP and cgMLST strategies were performed by our recently updated BacWGSTdb 2.0 server (Ruan and Feng, 2016). A publicly available cgMLST scheme for *A. baumannii* (2390 targets genes) was used to characterize the gene-by-gene allelic profile of *A. baumannii* ST195 isolates (Higgins et al., 2017). An *A. baumannii* ST195 strain, AC30 (NCBI Reference sequence: CP007577), was used as the reference genome for all sequence analysis. The resolution power of cgMLST and cgSNP analyses was assessed using Simpson's diversity index and the adjusted Wallace coefficient with 95% confidence intervals (Carrico et al., 2006; Severiano et al., 2011). Isolates were considered the same type if there were 0 SNPs or 0 alleles between them. Further comparison of the phylogenetic trees generated by cgMLST and cgSNP strategy was performed using the tanglegram algorithm deployed in Dendroscope 3 (Huson and Scornavacca, 2012; Scornavacca et al., 2011).

The genome alignment was parsed through Gubbins which identified and remove recombination regions (Croucher et al., 2015). A phylogenetic tree was constructed using the resulting SNPs with recombination regions removed using the maximum parsimony algorithm. This analysis returned an alignment of the non-recombinant SNPs (i.e. substitution mutations) and a maximum-likelihood (ML) phylogeny inferred from these SNPs, and assigns each SNP to either a recombination block or a substitution event and to a branch on the phylogenetic tree. SNP distance matrixes were calculated using snp-dist 0.6.3. Visualization of the phylogenetic tree was performed using the Interactive Tree of Life (iTOL) web server (Letunic and Bork, 2019). The minimum spanning tree based on cgMLST allelic profiles of ST195 *A. baumannii* isolates was created by PHYLOViZ 2.0 (Nascimento et al., 2017). The pan-genome analysis of the ST195 *A. baumannii* isolates was performed by Roary and OrthoVenn to identify orthologous genes (Page et al., 2015; Wang et al., 2015). The circular map including the BLAST-based comparison with the genome sequences of five representative *A. baumannii* ST195 isolates was generated using the BLAST Ring Image Generator (BRIG) (Alikhan et al., 2011).

2.3. Temporal analysis

Temporal signal was assessed by plotting root-to-tip divergence versus year of isolation with TempEst v1.5.1 (http://tree.bio.ed.ac.uk/software/tempest/) of the maximum likelihood (ML) tree including all aligned sequences (Rambaut et al., 2016). Given the evidence of temporal signal in the data, we proceeded with time-calibrated Bayesian phylogenetic inference using BEAST 1.10.4 to analyse the alignment of putative substitution mutations identified by Gubbins (Suchard et al., 2018). A Markov Chain Monte Carlo (MCMC) was run for 200 million generations, sampling every 20 million generations. Analysis files (xml) were generated in BEAUti 1.10.4 using: collection year tip dating, site heterogeneity gamma model with four categories and ascertainment bias correction. Both strict and uncorrelated relaxed clock model were tested with the following tree priors: constant size, exponential growth, GMRF Bayesian Skyride and Bayesian skyline. The substitution model HKY and GTR were also tested. Proper sampling of the Markov chain was evaluated by calculating the effective sampling size (ESS) with Tracer 1.7.1. The posterior distribution of trees was summarized into the maximum clade credibility (MCC) tree with TreeAnnotator v1.10.4 after 10% burn-in and the final tree was edited by FigTree 1.4.3 (http://tree.bio.ed.ac.uk/software/figtree/).

3. Results

Interrogation of the international genomes for the presence of acquired antimicrobial resistance genes revealed almost identical

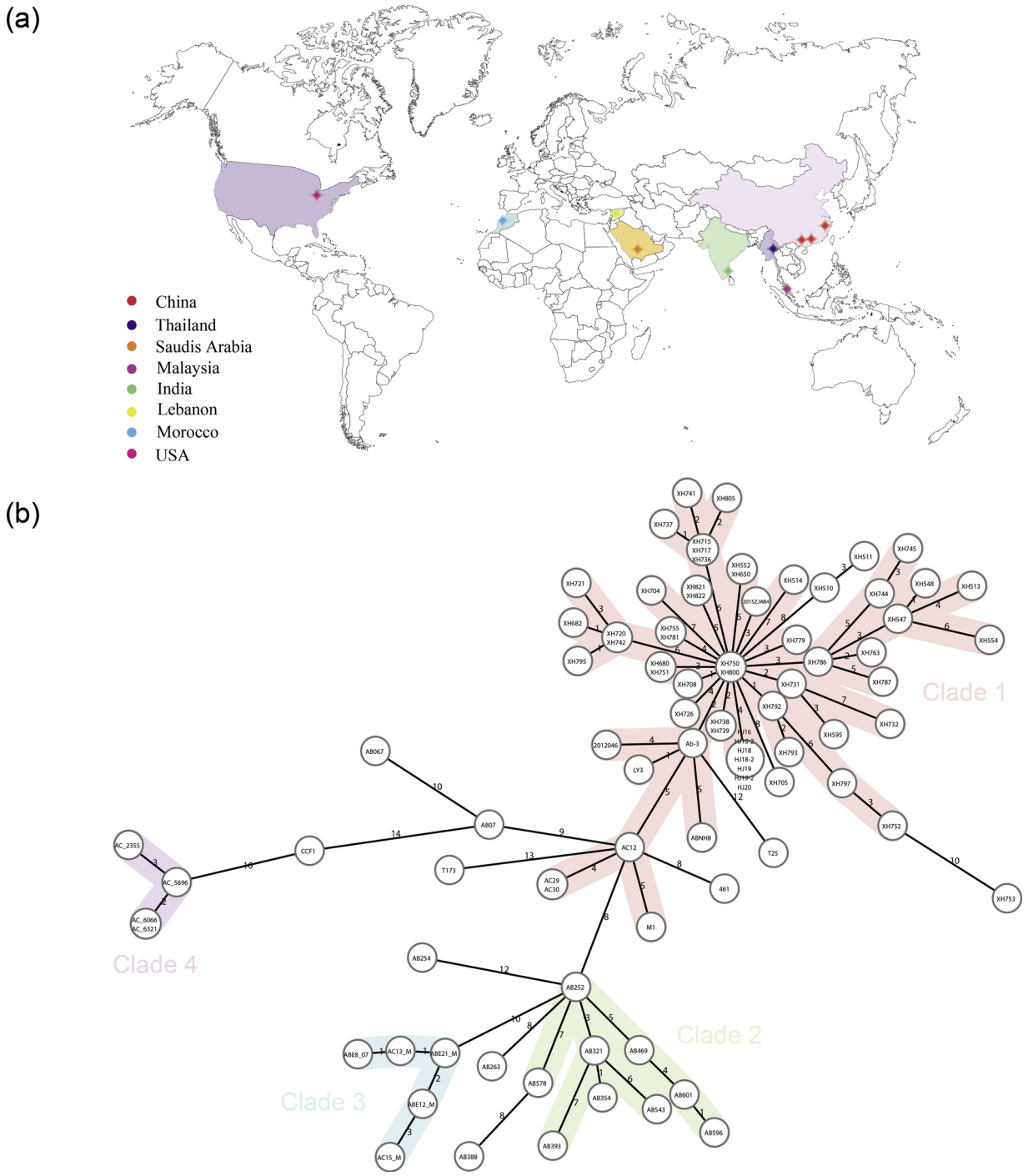

Fig. 1. Whole-genome sequencing (WGS) for tracking global bacterial transmissions, giving *A. baumannii* ST195 isolates as an example. (A) Diverse geographical locations for the 91 *A. baumannii* ST195 isolates. (B) A minimum spanning tree analysis of the *A. baumannii* ST195 isolates involved in this study. The lines connecting the circles indicate the clonal relationship between the different isolates and the digital numbers on the lines illustrate the number of allelic differences.

resistance gene profiles for all isolates: *armA* gene encoding for the 16S rRNA methylase, *aph(3′)-VI-a*, *aph(6′)-Id* for aminoglycoside-3′, 6′-phosphotransferases and *strA* gene all conferring resistance to aminoglycosides, *tet(B)* gene encoding for tetracycline resistance, *mph(E)* and *mrs(E)* genes, both responsible for macrolide resistance, *sul1* and *sul2* genes for sulphonamide resistance. The presence of the following β-lactamase genes was also detected: $bla_{\text{OXA-66}}$ (an OXA enzyme belonging to the intrinsic OXA-51-like enzymes), $bla_{\text{OXA-23}}$ carbapenemase, and the cephalosporinase-encoding $bla_{\text{ADC-25}}$. The most common carbapenemase gene was $bla_{\text{OXA-23}}$, which was present in 89% of the *A. baumannii* ST195 isolates. In addition, several other resistance determinants were absent in subsets of the isolates, suggesting they are possibly plasmid encoded and some isolates have lost the plasmid (Fig. 2).

The minimum spanning tree based on cgMLST profiles showed that the isolates belonging to ST195 that were temporally (with an interval of 7 years) and spatially distinct were grouped into a closely related cgMLST cluster (Fig. 1). The ST195 isolates differed from each other by 0–14 alleles, and included 53 isolates in clade 1, which differed by a maximum of 7 alleles suggesting that they are highly related. ST195 also contained clade 2, 3 and 4 which comprised nine, five and three isolates differing in seven, three and three alleles, respectively. There

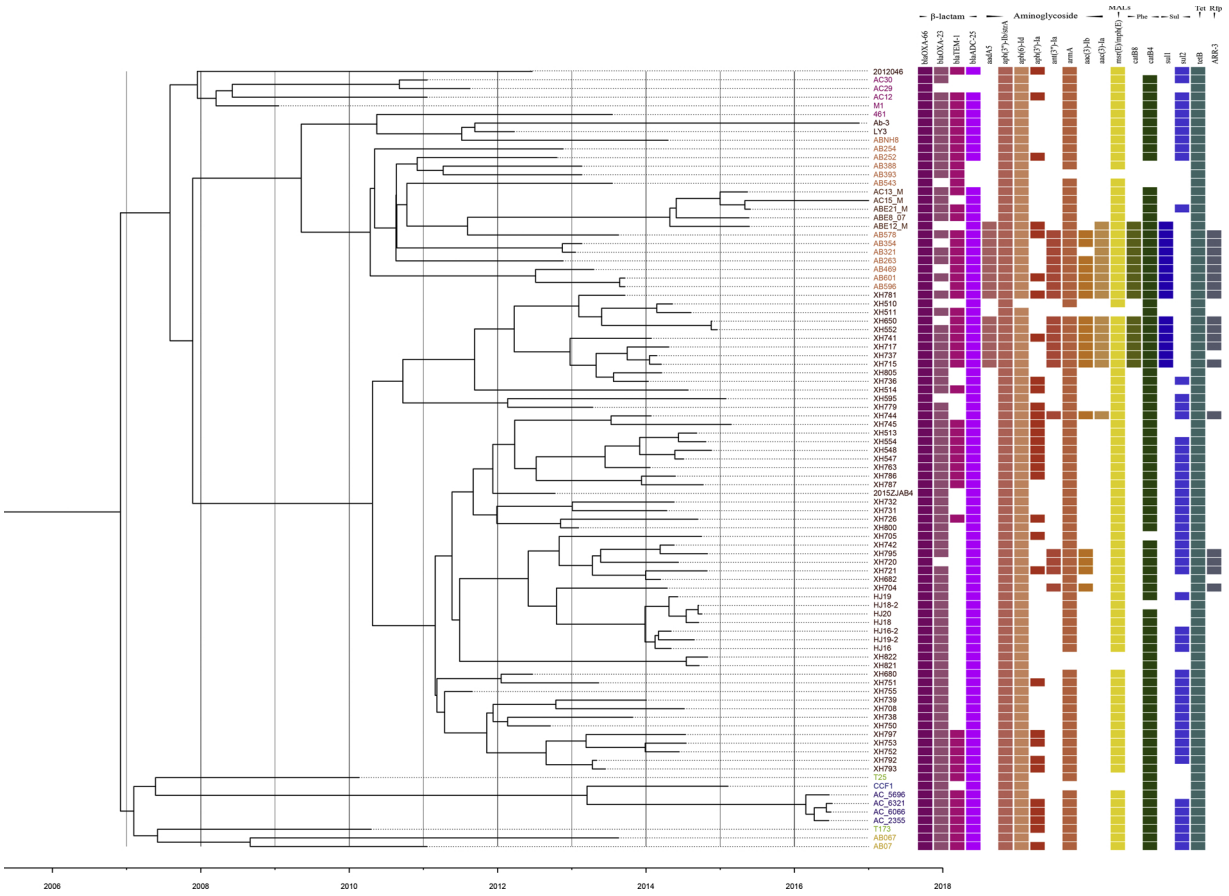

Fig. 2. Recombination-filtered core genome phylogeny and the distribution of antimicrobial resistance genes for ST195 *A. baumannii*. The cell in different colour indicates the presence of the gene while the blank cell indicates absence of the gene. Identical colours of strain name represents these are recovered from the same geographic location. Timescale indicated in years.

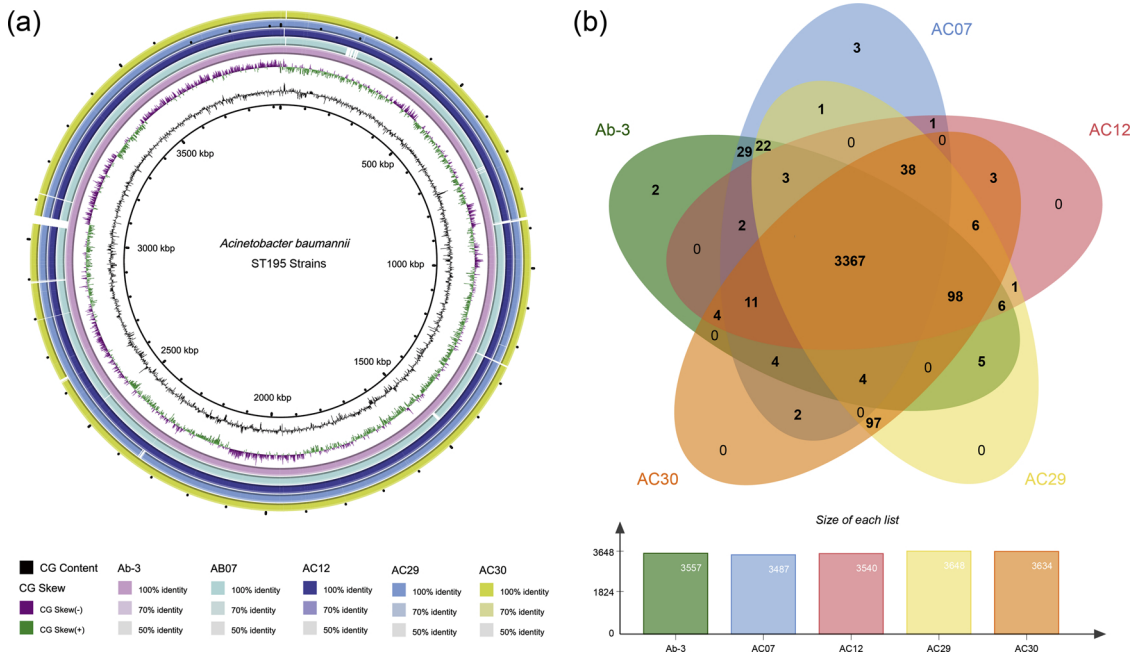

Fig. 3. Comparative genomic analyses of five representative *A. baumannii* ST195 isolates. (A) Genome sequence alignment of five *A. baumannii* ST195 isolates that revealed high degree of sequence similarity between the genomes of intercontinental isolates. (B) Venn diagram showed the orthologous groups in the five representative ST195 *A. baumannii* isolates. Numbers inside the Venn diagrams indicated the number of genes found to be shared among the given genomes.

were several instances where some ST195 isolates were closely related to those from geographically distant locations including Malaysia, Saudi Arabia and China. A 2016 nosocomial blood stream infection isolate recovered from Zhejiang, China (Ab-3) was separated from a cluster of isolates collected from patients entering an ICU in Saudi Arabia (ABNH8) in 2014 by 5 alleles, and was also separated by 5 alleles from a 2011 carbapenem-resistant bloodstream isolate from Malaysia (AC12). The patients infected with these isolates had no known epidemiological links to these international locations, and some of the patients were from very remote locations. This finding can also be evidenced by the comparative genomic analyses of five representative *A. baumannii* ST195 isolates, as they shared a large number of genes (Fig. 3).

The 91 *A. baumannii* ST195 isolates were resolved into 86 different types using the cgSNP strategy (Simpson's diversity index = 0.998; 95% confidence interval (CI): 0.995–1.000). In comparison, the cgMLST strategy generated fewer profiles, resolving into 74 unique types (Simpson's diversity index = 0.992; 95% CI: 0.985-0.999). The cgSNP strategy therefore provided a greater resolution power than cgMLST (adjusted Wallace coefficient = 0.874; 95% CI: 0.748–1.000). A visual comparison of the two phylogenetic trees indicated that although there were some minor differences in the two phylogenies, the majority of isolates were grouped into the same clusters, whether analysed with cgSNP or cgMLST strategy (**Figure S1**).

The correlation coefficient for the root-to-tip genetic divergence versus time in the TempEst analysis ($R^2 = 0.75$) indicated a strong linear relationship between accumulated mutations and sampling time, which suggested that enough signal was present to calibrate a strict clock. Bayesian model comparison through Bayes factors confirmed the strict clock model with constant size tree prior was the best fitting for the alignment. The median molecular clock rate was estimated to 7.6×10^{-3} (95% HPD interval 5.7×10^{-3} to 9.6×10^{-3}) substitutions per site per year which translates to approximately 2.8 mutations per year per genome. The most recent common ancestor (MRCA) for ST195 was estimated to date back to 2005 (95% highest posterior density, HPD, 2003–2008). There were five phylogenetically distinct clades, each including isolates from North America, Southeast Asia and North Africa, suggesting multiple introduction events between then and the present day. The lack of any discernible geographical signal and the close evolutionary relationships between isolates from geographically distant locations suggests that international dissemination of *A. baumannii* ST195 is occurring on a contemporary timescale (Fig. 2).

4. Discussion

The advent of WGS technologies has greatly increased the volume of genetic information available for characterizing the relatedness of bacterial isolates at the highest resolution level (Deng et al., 2016). This advantage makes inevitable the replacement of traditional typing techniques with WGS to become the new optimal standard of molecular epidemiology for the future surveillance of either local or global infectious diseases (Ruan et al., 2019). The evidence presented herein offers a deepened insight into the genomic epidemiology of internationally disseminating *A. baumannii* ST195 lineage. It is evident that the ST195 lineage in China is part of the international lineages and that several introductions combined with national transmission have formed the strain population. The clonal nature of the ST195 lineage complicates the estimation of local circulation and transmission, and highlights the importance of temporospatial epidemiological links even in the genomic era.

Clonally related isolates can be recovered at various geographic scales, and even disseminated worldwide, but without any definitive epidemiologic evidence. This phenomenon not only appears with *A. baumannii* but also in other bacterial pathogens, such as *S. pneumoniae* and *S. aureus*, although this point has not been directly raised hitherto (Croucher et al., 2014; Harris et al., 2010). In this study, the global identification of *A. baumannii* ST195 lineage over a large time span displayed an increasing clonal homogeneity, because of the allelic differences between these global isolates did not even pass the outbreak threshold for clonality (< 10 alleles) proposed in a previous study (Higgins et al., 2017). This could be the consequences of true global dissemination, however, there was a lack of epidemiological evidence for outbreak reconstruction, especially when associated with individual transmission events. Convenient and frequent global travels increase the likelihood for rapid and widespread transmissions of hospital-acquired and food-borne bacterial pathogens, allowing these easily cross geographical barriers. In addition, the sparsity of direct epidemiological evidence of asymptomatic carriers makes interpreting the putative transmission events and uncovering the true details of the natural history of infectious diseases difficult (Chisholm et al., 2018). Despite the enhanced surveillance practiced nowadays in response to the outbreak of infectious diseases, transmission links are inevitably absent due to missed sampling, suppression from antimicrobial therapy, or delays in identifying contacts. On this occasion, it is not a simple task to determine the clonal outbreaks rely solely on genomic data without an apparent epidemiological link. This finding illustrates the challenges when investigating outbreaks with conserved clonal lineages, as few methods can provide appropriate resolution to draw conclusions on strain transmission based on molecular typing data.

A more serious concern is that of determining whether the threshold for clonality can consistently be applied to the lineage, or whether this threshold can be influenced by other factors such as human host conditions, and hospital or geographic environments. The allelic differences between these global isolates do not pass the threshold for clonality, may also be due to different levels of purifying selection of slightly deleterious mutations. During an outbreak situation arising slightly deleterious mutations, while on evolutionary times-scales will be removed from the population, and will result in a decrease in allelic differences. This is the case because due to the short time-frame of sampling during a local outbreak, these mutations (allelic differences) have not been subjected (yet) to purifying selection. However, these slightly deleterious mutations will be removed from the populations through purifying selection in global isolates (longer timeframes). Similarly, lineages undergoing adaptive evolution are expected to accumulate mutations more rapidly than those subject to purifying selection. A recent analysis estimated the divergence date and the substitution rate within *A. baumannii* GC1 (Hamidian et al., 2019). We saw different values for the divergence date (2005 for lineage ST195 of GC2 in our study versus 1982 for GC1) and substitution rate (substitutions $site^{-1}\,year^{-1}$ of 7.6×10^{-3} in our study vs 1.26×10^{-6} for GC1). These values are naturally connected since a higher substitution rate (our study) leads to more variations in a shorter time period and therefore a later divergence date. It would be difficult to define the fixed criteria as there will be always cases that during a chain of transmission, the number of allelic differences may pass the threshold for clonality due to continuous genetic drift of the outbreak clone. A cut-off for determining a clonal outbreak can therefore be dynamic, which means for short-term local epidemiological purposes one probably should consider using another threshold than for long-term global epidemiological comparisons.

Both cgSNP and cgMLST analyses are restricted to regions of the genome present in all analysed isolates, which means that some potentially important components of clinically relevant phenotypic diversity in the accessory genome is discarded. Recently the combined analysis of variation in both core and accessory genome regions has been advocated and applied as an additional approach to study the genomic epidemiology of bacterial pathogens (McNally et al., 2016; Rouli et al., 2015; Schurch et al., 2018). Therefore, in cases where the cgSNP and cgMLST strategy fail to provide the sufficient resolution necessary for the epidemiological investigations of bacterial pathogens, inclusion of accessory genome may provide the additional discrimination to illuminate epidemiological linkages of bacterial populations.

In conclusion, we believe that WGS is still the most promising solution for undertaking the global surveillance of infectious diseases; however, the increasing clonal homogeneity of intercontinental isolates may complicate this task in the near future. Before WGS can be routinely employed for outbreak investigations, a series of parameters must be standardized for the data analysis procedure. This makes it imperative that, numerous simulations and optimizations be conducted, and that an enormous body of clinical genomic data be compiled to form the mainspring for future guidelines and standard procedures in this area.

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Declaration of Competing Interest

The authors declare that there are no conflicts of interest.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.ijmm.2019.151339.

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