Journal of Global Antimicrobial Resistance 17 (2019) 84–89

Contents lists available at ScienceDirect

Journal of Global Antimicrobial Resistance

journal homepage: www.elsevier.com/locate/jgar

Molecular epidemiology and genetic characterisation of carbapenem-resistant *Acinetobacter baumannii* isolates from Guangdong Province, South China

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ARTICLE INFO

Article history:
Received 2 August 2018
Received in revised form 21 September 2018
Accepted 5 November 2018
Available online 13 November 2018

Keywords:
Acinetobacter baumannii
Carbapenem resistance
Oxacillinase
Mobile genetic element
Molecular typing

ABSTRACT

Objectives: Carbapenem-resistant *Acinetobacter baumannii* (CRAB) has become a worldwide issue. This study aimed to characterise the epidemiology and genetic relationships of *A. baumannii* isolates in Guangdong Province, China.
Methods: CRAB isolates were collected from five municipal hospitals from June–December 2017. The 16S–23S rRNA intergenic spacer region was used for confirmation of strain identity. Antimicrobial susceptibility testing and the CarbAcineto NP test were performed to analyse the resistance spectrum and carbapenemase production of the isolates. PCR-based assays were used to detect β-lactamase genes and related mobile genetic elements. Genetic diversity among the isolates was analysed by enterobacterial repetitive intergenic consensus (ERIC)-PCR, multilocus sequence typing (MLST) and multiplex PCR.
Results: A total of 122 isolates were confirmed as *A. baumannii*; all were resistant to the tested antibiotics except for tigecycline and colistin. The CarbAcineto NP test showed that 93.4% of the isolates produced a carbapenemase. $bla_{OXA-23-like}$ and extended-spectrum β-lactamase-encoding genes were found by PCR in 94.3% and 91.8% of the isolates, respectively. Furthermore, the genetic environment of $bla_{OXA-23-like}$ was mainly associated with transposons Tn*2008* (46.1%), Tn*2006* (27.0%) and Tn*2009* (20.9%). MLST identified six existing sequence types (STs) and three novel STs, of which ST195 (35.7%) and ST208 (32.1%) were the most common, belonging to clonal group 92 and European clone II.
Conclusion: This study suggests that co-production of β-lactamases was the major resistance mechanism of CRAB isolates. Dissemination of $bla_{OXA-23-like}$ may be facilitated by transposable elements. ST195 and ST208 were the predominant epidemic types of *A. baumannii* in Guangdong Province.

1. Introduction

Acinetobacter baumannii is an important opportunistic pathogen responsible for a variety of nosocomial infections. Many of these infections show increasing morbidity and mortality, especially in intensive care units (ICUs) [1]. Carbapenems are usually considered to be the antibiotics of last resort for treating infections caused by this organism [2]. However, over the last decade, numerous nosocomial outbreaks caused by carbapenem-resistant *A. baumannii* (CRAB) have been reported worldwide and the spread of CRAB has become a global concern [3,4].

Carbapenem resistance in *A. baumannii* is primarily mediated through the production of carbapenem-hydrolysing class D β-lactamases, also called oxacillinases (OXAs). Four groups are most frequently reported, including the intrinsic chromosomal OXA-51-like β-lactamase and three acquired OXAs represented by OXA-23-like, OXA-24/OXA-40-like and OXA-58-like β-lactamases [4–6]. Several studies have reported that metallo-β-lactamases (MBLs), particularly IMP-, SIM-, NDM- and VIM-type carbapenemases, as well as some extended-spectrum β-lactamases (ESBLs) are produced by *A. baumannii* isolates [7,8].

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https://doi.org/10.1016/j.jgar.2018.11.002

Bacterial insertion sequence (IS) elements often flank one or both sides of an antimicrobial resistance gene, forming a complex structure called a composite transposon. These structures are mobile and play an important role in the regulation and dissemination of antimicrobial resistance genes [9]. In *A. baumannii*, IS*Aba1*, IS*Aba3* and IS*Aba4* can enhance the expression of $bla_{\text{OXA-like}}$ genes by providing a strong promoter [10–12]. Transposable elements played an essential role in the dissemination of $bla_{\text{OXA-23}}$ between different isolates [13], whilst composite transposons Tn*2006*, Tn*2007*, Tn*2008* and Tn*2009* are also associated with the transfer of $bla_{\text{OXA-23}}$ in *A. baumannii* [14,15].

Molecular typing is important for monitoring strain composition and the spread of outbreaks caused by bacterial pathogens. *A. baumannii* isolates obtained from various locations in Europe have been divided into three distinct lineages, termed European clone I (EUI), EUII and EUIII [16]. As *A. baumannii* isolates from different locations share some common genetic characteristics, multilocus sequence typing (MLST) has been widely used to molecularly subtype pathogenic strains [17]. Pulsed-field gel electrophoresis (PFGE) and PCR-based fingerprinting methods such as enterobacterial repetitive intergenic consensus (ERIC)-PCR are also frequently used in genetic typing analyses [18,19].

Guangdong Province in South China has a high population density and large population flow. It is ideally located in terms of trade and communication with inner China as well as overseas countries. Therefore, the mechanisms and prevalence of carbapenem resistance in this area are likely representative of many other locations. However, the epidemiology and genetic relationships of CRAB isolates in Guangdong Province remain to be determined.

This study aimed to analyse the antimicrobial resistance determinants and related transposable elements in CRAB isolates in an attempt to better understand the resistance mechanisms and the dissemination of $bla_{\text{OXA-23}}$ among these isolates. In addition, clonal relationships amongst the *A. baumannii* isolates were analysed using molecular typing methods to identify the most highly distributed clones in the study area. The results obtained will provide information for future epidemiological studies of CRAB in China and even worldwide.

2. Materials and methods

2.1. Collection and identification of bacterial isolates

A total of 130 non-repetitive, carbapenem-resistant *A. baumannii–calcoaceticus* (ABC) complex isolates were recovered from clinical specimens from five hospitals in Guangdong Province, China, from June–December 2017. Species identification was initially performed using a VITEK®2 COMPACT system (bioMérieux, Marcy-l'Étoile, France) and was further confirmed as *A. baumannii* by PCR-based amplification and sequencing of the highly conserved 16S–23S rRNA intergenic spacer (ITS) region of the *A. baumannii* genome [20]. *A. baumannii* isolates were then preserved in sterile skimmed milk at −80 °C for further study [21].

2.2. Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was performed for the following antimicrobial agents: imipenem; meropenem; cefoperazone/sulbactam (CSL); ceftazidime; gentamicin; ciprofloxacin; trimethoprim/sulfamethoxazole (SXT); colistin; and tigecycline. Resistance to tigecycline and colistin was examined by the broth microdilution method, whereas resistance to the other antimicrobial agents was determined by the Kirby–Bauer disk diffusion method. Susceptibility was interpreted based on Clinical and Laboratory Standards Institute (CLSI) breakpoints [22]. As there are no exclusive breakpoints for tigecycline against *Acinetobacter* spp., tigecycline breakpoints for Enterobacteriaceae from the US Food and Drug Administration (FDA) were used (susceptible, ≤2 mg/L; resistant, ≥8 mg/L) [23]. *Escherichia coli* ATCC 25922 and *A. baumannii* ATCC 17978 were used as controls strains.

2.3. Determination of carbapenemase production

The CarbAcineto NP test was used to rapidly identify carbapenemase-producing *A. baumannii* isolates using 5 M NaCl solution as lysis buffer and a full calibrated loop (10 μL) of bacterial inoculum [24]. Carbapenemase activity was detected by a colour change of the phenol red solution (red to yellow/orange) resulting from the hydrolysis of imipenem to a carboxylic derivative, leading to a decrease in the pH. An *Enterobacter cloacae* strain expressing New Delhi metallo-β-lactamase type 1 (NDM-1) and carbapenemase-free *A. baumannii* ATCC 17978 were included as positive and negative controls, respectively.

2.4. Detection of antimicrobial resistance genes and related mobile genetic elements

Putative carbapenemase-, ESBL- and AmpC-encoding resistance genes were screened by PCR [25–27]. Regions flanking the gene cassettes were detected as follows. IS elements (IS*Aba1*, IS*Aba3* and IS*Aba4*) were amplified before PCR mapping experiments were performed to map the position of the IS element relative to the $bla_{\text{OXA-like}}$ gene [10–12]. Subsequently, the surrounding genetic structure of $bla_{\text{OXA-23}}$ was examined for the presence of transposons Tn*2006*, Tn*2007*, Tn*2008* and Tn*2009* [15,28]. The gene arrangements of the $bla_{\text{OXA-23}}$ regions as well as the primer locations are shown in Fig. 1.

2.5. Molecular typing by ERIC-PCR, MLST and multiplex PCR

The genotypes and homology among the CRAB strains were analysed by ERIC-PCR as previously described [18,29]. Briefly, ERIC-

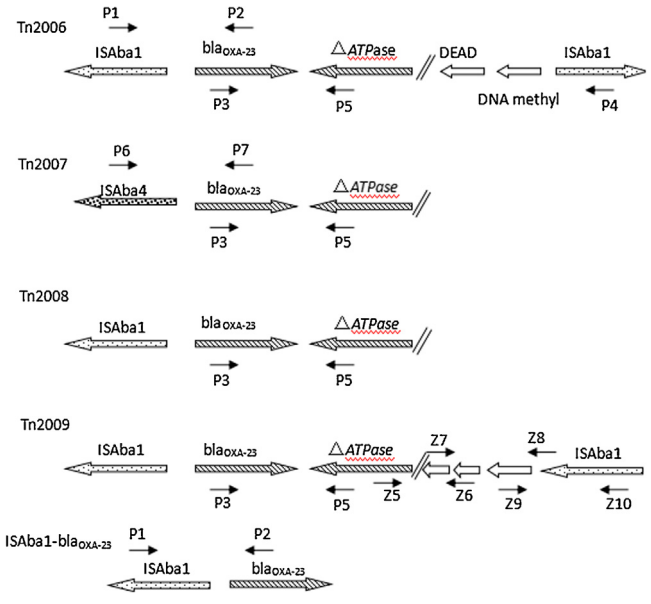

Fig. 1. Gene arrangements and primer locations in the IS*Aba1*–$bla_{\text{OXA-23}}$ composite transposons Tn*2006*, Tn*2008* and Tn*2009* and the IS*Aba4*–$bla_{\text{OXA-23}}$ composite transposon Tn*2007* of *Acinetobacter baumannii*. Primer set P1/P2 was used to amplify the IS*Aba1*–$bla_{\text{OXA-23-like}}$ region, primer set P3/P5 amplified the common region $bla_{\text{OXA-23}}$–ΔATPase in these four transposons, and primer set P3/P4 was specific for the Tn*2006* region. Three sets of nested primers (Z5/Z6, Z7/Z8 and Z9/Z10) were specific for the Tn*2009* region, whilst primers P6/P7 amplified IS*Aba4* (indication of the existence of Tn*2007*).

PCR products were subjected to electrophoresis and the fingerprints were classified into different band patterns according to the position and number of each band.

MLST was performed according to the protocols described on the *A. baumannii* MLST website (http://pubmlst.org/abaumannii/) using seven conserved housekeeping genes (*gltA*, *gryB*, *gdhB*, *recA*, *cpn60*, *rpoD* and *gpi*). The allelic numbers and sequence types (STs) were identified by means of the *A. baumannii* MLST database. Clusters of related STs [defined as clonal complexes (CCs)] were analysed using the eBURST website (http://eburst.mlst.net).

Identification of clonal lineages of the isolates was carried out using a multiplex PCR-based assay [30]. *A. baumannii* strains were divided into three CCs (designated Groups 1–3) based on two sets of primers (Set 1 and Set 2) targeting the *ompA* (outer membrane protein A), *csuE* (part of a pilus assembly system required for biofilm formation) and $bla_{\text{OXA-51-like}}$ (intrinsic carbapenemase gene in *A. baumannii*) genes (see Supplementary Table S1 in the online version at DOI: 10.1016/j.jgar.2018.11.002) [30]. The three CCs (Groups 1, 2 and 3) were found to correspond to EUII, EUI and EUIII, respectively.

Primers used in this study are outlined in Supplementary Table S1 in the online version at DOI: 10.1016/j.jgar.2018.11.002. Amplicons were analysed by 1.2% agarose gel electrophoresis and were then sequenced and subjected to BLAST analysis.

3. Results

3.1. Identification of isolates and antimicrobial susceptibility profiles

A total of 130 non-repetitive ABC complex isolates were collected, among which 122 (93.8%) were confirmed as *A. baumannii* by PCR-based amplification of the 16S–23S rRNA ITS region. Most of the isolates were recovered from patients in ICUs (49.2%; 60/122) and respiratory medicine wards (28.7%; 35/122), and most were isolated from respiratory specimens (85.2%; 104/122). All of the isolates were resistant to imipenem, meropenem, ceftazidime, CSL, gentamicin, ciprofloxacin and SXT. The resistance rate for tigecycline was 49.2% (60/122), whereas all isolates (100%) were susceptible to colistin.

3.2. Carbapenemase production

Rapid identification of carbapenemase-producing *A. baumannii* isolates was achieved using the CarbAcineto NP test. Overall, 114 isolates (93.4%) were positive for carbapenemase production, with only 8 isolates (6.6%) producing a negative result (see Supplementary Table S2 in the online version at DOI: 10.1016/j.jgar.2018.11.002). Representative results of this test are shown in Fig. 2.

3.3. Antimicrobial resistance genes and mobile genetic elements

PCR was used to detect the presence of relevant antimicrobial resistance genes. Overall, $bla_{\text{OXA-51-like}}$ genes, $bla_{\text{OXA-23}}$, bla_{AmpC} and bla_{TEM} were identified in 100% (122/122), 94.3% (115/122), 90.2% (110/122) and 87.7% (107/122) of the 122 CRAB isolates, respectively. Comparatively, the detection rates of bla_{VIM}, bla_{SIM}, $bla_{\text{CTX-M}}$, $bla_{\text{PER-1}}$, $bla_{\text{PER-1}}$ and bla_{SHV} were much lower, ranging from 0.8–6.6% (see Supplementary Table S2 in the online version at DOI: 10.1016/j.jgar.2018.11.002). The $bla_{\text{OXA-24/58-like}}$, bla_{IMP} and $bla_{\text{NDM-1}}$ genes were not detected in any of the isolates (see Supplementary Table S2 in the online version at DOI: 10.1016/j.jgar.2018.11.002).

The IS*Aba1* element was detected in all isolates, whereas none of the isolates carried IS*Aba3* or IS*Aba4*. PCR-based mapping experiments showed that IS*Aba1* was located upstream of $bla_{\text{OXA-51-like}}$ and $bla_{\text{OXA-23-like}}$ in 24.6% (30/122) and 93.9% (108/115) of the isolates, respectively (see Supplementary Table S3 in the online version at DOI: 10.1016/j.jgar.2018.11.002). As can be seen in the gene arrangement diagram shown in Fig. 1, the gene region surrounding $bla_{\text{OXA-23}}$ contained five structures. Overall, 115 $bla_{\text{OXA-23}}$-positive isolates were identified in this study, among which 108 (93.9%) contained the conserved $bla_{\text{OXA-23}}$–ATPase gene arrangement found in the four transposons, whilst the remaining 7 (6.1%) contained only the IS*Aba1*–$bla_{\text{OXA-23}}$ structure. IS*Aba4* was not detected, indicating that none of the isolates contained Tn*2007*. Furthermore, among the 108 strains containing the $bla_{\text{OXA-23}}$–ATPase structure, Tn*2006*-specific and Tn*2009*-specific regions were identified in 27.0% (31/115) and 20.9% (24/115), respectively, meaning that the remaining 46.1% (53/115) contained Tn*2008*-specific regions. Therefore, dissemination of the acquired $bla_{\text{OXA-23}}$ gene was associated with transposons Tn*2008* (46.1%; 53/115), Tn*2006* (27.0%; 31/115) and Tn*2009* (20.9%; 24/115) (see Supplementary Table S3 in the online version at DOI: 10.1016/j.jgar.2018.11.002).

3.4. Molecular typing

ERIC-PCR fingerprint analysis revealed seven distinct patterns, designated A–G (Fig. 3). In total, 4.1% (5/122) of the isolates showed pattern A, 36.9% (45/122) showed pattern B, 2.5% (3/122) showed pattern C, 7.4% (9/122) showed pattern D, 12.3% (15/122) showed pattern E, 23.0% (28/122) showed pattern F and 13.9% (17/122) showed pattern G (see Supplementary Table S4 in the online version at DOI: 10.1016/j.jgar.2018.11.002). Among the seven patterns, patterns B and F were the main epidemic genotypes.

On the basis of the ERIC-PCR fingerprints and the hospitals from which the isolates were collected, a set of 28 isolates were selected for further MLST analysis. The analysis identified six existing STs and three novel STs among the isolates. ST195 and ST208 were the predominant STs, comprising 35.7% (10/28) and 32.1% (9/28) of the isolates, respectively. Three isolates belonged to ST1633 (3/28; 10.7%), whilst ST345, ST381 and ST457 were represented by only one isolate each (1/28; 3.6%). Three novel STs (ST1849, ST1850 and ST1853) and 1 new allele (gpi 334) were identified. ST1853 has a new allele (gpi 334), which represents an A→T mutation at

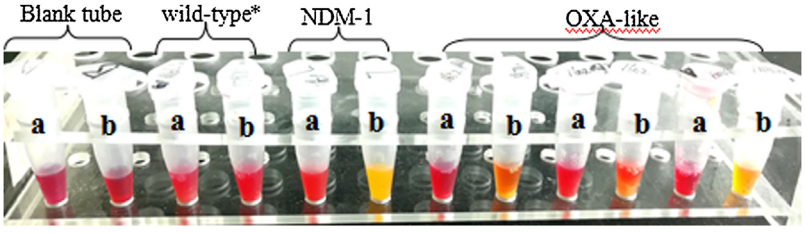

Fig. 2. CarbAcineto NP test results for *Acinetobacter* spp. isolates. Blank tube, no bacteria added (blank control); wild-type*, *A. baumannii* ATCC 17978 (negative control); NDM-1, Ambler class B (NDM-type) carbapenemase-producing *Enterobacter cloacae* (positive control); OXA-like, Ambler class D carbapenemase, clinical carbapenem-resistant *A. baumannii* isolates. Tube 'a' contains phenol red solution, 0.1 mM $ZnSO_4$ (pH 7.8) and 5 M NaCl; tube 'b' contains phenol red solution, 0.1 mM $ZnSO_4$ (pH 7.8) and 5 M NaCl supplemented with 12 mg/mL imipenem and cilastatin sodium.

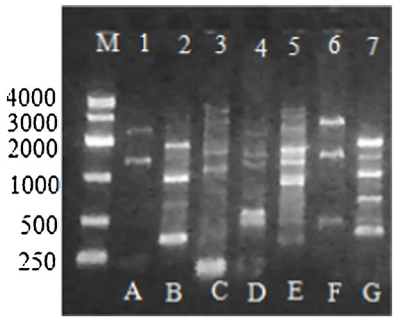

Fig. 3. Representative results showing the seven identified enterobacterial repetitive intergenic consensus (ERIC)-PCR fingerprints. Amplicons were analysed by 1.2% agarose gel electrophoresis. The fingerprints were divided into seven patterns, designated A–G.

position 120 of the gpi 222 locus. eBURST analysis showed that ST195, ST208, ST457, ST1633, ST1849 and ST1850 belonged to CC92. ST345, ST381 and ST1853 belonged to singleton STs not present in the MLST database and were not associated with any CC.

Clonal lineage analysis of the clinical isolates was performed by sequence type multiplex PCR. Analysis showed that 90.2% of the isolates (110/122) belonged to the Group 1 clonal complex and 1.6% (2/122) belonged to the Group 3 clonal complex, corresponding to EUII and EUIII, respectively (see Supplementary Table S4 in the online version at DOI: 10.1016/j.jgar.2018.11.002). Moreover, 8.2% of the isolates (10/122) could not be typed into any of the three groups, and none of the isolates belonged to the Group 2 clonal complex.

4. Discussion

CRAB-related nosocomial infection outbreaks are being increasingly reported in many countries, with the prevalence and epidemic spread of this pathogen arousing worldwide concern [1,4]. Compared with other members of the ABC complex, *A. baumannii* is more often associated with human infection and demonstrates higher levels of antimicrobial resistance, resulting in higher mortality rates [31]. Thus, it is imperative to accurately identify *A. baumannii* infection in order to effectively control the spread of these isolates.

In this study, 130 ABC complex isolates were collected from five hospitals, with the majority (93.8%) being confirmed as *A. baumannii*. Most of the CRAB isolates were recovered from respiratory specimens from patients in the ICU and respiratory departments, and all of the isolates were resistant to the tested antibiotics except for tigecycline (49.2% resistant) and colistin (100% susceptibility). These results suggest that most *A. baumannii* infections in hospitalised respiratory patients are caused by multidrug-resistant strains. In addition, colistin is likely to be an effective antibacterial agent for the treatment of these infections, whereas tigecycline may only be of limited use for *A. baumannii* infection in hospitalised patients.

The CarbAcineto NP test showed that 93.4% of isolates produced a carbapenemase, which was supported by PCR-based analysis confirming that all of the positive isolates carried $bla_{\text{OXA-23}}$. The correlation between biochemical and molecular testing methods confirmed that the CarbAcineto NP test has an important role in detecting potential carbapenemase-producers and guiding real-time treatment of *A. baumannii* infections [24].

In the current study, 94.3% of the isolates produced an OXA-23-like oxacillinase, but OXA−24/58-like oxacillinase production was not detected. Therefore, we predict that production of OXA−23-like oxacillinases is the most prevalent factor leading to carbapenem resistance across the five research hospitals in Guangdong Province, South China. Previous studies have reported that the predominant oxacillinase was OXA-23-like in *A. baumannii* in Beijing, Shanghai and Guangzhou City and Henan Province of China [28,32–34]. Similarly, this prevalence was also reported in other countries, such as the USA (New York City), Egypt, and India [35–37]. The $bla_{\text{OXA-51-like}}$ gene was detected in all CRAB isolates in the current study, supporting the assumption that that they are intrinsic genes in *A. baumannii* and underscoring their use as target genes for species identification of *A. baumannii* [25,38].

The chromosomally-encoded AmpC-type cephalosporinase gene was detected in 90.2% of the *A. baumannii* isolates in the current study. This result is similar to the findings of Huang et al. who reported that bla_{AmpC} was present in 84.0% (147/175) of *A. baumannii* strains isolated from a burn institute in Chongqing, China [39]. Of the MBL-encoding genes screened for in the current study, bla_{VIM} and bla_{SIM} were identified in only 5.7% (7/122) and 1.6% (2/122) of isolates, respectively. In comparison, ESBL-encoding genes were found in 91.8% of the isolates (112/122), with identification rates for $bla_{\text{CTX-M}}$, $bla_{\text{PER-1}}$, bla_{SHV} and bla_{TEM} ranging from 0.8–87.7%. This finding differs from the report of Safari et al. who identified prevalence rates for bla_{SHV}, bla_{TEM} and bla_{VIM} in *A. baumannii* isolates of 58% (58/100), 20% (20/100) and 30% (30/100), respectively [7]. In addition, a high proportion of isolates in the current study were found to contain more than one ESBL-encoding gene. For example, bla_{TEM} and $bla_{\text{OXA-23}}$ co-existed in 93 isolates; bla_{SHV}, bla_{TEM} and $bla_{\text{OXA-23}}$ were found in 3 isolates; bla_{VIM}, bla_{PER} and $bla_{\text{OXA-23}}$ co-existed in 3 isolates; and bla_{VIM}, bla_{TEM} and $bla_{\text{OXA-23}}$ were located in 1 isolate. These findings suggest that ESBLs, MBLs and AmpC-type cephalosporinases may work together with other resistance mechanisms in CRAB isolates to mediate multidrug resistance.

PCR assays showed that IS*Aba1* was located upstream of $bla_{\text{OXA-51}}$ and $bla_{\text{OXA-23-like}}$ in 24.6% (30/122) and 94.3% (115/122) of isolates in the current study, respectively. This result suggests that IS*Aba1* can simultaneously exist in these two genes to provide promoters that enhance gene expression [10]. Furthermore, analysis of the surrounding genetic environment of $bla_{\text{OXA-23}}$ showed that this acquired gene was mainly associated with transposons Tn*2008*, Tn*2006* and Tn*2009*, which facilitate the dissemination of the resistance gene. This is consistent with a previous report that Tn*2008* is vital for the dissemination of $bla_{\text{OXA-23}}$ among CRAB isolates in Shanghai, China [28]. However, other studies have reported that $bla_{\text{OXA-23}}$ is more commonly associated with Tn*2009* in China [15] and Tn*2006* in Taiwan and several other countries [14,40]. This study is the first to show that dissemination of the acquired $bla_{\text{OXA-23}}$ gene is mediated by transposons Tn*2008*, Tn*2006* and Tn*2009* in *A. baumannii* isolates from South China.

It is important to analyse clonal relationships among pathogens to better understand their molecular epidemiology. In the present study, ERIC-PCR, MLST and multiplex PCR were performed for molecular typing of the *A. baumannii* isolates. ERIC-PCR fingerprint analysis revealed the genotype diversity of isolates and the clonal expansion of certain CRAB strains in the research hospitals. MLST results showed that ST195 and ST208, belonging to the widespread CC92 [41], were the predominant STs of *A. baumannii* in Guangdong Province. In addition, the novel alleles and STs identified in this study indicated that the bacterial genome underwent rapid evolution. Previous studies showed that there were regional difference in the prevalence of STs. ST191 and ST195 prevailed in Beijing City, ST208 and ST368 in Chongqing City and ST92 in Henan Province [32,33,39]. The predominant CRAB strains belonged to ST101 in Central Greece, ST391 in Egypt and ST2 in Lebanon [36,42,43]. Regional differences in STs of *A. baumannii* revealed the genotyping diversity and genomic evolution. Multiplex PCR analysis showed that the majority (90.2%; 110/122) of CRAB isolates in the current study belonged to EUII, which is

consistent with previous reports that EUII strains are the most highly disseminated *A. baumannii* strains in European and Asian countries [44,45]. The above data show that ST195 and ST208 strains, belonging to CC92 and EUII, are the most prevalent CRAB STs in Guangdong Province, South China.

In conclusion, these observations suggest that co-production of OXA-23-like oxacillinase, ESBLs and AmpC-type cephalosporinase is the major mechanism of multidrug resistance among *A. baumannii* isolates in Guangdong Province, China. In addition, related transposable elements may aid in the dissemination of $bla_{\text{OXA-23}}$ among these isolates. The predominant STs of the *A. baumannii* isolates were ST195 and ST208, both of which belong to global CC92 and EUII. The findings of this study will be useful in determining how certain types of *A. baumannii* become dominant in specific environments and will aid in the development of appropriate measures to control this process.

Funding

This research was supported by the Natural Science Foundation of Guangdong Province [2015A030313522] and the Science and Technology Plan projects of Guangdong [2016A020215150].

Competing interests

None declared.

Ethical approval

Not required.

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