Development and evaluation of a LAMP assay for differentiating Carbapenem-Resistant Acinetobacter baumannii clinical strains harboring blaOXA-23

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Abstract

Background

Acinetobacter baumannii (A. baumannii) is an important nosocomial pathogen in hospital-acquired infections, and the resistance to carbapenems has been observed increasingly worldwide. Oxacillinase produced by blaOXA-23 is one of the predominant carbapenem resistance mechanisms in A. baumannii, which is highly prevalent worldwide, especially in China. The rapid identification of blaOXA-23 may give a valuable hint for the administration of directed antimicrobial therapy.

Method

In this study, we aimed to develop a LAMP-based detection for the blaOXA-23 gene; clinical samples of A. baumannii were used to determine the sensitivity and specificity of this method compared to phenotypic antimicrobial susceptibility testing and traditional PCR method. MLST was performed to investigate the epidemiology of A. baumannii bacterial population.

Results

Compared to the antimicrobial susceptibility testing, the sensitivity and specificity of LAMP in detecting blaOXA-23 was 88.4% and 97.7%, respectively. However, the LAMP method was found to be much simpler and the result could be available in a shorter period (within 60 minutes) when compared to conventional PCR and phenotypic susceptibility testing. The 113 isolates could be clustered into 30 sequence types (STs), and majority (83/113) of these strains belong to clonal complex 92 (CC92), which is also the dominant CC in the China.

Conclusion

The LAMP-based method detected blaOXA-23 in a much simpler way, by which could provide timelier results for differentiating the carbapenem-resistant Acinetobacter
baumannii than conventional methods. Consequently, blaOXA-23 may potentially serving as surrogate marker for the presence of CRAB in patients with serious infections in clinic.

Background

Recently, *Acinetobacter baumannii* (*A. baumannii*) has emerged as one of the important opportunistic nosocomial pathogens, and is known to be involved in a variety of infections such as ventilator-associated pneumonia, urinary tract infections, pneumonia and bacteraemia [1] This bacterium has a particular predilection for immunocompromised patients and severely ill patients in Intensive Care Units (ICUs), and is associated with substantial morbidity and mortality rates and high-cost health care [2]

One reason for the emergent interest in *A. baumannii* due to its great capability of acquiring new mechanisms of resistance adapt to any environment and eventually exhibiting multidrug resistance, which have been of significant medical concern [3]

Carbapenems represent the most versatile family of β-lactamases, and are recommended as last choice options for the treatment of serious infections. However, with the overuse of carbapenems, the carbapenem-resistant *A. baumannii* (CRAB) isolates have been reported frequently in recent years, and the resistance rate has increased to about 60% or even higher [4]

Although the resistance mechanisms of *A. baumannii* to antibiotics are complicated, the acquisition of β-lactamases with carbapenem hydrolyzing activity has been attributed mainly to the resistance against these agents [5]

Among the most widely spread carbapenem-resistant oxacillinase (prefix OXA)
β-lactamases genes, the plasmid-mediated $bla_{OXA-23}$ is the predominant oxacillinase responsible for the majority of phenotypic resistance to carbapenems in A. baumannii detected around the world [6-10].

The production of a low-copy-number plasmid vector carrying $bla_{OXA-23}$ gene by an A. baumannii strain is enough to confer resistance to the carbapenems [11]. Therefore, the $bla_{OXA-23}$ gene could be potentially considered as a surrogate marker for detecting the dissemination of CRAB strains carrying the $bla_{OXA-23}$ in clinic.

Rapid detection of pathogens and their resistance characterization is of suggestive significance, since it may lead to administration of directed antimicrobial therapy at an early stage, reduce the unnecessary earlier use of broad-spectrum agents, potentially result in better outcomes, and less emergence of antimicrobial-resistant organisms [12].

In present study, we aim to develop a Loop Mediated Isothermal Amplification (LAMP)-based method for the rapid differentiation of CRAB harboring the $bla_{OXA-23}$, and then evaluate the sensitivity and specificity of this method in clinical samples. Then the molecular epidemiology of these isolated strains was also investigated.

Methods

Bacterial strains and clinical isolates

All the 113 non-repetitive A. baumannii strains were obtained from the ICU and Respiratory hospitalized patients with clinically suspected infections in the former 307th Hospital of PLA. This study was approved by the Ethics Committee of the fifth Medical Centre of Chinese PLA General Hospital, and exemption of informed consent was obtained. The species identification was determined by both the LAMP method described previously [13] and the 16S rRNA sequencing [14].

A. baumannii bacteria were cultured in Luria-Bertani (LB) broth at $37^\circ C$ for 10-
12 h, while non-*Acinetobacter* species were grown at 37°C in brain heart infusion (BHI) broth overnight. The antimicrobial susceptibility testing was conducted by the VITEK 2 System (Biomerieux Vitrek, Inc., Hazelwood, MO, USA) using the AST-GN09 panels (bioMerieux Inc, 100 Rodolphe Street, Durham NC 27712 USA). Modified Hodge test (MHT) and Imipenem-EDTA double disk synergy test (DDS) were performed for carbapenemase production in accordance with the guidelines of the Clinical and Laboratory Standards Institute (CLSI). *A. baumannii* ATCC 22933 was used as control strains.

Preparation of sputum samples

Genomic DNA from sputum samples were prepared using the QIAamp® UCP Pathogen Mini kit (QIAGEN, Germany) according to the manufacturer’s instructions. DNA concentration was measured by OD260 measurements (ND-1000 spectrophotometer, NanoDrop Technologies, Inc, Wilmington, DE, USA). For the sensitivity and specificity of the LAMP assay, the concentration of the plasmid $bla_{OXA-23}$ was prepared by serial 10-fold dilutions to yield concentrations ranging from 324 ng/μl to 0.03 pg/μl.

Primer Design for LAMP Assay

To design $bla_{OXA-23}$ specific LAMP primers, the sequence of $bla_{OXA-23}$ with accession number CP030083.1 was downloaded from the NCBI GenBank database. The outer forward primer (F3), outer backward primer (B3), forward inner primer (FIP), and backward inner primer (BIP), and additional loop primers (loops F and loop B) were designed by PrimerExplorer (version 5) software (http://primerexplorer.jp/e/) with the acquired sequences. Four sets of primers were automatically designed. The sequence of $bla_{OXA-23}$ (used as the positive control) as well as the $bla_{OXA-58}$, $bla_{OXA-24}$, $bla_{KPC}$, $bla_{NDM}$, $bla_{SIM}$, $bla_{VIM}$, $bla_{IMP}$ (which were used in the specificity assay), and all the primers were synthesized commercially by Beijing Liuhe BGI Co., Ltd. (Beijing, China).
TABLE 1. Sequence of LAMP primers and PCR primers used for specific amplification and detection of \( \text{bla}_{\text{OXA-23}} \).

Development of the \( \text{bla}_{\text{OXA-23}} \)-LAMP

LAMP reactions were performed in a total volume of 25 \( \mu l \) in a LA-320CE instrument (Eiken Chemical Co., Ltd., Tochigi, Japan) for 60 min at 65\(^\circ\)C as described before [13]. To confirm the resistant genes detected in LAMP assay, normal PCR based on the \( \text{bla}_{\text{OXA-23}} \)-F and \( \text{bla}_{\text{OXA-23}} \)-R primers (Table 1) were performed as described previously [14] and the amplified products were sequenced by Beijing Liuhe BGI Co., Ltd. (Beijing, China) and blast against the PubMed database.

MLST for the clinical \( \text{A. baumannii} \) strains

The MLST scheme was performed based on the \( \text{A. baumannii} \) MLST (Oxford) methodology (http://pubmlst.org/abaumammii) [15]. The seven housekeeping genes (\( \text{gltA}, \text{gyrB}, \text{gdhB}, \text{recA}, \text{cpn60}, \text{gpi}, \text{and} \text{rpoD} \)) were amplified for all isolates, and the assembled sequences were aligned by using BLAST to assign the allelic numbers and sequence types (STs). Then the results were compared with the available alleles in the \( \text{A. baumannii} \) MLST (Oxford) database. eBURST analysis (http://eBURST.mlst.net/) was further conducted to investigate the genetic relationships and clonal complexes (CCs) of these isolates.

Statistics

SPSS version 18.0 (SPSS, Inc., Chicago, IL, USA) was applied for the statistical analysis. The Pearson Chi-Squared test was performed to compare the consistency between LAMP based method VITEK 2 system and a conventional PCR assay.

Results
LAMP reactions for $bla_{OXA-23}$

LAMP reactions were carried out according to the manufacturer’s instructions, and all the procedures were standardized at 65°C for 60 min. To select the optimal primers for the $bla_{OXA-23}$, the turbidity curves of the four sets of designed primers under the same condition with synthesized $bla_{OXA-23}$ DNA templates were observed. All the primer sets enabled successful amplification, of which the $bla_{OXA-23}$-1 primer set (Table 1) amplified the target sequence within the shortest time as shown in Figure 1, therefore, was chosen as the optimal primer set.

Figure 1. Four sets of primers were used to amplify $bla_{OXA-23}$ genes under the same conditions (at 65°C for 60min). The assay was monitored for the product of LAMP reaction, magnesium pyrophosphate, at optical density 650nm every 6s. The $bla_{OXA-23}$-1 primer set was chosen as the most appropriate primers for the rapid detection of $bla_{OXA-23}$.

To evaluate the specificity of LAMP detection for $bla_{OXA-23}$, genomic DNA extracted from a fully sequenced A. baumannii strains HRAB-85 carrying the $bla_{OXA-23}$ gene [16] and A. baumannii DNA (ATCC 22933) as well as other 8 synthesized drug resistant genes were tested using realtime turbidity. All other drug resistant genes templates including the A. baumannii strain 22933 tested negatively, whereas the HRAB-85 and the $bla_{OXA-23}$ DNA (positive control) were successfully amplified (Figure 2), indicating that the LAMP assay was specific for $bla_{OXA-23}$ within this research.

Figure 2. Effect of differing temperatures on the efficiency of detection of $bla_{OXA-23}$ by LAMP assay. Amplification was performed at 65°C for 60min, and the turbidity was monitored with a Loopamp real-time turbidimeter at a 650nm -absorbance every 6s. The detection limit of LAMP reaction was performed using 10-fold serial diluted $bla_{OXA-23}$
DNA from 324 ng/μl to 0.032 pg/μl. As shown in Figure 3, the detection limits of the real-time turbidity was about 32.4 pg/μl.

Figure 3. Specificity of the LAMP reactions in detecting the bla_{OXA-23} gene. Turbidity was monitored using a Loopamp real-time turbidimeter by measuring the absorbance at 650nm every 6s.

Evaluation of bla_{OXA-23} LAMP with collected A. baumannii strains

Totally, 113 A. baumannii strains were collected for evaluation the sensitivity and specificity of the LAMP based bla_{OXA-23} assay, and the consistency test with the phenotypic antimicrobial susceptibility testing by VITEK-2 system was also performed. The results were further confirmed by the classic broth microdilution and conventional PCR (listed in Table 2). Among all the A. baumannii strains, the bla_{OXA-23} LAMP assay detected 62 positive samples and 51 negative samples. Whereas the antimicrobial susceptibility testing showed that 69 A. baumannii strains were resistant to carbapenem (Imipenem and Meropenem), and 44 strains were susceptible to carbapenem. Broth microdilution method and phenotypic detection of carbapenemase was conducted for the 9 strains with inconsistent results among antibiotic susceptibility testing and bla_{OXA-23} LAMP assay. The results indicated that one bla_{OXA-23} LAMP positive strain was susceptible to carbapenem, while the rest 8 bla_{OXA-23} negative strains were resistant to Imipenem and Meropenem. Conventional PCR for bla_{OXA-23} was performed in 113 A. baumannii strains, and of all the 62 strains tested positively in LAMP assay 61 strains could amplify the aimed fragment successfully. Neither the single bla_{OXA-23} LAMP positive strain nor the 8 bla_{OXA-23} LAMP negative CRAB strains could amplify the bla_{OXA-23} fragment in PCR. The bla_{OXA-23} LAMP assay showed 88.4% sensitivity and 97.7% specificity compared to VITEK 2 system, and showed 100% sensitivity and 98.1% specificity when compared to the PCR. The Kappa
values between $bla_{OXA-23}$ LAMP assay and VITEK 2 system was 0.837, between $bla_{OXA-23}$ LAMP assay and PCR was 0.982, respectively. The consistency of these assays was quite satisfied. However, the procedure of the LAMP assay was less complicated when compared to the PCR, more rapid (available within 60 minutes) compared with the VITEK 2 results (18-24 hours). The results of LAMP assay and conventional PCR was highly consistent in identification of $bla_{OXA-23}$ in this research, both of which could potentially serve as a surrogate marker for the presence of CRAB carrying the $bla_{OXA-23}$.

**TABLE 2.** Sensitivity and Specificity of LAMP assay for detection of $bla_{OXA-23}$ against VITEK 2 system and PCR assay.

Molecular Epidemiology of the Clinical *A. baumannii* Isolates

All the 113 *A. baumannii* strains were typed by MLST and analyzed by eBURST. It was revealed that the strains could be clustered into 30 STs, and CC92 was the main clonal complex with 10 STs, of which ST208 was accounted for the dominant ST type (35/113, 31.0%), followed by ST195 (27/113, 23.9%), ST191 (6/113, 5.31%), ST218 (5/113, 4.42%), ST369 (3/113, 2.65%), ST445 (2/113, 1.77%), ST469 (2/113, 1.77%) and one strain of ST350, ST451 and 1117, respectively. Eighteen unrelated STs were considered as singletons.

**Figure 4.** Sequence types (STs) and clonal groups (CGs) of 113 *A. baumannii* isolates determined by eBURST analysis. A CC was defined as a group of STs sharing at least 5 identical loci among the 7 housekeeping genes tested.

**Discussion**

In 2017, WHO pronounced the Carbapenem-resistant gram-negative bacteria, namely, carbapenem-resistant *Enterobacteriaceae* (CRE), *Acinetobacter baumannii* (CRAB) and *Pseudomonas aeruginosa* (CRPA), who are the predominant pathogens causing Health
care-associated infections (HAI), have posed a significant threat to public health [17]. The choices of antimicrobial treatment against these organisms caused infection are severely limited; additionally, they have great potential for leading significant outbreaks in healthcare settings [18]. In the case of *Acinetobacter* infections, antibiotic resistance has became the most important determinant in clinical outcome, although it may not be a traditional virulence factor, it is by far the biggest driver of clinical outcome by precluding the clinician’s ability to kill the infecting strain [19].

CRAB is also an emerging problem in China, data from China Antimicrobial Surveillance Network (CHINET) involving 19 major hospitals demonstrated that the resistance to carbapenems increased rapidly from 31% in 2005 to 66.7% in 2015 [19] and with an average of 60% among varied provinces according to the results of China Antimicrobial Resistance Surveillance System (CARSS) in 2016 [20].

OXA-23 enzyme is the predominant oxacillinase responsible for the majority of phenotypic resistance to carbapenems, and the prevalence of acquired $bla_{OXA-23}$ detected in CRAB isolates was extremely high in recent published papers, including, but not limited to 100% of the 56 CRAB isolates [21], 95% of the 122 isolates [22] and 96.9% of the 194 isolates [23]. In this study, 69 strains out of the 113 *A. baumannii* were CRAB, and 88.4% (61 strains) harbored the $bla_{OXA-23}$. Hence, the clinical significance of OXA-23 isolates is of great importance, since the $bla_{OXA-23}$ gene having the potential of being the surrogate marker for the detection of the CRAB strains, by which the clinicians are advised not to
use the unnecessary extended-spectrum or antibiotics.

Conventional methods for identification of common pathogen and phenotypic susceptibility testing requiring cultured bacteria takes at least 2 days, during which time patients may be receiving ineffective or unnecessarily broad-spectrum antibiotics [12].

More recently, quite a few molecular diagnostic assays are explored for rapid identification of pathogens, providing timelier results than conventional subculture and phenotypic susceptibility testing [24-27]. For *A. baumannii* and carbapenemase resistance genes, recent advances in molecular technologies including the MALDI-TOF/MS [28], q-PCR [29], multiplex real-time PCR assays [30], DNA microarray [31], and even next generation sequencing (NGS) [32] were used to establish the rapid detection assays.

In this research, we described a LAMP-based diagnosis assay for the rapid detection of CRAB harboring the most prevalent carbapenemase gene *bla*_{OXA-23}. Sample for this assay was culture-independent, and genomic DNA extracted from sputum samples by commercial kits or just by direct boiling method greatly shorts the preparing work. When applied the LAMP assay, a positive reaction could be completed within an hour. 113 clinical samples of *A. baumannii* were used to determine the sensitivity and specificity of this method, and by comparing to phenotypic susceptibility testing, the sensitivity and specificity of LAMP in detecting *bla*_{OXA-23} was 88.4% and 97.7%, respectively. Eight phenotypic carbapenem-resistance strains were *bla*_{OXA-23}-negative in both LAMP assay
and PCR. *A. baumannii* resistant is highly complex, it is possible that other mechanisms, such as the production of other carbapenemases, hyperexpression of efflux pump, changes in outer membrane proteins (OMPs) were attributed to the carbapenem-resistance in these eight isolates. One carbapenem susceptible *A. baumannii* strain was presented $bla_{OXA-23}$-positive result in the LAMP assay. Very likely it was a false-positive result, however, it is possible that the production of the carbapenemases/ the copy number of $bla_{OXA-23}$ gene was too low to be detected by the available phenotypic susceptibility testing/ the conventional PCR.

It has been demonstrated that the epidemiology of the *A. baumannii* bacterial population closely related to their antimicrobial resistances. The great ability of acquiring resistance determinants and propensity to adapt to any environment resulted by their extraordinary genomic plasticity and powerful adaptability has led to the simultaneous presence of dominant *A. baumannii* clonal lineages, such as international clones I and II, and increasing prevalence in international hospitals all over the world [33].

A need to monitor the epidemiology of *A. baumannii* and its associated antimicrobial resistances is reinforced. So, MLST was employed to investigate the epidemiology of *A. baumannii* bacterial population. The 113 isolates could be clustered into 30 sequence types (STs), and majority (83/113) of these strains belong to clonal complex 92 (CC92), which was corresponded to international clonal lineage II. CC92 is the dominant CC in the Chinese mainland [34].

Furthermore, CC92 *A. baumannii carrying* $bla_{OXA-23}$ is a major drug-resistant strain spreading in China.

Definitely, this is not the first report about the detection method for $bla_{OXA-23}$ based on
the rapid molecular testing. What really interested us is to seek for a potential marker for the presence or absence of CRAB in patients with serious infections. Rapid identification of antibiotic resistance is central to timely isolation of patients harboring drug-resistant organisms [36].

Antimicrobial management strategies predicated on pathogens and their resistance characterization are associated with appropriate antibiotic usage in critically ill or immunocompromised patients, and have the potential to result in better outcomes and less emergence of antimicrobial-resistant. Unfortunately, the rapid test for identification carbapenemases produced organisms are still under explored, lacking of commercial molecular diagnostic kits such as for MRSA and VRE. The Kappa values between \( \text{bla}_{OXA-23} \) LAMP assay established in this study and phenotypic susceptibility testing was 0.837, which was quite satisfied. By combination with the \( \text{bla}_{OXA-51} \)-like LAMP method, we could distinguish \textit{A.baumannii} rapidly in several hours, much faster but less complicated. One limitation of the present research is that we could only detect the CRAB strains harboring the \( \text{bla}_{OXA-23} \), leading the miss identification of strains caused by other carbapenem-resistance mechanisms. In conjunction with detecting other carbapenem produced genes, such as the metallo-beta-lactamase (MBL), or mutation in porin genes, or key genes changes in their expression of efflux pumps, the detection rate of CRAB might be improved. However, with a extremely high \( \text{bla}_{OXA-23} \) prevalence of CRAB in China and the excessive empiric usage of carbapenem, the \( \text{bla}_{OXA-23} \)-positive results from this assay are sufficient to help clinicians to avoid choosing unnecessarily carbapenem in a meaningful time frame, to monitor the efficacy of therapy, and having the potential to improve patient care and outcomes.

Abbreviations
CRAB: carbapenem-resistant Acinetobacter baumannii; VAP: ventilator-associated pneumonia; UTI: urinary tract infections; ICUs: Intensive Care Units; OXA: oxacillinase; LAMP: Loop Mediated Isothermal Amplification; LB: Luria-Bertani; BHI: brain heart infusion; MHT: Modified Hodge test; DDS: double disk synergy test; CLSI: Clinical and Laboratory Standards Institute; ATCC: American Type Culture Collection; F3: outer forward primer; B3: outer backward primer; FIP: forward inner primer; BIP: backward inner primer; LF: loop (forward) primers; LB: loop (backward) primers; MLST: multi-locus sequence typing; STs: sequence types; CC: clonal complexe; CRE: carbapenem-resistant Enterobacteriaceae; CRPA: carbapenem-resistent Pseudomonas aeruginosa; HAI: Health care-associated infections; CHINET: China Antimicrobial Surveillance Network; CARSS: China Antimicrobial Resistance Surveillance System; MALDI-TOF/MS: matrix-assisted laser desorption/ionization time of flight; NGS: next generation sequencing; OMPs: outer membrane proteins; MBL: metallo-beta-lactamase.

Declarations

Ethics approval and consent to participate

This study was approved by the Ethics Committee of the fifth Medical Centre of Chinese PLA General Hospital (Former 307th Hospital of PLA), and exemption of informed consent was obtained.

Consent for publication

Not applicable.

Declarations

I can confirm I have included a statement regarding data and material availability in the declaration section of my manuscript.

Availability of data and material

All data generated or analysed during this study are included in this published article.
Competing interests

The authors declare that they have no competing interests.

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Author Contributions

Prof. Changqing Bai and Prof. Liuyu Huang helped conceive the project and design the study. Prof. Xin Yuan, Prof. Xiuyun Yin, Huiying Liu, Yanhong Qin and Jing Zheng collected the strains. Puyuan Li wrote the whole manuscript text and prepared all the tables and figures. Wenkai Niu, Yun Fang, Dayang Zou, Fengjiang Li and Yannan Liu executed the experiments. All the authors reviewed the manuscript and agreed with the publication.

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**Tables**

**TABLE 1.** Sequence of LAMP primers and PCR primers used for specific amplification and detection of *blaOXA-23*.

| primers    | sequences[5-3]                              | length[bp] |
|------------|--------------------------------------------|------------|
| *blaOXA-23-F3* | CAGAATATGTGCCAGCCTCT                      | 20         |
| *blaOXA-23-B3* | CGATACGTCGCGCAAGTT                       | 18         |
| *blaOXA-23-FIP* | TGACCTTTTTCGCCTCCATGGTG AATGCCcct GATCGGA | 41         |
| *blaOXA-23-BIP* | CCGCTTGGGAAAAAGACATGACACC CTGATAG ACTGGGAATCG | 43         |
| *blaOXA-23-LF* | AGGAGAAGCCATGAAGCTTTC                      | 21         |
| *blaOXA-23-F*  | ATGAATAATATTCTCTAGTTG                      | 20         |
| *blaOXA-23-R*  | TTAATAATATTCCAGCTGTT                      | 20         |
TABLE 2. Sensitivity and Specificity of LAMP assay for detection of \textit{blaOX23} against VITEK 2 system and PCR assay.

| phenotype testing by the VITEK 2 | \textit{blaOX23} LAMP assay | PCR for \textit{blaOX23} |
|----------------------------------|---------------------------|------------------------|
| CRAB n=69                        | 61                        | 61                     |
| CSAB n=44                        | 1                         | 0                      |
| total                            | 62                        | 61                     |

|                          | \textit{blaOX23}+ | \textit{blaOX23}- |
|--------------------------|------------------|------------------|
| CRAB n=69                | 61               | 8                |
| CSAB n=44                | 1                | 43               |
| total                    | 62               | 51               |

Sensitivity 88.4% 100% (LAMP compared to PCR)

Specificity 97.7% 98.1% (LAMP compared to PCR)

Kappa value 0.837 0.982

Figures
Four sets of primers were used to amplify blaOXA-23 genes under the same conditions (at 65 °C for 60 min). The assay was monitored for the product of LAMP reaction, magnesium pyrophosphate, at optical density 650 nm every 6 s. The blaOXA-23-1 primer set was chosen as the most appropriate primers for the rapid detection of blaOXA-23.
Effect of differing temperatures on the efficiency of detection of blaOXA-23 by LAMP assay. Amplification was performed at 65°C for 60min, and the turbidity was monitored with a Loopamp real-time turbidimeter at a 650nm absorbance every 6s.
Figure 3

Specificity of the LAMP reactions in detecting the blaOXA-23 gene. Turbidity was monitored using a Loopamp real-time turbidimeter by measuring the absorbance at 650nm every 6s.
Sequence types (STs) and clonal groups (CGs) of 113 A. baumannii isolates determined by eBURST analysis. A CC was defined as a group of STs sharing at least 5 identical loci among the 7 housekeeping genes tested.