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

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Research article

Keywords: Carbapenem-Resistant Acinetobacter baumannii, blaOXA-23, rapid detection, LAMP, differentiate

Posted Date: June 13th, 2019

DOI: https://doi.org/10.21203/rs.2.10254/v1

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.

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◦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 Vitek, 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 bla_{OXA-23}.

Development of the bla_{OXA-23}-LAMP

LAMP reactions were performed in a total volume of 25 μl in a LA-320CE instrument (Eiken Chemical Co., Ltd., Tochigi, Japan) for 60 min at 65◦C as described before [13]. To confirm the resistant genes detected in LAMP assay, normal PCR based on the bla_{OXA-23}-F and bla_{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 *A. baumannii* strains

The MLST scheme was performed based on the *A.baumannii* MLST (Oxford) methodology (http://pubmlst.org/abaumammii) [15]. The seven housekeeping genes (gltA, gyrB, gdhB, recA, cpn60, gpi, and 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 *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 positivelyin 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.

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-resistant *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.

Funding

This present work was supported by the Capital Health Research and Development of Special (grant no. 2016-2-5062), and the Capital Characteristic Clinic Project of Beijing (grant no. Z161100000516181).

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 agreeed with the publication.

Acknowledgements

We are grate to Liu Wei from the Institute for Disease Prevention and Control of PLA for constructive comments on this research.

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Tables

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

primers	sequences（5-3）	length（bp）
*bla*OXA-23-F3	CAGAATATGTGCCAGCCTCT	20
*bla*OXA-23-B3	CGATACGTCGCGCAAGTT	18
*bla*OXA-23-FIP	TGACCTTTTCTCGCCCTTCCATGTTGAATGCCct GATCGGA	41
*bla*OXA-23-BIP	CCGCTTGGGAAAAAGACATGACACCCTGATAG ACTGGGACTGC	43
*bla*OXA-23-LF	AGGAGAAGCCATGAAGCTTTC	21
*bla*OXA-23-F	ATGAATAAATATTTTACTTG	20
*bla*OXA-23-R	TTAAATAATATTCAGCTGTT	20

TABLE 2. Sensitivity and Specificity of LAMP assay for detection of *bla*OXA-23 against VITEK 2 system and PCR assay.

phenotype testing	*bla*OXA-23 LAMP assay		PCR for *bla*OXA-23	
by the VITEK 2	*bla*OXA-23+	*bla*OXA-23-	*bla*OXA-23+	*bla*OXA-23-
CRAB n=69	61	8	61	8
CSAB n=44	1	43	0	44
total	62	51	61	52
Sensitivity	88.4%		100% (LAMP compared to PCR)	
Specificity	97.7%		98.1%(LAMP compared to PCR)	
Kappa value	0.837		0.982	

Figures

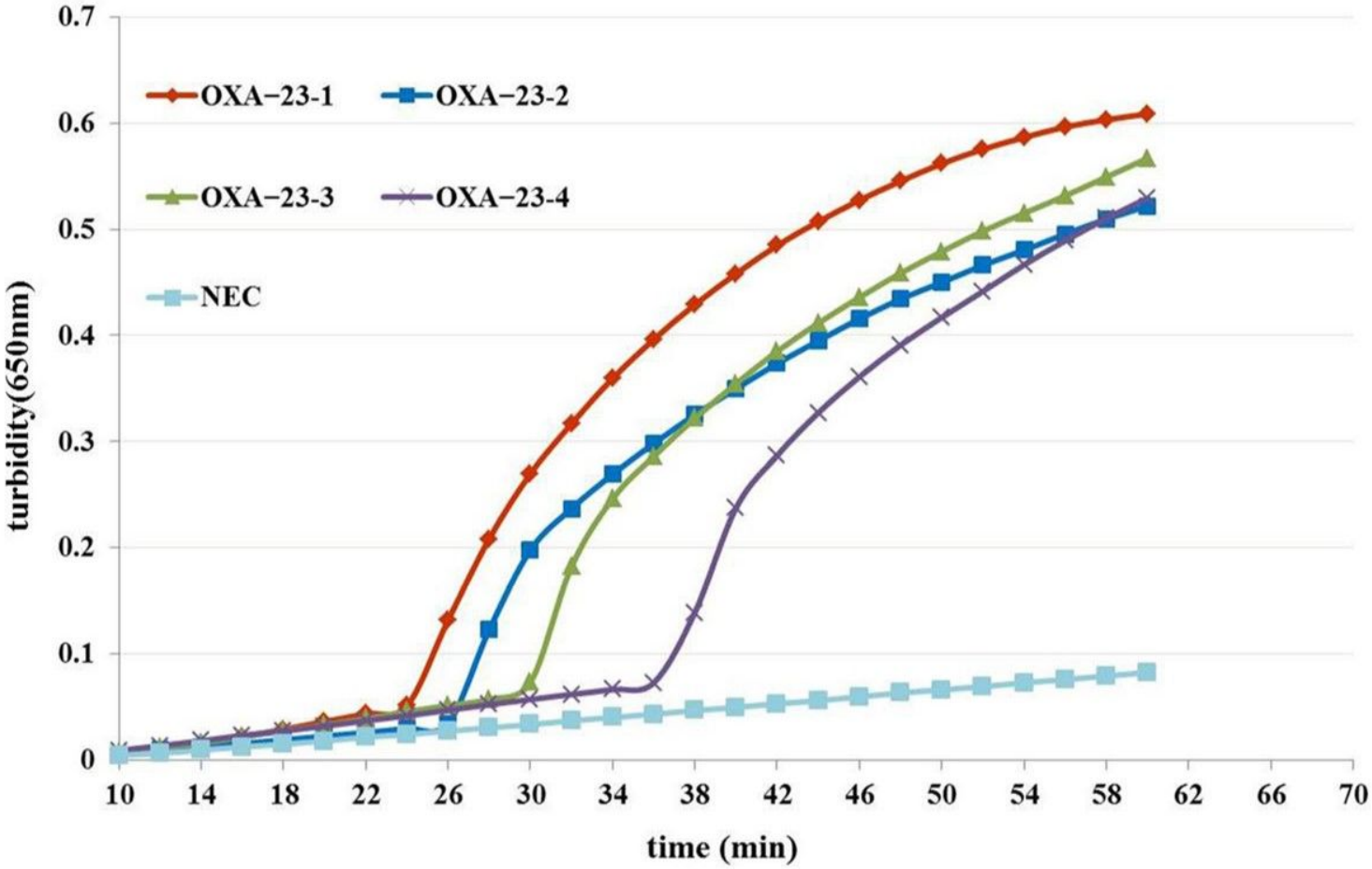

Figure 1

Four sets of primers were used to amplify blaOXA-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 blaOXA-23-1 primer set was chosen as the most appropriate primers for the rapid detection of blaOXA-23.

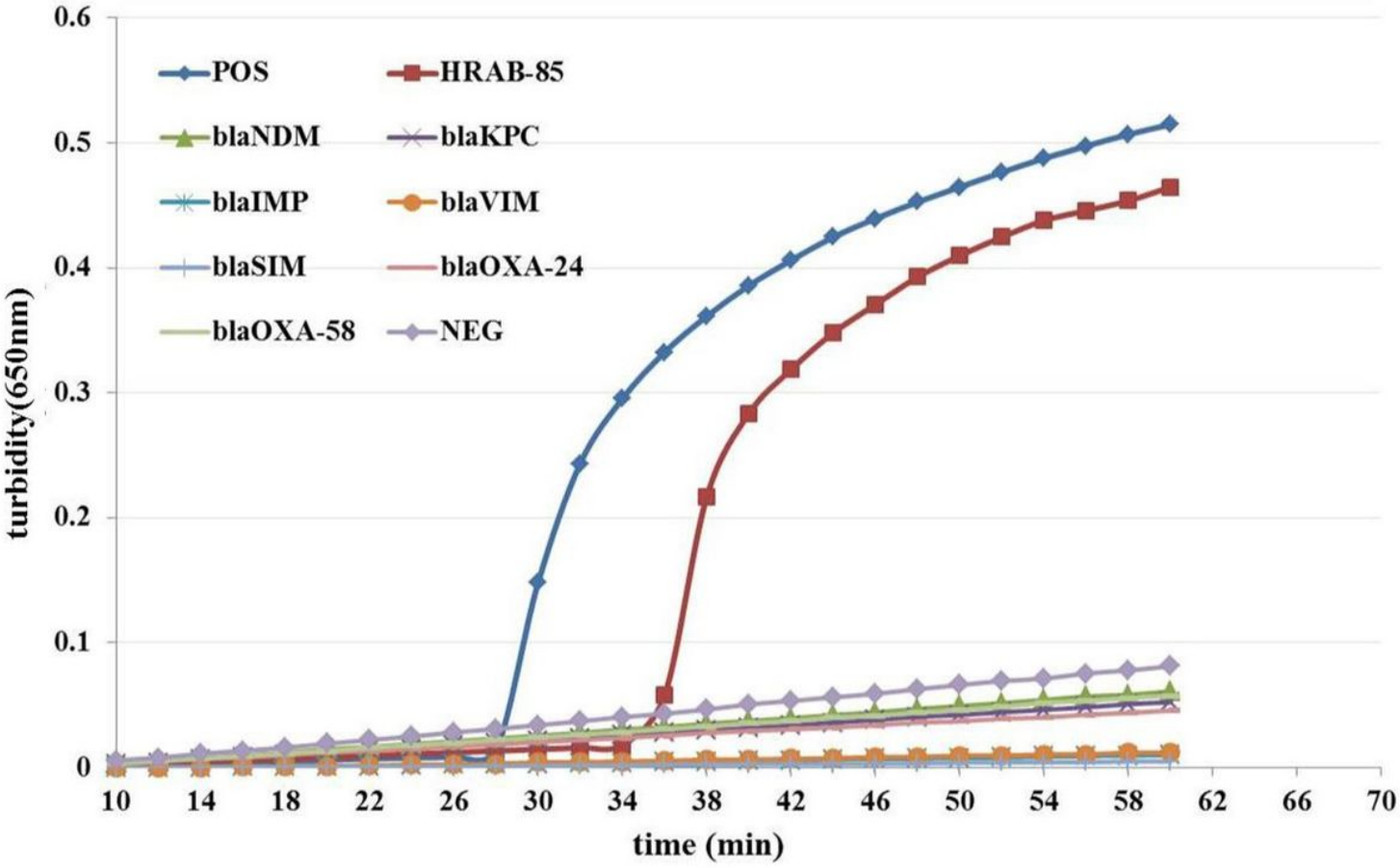

Figure 2

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.

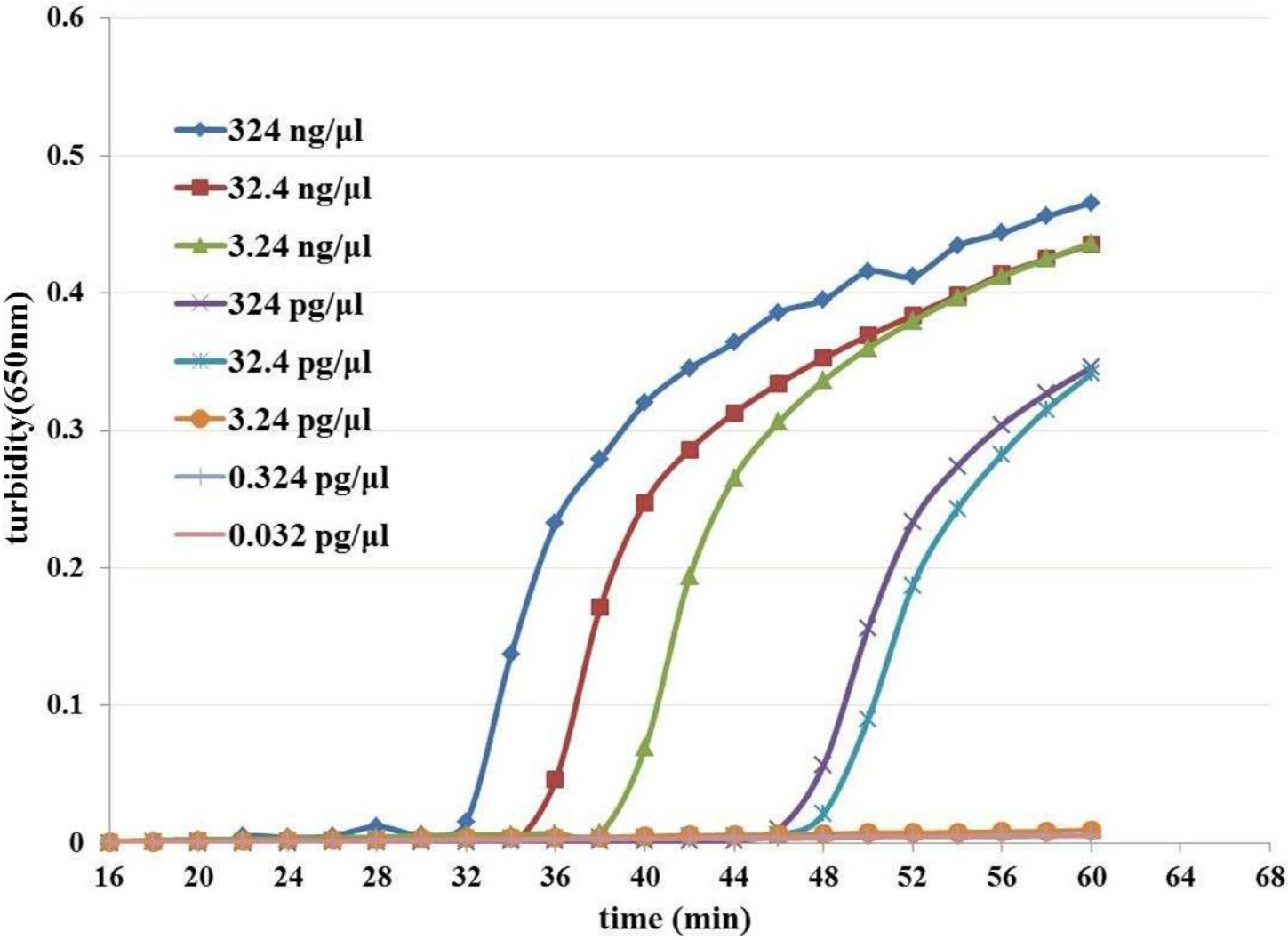

Figure 3

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.

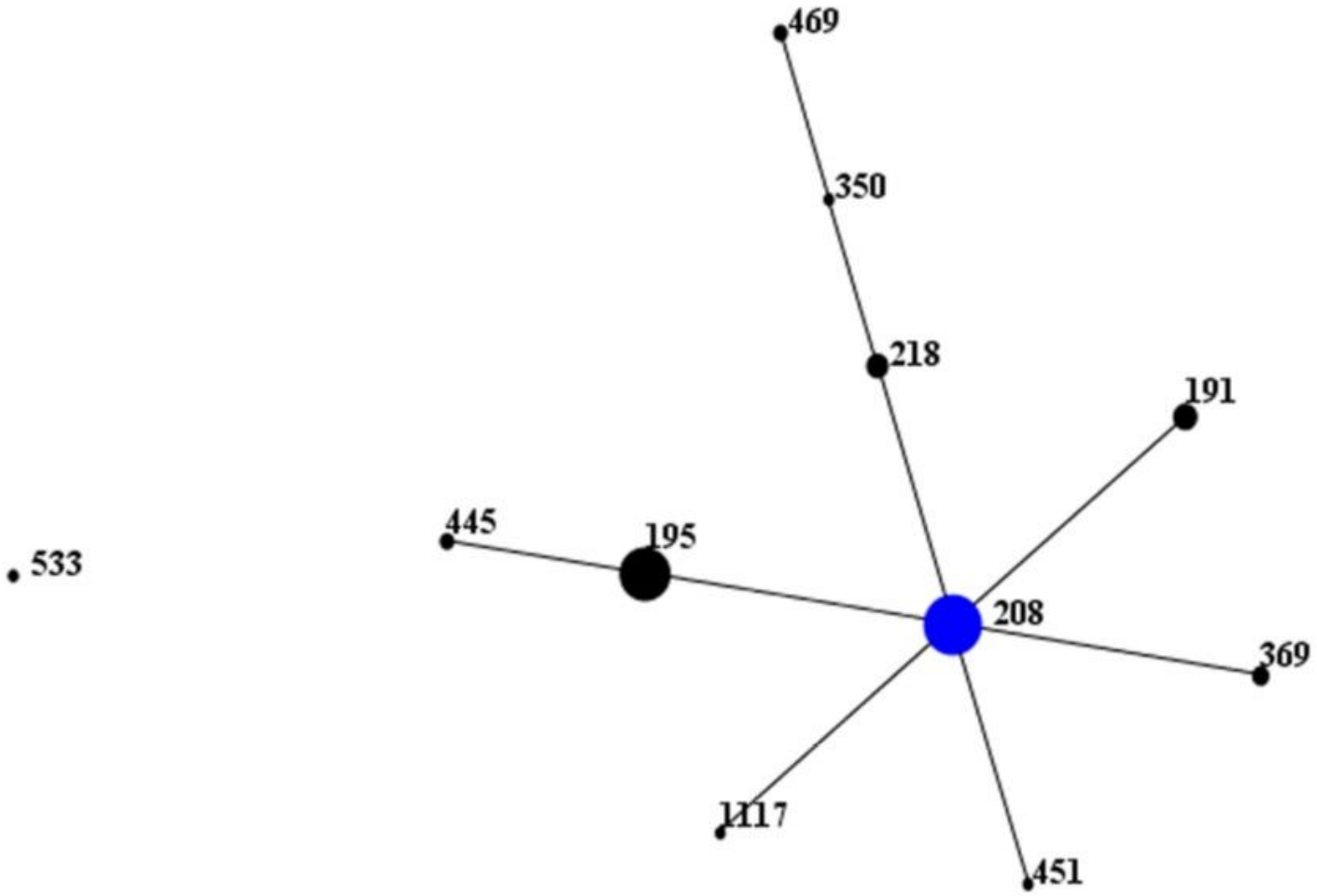

Figure 4

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.