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Loop-mediated isothermal amplification: Rapid and sensitive detection of the antibiotic resistance gene IS*Aba1*-*bla*$_{\text{OXA-51-like}}$ in *Acinetobacter baumannii*

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ABSTRACT

Carbapenem-resistant *Acinetobacter baumannii*, which are mainly induced by the production of OXA-type β-lactamases, are among the leading causes of nosocomial infections worldwide. Among the β-lactamase genes, the presence of the OXA-51-like gene carrying the upstream insertion sequence, IS*Aba1*, was found to be one of the most prevalent carbapenem resistance mechanisms utilized by these bacteria. Consequently, it is necessary to develop a rapid detection method for IS*Aba1*-*bla*$_{\text{OXA-51-like}}$ sequence for the timely and appropriate antibiotic treatment of *A. baumannii* infection. In this study, a loop-mediated isothermal amplification (LAMP) assay was optimized for IS*Aba1*-*bla*$_{\text{OXA-51-like}}$ detection. The LAMP primer set was designed to recognize distinct sequences in the IS*Aba1*-*bla*$_{\text{OXA-51-like}}$ gene and could amplify the gene within 25 min at an isothermal temperature of 60 °C. This LAMP assay was able to detect the IS*Aba1*-*bla*$_{\text{OXA-51-like}}$ gene with high specificity; in addition, no cross-reactivity was observed for other types of β-lactamase producers (OXA-23-like, OXA-40-like, OXA-58-like, and IMP-1), as indicated by the absence of false positive or false negative results. The detection limit for this assay was found to be 10^0 CFU per tube which was 100-fold more sensitive than a polymerase chain reaction assay for IS*Aba1*-*bla*$_{\text{OXA-51-like}}$ detection. Furthermore, the LAMP assay provided swift detection of the IS*Aba1*-*bla*$_{\text{OXA-51-like}}$ gene, even directly from clinical specimens. In summary, we have described a new, rapid assay for the detection of the IS*Aba1*-*bla*$_{\text{OXA-51-like}}$ gene from *A. baumannii* that could be useful in a clinical setting. This method might facilitate epidemiological studies and allow monitoring of the emergence of drug resistant strains.

1. Introduction

Acinetobacter baumannii is a major pathogen of nosocomial infections, which mainly cause respiratory, urinary tract, and surgical site infections, as well as ventilator-associated pneumonia, secondary meningitis, and even bacteremia (Rahal and Urban, 2000; Cerqueira and Peleg, 2011). *A. baumannii* poses a great threat to patients in intensive care units and in epidemic outbreaks of infection as they are often associated with multidrug-resistance to different antimicrobial classes (Alvargonzalez et al., 2014; Dettori et al., 2014; Nhu et al., 2014). Carbapenems are known as the last remaining effective antibiotics for treating infections caused by multidrug-resistant *A. baumannii*; however there are increasing numbers of reports of *A. baumannii* exhibiting resistance to carbapenems through the acquisition of carbapenemase.

Carbapenem-hydrolysing class D (OXA-type) β-lactamases are the most commonly identified carbapenemases in *A. baumannii* (Bonnin et al., 2013; Evans and Amyes, 2014). These include the acquired OXA-23-like, OXA-40-like, and OXA-58-like carbapenemases as well as the intrinsic OXA-51-like form (Evans and Amyes, 2014), which occurs naturally in the *A. baumannii* genome and exhibits weak catalytic activity (Evans et al., 2013). However, only those isolates with the insertion sequence IS*Aba1* adjacent to the *bla*$_{\text{OXA-51-like}}$ gene were resistant to carbapenems (Turton et al., 2006; Figueiredo et al., 2009). IS*Aba1* belongs to the IS4 family, appears to be relatively unique to *A. baumannii* (Segal et al., 2005), and contains promoter regions to induce the overexpression of down-stream resistance determinants. After insertion of IS*Aba1*, the minimum inhibitor concentration (MIC) of imipenem and meropenem increased from 1 and 2 mg·ml^{-1} to 8 and 32 mg·ml^{-1}, respectively, in *A. baumannii* isolates carrying the *bla*$_{\text{OXA-51-like}}$ gene (Turton et al., 2006). Previously, the presence of the IS*Aba1*-*bla*$_{\text{OXA-51-}}$

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like gene was found to be one of the most prevalent carbapenem resistance mechanisms in *A. baumannii* isolates worldwide (Higgins et al., 2010; Endo et al., 2012). For the detection of carbapenem-resistant organisms, phenotypic detection by culture-based methods (e.g., the modified Hodge test or susceptibility testing) are required prior to the application of routine diagnostic methods. However, phenotypic detection is time-consuming and often requires additional confirmation (Nordmann et al., 2012). Therefore, the rapid identification of the IS*Aba1*-$bla_{\text{OXA-51-like}}$ resistance gene is critical for the appropriate application of antibiotic therapy and infection control, especially in an outbreak setting.

Loop-mediated isothermal amplification (LAMP) is a simple and cost-effective assay that amplifies target sequences with high speed, specificity, and sensitivity under isothermal conditions. LAMP is based on auto-cycling strand displacement DNA synthesis in the presence of *Bacillus stearothermophilus* (*Bst*) polymerase (Tomita et al., 2008). This study aimed to develop and evaluate a rapid detection method for the resistance gene IS*Aba1*-$bla_{\text{OXA-51-like}}$ based on LAMP assays, and to apply this assay to the detection of IS*Aba1*-$bla_{\text{OXA-51-like}}$-positive strains in *A. baumannii* isolates and clinical samples.

2. Materials and methods

2.1. Bacterial isolates and DNA extraction

A total of 70 *A. baumannii* isolates obtained from various hospitals in different geographical regions of Japan between 2009 and 2015, were used in this study. These non-duplicate isolates were randomly selected from different patients and periods and were identified as *A. baumannii* by polymerase chain reaction (PCR) amplification of the *recA* gene (Krawczyk et al., 2002) and by DNA sequencing of a partial RNA polymerase β-subunit (*rpoB*) gene (La Scola et al., 2006). In order to evaluate the specificity and sensitivity of the LAMP assay, Ab14, which produces OXA-66, one of the predominant members of closely related OXA-51-like subgroups, was used as a reference strain for IS*Aba1*-$bla_{\text{OXA-51-like}}$-positive isolates. The antimicrobial susceptibility to imipenem was determined by the microdilution method according to the Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI, 2012).

The boiling method was utilized to extract the total bacterial DNA. Cells were heated at 99 °C for 10 min and the supernatants were obtained by centrifugation at 10,000 rpm for 5 min.

2.2. PCR assay

The presence of OXA-carbapenemase genes ($bla_{\text{OXA-23-like}}$, $bla_{\text{OXA-40-like}}$, $bla_{\text{OXA-51-like}}$, $bla_{\text{OXA-58-like}}$, $bla_{\text{OXA-143-like}}$, and $bla_{\text{OXA-235-like}}$) and of IS*Aba1* was assessed by PCR amplification (Woodford et al., 2006; Valenzuela et al., 2007; Higgins et al., 2013). PCR mapping experiments and DNA sequencing to confirm the position of IS*Aba1* relative to $bla_{\text{OXA-51-like}}$ were carried out using combinations of the IS*Aba1* forward primer (ISAba1 F) and $bla_{\text{OXA-51-like}}$ reverse primer (OXA-51-like R) as described previously (Valenzuela et al., 2007).

2.3. ***LAMP*** *primer design and* ***assay***

The LAMP primers were designed using PrimerExploreV4 software (http://primerexplorer.jp/elamp4.0.0/index.html) as described previously (Nakano et al., 2015). A set of outer primers (F3 and B3), inner primers (FIP and BIP), and one loop primer (LB, to accelerate the reaction) were designed specifically to recognize seven distinct regions including both the IS*Aba1* and $bla_{\text{OXA-51-like}}$ genes (Fig. 1). The IS*Aba1*-$bla_{\text{OXA-51-like}}$ gene reference sequence was taken from the DNA sequence of *A. baumannii* isolates (GenBank accession number HM545089).

The LAMP assay was carried out in 25 μl reaction mixtures using a Loopamp DNA amplification kit (Eiken Chemical Co. Ltd., Tokyo, Japan) containing the following reagents: 12.5 μl 2× Reaction Mix, 40 pmol each FIP and BIP, 5 pmol each F3 and B3, 20 pmol LB, 1 μl *Bst* DNA Polymerase, 1 μl DNA, and supplementary deionized water (Notomi et al., 2000). The LAMP reaction was carried out at optimal temperature for 60 min while recording the optical density at 650 nm every 6 s using a Loopamp Realtime Turbidimeter (LoopampEXIA; Eiken Chemical Co). To determine the optimal temperature, the LAMP reaction was initially performed at a range of temperatures varying from 58 to 70 °C.

To determine the sensitivity of the LAMP assay, serial dilutions of cells from the cultivated IS*Aba1*-$bla_{\text{OXA-51-like}}$-positive isolate Ab14 (10^0–10^6 CFU per reaction) were used. For sensitivity comparison between the two methods, PCR amplification targeting the IS*Aba1*-$bla_{\text{OXA-51-like}}$ gene was also performed on the serial dilutions, as previously described (Valenzuela et al., 2007).

2.4. Direct LAMP detection from human specimens

The sensitivity of the LAMP assay for direct detection of the IS*Aba1*-$bla_{\text{OXA-51-like}}$-positive isolate from human specimens was next evaluated. Sputum specimens were collected from healthy volunteers. The protocol was approved by the Ethical Review Committee at the Teikyo University School of Medicine (No. 14–128) and written informed consent was obtained from all participants.

Serial dilutions of cultivated IS*Aba1*-$bla_{\text{OXA-51-like}}$-positive isolate cells (10^0–10^7 CFU per ml sample) were prepared and added to the 2 ml sputum specimens. DNA was extracted from these samples directly using the MORA-EXTRACT kit (Kyokuto Pharm Co. Ltd., Japan) according to the manufacturer instructions.

3. Results

3.1. Carbapenemase gene analysis by PCR

Molecular characterization of the 70 clinical *A. baumannii* isolates was performed by PCR and DNA sequencing analysis to detect the presence of carbapenemase genes and PCR mapping was used to confirm the association between the IS*Aba1* sequence and the $bla_{\text{OXA-51-like}}$ gene. All 70 isolates were found to possess the intrinsic $bla_{\text{OXA-51-like}}$ genes including $bla_{\text{OXA-51}}$, $bla_{\text{OXA-66}}$, $bla_{\text{OXA-80}}$, and $bla_{\text{OXA-83}}$ genes. Of the 70 isolates, 60 were found not having acquired OXA genes (i.e., $bla_{\text{OXA-23-like}}$, $bla_{\text{OXA-40-like}}$, and $bla_{\text{OXA-58-like}}$). Of the 60 isolates possessing only intrinsic $bla_{\text{OXA-51-like}}$ genes, 36 carbapenem non-susceptible isolates (MIC of imipenem, ≥8 μg/ml) were positive for an IS*Aba1*-$bla_{\text{OXA-51-like}}$ gene in which the IS*Aba1* sequence was present upstream of and adjacent to the $bla_{\text{OXA-51-like}}$ gene (Table 1). In contrast, all 24 carbapenem-susceptible isolates were negative for IS*Aba1*-$bla_{\text{OXA-51-like}}$. Additionally, nine of the susceptible isolates were determined to be positive for IS*Aba1* and $bla_{\text{OXA-51-like}}$ sequences; however, in these isolates, the IS*Aba1* sequence was not located upstream of the $bla_{\text{OXA-51-like}}$ gene and thus, did not confer resistance.

The remaining 10 of the 70 isolates were negative for the IS*Aba1*-$bla_{\text{OXA-51-like}}$ gene but had, however, acquired other OXA genes (6 $bla_{\text{OXA-23-like}}$, 1 $bla_{\text{OXA-40-like}}$, or 3 $bla_{\text{OXA-58-like}}$) and were resistant to carbapenem. The IS*Aba1* sequence therein was located upstream of and adjacent to the acquired OXA genes.

3.2. Relative sensitivity of the LAMP and PCR assays

For the rapid and specific detection of IS*Aba1*-$bla_{\text{OXA-51-like}}$, LAMP primers were designed as shown in Fig. 1. The LAMP reaction was accomplished using a carbapenem-resistant isolate, Ab14, which was previously demonstrated to be positive for IS*Aba1*-$bla_{\text{OXA-51-like}}$ using PCR. Turbidity measurements indicated that the optimal temperature for successful LAMP amplification was 60 °C (data not shown).