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Original Article

Rapid identification of imipenem-resistant *pseudomonas aeruginosa* by loop-mediated isothermal amplification based detection of *OprL* and *OprD2* genes

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Abstract: Objective: The incidence of imipenem-resistant *Pseudomonas aeruginosa* (IRPA) continues increasing rapidly. Sensitive approaches for detecting IRPA infection are warranted to improve the ability to select appropriate antibiotic treatment. Methods: In this study, we optimized a rapid DNA-based diagnostic technique, the visual loop-mediated isothermal amplification (LAMP) technique for detecting of the outer membrane lipoprotein (*OprL*) gene and the loop 2 of outer membrane protein (*OprD2*) gene. Results: The target DNA was amplified and detected by LAMP method within 60 min at an isothermal temperature of 65°C. The sensitivity of LAMP, which had a detection limit of 17.41 μg/L DNA, was 10-fold greater than that of conventional PCR. Furthermore, positive LAMP results were directly read by the naked-eye. Thus, the visual LAMP method can be suggested as a simple and rapid diagnostic assay. Finally, 62 clinical isolates including 39 *P. aeruginosa* and 23 negative isolates were detected for *OprL* and *OprD2* genes by visual LAMP, which was showed to be more specific than conventional PCR. Conclusion: The LAMP method is a rapid, simple, sensitive and specific assay for the detection of *OprL* and *OprD2* genes.

Keywords: Loop-mediated isothermal amplification (LAMP), imipenem-resistant *pseudomonas aeruginosa* (IRPA), the outer membrane lipoprotein (*OprL*), the loop 2 of outer membrane porins (*OprD2*)

Introduction

Pseudomonas aeruginosa is a major opportunistic pathogen found in field trauma hospitals. It has a high prevalence of antibiotic-resistant strains, which can lead to sepsis and even death if not treated with appropriate and effective antibiotics timely [1]. Carbapenems are overused for their potency, leading to the high frequency of carbapenems resistance, particularly to imipenem, in *P. aeruginosa* strains [2]. Currently, the traditional techniques used to identify imipenem-resistant *P. aeruginosa* (IRPA) infection involve bacterial growth in selective media and antibiotic susceptibility testing [3, 4]. However, these traditional methods not only require professional technical personnel but also take 3-5 days. Although the Polymerase chain reaction (PCR) method has been widely employed to detect the above resistant-gene of bacteria in the past decades, there are still some drawbacks, such as expensive equipment, the dependence of the specificity on primer design and reaction conditions, and electrophoretic analysis. So it is not suitable for use in primary clinical health team or field. Thus, the development a simple, rapid, and sensitive detection for IRPA infection is urgently needed.

In the past decades, many studies have showed that imipenem specifically targets penicillin-binding protein of bacterial inner membrane in an *OprD* porin-dependent manner. It has been demonstrated that the loop2 of outer membrane porins (*OprD*) is a specific channel which is associated with a basal level of resistance to Carbapenem antibiotics, especially imipenem

Table 1. Strains used in this study

Specics	No. of strains	Source
P. aeruginosa	1	ATCC27853[a]
P. aeruginosa	39	Clinical isolate
P. fluorescens	1	Clinical isolate
P. putida	1	Clinical isolate
P. stutzeri	1	Clinical isolate
P. mendocina	1	Clinical isolate
P. luteola	1	Clinical isolate
Non-Pseudomonas strain		
Acinetobacter baumannii	6	Clinical isolate
Acinetobacter junii	1	Clinical isolate
Leclercia adecarboxylata	1	Clinical isolate
Stenotrophomonas maltophilia	1	Clinical isolate
Klebsiella pneumoniae	1	Clinical isolate
Streptococcus pneumoniae	1	Clinical isolate
Citrobacter freundii	1	Clinical isolate
Staphylococcus aureus	1	ATCC25923[a]
Staphylococcus aureus	1	Clinical isolate
Enterococcus faecalis	1	ATCC29212[a]
Enterococcus faecalis	1	Clinical isolate
Escherichia coli	1	ATCC25922[a]
Escherichia coli	1	Clinical isolate

[a]American Type Culture Collection, USA.

[5, 6]. Deletion of *OprD2* gene could inactivate *OprD* and lead to imipenem resistance [7]. Moreover, De Vos et al have studied and found the outer membrane lipoprotein (*OprL*) gene can be only amplified from *P. aeruginosa*, while positivity was not found in other bacteria [8]. We therefore hypothesized that a sensitive and rapid molecular method of detecting *OprL* and *OprD2* genes can be built to identify IRPA isolates, which will help the patient to achieve individualized treatment.

The loop-mediated isothermal amplification (LAMP) method is a one-step amplification method to identify the target genes in biological samples. A set of 4 oligonucleotide primers recognizing 6 distinct regions were found to amplify target DNA in the presence of the large fragment of *Bst* DNA polymerase at a constant temperature 65°C within 60 min [9]. Compared with the traditional method, LAMP has the following advantages: First, it can specifically detect target sequences under isothermal conditions, which can be performed by a simple water bath; Second, positive reactions can be identified by turbidity from pyrophosphate precipitation or color change by an intercalating dye, the metal ion indicators hydroxy naphthol blue (HNB) or calcein are also useful indicators, allowing easy visualization of positive reactions that turn from violet to sky blue or from orange to yellow [10, 11]. Besides the relative ease of performance LAMP, its sensitivity and specificity to detection is equal to or higher than conventional PCR, and it is faster [12-14].

In this study, we tested whether the visual LAMP assay (HNB or calcein) could effectively detect the presence or absence of the *OprL* and *OprD2* gene in *P. aeruginosa,* and we subsequently compared these results with the conventional PCR method. This study establishes a theoretical basis for using LAMP as a simpler and faster colour-based technique for early diagnosis of IRPA, allowing for easier determination of appropriate and effective antibiotics for infected patients that are accessible to a wide range of clinical settings.

Materials and methods

Ethics

In this study, the use of the clinical specimens (including 50 sputum, 5 blood, 3 urine, and 4 others) was performed after all patients had provided written informed consent. All these clinical specimen's data were analyzed anonymously. The study was approved by the Ethics Committee of Southwest hospital, Third Military Medical University.

Bacterial strains and antimicrobial susceptibility test

All the bacteria strains used in this study were obtained between January 2014 and March 2015 from the Department of Microbiology laboratory at the Southwest Hospital of Third Military Medical University (Chongqing, China). *P. aeruginosa*. ATCC 27853 (Manassas, VA, USA) served as a standard control strain (**Table 1**). Identification and antimicrobial susceptibility testing were performed using traditional methods, including VITEK 2 (BioMerieux, France) and agar dilution method. Minimal inhibitory concentration (MIC) of imipenem was determined by the agar dilution method and

Table 2. Sequence of LAMP primers for specific amplification of the P. aeruginosa OprD2 gene

Primer	Type	Sequence (5'→3')	Length
F3	Forward outer	GGTGCGCATCTCCAAGAC	18 bp
B3	Backward outer	CGATTTGACGGTGGTCGG	18 bp
FIP (F1C+F2)	Forward inner	TCGCGGTCTGCGGGAACAGATGCTGAAGTGGGGCGAG	37 bp
BIP (B1C+B2)	Backward inner	CCGGCTTCCAGCTGCAGAGTGAAGTGGCCTGCCTCG	36 bp

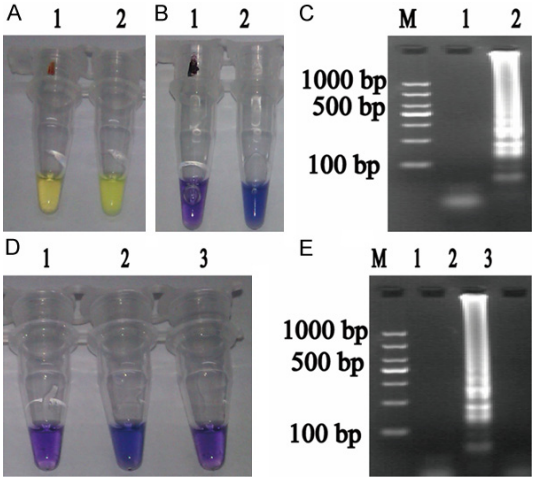

Figure 1. A, B. LAMP assay on *OprL* gene of *P. aeruginosa,* and visual detection of LAMP products in daylight. A. Calcein: colors changed from orange (negative) to yellow (positive). B. HNB: colors changed from violet (negative) to sky-blue (positive). C. M, DL1000 DNA ladder; 1, Negative control (no template); 2, Amplified *OprL* gene LAMP product exhibited a ladder-like pattern; D, E. LAMP assay on *OprD2* gene and visual detection of products in daylight. D. HNB: colors changed from violet (negative) to sky blue (positive). E. M, DL1000 DNA ladder; 1, Negative control (no template); 2, Amplified *OprD2* gene LAMP product exhibited a ladder-like pattern; 3, *OprD2*-deletion specimen.

interpreted by the Clinical and Laboratory Standards Institute guidelines.

DNA preparation

Specimens were cultured aerobically overnight at 37°C on nutrient agar. A loopful of cultured cells were placed in 100 μL of 5% chelex-100 and boiled for 10 min. Cell debris were removed by centrifugation at 12,000 *g* for 5 min, and supernatant containing the genomic DNA was stored at -20°C until use in the LAMP and PCR assays.

LAMP primer design

The LAMP primers of *P. aeruginosa OprL* (GenBank accession no. Z50191) was part of a set published by Goto et al [15] (data not shown). LAMP primers for the *OprD* (GenBank accession no. Z14065) including the 11-bp deletion were designed specifically for this study [16, 17]. A set of 4 primers-consisting of 2 outer (F3 and B3) and 2 inners (FIP and BIP) primers, collectively capable of recognizing 6 distinct regions on the target sequence-were designed with the PrimerExplorer V4 software (http://primerexplorer.jp/e/) (**Table 2**).

Visual LAMP assay and product detection

Visual LAMP assays were performed in a 25-μL reaction mixture containing: 1.0 μL DNA; 1.6 μM each of FIP and BIP; 0.4 μM each of F3 and B3; 1.0 mM of each dNTP; 1.0 μL of 8 U/μL *Bst* DNA polymerase large fragment (New England BioLabs, U.S.); 1.0 μL of 3 mM HNB trisodium salt (CAS: 63451-35-4, Sigma-Aldrich, U.S.) or 1.0 μL of 625 μM calcein (CAS:1461-15-0, Sigma-Aldrich) and 12.5 mM $MnCl_2$ in LAMP buffer (20 mM Tris-HCl [pH 8.8]; 8 mM $MgSO_4$, 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 0.8 M betaine (Sigma-Aldrich); and 0.1% Triton X-100 (New England BioLabs). Reactions were incubated at 60°C, 63°C, and 65°C for 1 h and then heated to 80°C for 5 min for termination. Results were considered negative if the reaction appeared violet for HNB or orange for calcein/Mn^{2+} and positive if it appeared sky blue or yellow, respectively. Positive assay was further approved by 2% agarose gel (Amresco, U.S.) electrophoresis.

PCR assay

PCR primers were provided by De Vos et al and synthesized by Shanghai Sangon [8] (data not shown). PCR amplification was carried out with a PCR system 9700 (Applied Biosystems, Foster City, CA, USA) with following conditions: 3 min of denaturation at 94°C and 30 cycles of denaturation at 94°C for 30 sec; annealing at 60°C for 30 secs; and extension at 72°C for 3 min. The PCR products were analysed by 2% agarose gel electrophoresis.

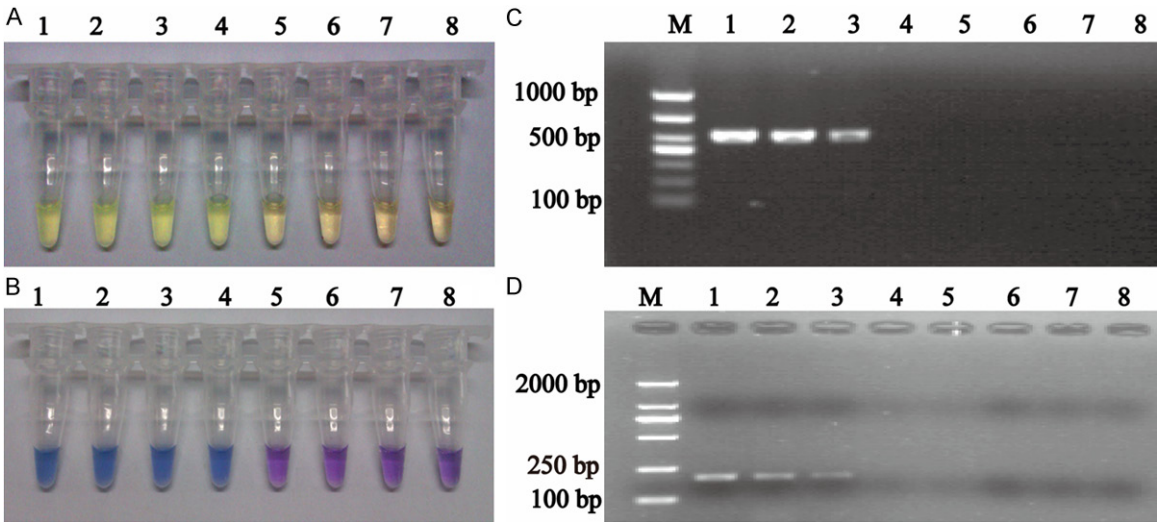

Figure 2. Evaluation of the sensitivity of detecting the *P. aeruginosa OprL* and *OprD2* genes using the visual LAMP assay vs. the PCR assay. A. Calcein-based LAMP assay: colors changed from orange (negative) to yellow (positive). B. HNB-based LAMP assay: colors changed from violet (negative) to sky blue (positive). C. *OprL* PCR assay. D. *OprD2* PCR assay. Samples in tubes/lanes 1-8 contained 10-fold serial dilutions of *P. aeruginosa* DNA. 1, 17.414 mg/L; 2, 1.7414 mg/L; 3, 174.14 μg/L; 4, 17.414 μg/L, 5, 1.7414 μg/L; 6, 174.14 ng/L; 7, 17.414 ng/L; 8, Negative control (no template); M, DL1000, 2000 DNA ladder.

Specificity and sensitivity of visual LAMP assay

The specificity of the *P. aeruginosa* primers was determined using DNA from the standard *P. aeruginosa* strain as a positive control and non-*P. aeruginosa* DNA isolates as negative controls. Sterile double-distilled water was used as a blank control for LAMP. DNA templates were prepared by boiling for 10 min. To determine sensitivity, stock *P. aeruginosa* genomic DNA (17.41 mg/L) was serially diluted in 10-fold increments and assayed by LAMP and PCR, separately.

Statistical analysis

All statistical analyses were performed using SPSS 19.0. Compared with the *OprD2*-positive strains, the imipenem resistance rate of *OprD2*-negative strains was used the chi-square test. $P < 0.05$ was considered statistically significant.

Results and discussion

Establishment and Optimization of a visual LAMP reaction

The LAMP reaction conditions were optimized and accomplished using the extracted DNA. Results showed that when 1.0 μL of 625 μM calcein and 12.5 mM $MnCl_2$ were mixed with DNA prior to the reaction and incubated at 65°C for 1 h. Positive tubes displayed bright yellow fluorescence, while negative tubes remained orange (**Figure 1A**). When 1.0 μL of 3 mM HNB was added before the reaction and incubated for 1 h at 65°C, positive tubes were bright blue, and negative tubes remained violet in natural light (**Figure 1B**). Results were verified by electrophoresis and were consistent with the color-based results (**Figure 1C**). HNB-based LAMP amplification of the *OprD2* gene is shown in **Figure 1D** and **1E**. In this way, the color-based LAMP assay was able to detect the deletion of *OprD2* in IRPA.

Sensitivity of a visual LAMP assay

To determine the sensitivity of the LAMP for *OprL* and *OprD2* genes, genomic DNA templates from pure cultures of stock *P. aeruginosa* were serially diluted in 10-fold increments, and each dilution was assayed by LAMP and conventional PCR. As shown in **Figure 2**, the detection limit of the LAMP method for the *OprL* and *OprD2* genes was 17.41 μg/L, while we observed that the detection limit of the conventional PCR method was 174.14 μg/L. The detection limit of the LAMP method was 10 times higher than that of the PCR method.

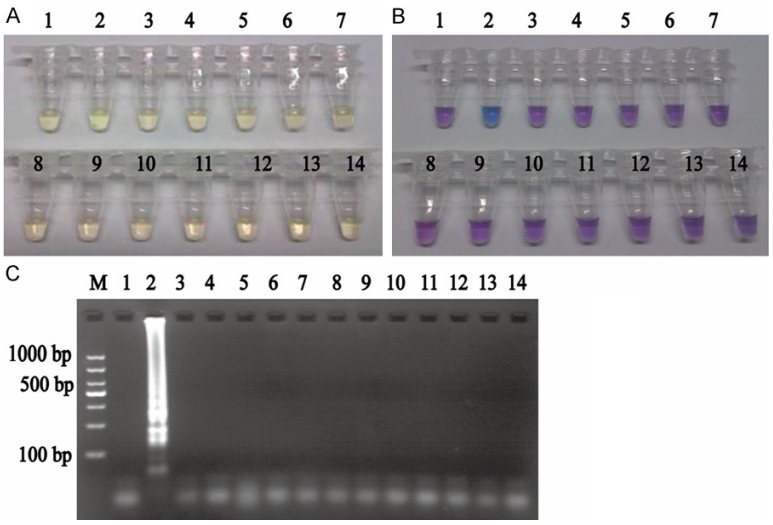

Figure 3. Specificity analyses of the visual LAMP assay for the *OprL* gene. A. Calcein: colors changed from orange (negative) to yellow (positive). B. HNB: colors changed from violet (negative) to sky blue (positive). C. Agarose gel electrophoresis visualization of LAMP results. A ladder-like pattern is observed only in the second lane. M, DL1,000 DNA ladder; 1, Negative control (no template); 2, *P. aeruginosa*; 3, *P. fluorescens*; 4, *P. putida*; 5, *P. luteola;* 6, *P. mendocina*; 7, *Acinetobacter baumannii*; 8, *Stenotrophomonas maltophilia*; 9, *Klebsiella pneumoniae*; 10, *Streptococcus pneumoniae*; 11, *Citrobacter freundii*; 12, *Staphylococcus aureus*; 13, *Enterococcus faecalis*; 14, *Escherichia coli.*

Specificity of a visual LAMP assay

To determine the specificity of this LAMP assay for *OprL* gene, genomic DNA from various bacterial species were tested using two dyes LAMP. As showed in **Figure 3A**, **3B**, only the tube containing *P. aeruginosa* changed color in both the HNB- and calcein/Mn^{2+}-based LAMP assays, while the blank control (double-distilled water) and other tubes remained negative. Gel electrophoresis analysis of the LAMP products were consistent with the results of the visual LAMP assay, where amplification products were only detected in the *P. aeruginosa* isolates (**Figure 3C**). To determine the specificity of this LAMP assay for *OprD2* gene, the *OprD2*-positive and *OprD2*-negative *P. aeruginosa* were tested using HNB-LAMP. Only the *OprD2*-positive tube changed color, while the blank control (double-distilled water) and other tubes remained negative (data not shown).

Clinical isolates detection

To evaluate the performance of the visual LAMP method for clinical isolates, the traditional method (bacterial culture, VITEK 2 system, and agar dilution method) were used to test the collected clinical isolates while simultaneously using 1.0 μL of DNA for visual LAMP and conventional PCR methods. 62 clinical isolates including 39 *P. aeruginosa* and 23 negative isolates obtained from hospital rooms by the traditional method were tested. For all 39 *P. aeruginosa*-positive isolates, the detection rate of *OprL gene* by LAMP was 39/39 (100%), and the detection rate of *OprL gene* by conventional PCR was 37/39 (94.9%). Among these 39 *P. aeruginosa*, 19 were determined to be imipenem-resistant based on the agar dilution method (MIC ≥ 16 μg/mL), and the other 20 strains were found to be sensitive to imipenem (MIC ≤ 2 μg/mL). Of the 19 imipenem-resistant strains, 14 were negative for OprD2 gene by LAMP and conventional PCR. Of the 20 imipenem-sensitive strains, 15 were positive for OprD2 gene by LAMP and 14 were positive by conventional PCR (**Table 3**). Statistical analysis showed that there was significant difference in imipenem resistance between OprD2-negative strains and -positive strains ($P < 0.05$).

In recent years, imipenem-resistant *P. aeruginosa* strains (IRPA) have become increasingly prevalent and now pose a significant public health problem [18-20]. In this study, we describe a visual LAMP method for the identifying IRPA infection for the first time. The *OprL* and *OprD2* genes were targeted. These results show that target DNA was specifically amplified and visualized by LAMP method within 60 min after DNA extraction at an isothermal temperature of 65°C. Besides, both calcein and HNB dyes were used to qualitatively detect the *OprL* gene of *P. aeruginosa* through visual colour changes under ambient light. These dyes were added before the LAMP reaction and did not affect LAMP biochemistry. The results showed that, when used with two dyes, the LAMP method had the higher sensitivity than the tradition-

Table 3. Sensitivity and specificity of the visual LAMP method compared to traditional method and PCR method

Traditional method	LAMP		PCR	
	Positive	Negtive	Positive	Negative
OprL				
Positive (39)	39	0	37	2
Negative (23)	0	23	3	20
Sensitivity	100%		94.9%	
Specificity	100%		87.0%	
PPV	100%		92.5%	
NPV	100%		90.9%	
OprD2				
Imipenem-resistant (19)[1]	5	14	5	14
Imipenem-sensitive (20)[2]	15	5	14	6
Sensitivity	75%		70%	
Specificity	73.7%		73.7%	
PPV	75%		73.7%	
NPV	73.7%		70%	

Traditional method: bacterial culture, VITEK 2 system, and agar dilution method. VITEK 2 system: a fluorescence-based automated identification system. LAMP: loop-mediated isothermal amplification. PCR: polymerase chain reaction. PPV, positive value. NPV, negative value. [1]: imipenem MIC ≥ 16 μg/mL. [2]: imipenem MIC ≤ 2 μg/mL.

al PCR. Because HNB was easier to prepare and the resulting color change was more obvious to the naked eye than that of calcein, HNB dye was selected for detection of the OprD2 gene in subsequent studies.

The sensitivity of LAMP, which showed a detection limit of 17.41 μg/L DNA, was 10-fold higher than that of conventional PCR. Several possible factors may contribute to this fact. LAMP is inherently tolerant of inhibitors, and DNA does not need to be purified if it is meant to be applied to crude sample matrices [21]. However, the sensitivity of PCR can be greatly reduced if the sample contains DNA inhibitors. In addition, four primers targeting six independent target sequence regions were used for the LAMP reaction, but only two primers targeting two independent target sequence regions were used in the PCR reaction. The specificity and sensitivity of LAMP reaction were greatly enhanced. During the study, of the 39 P. aeruginosa-positive isolates detected by traditional method, none was negative by LAMP and 2 were negative by PCR method. Of the 23 P. aeruginosa-negative isolates, none was positive by LAMP and 3 were positive by PCR. The LAMP method was demonstrated to be a specific and sensitive assay. Of the 19 imipenem-resistant isolates, 14 were negative for *OprD2* gene by LAMP and PCR. Of the 20 imipenem-sensitive strains, 15 were positive for *OprD2* gene by LAMP and 14 were positive by PCR. The results are consistent with previous study showing that *OprD* mutations were the major determinant of imipenem resistance [4]. Then we did a statistical analysis for the imipenem resistance rate between *OprD2*-negative strains and *OprD2*-positive strains, and the results suggested that *OprD2*-negative strains have a higher risk of imipenem resistance than *OprD2*-positive strains.

There are many advantages of LAMP method. However, it also has some disadvantages, such as false-positive results, and the laboratory cross-contaminations in early experiment. This is that the amplification sensitivity of the LAMP reaction is extremely high. To prevent the undesirable results, the reagent preparation and performance of the test should be done in different lab rooms. Moreover, don't open the tube cover after reaction.

Conclusion

In conclusion, a rapid, sensitive and simple LAMP method for *OprL* and *OprD2* genes detection in imipenem-resistant *P. aeruginosa* strains was established. Early identifying the *OprL* and *OprD2* gene will help us select a better antibiotic treatment proposal for IRPA infection, which will reduce costs and time. This diagnostic assay is more suitable for use in primary clinical health team or field.

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Disclosure of conflict of interest

None.

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