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Short Communication

Rapid and sensitive detection of the *vanA* resistance gene from clinical *Enterococcus faecium* and *Enterococcus faecalis* isolates by loop-mediated isothermal amplification

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ABSTRACT

Objectives: Vancomycin resistance in *Enterococcus* spp., mediated mainly by the *vanA* resistance gene, has become a major health concern as it has spread worldwide. Therefore, a rapid method is urgently required to detect the *vanA* gene for timely and appropriate antimicrobial control of resistant *Enterococcus* infections.
Methods: The loop-mediated isothermal amplification (LAMP) assay was optimised for *vanA* detection in *Enterococcus* spp. isolates.
Results: The LAMP primer set designed in this study could reliably recognise seven distinct regions of the *vanA* gene and amplify the gene within 25 min at an isothermal temperature of 65 °C with high specificity. The sensitivity of the optimised assay was high, with a detection limit for *vanA* as low as 100 pg/μL, which is 100-fold more sensitive than the PCR assay. A special advantage of this optimised LAMP method is that the *vanA* gene could be detected directly from clinical specimens.
Conclusion: This optimised LAMP assay has great application potential for efficient detection of *vanA* in clinical diagnosis and epidemiological studies.

1. Introduction

Vancomycin-resistant enterococci (VRE) strains were isolated first in Europe in 1988 [1] and were then reported in North America [2], Asia [3] and other regions. By 2003, the vancomycin resistance rates of *Enterococcus* spp. had increased to 19% in Ireland, 23% in Greece, 28.5% in the USA and as high as 50% in Portugal [4,5]. *Enterococcus faecalis* and *Enterococcus faecium* are the two leading human-infecting *Enterococcus* spp. [6]. In China, VRE was first reported in 2007 [7]. In Beijing, the isolation rates of vancomycin-resistant *E. faecium* and *E. faecalis* were 3.6% and 0.6% in 2010 but increased to 14.3% and 1.3%, respectively, in 2013 [8]. Globally, VRE have become an increasingly serious health problem owing to rapid spread of the vancomycin resistance genes.

There are eight types of vancomycin resistance genes known to date, including *vanA*, *vanB*, *vanC*, *vanD*, *vanE*, *vanG*, *vanL* and *vanN* [9,10], among which the transmissible genes *vanA* and *vanB* are the most prevalent [11–13]. On the other hand, *vanC* is an inherent resistance gene and is seldom found even in susceptible *E. faecalis* or *E. faecium* strains, whereas *vanD*, *vanE*, *vanG*, *vanL* and *vanN* encode medium-low levels of resistance [10,14–16]. Therefore, detection of *vanA* and *vanB* by rapid and sensitive assays is of great importance for timely and appropriate therapy, especially in outbreaks.

Conventional detection of vancomycin resistance by culture-based methods, such as the Kirby–Bauer and broth dilution methods, is both technically complicated and time-consuming.

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Molecular diagnostic techniques, such as conventional and real-time PCR methods, require specialised and costly instruments and consumables. Therefore, a rapid, sensitive and cost-effective assay is urgently required to detect vancomycin resistance genes, such as the loop-mediated isothermal amplification (LAMP) method, which amplifies DNA with high specificity and sensitivity [17]. LAMP requires only a temperature-controlled water- or dry-bath and the results can be analysed directly by the naked eye. So far, the LAMP technique has been used in the detection of *Enterococcus* bacteria in water [18] and the resistance gene *vanA* of enterococci from stools [19]. In this study, the LAMP assay was optimised for rapid and sensitive detection of the *vanA* gene in *Enterococcus* spp. bacteria directly from clinical samples.

2. Materials and methods

2.1. Bacterial isolation, identification and DNA extraction

A total of 210 *E. faecalis* strains and 8 *E. faecium* strains isolated from urine samples between 2014–2016 were collected in this study (Supplementary Table S1). All strains were grown at 37 °C on blood agar. Total bacterial DNA was extracted by the boiling method. Cells were heated at 99 °C for 5 min and the supernatant containing bacterial DNA was then collected by centrifugation at 10,000 rpm for 5 min and was stored at 4 °C for the assay.

2.2. PCR assay to detect the vancomycin resistance gene vanA

PCR as a comparative method for VRE resistance gene detection was performed using a TaKaRa PCR Amplification Kit (Takara Bio Inc., Shiga, Japan) in a 50 μL volume containing 5 μL of 10× buffer, 4 μL of dNTPs, 15 nmol forward primer, 15 nmol reverse primer, 1 μL of *Taq* DNA Polymerase (Takara Bio Inc.), 5 μL of sample DNA and 32 μL of distilled water. PCR cycling conditions were 94 °C for 5 min, followed by 30 cycles of 94 °C for 45 s, 55 °C for 45 s and 72 °C for 75 s, and a final extension at 72 °C for 2 min. All PCR products were analysed by 1% agarose gel electrophoresis and were visualised using an ultraviolet (UV) transilluminator (Bio-Rad, Hercules, CA). The primers for the *vanA* gene were F, 5′-CATGAATAGAATAAAAGTTGCAATA-3′ and R, 5′-CCCCTTTAACGC-TAATACGATCAA-3′.

2.3. LAMP primer design and LAMP assay

Sequences of the *vanA* gene were downloaded from the National Center for Biotechnology Information (NCBI) database. Consensus sequences were chosen for primer design using the LAMP primer designing software PrimerExplorer v.4 (http://primerexplorer.jp/e/). As shown in Table 1, a set of outer primers (F3 and B3), inner primers (FIP and BIP) and loop primer (LB, to accelerate the reaction), which specifically recognise seven distinct regions of the *vanA* gene, were determined based on their reaction temperatures and dG values (stability values). LAMP reactions were performed in a 20 μL reaction mixture containing 10 μL of 2× Reaction Mix (Eiken China Co., Ltd., Shanghai, China), 5 pmol each of F3 and B3, 40 pmol each of FIP and BIP, 20 pmol of LB, 1 μL of Bst DNA Polymerase (Eiken China Co., Ltd.), 1 μL of sample DNA and supplementary deionised water. The reaction was carried out in a dry-bath. The products were analysed by 1% agarose gel electrophoresis and were visualised using a UV transilluminator.

Table 1
Loop-mediated isothermal amplification (LAMP) primer set for the *vanA* gene.

Primer	Sequence
F3	5′-TTTCAGCTTTGCATGGCA-3′
B3	5′-AGGTAAACGTAGCTGCCA-3′
FIP	5′-TCGCAGCCTACAAAAGGGATTGAAGATGGATCCATACAAGG-3′
BIP	5′-AAGCTCAGCAATTTGTATGGACAAATAACCCAAAAGGCGGGA-3′
LB	5′-CATCGTTGCGAAAAATGCTGG-3′

2.4. DNA sequencing

The shortest band of the LAMP product on the gel was excised and purified using a QIAEX® II Gel Extraction Kit (QIAGEN, Shanghai, China) and was then sent to GENEWIZ Inc. (Suzhou, China) for sequencing.

2.5. Direct LAMP detection of the vancomycin resistance gene vanA from human specimens

Direct detection of the *vanA* resistance gene was performed on urine samples. Bacterial DNA from these samples was extracted using a QIAamp DNA Microbiome Kit (QIAGEN). The LAMP assay was performed under optimised temperature and reaction time.

3. Results

3.1. Detection of vanA by LAMP: optimal temperature and reaction time

To determine the optimal temperature for *vanA* detection by LAMP, total DNA was extracted from a vancomycin-resistant isolate (Ef1) that was previously demonstrated to be positive for *vanA* by PCR. LAMP assays were performed by running multiple reactions at different temperatures ranging from 58–70 °C. All of the products were then analysed by electrophoresis in one gel. As shown in Fig. 1A, the ladder-like bands were present at temperatures from 58–68 °C. No further bands were observed at temperatures beyond 68 °C. The brightest bands were observed at temperatures of 64, 65 and 66 °C.

To determine the optimal reaction time for *vanA* detection, LAMP assays were performed by running multiple reactions for various lengths of time, including 20, 25, 30, 40, 50 and 60 min. As shown in Fig. 1B, bands began to appear when the amplification was run for 25 min and the band intensity was enhanced with the increase in reaction time.

3.2. Sensitivity of vanA detection by LAMP assay

To evaluate the sensitivity of the LAMP assay for detection of the *vanA* gene, a series of 10-fold dilutions of template DNA was used in the amplification reactions, starting from 100 ng/μL down to 0 pg/μL. The detection limit of the LAMP assay for *vanA* was 100 pg/μL (Fig. 1C) compared with 10 ng/μL sensitivity of the PCR assay (Fig. 1D). Therefore, the LAMP assay was 100-fold more sensitive than the PCR assay for the *vanA* gene.

3.3. Specificity of vanA detection by LAMP assay

To evaluate the specificity of the LAMP assay for detection of the *vanA* gene, reactions were performed using DNA template from the reference strain *E. faecalis* ATCC 29212 and 68 clinical *Enterococcus* strains. As shown in Table 2, the LAMP assay successfully detected 30 *vanA*-positive *E. faecalis* and 4 *vanA*-positive *E. faecium* isolates. No products were amplified in the other 34 isolates or the reference strain *E. faecalis* ATCC 29212 that were *vanA*-negative by PCR assay.

3.4. Direct detection of vanA from urine samples by LAMP

To further evaluate the specificity of the LAMP assay for direct detection of the *vanA* gene from human specimens, urine samples

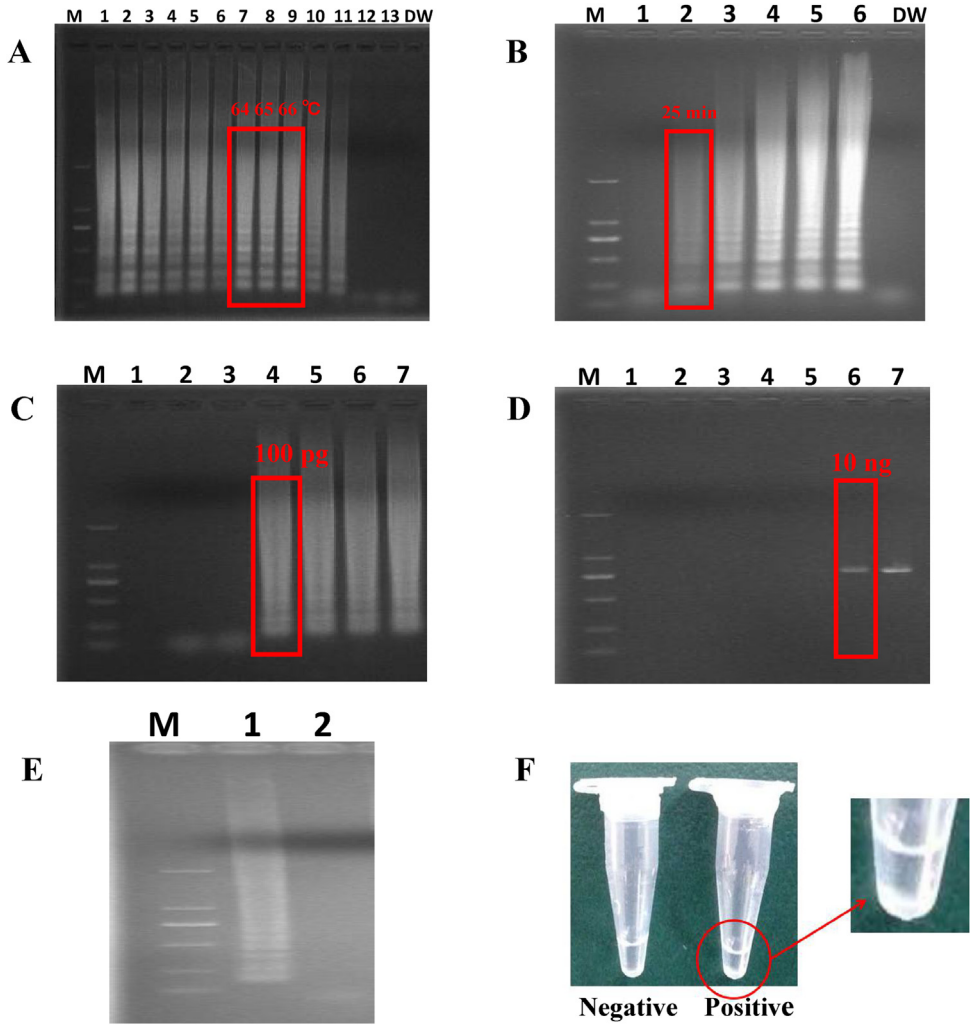

Fig. 1. Optimum conditions, sensitivity and specificity evaluations of the loop-mediated isothermal amplification (LAMP) assay for detection of the *vanA* resistance gene. (A) Determination of the optimum reaction temperature. Lanes 1–13, temperature points at 58–70 °C. Each LAMP assay was run for 60 min with 100 ng of DNA template. (B) Determination of the optimum reaction time. The LAMP assay was run at 65 °C with 100 ng of DNA template. Lanes 1–6, reaction times of 20, 25, 30, 40, 50 and 60 min, respectively. Lane M, 2000-bp DNA ladder marker; lane DW, negative control containing distilled water and reaction mixture but no template DNA. (C,D) Sensitivity evaluation of the LAMP assay (C) and PCR (D) for detection of the *vanA* gene. The LAMP assay was performed at 65 °C for 60 min. Lanes 1–7, template DNA at concentrations of 0 pg, 1 pg, 10 pg, 100 pg, 1 ng, 10 ng and 100 ng, respectively. The PCR-amplified product of the *vanA* gene was 1030 bp in size. (E) Specificity evaluation of the LAMP assay for direct detection of the *vanA* gene from urine samples. Amplification was performed at 65 °C for 40 min. Lane 1, urine sample with *vanA*-positive bacterial cells; lane 2, urine sample with *vanA*-negative bacterial cells. (F) Result of the LAMP assay identified by the naked eye. Magnesium pyrophosphate precipitate was produced in the positive reaction.

were supplemented with cultivated *vanA*-positive or *vanA*-negative bacterial cells. As shown in Fig. 1E, the LAMP assay could amplify the *vanA* gene directly from human specimens in 40 min. No product was seen in urine specimens supplemented with cultivated *vanA*-negative bacterial cells. Furthermore, the positive result could be inspected by the naked eye as some white precipitate was present at the bottom of the tube (Fig. 1F).

Table 2
Specificity evaluation of the loop-mediated isothermal amplification (LAMP) assay for the *vanA* gene compared with PCR in *Enterococcus* spp.

Enterococcus strains	PCR		LAMP	
	Positive	Negative	Positive	Negative
E. faecalis ATCC 29212	0	1	0	1
E. faecalis	30	30	30	30
E. faecium	4	4	4	4

4. Discussion

Vancomycin is considered to be the last effective antibiotic as it is still active against some multidrug-resistant bacteria. However, VRE, which first appeared three decades ago, is now a serious threat to public health as it has rapidly spread to many regions of the world. The *vanA* gene is the most common mediator of vancomycin resistance and is increasingly predominant in VRE isolates [2,20]. The fact that only the *vanA* gene was identified in all analysed vancomycin-resistant *Enterococcus* strains isolated from a broad range of Northeastern Chinese hospitals in this study suggests that the resistance of VRE is mainly mediated by the *vanA* gene in Northeastern China (Supplementary Table S2). The rapid and sensitive LAMP assay optimised in this study can efficiently detect the *vanA* gene with high sensitivity and specificity, which can greatly facilitate timely diagnosis, appropriate therapy and proper outbreak control.

The LAMP assay optimised here was able to specifically detect the resistance gene *vanA* in VRE strains within only 25 min under isothermal conditions of 65 °C using a dry-bath. The detection limit of the LAMP assay for *vanA* was 100 pg/μL, which is 100-fold more sensitive than the corresponding PCR reaction. Furthermore, the LAMP method could amplify the *vanA* gene directly from urine specimens, and the result of the LAMP assay could be quickly inspected by the unaided eye as a large quantity of the by-product magnesium pyrophosphate aggregate was generated in the reaction. Therefore, the LAMP assay is more appropriate and convenient than PCR for detection of *vanA* in the clinic, especially for rapid diagnostic and infection control purposes.

Although the LAMP assay has many advantages as reported above, its supersensitivity may lead to false-positive results due to the environmental contamination of amplicon aerosols. Strict spatial separation of reagent preparation and amplifying reactions would be necessary to guarantee its accuracy and reproducibility, such as by wax pellets that can seal the reaction system and reduce contamination [21]. In addition, without opening the tube lid, LAMP results could be easily identified by instantaneous low-speed centrifugation or addition of fluorescent dyes in the reaction mixture. All of these strategies can effectively inhibit the occurrence of false-positives results.

Since in this study only 69 enterococci strains were analysed by LAMP for the *vanA* gene, a larger sample size would be desirable to determine whether this assay will be ideally accurate when it is applied in the clinic. Therefore, further research should be carried out on larger pools of clinical samples including various *vanA*-positive bacterial strains from different types of infections.

In conclusion, a rapid, specific, sensitive and cost-effective LAMP assay was established for *vanA* gene detection in this study. This method has great potential to be applied in the clinical setting as it can detect the *vanA* gene directly from clinical specimens.

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Competing interests

None declared.

Ethical approval

Not required.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.jgar.2018.10.012.

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