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A TaqMan Duplex Quantitative PCR Method for Detecting *Klebsiella pneumoniae* has been Developed Based on Pan-Genome Analysis

(Kaedah PCR Kuantitatif Dupleks TaqMan untuk Mengesan *Klebsiella pneumoniae* telah Dibangunkan Berdasarkan Analisis Pan-Genom)

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ABSTRACT

Klebsiella pneumoniae is a significant pathogen capable of causing infections in the respiratory, urinary, and bloodstream. In this study, bioinformatics-based pan-genome analysis identified specific and conserved LptD and MerR protein gene sequences in *K. pneumoniae*. Based on these sequences, specific primers and probes were designed, and the reaction system and conditions were optimized to establish a TaqMan probe-based duplex real-time quantitative PCR method for detecting *K. pneumoniae*. The method was tested on genomic DNA from 14 common pathogens and negative controls. The results showed that only the genomic DNA of *K. pneumoniae* was positive, while all other samples were negative. The detection limits for LptD and MerR gene-positive standard plasmid DNA were 5.28×10^1 copies/μL and 5.78×10^1 copies/μL, respectively, and the coefficient of variation for Ct values between intra- and inter-gene groups was less than 3%. These results indicate that the established TaqMan probe-based duplex real-time quantitative PCR method can specifically and rapidly detect *K. pneumoniae*, which is of significant importance for clinical diagnosis and treatment.

Keywords: Duplex quantitative PCR; *Klebsiella pneumoniae*; LptD gene; MerR gene; pan-genome analysis

ABSTRAK

Klebsiella pneumoniae adalah patogen yang signifikan dan mampu menyebabkan jangkitan pada sistem pernafasan, sistem kencing dan aliran darah. Dalam kajian ini, analisis pan-genom berasaskan bioinformatik telah mengenal pasti urutan gen protein MerR dan LptD yang khusus dan terpelihara dalam *K. pneumoniae.* Berdasarkan urutan ini, primer dan prob khusus telah direka bentuk dan sistem serta syarat reaksi telah dioptimumkan untuk membangunkan kaedah PCR kuantitatif masa nyata dwi-fluoresen TaqMan untuk mengesan *K. pneumoniae.* Kaedah ini telah diuji pada DNA genom daripada 14 patogen biasa dan kawalan negatif. Keputusan menunjukkan bahawa hanya DNA genom *K. pneumoniae* yang didapati positif, manakala semua sampel lain adalah negatif. Had pengesanan untuk DNA plasmid piawai positif gen LptD dan MerR masing-masing adalah 5.28×10^1 salinan/μL dan 5.78×10^1 salinan/μL dan pekali variasi untuk nilai Ct antara kumpulan intra- dan inter-gen adalah kurang daripada 3%. Keputusan ini menunjukkan bahawa kaedah PCR kuantitatif masa nyata dwi-fluoresen TaqMan yang dibangunkan boleh mengesan *K. pneumoniae* secara khusus dan pantas, yang mempunyai kepentingan besar untuk diagnosis dan rawatan klinikal.

Kata kunci: Analisis pan-genom; gen MerR; gen LptD; *Klebsiella pneumoniae*; PCR kuantitatif dwi

INTRODUCTION

Klebsiella pneumoniae is a common opportunistic pathogen that frequently causes hospital-acquired infections, including pneumonia, meningitis, bloodstream infections, and urinary tract infections (Bengoechea & Pessoa 2019; Togawa et al. 2015). Additionally, *K. pneumoniae* can cause community-acquired infections in healthy individuals, such as liver abscesses, endophthalmitis, and meningitis (Paczosa & Mecsas 2016; Russo & Marr 2019). In recent years, the increasing antibiotic resistance and the emergence of hypervirulent strains of *K. pneumoniae* have rendered its clinical management particularly challenging (De Oliveira et al. 2020; Pitout, Nordmann & Poirel 2015; Zhu et al. 2021). Therefore, developing a rapid, accurate, and simple method for detecting *K. pneumoniae* is of significant importance for clinical diagnosis and treatment.

Bacterial culture identification is a commonly used clinical diagnostic method, but the process is time-consuming (48-72 h) and does not meet the demands for rapid diagnosis. Real-time fluorescence quantitative PCR

technology, as a molecular diagnostic method, offers advantages such as high specificity, good reproducibility, accurate quantification, large-scale detection, and high automation, making it widely applied in the field of microbiological testing (Romero-Alvarez et al. 2021).

Previous primers and probes were designed based on reported gene sequences of *K. pneumoniae* in databases. Due to the genetic diversity of *K. pneumoniae*, this may lead to false-negative results (Kim et al. 2022; Kurupati et al. 2004; Liu et al. 2008). Therefore, this study performed pan-genome analysis on 100 *K. pneumoniae* whole genome sequences from the NCBI database and identified two genes (LptD and MerR) with high conservation and specificity in *K. pneumoniae*. Specific primers and probes were designed based on these two genes, and using the same reaction protocols and systems, it is possible to simultaneously detect two targets genes of *K. pneumoniae*.

MATERIALS AND METHODS

PAN-GENOME ANALYSIS

Whole genome sequences of *K. pneumoniae* were downloaded from the National Center for Biotechnology Information. The data was imported into BPGA for pan-genome analysis, and clustering was performed using USEARCH to obtain the core sequences (Chaudhari, Gupta & Dutta 2016; Costa et al. 2020). Redundant sequences were removed through clustering similarity using CD-HIT (Cluster Database at High Identity with Tolerance) (Fu et al. 2012; Wei et al. 2023). Local BLAST analysis was conducted with Bioedit, using the downloaded *K. pneumoniae* whole genome as the nucleotide database and the core sequences from CD-HIT as the query sequences, to assess the conservation of the target genes. Specificity of the sequences was checked using Blastp on the NCBI website (Benson et al. 2013). Primers and probes with high conservation and specificity for *K. pneumoniae* were designed using Primer Express 3.0, and their specificity was validated using Primer Blast (NCBI) (Ye et al. 2012). Two probes were labeled with different fluorescent reporter groups: the LptD gene probe was tagged with 6-FAM, and the MerR gene probe was tagged with VIC, with BHQ1 as the quencher (Navarro et al. 2015). The primers were synthesized by Beijing Qingke Biotechnology Co., Ltd. (Wuhan), and the probes were synthesized by Shenggong Biological Engineering Co., Ltd. (Wuhan). The synthesized primers and probes were aliquoted and stored at -20 °C for future use.

OPTIMIZATION OF REACTION CONDITIONS

Optimal primer concentrations were tested at 0.1 μM, 0.2 μM, 0.4 μM, 0.6 μM, 0.8 μM, and 1 μM; probe concentrations were tested at 0.1 μM, 0.2 μM, 0.3 μM, 0.4 μM, and 0.5 μM; and annealing temperatures were tested at 60 °C, 62 °C, and 65 °C to determine the optimal conditions for the duplex TaqMan quantitative PCR.

SAMPLE PREPARATION

Cultivate the common respiratory pathogens listed in Table 1 using the following culture medium formulation: beef extract 3 g/L, NaCl 5 g/L, peptone 10 g/L, and sterile sheep blood 50 mL/L. Incubate the cultures at 37 °C for

TABLE 1. Bacterial names and preservation numbers of each strain at preservation institutions

Number	Species	Serial Number
1	*Klebsiella pneumoniae*	CCTCCPB2022081
2	*Pseudomonas aeruginosa*	CCTCCPB2022082
3	*Staphylococcus aureus*	CCTCCPB2022080
4	*Acinetobacter baumannii*	CCTCCPB2022084
5	*Streptococcus pyogenes*	CCTCCPB2022075
6	*Streptococcus salivarius*	CCTCCPB2022076
7	*Pseudomonas maltophilia*	CCTCCPB2022083
8	*Citrobacter freundii*	CCTCCPF2022078
9	*Neisseria meningitidis*	CCTCCPB2022074
10	*Nocardia Asteroides*	CCTCCPB2022079
11	*Klebsiella oxytoca*	CCTCCPB2022085
12	*Haemophilus influenzae*	BNCC359479
13	*Moraxella catarrhalis*	BNCC292096
14	*Streptococcus pneumoniae*	BNCC186345
15	*Proteus mirabilis*	BNCC370491

18 to 24 h. Subsequently, extract the genomic DNA of the pathogens according to the instructions provided with the CW0552S Bacteria Genomic DNA Kit (CWBio). Finally, adjust the concentration of the obtained DNA to 180 ng/μL and store it at -20 °C for future use.

PREPARATION OF STANDARD PLASMIDS AND ESTABLISHMENT OF A STANDARD CURVE

The genomic DNA of *K. pneumoniae* was used as a template for PCR amplification of the LptD (LptD-F; LptD-R) and MerR (MerR-F; MerR-R) gene sequences, as indicated in Table 2. The DNA polymerase 2xES Taq MasterMix (Dye) was purchased from Jiangsu Conway Century Biotechnology Co., Ltd. The amplification program was as follows: initial denaturation at 94 °C for 2 min, denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s, extension at 72 °C for 30 s, final extension at 72 °C for 2 min, with 35 cycles. The PCR products were verified on a 1% agarose gel for specific bands, and target gene fragments were recovered using the agarose gel DNA recovery kit from Shanghai Tuloport Biotechnology Co., Ltd.

Recovered gene fragments were cloned into the pMD-18T vector using the TaKaRa pMD™-18T Vector Cloning Kit according to the manufacturer's instruction and transformed into DH5 α competent cells. Positive colonies were selected and cultured, and plasmids were extracted using the Plasmid Extraction Kit (DP104) from TianGen Biochemical Technology Co., Ltd., followed by sequencing for verification. The purity and concentration of the plasmids were measured using a micro-spectrophotometer at wavelengths of 230 nm, 260 nm, and 280 nm.

Standard plasmids pMD18-T-LptD and pMD18-T-MerR were diluted to copy numbers ranging from 10^2 to 10^9, with 1 μL of each dilution used as template, resulting in a total template volume of 2 μL per reaction. TaqMan probe duplex quantitative PCR amplification was performed according to the optimized reaction conditions and system. A standard curve was constructed by plotting the logarithmic values of plasmid DNA copy numbers on the x-axis and the corresponding Ct values on the y-axis.

SENSITIVITY, SPECIFICITY AND REPRODUCIBILITY TESTS

A *K. pneumoniae* suspension was inoculated onto solid blood agar plates containing 5% sheep blood and incubated at 37 °C for 18-24 h. After washing with physiological saline and collecting the bacteria, the optical density of the suspension was adjusted to 0.5 at 460 nm, with a plate count of 2.17×10^9 CFU/mL. Genomic DNA was extracted from 1 mL of the bacterial suspension using the CW0552S Bacteria Genomic DNA Kit, and then subjected to a 10-fold serial dilution to achieve a final genomic DNA concentration corresponding to a bacterial concentration of 2.17×10^0 CFU/mL. Genomic DNA (2 μL) from *K. pneumoniae* suspensions with concentrations ranging from 2.17×10^7 CFU/mL to 2.17×10^0 CFU/mL was used as a template for quantitative real-time PCR amplification with optimized reaction conditions and system. The lowest bacterial concentration that could produce a positive result was defined as the detection limit.

1 μL of pMD18-T-LptD and pMD18-T-MerR standard plasmids, with copy numbers ranging from 10^0 to 10^9, were selected and used as templates, resulting in a total template volume of 2 μL per reaction. TaqMan duplex quantitative PCR amplification was performed according to the optimized reaction conditions and system to determine the sensitivity of the method.

Genomic DNA extracted from respiratory pathogens was used as the template, with 2 μL added per reaction. Sterile double-distilled water was used as a negative control. TaqMan probe duplex quantitative PCR was performed according to the optimized reaction conditions and system for verification.

Standard plasmids pMD18-T-LptD and pMD18-T-MerR with dilutions from 10^2 to 10^6 were used, with 1 μL of each dilution as the template, resulting in a total template volume of 2 μL per reaction. Duplex quantitative PCR amplification was performed according to the optimized reaction conditions and system. Three replicates were set for each concentration gradient for intra-group variability calculation. The experiment was independently repeated three times to assess inter-group differences. The coefficient of variation (CV) was calculated as (standard deviation SD / mean) × 100%.

RESULTS

CONSERVATIVE AND SPECIFIC ANALYSIS

Pan-genome analysis was conducted to derive the core and accessory genomes. Core genes are ubiquitous within species' genomes and are crucial for understanding genome evolution, gene homology, genomic complexity, and the identification of pathogenic and therapeutic sequences. As shown in Figure 1, we downloaded 100 *K. pneumoniae* genome sequences from the NCBI website. Data preprocessing was performed using BPGA, followed by clustering with USEARCH at a default sequence similarity threshold of 50%. This process resulted in 18,066 core sequences, with the GC content of these genomes ranging from 56.2% to 57.8%. Redundant sequences with less than 50% similarity were removed using CD-HIT, yielding a final set of 2,837 core gene sequences. A nucleotide database was constructed using 100 *K. pneumoniae* genome sequences to analyze the conservation of core gene sequences. Similarity searches were performed on the NCBI website using Blastp with the NR database to compare protein sequences against common respiratory pathogens. Ultimately, highly conserved, and specific LptD and MerR protein genes were selected.

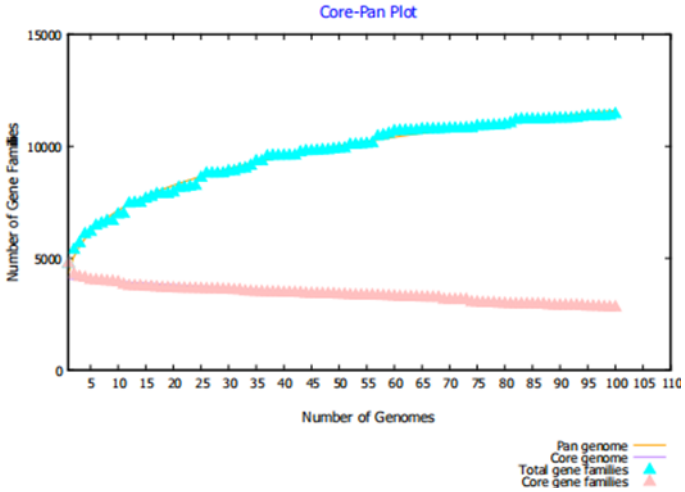

FIGURE 1. Pan-genome analysis results of *K. pneumoniae*

PRIMER, PROBE AND REACTION PROCEDURE

The gene sequences for the LptD and MerR proteins of *K. pneumoniae* were downloaded from NCBI. A multiple sequence alignment was performed to identify the conserved regions within the LptD (n=50) and MerR (n=50) genes (Figure 2). Specific primers and probes based on these conserved regions can be seen in Table 2. The LptD probe is labeled with the fluorescent reporter dye 6-FAM at the 5' end and the fluorescent quencher dye BHQ1 at the 3' end. The MerR probe is labeled with the fluorescent reporter dye VIC at the 5' end and the fluorescent quencher dye BHQ1 at the 3' end.

The final optimized reaction system is detailed in Table 3. The TaqMan duplex quantitative PCR amplification protocol included an initial denaturation at 95 °C for 20 s; followed by 40 cycles of denaturation at 95 °C for 1 s, annealing and extension at 65 °C for 20 s, with fluorescence data collected at the end of each step at 65 °C.

STANDARD CURVE

After sequencing and verification, the final concentrations of the LptD and MerR gene recombinant plasmids were determined to be 165 ng/μL and 182 ng/μL, respectively. The successfully constructed standard plasmids were named pMD18-T-LptD and pMD18-T-MerR. According to the standard plasmid copy number calculation formula, (copy number (copies/ μL) = (standard plasmid concentration (ng/ μL) × 6.02 × 10^{14}) / (plasmid total length × 660)), the copy numbers of the LptD and MerR gene recombinant plasmids were calculated to be 5.28 × 10^{10} copies/μL and 5.78 × 10^{10} copies/μL, respectively. The successfully constructed standard plasmids were designated as pMD18-T-LptD and pMD18-T-MerR. These standard plasmids, pMD18-T-LptD and pMD18-T-MerR, were diluted in a 10-fold gradient using Takara EASY Dilution to final concentrations of 5.28 × 10^{0} copies/μL and 5.78 × 10^{0} copies/ μL, respectively.

The standard curve equation for LptD was y = −3.289x + 43.778 with an R^2 value of 0.999 and an amplification efficiency of 101.4%. For MerR, the standard curve equation was y = −3.317x + 42.551 with an R^2 value of 0.999 and an amplification efficiency of 100.2% (Figure 3). These results indicate a strong linear relationship between the logarithm of plasmid DNA copy numbers and Ct values and confirm high efficiency and stability of the duplex real-time quantitative PCR reaction system.

SENSITIVITY, SPECIFICITY AND REPRODUCIBILITY ANALYSIS

The detection limits for bacterial concentrations were established as 2.17 × 10^{2} cfu/mL for the LptD gene and 2.17 × 10^{2} cfu/mL for the MerR gene (Figure 4(A)). For plasmid DNA copy numbers, the detection limits were determined to be 5.28 × 10^{1} copies/μL for LptD and 5.78 × 10^{1} copies/μL for MerR (Figure 4(B)). These results further underscore the high sensitivity of the method employed.

Within 40 cycles, only the reaction using *K. pneumoniae* genomic DNA as a template showed a positive amplification curve, while all other samples and the negative control were negative (Figure 5). This indicates that the designed primers and probes possess high specificity, and the method exhibits high specificity for the detection of *K. pneumoniae*.

The coefficient of variation for the LptD and MerR genes ranged from 0.12% to 1.73% and 0.19% to 2.85%, respectively (Table 4). The coefficient of variation for Ct values within and between groups for both genes was less than 3%, indicating that the method demonstrates good stability and reproducibility.

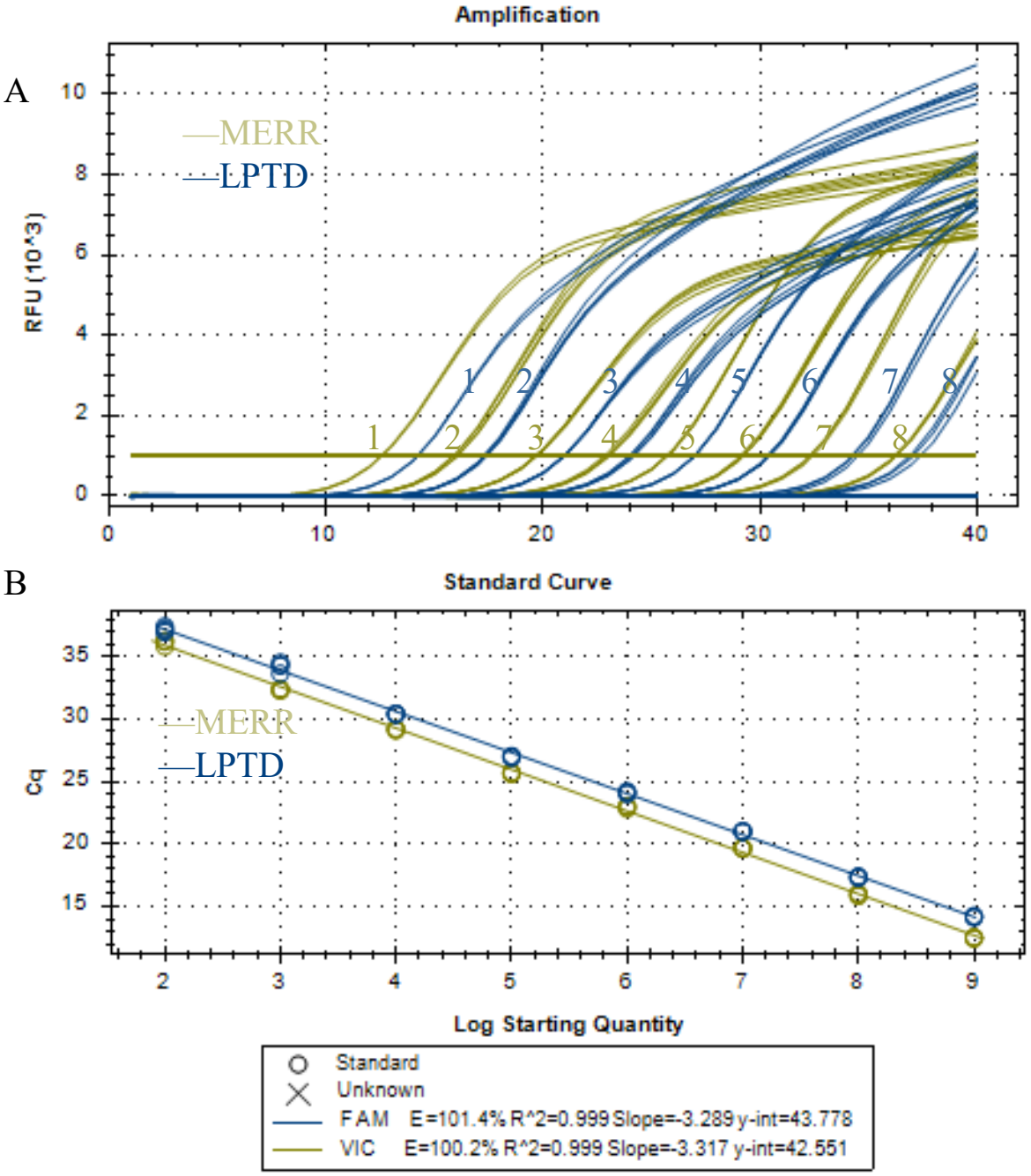

FIGURE 3. Standard curves for the duplex fluorescent quantitative PCR method for *K. pneumoniae*. Panel (A) shows the amplification curves for two plasmid standards. The blue curves (1-8) represent the amplification of the standard plasmid pMD18-T-LptD, with copy numbers ranging from 5.28×10^9 copies/μL to 5.28×10^2 copies/μL. The yellow curves (1-8) depict the amplification of the standard plasmid pMD18-T-MerR, with copy numbers ranging from 5.78×10^9 copies/μL to 5.78×10^2 copies/μL. Panel (B) displays the standard curves with the blue curve representing the LptD gene and the yellow curve representing the MerR gene

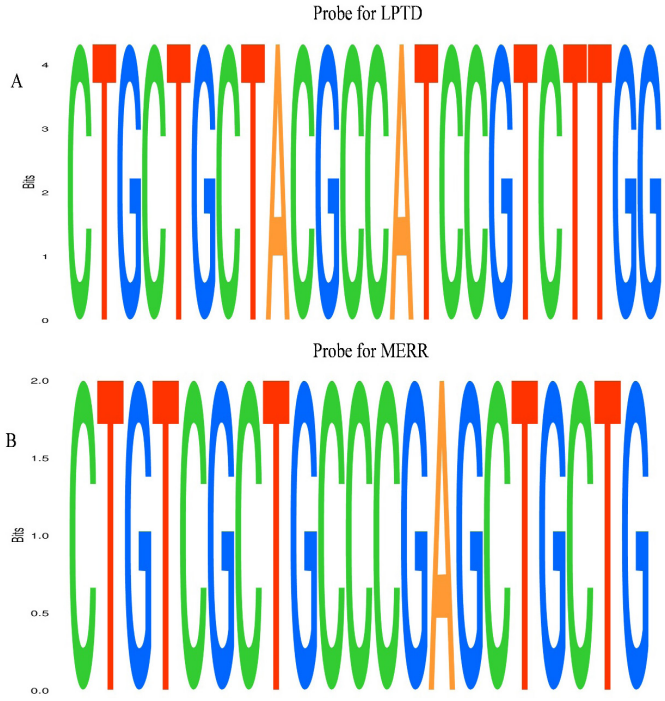

FIGURE 2. Probes designed in this study. The logo plots display the highly conserved regions of the LptD gene (A) and MerR gene (B) probes

TABLE 2. Specific primers

Primer	Sequence(5'-3')	Amplicon size(bp)
LptD-F	GCAGCAAACCAGATGTTGGG	157
LptD-R	TCAGACCGCGCAATTCGATA	
LptD-Probe	CTGCTGCTACGCCATCCGTCTTGG	
MerR-F	CTAACGCTCGCCACAATCAAG	178
MerR-R	AGGACTCCAGATCGGGTTCA	
MerR-Probe	CTGTCGCTGCCCGAGCTGCTG	

TABLE 3. Composition of the reaction mixture excluding template

Reaction components	Reaction system (25 μL)
Probe qPCR Mix MultiPlus	12.5 μL
LptD	F: 0.5 μL (10 μM)
	R: 0.5 μL (10 μM)
	Probe: 0.6 μL (10 μM)
MerR	F: 0.5 μL (10 μM)
	R: 0.5 μL (10 μM)
	Probe: 0.6 μL (10 μM)
Genomic DNA Template	2 μL
ddH_2O	Up to 25 μL

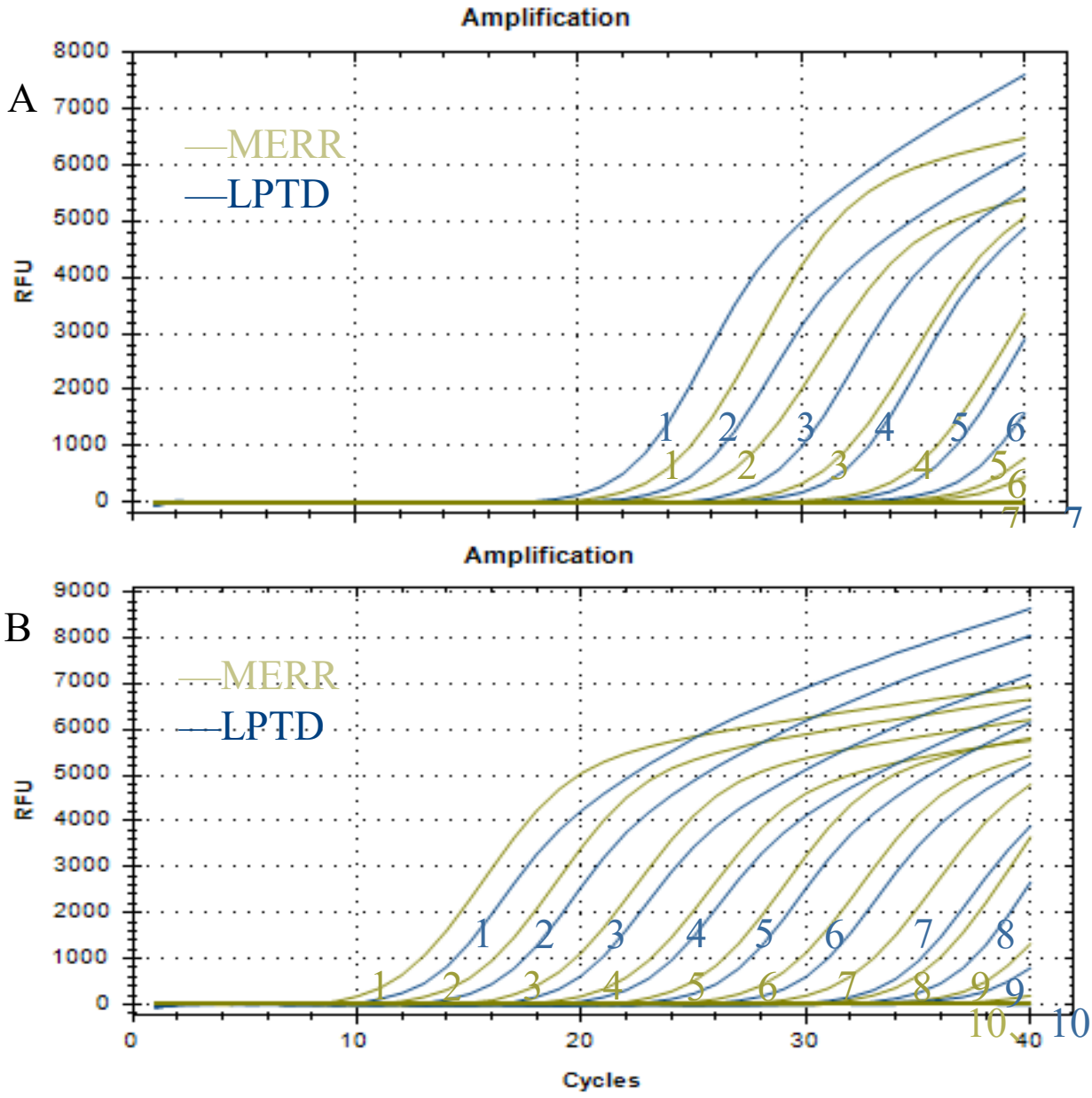

FIGURE 4. Detection limits of the duplex fluorescent quantitative PCR method for *K. pneumoniae*. Panel (A) displays the detection limits for plasmid standards. The blue curves (1-10) represent amplification using the standard plasmid pMD18-T-LptD with concentrations ranging from 5.28 × 10^9 copies/μL to 5.28 × 10^0 copies/μL. The yellow curves (1-10) show amplification with the standard plasmid pMD18-T-MerR, with concentrations ranging from 5.78 × 10^9 copies/μL to 5.78 × 10^0 copies/μL. Panel (B) presents the amplification curves for genomic DNA templates with concentrations ranging from 2.17 × 10^7 CFU/mL to 2.17 × 10^0 CFU/mL

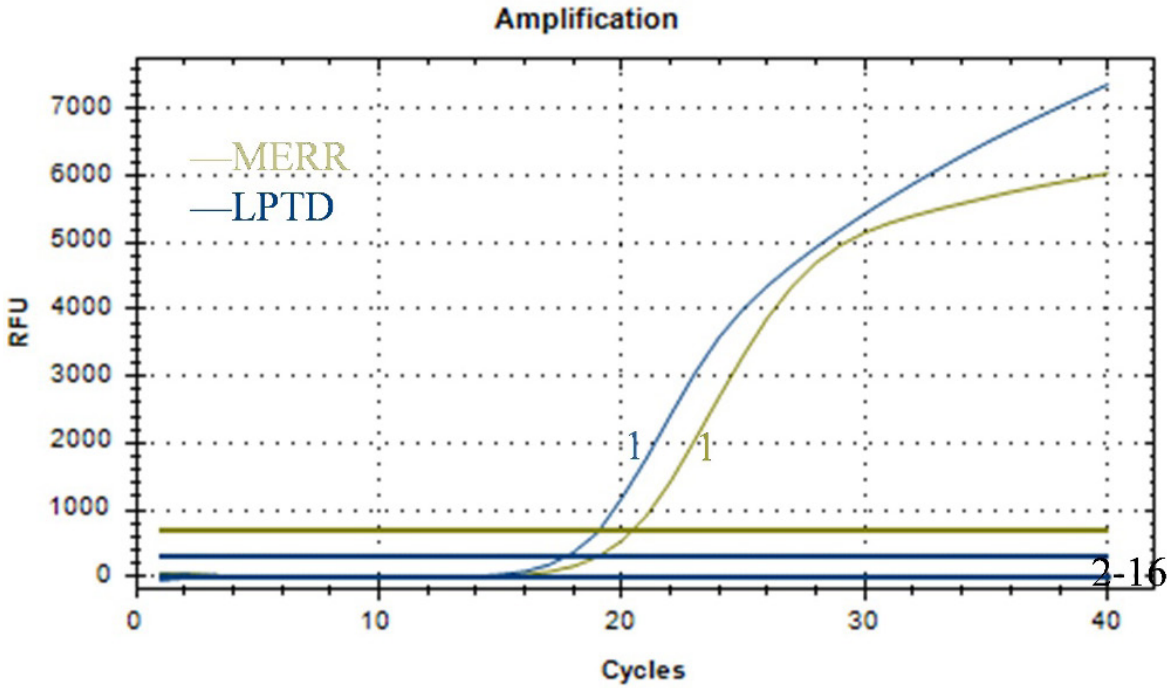

FIGURE 5. Specificity testing results of the duplex fluorescent quantitative PCR method. Curve (1) represents the amplification curve obtained using *K. pneumoniae* genomic DNA as the template. Curves 2-16 depict the amplification results using genomic DNA from various other pathogens and a negative control

TABLE 4. Reproducibility data

Plasmid standard	Template concentration (copies/μL)	Ct value of repeatability experiment					
		Intra-assay			Inter-assay		
		Average value	Standard deviation	Coefficient of Variation/% (CV)	Average value	Standard deviation	Coefficient of Variation/%(CV)
PMD-18T-LPTD	5.28×10^6	24.15	0.10	0.42	24.09	0.42	1.73
	5.28×10^5	26.09	0.08	0.30	27.06	0.22	0.80
	5.28×10^4	30.41	0.04	0.12	30.28	0.25	0.84
	5.28×10^3	34.38	0.14	0.40	34.60	0.54	1.57
	5.28×10^2	37.26	0.16	0.43	37.47	0.48	1.27
PMD-18T-MERR	5.78×10^6	22.97	0.10	0.44	23.12	0.66	2.85
	5.78×10^5	25.68	0.06	0.25	26.08	0.60	2.28
	5.78×10^4	29.18	0.05	0.19	29.54	0.56	1.88
	5.78×10^3	32.36	0.07	0.20	32.90	0.77	2.35
	5.78×10^2	36.14	0.21	0.59	36.38	0.80	2.19

DISCUSSION

A study on community-acquired pneumonia (CAP) in China showed that the detection rate of *K. pneumoniae* was 10.43%, with a particularly high rate observed in elderly patients, suggesting that *K. pneumoniae* is one of the risk factors for severe CAP (Zhang et al. 2023). Notably, *K. pneumoniae* -induced neonatal sepsis is increasingly becoming a serious public health issue (Ma et al. 2024). Due to the overuse of antibiotics, this pathogen has developed resistance to multiple antibiotics, with the infection rate of carbapenem-resistant *K. pneumoniae* continuing to rise, presenting significant challenges for clinical treatment (Logan & Weinstein 2017; Navon-Venezia, Kondratyeva & Carattoli 2017; Pitout, Nordmann & Poirel 2015). Therefore, establishing a rapid and accurate method for detecting *K. pneumoniae* is of great importance for early diagnosis and precise treatment.

Currently, the most commonly used detection methods for *K. pneumoniae* include bacterial culture and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). However, these methods suffer from drawbacks such as long detection cycles, low sensitivity, and poor specificity (Abdeta et al. 2021; Huang et al. 2022; Keshta et al. 2021; Marí-Almirall et al. 2021). To address these issues, this study utilized TaqMan fluorescence quantitative PCR technology. TaqMan PCR relies on the 5'-3' exonuclease activity of TaqMan polymerase to degrade the TaqMan probe that hybridizes with the target DNA sequence, thereby, releasing a fluorescent signal. The fluorescent signal is monitored in real-time to quantitatively analyze the target DNA, providing specific amplification curves and yielding results within one hour.

To ensure the specificity and sensitivity of the detection, we employed a pan-genome analysis approach to screen the detection targets. Pan-genome analysis is an effective method for studying microbial genome diversity and variability. By analyzing the genomes of multiple strains, commonalities and differences between strains can be identified, providing theoretical support for the selection of diagnostic targets (Chaudhari, Gupta & Dutta 2016). Lee et al. (2021) applied pan-genomics to show the conserved genetic diversity structures present in various microbial pathogens. In this study, we performed pan-genome analysis on 100 randomly selected *K. pneumoniae* genomes from the NCBI Genomes database. A total of 2,837 core genes were identified, from which two highly conserved and specific target genes—LptD and MerR—were further screened. Bioinformatic analysis showed that these two genes were present in all 100 *K. pneumoniae* genomes, with sequence similarity greater than 99%, indicating high conservancy. BLAST analysis further showed no similarity between these genes and those of common respiratory pathogens, demonstrating their high specificity and suggesting their potential as diagnostic targets.

LptD protein, an outer membrane lipopolysaccharide (LPS) transporter, plays a key role in transporting LPS from the inner to the outer membrane. It is highly conserved across various *K. pneumoniae* and is considered a potential vaccine antigen and target for immunotherapy (Romano & Hung 2023; Zha et al. 2016). MerR protein is a bacterial transcriptional regulator that responds to a wide range of signals, including toxins, metal ions, and endogenous metabolites. Tulin et al. (2024) explored the diversity of the MerR family and the structural and functional similarities among its members, reflecting its evolutionary conservancy. To date, application of LptD and MerR protein genes as target markers for *K. pneumoniae* detection have not been conducted.

In this study, we designed specific primers and probes for the LptD and MerR protein genes. As expected, these primers and probes stably and specifically amplified the LptD and MerR genes in *K. pneumoniae*. We further adopted a dual-fluorescent quantitative PCR strategy to simultaneously detect both genes in a single reaction channel. By using two different fluorescent reporters and distinguishing them by color, our method significantly reduced the risk of missed detection compared to the single-target detection methods used by Gadsby et al. (2015). Moreover, the standard curves for both genes demonstrated a high correlation coefficient ($R^2 \geq 0.999$), confirming the excellent linear relationship of the detection method. The within- and between-group coefficient of variation for the standard plasmids was less than 3%, indicating good stability and repeatability of the developed detection method.

Although the TaqMan probe dual-fluorescent quantitative PCR detection method established in this study shows significant advantages for *K. pneumoniae* detection, certain challenges remain in its clinical application. These include the risk of contamination during the operation and issues related to sample handling. Additionally, the current method cannot detect the antibiotic resistance genes carried by *K. pneumoniae*, which will require further bioinformatics analysis and molecular biology studies to improve. We look forward to exploring these issues in future research to provide more precise diagnostic and therapeutic tools for clinical practice.

CONCLUSIONS

This study focuses on *K. pneumoniae*, an important pathogenic bacterium, and employs bioinformatics analysis to successfully identify the MerR and LptD protein gene sequences as specific and conserved targets for detection. Based on these sequences, we designed specific primers and probes, optimized the reaction system and conditions, and established a TaqMan probe-based dual fluorescence quantitative PCR method. This method demonstrated exceptional specificity when tested against genomic DNA from 14 common pathogens and negative

controls, showing positive results only for *K. pneumoniae* genomic DNA. Moreover, the detection limits for the LptD and MerR genes were 5.28×10^1 copies/μL and 5.78×10^1 copies/μL, respectively, indicating high sensitivity. The variation coefficients of Ct values both within and between groups were less than 3%, confirming the stability and reproducibility of the method. In conclusion, the TaqMan probe-based dual fluorescence quantitative PCR method developed in this study provides a specific and rapid approach for detecting *K. pneumoniae* infections. This method holds significant potential for clinical diagnosis and rational antimicrobial therapy.

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