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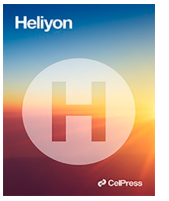

Research article

# Development and evaluation of a duplex real-time multienzyme isothermal rapid amplification assay for the detection of hypervirulent *Klebsiella pneumoniae* in clinical spiked blood specimens

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ABSTRACT

*Objectives:* Our objective was to establish a rapid and precise method for detecting hypervirulent *Klebsiella pneumoniae* (hvKP) by utilizing a duplex real-time multienzyme isothermal rapid amplification (real-time MIRA) and to evaluate its performance in clinical spiked blood specimens.

*Methods:* The research comprised two phases: an initial pilot study to establish the methodology and a clinical validation study to assess its effectiveness. In the pilot phase, we designed specific primers and probes targeting the hvKP *pg344* and *incA* genes and subsequently developed a duplex real-time MIRA assay to evaluate its detection limits, specificity, and efficiency. In the clinical validation phase, we analyzed thirty-three spiked blood specimens using the duplex real-time MIRA assay.

*Results:* The duplex real-time MIRA assay demonstrated no cross-reactivity with other strains. Sensitivity experiments confirmed that the assay had a detection limit as low as $8 \times 10^2$ CFU per reaction for hvKP. The analysis of clinical spiked blood specimens indicated that the sensitivity and specificity of the duplex real-time MIRA assay were on par with those of duplex real-time PCR.

*Conclusions:* These findings confirm that the duplex real-time MIRA assay is a fast, straightforward, and dependable method for detecting hvKP.

## 1. Introduction

*Klebsiella pneumoniae* is a common pathogen responsible for both community- and hospital-acquired infections, characterized by its thick capsule polysaccharide that effectively hinders neutrophil phagocytosis and antibiotic activity, thereby increasing the susceptibility to pneumonia, bloodstream infection, and urinary tract infection [1–4]. The presence of a robust capsular polysaccharide layer enables *K. pneumoniae* to evade neutrophil phagocytosis and antibiotic action efficiently, consequently enhancing the likelihood of developing lung inflammation, bloodstream infections, and urinary tract infections. Currently, due to variations in capsular polysaccharide species and alterations in bacterial virulence factors, there has been an escalating global prevalence of hypervirulent

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*K. pneumoniae* (hvKP) infections [5]. In comparison to nosocomial infections caused by classical *K. pneumoniae* (cKP), hvKP is associated with higher rates of severe complications and elevated mortality such as sepsis and metastatic infection [6–8]. Recently, the resistance patterns exhibited by hvKP strains have garnered significant attention worldwide due to their close association with bacterial virulence traits. Of particular concern is the emergence of carbapenem-resistant hvKP (CR-hvKP), which poses a formidable challenge for clinical management [9–11].

**Table 1**
Characteristics of the thirty-three isolates used for validation of the designed duplex real-time MIRA assay for the detection of hvKP.

| NO | Bacterial strain | Source | Type | Real-time MIRA | Real-time PCR |
|---|---|---|---|---|---|
| 1 | hvKP peg344 and incA | Neurology | Sputum | Positive | Positive |
| 2 | hvKP peg344 and incA | ICU | Lung | Positive | Positive |
| 3 | hvKP peg344 and incA | Endocrinology | Secretion | Positive | Positive |
| 4 | hvKP peg344 and incA | Neurology | Lung | Positive | Positive |
| 5 | hvKP peg344 and incA | Cardiovascular medicine | Sputum | Positive | Positive |
| 6 | hvKP peg344 and incA | ICU | Lung | Positive | Positive |
| 7 | hvKP peg344 and incA | ICU | Sputum | Positive | Positive |
| 8 | hvKP peg344 and incA | RICU | Lung | Positive | Positive |
| 9 | hvKP peg344 and incA | ICU | Sputum | Positive | Positive |
| 10 | hvKP peg344 and incA | Endocrinology | Lung | Positive | Positive |
| 11 | hvKP peg344 and incA | Cardiovascular medicine | Sputum | Positive | Positive |
| 12 | hvKP peg344 and incA | ICU | Lung | Positive | Positive |
| 13 | hvKP peg344 and incA | ICU | Lung | Positive | Positive |
| 14 | hvKP peg344 and incA | Neurology | Sputum | Positive | Positive |
| 15 | hvKP peg344 and incA | ICU | Lung | Positive | Positive |
| 16 | hvKP peg344 and incA | Rehabilitation | Sputum | Positive | Positive |
| 17 | hvKP peg344 and incA | ICU | Lung | Positive | Positive |
| 18 | hvKP peg344 | Cardiovascular medicine | Urine | Negative | Negative |
| 19 | hvKP incA | ICU | Lung | Negative | Negative |
| 20 | non-peg344/incA hvKP | ICU | Sputum | Negative | Negative |
| 21 | non-peg344/incA hvKP | Neurology | Urine | Negative | Negative |
| 22 | cKP | Oncology department | Urine | Negative | Negative |
| 23 | cKP | Endocrinology | Blood | Negative | Negative |
| 24 | *Escherichia coli* | Rehabilitation | Urine | Negative | Negative |
| 25 | *Escherichia coli* | Neurology | Blood | Negative | Negative |
| 26 | *Enterobacter cloacae* | Maxillofacial | Secretion | Negative | Negative |
| 27 | *Enterobacter cloacae* | Endocrinology | Urine | Negative | Negative |
| 28 | *Klebsiella oxytoca* | Trauma Surgery | Pus | Negative | Negative |
| 29 | *Klebsiella oxytoca* | Ophthalmology | Secretion | Negative | Negative |
| 30 | *Serratia marcescens* | GICU | Lung | Negative | Negative |
| 31 | *Serratia marcescens* | Trauma Surgery | Bile | Negative | Negative |
| 32 | *Morganella morganii* | Endocrinology | Urine | Negative | Negative |
| 33 | *Morganella morganii* | Trauma Surgery | Blood | Negative | Negative |

hvKP peg344 and incA: hypervirulent *Klebsiella pneumoniae* coharboring *peg344* and *incA* genes.
hvKP peg344: hypervirulent *Klebsiella pneumoniae* coharboring *peg344* gene.
hvKP incA: hypervirulent *Klebsiella pneumoniae* coharboring *incA* gene.
non-peg344/incA hvKP: hypervirulent *Klebsiella pneumoniae* coharboring non-*peg344* and non-*incA* genes.
cKP: classical *Klebsiella pneumoniae*.
ICU: Intensive Care Unit.
GICU: Gastroenterology Intensive Care Unit.
RICU: Respiratory Intensive Care Unit.

Advanced methods for detecting hvKP include colony morphology analysis, serum killing assays, mouse lethality tests, string tests, and real-time PCR [12] However, these traditional methods are time-intensive, often requiring several hours or even up to 2 days to produce results. In contrast, hvKP strains frequently carry virulence plasmids such as pK2044, pLVPK, and pVir-CR-hvKp4, which encode well-known virulence factors associated with hvKP [13].Importantly, plasmid-borne virulence genes can serve as specific biomarkers for detecting hvKP. Four such virulence genes namely *peg344*, *iucA*, *iroB*, and $_p$*rmpA* are considered molecular markers for detecting hvKP. Among these, *peg344* stands out for its accuracy, sensitivity, and specificity in detecting hvKP, as it encodes a metabolic transporter of unknown function located on the inner membrane [14]. Furthermore, *iucA* is also regarded as a hvKP-specific gene owing to its involvement in the production of aerobactin siderophore biosynthesis and pathogenicity. Consequently, we have chosen *peg344* and *iucA* as key genes for the rapid detection of hvKP.

Recently, the multienzyme isothermal rapid amplification (MIRA) has replaced traditional PCR approaches. Compared to conventional PCR technologies, MIRA technology offers several advantages, including enhanced speed and accuracy, reduced equipment dependency (even in the absence of equipment), simplified operation, minimized environmental and personnel requirements. Moreover, in comparison to existing isothermal amplification technologies, MIRA requires less sample material and eliminates the need for imported raw materials. It can efficiently amplify trace nucleic acid templates to detectable levels at 37–42 °C and can be combined with other detection technologies to broaden its application fields [15,16]. Real-time MIRA technology has garnered significant attention for its high sensitivity and specificity, rapid detection time, room-temperature isothermal reaction, versatility across various applications, and the use of freeze-dried reagents that are easy to transport and maintain stable enzyme activity. In this study, we assessed the efficacy of a duplex real-time MIRA assay targeting the *peg344* and *iucA* genes for the rapid detection of hvKP in clinical spiked blood specimens.

## 2. Materials and methods

### 2.1. Bacterial strains and genomic DNA preparation

In the present study, we collected a detailed list of thirty-three clinical isolates from Daping Hospital for duplex real-time MIRA performance. The list includes hvKP pg344 and incA, hvKP pg344, hvKP incA, non-pg344/incA hvKP, cKP, *E. coli*, *E. cloacae*, *K. oxyota*, *S. marcescens*, and *M. morganii* as shown in Table 1. International standard strain stored in our laboratory included *Klebsiella pneumoniae* ATCC700603, *Klebsiella oxyota* ATCC700324, *Escherichia coli* ATCC25922, *Enterobacter hoemaechei* ATCC700323, *Acinetobacter baumannii* ATCC19666, *Pseudomoas areuginosa* ATCC27853, and *Stenotrophomonas maltophilia* ATCC17666 were chosen as the negative reference strains for specificity experiment as shown in Table 2. The hvKP pg344 and incA strains, which were isolated from clinical patients, were used as positive reference strains for sensitivity and specificity experiments. The primary method utilized for quantifying bacterial colony forming units (CFU) involved a continuous tenfold dilution of the bacterial solution with a 0.35 McFarland standard, followed by the deposition of 10 μL of the appropriate dilution onto an LB plate. Each concentration was replicated thrice, incubated in a 35 °C chamber for 24 h, and subsequently subjected to CFU enumeration. For blood specimens spiked with bacteria, 900 μL of whole blood was mixed with 100 μL of bacterial strains at different concentrations. The spiked blood samples were processed using the TIANamp Bacteria DNA Kit (Tiangen Biotech Co., Ltd., Beijing, China) according to the manufacturer's instructions. The extracted DNA was then stored at −20 °C until needed.

### 2.2. Identification of hvKP peg344 and iucA strains by conventional PCR

All hvKP pg344 (Fig. S1a) and incA (Fig. S1b) strains in this study were verified using conventional PCR. The PCR products of the *pg344* gene (508 bp) and *incA* gene (583 bp) were purified using a PCR product purification kit from Tiangen Biotech (Beijing, China). The purified sequences were then compared with those in the GenBank database using the BLAST algorithm for alignment.

### 2.3. Primer and probe design

In this study, the major virulence genes *peg344* and *iucA* of hvKP were selected as target regions for designing primers and probes. These genes have been previously detected using real-time PCR-based assays [17]. Primers and probes were manually designed

**Table 2**
Specificity of duplex real-time MIRA assay for the detection of hvKP.

| NO | Strain name | Source of strain | Real-time MIRA |
|---|---|---|---|
| 1 | hvKP peg344 and incA | Laboratory collection | Positive |
| 2 | *Klebsiella pneumoniae* | ATCC700603 | Negative |
| 3 | *Klebsiella oxyota* | ATCC700324 | Negative |
| 4 | *Escherichia coli* | ATCC25922 | Negative |
| 5 | *Enterobacter hoemaechei* | ATCC700323 | Negative |
| 6 | *Acinetobacter baumannii* | ATCC19666 | Negative |
| 7 | *Pseudomoas areuginosa* | ATCC27853 | Negative |
| 8 | *Stenotrophomonas maltophilia* | ATCC17666 | Negative |

hvKP peg344 and incA: hypervirulent *Klebsiella pneumoniae* coharboring *peg344* and *incA* genes.

targeting the conserved regions of the *peg344* and *iucA* genes, adhering to real-time MIRA primer and probe design principles. Their specificity was verified using NCBI's Primer-BLAST. The primers and probes were synthesized and purified by BGI Biotechnology Corporation (Beijing, China) through high-performance liquid chromatography (HPLC). Their sequences are provided in Table 3.

### 2.4. Duplex real-time MIRA assay

Nine duplex real-time MIRA amplification groups were optimized using exo real-time MIRA kits (Amp-Future Biotech Co., Ltd., Weifang, China) and the CFX96 real-time PCR system (Bio-Rad, USA). The FAM and VIC two-channel method was employed with the primers and probes detailed in Table 3. Each reaction was conducted in a 50 μL final volume, containing 2 μL of DNA template, 29.4 μL of reaction buffer, 11.5 μL of water, 1 μL each of peg344-F and R primers (10 μM), 1 μL each of iucA-F and R primers (10 μM), 0.3 μL each of peg344 and iucA probes (10 μM), and 2.5 μL of 280 mM magnesium acetate. The mixture was vortexed briefly, centrifuged, and then analyzed in the CFX96 real-time PCR system to monitor fluorescence signals in real-time, with data collection every 30 s over 20 min (40 cycles).

### 2.5. Analytical sensitivity and specificity of the duplex real-time MIRA assay

The analytical sensitivity of the duplex real-time MIRA assay was assessed using a dilution series of purified DNA extracted from hvKP peg344 and iucA strains. Tenfold serial dilutions, ranging from $8 \times 10^6$ to $8 \times 10^2$ CFU per reaction, served as templates for genomic DNA. To verify the specificity of the duplex real-time MIRA assay, genomic DNA samples (0.5–2.0 ng) from *K. pneumoniae* ATCC700603, *Klebsiella oxyota* ATCC700324, *Escherichia coli* ATCC25922, *Enterobacter hoemaechei* ATCC700323, *Acinetobacter baumannii* ATCC19666, *Pseudomoas areuginosa* ATCC27853, and *Stenotrophomonas maltophilia* ATCC17666 were tested for potential cross-reactivity. This experiment was repeated thrice.

### 2.6. Duplex real-time PCR assay

Duplex real-time PCR analysis was used as the reference standard for detecting hvKP, following established protocols with the specific primers and probes listed in Table 3. Sensitivity tests were conducted using diluted DNA samples subjected to a 40-cycle protocol, consisting of denaturation at 95 °C for 10 s and annealing/extension at 60 °C for 40 s. The reaction mixture included Tris-HCl buffer, hot start Taq enzyme, dNTPs, 18 μL of PCR mix, and 2 μL of DNA sample. A sample was considered positive if the threshold cycle (Ct value) was less than 35.

### 2.7. Evaluation of the duplex real-time MIRA assay using clinical spiked blood specimens

The duplex real-time MIRA assay's effectiveness in detecting hvKP was assessed using thirty-three clinical blood specimens spiked with pathogens, and its performance was compared to that of a duplex real-time PCR assay for hvKP detection.

**Table 3**
Sequences of primers and probes.

| Assay | Name | Sequence (5′-3′) and modification | Length (bp) |
|---|---|---|---|
| Real-time MIRA | peg344-F1 | CCCTCCAGTCTTTGCTACCGGGATGAGATT | 30 |
| | peg344-F2 | TCCGCTCAATTATTAATCATTATCGCATGG | 30 |
| | peg344-F3 | TCCTGTTGGCCAGCGTCTATTTCAACTTGC | 30 |
| | peg344-R1 | ATTAGTCCAGTAATCTGTATTGAGTTTG | 28 |
| | peg344-R2 | CCTCCGTGATGAGGATGAACGAAAGTGAAG | 30 |
| | peg344-R3 | AAGAAAGGGCAATAACTCCCGTCCACTGG | 29 |
| | incA-F1 | ATCAATGGCTATTCCCGCTGCACCCGTGGC | 30 |
| | incA-F2 | TCTGTTGCAGCAGGAGTGGTGCCAGGAGCT | 30 |
| | incA-F3 | CAATGGCTATTCCCGCTGCACCCGTGGCAG | 30 |
| | incA-R1 | ATCAATGGCTATTCCCGCTGCACCCGTGGC | 30 |
| | incA-R2 | CTTTCACTGACAGGGTACGGACGGAGTTGG | 30 |
| | incA-R3 | ACGGAGTTGGTCAGGCGCACGCTCAGGGAG | 30 |
| | peg344-P1 | TCCTTTCTCTCTAATGATTTATGGTGAGGT[FAM-dT]A[THF][BHQ1-dT]GTAAACCCAGGACTT-[C3spacer] | 78 |
| | incA-P1 | TGGCTACCGACCACCTCTTCCCGCTCGCTC[VIC-dT]A[THF][BHQ1-dT]GCGCCACCAGCAGCG-[C3spacer] | 78 |
| Real-time PCR | peg344-F | AGCTTCACTTTCGTTCATCCT | 20 |
| | peg344-R | CTGCAGAAGAAAGGGCAATAAC | 22 |
| | peg344-P | FAM-TCCACTGGCTTTCTGTCCTTTCCC-BHQ1 | 33 |
| | incA-F | TTGTCGCTAAAGGGCTGATT | 20 |
| | incA-R | CACGCTCAGGGAGAATTTGA | 20 |
| | incA-P | VIC-ACCTCTTCCCGCTCGCTCTACT-BHQ1 | 31 |

F: forward primer; R:reverse primer; P: probe; FAM-dT: thymidine nucleotide carrying fluorescein FAM; VIC-dT: thymidine nucleotide carrying fluorescein VIC; THF: tetra hydro furan spacer; BHQ1-dT: thymidine nucleotide carrying Black Hole Quencher 1, 3′-block.

## 3. Results

### 3.1. Optimal condition of duplex real-time MIRA

Selecting appropriate primers and probes is crucial for effective duplex real-time MIRA in pathogen detection. In this study, we tested nine primer pairs and one probe targeting the conserved regions of *peg344* and *iucA* genes in hvKP. All combinations yielded positive signals, but primer pairs peg344-F1/R1 (Fig. S2a) and iucA-F3/R2 (Fig. S2b) exhibited the best performance in terms of amplification time and signal intensity. Therefore, peg344-F1/R1/P1 and iucA-F3/R2/P1 were chosen as the optimal sets for hvKP detection.

### 3.2. Specificity and sensitivity of duplex MIRA primers

The specificity of the duplex real-time MIRA assay was assessed using a panel of bacterial pathogens. Positive results were obtained exclusively with the hvKP peg344 and iucA strains, with no positives for non-hvKP peg344/iucA strains (Fig. 1). This confirms the assay's reliable specificity.

For sensitivity testing, genome copy dilutions ranging from approximately $8 \times 10^6$ CFU to $8 \times 10^2$ CFU were used as templates for amplification with both duplex real-time MIRA (Fig. 2a) and duplex real-time PCR (Fig. 2b). The amplification curves demonstrated that the lowest detectable concentration of hvKP peg344 and iucA strain DNA for duplex real-time MIRA matched that of duplex real-time PCR, reaching $8 \times 10^2$ CFU per reaction.

### 3.3. Evaluation of duplex real-time MIRA assay using clinical specimens and comparison with duplex real-time PCR

We evaluated thirty-three clinical spiked blood specimens using the duplex real-time MIRA assay and compared the results with those from the reference duplex real-time PCR method. Seventeen specimens spiked with clinical hvKP peg344 and iucA were positive for hvKP, showing complete agreement with the duplex real-time PCR assay (Table 1). There were no significant differences between the detection results of the duplex real-time MIRA and duplex real-time PCR assays.

## 4. Discussion

Traditional molecular epidemiology methods are often inadequate for controlling the rapid spread of nosocomial infections during an hvKP outbreak. This highlights the need for a rapid, specific, and convenient diagnostic tool capable of detecting both hvKP and other bacterial species. Real-time MIRA, an emerging isothermal amplification method, offers advantages in time efficiency and cost reduction compared to other techniques [18,19]. Based on these findings, we anticipate that real-time MIRA can accurately identify hvKP as well as other non-hvKP bacterial species.

The genome sequences of hvKP obtained from NCBI exhibited significant genetic diversity despite their overall similarity [20]. Specific primers play a crucial role in molecular diagnostic methods, and previous studies have identified *peg344* and *iucA* genes as suitable targets for hvKP detection. In this study, nine sets of duplex real-time MIRA primers and probes targeting *peg344* and *iucA* genes were designed, respectively. After rigorous testing, the primer pairs peg344-F1/R1 and iucA-F3/R2 were selected for the duplex real-time MIRA assay due to their high specificity towards hvKP strains coharboring *peg344* and *iucA* genes, without any cross-reactivity with non-peg344/iucA strains.

The analytical sensitivity of the hvKP peg344 and iucA assay was $8 \times 10^2$ CFU per reaction, consistent with that of the duplex real-time PCR assay. However, the novel duplex real-time MIRA assay offers fewer steps and faster processing while maintaining comparable sensitivity. We tested thirty-three clinical specimens with known pathogen concentrations using both assays, showing 100 % agreement between our duplex real-time MIRA and duplex real-time PCR, indicating its suitability for diagnosing hvKP in spiked blood specimens.

A limitation of this study is the small number and limited variety of clinical hvKP strains with *peg344* or *iucA* used for validation.

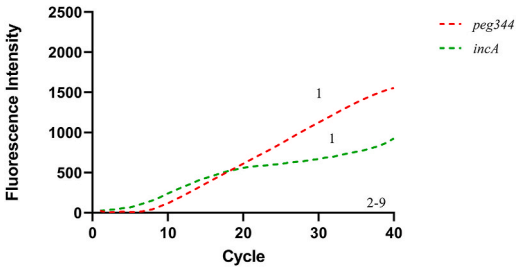


**Fig. 1.** Specificity of the duplex real-time MIRA assay for the detection of hvKP. Partial results display that only the hvKP peg344 and iucA strain (NO. 1) produced amplification signals, whereas the other non-peg344/iucA strains and the negative control (NO. 2–9) did not produce any amplification signals. This experiment was repeated three times with consistent results.

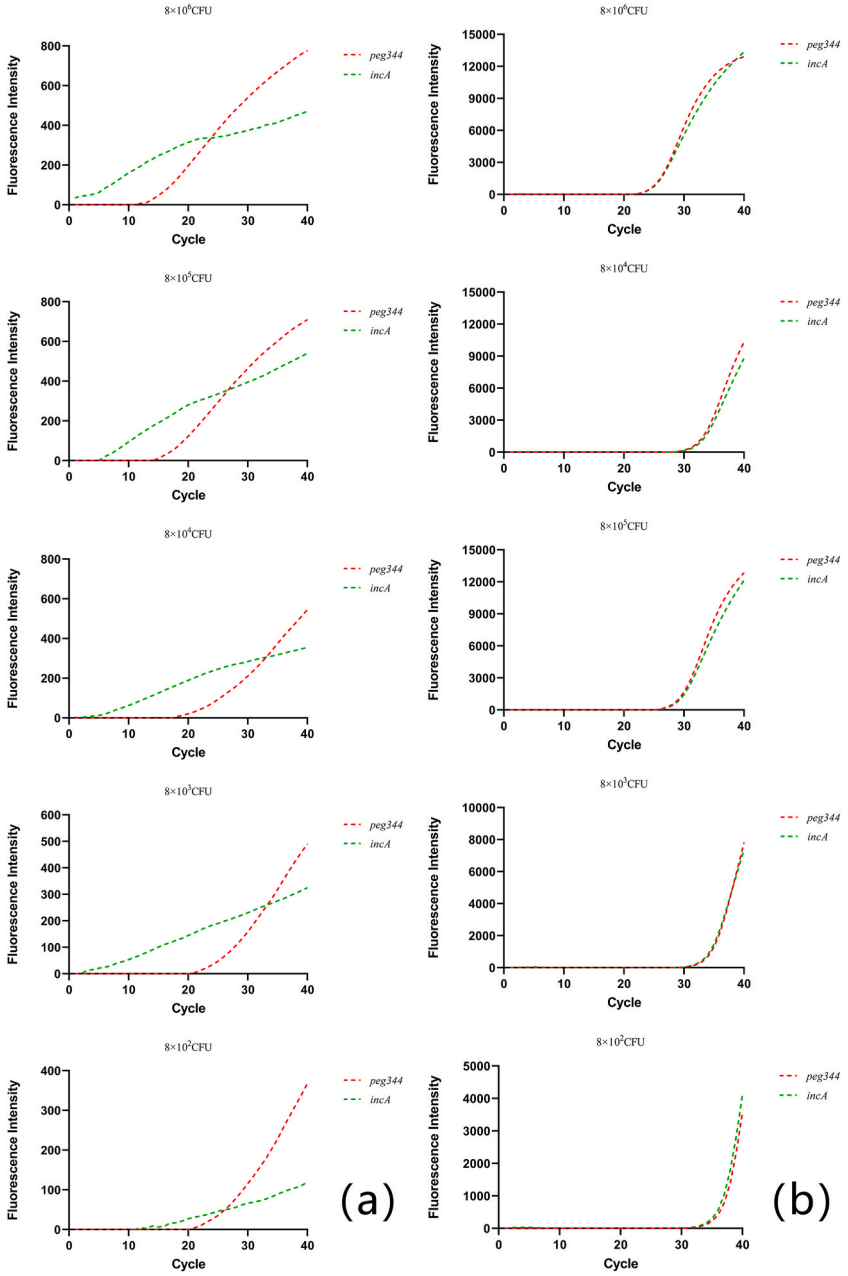


**Fig. 2.** Sensitivity of the duplex real-time MIRA and duplex real-time PCR. The analytical sensitivity of the duplex real-time MIRA assay was evaluated based on the quantity of genomic DNA from hvKP peg344 and iucA strains. Serial dilutions of targeted bacteria (at concentrations of $8 \times 10^6$ CFU, $8 \times 10^5$ CFU, $8 \times 10^4$ CFU, $8 \times 10^3$ CFU and $8 \times 10^2$ CFU per reaction) were tested using both duplex real-time MIRA at 39 °C with a setting of one cycle per 30 s for 20 min (40 cycles) for real-time monitoring of fluorescence signals (a) and duplex real-time PCR at 95 °C for 5 min followed by forty cycles of amplification at temperatures of 95 °C for 10 s and then at a temperature of 60 °C for 30 s (b). This experiment was repeated three times with identical conditions.

Increasing both the number and diversity of validation strains is crucial for ensuring accurate and reliable identification.

In conclusion, the duplex real-time MIRA assay demonstrates excellent specificity and sensitivity, making it a fast, straightforward, and dependable method for detecting hvKP, particularly in diagnostic laboratories with limited resources.

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## Data availability statement

The data that support the findings of the study is available from the corresponding author on reasonable request.

## Ethics tatement

The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the ethics committee of the Affiliated Dazu's Hospital of Chongqing Medical University (Approval No. 2023LLSC074).

## Consent for publication

Not applicable.

## CRediT authorship contribution statement

**Zhixiong Duan:** Software, Methodology, Formal analysis, Data curation, Conceptualization. **Shan Wang:** Project administration, Formal analysis. **Niqi Xie:** Project administration, Investigation. **Junying Zhao:** Resources, Project administration. **Jian Dong:** Validation, Supervision. **Jin Li:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Methodology, Funding acquisition, Formal analysis, Data curation, Conceptualization.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e37050.

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