Detection and Quantification of *Klebsiella pneumoniae* in Fecal Samples Using Digital Droplet PCR in Comparison with Real-Time PCR

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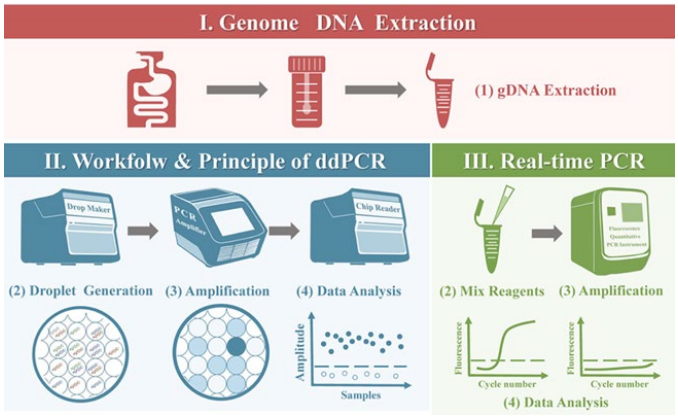
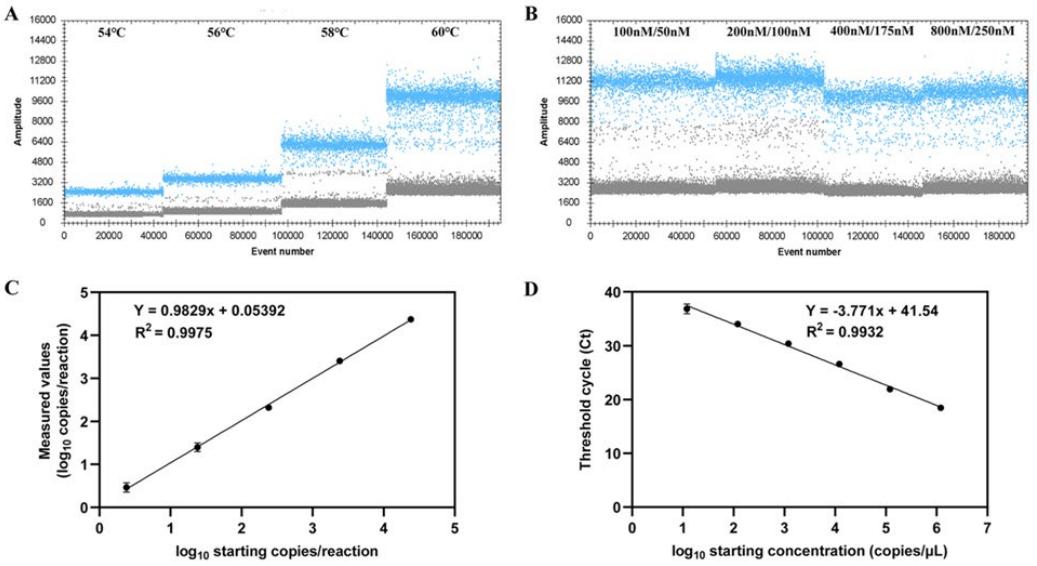
INTRODUCTION (Ahad jami)

- *Klebsiella pneumoniae* is a major opportunistic pathogen associated with various infections, including urinary tract infections and pneumonia. It exhibits high colonization rates in humans and increasing antibiotic resistance.
- Traditional culture methods are time-consuming and lack specificity, necessitating the development of more efficient detection methods for *K. pneumoniae* in fecal samples.
- While real-time PCR offers improved sensitivity and specificity over culture methods, it relies on standard curves for quantification, which can limit accuracy and reproducibility.
- **Droplet digital PCR (ddPCR)** is a new DNA quantitation technology that detects absolute copy numbers of a target without a calibration curve. It involves separating the mixture into thousands of small-volume reaction droplets, conducting endpoint PCR in each droplet individually, and counting positive and negative reactions. Using Poisson binomial distributions, the absolute copy number of a template can be calculated. ddPCR is widely used for diagnosing clinical infectious diseases due to its high sensitivity and accuracy.
- The researchers designed specific primers and probes targeting the *khe* gene of *K. pneumoniae* and evaluated the assay's sensitivity, specificity, and clinical application using 103 fecal samples.



Results (Ahad jami)

- ddPCR Assay Development: The optimal conditions for the ddPCR assay were established, with an elongation temperature of 60°C and primer/probe concentrations of 800 nM/250 nM.
- Sensitivity: The detection limit for ddPCR was found to be 1.1 copies/mL, which is approximately 10 times more sensitive than real-time PCR, which had a detection limit of 10 copies/mL.
- Specificity: The ddPCR assay demonstrated high specificity, detecting only *K. pneumoniae* and not any of the 13 other tested pathogens.
- Clinical Sample Testing: Out of 103 clinical fecal samples, ddPCR identified 99 positives for *K. pneumoniae*, compared to 91 by real-time PCR and 83 by culture methods.
- Clinical Sensitivity: The clinical sensitivity of the ddPCR assay was calculated at 100%, while that of real-time PCR was 98.8%.
- Inhibition Testing: The ddPCR assay showed significantly less inhibition from fecal sample matrix compared to real-time PCR, enhancing its reliability in clinical settings.



DISCUSSION (Shahad Alsubaie):

- *K. pneumoniae* can cause various diseases and easily develops resistance to most antibacterial drugs.
- curing *K. pneumoniae*-caused infections is becoming increasingly difficult. Early detection is thus essential for timely clinical diagnosis and effective treatment.
- Real-time PCR is used as a daily diagnostic tool to check for pathogens due to its good sensitivity and specificity.
- ddPCR, which is a method that quantifies the absolute copy number of a target DNA, is more sensitive and repeatable than real-time PCR for the detection of the pathogen.
- Compared with real-time PCR, ddPCR also has certain limitations, such as smaller sample processing volume than real-time PCR, higher false-positive rate of ddPCR, and higher cost due to the requirement of more expensive equipment and reagents than real-time PCR.
- In the present study, a ddPCR method was established for the detection of *K. pneumoniae* in fecal samples.
- The hemolysin gene of *K. pneumoniae*, which is often used for the detection of *K. pneumoniae* in various clinical samples by different methods, was chosen as the target gene.
- The intestinal tract is a common colonization location of *K. pneumoniae*, so the load of *K. pneumoniae* in feces reflects the situation in the gut to some degree.
- it is not possible to determine whether a single microbial infection or multiple microbial infections occur in a patient based solely on ddPCR detection results.
- Overall, copy numbers of *K. pneumoniae* in fecal samples can be accurately measured by ddPCR with excellent analytical specificity, sensitivity, repeatability, and reproducibility.
- It is a valuable method to identify causal pathogens and guide treatment decisions.

MATERIALS AND METHODS (Shahad Alsubaie):

Sample collection and culture:

- Thirteen hundred and three stool samples were collected from the Capital Institute of Pediatrics for fecal culture and molecular diagnosis.
- A 200-mg sample was taken from each fecal sample and diluted to the appropriate concentration, then fecal dilutions were plated on MacConkey agar plates and incubated for 16 h at 37°C.

Recombinant plasmid construction:

- The sequence of the khe gene from *K. pneumoniae* was downloaded from the NCBI GenBank.
- Then, the synthetic khe gene was cloned into vector pUC57 to obtain recombinant plasmid pUC57-khe.

DNA extraction:

- The total DNA of 200-mg fecal samples were extracted by using a QIAamp DNA stool minikit (Qiagen, Hilden, Germany) following the manufacturer's instructions.

Primers and probe:

- The primers and probe targeting the hemolysin gene of *K. pneumoniae* were designed using Primer Express software (Thermo Fisher Scientific).

TABLE 4 Primers and probe for specific amplification of *K. pneumoniae*[a]

Name	Sequence (5′–3′)
KP-F	CGATGCTACTTATCCCGACA
KP-R	AGCCGGTTGAGACGTAAAC
KP-probe	6FAM-CCGATTGAAAAACGCTCCGGGC-BHQ1

[a]KP, *klebsiella pneumoniae*; F, forward primer; R, reverse primer.

ddPCR assay:

- The ddPCR assay was performed using a TARGETING ONE droplet digital PCR system.

Real-time PCR assay:

- All real-time PCR assays were performed using a QuantStudio 7 Flex (Applied Biosystems) instrument.

Estimation of the limit of detection, limit of blank, and specificity:

- To analyze the limit of detection for ddPCR and real-time PCR assays, recombinant plasmid with DNase-free water.
- For the specificity evaluation, 13 pathogens' DNA.

Repeatability and reproducibility of ddPCR assay:

- For repeatability evaluation, 10-fold dilutions of recombinant plasmid pUC57-khe were examined in triplicate in one experiment run, and the intraassay coefficients of variation (CV) was calculated.

Detection of ddPCR and real-time PCR inhibition by residual matrix:

- To identify the inhibition effect of residual matrix, the capacity of the ddPCR and real-time PCR assays to quantify a constant amount of plasmid DNA in the presence of different quantities of fecal DNAs were compared.

Statistical analysis:

- PASS software was used for sample size calculation. IBM SPSS Statistics version 21.0 was used for statistical analyses.

Ethics statement:

- All fecal samples used in this study are part of routine patient management without any additional collection.