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# Development of a multiplex two-gene real-time PCR assay for accurate detection of *Klebsiella pneumoniae*

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*Klebsiella pneumoniae* is a pathogenic Gram-negative bacterium of the *Enterobacteriaceae* family [1]. Due to the resistance to carbapenems, *K. pneumoniae* has become an urgent threat to public health, so that rapid and accurate detection of this pathogen is required for the timely administration of accurate therapy and controlling outbreaks of carbapenem-resistant strains. Methods for detecting microbes are summarised in Table 1: many have shortcomings. Real-time PCR systems have been developed for identification of *K. pneumonias*, although these methods target single genes [2–4]. Moreover, all the designed primers were based on the reported gene sequences of *K. pneumoniae* in the database. Due to the high genetic diversity of *K. pneumoniae* epidemic strains, sensitivity and specificity of these methods could not satisfy the requirements of current precise treatment. Thus, an alternative PCR assay targeting additional *K. pneumoniae* genes based on gene sequencing of local strains would be a promising strategy to detect local strains with high sensitivity and specificity. *rcsA*, a gene specific to *K. pneumoniae* that regulates the synthesis of capsular polysaccharide, was selected as one target gene because it is more stable [3]. The *23S rRNA* gene, widely used in identification of the pathogen [5], was selected as another target. Our objective was to develop a multiplex real-time PCR assay for the rapid detection and accurate quantification of *K. pneumoniae* by targeting both *rcsA* and *23S rRNA*.

Ninety-five *K. pneumoniae* local isolates were randomly selected as positive controls, whilst 95 local isolates commonly found in the lower respiratory tract as pathogens were negative controls. The latter include *Streptococcus pneumoniae, Haemophilus influenza, Staphyloccoccus aureus, Moraxella catarrhalis, Escherichia coli, Pseudomonas aeruginosa* and *Acinetobacter baumannii*. All positive and negative control strains were isolated from respiratory samples and were characterized by colonial morphology, standard biochemical methods and VITEK-2 (bioMerieux, Basingstoke, UK). A total of 2754 sputum specimens were collected from patients with suspected lower respiratory tract infections between January 2015 and December 2016 following routine microbiological culture. Specimens with >25 white blood cells per high-power field and <10 squamous cell per high-power field through Gram smear detection were accepted. Ethical committee approval and informed consent from all participants were obtained.

Pure cultures of bacterial isolates were suspended in saline to 0.5 McFarland standard concentration, and total nucleic acid was extracted from 100 µL suspension with the DNA Extraction Kit according to the manufacturer's instructions (Huiyan Biotech, Shenzhen, China). For sputum specimens, liquified sputum [EDTA 10 mM containing N-acetyl-L-cysteine (v/v, 1.0%), emulsifier (v/v, 0.25%), sterilizing agent (v/v, 0.2%)] was added into aseptic phlegm culture bottle at a ratio of 1:1 and vortexed for 5 min. Total nucleic acid was extracted from 100 µL of this mixture with the DNA Extraction Kit. A 1000 bp DNA sequence was randomly generated with DNAMAN software (Lynnon Biosoft, San Ramon, CA, USA). The random DNA sequence was synthesized and introduced into plasmid pBSK resulting into pBSK-R by TaKaRa (TaKaRa, Dalian, China). The resulted pBSK-R was used as negative quality control template.

To amplify the *rcsA* and *23S rRNA* of *K. pneumoniae* isolates, primers were designed based on the sequence of the two genes in the GenBank database.

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Primers rcsAF (5′-GCCATCCACATTTGCAGCATA-3′ and 5′- GCGTTAAGCCTTCCACCCC-3′) and rcsAR (5′-GCGTTAAGCCTTCCACCCC-3′) were used for *rcsA*, and primers 23SF (5′-CAAGGCTGAGGTGTGATGAC-3′) and 23SR (5′-GTTACGCTTTGGGAGGAGAC-3′) were used for *23S rRNA*. PCR was carried out in a total volume of 50 µL containing 25 µL of iQ Multiplex Powermix (Bio-Rad Laboratories, Hercules, CA, USA), 0.5 µL of each primer (10 µM), 0.5 µL of dNTP (10 mM), 5 µL of template and 18.5 µL of nuclease-free water. PCR was run on the Roche LightCycler® L480 (Roche, Switzerland) for 95 °C for 2 min, followed by 30 cycles of 95 °C 10 s, 59 °C 10 s and 72 °C 30 s. All PCR products were sequenced by BGI (BGI, Shenzhen, China).

Primers and probes for multiplex PCR were designed based on the sequence of *rcsA* and *23S rRNA* of 95 *K. pneumoniae* isolates and sequences of plasmid pBSK-R, respectively. Primers rcsAQF (5′-GGCAACACGACGTACAGT-3′) and rcsAQR (5′- GGTTGGGATTGACGGGATAT-3′) and probe rcsAP (5′-FAM-AGATCCGCAGCACTGTTGACCTCA-BHQ1-3′) were used for *rcsA*. Primers 23SQF (5′-CACAGGTGGTCAGGTAGAGA ATAC-3′) and 23SQR (5′-CTGGTCTCAGCTCCATCCG-3′) and probe 23SP (5′-VIC-ACCAGCGTGCCTTCTCCCGA-BHQ1-3′) were used for *23S rRNA*. Primers RSF (5′-GCTAGTCTCAAGAGTCTGGAAG-3′) and RSR (5′-CTGGGCTGTCTAATGCTGC-3′) and probe RSP (5′-ROX-CCGTCAGCATCCTCGCATC(b)AAGCA-BHQ2-3′) were used for plasmid pBSK-R. The multiplex PCR was carried out in a total volume of 40 µL comprising: buffer (5×), 8 µL; dNTP (10 mM each), 0.5 µL; each primer (10 µM), 1.2 µL; each probe (10 µM), 0.4 µL; pBSK-R, 0.1 µL; and DNA template, 20.0 µL. The final volume was adjusted to 40 µL with nuclease-free water. The reaction was performed on Roche LightCycler® L480 at 95 °C for 2 min, followed by 40 cycles of 95 °C for 10 s and 60 °C for 40 s. Samples were classed as positive if the $C_T$ values were ≤36 and the angle between amplification curve and baseline ≥30 °C. Otherwise, samples were classed as negative. For quantification, a pure culture of a *K. pneumoniae* isolate in log phase was adjusted to $1.0 \times 10^8$ cfu/mL after its concentration was calculated by the plate count method. Dilution series ranging from $1.0 \times 10^2$ to $1.0 \times 10^8$ cfu/mL were prepared, and serial DNA was extracted from the dilution series and included in each run. Roche LightCycler L480 software was used to construct an eight-point standard curve and extrapolate a quantitative result. Conversion of cfu/mL to cfu/PCR was based on a 20 µL input per PCR reaction drawn from 100 µL of DNA concentrated from 1 mL culture.

The amplification efficiency of PCR ($E = 10^{(-1/\text{slope})-1}$) was calculated after testing several replicates of dilution series in different runs and was accepted when the values were between 0.90 and 1.10. Correlation coefficient ($R^2$) was determined when the standard curves were constructed. The quantitative range was determined from the range of 10-fold DNA dilutions, which gave optimal efficiency, linearity, standard deviation and coefficient of variation. Sensitivity and specificity were measured by testing DNA extracted from 95 positive *K. pneumoniae* isolates and 95 negative isolates.

To evaluate the clinical utility of the multiplex real-time PCR assay, 2754 clinical samples were tested. Positive samples by microbial culture were classed true positive, negative samples by microbial culture but identified as positive by both PCR assay and sequencing were also classified true positive. Negative samples by both microbial culture and PCR assay were classed true negative, whilst negative samples by microbial culture but identified as positive by PCR assay and negative by

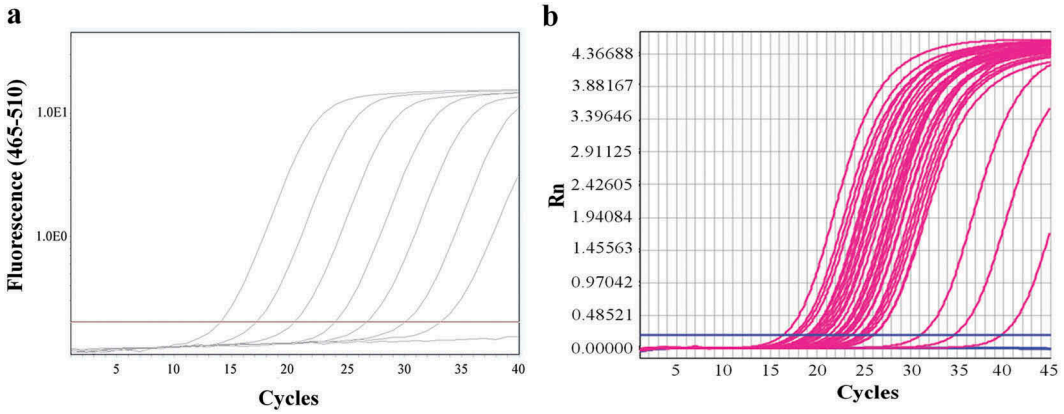


**Figure 1.** (a) The standard curve of *K. pneumoniae* in multiplex real-time PCR assay. Ten-fold serial dilutions of *K. pneumoniae* DNA were used for standard curve construction. DNA concentrations are (from left to right) $10^8$, $10^7$, $10^6$, $10^5$, $10^4$, $10^3$, $10^2$ and blank. (b) Amplification curves of the PCR assay on the culture-positive sputum specimens. From left to right: curve cluster for sputum specimens with high concentration of *K. pneumoniae*, low concentration of *K. pneumoniae* and negative control ($H_2O$).

sequencing were also classed true negative. The diagnostic sensitivity was calculated as true positive/(true positive + false negative) and diagnostic specificity as true negative/(true negative + false positive).

Specific primers were designed according to the gene sequences of 95 *K. pneumoniae* local isolates and used to develop the multiplex real-time PCR. Standard curves were constructed after quantification of the DNA dilution series by the multiplex real-time PCR (Figure 1(a)). The detection limit of the PCR assay was estimated as 2 cfu/reaction. Amplification of the PCR assay for *rcsA* and *23S rRNA* was linear and efficient within the quantitative range 2–2×$10^6$ cfu/reaction (*rcsA*, $E$ = 1.02, $R^2$ = 0.99960; *23S rRNA*, $E$ = 0.996, $R^2$ = 0.99982). Within the range, all the positive controls were positive with amplification efficiency ($E$ value) of 0.90–1.10 and correlation coefficient ≥0.98. Sensitivity and specificity were 98.9% (94/95) and 97.9% (93/95), respectively.

In the 2754 sputum specimens, 355 sputum specimens were *K. pneumoniae* positive and 2399 sputum specimens were *K. pneumoniae* negative with culture-based method. The 355 culture-positive sputum specimens were retrospectively tested with our assay, and the amplification curves of some positive samples were shown in Figure 1(b). In the 355 sputum specimens, 349 positive specimens and 6 negative specimens were detected by the PCR assay. In addition, the 2399 culture-negative sputum specimens were retrospectively tested with the PCR assay. In 104 of 2399 sputum specimens, *K. pneumoniae* was detected with the PCR assay. PCR products of the 104 PCR-positive specimens were sequenced, and 75 were confirmed to be *K. pneumoniae* by sequence alignment. Overall, the PCR assay showed a diagnostic sensitivity of 98.6% (424/430) and a diagnostic specificity of 98.8% (2295/2324) on 2399 clinical sputum specimens.

Table 1 compares the PCR assay developed here with alternative techniques, which have limitations [4,6-15]. Methods based on culture and macroscopic observation are time consuming, laborious, and, by definion, miss uncultivatable organisms. There is no application in the differentiation of highly related or fastidious microbes and in epidemiological investigations. Morphological methods (optical and electron microscope) suffer from non-specific identification, The sensitivity of these methods declines with lower colony counts, and some Gram-positive bacteria are identified as Gram-negative. Tissue is occasionally placed in fixative without consideration of the potential need for culture. Mass spectrometry methods rely on bacterial proteins being highly conserved and abundant in the particular bacterial cell, and demand experienced operators. Species with a lower rate of differences in their ribosomal protein sequences can be misidentified, and the accuracy of the identification depends greatly on the number of database entries. PCR analysis rely on conserved genes and species-specific sequences.

Primers used in previous reports were all designed according to the reported gene sequences of *K. pneumoniae* in database and were not specific to target genes of *K. pneumoniae* due to the high genetic diversity of *K. pneumoniae* strains. Primers used in this study were designed on sequence alignments of local isolates of *K. pneumoniae,* which enable the specificity of the PCR assay on local *K. pneumoniae* strains. For the precise detection of *K. pneumoniae*, two target genes were chosen, which is also different to the previous reports. In addition, application of the PCR assay in diagnosis of clinical specimens with a large sample size was evaluated, showing a better application potential in comparison to the previous reports with small specimen sizes. A total of 75 culture-negative sputum specimens retested with our PCR assay and gene sequencing were *K. pneumoniae* positive, which also justify the potential of our multiplex real-time PCR for *K. pneumoniae* detection.

**Table 1.** Comparison of the reported methods with the multiplex real-time PCR assay in this study.

| Method | Limit of detection, CFUs/PCR | Sensitivity (%) | Specificity (%) | Time (h) | Reference |
|---|---|---|---|---|---|
| Culture method and macroscopic observation | N.A. | Poor | N.A. | 2–4 days | [4] |
| Morphologic methods (optical microscope and electron microscope) | N.A. | Low | Low | N.A. | [6] |
| Serological methods | N.A. | N.A. | N.A. | 2–4 weeks | [7] |
| Automated Microbic System (metabolism activity and biochemical tests) | N.A. | N.A. | N.A. | 2–10 h | [8] |
| Mass spectrometry methods (analysis of bacterial proteome) | N.A. | Rapid, accurate and sensitive | N.A. | a few minutes | [9] |
| PCR (genetic analysis) | N.A. | 100% | 100% | N.A. | [10] |
| | $10^4$ CFU/mL | 100% | 100% | N.A. | [11] |
| | N.A. | 100% | 100% | N.A. | [12,13] |
| | N.A. | 67–97% | 89–100% | N.A. | [14] |
| | 4 CFU/PCR | 100% | 100% | ≤1.5 h | [15] |

N.A. = not applicable/available. CFU = colony forming units.

This work represents an advance in biomedical science because the multiplex PCR targeting both *rcsA* and *23S rRNA* of *K. pneumoniae* improves the diagnostic sensitivity and specificity.

## Disclosure statement

No potential conflict of interest was reported by the authors.

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