DEVELOPMENT OF A NOVEL MULTIPLEX PCR ASSAY FOR THE DETECTION OF MOBILE CARBAPENEM, TETRACYCLINE RESISTANCE GENES, AND CLASS 1 INTEGRON IN ENVIRONMENTAL AND CLINICAL SPECIMENS

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ABSTRACT

The rapid spread of antibiotic-resistant bacteria and antimicrobial resistance genes threatens the global health. Polymerase chain reaction based assays offer rapid detection of antibiotic-resistant bacteria and resistance determinants. This study develops a novel multiplex polymerase chain reaction assay for the simultaneous detection of genes: *tetM* (tetracycline resistance), *blaNDM* and *blaKPC* (carbapenem resistance), and *intI1* (class 1 integron-integrase gene). The assay demonstrated 100% sensitivity and specificity for the detection of these target genes, both individually and in mixtures. The assay's limit of detection was established at 10^1 copies/mL for *blaKPC* and *blaNDM*, and at 10^4 copies/mL for *tetM* and *intI1*. Furthermore, the multiplex polymerase chain reaction assay was successfully applied to the direct screening of target genes in environmental and clinical samples. In conclusion, the multiplex polymerase chain reaction assay developed in this study represents a valuable tool for the surveillance of *tetM*, *blaNDM*, *blaKPC*, and *intI1* genes, and for the detection of antibiotic-resistant bacteria.

PHÁT TRIỂN PHƯƠNG PHÁP MULTIPLEX PCR PHÁT HIỆN CÁC GEN KHÁNG KHÁNG SINH THUỘC NHÓM CARBAPENEM, TETRACYLINE VÀ INTERGON LOẠI 1 TRONG CÁC MẪU MÔI TRƯỜNG VÀ LÂM SÀNG

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TỪ KHÓA

Multiplex PCR
Intergon loại 1
Kháng carbapenem
Kháng tetracycline
Đa kháng thuốc

TÓM TẮT

Sự lan truyền nhanh chóng của vi khuẩn kháng kháng sinh và các gen kháng thuốc đe dọa sức khỏe cộng đồng. Các phương pháp dựa trên phản ứng chuỗi polymerase cho phép phát hiện nhanh vi khuẩn kháng thuốc và các yếu tố di truyền kháng kháng sinh. Nghiên cứu này phát triển một phương pháp phản ứng chuỗi polymerase đa mồi để phát hiện đồng thời các gene: *tetM* (kháng tetracycline), *blaNDM* và *blaKPC* (kháng carbapenem), và *intI1* (integron-integrase loại 1). Phương pháp này có độ nhạy và độ đặc hiệu 100% trong việc phát hiện các gene đích, cả riêng lẻ và trong hỗn hợp. Ngưỡng phát hiện của phương pháp được xác định ở mức 10^1 bản sao/mL đối với *blaKPC* và *blaNDM*, và 10^4 bản sao/mL đối với *tetM* và *intI1*. Hơn nữa, phương pháp phản ứng chuỗi polymerase đa mồi đã được áp dụng thành công trong việc sàng lọc các gene đích trực tiếp từ các mẫu môi trường và lâm sàng. Tóm lại, phương pháp phản ứng chuỗi polymerase đa mồi được phát triển trong nghiên cứu này có thể được sử dụng cho việc giám sát các gene *tetM*, *blaNDM*, *blaKPC* và *intI1*, và phát hiện vi khuẩn kháng kháng sinh.

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1. Introduction

Antibiotic resistance has emerged as a global public health threat, contributing to increased morbidity and mortality rates [1]. The overuse, misuse, and uncontrolled release of antibiotics in various environments have accelerated the emergence and dissemination of antibiotic-resistant bacteria (ARB) and antibiotic resistance genes (ARGs) [1]. These resistant pathogens are now widespread in diverse settings, including healthcare facilities, soil, sediment, aquatic environments, marine ecosystems, and the atmosphere. In 2017, the World Health Organization (WHO) published its first list of 12 priority pathogens, highlighting those for which new antibiotic development is urgently needed [2]. Among these, carbapenem-resistant organisms were classified in the "critical" priority group, underscoring the severity of resistance to last-resort antibiotics [2]. In 2024, the WHO updated its priority pathogens list, maintaining carbapenem-resistant *Acinetobacter baumannii* and *Enterobacterales* in the critical priority group, while also emphasizing the ongoing need for research and development of new antibiotics against these and other high-priority pathogens [3]. This update underscores the persistent and evolving threat of antibiotic resistance, reinforcing the urgent call for global action to combat this challenge. Among strategies, development of efficient and cheap assays for rapid detection of ARGs, facilitating timely and appropriate clinical interventions, and serving for surveillance of antimicrobial resistance (AMR) [4].

Carbapenem is one of the last sort antibiotics which is used to treat multidrug-resistant bacteria [5]. Nevertheless, the prevalence of carbapenem-resistant bacteria is rapidly increasing in healthcare settings globally [2] – [6]. Carbapenem resistance is very difficult to treat with a high rate of mortality and motility [2] – [6]. Furthermore, the high prevalence of carbapenem resistance in livestock and aquaculture, along with the widespread presence of carbapenem-resistant organisms in natural environments, highlights the growing concern over environmental dissemination [7]. The primary mechanisms of carbapenem resistance involve the acquisition of carbapenemase-encoding genes such as *blaKPC, blaNDM*, *blaIMP*, *blaOXA*, and *blaVIM*, with *blaKPC* and *blaNDM* being the most prevalent [8]. These genes are easily exchanged and transmitted among bacterial populations via horizontal gene transfers [8]. In addition, tetracyclines are a family of broad-spectrum antibiotics with an excellent safety profile, making them widely used in human and veterinary medicine, livestock and aquaculture [9]. However, the emergence and spread of tetracycline resistance, particularly through the *tetM* gene, pose a significant challenge to their clinical efficacy. The *tetM* gene is widely distributed and commonly found with transposons, which are mobile genetic elements that facilitate its transfer between bacteria [10]. This association with transposons contributes to the rapid dissemination of tetracycline resistance across diverse bacterial populations. The *tetM* was detected in various environments including soil, water, and food products, underscores the need for comprehensive strategies to monitor and control its spread [10], [11].

In this context, we present the development of a novel multiplex polymerase chain reaction (PCR) assay for the rapid detection and surveillance of common ARGs, including *tetM* (tetracycline resistance), *blaNDM*, and *blaKPC* (carbapenem resistance). Furthermore, the assay incorporates the integron class I gene (*intI1*), which is closely associated with the dissemination and amplification of ARGs. The assay was evaluated on two distinct sample types: environmental samples (water and sediment) and human clinical specimens. This multiplex PCR assay provides a valuable tool for monitoring the prevalence of these resistance genes across different environmental and clinical settings. It holds significant potential as a reference method for informing strategies aimed at combating the emergence and spread of AMR in Vietnam.

2. Materials and Methods

2.1. Antibiotic-resistant bacterial strains and targeted gene preparations

For the optimization of multiplex PCR, reference strains harboring carbapenem-resistance genes were used as positive controls, including *Klebsiella pneumoniae* NCTC 13438 (blaKPC-positive) and *Klebsiella pneumoniae* ATCC® BAA-2146™ (blaNDM-positive) provided by International Joint Laboratory LMI DRISA, Vietnam-Cambodia-France. These strains were cultured on Luria-Bertani (LB) agar plates, and genomic DNA was extracted using the Bacterial DNA Isolation Kit (QIAGEN, Germany). Additionally, *tetM* and *intI1* genes were directly amplified from positive samples as described previously [12]. The resulting PCR amplicons were purified and utilized for the optimization of the multiplex PCR assay.

2.2. Development of multiplex PCR assay

Initially, a singleplex PCR assay was performed to evaluate the specificity and sensitivity of each primer pair for target gene amplification. The primer sequences and corresponding amplicon sizes are presented in Table 1. PCR amplification was conducted in a thermal cycler (ProFlex™ PCR Systems, Applied Biosystems™, Themo Fisher Scientific) with a total reaction volume of 25 µL. Each reaction mixture contained 5 µl of 2x Taq Master Mix (Dye Plus, Vazyme), 8 µL of forward and reverse primers (10 µM each), nuclease-free water to a final volume of 23 µL, and 2 µL of DNA template. The thermal cycling conditions were as follows: initial denaturation at 95 °C for 5 min, followed by 35 cycles of 95 °C for 15 s, 59 °C for 1 min, and 72 °C for 1 min, with a final extension at 72 °C for 5 min.

Subsequently, a multiplex PCR assay incorporating four primer pairs targeting *tetM*, *blaKPC*, *blaNDM*, and *intI1* was optimized to detect individual target genes and their combinations within a single reaction. The multiplex PCR assay was thoroughly evaluated for its specificity in detecting combinations of two, three, and four target genes within a single reaction. Primer concentrations were further adjusted to enhance the specificity and sensitivity of the assay, while PCR conditions remained consistent with those used in the singleplex PCR. In addition, to determine the limit of detection (LoD) of the multiplex PCR assay, the DNA templates of corresponding target genes were diluted to create a series of concentrations ranging from 10^0 to 10^6 copies/mL. Amplified products were analyzed via 1.5% agarose gel electrophoresis. To ensure experimental reliability, non-template controls (NTCs) were included in all assays.

Table 1. *The primer sequences used for detection of target genes*

Primer	Sequence	Amplicon size (bp)	Reference
blaKPC-F blaKPC-R	5’-CTGTCTTGTCTCTCATGGCC-3’ 5’-CCTCGCTGTRCTTGTCATCC-3’	796	[13]
blaNDM-F blaNDM-R	5’-TGGCAGCACACTTCCTATC-3’ 5’-AGATTGCCGAGCGACTTG-3’	488	[14]
tetM-F tetM-R	5'-ACACGCCAGGACATATGGAT-3' 5'-GGGAATCCCCATTTTCCTAA-3'	126	[15]
IntI1-F IntI1-R	5-'GCTGGATAGGTTAAGGGCGG-3' 5'-CTCTATGGGCACTGTCCACATTG-3'	592	[16]

2.3. Environmental and clinical samples

The multiplex PCR assay was introduced to detect the target genes on different types of sample. Specifically, a total of 50 samples including 34 environmental samples (17 water and 17 sediment samples) from the Mekong River, and 16 clinical specimens (stool samples from colorectal cancer patients from 108 Military Centre Hospital, Hanoi). The total DNA was extracted from the samples using the DNeasy PowerSoil Pro kit (QIAGEN, Germany). The purity and concentration of DNA were examined using a Nanodrop 2000 spectrophotometer (Thermofisher Scientific, USA).

2.4. Statistical analysis

Data analysis was carried out employing a range of statistical methods to interpret and visualize the findings. Initially, descriptive statistical analysis was performed to summarize the key characteristics and distributions of the variables of interest. Following this, appropriate statistical tests were applied to evaluate the significance of the observed results. The analysis was conducted using R Studio (R Core Team, 2021) for data processing and visualization.

3. Results and Discussions

3.1. Specificity of multiplex PCR assay for the detection of target genes

The specificity of the multiplex PCR assay was initially confirmed by the successful detection of individual target genes, as shown in Figure 1a. The PCR amplicons corresponded to the expected sizes for the *tetM*, *blaNDM*, *intI1*, and *blaKPC* genes, as listed in Table 1. Although some non-specific bands were observed in lanes 1 and 4 (Figure 1a), the target amplicons appeared clearly and with strong intensity, indicating that the primer sets exhibit high specificity for their respective target genes. Subsequently, the multiplex PCR assay was developed to assess its specificity in simultaneous detection of multiple targets in combinations of two, three and four genes (Figure 1a and 1b). As anticipated, the multiplex assay successfully amplified the target genes, producing amplicons of sizes identical to those obtained in the singleplex PCR, demonstrating its reliability for detecting multiple resistance determinants in a single reaction. Finally, the optimal primer concentrations for the multiplex PCR master mix were determined for each primer pair as follows: *tetM* (0.88 μM), *intI1* (0.72 μM), and *blaNDM* and *blaKPC* (0.8 μM for each pair).

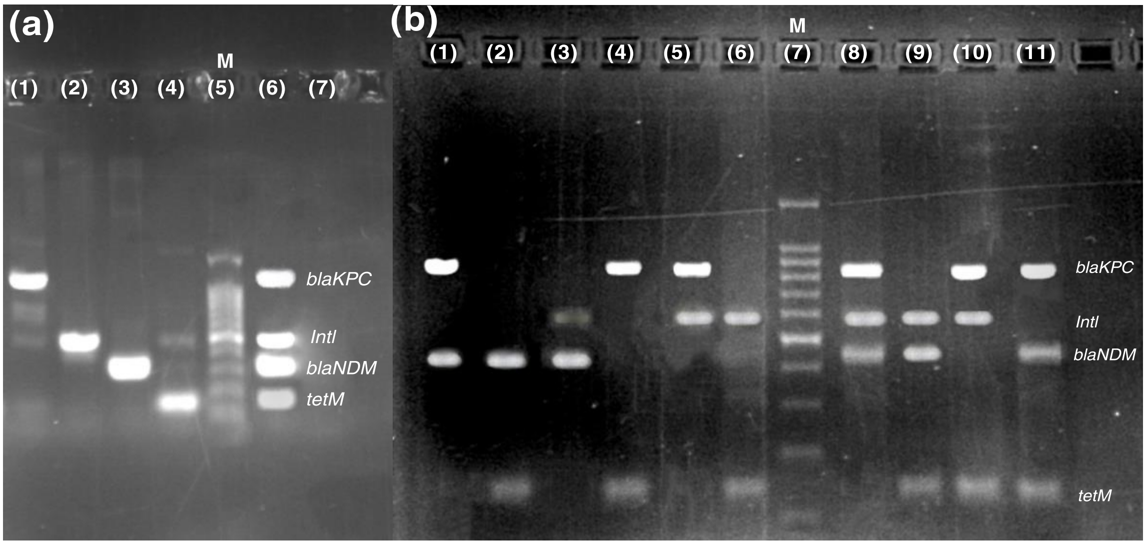

Figure 1. *Specificity of the multiplex PCR assay for the detection of target genes: (a) Lane assignments: Lane 1 – blaKPC; Lane 2 – intI1; Lane 3 – blaNDM; Lane 4 – tetM; Lane 5 – DNA 100 bp ladder; Lane 6 – mixture of all four target genes (blaKPC, intI1, blaNDM, tetM); Lane 7 – negative control. (b) Lane assignments: Lane 1 – blaKPC + blaNDM; Lane 2 – blaNDM + tetM; Lane 3 – intI1 + blaNDM; Lane 4 – blaKPC + tetM; Lane 5 – blaKPC + intI1; Lane 6 – intI1 + tetM; Lane 7 – DNA 100 bp ladder; Lane 8 – blaKPC + intI1 + blaNDM; Lane 9 – intI1 + blaNDM + tetM; Lane 10 – blaKPC + intI1 + tetM; Lane 11 – blaKPC + blaNDM + tetM.*

Previous study [17] developed multiplex real-time PCR assays for the detection of carbapenem-resistant genes, including *blaKPC* and *blaNDM*, in combination with *blaOXA-48*, *blaVIM*, and *blaIMP*, from cultured bacterial isolates, achieving 100% sensitivity and specificity.

Similarly, a subsequent study established a novel multiplex real-time PCR assay for the detection of *blaKPC*, *blaNDM*, *blaVIM*, and *blaOXA-48* directly from clinical isolates using colony PCR [18]. A more recent study [19] developed a multiplex real-time PCR for the simultaneous detection of common carbapenem and colistin resistance genes, including *blaKPC*, *blaNDM*, *blaIMP*, *blaOXA-48*, and *mcr-1*, also with 100% sensitivity and specificity. In contrast, our study presents the first multiplex PCR assay designed for the rapid detection of *blaKPC*, *blaNDM*, *tetM*, and *intI1* genes, demonstrating 100% sensitivity and specificity. This novel assay not only targets the commonly encountered mobile carbapenem-resistance and tetracycline-resistance genes but also enables the detection of the *intI1*, which play a crucial role in the propagation and dissemination of antibiotic resistance genes within bacterial populations.

3.2. Limit of detection of the multiplex PCR assay for target genes

The LoD of the multiplex PCR assay was determined for detecting individual target genes. Specifically, the LoD for *blaKPC* and *blaNDM* was 10^1 copies/mL (Figure 2a and 2b), while the LoD for the *tetM* and *intI1* genes was higher, at 10^4 copies/mL (Figure 2c and 2d). These findings highlight the assay's high sensitivity for detecting mobile carbapenem-resistant genes, consistent with previous studies that reported similar sensitivities for detecting *blaKPC* and *blaNDM* genes, with an LoD of 10^1 CFU/mL [20]. Although the assay demonstrated relatively lower sensitivity for *tetM* and *intI1*, it remains suitable for screening these resistance genes within bacterial populations. Importantly, *intI1* is a major genetic element involved in the dissemination of antibiotic resistance determinants, contributing significantly to the genomic plasticity and environmental adaptability of bacteria [21]. Given the global prevalence of *intI1*, ongoing monitoring and surveillance of its circulation are essential for effectively controlling the transmission and spread of antibiotic-resistant bacteria and their associated resistance genes.

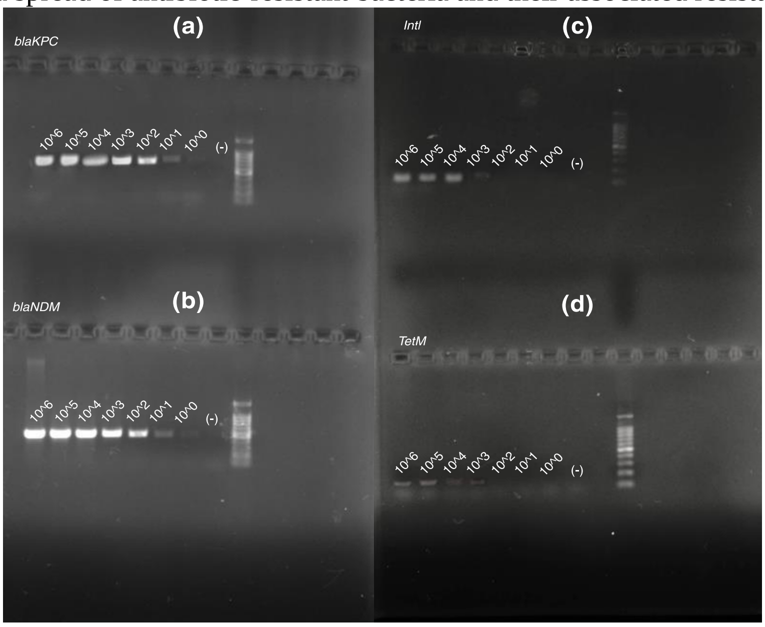

Figure 2. *Limit of detection of the multiplex PCR assay for individual target genes.*
Ten-fold serial dilutions ranging from 10^6 to 10^0 copies were prepared for each individual target gene: (a) blaKPC*; (b)* blaNDM*; (c)* intI1*; and (d)* tetM. *Lane (–) represents the negative control.*

3.3. Evaluation of the multiplex PCR assay for detection of target genes in environmental and clinical samples

The evaluation results of the multiplex PCR assay for detecting target genes in the selected samples are presented in Figure 3a. Specifically, in water samples, *tetM* was detected in all samples (100%), followed by *intI1* (16/17, 94.1%) and *blaNDM* (10/17), while *blaKPC* was absent in all samples (Figure 3b). In sediment samples, the three genes *tetM*, *blaKPC*, and *intI1* were present in all samples (100%), while *blaNDM* was detected in 16 out of 17 samples (94.1%) (Figure 3c). These results underscore the high prevalence of ARGs in the Mekong River ecosystem, highlighting a significant public health concern [22].

In clinical samples, all (100%) tested positive for the presence of *tetM*, *blaKPC*, *blaNDM*, and *intI1* genes, reinforcing the notion that patients undergoing treatment for colorectal cancer are at heightened risk of exposure to antibiotic resistance (Figure 3d). This is likely due to the extensive use of antibiotics before, during, and after treatment, which can significantly alter the microbiome and increase the susceptibility to infections caused by MDR bacteria.

The DNA concentrations observed in the water samples ranged from 21 ng/µL to 96 ng/µL, while sediment samples exhibited concentrations between 29.2 ng/µL and 380.9 ng/µL, and clinical specimens ranged from 20.8 ng/µL to 276.2 ng/µL. These results demonstrate the multiplex real-time PCR assay's robust capacity to detect target genes across a wide concentration range in diverse substrates, including water, sediment, and clinical specimens. The assay’s high sensitivity and specificity make it an invaluable tool for the rapid detection of carbapenemase genes, providing critical, real-time data that can inform timely treatment decisions in clinical practice. Additionally, its applicability for surveillance of ARGs across various environmental matrices highlights its utility in monitoring the spread of AMR in different ecosystems.

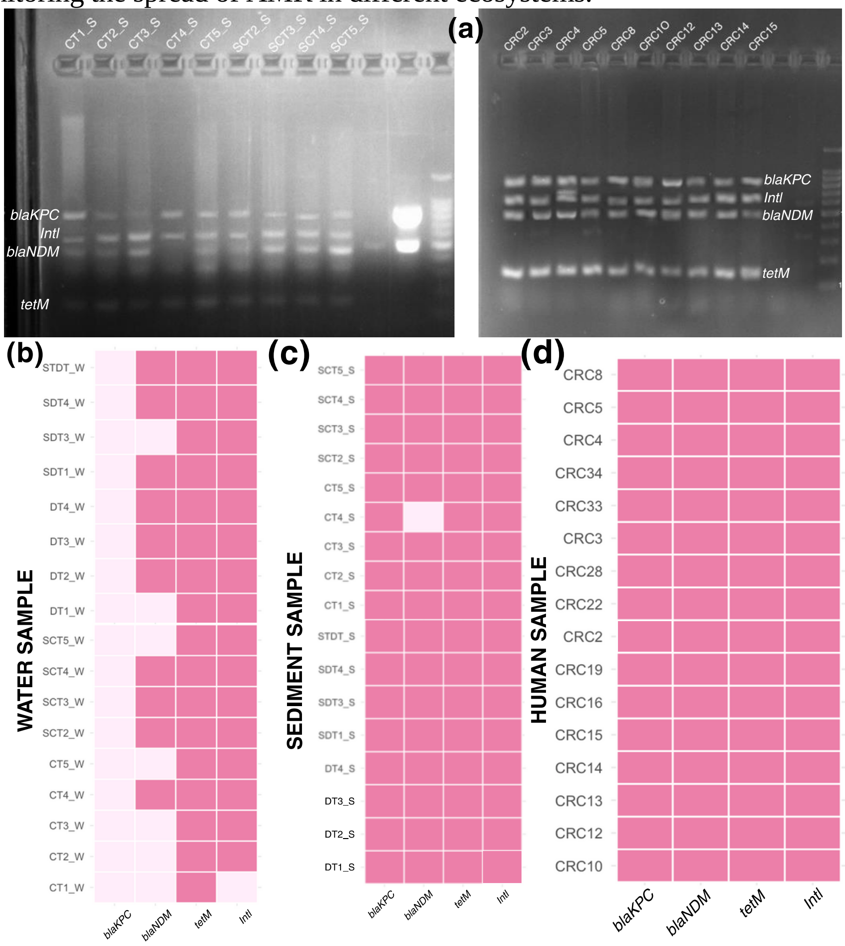

Figure 3. *Results of the multiplex PCR assay for detection of target genes in different environmental samples and in clinical specimens: (a) The results of agarose gel electrophoresis for selected samples; (b) Water samples; (c): Sediment samples; (d): Colorectal cancer patients specimens*

4. Conclusion

This study successfully developed a novel multiplex PCR assay capable of simultaneously detecting four key antibiotic-resistance genes *tetM*, *blaKPC*, *blaNDM*, and *intI1* within a single reaction. The assay demonstrated effective amplification and specificity across the target genes, highlighting its potential application in AMR surveillance. Despite the assay's overall effectiveness, the LoD for *tetM* and *intI1* remains relatively high, which may compromise the sensitivity in low-abundance scenarios. Further optimization is required to improve detection thresholds for these targets.

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