BioTechniques

ISSN: 0736-6205 (Print) 1940-9818 (Online) Journal homepage: www.tandfonline.com/journals/ibtn20

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To cite this article: Thitima Srathongneam, Phongsawat Paisantham, Andrew C. Singer, Rojana Sukchawalit, Skorn Mongkolsuk & Kwanrawee Sirikanchana (2026) Comparative performance of high-throughput qPCR, qPCR, and digital PCR for antibiotic resistance gene monitoring in tropical water systems, BioTechniques, 78:1-12, 158-169, DOI: 10.1080/07366205.2026.2649861

To link to this article: https://doi.org/10.1080/07366205.2026.2649861

BIOTECHNIQUES
2026, VOL. 78, NO. 01-12, 158–169
https://doi.org/10.1080/07366205.2026.2649861

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REPORT

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Comparative performance of high-throughput qPCR, qPCR, and digital PCR for antibiotic resistance gene monitoring in tropical water systems

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ABSTRACT

Antibiotic resistance genes (ARGs) are important contaminants in water systems, and their detection depends strongly on methodological sensitivity. This study compared three molecular platforms, high-throughput quantitative polymerase chain reaction (HT-qPCR), hydrolysis probe-based qPCR, and droplet digital PCR (ddPCR), for detecting ARGs in wastewater, river water, and seawater in Thailand. HT-qPCR enabled broad resistome profiling, detecting 325–336 ARGs out of 373 targets (87.1–90.1%), with aminoglycoside, beta-lactam, macrolide–lincosamide–streptogramin B, sulfonamide, mobile genetic element, and integron genes most prevalent. qPCR quantified selected ARGs using standard curves constructed from plasmid standards whose absolute copy numbers were calibrated by ddPCR, yielding high efficiency and strong linearity. ddPCR detected the same target genes as qPCR with comparable concentration estimates and additionally identified bla_{IND}, which was not observed by HT-qPCR or qPCR. Quantifications from qPCR and ddPCR showed high rank-based concordance across matrices. Combining HT-qPCR with qPCR improved coverage by about 1% relative to HT-qPCR alone, while HT-qPCR with ddPCR offered nearly identical values. In conclusion, HT-qPCR was most effective for comprehensive profiling, qPCR ensured reliable quantification, and ddPCR enabled detection of rare targets, supporting a tiered and cost-effective framework for smart ARG monitoring in tropical aquatic environments.

PLAIN LANGUAGE SUMMARY

Antibiotic resistance is a growing global concern, but detecting resistance genes in environmental samples remains challenging because they often occur at very low concentrations and in complex matrices. In this study, we compared three DNA-based molecular approaches, high-throughput quantitative polymerase chain reaction (HT-qPCR), hydrolysis probe-based quantitative PCR (qPCR), and droplet digital PCR (ddPCR), to examine how each performs when applied to different water environments. Untreated and treated wastewater, river water, and seawater samples were collected in Thailand and analyzed using the same genetic targets across platforms. HT-qPCR allowed simultaneous screening of a large number of resistance genes, providing an overview of resistance diversity across all sample types. qPCR was used for targeted measurements of selected genes and showed consistent performance suitable for routine analysis. ddPCR enabled absolute quantification without standard curves and was able to detect a very low-abundance target that was not observed by the other methods. When resistance genes were present at moderate levels, results from qPCR and ddPCR were highly comparable. By directly comparing these platforms, this work illustrates how different PCR-based methods can be combined in a complementary way, offering practical guidance for laboratories working with complex environmental DNA samples.

ARTICLE HIGHLIGHTS

- Comparative assessment of high-throughput quantitative PCR (HT-qPCR), hydrolysis probe-based qPCR, and droplet digital PCR (ddPCR) for simultaneous detection of multiple antibiotic resistance genes (ARGs) across diverse environmental matrices

ARTICLE HISTORY

Received 23 December 2025
Accepted 19 March 2026

KEYWORDS

Antimicrobial resistance surveillance; multi-source water monitoring; microbial biomarkers; smart monitoring; tropical water systems

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Supplemental data for this article can be accessed online at https://doi.org/10.1080/07366205.2026.2649861.

- Broad multi-gene screening capability of HT-qPCR, detecting 325–336 ARGs out of a 373 gene panel in wastewater, river water, and seawater
- Approximately 1% increase in ARG detection rates within the evaluated sample set when HT-qPCR results were combined with qPCR or ddPCR
- Identification of a low-abundance carbapenemase gene (bla_{IND}) by ddPCR that was not detected by HT-qPCR or qPCR
- Use of ddPCR-calibrated plasmid standards to support accurate qPCR quantification across multiple gene targets

1. Introduction

Antimicrobial resistance (AMR) is recognized as one of the most pressing global health threats, with the potential to undermine decades of progress in infectious disease treatment [1]. Antibiotic resistance genes (ARGs) are central to this problem because they can persist in environmental reservoirs and transfer between bacteria [2,3]. Aquatic environments, including wastewater treatment plants (WWTPs), rivers, and coastal waters, act as major pathways for ARG dissemination due to continuous inputs from municipal, agricultural, and industrial sources [4,5]. These pathways link human, animal, and environmental health. In tropical regions, seasonal fluctuations, rapid urbanization, and high population density further shape water quality and accelerate ARG dissemination, making effective surveillance especially urgent [6,7]. Traditional monitoring of AMR has relied on culture-based methods, which are limited because most environmental bacteria are uncultivable [8,9]. Molecular tools now provide more sensitive and comprehensive approaches. Quantitative polymerase chain reaction (qPCR) has been widely used to quantify ARGs, providing high sensitivity and specificity for selected targets [10]. High-throughput quantitative PCR (HT-qPCR) extends this capability by enabling the simultaneous screening of hundreds of ARGs, mobile genetic elements (MGEs), and integrons, thereby providing broad resistome profiles across environmental matrices [11]. Yet, HT-qPCR remains semi-quantitative and less reliable for rare-gene detection, highlighting the need for complementary methods.

Droplet digital PCR (ddPCR) has emerged as an advanced alternative, offering absolute quantification without the need for calibration curves, improved tolerance to inhibitors, and a detection limit as low as 1–2 copies per reaction [12]. These features make ddPCR particularly suitable for detecting low-abundance ARGs or clinically significant targets. Despite these advantages, systematic comparisons of ddPCR with HT-qPCR and conventional qPCR across multiple water sources remain limited, particularly in tropical environments where surveillance needs are most pressing.

Here, the objective of this study was to systematically compared the analytical performance of HT-qPCR, qPCR, and ddPCR for the detection and quantification of ARGs in selected aquatic environments in Thailand. By assessing their performance in different water matrices, from municipal wastewater to freshwater and seawater, and evaluating their complementarity, we aimed to determine how these platforms can be integrated into a practical tiered monitoring framework. Total coliforms and *E. coli* were included as conventional fecal indicator bacteria to estimate fecal contamination intensity in each matrix, as fecal pollution is a recognized contributor to environmental ARG inputs [7,13,14]. Through this methodological comparison, we evaluated the strengths and limitations of each platform and explored their combined application for environmental AMR surveillance.

2. Materials and methods

2.1. Study design and sampling

Four water samples were collected from three types of aquatic environments in Thailand: municipal wastewater and river water in January 2023, and seawater in February 2023. Wastewater samples were obtained from a WWTP, including one influent sample collected after bar screening (NK-IN) and one effluent sample collected after secondary sedimentation (NK-EF). One freshwater sample was collected from a river in Nonthaburi Province (WSN), and one seawater sample was collected from a tourist beach in Chonburi Province (PTB) (Figure 1 and Table S1). For each site, two liters of water samples were collected approximately 30 cm below the water surface, stored on ice during transport, and kept at 4 °C

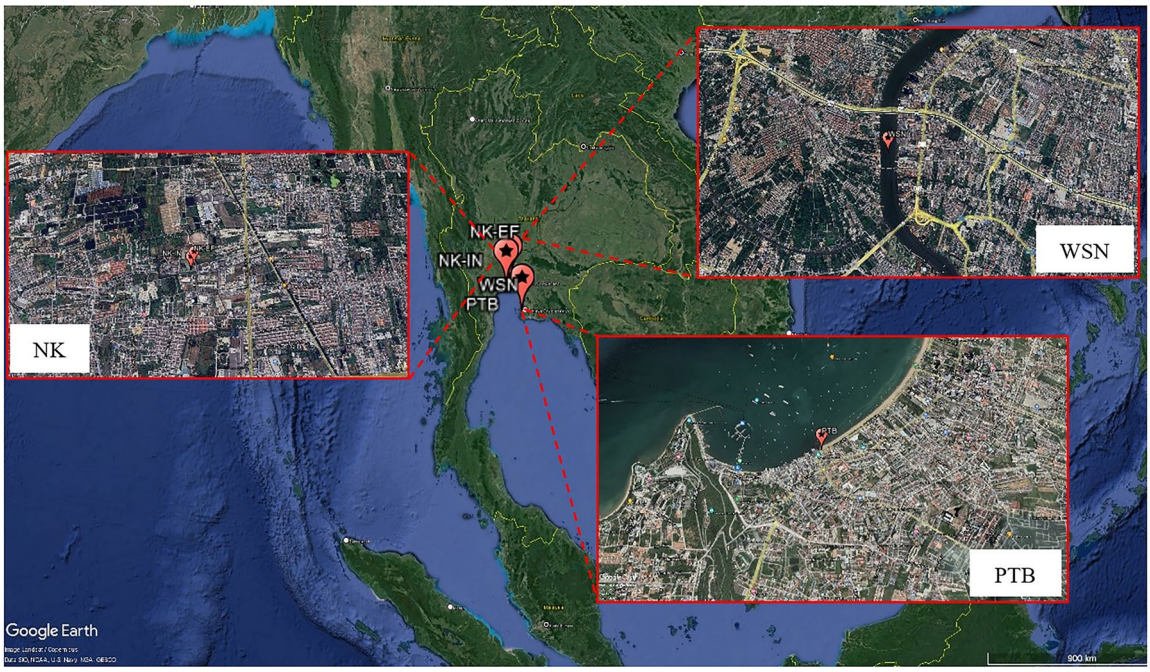

Figure 1. Sampling sites for influent wastewater (NK-IN), effluent wastewater (NK-EF), freshwater (WSN), and seawater (PTB). Basemap imagery from Google Earth (Image © Google; data © Maxar Technologies).

until analysis. These sites were selected based on our previous studies, which identified them as locations with high levels of fecal pollution and enriched ARG contamination [7,13]. Because the objective of this study was to compare analytical performance among PCR platforms, we intentionally selected matrices expected to contain diverse and detectable ARG profiles. In total, four representative samples were analyzed in the platform comparison: influent wastewater, effluent wastewater, river water, and seawater.

2.2. Microbial indicators

Escherichia coli and total coliforms were enumerated by membrane filtration using 0.45 µm pore size cellulose ester membrane and plating on 4-methylumbelliferyl-β-D-galactopyranoside and indoxyl-β-D-glucuronide (MI) agar [15], following serial dilutions appropriate for each sample type. Colony forming units (CFU) were expressed per 100 mL of water. A method blank was performed as a quality control.

2.3. DNA extraction

For influent wastewater, 200 mL of sample was filtered, while 1 L was filtered for effluent, river water, and seawater, using 0.22 µm mixed cellulose ester filters (Merck, Germany). DNA was extracted using the ZymoBIOMICS DNA Miniprep Kit (Zymo Research, USA) and eluted in 50 µL. DNA concentration and purity were checked by NanoDrop spectrophotometry (Thermo Fisher Scientific, USA). Extracts were stored at −80 °C prior to molecular analysis.

2.4. Primers, probes, and plasmid standards

Eight ARGs were selected for detailed quantification based on the results of HT-qPCR screening. These included genes detected only in specific sample types: $bla_{\text{CTX-M-8}}$ was detected in influent wastewater, *catB2* in effluent wastewater, *vanXB* in river water, and *catQ* only in seawater. In addition, ARGs that were not detected in any samples, *aac(6)ij*, bla_{IND}, *mcr-2*, and *dfrC*, were also selected (Table S2). Together, these genes represent different resistance classes and detection patterns (matrix-specific detection and non-detection), allowing evaluation of platform performance across varying abundance levels and

environmental contexts. To ensure comparability across methods, the same set of primers and probes was used for HT-qPCR, qPCR, and ddPCR. Primers were obtained from Resistomap (Helsinki, Finland) and probe sequences were designed in this study and confirmed in silico using BLAST to avoid nonspecific amplification. Eight synthetic plasmids, each carrying one ARG, were constructed (GeneArt, Thermo Fisher, USA) for generating standard curves and assay validation.

2.5. High-throughput qPCR (HT-qPCR)

HT-qPCR was performed using the SmartChip Real-Time PCR system (Takara Bio, USA) as a service provided by Resistomap (Helsinki, Finland). Each chip contained 5184 wells (100 nL per reaction). The reaction mix consisted of SmartChip TB Green Gene Expression Master Mix, primers (300 nM), and DNA template (2 ng/μL). Cycling conditions were 95 °C for 10 min, followed by 40 cycles of 95 °C for 30 s and 60 °C for 30 s. A cycle threshold of 27 was applied as the limit of quantification [11,16]. Melting curve analysis was used to confirm specificity. The SmartChip array included 384 primer sets covering 373 resistance genes, ten taxonomic marker genes, and one 16S rRNA gene (Table 1).

2.6. qPCR

qPCR assays targeting *aac(6)-ij*, bla_{IND}, *dfrC*, $bla_{CTX\text{-}M\text{-}8}$, *catB2*, *vanXB*, and *catQ* were conducted in 20 μL reaction volumes using 10 μL Luna Universal qPCR Master Mix (New England Biolabs, USA), 0.8 μL of each primer (10 μM), 0.4 μL of probe (10 μM), 3 μL of bovine serum albumin (1 μg/μL), and 5 μL of DNA template. The *mcr-2* assay was performed in 20 μL reactions containing 10 μL Luna Universal qPCR Master Mix, 0.4 μL of each primer (10 μM), 0.2 μL of probe (10 μM), 4 μL bovine serum albumin (1 μg/μL), and 5 μL DNA template. Cycling was performed with initial denaturation at 95 °C for 3 min, followed by 40 cycles of 95 °C for 15 s and annealing at 58–62 °C for 1 min, depending on the primer set (Table S2). For each target gene, standard curves were generated from five ten-fold serial dilutions of linearized synthetic plasmids. The absolute concentrations of plasmid standards were first calibrated by ddPCR to ensure accuracy before plotting Ct values against the $\log_{10}$ of copy number. Standard curves showed slopes between −3.20 and −3.81, corresponding to amplification efficiencies of 82–104% and high linearity of the standard curves (Table S3).

2.7. ddPCR

ddPCR assays were carried out using the QX200 Droplet Digital PCR System (Bio-Rad, USA). For *aac(6)-ij*, bla_{IND}, *dfrC*, $bla_{CTX\text{-}M\text{-}8}$, *catB2*, *vanXB*, and *catQ*, 20 μL reactions volume contained 10 μL 2× ddPCR Supermix

Table 1. Detected ARGs of each sample.

		Detected ARGs			
Gene group	Total ARGs[a]	NK-IN	NK-EF	WSN	PTB
Aminoglycoside	60	53	52	52	53
Beta Lactam	54	45	45	42	47
MGE[b]	48	46	44	44	46
MLSB[c]	47	43	43	42	44
MDR[d]	37	34	33	34	35
Tetracycline	26	24	23	23	23
Vancomycin	24	18	17	19	18
Phenicol	22	19	20	19	20
Other[e]	17	16	16	16	16
Trimethoprim	17	14	13	13	13
Quinolone	11	11	11	11	11
Sulfonamide	6	6	6	6	6
Integrons	4	4	3	4	4
Total detected ARGs	373	333	326	325	336
Not detected		40	47	48	37
% detected ARGs		89.3	87.4	87.1	90.1

[a]A total of 373 resistance genes, 10 taxonomic marker genes, and 1 16S rRNA gene, comprising 384 assays.
[b]Mobile genetic elements.
[c]Macrolide–lincosamide–streptogramin B.
[d]Multidrug resistance gene.
[e]Others comprise *qacEΔ1_1*, *merA*, *qacEΔ1_3*, *bacA*, *arr3*, *arr2*, *mcr-3*, *sat4*, *mcr-4*, *mcr-1*, *ttgB*, *nisB_1*, *fabK*, *nimE*, *fosb*, *fosX*, and *mcr-2*.

for Probes (no dUTP), each primer at a final concentration of 900 nM, the probe at a final concentration of 250 nM, and 2 µL template DNA. For *mcr-2*, 20 µL reactions contained 10 µL 2× ddPCR Supermix for Probes (no dUTP), each primer at a final concentration of 350 nM, the probe at a final concentration of 125 nM, and 2 µL template DNA. Droplets were generated using a QX200 droplet generator, and PCR was performed with initial denaturation at 94 °C for 30 s, followed by 40 cycles of 94 °C for 30 s and annealing/extension at 56–60 °C for 1 min (Table S2). Plates were read with the QX200 droplet reader and analyzed with QuantaSoft software.

2.8. Data analysis

Relative abundances of ARGs were normalized against the 16S rRNA gene. Comparative analyses were performed across platforms using Spearman's rank correlation test between qPCR and ddPCR quantifications. ARG diversity profiles from HT-qPCR were visualized by a Venn diagram and heatmap created by R studio (version 4.3.1).

3. Results

3.1. Microbial indicators and DNA yield

Microbial indicator analysis showed the highest levels in influent wastewater (NK-IN), with total coliforms of 7.92×10^{6} CFU/100 mL and *E. coli* of 6.70×10^{5} CFU/100 mL (Table 2). Effluent wastewater (NK-EF) contained markedly lower counts, with 6.67×10^{4} total coliforms and 1.52×10^{4} *E. coli* CFU/100 mL. River water (WSN) had 4.89×10^{3} total coliforms and 9.40×10^{2} *E. coli* CFU/100 mL, while seawater (PTB) showed 6.38×10^{3} total coliforms and 9.30×10^{2} *E. coli* CFU/100 mL. DNA yield varied among sample types. River water had the highest DNA concentration (217.3 ng/µL), followed by effluent wastewater (189.5 ng/µL), influent wastewater (167 ng/µL), and seawater (101.3 ng/µL). These results indicate that although influent wastewater carried the largest coliform loads, river water samples provided the highest DNA yields, while seawater contained the least.

3.2. High-throughput qPCR detection of ARGs

HT-qPCR detected a broad range of ARGs in all water samples, with 325–336 ARGs identified out of 373 targets, corresponding to detection rates between 87.1% and 90.1% (Table 1). Seawater (PTB) showed the highest number of detected ARGs (336; 90.1%), followed by influent wastewater (NK-IN, 333; 89.3%), effluent wastewater (NK-EF, 326; 87.4%), and river water (WSN, 325; 87.1%). The number of undetected ARGs ranged from 37 in seawater to 48 in river water. Across resistance categories, aminoglycoside resistance genes were the most frequently detected (52–53 out of 60), followed by beta-lactam (42–47 out of 54), MGE (44–46 out of 48), macrolide–lincosamide–streptogramin B (MLSB; 42–44 out of 47), MDR (33 to 35 out of 37), and tetracyclin (23 to 24 out of 26). Lower detection levels were found for vancomycin (17–19 out of 24), trimethoprim (13–14 out of 17), and phenicol groups (19–20 out of 22). Sulfonamide (6/6), quinolone (11/11), and integron genes (3–4 out of 4) were consistently detected across all samples. Notably, seawater contained the broadest ARG profile, exceeding even influent wastewater, while effluent wastewater and river water exhibited slightly lower detection coverage. Comparison across sample types showed that 312 genes were shared among all four environments, defined as genes detected by HT-qPCR above the established detection threshold (Cq < 27) in all four environmental DNA extracts. In contrast,

Table 2. Microbial indicator and total DNA concentrations of samples.

Sample	Total coliforms (CFU/100 mL)	*E. coli* (CFU/100 mL)	DNA conc. (ng/µL)
NK-IN	7.92×10^{6}	6.70×10^{5}	1.67×10^{2}
NK-EF	6.67×10^{4}	1.52×10^{4}	1.90×10^{2}
WSN	4.89×10^{3}	9.40×10^{2}	2.17×10^{2}
PTB	6.38×10^{3}	9.30×10^{2}	1.01×10^{2}
Method blank	<10	<10	<1

26 ARGs, including *bla*$_{IND}$, *aac(6)-ij*, *mcr-2*, and *dfrC*, were not detected in any sample (Table 3 and Figure 2). The relative abundance of each gene is shown in Figure S1.

3.3. qPCR and ddPCR comparison of selected ARGs

Eight ARGs were selected for cross-platform comparison, including genes not detected by HT-qPCR in any environment (*aac(6)-ij*, *bla*$_{IND}$, *dfrC*, *mcr-2*) and genes detected in only one environment (*bla*$_{CTX-M-8}$, *catB2*, *catQ*, *vanXB*) (Table 3). *aac(6)-ij* and *mcr-2* remained undetected by both qPCR and ddPCR across all samples, whereas *dfrC*, although absent from HT-qPCR screening, was consistently quantified by qPCR and ddPCR in every matrix, with qPCR concentrations ranging from 1.26×10^3 copies/100 mL in influent

Table 3. Genes detected from different samples.

Samples	Genes	Number of genes
All four samples	**Aminoglycoside**; *aadA7*, *spcN*, *aadA2_3*, *aac3-IVa*, *aadA1_2*, *aac(3)-iid_iia*, *aadA_1*, *aph6-ia*, *aadB*, *aph4-ib*, *aadA2_1*, *aph3-ib*, *aac(3)-ib*, *aac(6′)-Ib_1*, *aac(6)-ir*, *aadA16*, *strB*, *aadA6*, *ant6-ia*, *aph3-iii*, *aac(6)-iic*, *aac(3)-id_ie*, *aacC4*, *aadA5_2*, *rmtB*, *aac(6)-iv_ih*, *aac(3)-xa_1*, *aac(6)-iz*, *aadA10*, *ant4-ib*, *ant6-ib*, *aph(3′′)-ia*, *aadA9_1*, *aac6-aph2*, *aphA3_1*, *aac(6)-ig*, *aphA1/7*, *aac(6′)-II*, *aac(6′)-Iy*, *aacA/aphD*, *aph_viii*, *aac(6′)I1*, *aac(6)-is_iu_ix*, *strA*, *armA_1*, *aacC2*, *aph4-ia*, *aadD*, *aac(6)-im*, and *apmA*. **Beta Lactam**; *blaOXY*, *penA*, *blaSFO*, *blaVEB*, *blaGOB*, *blaMIR*, *blaOXY1*, *blaCARB*, *blaACT*, *blaCTX-M*, *blaFOX*, *cfxA*, *blaGES*, *cphA_1*, *blaTEM*, *blaCMY_2*, *blaSHV11*, *blaOXA48*, *blaMOX/blaCMY*, *blaACC*, *cfiA*, *blaOCH*, *blaNDM*, *blaPSE*, *ampC/blaDHA*, *blaROB*, *beta_B2*, *blaPAO*, *blaCTX-M_5*, *blaPER*, *blaKPC*, *bla-L1*, *blaBEL-nonmobile*, *blaVIM*, *blaLEN*, *blaIMI*, *blaHERA*, *blaOXA51*, *imiR_2*, *blaSME*, and *pbp*. **MLSB**; *ereA*, *ermX_2*, *pncA*, *ermE*, *mphA*, *erm35*, *msrE*, *lnuB*, *ermF*, *erm42*, *oleC*, *mefA_1*, *pikR2*, *ermF_1*, *ermO*, *lnuF*, *mefA*, *erm36*, *lnuC*, *vgaB_1*, *vat(A)*, *ermB_3*, *ermB_2*, *ermD/K*, *vat(B)*, *carB*, *ermX_1*, *mphB*, *ermD_1*, *ermA*, *mefB*, *lmrA_1*, *msrC_1*, *erm34*, *vgaA_1*, *ermA/ermTR*, *ermD*, *vga(A)LC_1*, and *vatE_2*. **MDR**; *cefa_qacelta*, *oprD*, *pbrT*, *arsA*, *czcA*, *qacF/H*, *mdtA*, *sugE*, *mdtH*, *tolC_2*, *mepA*, *emrD_1*, *cadC*, *copA*, *acrR_1*, *mexB*, *ttgA*, *mdtE*, *pcoA*, *acrF*, *qacA/B*, *adeA*, *acrA_1*, *oqxA*, *acrB_1*, *terW*, *mexA*, *mexE*, *tcrB*, *marR_3*, *bexA/norM*, *adeI*, and *mdsA*. **Tetracycline**; *tetD*, *tetG*, *tetR*, *tetL_2*, *tet39*, *tetM*, *tetA_2*, *tetR_1*, *tetX*, *tetE*, *tetQ*, *tetW*, *tetO_2*, *tetJ*, *tet32*, *tet44*, *tetS*, *tet36_1*, *tetA/B_1*, *tet38*, *tetC_2*, and *tetPB_1*. **Vancomycin**; *vanTC_2*, *vanA*, *vanHB*, *vanYD_1*, *vanB_1*, *vanYB*, *vanRB*, *vanC2*, *vanTE*, *vanSC_2*, *vanHD*, *vanC_2*, *vanTG*, *vanWB*, *vanRC*, and *vanG*. **Phenicol**; *cmlA_2*, *cmlV*, *cmlA_4*, *floR_1*, *catB3*, *catA3*, *ceoA*, *cmxA*, *catB8*, *yidY/mdtL*, *cat*, *catP*, *floR*, *catB9*, *optrA*, *mdtL*, *cat(pC221)*, *catA2*, and *catA1*. **Trimethoprim**; *dfrA27*, *dfrA22*, *dfra21*, *dfrA25*, *dfrA10*, *dfra17*, *dfrA1_1*, *dfrA8*, *dfrA15*, *dfrB*, *dfrA12*, and *dfrA19*. **Quinolone**; *qepA*, *qnrS2*, *qnrB4*, *qnrVC_2*, *qnrVC1_VC3_VC6*, *qnrB*, *qnrS_1*, *qnrB_2*, *qnrA*, *qnrD*, and *norA*. **Sulfonamide**; *sul1_2*, *sul2_2*, *sul3_1*, *sul4*, *folA_1*, and *folP_2*. **Other**; *qacEΔ1_1*, *merA*, *qacEΔ1_3*, *bacA*, *arr3*, *arr2*, *mcr-3*, *sat4*, *mcr-4*, *mcr-1*, *ttgB*, *nisB_1*, *fabK*, *nimE*, *fosb*, and *fosX*. **MGE**; trbC, IS26_1, IS1111, Tn5403, IS6100, orf37-IS26, tnpA_1, tnpA_2, IS21-ISAs29, IS1247_1, IS1133, IS630, IS1247_2, ISCR1, ISPps, tnpA_3, IS613, IncI1_repI1, IncP_oriT, tnpA_4, EAE_05855, ISEcp1, tnpA_5, ISAba3, Tn5, tnpA_6, IncN_rep, tnpA_7, IncW_trwAB, Tn3, IS3, IncQ_oriT, traN, Tp614, cro, IncF_FIC, IS200_2, IS6/257, IS200_1, pAKD1, trfA, IS256, IncHI2-smr0018, ISEfm1 **Integrons**; intI1_1, intI3, intI1_2	312
NK-IN, NK-EF and WSN	**Tetracycline**; *tetH*, **Vancomycin**; *dfrK*, **Trimethoprim**; *vanRD*.	3
NK-IN, NK-EF and PTB	**Aminoglycoside**; *aadE*, **Beta Lactam**; *blaTLA and cepA*, **MLSB**; *ermB_1* and *lnuA_1*.	5
NK-IN, WSN and PTB	**Aminoglycoside**; *aph9-ia*, **Vancomycin**; *vanSB* **Integrons**; *intI2_2*.	3
NK-EF, WSN and PTB	**Beta Lactam**; *ampC_cefa*, **MLSB**; *lsaC* and *ermY*.	3
NK-IN and NK-EF	-	0
NK-IN and WSN	**MLSB**; *ermC_2*	1
NK-IN and PTB	**Aminoglycoside**; *str*, **MLSB**; *ermT_1*, **MDR**; *mdtG_1*, **Tetracycline**; *tetT*, **Trimethoprim**; *dfrG*, **MGE**; *IncN_korA*.	6
NK-EF and PTB	**Beta Lactam**; *bla1*	1
WSN and PTB	**MDR**; *cmr*	1
Only NK-IN	**Beta Lactam**; *blaADC-nonmobile* and *bla*$_{CTX-M-8}$[a], **MGE**; *pAMBL*.	3
Only NK-EF	**Aminoglycoside**; *aacA43*, **Phenicol**; *catB2*[a]	2
Only WSN	**Aminoglycoside**; *aph3-via*, **Vancomycin**; *vanXB*[a]	2
Only PTB	**Beta Lactam**; *bl1acc* and *pbp5*, **Vancomycin**; *vanRC4*, **Phenicol**; *catQ*[a], **MGE**; *IncN_oriT*.	5
Not detected in any samples	**Aminoglycoside**; *aac(6)-ij*[a], *aph(2′)-Ib*, *armA_2*, *aac(6)-iw*, and *aph3-viia*, **Beta Lactam**; *bla*$_{IND}$[a], *blaB*, *blaZ*, *mecA*, *beta_ccra*, **MLSB**; *msrA_1* and *msrD*, **MDR**; *cfr* and *pmrA*, **Tetracycline**; *tetK*, *tetPA*, **Vancomycin**; *vanRA_1*, *vanD*, *vanSA*, *vanXA*, **Phenicol**; *fexA*, **Trimethoprim**; *dfrAB4*, *dfrA7*, and *dfrC*[a], **Other**; *mcr-2*[a], **MGE**; *IS5/IS1182*.	26

[a]Genes selected for further comparison among HT-qPCR, qPCR, and ddPCR.

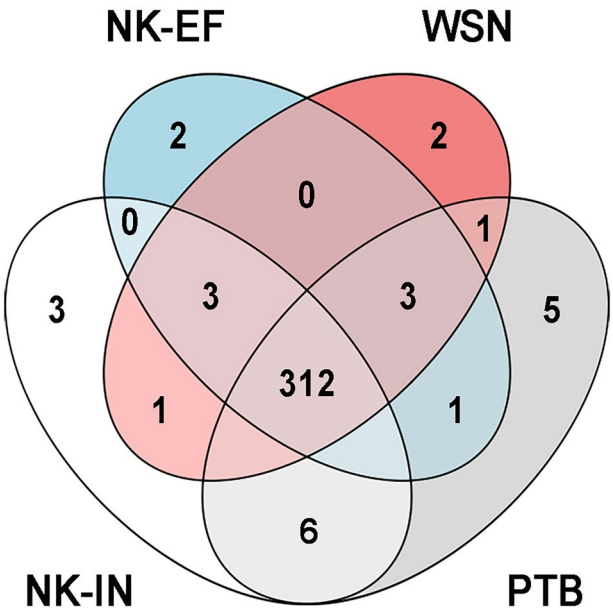

Figure 2. Venn diagram showing the numbers of antibiotic resistance genes from influent wastewater (NK-IN), effluent wastewater (NK-EF), freshwater (WSN), and seawater (PTB).

wastewater (NK-IN) to 1.81×10^{1} in river water (WSN) (Table 4). ddPCR results were comparable (1.23×10^{3} to 1.65×10^{1} copies/100 mL), and relative abundances normalized to 16S rRNA were on the order of 10^{-8} to 10^{-6}. bla_{IND}, another gene absent in HT-qPCR, was uniquely detected by ddPCR in influent wastewater at an extremely low level (37.5 copies/100 mL; 2.71×10^{-8} relative to 16S rRNA gene), while qPCR reported no amplification.

Among the four genes identified by HT-qPCR in a single environment, $bla_{CTX\text{-}M\text{-}8}$ was quantified by qPCR in all matrices at 8.05×10^{4} (NK-IN) to 1.41×10^{2} (WSN) copies/100 mL, with ddPCR producing comparable values (5.10×10^{4} to 1.95×10^{2} copies/100 mL). *catB2*, initially detected in effluent wastewater by HT-qPCR, was measured by qPCR in all matrices (3.53×10^{5} in NK-IN to 3.63×10^{3} in WSN) and at similar concentrations by ddPCR (5.20×10^{5} to 4.65×10^{3} copies/100 mL). *catQ*, first detected in seawater by HT-qPCR, showed strong agreement across platforms, with qPCR concentrations of 1.81×10^{6} in NK-IN and 3.32×10^{4} in WSN and ddPCR values of 1.35×10^{6} to 4.45×10^{4} copies/100 mL. *vanXB*, detected only in river water by HT-qPCR, was measured by qPCR in all samples (3.10×10^{3} in NK-IN to 9.46 in PTB) and by ddPCR at comparable levels (1.15×10^{3} in NK-IN to 2.3×10^{1} copies/100 mL in NK-EF). Across the four matrices, qPCR and ddPCR quantification showed high concordance in relative ranking of concentrations (Figure S2). Spearman's rank correlation coefficients were $\rho=1$ for *dfrC*, $bla_{CTX\text{-}M\text{-}8}$, *catB2*, and *catQ*, indicating identical rank ordering of concentrations between platforms across matrices. In contrast, *vanXB* showed lower agreement ($\rho=0.4$), suggesting greater variability in relative quantification at low concentration levels. The associated p-values (e.g., $p=0.08$ when $\rho=1$) reflect the limited statistical power inherent to the small sample size ($n=4$) rather than the absence of concordance. ddPCR additionally identified bla_{IND}, a target not detected by the other platforms.

3.4. Combination of platforms improves detection coverage

When overall detection rates were calculated across the three platforms, combining HT-qPCR with qPCR improved the proportion of detected ARGs compared with HT-qPCR alone (Table 5). In influent wastewater (NK-IN), the detection rate increased from 89.3% to 90.4%. Effluent wastewater (NK-EF) rose from 87.4% to 88.5%, river water (WSN) from 87.1% to 88.2%, and seawater (PTB) from 90.1% to 91.2%. By contrast, combining HT-qPCR with ddPCR yielded nearly identical detection rates to the HT-qPCR plus qPCR approach: 90.6% in NK-IN, 88.5% in NK-EF, 88.2% in WSN, and 91.2% in PTB. These results indicate that HT-qPCR complemented by qPCR provides a small improvement in coverage, while the addition of

Table 4. Gene quantification using HT-qPCR, qPCR, and ddPCR.

Genes	Samples	qPCR[a] (copies/100 mL)	ddPCR[b] (copies/100 mL)	Relative abundance normalized by 16S HT-qPCR	qPCR	ddPCR
16S rRNA	NK-IN		1.4×10^{9}	1	1	1
	NK-EF		1.5×10^{8}	1	1	1
	WSN		1.9×10^{8}	1	1	1
	PTB		1.2×10^{8}	1	1	1
aac(6)-ij	NK-IN	ND[c]	ND[c]	ND	ND	ND
	NK-EF	ND	ND	ND	ND	ND
	WSN	ND	ND	ND	ND	ND
	PTB	ND	ND	ND	ND	ND
bla_{IND}	NK-IN	ND	3.75×10^{1}	ND	ND	2.71×10^{-8}
	NK-EF	ND	ND	ND	ND	ND
	WSN	ND	ND	ND	ND	ND
	PTB	ND	ND	ND	ND	ND
dfrC	NK-IN	1.26×10^{3}	1.23×10^{3}	ND	9.08×10^{-7}	8.84×10^{-7}
	NK-EF	4.70×10^{1}	7.50×10^{1}	ND	3.10×10^{-7}	4.95×10^{-7}
	WSN	1.81×10^{1}	1.65×10^{1}	ND	9.43×10^{-8}	8.57×10^{-8}
	PTB	1.48×10^{2}	1.25×10^{2}	ND	1.26×10^{-6}	1.06×10^{-6}
mcr-2	NK-IN	ND	ND	ND	ND	ND
	NK-EF	ND	ND	ND	ND	ND
	WSN	ND	ND	ND	ND	ND
	PTB	ND	ND	ND	ND	ND
$bla_{CTX\text{-}M\text{-}8}$	NK-IN	8.05×10^{4}	5.10×10^{4}	8.73×10^{-5}	5.81×10^{-5}	3.68×10^{-5}
	NK-EF	2.76×10^{3}	1.99×10^{3}	ND	1.82×10^{-5}	1.31×10^{-5}
	WSN	1.41×10^{2}	1.95×10^{2}	ND	7.34×10^{-7}	1.01×10^{-6}
	PTB	2.69×10^{2}	2.15×10^{2}	ND	2.29×10^{-6}	1.83×10^{-6}
catB2	NK-IN	3.53×10^{5}	5.20×10^{5}	ND	2.55×10^{-4}	3.75×10^{-4}
	NK-EF	2.38×10^{4}	3.11×10^{4}	4.42×10^{-4}	1.57×10^{-4}	2.05×10^{-4}
	WSN	3.63×10^{3}	4.65×10^{3}	ND	1.89×10^{-5}	2.42×10^{-5}
	PTB	9.56×10^{3}	1.38×10^{4}	ND	8.14×10^{-5}	1.17×10^{-4}
vanXB	NK-IN	3.10×10^{3}	1.15×10^{3}	ND	2.24×10^{-6}	8.30×10^{-7}
	NK-EF	9.47×10^{1}	2.30×10^{1}	ND	6.25×10^{-7}	1.52×10^{-7}
	WSN	1.83×10^{2}	2.90×10^{1}	4.59×10^{-5}	9.50×10^{-7}	1.51×10^{-7}
	PTB	9.46×10^{0}	4.00×10^{1}	ND	8.05×10^{-8}	3.40×10^{-7}
catQ	NK-IN	1.81×10^{6}	1.35×10^{6}	ND	1.30×10^{-3}	9.73×10^{-4}
	NK-EF	8.55×10^{4}	1.46×10^{5}	ND	5.65×10^{-4}	9.60×10^{-4}
	WSN	3.32×10^{4}	4.45×10^{4}	ND	1.73×10^{-4}	2.31×10^{-4}
	PTB	3.34×10^{4}	5.74×10^{4}	2.83×10^{-3}	2.84×10^{-4}	4.89×10^{-4}

[a]Limit of detection of qPCR is C_q of 40 cycles.
[b]Limit of detection of ddPCR is 1.4 copies per reaction.
[c]ND is not detected.

Table 5. Overall positive detection rates of samples from a combination of HT-qPCR, qPCR, and ddPCR platforms (a total of 373 genes).

Technique	NK-IN	NK-EF	WSN	PTB
HT-qPCR	89.3%	87.4%	87.1%	90.08%
HT-qPCR and qPCR	90.4%	88.5%	88.2%	91.15%
HT-qPCR and ddPCR	90.6%	88.5%	88.2%	91.15%

ddPCR adds limited additional benefit within the conditions and sample set evaluated in this study, relative to its higher cost.

4. Discussion

4.1. Breadth of ARG detection with HT-qPCR

Our results confirm that HT-qPCR is a powerful tool for profiling the diversity of ARGs in aquatic environments, consistent with previous reports in wastewater and surface waters [17–19]. In this study, between 325 and 336 ARGs were consistently detected across wastewater, river water, and seawater, corresponding to 87.1–90.1% of all targets tested. This high coverage demonstrates the capacity of HT-qPCR to capture a wide range of resistance groups, many of which are underrepresented in culture-based approaches or low-throughput assays. The frequent occurrence of aminoglycoside, beta-lactam, MLSB, and tetracycline resistance genes in our samples is in line with their known dominance in environments receiving anthropogenic inputs such as municipal effluents and agricultural

discharges [14,20,21]. The enumeration of total coliforms and *E. coli* provided supporting information on fecal contamination levels in the studied matrices. As expected, higher counts were observed in wastewater samples compared to river and seawater. However, it should be emphasized that ARGs are not restricted to coliform-associated bacteria and may be distributed across diverse environmental taxa. Therefore, the culture-based indicators in this study were used to provide general contamination context rather than to infer specific ARG hosts. Importantly, the inclusion of MGEs and integrons underscores the strength of HT-qPCR in tracking genetic determinants that mediate horizontal transfer of resistance, which is a central driver of AMR dissemination in aquatic systems [22,23]. By incorporating ARGs, MGEs, and integrons, HT-qPCR demonstrates its role as a true multi-biomarker tool. Moreover, applying it across wastewater, river, and seawater highlights its capacity for multi-source surveillance, both at the point of discharge and in receiving environments. By combining broad coverage with semi-quantitative outputs, HT-qPCR can be positioned as a cornerstone method for environmental AMR surveillance, particularly in tropical regions where systematic monitoring programs are still emerging and resources are limited [24].

4.2. Comparative performance and practical tradeoffs of qPCR and ddPCR

Although HT-qPCR enabled comprehensive screening, the platform is semi-quantitative and not suited for absolute quantification, which represents an inherent limitation when precise quantification is required. qPCR assays in this study provided robust quantification, in agreement with international quality benchmarks and previous evaluations of qPCR performance in wastewater monitoring [10,25]. However, ddPCR showed greater sensitivity, enabling the detection of very low-abundance ARGs, consistent with evidence from hospital wastewater analyses, where ddPCR consistently outperformed qPCR in identifying low-abundance carbapenemase genes and proved more robust to inhibitors in complex environmental matrices [26]. Importantly, for genes present at moderate concentrations, qPCR and ddPCR produced high rank concordance across matrices, as indicated by Spearman's rank correlation coefficients (Figure S2), although interpretation should consider the limited number of paired observations ($n = 4$). These findings are consistent with previous work showing strong agreement between the two methods despite occasional differences in absolute values [26]. Compared with qPCR, ddPCR provides absolute quantification without the need for a standard curve, offers improved accuracy at low copy numbers, and is less affected by inhibitors. On the other hand, qPCR is more suitable for large-scale surveillance because it is faster, lower in cost, and allows higher sample throughput per run, whereas ddPCR is limited by higher instrument and reagent costs, narrower dynamic range, and lower sample throughput, which may constrain its application in routine large-scale environmental monitoring [12,27].

When overall detection rates were compared, HT-qPCR alone detected 87.1–90.1% of the ARG panel across matrices. Pairing HT-qPCR with qPCR increased coverage by about 1%, reaching 90.4% in influent wastewater and 88.5% in effluent. Importantly, certain genes absent in HT-qPCR, such as *dfrC*, were consistently recovered by qPCR, demonstrating the added value of combining the two methods where such targets are deemed "key" for interpretation and further action. By contrast, adding ddPCR to HT-qPCR produced nearly identical coverage, with only a slight improvement observed in influent wastewater (90.6%). This limited gain suggests that ddPCR contributes little additional coverage under the tested conditions, particularly for broadly distributed ARGs. From a practical perspective, qPCR thus provides a more favorable balance between sensitivity, cost, and throughput for large-scale surveillance, whereas ddPCR should be applied strategically in specific contexts. Together, these results point to a tiered monitoring framework that aligns with the concept of smart monitoring: HT-qPCR for broad resistome profiling, qPCR for reliable and cost-effective quantification, and ddPCR for rare-gene validation. This multi-platform integration maximizes surveillance efficiency across different water sources and supports the development of scalable monitoring systems in tropical and resource-limited settings. In particular, while HT-qPCR is well suited for simultaneous detection of a large number of ARG targets, low-throughput platforms such as qPCR and ddPCR require selective targeting and therefore are less practical for broad simultaneous screening in complex environmental matrices.

4.3. Limitations of this study

This study has some limitations that should be acknowledged. Only one representative sample was collected from each environment, and therefore the results do not reflect spatial or temporal variability of ARG distribution. ARG levels in aquatic systems may vary depending on hydrological conditions, pollution inputs, and seasonal factors [2,5]. However, the main objective of this work was to compare the analytical performance of different PCR platforms using the same DNA extracts. The selected sites had been previously identified in our earlier investigations as heavily impacted by fecal pollution and ARG contamination, which ensured that sufficient target genes were present for cross-platform comparison. Therefore, the study design prioritized methodological evaluation rather than environmental representativeness. Future studies including replicate sampling across time and space would be necessary to further validate the proposed monitoring framework under variable environmental conditions.

5. Conclusions

This study provided a systematic comparison of HT-qPCR, qPCR, and ddPCR for the detection of ARGs in tropical aquatic environments. HT-qPCR enabled comprehensive profiling, identifying more than 325 ARGs per sample and capturing the dominant resistance groups in wastewater, river water, and seawater. qPCR delivered reliable quantification with high amplification efficiency and strong linearity, while ddPCR revealed bla_{IND}, a rare gene undetected by the other methods. Despite this added sensitivity, the quantitative results of qPCR and ddPCR were highly consistent for abundant targets, and the overall improvement in detection coverage from ddPCR was limited within this dataset. In contrast, combining HT-qPCR with qPCR provided broader coverage with minimal additional cost, representing a practical approach for routine monitoring. These findings highlight the complementary strengths of each platform and suggest that HT-qPCR together with qPCR may offer a feasible strategy for wastewater-based resistance surveillance, while ddPCR may be applied selectively for confirmatory detection of rare or clinically significant ARGs.

6. Future perspective

As multi-gene detection becomes more central to environmental and molecular biology research, the strategic integration of different PCR platforms will be increasingly important. This study shows that broad screening, routine quantification, and ultra-sensitive detection are best addressed using complementary rather than standalone methods. Future work should focus on developing standardized decision frameworks that guide platform selection based on study objectives, target abundance, and sample complexity. Additional inter-laboratory comparisons and expansion to other gene groups will be essential for improving reproducibility and data comparability. As high-throughput and digital PCR technologies continue to mature, their combined application is likely to play a key role in advancing reliable and scalable multi-gene detection workflows.

Author contributions

CRediT: **Thitima Srathongneam**: Formal analysis, Investigation, Visualization, Writing – original draft, Writing – review & editing; **Phongsawat Paisantham**: Investigation; **Andrew C. Singer**: Supervision, Writing – review & editing; **Rojana Sukchawalit**: Supervision, Writing – review & editing; **Skorn Mongkolsuk**: Funding acquisition, Resources; **Kwanrawee Sirikanchana**: Conceptualization, Methodology, Supervision, Writing – original draft, Writing – review & editing.

Disclosure statement

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

No writing assistance was utilized in the production of this manuscript.
Peer reviewers on this manuscript have no relevant financial or other relationships to disclose.

Funding

This study was financially supported by Thailand Science Research and Innovation, Thailand (TSRI), Chulabhorn Research Institute, Thailand (Grant No. 53500/4821855).

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Data availability statement

All relevant data are included in the paper or its Supplementary Information.

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