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Defining the clinical utility of dengue RT-iiPCR: stratification by days post-onset and primary vs. secondary infection during the 2023 Taiwan outbreak

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ABSTRACT

Background: Rapid molecular diagnostics are critical for timely dengue virus (DENV) detection, especially in resource-limited settings. Reverse transcription-insulated isothermal polymerase chain reaction (RT-iiPCR) enables sample-to-answer amplification without thermocyclers. While this platform was previously validated, its diagnostic performance against contemporary viral genotypes and, critically, across different host immune statuses (primary vs. secondary infection) remains largely undefined. We assessed a DENV iiPCR assay against quantitative real-time reverse transcription PCR (qRT-PCR) during the 2023 outbreak in southern Taiwan.
Methods: Acute-phase sera (n = 185) from febrile patients were tested by qRT-PCR, pan-DENV iiPCR, IgM/IgG ELISAs, and serotype-specific RT-PCR/iiPCR. Diagnostic performance was evaluated with qRT-PCR as a reference. Subgroup analyses examined effects of days post-symptom onset (PSO), immune status (primary vs secondary infection), and serotype on iiPCR sensitivity.
Results: Pan-iiPCR achieved 88.4 % sensitivity and 100 % specificity versus qRT-PCR. Sensitivity peaked within 3 days PSO (96.8 %) and in primary infections (95.0 %) but declined after day 3 (53.3 %) and in secondary infections (82.2 %). Combining pan-iiPCR with IgM testing raised sensitivity to ≥99 % beyond the viremic window. iiPCR correctly typed 83.5 % (71/85) of DENV-1 and 94.3 % (66/70) of DENV-2. Because pan-iiPCR served as the screening step, 18 RT-PCR–positive specimens were pan-iiPCR negative and therefore did not proceed to iiPCR serotyping.
Conclusions: iiPCR enables rapid early-phase detection and is useful when paired with serology. However, reduced sensitivity in low-viremia and secondary infections, as well as incomplete serotyping, limit standalone use. Primer optimization and integration with IgM testing may enhance outbreak utility.

1. Introduction

Dengue virus (DENV) is a positive-sense, single-stranded RNA virus of the genus Flavivirus (family Flaviviridae). Its ~11-kb genome encodes a single polyprotein that is cleaved into three structural (C, prM/M, E) and seven non-structural (NS1-NS5) proteins [1]. Four antigenically distinct serotypes (DENV-1-4) co-circulate worldwide and can confer lifelong homologous immunity but only transient, partial cross-protection [2]. Clinical manifestations range from asymptomatic infection or self-limited febrile illness (dengue fever) to severe forms

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such as dengue hemorrhagic fever and dengue shock syndrome, often precipitated by secondary heterotypic infection and antibody-dependent enhancement [3,4]. The global burden is estimated at 390 million infections annually across more than 120 countries, concentrated in tropical and subtropical regions with competent Aedes vectors [5,6].

Conventional diagnosis relies on quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR), NS1 antigen detection, and IgM/IgG ELISA. qRT-PCR provides high sensitivity but requires expensive thermocyclers, cold-chain reagents, and trained personnel. Serology is inexpensive but insensitive during early infection and prone to flavivirus cross-reactivity, while NS1 assays show variable serotype performance [2,7]. Insulated isothermal PCR (iiPCR) was developed to address these constraints by harnessing Rayleigh-Bénard convection in capillary tubes. Early systems used thermal baffles for environmental stability [8] and later incorporated TaqMan probes with on-board optics for contamination-free detection [9]. Field-deployable iiPCR kits have since shown high accuracy for DENV [10–14] and for numerous other pathogens, including influenza A [15], duck hepatitis A virus [16], Seneca Valley virus [17], avian influenza virus [18], and Toxoplasma gondii [19], thereby demonstrating versatility at the point of need. Comparative studies consistently report ≥90 % sensitivity versus reference RT-PCR, with results available in under 2 h and minimal instrumentation. However, few dengue iiPCR evaluations have examined performance by illness timing, immune status, or circulating serotype/genotype, motivating the present study.

Taiwan has experienced cyclical dengue epidemics over the past three decades, with major outbreaks in 2002, 2007, 2014, 2015, and 2023, largely centered in the southern metropolitan areas of Kaohsiung and Tainan [20,21]. The 2015 epidemic recorded more than 43,000 confirmed cases, the largest in Taiwan's history, and was driven predominantly by DENV-2, being associated with significant morbidity and strained healthcare resources [20]. In 2023, Kaohsiung again reported >20,000 infections, this time with co-circulation of DENV-1 genotype I and DENV-2 genotype II, highlighting the dynamic serotype/genotype landscape and the need for rapid, field-deployable diagnostics [21].

Our laboratory previously developed three iiPCR assays that achieved 95–100 % sensitivity and specificity for DENV detection and rapid serotyping using sera from the 2012 Kaohsiung outbreak (DENV-1 genotype III; DENV-2 cosmopolitan) [11–13]. However, the robustness of this assay against the different viral genotypes circulating in the 2023 outbreak (DENV-1 genotype I and DENV-2 genotype II) remained unknown. Furthermore, a critical gap in the previous validation was the lack of performance stratification by (i) days post-symptom onset (PSO) and (ii) the patient's immune status (primary vs. secondary infection), which is a crucial factor known to influence viremia dynamics. Therefore, this study was warranted to provide a rigorous assessment of the DENV RT-iiPCR assay across these critical variables using specimens from the 2023 outbreak. This study therefore assesses DENV RT-iiPCR across these critical variables to define its diagnostic performance under outbreak conditions. Our findings provide practical insights into the strengths and limitations of iiPCR as a frontline diagnostic tool and its potential integration into dengue diagnostic algorithms.

2. Materials and Methods

2.1. Ethics statement and sample collection

This study was conducted at Kaohsiung Medical University Hospital (KMUH), Kaohsiung, Taiwan. The study protocol was reviewed and approved by the Institutional Review Board of Kaohsiung Medical University Hospital (KMUHIRB-960195), and written informed consent was obtained from all participants, in accordance with the Declaration of Helsinki. Between July and December 2023, patients presenting with dengue-like symptoms [21] were screened for enrollment. Acute-phase sera (0–12 days PSO) [2,20] were collected at presentation and tested by qRT-PCR for DENV RNA (PSO as recorded in Table 1; subgroup analyses further restricted PSO to ≤ 3 days to capture peak viremia, and to 0–7 days as a necessary methodological constraint to ensure valid serological classification, as detailed in the Statistical analysis subsection below). All patients with positive qRT-PCR results (n = 155) formed the dengue cohort. In addition, 30 qRT-PCR-negative patients with other febrile illnesses (OFI) were selected from the same screening pool and matched by age and sex to serve as controls. Written informed consent was obtained from all participants prior to sample collection.

Table 1
Characteristics of the study cohort.[a]

Variables	Dengue	Other febrile illness	*p* value[c]
	n = 155	n = 30	
Age	50 (1–92)	50 (5–84)	NS
Sex			NS
Male	75 (48.4)	17 (56.7)	
Female	80 (51.6)	13 (43.3)	
Days PSO	1 (0–12)	2 (0–9)	NS
Serotype			NA
DENV-1	85 (54.8)	–	
DENV-2	70 (45.2)	–	
Infection status			NA
Primary infection	99 (63.9)	–	
Secondary infection	45 (29)	–	
Undetermined[b]	11 (7.1)	–	

Abbreviations: qRT-PCR, quantitative real-time reverse transcription polymerase chain reaction. PSO, post-symptom onset. DENV, dengue virus.

[a] Presented as the numbers (%) except for age and days PSO, which are shown as the median (range). Diagnosis of dengue was based on a positive DENV qRT-PCR result. Diagnosis of other febrile illness was based on a negative DENV qRT-PCR result with dengue-like illness.

[b] Two cases with PSO >7 days and 9 cases with inconclusive ELISA results (PSO <7 days).

[c] For statistical analysis, the Mann-Whitney *U* test was applied to compare age and days PSO, while the Chi-squared test was used to assess differences in sex distribution. NS, not significant. NA: not available.

2.2. Viral RNA extraction, quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR), and serotype-specific RT-PCR

DENV genome was detected according to methods described in our previous studies. In brief, DENV RNA was extracted from 200 μL of serum using the PureLink Viral RNA Mini Kit (Invitrogen, Thermo Fisher Scientific Inc., USA). Real-time qRT-PCR was conducted in a 25 μL reaction using the Brilliant II SYBR Green system (Agilent Technologies, Inc., USA) on an Mx3000P Real-Time PCR System (Agilent Technologies, Inc., USA). Dengue-specific and serotype-specific primers targeting the nucleocapsid gene, as well as interpretation criteria for qRT-PCR, were described previously [20–22]. The high specificity and validity of this serotype-specific RT-PCR reference method are consistently confirmed by external proficiency testing from the Taiwan Centers for Disease Control (TCDC) and routine internal quality control checks against sequencing results.

2.3. Pan-DENV reverse transcription-insulated isothermal PCR (RT-iiPCR) assay and molecular serotyping by serotype-specific RT-iiPCR

The pan-DENV RT-iiPCR assay targets the 3′ untranslated region (3' UTR) of the viral genome and was conducted using the POCKIT Central Dengue Virus Premix Reagent on the fully automated POCKIT Central system (GeneReach Biotechnology, Inc., Taiwan). The reagent premix tube was loaded into the Transfer Cartridge, and 200 μL of clinical specimen was added to the Extraction Cartridge. After entering sample and reagent information into the system, the cartridges were inserted into the POCKIT Central device. The integrated workflow, which included RNA extraction, RT-iiPCR amplification, and real-time detection, was completed automatically within 85 min and generated

qualitative diagnostic results on the instrument interface. Molecular serotyping was performed using four RT-iiPCR reagent sets specific for DENV-1 to DENV-4 (POCKIT Dengue Virus Serotype 1–4 Reagent Sets, GeneReach Biotechnology, Inc., Taiwan), following previously described protocols [11–13]. Pan-DENV RT-iiPCR was used as an entry screen; only pan-iiPCR–positive specimens proceeded to serotype-specific assays.

2.4. Detection of anti-dengue IgM

Anti-dengue IgM antibodies were measured using the Bioline Dengue IgM Capture ELISA (Abbott Diagnostics Korea Inc., Korea) according to the manufacturer's protocol. Briefly, patient sera were diluted 1:100 and added (100 μL per well) to microplate wells pre-coated with mouse monoclonal anti-human IgM antibodies, followed by incubation for 1 h at 37 °C. Wells were washed five times with 350 μL of diluted washing solution, ensuring each well was soaked for at least 10 s and that all residual liquid was removed. Subsequently, 100 μL of a 1:1 mixture of anti-dengue horseradish peroxidase (HRP) conjugate and dengue antigen was added, incubated for 1 h at 37 °C, and washed again. 100 μL of the tetramethylbenzidine (TMB) substrate was added and incubated for 10 min at room temperature, and the reaction was stopped with 100 μL of stopping solution. Absorbance was read at 450 nm using an Infinite 200 PRO microplate reader (TECAN Life Sciences, Switzerland). Results were interpreted using a cut-off defined as the mean optical density (OD) of negative controls plus 0.3; values greater than or equal to the cut-off were considered positive, and those below were negative.

2.5. Detection of preexisting anti-dengue IgG

To distinguish primary from secondary DENV infection, anti-DENV IgG was measured in acute-phase sera using the InBios DENV Detect IgG ELISA kit (InBios International, Inc., USA) according to the manufacturer's instructions. Absorbance was read on an Infinite 200 PRO microplate reader (TECAN Life Sciences, Switzerland). Detailed procedures and interpretation criteria have been described in our previous studies [20,21]. A positive IgG indicated recent secondary infection, whereas a negative result indicated primary infection. All samples were tested in duplicate, with no discordant results observed.

2.6. Statistical analysis

All statistical analyses were performed using SPSS version 20.0 (IBM Corp., Armonk, NY, USA). Continuous variables are presented as medians (range) and compared using the Mann-Whitney *U* test; where normality was confirmed, results were cross-checked with the independent *t*-test. Categorical variables are expressed as counts and percentages and compared using the Chi-squared test or Fisher's exact test, as appropriate. Diagnostic performance, including sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and accuracy, was calculated from 2 × 2 contingency tables with qRT-PCR as the reference standard. Sensitivity analyses were stratified by PSO (≤3 days vs > 3 days) and by immune status. For the latter, the analysis was restricted to a 0–7 days window as a methodological necessity to exclude samples with indeterminate IgG results, thereby ensuring a valid comparison between conclusively classified primary and secondary infections. Paired proportions (iiPCR vs qRT-PCR) were compared using McNemar's test. Cycle threshold (Ct) values were analyzed with the Mann-Whitney *U* test and confirmed with the independent *t*-test when appropriate. Diagnostic indices are reported with 95 % confidence intervals (CIs). Agreement between assays (overall detection and molecular serotyping) was assessed using raw concordance and Cohen's kappa (κ) with 95 % CIs. A p value < 0.05 was considered statistically significant.

3. Results

3.1. Baseline characteristics of the study population

A total of 185 patients were included, comprising 155 qRT-PCR-confirmed dengue cases and 30 qRT-PCR-negative patients with OFI (Fig. 1). As shown in Table 1, median age and sex distribution were similar between the dengue and OFI groups (age: $p = 0.92$, Mann-Whitney U; sex: $p = 0.41$, Chi-squared test). The median interval from symptom onset to sample collection was slightly shorter in dengue patients (1 day; range, 0–12) than in OFI patients (2 days; range, 0–9), though not statistically significant ($p = 0.15$, Mann-Whitney U). Serotype-specific RT-PCR identified DENV-1 in 54.8 % (85/155) and DENV-2 in 45.2 % (70/155) of dengue cases. Most infections were primary (63.9 %), followed by secondary (29 %) and undetermined (7.1 %).

3.2. Comparison between qRT-PCR and iiPCR for dengue detection

All 185 serum samples, comprising 155 qRT-PCR-confirmed dengue cases and 30 age- and sex-matched febrile controls, were tested in parallel by qRT-PCR and iiPCR (Table 2). Matching for age and sex ensured comparable demographic profiles, providing an appropriate basis for assessing iiPCR diagnostic performance against the reference assay. Using qRT-PCR as the reference, iiPCR demonstrated 88.4 % (137/155) sensitivity and 100 % (30/30) specificity, with a PPV of 100 %, NPV of 62.5 %, and substantial agreement (κ = 0.71, 95 % CI 0.59–0.83) (Table 2). Paired comparison by McNemar's test showed significant discordance driven by more iiPCR false-negatives than false-positives ($p < 0.001$). These results confirm that iiPCR broadly aligns with qRT-PCR in classifying dengue-positive and -negative samples, reinforcing its suitability as a rapid, frontline diagnostic tool in decentralized settings.

3.3. Performance of iiPCR in relation to days post symptom onset

To evaluate the diagnostic performance of iiPCR over the clinical course, samples were stratified by days post-symptom onset (PSO). Among 125 qRT-PCR-positive samples collected within 3 days PSO, iiPCR sensitivity was 96.8 % (121/125). In contrast, sensitivity declined to 53.3 % (16/30) for samples collected beyond 3 days PSO (Fisher's exact test, $p < 0.001$) (Table 3 and Fig. 2A). Combining iiPCR with IgM testing increased sensitivity to 99.2 % (124/125) for PSO ≤3 and 100 % (30/30) for PSO >3, indicating that serologic testing effectively complements iiPCR in later stages of infection.

3.4. Diagnostic performance of qRT-PCR and iiPCR in primary and secondary infections

The diagnostic sensitivity of qRT-PCR and iiPCR was further analyzed by infection type. To perform a valid comparison between conclusively classified primary and secondary infections, we applied a necessary methodological constraint. Because the acute-phase IgG ELISA used for classification cannot reliably determine infection status in samples collected >7 days PSO (see Table 1, footnote b), we excluded these samples from this specific analysis. This exclusion left 144 qRT-PCR cases (down from 155) and 131 iiPCR cases (down from 137). Among these, qRT-PCR identified 99 primary and 45 secondary infections, whereas iiPCR detected 94 primary and 37 secondary infections (Table 4). Viremia was significantly lower in secondary infections than in primary infections (Mann-Whitney U, $p < 0.001$). Median PSO was similar across infection types for both methods: 1 day (range, 0–7) for qRT-PCR and 1 day (range, 0–6 for primary; 0–5 for secondary) for iiPCR, indicating minimal confounding by sampling time. These results were based on partially overlapping but not identical sample sets, reflecting minor differences in sample availability and test applicability between the two methods (Table 2; total n = 185). Using

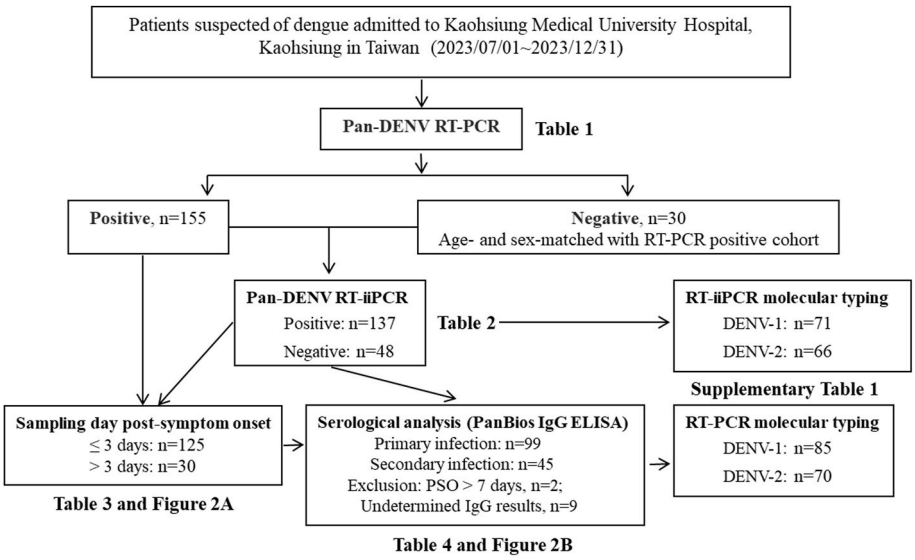

Fig. 1. Flow diagram for recruitment and assessment of study patients. Abbreviations: DENV, dengue virus. RT-PCR, real-time reverse transcription polymerase chain reaction. iiPCR, insulated isothermal polymerase chain reaction. ELISA, Enzyme-linked immunosorbent assay.

Table 2
Comparison of qRT-PCR and iiPCR in detecting dengue virus infection.

Methods		qRT-PCR		
	Results	Positive	Negative	**Total**
iiPCR	Positive	137	0	137
	Negative	18	30	48
	Total	155	30	185

Abbreviations: qRT-PCR, quantitative real-time reverse transcription polymerase chain reaction. iiPCR, insulated isothermal polymerase chain reaction.

qRT-PCR-positive cases as the reference, iiPCR sensitivity was 95.0 % (94/99) for primary infections and 82.2 % (37/45) for secondary infections, indicating a significant reduction in diagnostic sensitivity for secondary dengue infections relative to primary infections ($p = 0.024$, Fisher's exact test) (Fig. 2B).

3.5. Comparison of molecular serotyping by RT-PCR and iiPCR

Molecular serotyping by iiPCR was compared with serotype-specific RT-PCR as the reference (see web-only Supplementary Table S1). Among 85 DENV-1 cases confirmed by serotype-specific RT-PCR, serotype-specific RT-iiPCR correctly classified 71 (83.5 % concordance), while 66 of 70 DENV-2 cases were correctly identified (94.3 %). Pan-DENV iiPCR served as a screening step; 18 RT-PCR-positive specimens were iiPCR-negative and thus did not undergo serotype-specific iiPCR. Across the entire RT-PCR-positive cohort (n = 155), the overall serotype ascertainment rate by iiPCR was 88.4 % (137/155), with undetermined cases largely reflecting specimens that did not pass the pan-DENV iiPCR screening step. Among the screen-positive samples that underwent serotype-specific iiPCR, concordance with serotype-specific RT-PCR was 100 % (137/137). No DENV-3 or DENV-4 cases were detected in this cohort. Notably, 18 samples (11.6 %) were negative in the pan-DENV iiPCR screening assay and therefore did not proceed to serotype-specific testing; all 18 were successfully serotyped by RT-PCR, including 14 DENV-1 and 4 DENV-2 cases. Across the RT-PCR–positive cohort, the serotype ascertainment rate was 88.4 % (137/155). Among screen-positive specimens that underwent iiPCR serotyping, concordance with serotype-specific RT-PCR was 100 % (137/137) (Table 2 & Supplementary Table S1). These findings indicate that while iiPCR achieves highly accurate serotyping when the screening assay is positive, a notable fraction of qRT-PCR-confirmed cases remain undetected, reflecting limitations in analytical sensitivity for serotype determination.

4. Discussion

This study evaluated the clinical and diagnostic performance of iiPCR for DENV detection and serotyping, using qRT-PCR as the reference standard. A cohort of 185 age- and sex-matched febrile patients was analyzed. Overall, iiPCR showed promising diagnostic performance relative to qRT-PCR, with a sensitivity of 88.4 % and specificity of 100 %, and a PPV of 100 %. These results indicate that iiPCR is useful for rapid confirmation in early-phase or primary infections and is suitable for decentralized testing settings. This overall sensitivity (88.4 %) is notably lower than the 95–100 % reported in our previous validation on 2012 outbreak samples, a key finding we attribute to differences in circulating genotypes and host immune status. However, the NPV was limited to 62.5 %, with 18 false-negative results observed, emphasizing the need for cautious interpretation of iiPCR-negative cases when clinical suspicion remains high. Notably, specimens that were iiPCR-

Table 3
Sensitivity of the dengue iiPCR for samples collected at different time periods using qRT-PCR as a reference method.[a]

Days PSO	qRT-PCR positive			iiPCR positive		
	Total (n)	Ct	IgM positive (n)	Total (n)	IgM positive (n)	iiPCR or IgM positive (n)
≤3 days	125	19.42 (12.74–38.54)	19	121	16	124
>3 days	30	29.26 (18.26–36.09)	24	16	10	30

Abbreviations: iiPCR, insulated isothermal polymerase chain reaction. qRT-PCR, quantitative real-time reverse transcription polymerase chain reaction. PSO, post-symptom onset. iiPCR sensitivities were calculated among qRT-PCR-positive specimens.
[a] Presented as the numbers (n) except for Ct, which was shown as the median (range).

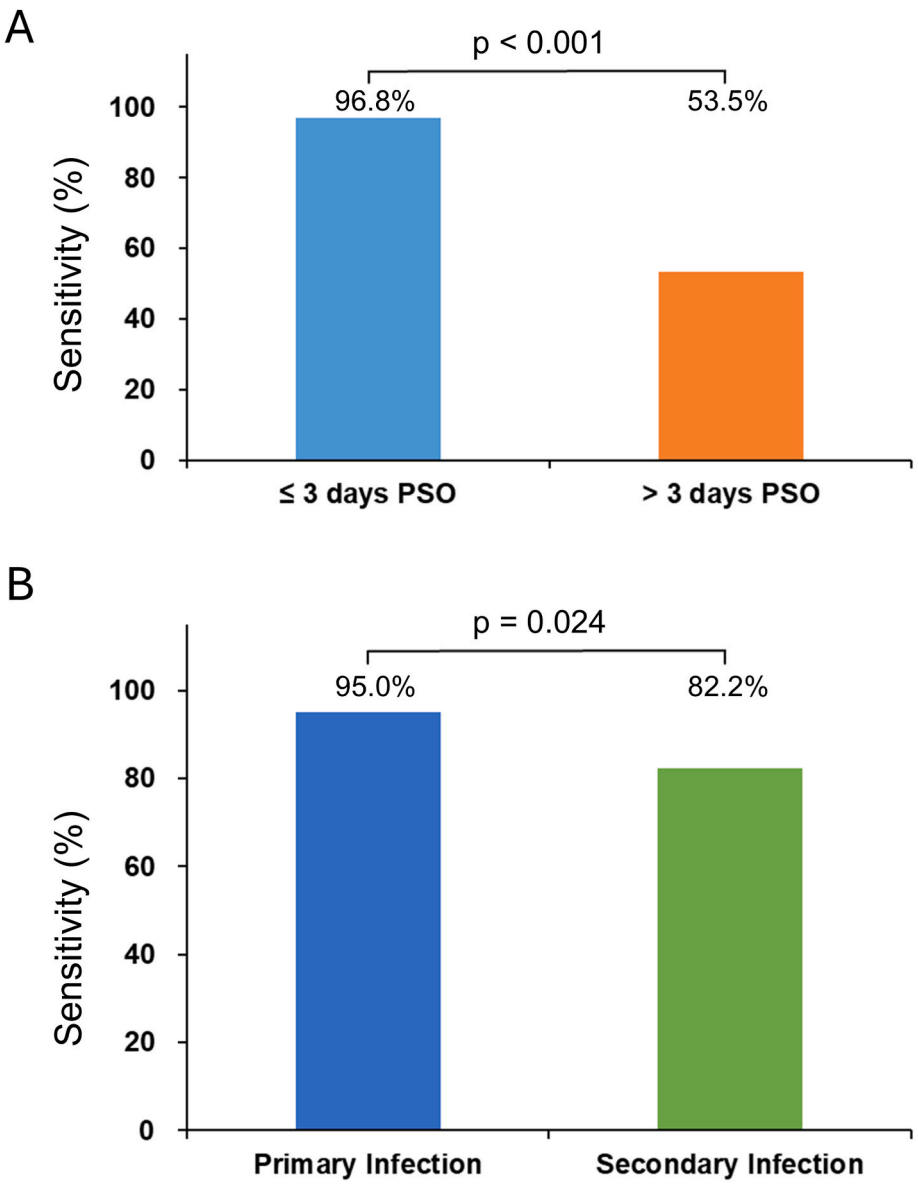

Fig. 2. Stratified diagnostic sensitivity of the dengue RT-iiPCR assay. (A) RT-iiPCR sensitivity in acute-phase samples collected ≤3 days post-symptom onset (PSO) (96.8 %; 121/125) versus those collected >3 days PSO (53.3 %; 16/30). (B) RT-iiPCR sensitivity in patients classified with primary dengue infection (95.0 %; 94/99) versus those with secondary dengue infection (82.2 %; 37/45). Percentages are indicated above the bars. *p*-values are shown; significance in both (A) and (B) was determined by Fisher's exact test.

Table 4
Sensitivity of qRT-PCR and iiPCR for detection of primary and secondary dengue virus infection.[a]

Infection status	qRT-PCR positive			iiPCR positive	
	Cases (n)	Ct	Days PSO	Cases (n)	Days PSO
Primary infection	99	19.04 (12.74–38.54)	1 (0–7)	94	1 (0–6)
Secondary infection	45	24.3 (12.75–37.91)	1 (0–7)	37	1 (0–5)
p value[b]	–	<0.001	–	–	–

Abbreviations: qRT-PCR, quantitative real-time reverse transcription polymerase chain reaction. iiPCR, insulated isothermal polymerase chain reaction. Ct, cycle threshold. PSO, post-symptom onset.

[a] Presented as the numbers (n) except for Ct and days PSO, which were shown as the median (range).

[b] Differences in Ct values were assessed using the Mann-Whitney *U* test.

positive exhibited a much lower viral-load proxy (mean Ct = 20.36; median = 19.65; range 12.74–37.91) compared with iiPCR-negative but qRT-PCR-positive samples (mean Ct = 32.38; median = 31.99; range 26.64–38.54). This 12-cycle difference indicates that iiPCR sensitivity declines as viremia decreases and samples approach the diagnostic threshold. Consequently, most discrepancies occur in low-viremia settings, highlighting the need for follow-up testing or complementary assays in cases with negative iiPCR results. Although iiPCR facilitates rapid, field-deployable diagnosis [11], the observed discordance with qRT-PCR ($p < 0.001$) underscores the risk of missed cases, reinforcing the importance of confirmatory testing when clinical suspicion persists. In comparison with previous studies, where pan-DENV RT-iiPCR achieved 95.2–100 % sensitivity and 95–100 % specificity for DENV-1, -2, and -3 detection [11,12], the sensitivity observed in the current cohort was lower. For DENV-1, iiPCR correctly identified 71 of 85 qRT-PCR-confirmed cases (83.5 %), with iiPCR-positive specimens showing a mean Ct of 20.7 (median 20.42; range 13.08–34.74), whereas iiPCR-negative samples had a mean Ct of 32.18 (median 31.99; range

26.64–38.54). DENV-2 detection remained higher at 94.3 % (66/70), yet a similar Ct gap was evident, with iiPCR-positive cases showing a mean of 19.99 (median 19.28; range 12.74–37.91) compared to 33.08 (median 32.56; range 31.09–36.09) in iiPCR-negative cases, highlighting that most missed detections occurred in low-viremia specimens. This reduced sensitivity may also be influenced by differences in circulating viral genotypes. The 2023 outbreak primarily involved DENV-1 genotype I and DENV-2 genotype II [21], whereas the earlier validation study used samples with DENV-1 genotype III and DENV-2 of the cosmopolitan lineage [12,23]. It is likely that the primers originally optimized for 2012 viral genotypes contributed to the lower sensitivity observed in the current study [12]. This finding itself is a key contribution of our study, as it highlights that molecular assays validated against historical genotypes (e.g., those from 2012) require continuous, real-world re-evaluation to ensure their efficacy against evolving viral strains (e.g., those in 2023).

Fever, which typically lasts 2–7 days, is one of the earliest and most prominent clinical manifestations of dengue virus infection. This febrile phase is closely associated with high circulating levels of viral RNA, which generally peak around symptom onset and decline rapidly as the host immune responses are activated [2,24,25]. In our study, qRT-PCR-positive samples collected within 3 days PSO exhibited a mean Ct value of 20.23 (range, 12.74–38.54), indicative of high viral load. Within this early window, iiPCR demonstrated excellent sensitivity (96.8 %), reinforcing its clinical utility in the acute phase. However, beyond day 3 PSO, viral RNA levels declined substantially, with the mean Ct value increasing to 28.37 (range, 18.26–36.09), reflecting reduced viremia. Correspondingly, iiPCR sensitivity decreased to 53.3 %. These findings are consistent with previous reports that iiPCR sensitivity is compromised in samples with lower viral titers or higher Ct values [11,26]. The kinetics of serologic responses followed the expected temporal pattern. IgM antibodies were detectable in only 15.2 % (19/125) of samples collected within 3 days PSO but increased markedly to 80 % (24/30) in samples collected thereafter. This trend is consistent with the well-documented delay in IgM seroconversion, which typically begins around 4–5 days after symptom onset [25,27]. Therefore, exclusive reliance on serologic testing in the early febrile phase may result in underdiagnosis, whereas molecular assays such as qRT-PCR and RT-iiPCR remain the preferred modalities for early case detection (iiPCR within 3 days PSO) [25,27]. Our findings further highlight the importance of integrating serologic testing into diagnostic algorithms, with iiPCR employed during the acute viremic phase and IgM serology used thereafter, particularly in clinical settings where patients often present beyond the viremic window or where molecular testing alone may not provide sufficient diagnostic confirmation.

We evaluated the diagnostic performance of iiPCR in primary and secondary dengue infections, using qRT-PCR as the reference standard. iiPCR demonstrated higher sensitivity for primary infections (95.0 %) than for secondary infections (82.2 %). Among qRT-PCR-positive cases, the average Ct value was significantly lower in primary infections than in secondary infections, at 19.04 (range: 12.74–38.54) versus 24.3 (range: 12.75–37.91), respectively (Mann–Whitney U, $p < 0.001$), consistent with higher viral loads in primary infections [28–30]. This observation aligns with prior evaluations of isothermal amplification platforms, which report reduced sensitivity under low-viremia conditions [11,12]. Although qRT-PCR remains the diagnostic gold standard for dengue, most existing iiPCR evaluations have not examined performance across immune status categories (primary vs secondary infections). To our knowledge, this is the first study to assess iiPCR performance in both primary and secondary dengue infections under real-world outbreak conditions, providing novel insights into its diagnostic reliability across varying host immune backgrounds and illness phases.

Despite its advantages, including portability, simplicity, and rapid turnaround, several limitations of iiPCR were identified. False negatives and non-typeable results are most likely attributable to lower viremia in secondary infections and potential genotype-primer mismatches. First, reduced sensitivity in secondary infections reflects its vulnerability in low- or transient-viremia settings, leading to reduced RNA detectability. Second, a notable proportion of undetermined cases during molecular serotyping suggests assay limitations, potentially related to primer-genotype mismatch or suboptimal sensitivity, which may limit its utility for molecular epidemiology. Third, false-negative results were observed near the detection threshold, underscoring the need for confirmatory testing in clinically suspected cases.

Taken together, these findings indicate that while iiPCR is a valuable frontline diagnostic tool, its optimal application lies in conjunction with established methods such as qRT-PCR and serologic or antigen-based assays, particularly in diverse epidemiologic contexts. Further optimization of RT-iiPCR primers and assay conditions is warranted, and future studies should specifically address its performance for DENV-3 and DENV-4 detection.

5. Conclusions

This study provides a crucial performance update on the iiPCR assay in the context of a contemporary outbreak, demonstrating it offers a promising point-of-care platform for early dengue diagnosis, particularly during the acute febrile phase and in primary infections. Crucially, by stratifying performance by immune status and circulating genotype—analyses not included in its original validation—we demonstrate that its sensitivity is reduced in secondary infections and that its performance in serotyping is limited in low-viremia samples, likely due to both reduced RNA abundance and genotype-primer mismatches. These limitations underscore the need for complementary diagnostic strategies that combine molecular, antigen-based, and serologic approaches (e.g., anti-DENV IgM) to ensure optimal case detection across diverse clinical and epidemiological contexts. Future refinements in assay design, such as improved primer coverage and multiplexing capabilities, may help overcome current limitations and enhance the utility of iiPCR in dengue surveillance and clinical management.

CRediT authorship contribution statement

Li-Teh Liu: Writing – review & editing, Writing – original draft, Visualization, Formal analysis, Data curation, Conceptualization. **Po-Chih Chen:** Methodology, Investigation, Conceptualization. **Ping-Chang Lin:** Validation, Methodology, Formal analysis, Data curation. **Ching-Yi Tsai:** Validation, Methodology, Formal analysis, Data curation. **Chun-Hong Chen:** Methodology, Investigation. **Chen-Hsuan Lin:** Writing – review & editing, Formal analysis. **Jih-Jin Tsai:** Writing – review & editing, Writing – original draft, Validation, Supervision, Resources, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Human ethics and consent to participate

The study protocol was reviewed and approved by the Institutional Review Board of Kaohsiung Medical University Hospital (KMUHIRB-960195), and written informed consent was obtained from all participants, in accordance with the Declaration of Helsinki.

Consent for publication

All authors confirm that they take responsibility for its integrity and the accuracy of the analysis. All authors approved the final version for submission and publication.

Data availability statement

All authors confirm that they had full access to all the data in the study. All summary data appeared in the article and Supplementary

Material; assay protocols are provided in Materials and Methods and cited references.

Clinical trial number

Not applicable.

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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.tmaid.2025.102945.

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