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Rapid and quantitative loop-mediated isothermal amplification (LAMP) assays for discriminatory detection of *Vibrio cholerae*

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Cholera outbreaks continue to pose significant public health challenges, particularly in resource-constrained regions of Africa and Southeast Asia. Limitations in existing surveillance tools and approaches are one impediment to rapid and effective public health responses. To address this, we developed molecular beacon-based loop-mediated isothermal amplification (LAMP) assays targeting *Vibrio cholerae* (*V. cholerae*) markers *ompW*, *O1rfb*, *O139rfb*, and *tcpA* relevant to species-, serogroup-, and biotype-level detection. The assays achieved high analytical sensitivity with limits of detection ranging from ~99 to 487 gene copies per reaction, performed in duplex with minimal change in non-specific background, and were robust when tested on wastewater as a complex environmental matrix. Quantitatively, the assays showed a strong monotonic association between the logarithmic target concentration and the assay's time-to-threshold (T_t) (Spearman's ρ: consistently high across targets, $\rho \leq 0.53$), but only moderate linearity (singleplex R^2 = 0.59 to 0.74, duplex R^2 = 0.54 to 0.72). While the monotonic relationship is strong, concentration estimates remain uncertain, constraining precise linear quantification. We outline assay design and analysis features that help reconcile these differences and may guide future improvements. Despite some limitations, particularly variability in time-to-reach threshold at low target concentrations, these validated LAMP assays show potential as tools for cholera surveillance and outbreak response. However, further optimization, including improving reproducibility at low concentrations and minimizing false positives during extended reaction times, would enhance their reliability for routine field deployment.

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Water impact

Vibrio cholerae (*V. cholerae*) is a major concern in fecal-related water environments. This work demonstrates molecular beacon-based LAMP for discriminatory detection and robust monotonic quantification of *V. cholerae* in wastewater. More broadly, LAMP's speed, simplicity, and field-compatibility make it well-suited for pathogen surveillance in resource-limited contexts.

Introduction

Vibrio cholerae (*V. cholerae*) has consistently caused regional and national outbreaks of acute and watery diarrheal disease and can ultimately lead to dehydration and death in severe cases.[1] According to the World Health Organization (WHO), there were 804 721 cholera cases and 5805 deaths reported in 33 countries in 2024.[2] The classification of *V. cholerae* strains based on their lipopolysaccharide O antigen has revealed over 200 serogroups, highlighting the diversity within this pathogen. Among these serogroups, O1 and O139 are known to be toxigenic and responsible for major outbreaks.[3] The strain of *V. cholerae* O1 El Tor biotype (7PET) caused the seventh of seven historic cholera pandemics in African countries, replacing the O1 classical biotype responsible for the sixth pandemic.[4] O139 serogroup emerged in Southeast Asia countries in the early 1990s and was responsible for multiple outbreaks,[5] but did not cause a widespread pandemic as O1 did.[6] At present, all cholera outbreaks worldwide are caused by the 7PET lineage across Africa, Asia, and the Middle East. The classical O1 biotype is no longer circulating, and O139 now appears only rarely as sporadic events in Asia.[7]

Early detection and subsequent rapid response, including safe drinking water provision, sanitation, and basic medical

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care, including vaccination,[8] can minimize the public health impacts of cholera outbreaks. Conventional culture methods, while selective, require additional biochemical and serological tests to identify the toxigenic strains, often requiring several days until results are available.[9,10] Polymerase chain reaction (PCR)-based molecular assays offer high sensitivity and accuracy but generally rely on intensive resources and centralized facilities, limiting their practical deployment during outbreaks.[11]

Despite technological advances in cholera detection, the majority of cholera-prone regions continue to face significant challenges in outbreak detection and response due to weak surveillance systems, insufficient laboratory capacity, and limitations in availability of skilled personnel.[2,12] Consequently, there is a pressing need to develop simple, rapid, cost-effective assays for cholera detection. Loop-mediated isothermal amplification (LAMP) has emerged as an attractive approach due to its fast turnaround and ease of operation.[13] A single LAMP assay targeting one gene region uses six primers that recognize eight distinct regions of the target DNA. The strand displacement activity of a *Bst* DNA polymerase between primers and the target region induces the formation of a dumbbell-shaped loop complex of the product, which is rapidly replicated (Fig. 1). Such rapid reaction happens under isothermal conditions, allowing LAMP assays to eliminate the need for repeated thermocycles required for traditional polymerase chain reaction (PCR)-based amplification.

To date, several LAMP assays targeting *V. cholerae* have been developed. Srisuk *et al.* developed a colorimetric LAMP assay targeting the *ompW* gene, encoding the outer membrane protein, in water using hydroxy naphthol blue (HNB) dye.[14] Similarly, LAMP assays targeting multiple molecular markers of *V. cholerae* have been developed using colorimetric dyes like HNB and SYBR green under UV light.[15] A real-time turbidity detection method has also been applied to develop an assay targeting *ctxB*.[16] However, these colorimetric and turbidity-based, or gel electrophoresis readouts often suffer from low sensitivity and poor specificity, as they cannot distinguish non-specific amplification from true positives. They also lack quantitative capability, typically providing only diagnostic presence or absence results. Quantification of *V. cholerae* enables comparison of target abundance across samples and improves interpretation of results near the detection limit, particularly in surveillance applications.

In this study, we developed rapid, quantitative LAMP assays targeting several molecular markers of *V. cholerae*. The *ompW* gene was selected as a pan-*V. cholerae* target, as it is present on most *V. cholerae* strains.[17] Additionally, the assays were developed targeting the *O1rfb* and *O139rfb* genes, specific to their respective serogroup surface antigens.[18] Lastly, the *tcpA* gene, which encodes the toxin-coregulated pilus, serving as a marker for the 7PET-specific biotype, O1 El Tor, and allowing differentiation from other cholera biotypes due to a sequence deletion in the classical allele, was selected as a target.[19,20]

Molecular beacons, a class of highly specific fluorescent DNA hairpin probe,[21] offer a sequence-specific alternative to common LAMP readouts, colorimetric dyes, turbidity, and gel electrophoresis, which are largely qualitative and cannot reliably distinguish target amplicons from non-specific

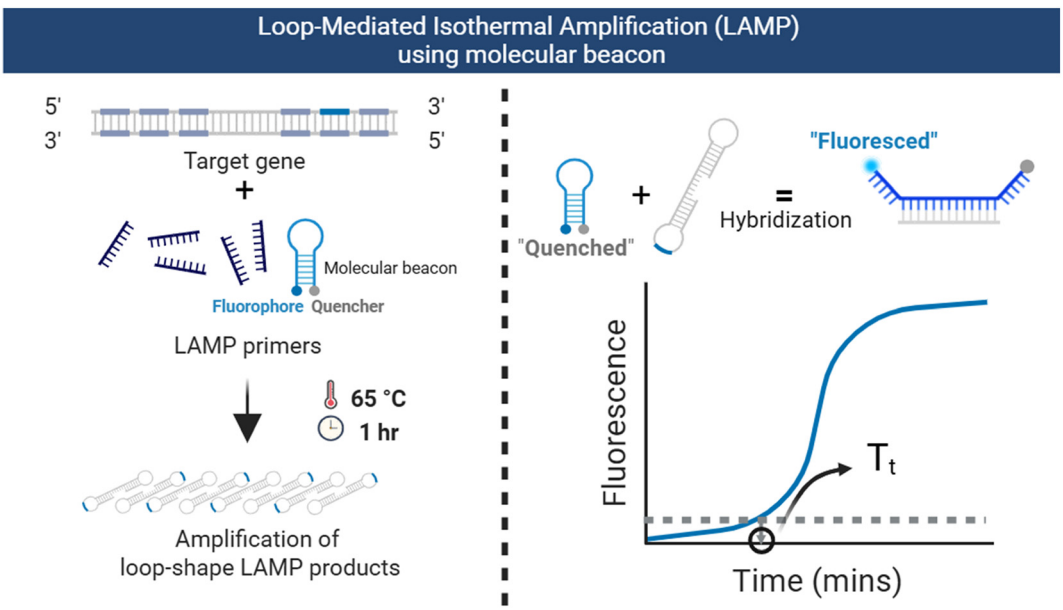

Fig. 1 Real-time monitoring of loop-mediated isothermal amplification (LAMP) assay was enabled using a molecular beacon, which is an elongated loop primer for hairpin formation. A molecular beacon was prepared with a fluorophore and quencher at the termini. Upon the formation of loop complexes by LAMP, the molecular beacon was extended by hybridization to it, increasing the fluorescence signal as it distances from the quencher. The relative fluorescence signal in the reaction is monitored over time to confirm amplification, and the time to reach the threshold, T_t, is estimated.

products.[22,23] Molecular beacon fluoresces only upon hybridizing to its complementary target, enabling real-time signal acquisition and more specific, quantitative readouts. Here, we develop and validate beacon-based LAMP assays for molecular markers of *V. cholerae*, *ompW*, *O1rfb*, *O139rfb*, and *tcpA*, to address the interpretive and quantitation limitations of conventional formats. In our design, the loop primer was converted into a hairpin-structured probe by adding a short complementary stem and placing a fluorophore and quencher at the 5′ and 3′ termini. Target-mediated opening of the hairpin separates these functional elements and generates fluorescence, thereby enhancing assay specificity. Although the current implementation relies on fluorescence-based detection, this work establishes a robust assay framework that can be adapted to alternative, lower-cost readout formats in future studies. Furthermore, we explored multiplexing potential by employing probes labeled with distinct fluorophores for the simultaneous detection of multiple targets. The robustness of the assays was validated in stored wastewater as a prototypical environmental media, as it serves as a pathogen reservoir and is frequently surveilled in regions with non-sewered sanitation systems for disease monitoring.[24–26]

Materials and methods

LAMP primers

The LAMP primer set consists of two outer primers (F3 and B3) and two inner primers (FIP and BIP) (Table 1). Additionally, two loop primers (LF and LB) were added to accelerate the LAMP reaction (Table 1).[27] Primers targeting *O1rfb*, *O139rfb*, and *tcpA* were designed *de novo* using the 'PrimerExplorer V5' software (http://primerexplorer.jp/, Eiken Chemical Co., Ltd., Tokyo, Japan). The *ompW* primer set was sourced from previous literature.[28] For each assay, the forward loop primer (LF) was modified into a molecular beacon by adding a fluorophore and quencher at the 5′ and 3′ ends, respectively, and extending six complementary nucleotides to form a hairpin structure (Table 1, Fig. 1). LAMP primers for all four targets were aligned against the reference genome of the strains used in this study using SnapGene software (v. 7.0.1, GSL Biotech LLC, Boston, MA) (Fig. S1), and no mismatches were identified confirming primer specificity to the intended target regions. To further evaluate primer inclusivity, LAMP primer sets were aligned against publicly available *V. cholerae* genomes retrieved from the NCBI database (https://www.ncbi.nlm.nih.gov/datasets/genome). A total of 514 out of 562 (91.46%) complete *V. cholerae* genomes matched the *ompW* primer set. Out of 26 *V. cholerae* O1 serogroup genomes, 26 (100%) and 24 (92.31%) matched the *O1rfb* and *tcpA* primers without mismatches within primer binding regions. For the O139 serogroup, there was no complete genome available from the database. Out of 3700 contigs from 32 O139 serogroup strains, 26 (81.25%) matched the *O139rfb* primers. The results support the sequence conservation of the selected targets. Detailed alignment results are provided in the **Annex**. Primers were synthesized by Microsynth AG (Balgach, Switzerland). Standard desalting was applied to all primers, except the fluorophore-labeled LF, which underwent HPLC purification. Primers and probes were dissolved in nuclease-free water and stored at −20 °C until use. To investigate the thermodynamic stability of primer

Table 1 Sequences of primers for loop-mediated isothermal amplification (LAMP) assays for detection of molecular markers of *V. cholerae: ompW*, *O1rfb*, *O139rfb*, and *tcpA*. Six bases were added to LF at 5′ for hairpin shape formation, and it was functionalized with a fluorophore and a black hole quencher, BHQ. The extended six bases, fluorophore, and quencher are bolded

Target	Primer	5′-Sequence-3′	Ref.
ompW	F3	CGGTAGTACCTAATGACAGTAG	28
	B3	GCAAATGTTTTGTTTCACCAAT	
	FIP	CAAGCGTTAACCCTAAGTGGGTATTTTTTTAAAGTGTTAAACACTCAAAGTGAG	
	BIP	ACATCAGTTTTGAAGTCCTCGCTTTTTATCACCAAGGCTACCTAAC	
	LF	**FAM/TTGGCA**CTGGGTATTACTATTAACTGCCAA/**BHQ-1**	
	LB	ACATAAGATTTCTACCTCTGGTGGT	
O1rfb	F3	TCCAGCTTTACCACACTC	Self-design
	B3	GGATGGAAACATATTCATGCC	
	FIP	CATTCATATCCGGGGAATGTTGTACAAAGACTTTCTTCAATCACA	
	BIP	AACTCAAGTAAGCCTACTTTACCTGTATTCTGACGTAATTATTCGTGA	
	LF	**ATTO590/TCACAC**TTACAGATGACCTTGGTGTGA/**BHQ-2**	
	LB	CACACACTTCTAGGTTCGATT	
O139rfb	F3	GTTTTGACCGGACGAGTA	Self-design
	B3	TCATGCTGTTTCTCTGCA	
	FIP	TAGGGGCTTTTTTATCCGGAGGAGAGGGATTGTAAAATACCCA	
	BIP	ACGGAACATCCGATAACGCTTTTTCCGATCATGATGCCG	
	LF	**TAMRA/CCACAG**GGTTGATTCGTCCACTGTGG/**BHQ-2**	
	LB	GATCTTGAATAGACTGCTTAAT	
tcpA	F3	GCTTGACCCAAGCACAATGT	Self-design
	B3	AGCTTCTCAACATGCGTGAT	
	FIP	ACAGCAGCGAAAGCACCTTCTTTTGGTTACAAGCGTAGGGGA	
	BIP	AACGAGTGTCGCAGATGCTGCCACTTCCTGGTGCAATGGAC	
	LF	**FAM**/CACGTTGATAAATGGAAACA**AACGTG/BHQ-1**	
	LB	CTGGCGCTGGCGTAATT	

mixes, heterodimer analysis of Gibbs free energy (ΔG, kcal mol^{-1}) was conducted using an OligoAnalyzer (https://eu.idtdna.com/calc/analyzer, accessed on August 7th, 2025) from Integrated DNA Technologies (IDT; Coralville, IA, USA). The risk level for heterodimerization was classified by ΔG from low ($\Delta G > -9$), moderate ($-12 < \Delta G < -9$), and high ($\Delta G < -12$).[29,30]

Real-time LAMP assay

LAMP reagents, including *Bst* DNA polymerase (Cat. No. M0374) and the dNTP mix (Cat. No. N0447S), were purchased from New England Biolabs (NEB, Ipswich, MA, USA). Buffer components, Tris-HCl, $(NH_4)SO_4$, KCl, $MgSO_4$, and betaine (Cat. No. B0300) and Tween20 (Cat. No. P1379) were acquired from Sigma-Aldrich (St. Louis, MO, USA).

The 10× LAMP reaction buffer was composed of 200 mM Tris-HCl, 100 mM $(NH_4)_2SO_4$, 1500 mM KCl, and 80 mM $MgSO_4$. The 25 μL reaction mixture contained 1× LAMP reaction buffer, 8 U *Bst* polymerase, 1.4 mM dNTPs, 0.8 M betaine, 0.1% Tween20, primers (outer: 0.2 μM, loop: 0.8 μM, inner: 1.6 μM), and 5 μL DNA template. No-template control (NTC) included 5 μL nuclease-free water instead of the DNA template. Components were stored at −20 °C, thawed on ice, mixed separately from the DNA template to avoid contamination, gently vortexed, and centrifuged briefly. Reactions were performed in a QuantStudio 3 system (Applied Biosystems, Waltham, MA, USA) at 65 °C for 60 minutes. Reaction conditions were partially optimized during assay development, with emphasis on reagent composition. Betaine was included at a final concentration of 0.8 M to enhance amplification efficiency and probe-target hybridization,[31] while the commercial reaction buffer was maintained for robustness. The reaction temperature was fixed at 65 °C based on the manufacturer's recommendation. Further optimization of reaction conditions may improve assay efficiency and reduce the limit of detection. Relative fluorescence signal (ΔR) was recorded every minute using FAM, ROX, and TAMRA channels. Assays were performed in replicates (n = 2 to 6) to confirm reproducibility. Reactions were performed across multiple experimental runs to assess both repeatability and reproducibility, with duplicate measurements conducted per concentration in each run. Consequently, the total number of technical replicates varied among concentrations. Higher replicate numbers were allocated to conditions exhibiting greater variability in amplification, whereas fewer replicates were sufficient for concentrations with consistently low variability. Nonetheless, increased replication would further improve confidence in quantitative accuracy and assay sensitivity.

Reference digital PCR (dPCR) analysis

dPCR analysis was conducted to quantify gene copies of *ompW*, *O1rfb*, *O139rfb*, and *tcpA*. Details are described in the SI.

Sample preparation

Synthetic DNA segments of *ompW*, *O1rfb*, *O139rfb*, and *tcpA* were purchased from IDT (Coralville, IA, USA) and quantified *via* dPCR. Bacterial suspension included four toxigenic *V. cholerae* strains (A1552, N16961, O395, and MO10), and one environmental strain (Sa5Y), provided by Prof. Melanie Blokesch (École Polytechnique Fédérale de Lausanne (EPFL), Switzerland). A1552 and N16961 strains are O1 EI Tor biotype (7PET), O395 is O1 classical biotype, and MO10 is an O139 strain. Strains were cultured on Lysogeny broth (LB) agar, and single colonies were grown in LB broth at 37 °C overnight and with a shaking speed of 200 rpm. Stocks were prepared with 20% glycerol and frozen at −80 °C. For assay use, bacteria were freshly cultured by spiking 100 μL of stock in 5 mL of LB broth and incubating at 37 °C for 6 hours at 200 rpm. The bacteria suspension was centrifuged (3000 × g, 15 min), washed thrice in 1× phosphate-buffered saline (PBS; pH 7.4, Thermo-Fisher, Cat. No. AM9625), and resuspended. The viable bacterial concentrations (CFU mL^{-1}) were enumerated on a CHROMagar™ Vibrio agar plate (VB912, France). DNA was extracted from 1 mL of bacterial suspension using the QIAamp Fast DNA Stool Mini Kit (Qiagen, Germany; Cat. No. 51604). Finally, 100 μL of eluted extract was purified using the Zymo OneStep PCR Inhibitor Removal Kit (Zymo Research, Cat. No. D6030) and stored at −80 °C. The amounts of *ompW*, *O1rfb*, *O139rfb*, and *tcpA* were quantified by dPCR.

To simulate an environmental sample, 10 μL of bacterial suspension of each strain, A1552, N16961, MO10, and Sa5Y, at a concentration of ~10^8 CFU mL^{-1} was spiked into 990 μL of stored wastewater collected from a household septic tank in Kampala, Uganda, in January 2023. DNA extraction and quantification followed the same procedure as previously described for the bacterial suspension.

Data analysis

Fluorescence was acquired on a QuantaStudio 3. Data were analyzed with the baseline threshold algorithm using automatic baseline and automatic threshold. The software determined the early flat baseline region and placed the threshold within the exponential phase of amplification. The fluorescence signal indicated DNA amplification, as the fluorophore-labeled LF primer was designed to be linearized by the amplified product. The threshold time (T_t; in minutes) for amplification was determined as a quantifiable indicator, analogous to cycle threshold (Ct values) in PCR. To assess quantitative behavior, we computed Spearman's rank correlation (ρ) between T_t values and logarithmic target DNA concentration for each assay. Additionally, R^2 values were calculated from the best-fit regression lines. T_t values exceeding those of average NTCs were excluded from the regression as false positives. The limit of detection (LOD) was estimated using a probabilistic binary-response model based on replicate testing of serially diluted target concentrations. Detection outcomes were recorded as binary

variables (detection = 1, non-detection = 0) and modeled as a function of target concentration using a logistic regression framework:

$$P = \left(1 + e^{-(\beta_0+\beta_1 \log C)}\right)^{-1}$$

where P is the detection probability, β_0 and β_1 are the regression coefficients, and $\log C$ is the logarithmic concentration (gc in the reaction). For each concentration, 2–6 replicates were tested. The LOD was defined as the concentration corresponding to a 95% detection probability (P = 0.95). This approach is mathematically analogous to probit analysis, differing only in the choice of link function, and yields comparable LOD estimates when applied to replicate detection data near the threshold.[32]

Results

LAMP assays for the detection of *V. cholerae* at species- and serogroup-level

Initially, singleplex assays targeting *ompW*, *O1rfb*, and *O139rfb* were analyzed in LAMP reactions using respective primer sets (Table 1). Serial dilutions of synthetic DNA gene segments ranging from ~10 to 10^6 gene copies (gc) per reaction, as confirmed by dPCR, were tested, and fluorescence signals were monitored in real-time (Fig. 2A). All three LAMP assays displayed an exponential increase in their relative fluorescence signal, ΔR, at specific time points in positive samples with higher target concentration, typically $>10^2$ to 10^3 gc per reaction, indicating successful gene amplification. As the target concentration increased, fluorescence signals appeared earlier, whereas fluorescent

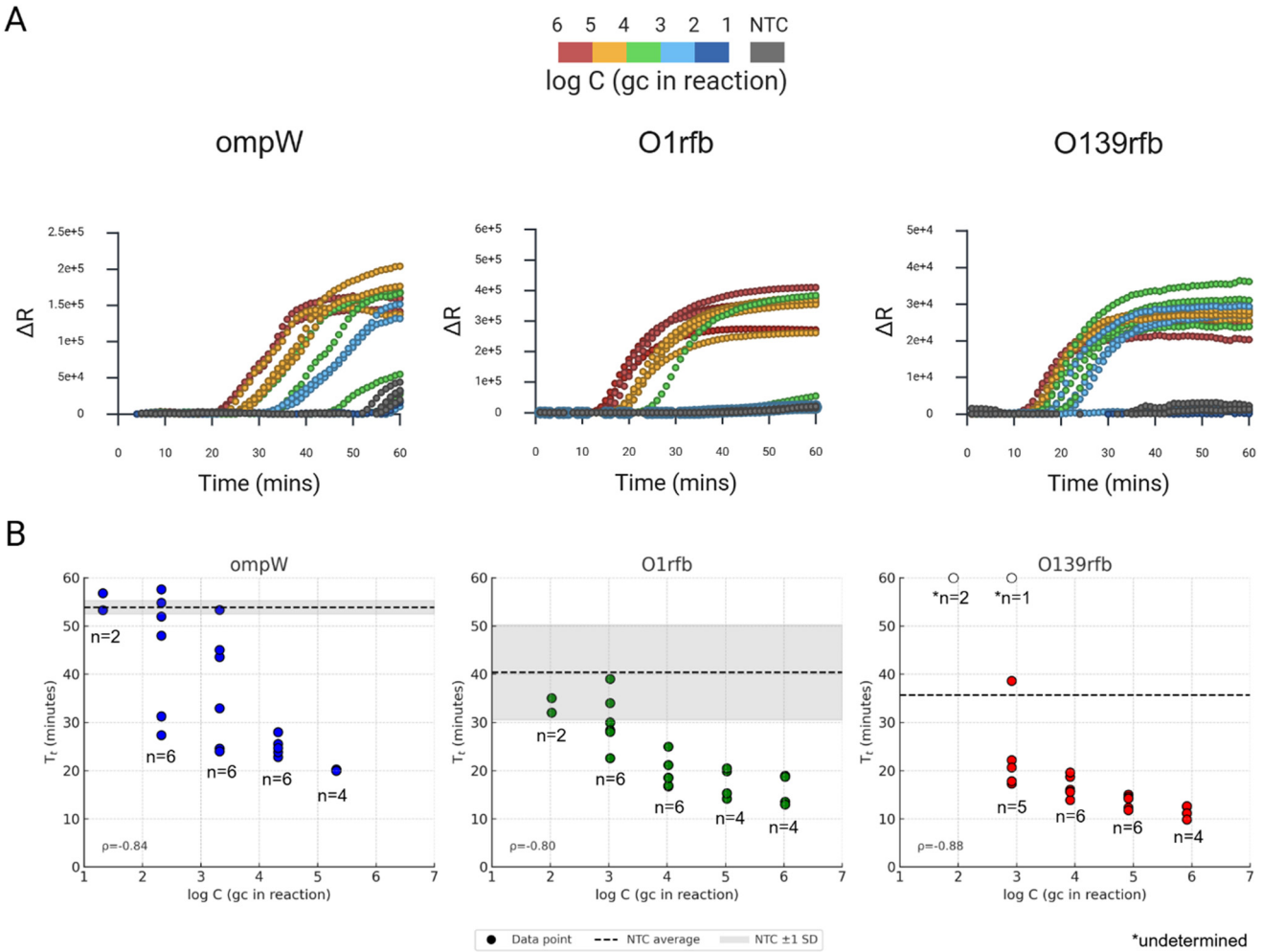

Fig. 2 Three loop-mediated isothermal amplification (LAMP) assays for detection of *Vibrio cholerae* (*V. cholerae*) O1 and O139 serogroups. Three molecular markers, *ompW* for *V. cholerae*, *O1rfb* and *O139rfb* for respective serogroups, were targeted by each assay. (A) Real-time monitoring of relative fluorescence signal, ΔR, over 60 minutes, measured every minute with serially diluted synthetic segments of targets and no-target controls (NTCs). (B) The plots between the logarithmic concentration and the time to reach threshold, T_t, values for each assay were established. Data points indicate different LAMP reactions. Open circle data points indicate no amplification with the reaction period. Dashed horizontal lines and the shaded region indicate the T_t mean for NTCs and its standard deviation. Monotonic relationships are evaluated using Spearman's analysis, indicated by ρ values.

signals for NTCs remained stable or showed slight increases at later time points, >35 minutes.

To evaluate quantitative capability, T_t were plotted against logarithmic target concentrations estimated by dPCR (Fig. 2B). Smaller T_t values, implying faster reactions, corresponded to higher target concentrations, demonstrating a clear monotonic relationship. Specifically, the *ompW* assay showed T_t values ranging from 20.2 (± 0.1, n = 2, ~10^5 gc per reaction) to 55.0 (±1.8, n = 2, ~10 gc per reaction) minutes. The *O1rfb* assay exhibited T_t values from 16.0 (±3.2, n = 4, ~10^6 gc per reaction) to 33.5 (±2.1, n = 2, ~10^2 gc per reaction) minutes. Similarly, the *O139rfb* assay displayed T_t values ranging from 11.5 (±1.3, n = 4, ~10^6 gc per reaction) to 23.3 (±8.7, n = 5, ~10^3 gc per reaction) minutes. At ~10^2 gc per reaction, all replicates (n = 2) showed no amplification within 60 minutes.

Some replicates of NTCs showed non-specific amplification: for *ompW*, 4/6 NTCs exhibited T_t values of 53.8 ± 1.4 minutes; for *O1rfb*, 6/6 NTCs had T_t values of 40.4 ± 9.9 minutes, and for *O139rfb*, 1/6 NTCs had a T_t value of 35.6 minutes. The T_t values of the remaining NTC replicates were undetermined due to the absence of amplification in 60 minutes, which is expected for NTC reactions. Despite primer specificity, prolonged reaction times up to 60 minutes occasionally led to non-specific amplification, linearizing the molecular beacon probe and causing false-positive signals. Thus, any fluorescence appearing after the mean T_t value of NTC could not be reliably considered a true positive. For instance, in the *ompW* assay, 50% of reactions at ~10 gc per reaction (1 of 2 replicates) and 33.3% at ~10^2 gc per reaction (2 of 6 replicates) produced T_t values smaller than those of the NTC mean.

Spearman analysis showed a strong negative monotonic association between the T_t values and the estimated logarithmic concentration of the target for all assays with ρ values of −0.84 (*ompW*), −0.80 (*O1rfb*), and −0.88 (*O139rfb*). Variability was greatest at low concentrations, limiting quantitative reliability, whereas measurements were comparatively consistent at higher concentrations. Regression analysis resulted in moderate linear relationships with R^2 values of 0.59 (*ompW*), 0.60 (*O1rfb*), and 0.74 (*O139rfb*) (Fig. S2).

Gel electrophoresis of LAMP products from positive samples and no-template controls produced similar ladder-like patterns (Fig. S3), limiting its utility for distinguishing specific amplification or probe annealing in this assay.

Duplex LAMP assays for the detection of *V. cholerae* at species- and serogroup-level

Duplex assays were developed by combining the *ompW* assay (FAM-labeled molecular beacon) with either *O1rfb* (ATTO590-labeled) or *O139rfb* (TAMRA-labeled), allowing simultaneous detection of two targets using distinct fluorescence channels. The duplex assay configurations targeting *ompW*/*O1rfb* and *ompW*/*O139rfb* were selected as representative examples to demonstrate multiplexing capability while maintaining assay robustness. Increasing the number of targets in a single reaction elevates the risk of primer–primer interactions and non-specific amplification. To minimize these risks, multiplexing was limited to duplex formats in this study. Nonetheless, the assay design remains flexible, and alternative target combinations may be implemented depending on specific diagnostic or surveillance requirements. Serially diluted mixtures of two synthetic gene segments in the same magnitude were tested, and signals for both targets in each duplex assay were monitored concurrently.

Both duplex assays demonstrated associations with earlier fluorescence signal detection (lower T_t) with increasing target concentrations, consistent with single-target assays (Fig. S4). Specifically, the *ompW–O1rfb* duplex assay displayed *ompW* T_t values of 24.9 (±4.2, n = 4, ~10^5 gc per reaction) to 47.6 (±5.5, n = 2, ~10 gc per reaction) minutes, and *O1rfb* T_t values of 15.7 (±2.8, n = 4, ~10^6 gc per reaction) to 47.6 (±4.9, n = 2, ~10^2 gc per reaction) minutes (Fig. 3, top). Similarly, the *ompW–O139rfb* duplex assay showed *ompW* T_t values ranging from 26.0 (±1.3, n = 6, ~10^5 gc per reaction) to 36.4 (±9.6, n = 5, ~10^2 gc per reaction) minutes, and *O139rfb* T_t values ranging from 13.8 (±2.4, n = 6, ~10^6 gc per reaction) to 28.1 (±8.2, n = 4, ~10^3 gc per reaction) minutes (Fig. 3, bottom).

Spearman's analysis indicated a high monotonic relationship with ρ values of −0.71 (*ompW*) and −0.74 (*O1rfb*) for the *ompW–O1rfb* assay, and −0.53 (*ompW*) and −0.88 (*O139rfb*) for the *ompW–O139rfb* assay. Regression analysis indicated moderate quantitative performance: R^2 values were 0.72 (*ompW*) and 0.68 (*O1rfb*) for the *ompW–O1rfb* assay, and 0.54 (*ompW*) and 0.63 (*O139rfb*) for the *ompW–O139rfb* assay (Fig. S5). Logistic regression-derived LODs were estimated to be 132 gc (*ompW*) and 135 gc (*O1rfb*) per reaction for the *ompW–O1rfb* assay, and 99 gc (*ompW*) and 138 gc (*O139rfb*) per reaction for the *ompW–O139rfb* assay (Fig. S6).

Conceptually, even with thermodynamic optimization to minimize unwanted interactions,[33] combining two targets in a duplex appeared to increase the risk of primer–primer pairing and thus non-specific amplification. The potential risk of heterodimerization between pair-wise primers targeting *ompW*, *O1rfb*, and *O139rfb* was evaluated *in silico* using ΔG (Table S1). For single-target assays, there was one moderate-risk pair (*ompW*), no risk pairs (*O1rfb*), and two moderate-risk pairs (*O139rfb*). Meanwhile, there were three moderate-risk pairs (*ompW*) and one high-risk pair (*O1rfb*) for *ompW–O1rfb* duplex and one moderate-risk pair (*ompW*) and three high-risk pairs (*O139rfb*) for *ompW–O139rfb* duplex. The *in silico* analysis indicated that there were greater risks of heterodimerization when duplexing, potentially causing non-specific amplification.

Both duplex assays occasionally exhibited non-specific amplification in NTCs, with T_t values of 50.9 (*ompW*; ±4.5, n = 3) and 54.3 (*O1rfb*; ±4.0, n = 4) minutes for the *ompW–O1rfb* assay, and 46.2 (*ompW*; ±9.0, n = 4) and 33.1 (*O139rfb*; ±5.6, n = 4) minutes for the *ompW–O139rfb* assay. When compared to

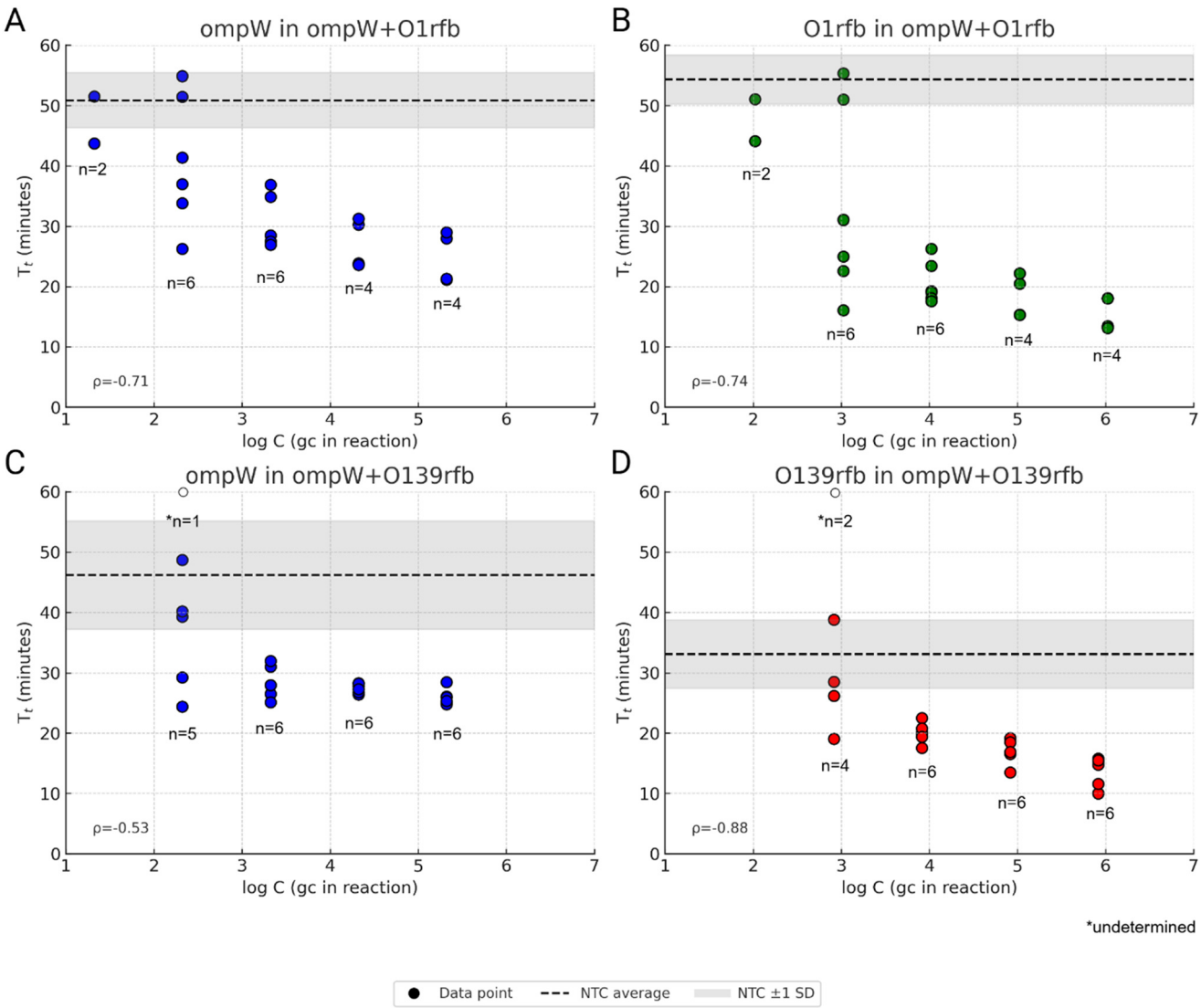

Fig. 3 Two duplex loop-mediated isothermal amplification (LAMP) assays for detection of *Vibrio cholerae* (*V. cholerae*) O1 and O139 serogroups. Three molecular markers, *ompW* for *V. cholerae*, *O1rfb* and *O139rfb* for respective serogroups, were targeted by each assay. (A and B) *ompW–O1rfb* duplex and (C and D) *ompW–O139rfb* duplex LAMP assays were developed. The plots between the logarithmic concentration of the synthetic segments and the time to reach threshold, T_t, values for each assay were established. Data points correspond to different LAMP reactions, while open circle data points indicate no amplification within the reaction period. Dashed horizontal lines and the shaded region represent the T_t mean for NTCs and its standard deviation. Monotonic relationships were evaluated using Spearman's analysis, indicated by ρ values.

single-target assays, duplexing did not significantly alter or suppress non-specific amplification for *ompW* in either assay (*ompW–O1rfb*, p = 0.93; *ompW–O139rfb*, p = 0.57) (Fig. S7). Notably, NTC T_t values for *O1rfb* significantly increased (p = 0.013) in duplex reactions compared to the single-target assay. Single-target *O139rfb* assay showed no significant difference in NTC T_t values with the duplex assay (p = 0.13). Overall, despite shifts in NTC T_t values when duplexed, non-specific amplification occurred at comparable extent in duplex and single-plex formats.

The duplex assays were further validated with DNA extracted from bacterial suspensions of two O1 strains (A1552 and N16961) and one O139 strain (MO10). Positive detection was consistent across all strains at concentrations ranging from ~10^3 to 10^6 gc per reaction, estimated by dPCR. However, most T_t values deviated from those observed with synthetic gene segments, highlighting the need for strain-specific calibration curves (Fig. 4). Spearman's analysis indicated a high monotonic relationship with the ρ values of −0.89 (*ompW*) and −0.94 (*O1rfb*) for the *ompW–O1rfb* assay, and −0.91 (*ompW*) and −0.98 (*O139rfb*) for the *ompW–O139rfb* assay. The observed linearity was high for DNA extracts from bacterial suspension, as indicated by an R^2 ranging from 0.75 to 0.95, though the slope differed from those of synthetic segments (Fig. S8). Similar results were obtained when the strains were spiked into stored wastewater (represented by

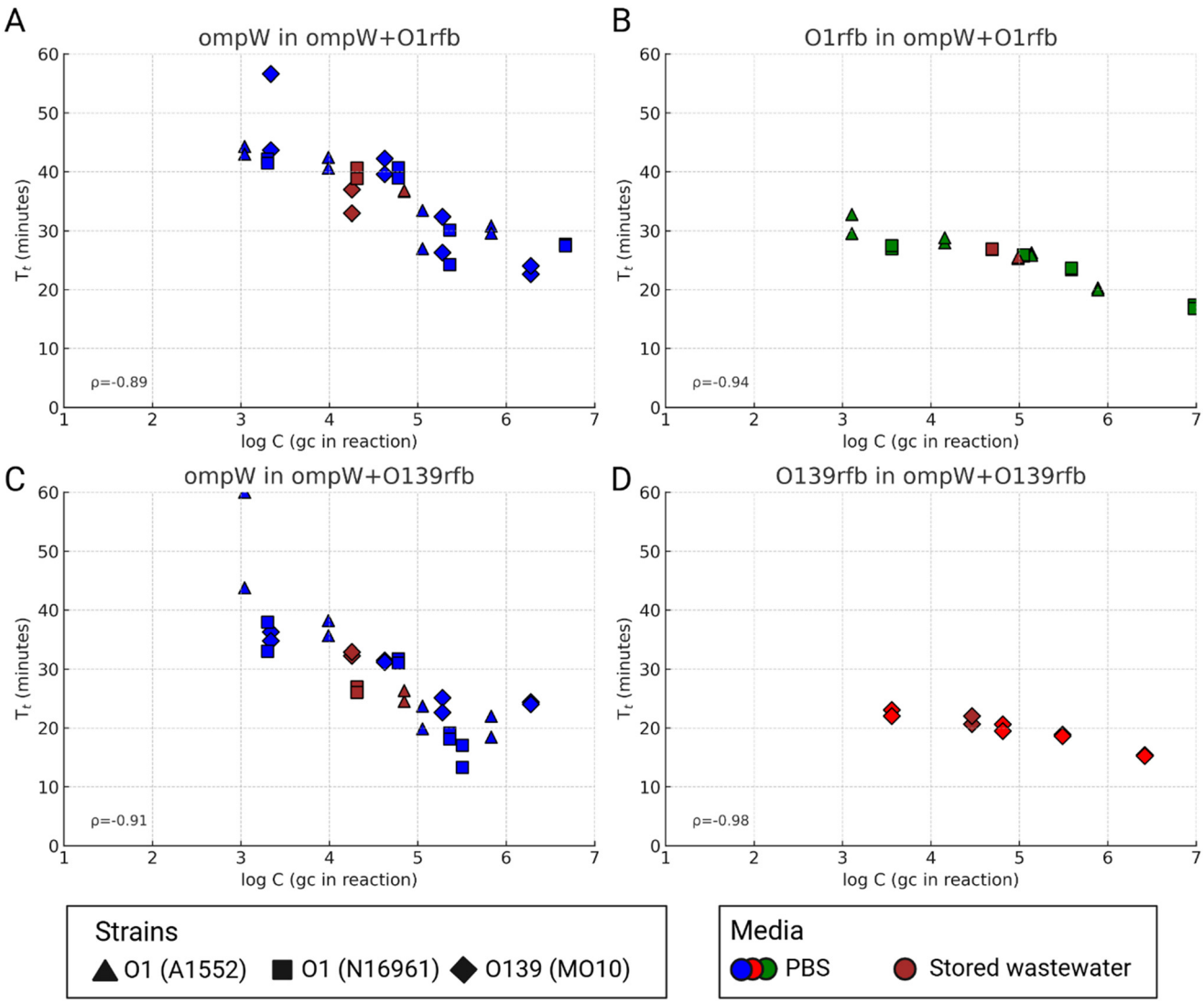

Fig. 4 Validation of (A and B) *ompW–O1rfb* and (C and D) *ompW–O139rfb* duplex loop-mediated isothermal amplification (LAMP) assays against three *Vibrio cholerae* (*V. cholerae*) strains; two O1 serogroups, A1552 (triangle) and N16961 (square), and one O139 serogroup, MO10 (diamond). Three molecular markers, *ompW* for *V. cholerae*, *O1rfb* and *O139rfb* for respective serogroups, were targeted by each assay. The plots between the logarithmic concentration of the synthetic segments and the time to reach threshold, T_t, values for each assay were established. Data points indicate different LAMP reactions for DNA extracts from *V. cholerae* strain suspensions in phosphate-buffered saline (PBS) (blue, green, and red) and in stored wastewater (dark brown).

brown symbols), suggesting that the stored wastewater used as a proxy environmental matrix did not cause interference with assay quantification (Fig. 4).

LAMP assay for the detection of *V. cholerae* at the biotype level (O1 El Tor; 7PET)

A biotype-specific LAMP assay targeting the *tcpA* gene, characteristic of the O1 El Tor biotype (7PET responsible for the ongoing pandemic), was also developed. In this study, *tcpA* was evaluated as a singleplex assay to prioritize independent assessment of biotype-specific detection and limit assay complexity. Duplex or higher-order multiplex formats could be implemented in future work if necessary. Synthetic target testing showed T_t values ranging from 22.2 (±0.1, n = 2, ~10^4 gc per reaction) to 38.8 (±7.8, n = 2, ~10^1 gc per reaction) minutes (Fig. 5). NTC reactions exhibited non-specific amplification at 48.1 (±2.2, n = 4) minutes.

Spearman analysis indicated a strong negative monotonic association between T_t and the logarithmic target concentration, with a ρ value of −0.83. Regression analysis indicated moderate quantitative performance with an R^2 value of 0.60 (Fig. S9). The LODs were estimated to be 487 gc per reaction (Fig. S10). Validation using DNA extracted from the cultivated strain A1552 (~10^3 gc per reaction) yielded consistent results, T_t = 25.9 (±0.8, n = 4) minutes. Similar detection reliability was maintained in wastewater samples spiked with the cultivated strain A1552 (~10^3 gc per reaction), T_t = 27.8 (±1.2, n = 2) minutes, demonstrating assay robustness in complex environmental matrices.

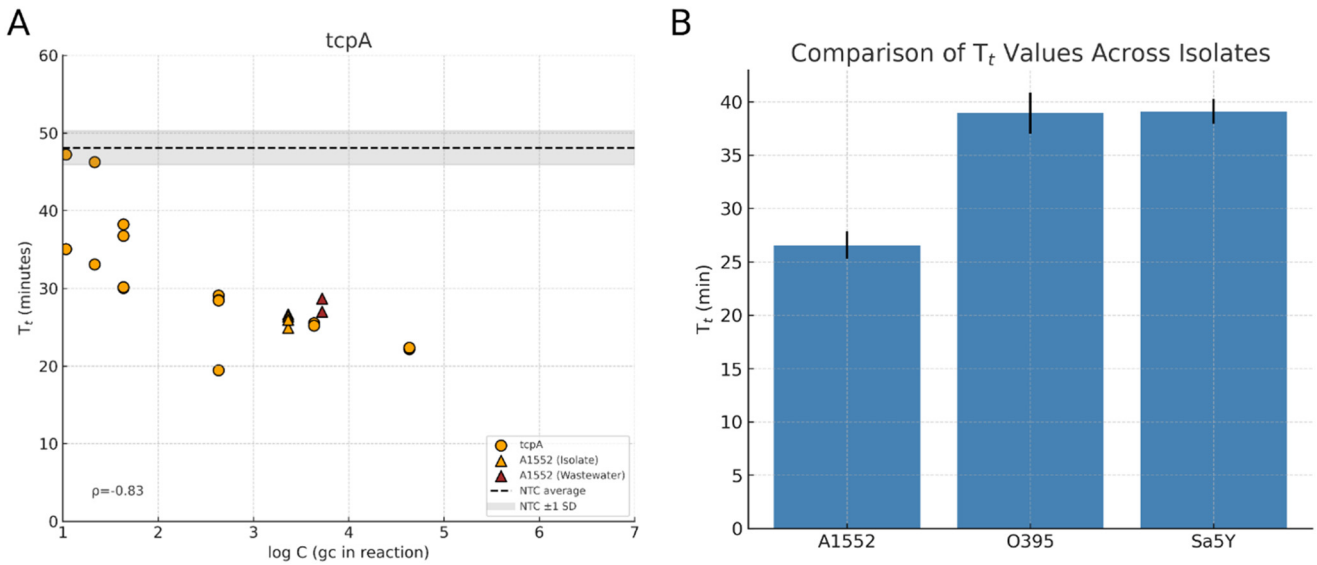

Fig. 5 Loop-mediated isothermal amplification (LAMP) assays for detection of *Vibrio cholerae* (*V. cholerae*) O1 El Tor biotype (7PET). A molecular marker, *tcpA*, was targeted by the assay. (A) The plot between the logarithmic concentration of the synthetic segment and the time to reach threshold, T_t, values for the assay was established. Data points indicate different LAMP reactions for synthetic segments (orange circle), DNA extracts from the *V. cholerae* A1552 strain in phosphate-buffered saline (PBS) (orange triangle), and in stored wastewater (dark brown triangle). Dashed horizontal lines and the shaded region represent the T_t mean for NTCs and its standard deviation. (B) T_t values for *tcpA* LAMP assays against three *V. cholerae* strains: A1552, O395, and Sa5Y.

Specificity testing using A1552 (positive control), O395 (classical biotype), and Sa5Y (environmental strain) revealed clear discrimination based on T_t values: A1552 had significantly lower T_t values, 25.4 (±0.7, n = 2) compared to O395, 39.8 (±0.7, n = 2) and Sa5Y, 41.1 (±1.9, n = 2) minutes (Fig. 5B). Despite their lower T_t values compared to NTCs, O395 and Sa5Y remained distinguishable from the true positive (A1552), highlighting the assay potential in discriminatory power.

Discussion

Rapid detection of *V. cholerae* at various classification levels (species, serogroup, and biotype) is critical for effective cholera surveillance and outbreak response. In this study, we developed and validated multiple LAMP assays targeting diverse molecular markers of *V. cholerae*, *ompW*, *O1rfb*, *O139rfb*, and *tcpA*, to enable comprehensive detection. These assays were evaluated using five representative *V. cholerae* strains: three O1 serogroup strains (two El Tor biotypes and one classical biotype), one O139 serogroup strain, and one environmental strain.

We adopted molecular beacon-based probes to obtain sequence-specific LAMP readouts that discriminate true targets from non-specific products, an approach reported to reduce false-positives compared with turbidity/color dyes or intercalating dyes and to enable real-time quantification. Our results of LODs, ~10^2–10^3 gc per reaction, are consistent with prior probe-based LAMP reports, which emphasize improved specificity/readout clarity and maintained analytical sensitivity.[34–36] Thus, in line with previous studies and demonstrations of probe-based LAMP, we find that molecular beacons mitigate false-positive interpretation and support quantitative analysis, while overall sensitivity remains similar to prior studies.

Quantitatively, T_t showed a strong monotonic association with the logarithmic target concentration, exhibiting high Spearman's ρ across targets, but only moderate linear fit (single-plex R^2 = 0.59 to 0.74; duplex R^2 = 0.54–0.72). Practically, this indicates robust rank-ordering over several logs while precise linear quantification is limited under current conditions. Amplification behavior differed between synthetic segments and bacterial DNA, motivating strain- or matrix-matched calibration curves for accurate quantification. This aligns with the report of strain-dependent LODs for identical targets, likely reflecting primer-target and chemistry effects.[15] While the LAMP assays effectively detected targeted gene regions, reproducibility at low DNA concentrations was limited, and false positives emerged when reaction times were extended to one hour. To mitigate variability at low DNA concentrations and reduce late, spurious positives, a short-pre-enrichment (~6–8 hours) in alkaline peptone water (APW) before LAMP can increase target load and improve detection specificity, as recommended in CDC/WHO laboratory guidance.[37–39] Although pre-enrichment extends the incubation time and reduces the quantitative utility of the assay, it has been shown to enhance diagnostic performance for cholera assays. Further optimization efforts should focus on additional approaches that enhance the overall LAMP performance while maintaining diagnostic precision. Assay robustness was confirmed by testing in complex environmental matrices,

specifically stored wastewater, which showed minimal environmental interference. It is consistent with prior LAMP applications in environmental matrices, including studies that assessed LAMP for *V. cholerae* in environmental waters. For example, Srisuk *et al.* applied it to water and wastewater with performance comparable to PCR.[14] More broadly, recent reviews highlight the feasibility of wastewater or environmental surveillance for cholera and the successful use of LAMP for pathogen detection in wastewater.[40] Additionally, duplex assays employing two fluorophores demonstrated multiplex capability. However, high variability in threshold time, T_t, at lower target concentrations highlighted the need for further investigation to enhance assay sensitivity and quantitative reliability. For accurate and reliable quantification of samples and assessment of intra-sample variation, including determination of LOD, replicates can be increased. Expanding the number of replicates near the detection threshold would help refine LOD estimates and strengthen confidence in quantitative performance during future validation studies.

Identifying specific serogroups and biotypes requires multiple molecular markers, and multiplexing these assays involves numerous primers at high concentrations, raising concerns about increased primer interactions and non-specific amplification. Despite higher predicted heterodimer risk, duplex assays showed minimal practical impact on specificity compared to single-target assays. This aligns with prior reports that multiplex probe-based LAMP can preserve specificity, with only minor sensitivity trade-offs from primer competition.[41–43] Nonetheless, the persistent issue of false positives due to non-specific amplification requires continued attention when multiplexing LAMP assays.

Despite the high analytical specificity and improved performance demonstrated by this molecular beacon-based LAMP assay, some limitations remain that are important to consider, particularly in the context of deployment in resource-constrained settings. The primary trade-off associated with the use of molecular beacons is their dependence on fluorescence-based detection, which currently requires access to fluorometers or qPCR platforms. While this requirement may limit immediate field deployment, real-time fluorescence monitoring was critical during assay development to characterize amplification kinetics, determine the onset of non-specific amplification, and define optimal reaction time windows that ensure specificity. Such validation is an essential step in establishing assay reliability and is widely regarded as best practice in point-of-care assay development. Once performance characteristics are well defined, this assay framework can be adapted to alternative, lower-cost readout formats, such as lateral flow systems, as demonstrated in our previous assay development efforts.[34] Further work will therefore focus on translating this validated assay into more accessible detection modalities and evaluating its performance under field conditions. Addressing these limitations is a necessary step toward transforming this high-specificity diagnostic approach into a practical surveillance and diagnostic tool suitable for low-income and outbreak-prone regions.

Conclusions

This study presents a suite of rapid, robust, and semi-quantitative LAMP assays for the detection of *V. cholerae* at species, serogroup, and biotype levels. The assays demonstrated specificity across key *V. cholerae* strains and retained performance in environmental matrices such as stored wastewater. Despite some limitations, particularly variability at low concentrations and the potential for false positives during extended reaction times, the assays are proven to be suitable for diagnostic purposes and field surveillance. A significant limitation to widespread adoption remains the dependence on fluorometer-based detection, which may be cost-prohibitive in resource-constrained settings. Future work should focus on adapting these assays to low-cost, easy-to-use formats, such as lateral flow devices, to increase accessibility. Importantly, the validated primer sets and real-time assay designs in this study provide a strong foundation for transitioning to such alternative detection platforms.

Author contributions

S. K.: conceptualization, methodology, data curation, formal analysis, visualization, supervision, writing – original draft, review & editing, funding acquisition, project administration; M. Z.: methodology, data curation, review & editing; M. R.: methodology, data curation, review & editing; T. R. J.: conceptualization, methodology, supervision, project administration, review & editing.

Conflicts of interest

There are no conflicts to declare.

Data availability

All data have been deposited in the Eawag Research Data Institutional Collection (ERIC) for public availability at https://doi.org/10.25678/000FQP.

Supplementary information (SI): Text. Digital PCR (dPCR) assay; Fig. S1 Alignment of loop-mediated isothermal amplification (LAMP) primers for four molecular indicators of *Vibrio cholerae* (*V. cholerae*) against reference genomes, *ompW*, *O1rfb*, *O139rfb*, and *tcpA*; Fig. S2 The $\log C$ (synthetic segments) *vs.* T_t plots with a best-fit line, R^2 value, and 95% confidence interval (CI). Three single-plex LAMP assays for the detection of *ompW*, *O1rfb*, and *O139rfb*; Fig. S3 Gel electrophoresis of LAMP products from positive controls ($\sim 10^4$ gc μL^{-1}) and no-template control (NTC) for three singplex LAMP assays: ompW, O1rfb, and O139rfb; Fig. S4 Real-time monitoring of relative fluorescence signal, ΔR, over 60 minutes for two duplex LAMP assays: the *ompW–O1rfb* and *ompW-O139rfb*; Fig. S5 The $\log C$ (synthetic segments) *vs.* T_t

plots with a best-fit line, R^2 value, and 95% confidence interval (CI). Two duplex LAMP assays: the *ompW–O1rfb* and *ompW–O139rfb*; Fig. S6 Calculation of limit of detection (LOD) using a logistic regression-based method for two duplex LAMP assays: *ompW–O1rfb* and *ompW–O139rfb*; Fig. S7 Pair-wise comparison of mean of time to reach threshold, T_t, values for no-template controls (NTCs) for single and duplex LAMP assays targeting *ompW*, *O1rfb* and *O139rfb*; Fig. S8 The log C (DNA extracts) *vs.* T_t plots with a best-fit line, R^2 value, and 95% confidence interval (CI). Two duplex LAMP assays: the *ompW–O1rfb* and *ompW–O139rfb*; Fig. S9 The log C *vs.* T_t plots with a best-fit line, R^2 value, and 95% confidence interval (CI). The LAMP assay for *tcpA*; Fig. S10 Calculation of limit of detection (LOD) using a logistic regression-based method for LAMP assay for *tcpA*; Table S1 Hetero-dimer analysis across loop-mediated isothermal amplification (LAMP) forward, inner, and loop (F3, B3, FIP, BIP, LF, and LB) primers targeting *ompW*, *O1rfb*, and *O139rfb*. [Annex] Results of LAMP primer alignment against publicly available database. See DOI: https://doi.org/10.1039/d5ew01147g.

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