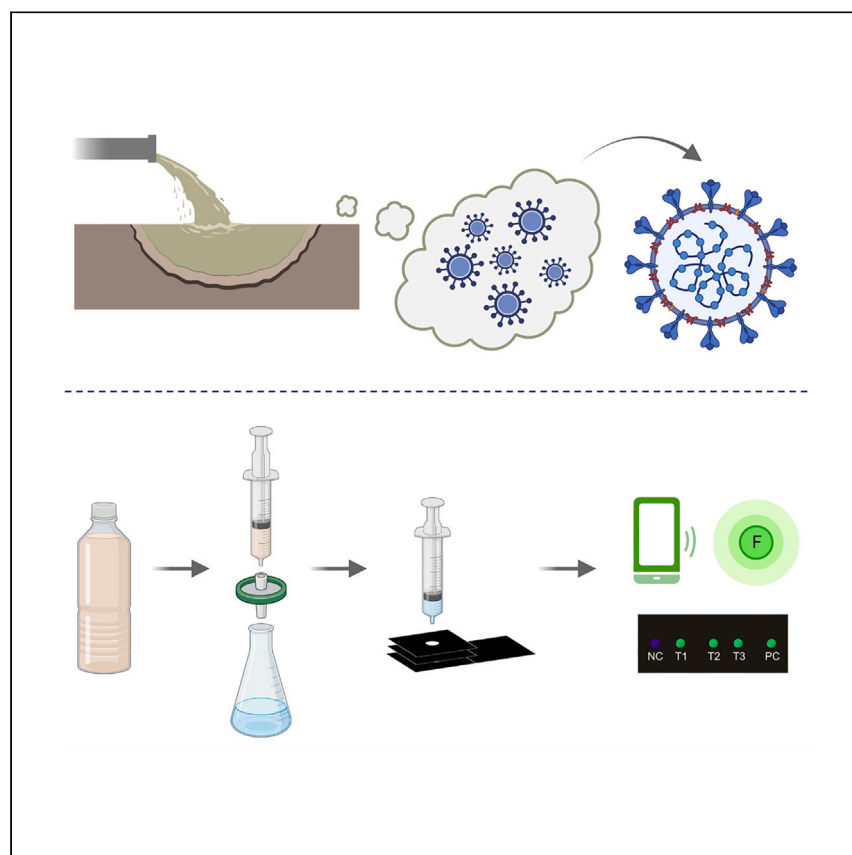


Article

Paper microfluidic sentinel sensors enable rapid and on-site wastewater surveillance in community settings



Infectious diseases remain a global health concern, and wastewater surveillance provides early warning for pandemic preparedness and public health management. Pan et al. develop a low-cost, rapid, and user-friendly paper microfluidic platform for virus detection and develop an on-site enrichment method for pathogen concentration in wastewater.

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Highlights

Low-cost and rapid paper
microfluidic device for on-site
wastewater surveillance

Sensing platform for pandemic
preparedness and public health

Field testing of SARS-CoV-2 in
wastewater from quarantine
hotels

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Article

Paper microfluidic sentinel sensors enable rapid and on-site wastewater surveillance in community settings

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SUMMARY

Tracking genomic sequences as microbial biomarkers in wastewater has been used to determine community prevalence of infectious diseases, contributing to public health surveillance programs worldwide. Here, we report upon a low-cost, rapid, and user-friendly paper microfluidic platform for severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) and influenza detection, using loop-mediated isothermal amplification, with signal read using a mobile phone camera. Sample-to-answer results were collected in less than 1.5 h, providing rapid multiplexed detection of viruses in wastewater, with a detection limit of <20 copies mL^{-1} . The device was subsequently used for on-site testing of SARS-CoV-2 in wastewater samples from four quarantine hotels at London Heathrow Airport, showing comparable results to those obtained using polymerase chain reaction. This sensing platform, which enables rapid and localized testing without requiring samples to be sent to centralized laboratories, provides a potentially important public health tool for pandemic preparedness, with a variety of future wastewater surveillance applications in community settings.

INTRODUCTION

Coronavirus disease 2019 (COVID-19), caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), brought about significant public health and socioeconomic challenges across the globe.¹ While the healthcare outcomes of the disease in severe cases are well documented, the Centers for Disease Control and Prevention (CDC) noted that in mild cases, the symptoms of the disease are similar to a number of other respiratory infections, and they emphasized the importance of distinguishing between infections caused by SARS-CoV-2 and other respiratory viruses, particularly those causing influenza.²

For many infectious diseases,³ including both influenza and COVID-19,⁴ during an active infection, microbial materials are excreted in feces and survive within sewage for several days. Wastewater surveillance has previously been shown to be capable of detecting a wide range of such diseases within community settings, acting as an early warning tool through the analysis of readily available aggregated samples.⁵ However, limitations in the technologies remain, preventing their more widespread use, including the local availability of diagnostic centers, with appropriately trained staff.

Currently, wastewater surveillance samples are sent to centralized analytical laboratories, with analysis involving using either polymerase chain reaction (PCR) assay,^{6,7}

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as a gold standard analytical method, and/or genome sequencing for the identification of new variants with associated lengthy times for results to be reported (typically 24–48 h). Notwithstanding such delays, in one example, wastewater-based epidemiology (WBE) was successfully performed across 45 sewage sites in the UK, demonstrating the emergence of SARS-CoV-2, 4–5 days in advance of the formal diagnosis of individual clinical cases.⁸ In a second example, deep sequencing of 94 urban water catchments was used to infer the spatiotemporal abundance of predefined variants of SARS-CoV-2 from a large number of wastewater samples.⁹

In addition to challenges around the length of the analysis time (during which period, nucleic acid degradation may occur during sample storage and transport), the presence of PCR inhibitors in sewage may also lead to inconsistent results. Point-of-need *in situ* testing with rapid target amplification is needed to overcome these limitations, enabling the sensitive detection of low viral loads (often associated with sample dilution by rainwater runoff in the wastewater).

As an alternative to laboratory-based PCR, isothermal amplification methods such as loop-mediated isothermal amplification (LAMP) have attracted increasing attention for the detection of a range of pathogens,¹⁰ providing a less complex assay workflow by negating the demand for complex thermocycling equipment. Using such isothermal amplification protocols, RNA viral biomarkers can be easily integrated in the form of reverse-transcription loop-mediated isothermal amplification (RT-LAMP), allowing detection of SARS-CoV-2¹¹ as well as influenza A and influenza B.¹² However, many of these LAMP-based detection methods still rely upon centralized laboratories, preventing their wider application in point-of-care diagnostics.

Paper-based microdevices have been proposed as a method of providing point-of-need diagnostics that are affordable, sensitive, specific, user-friendly, rapid, robust, equipment-free, and deliverable to end users (ASSURED criteria).¹³ When such assays include real-time connectivity and ease of specimen collection, tests have come to be known as RE-ASSURED.¹⁴ Proof-of-concept methods have been developed to integrate nucleic acid extraction, purification, elution, amplification, and detection into affordable and easy-to-use tests.¹⁵ The whole analysis process can be completed by “origami” folding of the paper device, without the need for fixed power supplies.

With advances in microfluidic technology, LAMP-based assays have now been implemented on paper-based tests, enabling the detection of a variety of pathogens.¹⁶ For example, the paper-origami technique was used to enable DNA extraction, amplification, and array-based fluorescence detection of malaria species,¹⁷ and it was applied for the multiplexed detection of bovine infectious reproductive diseases in rural India.¹⁸ Such devices have been further developed to perform multiplexed lateral flow test (LFT) detection for malaria field testing in Uganda, Africa,^{19,20} demonstrating that paper-based devices can be readily integrated into field-ready modules and provide excellent detection capabilities comparable to laboratory-based instruments. In addition, RT-LAMP has been integrated with LFT for the clinical detection of RNA viruses, including hepatitis C.²¹

Most recently, the combination of paper microfluidic devices and WBE has provided new insights into SARS-CoV-2 detection using a paper device based on clustered regularly interspaced short palindromic repeats (CRISPR)-Cas12a and RT-LAMP for the detection of N, E, and S genes in the laboratory using wastewater spiked with genomic sequences and/or viruses.²² The concept of the potential use of

SARS-CoV-2 testing in sewage has also been described, to trace the source of COVID-19 and to determine the presence of SARS-CoV-2 carriers in a community.²³

Early warning sensing systems based upon WBE and paper tests are now expected to act as *sentinels* to highlight the emergence of new disease outbreaks and to help monitor prevalence and control future outbreaks.⁷ In order to demonstrate the power of community sensing, at the point of need, we now describe a device using RT-LAMP assay for rapid detection of SARS-CoV-2 and influenza A/B in community wastewater. The method comprised a simple hand-held syringe-based sample preparation system to enrich pathogen samples from wastewater, enabling the extraction, purification, amplification, and *in situ* detection.

The paper microfluidic device was first optimized using a model surrogate virus porcine reproductive and respiratory syndrome virus (PRRSV) in order to define channel geometries and sensing area sizes. To assess the sensitivity and specificity of the test for pathogen detection, transcribed RNA at different concentrations was spiked into water to mimic real samples in a laboratory setting. Subsequently, to optimize the platform in real samples, we tested 20 wastewater samples collected by Anglian Water (using a laboratory-based RT-qPCR assay as a gold standard reference).

Finally, we implemented the test for the local analysis of wastewater within a government-designated quarantine hotel near London Heathrow Airport, showing its potential for rapid and on-site wastewater surveillance. The technique not only shows the value of the methodology for localized community testing in well-resourced environments, but it also illustrates the potential application for the paper microfluidic device for pandemic preparedness in the resource-limited settings, where there is an increasing need to develop rapid, portable, low-cost, and user-friendly platforms to detect pathogens.

RESULTS AND DISCUSSION

Optimization of a paper microfluidic device for virus detection

During the COVID-19 outbreak between 2020 and 2023, the spread of the pandemic was monitored and mitigated by designing detection platforms to distinguish viruses in wastewater.²⁴ In general, these systems only provided infection trends, with the effectiveness of the methods being limited by samples having to be transported to centralized laboratories for analysis (and data generated was retrospective and not “real time”). We chose to overcome these limitations by developing a paper-based LFT for wastewater epidemiology, measuring nucleic acid biomarkers *in situ*, at the point of sampling.

We first optimized the paper microfluidic device based upon a general testing framework (Figure 1). Initial device design was studied using PRRSV as a surrogate virus (Figure S1). The cycle threshold (C_T) is the number of cycles at which the intensity of the fluorescent signal exceeds the threshold signal for positivity.²⁵ The optimal pore size, channel width, and channel length of the paper device were concluded to be 4.0 mm, 1.5 mm, and 2.5 mm, respectively. We subsequently confirmed the validity of the developed device for virus detection in wastewater, using spiked genomic samples, samples from a wastewater company, and finally in a community setting within four quarantine hotels during pandemic.

Real-time and paper-based LAMP assays for virus detection

In the current COVID-19 pandemic and similar outbreaks, RT-LAMP has shown potential as a technology for epidemiological monitoring of virus infections in

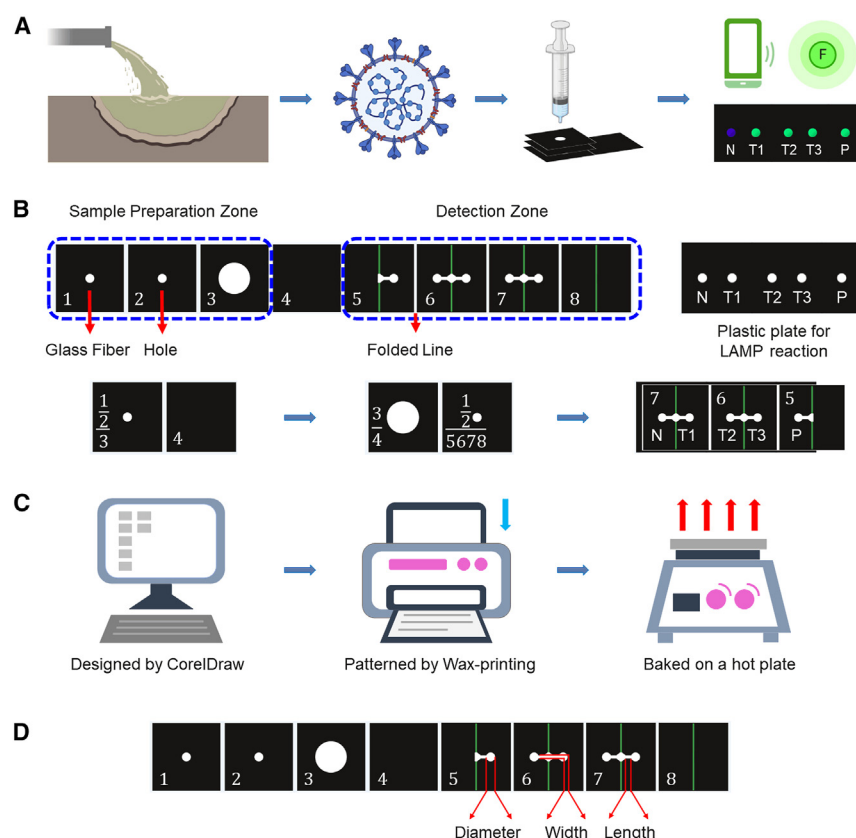


Figure 1. Design and characterization of paper microfluidic device with high-throughput detection

(A) Schematic showing the sample-to-answer diagnostic process for virus detection in wastewater using a paper microfluidic device.

(B) Design of the paper microfluidic device. The device contains three components, comprising a filter paper-based microfluidic device, a plastic device sealed with a single-sided optical film, and one glass-fiber circular disc (4 mm in diameter). The paper unit for each panel shows a footprint of 3 cm × 3 cm. N represents the internal negative control, T1, T2 and T3 represent the targets, and P represents the internal positive control.

(C) Fabrication of the paper microfluidic device. The device was designed in CorelDraw, then patterned by wax-printing, and finally baked on a hot plate.

(D) Illustration of the paper microfluidic device, indicating the specific dimensions: pore size, channel width, and channel length.

community settings. The nucleic acids released into wastewater from SARS-CoV-2 virus are RNA sequences/fragments, and the strategy for their amplification and detection involved performing reverse-transcription (RT) production of cDNA, prior to an isothermal amplification using LAMP. The LAMP assay was based upon using 4–6 different primers to identify 6–8 different regions on the target sequence, which were amplified by DNA polymerase at a constant temperature. In addition, the use of loop primers was used to reduce amplification time.²⁶ The LAMP assay can amplify a small amount of target DNA, generating a large amount of amplified DNA in less than 1 h.

ORF1ab, S, and N genes are three major regions in the SARS-CoV-2 genome, encoding the viral replicase, spike protein, and nucleocapsid protein, respectively. They play significant roles in the viral replication and infection process and thus were selected as target genes for SARS-CoV-2 detection. For LAMP primer selection,

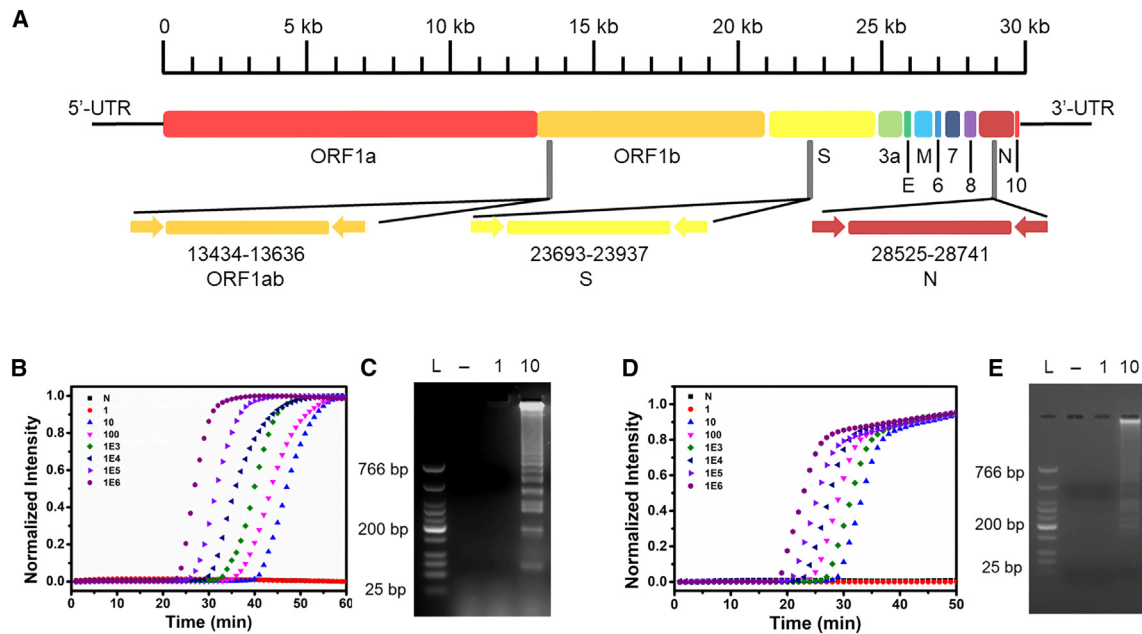


Figure 2. Design and optimization of LAMP assay for viral RNA detection including SARS-CoV-2 and influenza
(A) Map showing LAMP primers for SARS-CoV-2 detection. Visualization of primers on the SARS-CoV-2 genome (GenBank: MN908947).
(B) RT-LAMP assay for N gene.
(C) Gel electrophoresis for N LAMP products (L, ladder; –, ddH₂O; 1, 1 copy μL^{-1} ; 10, 10 copies μL^{-1}).
(D) RT-LAMP assay for influenza B genotype.
(E) Gel electrophoresis for influenza B LAMP products (L, ladder; –, ddH₂O; 1, 1 copy μL^{-1} ; 10, 10 copies μL^{-1}).

the target gene sequences were first obtained, and then the primer design software PrimerExplorer was used to design primers for specific sites. With the BLAST sequence comparison tool, primers were checked for similarity with other gene sequences to avoid non-specific amplification.

As stated, our assays were designed based upon three well-established target biomarker genes (GenBank: MN908947), the locations of which are shown in Figure 2A. The ORF1ab LAMP primers were directed against a 203-nt-long sequence from nucleotide positions 13434 to 13636 of the ORF1ab gene. The S LAMP primers spanned a 245-nt-long sequence from nucleotide positions 23693 to 23937 of the S gene, while N LAMP primers covered a 217-nt-long sequence from nucleotide positions 28525 to 28741 of the N gene.

Sensitivity and specificity of real-time RT-qPCR assays on viruses

The 10-fold serial dilutions (10^6 copies μL^{-1} to 1 copy μL^{-1}) of *in vitro* transcribed viral RNA was prepared to evaluate the sensitivity of real-time RT-qPCR assay. In real-time qPCR assay, the reaction products of each cycle provide a fluorescent signal from the probe binding to the target sequence. As illustrated in Figure S2, the ORF1ab RT-qPCR assay demonstrated a linear range of 10^{-10} to 10^6 copies μL^{-1} with a limit of detection (LOD) at 10 copies μL^{-1} . The RT-qPCR amplicons were then analyzed on 3% agarose gel for the presence of DNA bands. The detection limits for ORF1ab, S, and N RNA were around 10 copies μL^{-1} . For real-time RT-qPCR assays of three influenza genotypes and associated agarose gel electrophoresis, similar trends were observed (Figure S3). Detection limits were as low as 10 copies μL^{-1} for H1N1, H7N9, and influenza B genotypes. The standard curve of the RT-qPCR assay that we generated was also used as a reference standard for the corresponding RT-LAMP assay.

Sensitivity and specificity of real-time RT-LAMP assays

The concentration ratio of calcein to Mn^{2+} was optimized by real-time LAMP assay. The optimal concentration ratio of calcein to Mn^{2+} was 1:20 (Figure S4), a ratio that was used in all subsequent LAMP experiments.

The 10-fold serial dilutions (10^6 copies μL^{-1} to 1 copy μL^{-1}) of *in vitro* transcribed SARS-CoV-2 viral RNA were prepared to evaluate the sensitivity of real-time RT-LAMP assay. The real-time RT-LAMP assay for the detection of viruses was performed on a Quant Studio 3 Real-Time PCR System. The LAMP assays showed detection limits of 10 copies μL^{-1} for the detection of ORF1ab, S, and N RNA (Figure S5). The LAMP amplicons were observed under UV light and then analyzed on 3% agarose gel for the presence of ladder pattern. The detection limits for H1N1, H7N9, and influenza B genotypes were also 10 copies μL^{-1} (Figure S6).

As also illustrated in Figure S5, the time required for positive detection with the ORF1ab primer set ranged from 25 min for 10^6 copies μL^{-1} to 35 min for 10 copies μL^{-1} , while for the S primer set, the time required for positive detection ranged from 20 min for 10^6 copies μL^{-1} to 30 min for 10 copies μL^{-1} . For the N primer set, the time required for positive detection ranged from 35 min for 10^6 copies μL^{-1} to 60 min for 10 copies μL^{-1} . The RT-LAMP assay based on these primers indicated an LOD of 10 copies μL^{-1} within 60 min, so the reaction time for RT-LAMP assay was set as 60 min. In contrast, the RT-qPCR assay achieved an LOD of 10 copies μL^{-1} within 2 h. The detection limit obtained using a visual readout was consistent with the LOD obtained through analyzing real-time RT-LAMP fluorescence amplification curves, indicating that the calcein-based RT-LAMP assay could be applied simply in subsequent paper-based tests.

The LOD for SARS-CoV-2 (10 copies μL^{-1}) was similar to or lower than a nanoparticle-based lateral flow biosensor integrated with multiplexed RT-LAMP assay for ORF1ab detection (12 copies μL^{-1}),²⁷ an RT-LAMP assay based on the Loopamp RNA amplification kit for S detection (100 copies μL^{-1}),²⁸ and a colorimetric RT-LAMP assay based on the WarmStart Colorimetric RT-LAMP Master Mix for N detection (100 copies μL^{-1}).²⁹ In another study, a modified two-step RT-LAMP assay for SARS-CoV-2 detection,³⁰ adding an RT incubation step (55°C, 10 min) prior to the RT-LAMP reaction, was reported to achieve a detection limit as low as 1 copy μL^{-1} . Thermal lysis (95°C, 10 min) inactivated nucleases in the crude samples, while RT (55°C, 10 min) improved annealing between RNA template and primers to allow efficient complementary strand synthesis. Our assay thus showed very good LODs compared with published reports, with a higher detection limit when compared to RT droplet digital PCR assay.³¹

Field testing of wastewater with paper microfluidic device

A general scheme for our paper-based RT-LAMP assay is shown in Figure 3, with the wastewater sample being first filtered twice using syringe filters (0.45 μm and 0.025 μm) to remove debris and impurities while retaining virus particles on the membrane filter. As illustrated in Figure 3B schematically, fluorescent labels indicate different target-specific reactions. The results are the representative images for single (target 1), duplex (target 1–2), and triplex (target 1–3) target detection, together with internal negative control and internal positive control to determine the effectiveness of the test. In the paper-based RT-LAMP assay, green color indicates a positive test result, while no green color indicates a negative test result.

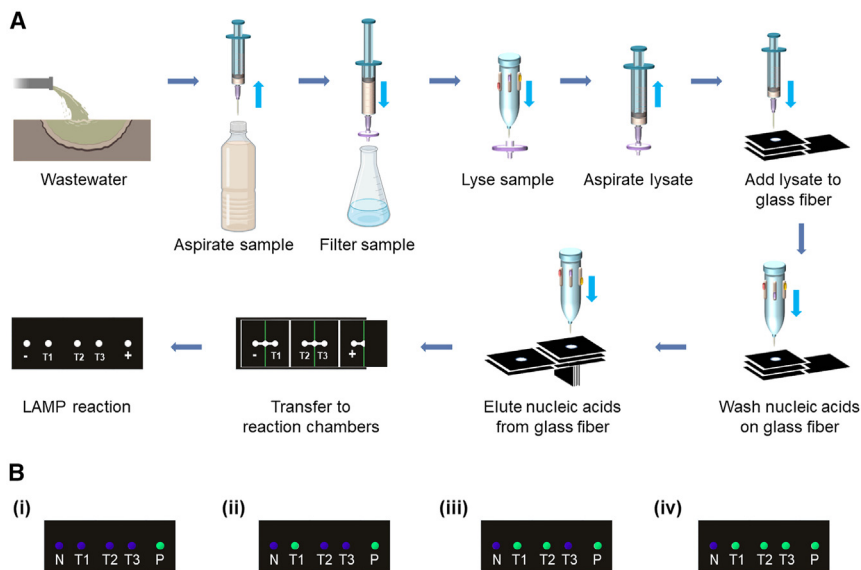


Figure 3. The on-site enrichment workflow and paper microfluidic device for multiplexed detection of SARS-CoV-2

(A) Figure showing a schematic illustrating the analytical workflow. The water sample was first filtered twice, followed by sample lysis, expanding upon the schematic shown in Figure 1A. The water sample was first filtered through syringe filters (0.45 μm and 0.025 μm), followed by the addition of lysis buffer. The lysate was introduced onto the glass fiber punched into the printed panel. Any debris, including cell membrane residues, were rinsed off using 70% ethanol (25 μL). The paper device was then folded for elution, and nucleic acids were released from the glass fiber using ddH₂O (30 μL), reaching the printed hydrophilic channels. The circular discs defined as the detection zone by punching were placed in the sample chambers, and LAMP reagents (20 μL) were subsequently added to each sample chamber. An optical adhesive film was later used to cover the plastic plate to avoid reagent evaporation during isothermal amplification in a digital dry bath. The results were read using a hand-held UV torch or semi-quantitatively using a mobile phone camera. (B) The labels indicate different target-specific reactions. N, internal negative control; P, internal positive control; T1, target 1; T2, target 2; T3, target 3. The results are the representative images for single (target 1), duplex (target 1–2), and triplex (target 1–3) target detection, together with negative control and positive control to determine the effectiveness of the test (green color for positive tests and no green color for negative tests).

To analyze the feasibility of the paper-based platform to detect SARS-CoV-2 in real wastewater samples, we performed RT-LAMP assay on 20 wastewater samples collected by a UK-based water company, Anglian Water, and then we compared the detection results with those from standard RT-qPCR assay. Reference analysis involved using both a kit-based RNA extraction and RT-qPCR assay. The different relationships between C_t and date are presented in Figures 4A–4C, respectively. New cases by specimen date (between October 20 and November 30, 2020) are also shown in three figures. Lower C_t values are indicative of more new cases according to the specimen date. Table S1 shows testing of a cohort of wastewater samples in local wastewater treatment plants (WWTPs) in 2020, including RT-qPCR testing and paper-based RT-LAMP assay of ORF1ab gene, S gene, and N gene of SARS-CoV-2 collected from local WWTPs.

The RT-qPCR results demonstrated that among the 20 wastewater samples, 6 samples tested positive for ORF1ab gene, 17 samples tested positive for S gene, and 16 samples tested positive for N gene. It can be inferred that the detection efficiency of ORF1ab gene in wastewater was lower than S and N genes, which is consistent with a previous report.³² Current detection of SARS-CoV-2 RNA primarily relies on the

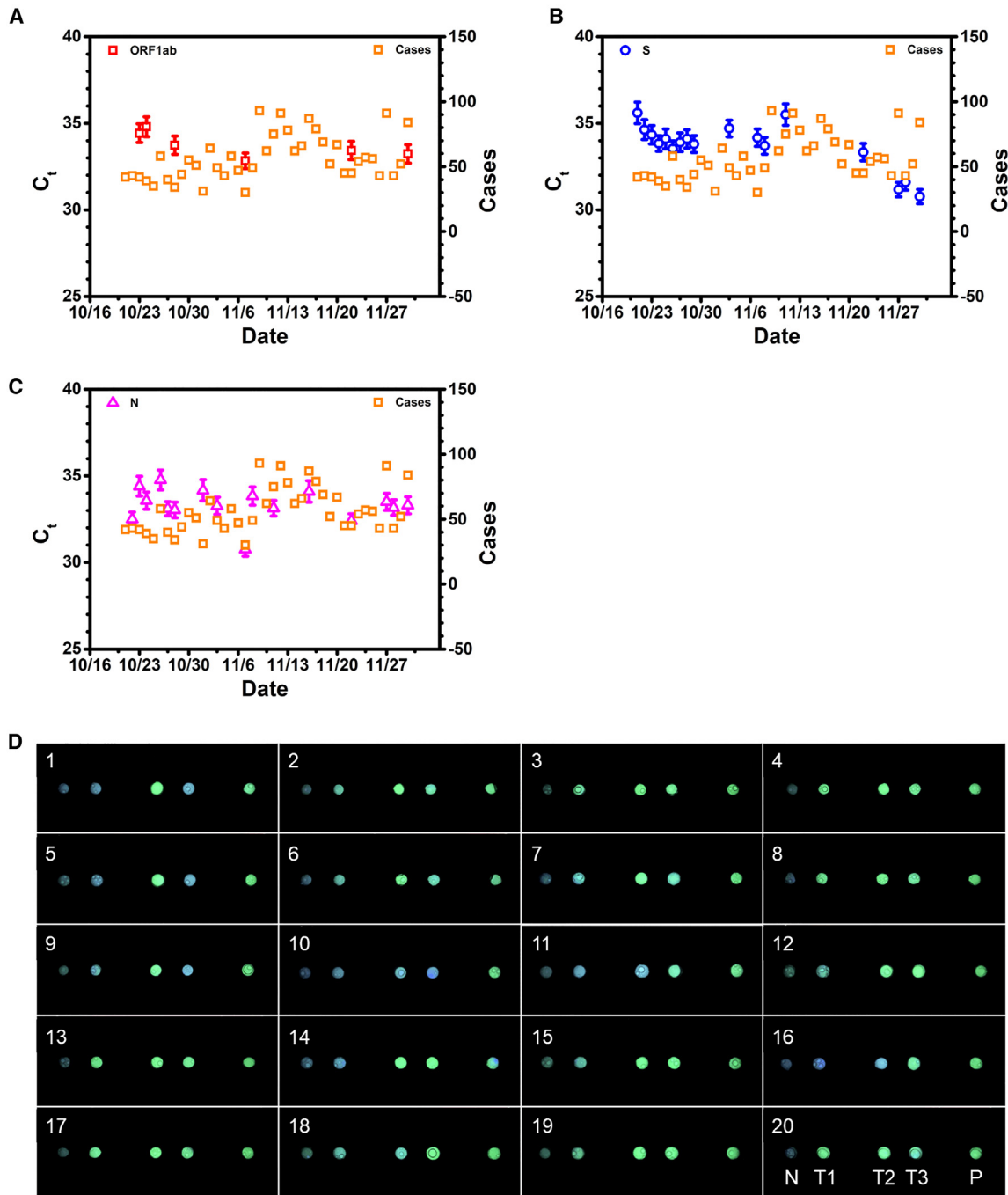


Figure 4. Testing of a cohort of wastewater samples in local WWTPs in 2020

(A–C) RT-PCR testing of (A) ORF1ab gene, (B) S gene, and (C) N gene of SARS-CoV-2 collected from local WWTPs in November 2020 at Anglian Water Treatment plant with the confirmed positive data disclosed from local city council within the catchment of the populations. Error bars indicate standard deviations, and each experiment was replicated three times.

(D) Images of the paper-based RT-LAMP assay were illuminated with a hand-held UV torch and captured by mobile phone camera for SARS-CoV-2 detection in wastewater. N, ddH₂O; T1, ORF1ab; T2, S; T3, N; P, mtDNA.

determination of ORF1ab and N genes. N protein provides a highly immunogenic and conserved amino acid sequence, indicating that N gene is a suitable target for monitoring SARS-CoV-2. In detail, it has also been found that N gene was the

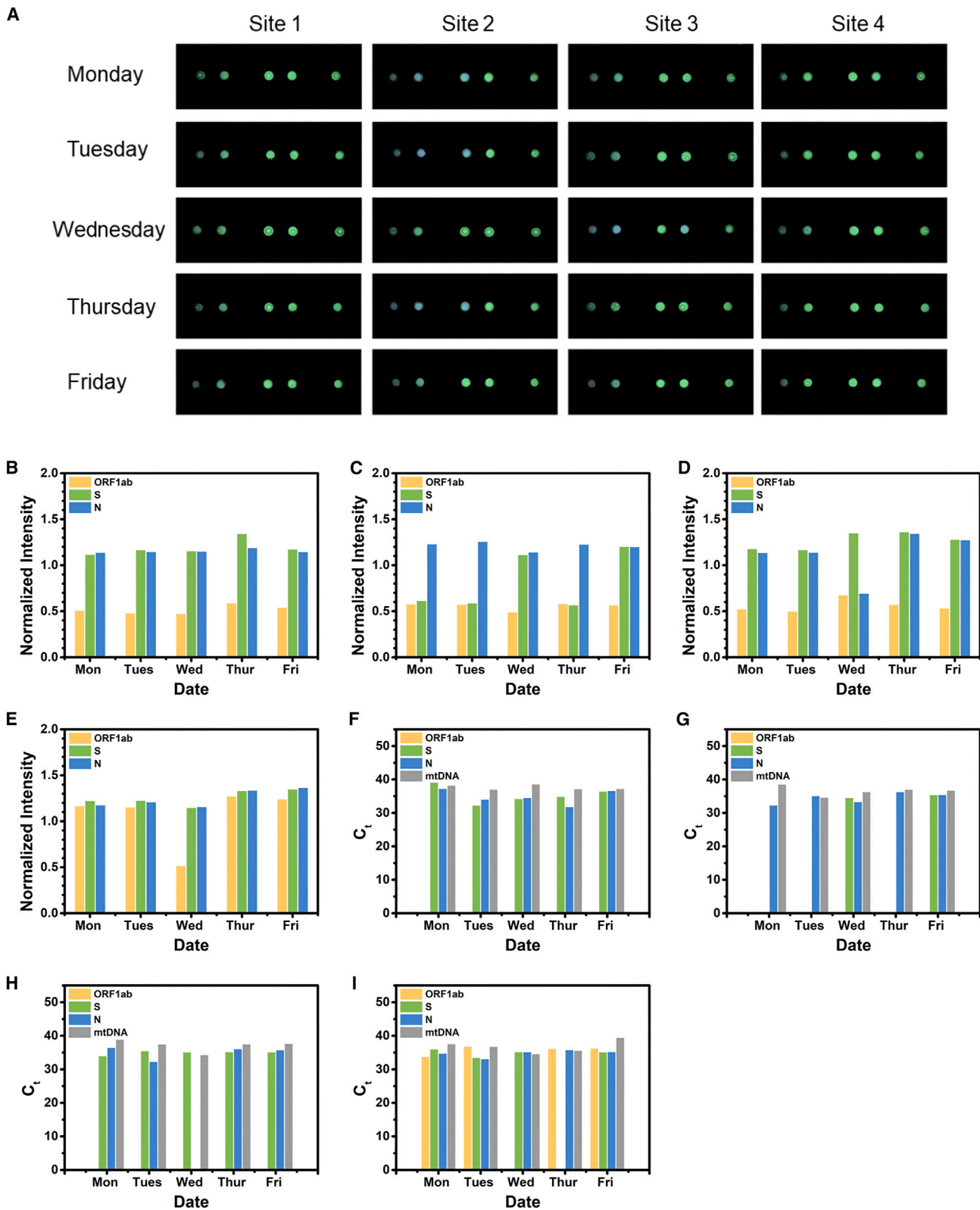


Figure 5. Hotel testing of wastewater samples within the paper microfluidic device

(A) Paper-based RT-LAMP assay for SARS-CoV-2 detection during field testing at four quarantine hotels near Heathrow Airport in London. The wastewater samples were sampled daily from these four locations near the airport from Monday through Friday (5 days). The images of the paper-based RT-LAMP assays were illuminated using a hand-held UV torch and were captured by a mobile phone camera for quantification. N, ddH₂O; T1, ORF1ab; T2, S; T3, N; P, mtDNA.

(B–E) Normalized fluorescence intensity of paper-based RT-LAMP assay for (B) site 1, (C) site 2, (D) site 3, and (E) site 4.

(F–I) Laboratory-based RT-qPCR detection of SARS-CoV-2 performed on wastewater samples collected daily from 4 sites near the airport from Monday to Friday, including (F) site 1, (G) site 2, (H) site 3, and (I) site 4.

predominant positive fragment in COVID-19 patients, while ORF1ab gene presented a lower percentage of positive patient infections.³³ According to the observation of the dynamic changes of ORF1ab and N genes in COVID-19 patients, the positive duration of N gene was longer than that of ORF1ab gene.

The above-mentioned wastewater samples were also tested by the paper-based RT-LAMP assay. The results of the paper-based platform for 20 wastewater samples, analyzed in triplicate, are presented in Figure 4D. For ORF1ab gene, 7 sample chambers showed obvious fluorescence, while no fluorescence was observed in the remaining 13 samples. For S and N genes, 16 sample chambers showed obvious fluorescence, while no fluorescence was observed in the remaining 4 samples. The results obtained by our platform were in good agreement with the laboratory-based RT-qPCR assay (analysis of variance [ANOVA], $p > 0.05$). Sixteen of the 17 RT-qPCR S-positive samples and all the RT-qPCR N-positive samples ($n = 16$) were detected as positive by our platform. Of the 17 RT-qPCR S-positive samples, only one sample was detected as negative. In addition, one sample tested by the platform showed a false-positive result for ORF1ab gene, indicating possible carry-over contamination of the LAMP amplicons. Contamination may in future be reduced by adding uracil-DNA glycosylase and deoxyuridine triphosphate to the RT-LAMP reaction.³⁴ Overall, we demonstrated that the paper-based platform could be used for sensitive and specific detection of SARS-CoV-2 in wastewater.

To demonstrate the feasibility of the platform for field applications, we used the paper microfluidic device for on-site SARS-CoV-2 detection at a hotel near Heathrow Airport from July 12 to 17, 2021, with samples collected using industry-standard autosamplers. The wastewater samples were collected daily from 4 locations near the airport from Monday to Friday, over 5 days. Figure S7 presents the associated equipment, consumables, and reagents, including wax-printed paper device, syringe filter, heat blocker, and UV torch. A syringe-based system was designed to enrich pathogen samples from wastewater, allowing for the subsequent extraction, purification, amplification, and detection (Figure S8). The enrichment step does not require any electricity or laboratory-based equipment, thus offering the potential for field testing in resource-limited areas. Nucleic acids were extracted from pathogens, amplified by RT-LAMP, and detected using fluorescent signal. Results were read by illumination using a hand-held UV torch and/or collected by a mobile phone camera (to enable quantification).

The platform successfully detected ORF1ab, S, and N genes of SARS-CoV-2 in wastewater samples. RT-qPCR detection of SARS-CoV-2 was also performed on wastewater samples collected from four sites from Monday to Friday (Figure 5), including site 1 (Figure 5F), site 2 (Figure 5G), site 3 (Figure 5H), and site 4 (Figure 5I). The RT-LAMP results were largely consistent with the RT-qPCR results (ANOVA, $p > 0.05$). Table S2 shows hotel testing of wastewater samples within the paper microfluidic device, including RT-qPCR testing and paper-based RT-LAMP assay of

ORF1ab, S, and N genes during field testing at four quarantine hotels in London. N gene presented the highest detection rate in wastewater samples, followed by S and ORF1ab genes.

Molecular diagnosis of SARS-CoV-2 currently relies on the detection of specific and conserved ORF1ab, S, N, RdRp, and E genes. Despite the high specificity of ORF1ab gene, clinical applications suggest that ORF1ab gene is less sensitive than other genes.³⁵ ORF1ab gene shows less sensitivity than N and S genes due to a shorter sequence, which is likely to be degraded in the wastewater system. On the other hand, nucleotide variations are more frequent in ORF1ab gene than in N gene.³⁶ Nucleotide variations in the published ORF1ab-related qPCR primer sequences are also more frequent than in N gene. With the emergence of new SARS-CoV-2 variants, the detection of N gene in wastewater was more stable than ORF1ab gene because N gene has fewer nucleotide variations. Higher abundance of sub-genomic N gene messenger RNAs also resulted in N gene being more sensitive than ORF1ab and S genes.³⁷

Wastewater samples collected from site 4 showed the most positive results for ORF1ab gene, possibly due to the highest number of hotel guests or the highest infection rate among guests. In contrast, site 2 wastewater samples presented the least number of positive tests for S gene, with one important possibility being that this may be due to dilution of viral load or a decrease in infection cases during testing. Two results from the paper microfluidic device were inconsistent with RT-qPCR results, which may be due to a potential carry-over of inhibitors from the syringe-based system and paper-based extraction in comparison to kit-base RNA extraction.

Common inhibitors in wastewater include organics (e.g., formic acid) and ions. To improve the tolerance of a paper microfluidic device to these inhibitors, wastewater samples have been pre-treated by removing the dust with filtration. In our protocol, the target gene has been extracted with exhaustive washing and elution to remove the potential inhibitors (including twice washing with ethanol to remove the organics effectively). The purified nucleic acids were then transferred to mix with LAMP reagents for reaction. If community testing strategies are to be developed in the future, great care must be taken around the consideration of how samples are collected, consistently within a given site or between sites.

During the SARS-CoV-2 pandemic, British authorities used hotels near Heathrow Airport to isolate travelers, including those who had tested positive for COVID-19. This activity was consistent with the World Health Organization's advice, which identified WBE as a potential tool for COVID-19 monitoring.

The sensitivity and specificity of our paper microfluidic device for pathogen detection are comparable to the current centralized laboratory methods. Whereas centralized laboratories use sophisticated equipment and multiple steps to analyze complex samples, potentially providing higher accuracy, our device excels at delivering rapid results, with on-site testing (typically within 90 min) from sample collection to display. This protocol avoids the time-consuming transfer of samples from remote areas to centralized laboratories, which often takes from several hours to a full day. In addition, our device is cost-effective compared to the expense of large instruments (and their associated operation and maintenance) in centralized laboratories. With equivalent diagnostic capabilities of centralized laboratories, but with the additional advantages of portability, affordability, rapid analysis, ease of use,

and environmental friendliness, we demonstrate that our device holds great promise for on-site testing, especially in low-resource settings, enabling public health officials to make timely interventions.

The potential for cross-reactivity between SARS-CoV-2 and influenza viruses is an important consideration for multiplexed assays. These viruses are similar to each other, both having spike proteins that play a crucial role in viral entry into host cells (which are thus the primary targets for diagnostic testing). To mitigate the possibility of cross-reactivity, LAMP primers are designed to target the region of lowest homology between SARS-CoV-2 and influenza virus proteins, thereby minimizing cross-reactivity. We also note that the device was designed in such a manner, including the optimization of the spacing between channels, so as to minimize the cross-contaminants of each target.

Positive and negative controls are included in the test device, so as to indicate if the assay is valid. Additional targets, specific to individual viruses, may be included, such as the nucleocapsid protein of SARS-CoV-2 or the matrix protein of influenza virus, as required. Extensive cross-reactivity assays can be conducted using a panel of influenza virus and SARS-CoV-2 strains. It is also critical to perform continuous optimization to maintain appropriate analytical performance.

With the aim of making the paper microfluidic device more user-friendly and accessible in low-resource settings, we propose several future strategies to improve the device. Firstly, all the reagents will be lyophilized on paper, eliminating the need for refrigeration, thereby improving stability and portability of the reagents, which will facilitate the operational step. One can easily use a mobile camera (a mobile phone has been very common even at resource-limiting areas) to record the video, documenting the dynamic changes of colorimetric detection, integrating a wireless communication module to enable remote result transmission for data analysis, with a potential to connect with a mobile phone for fast data interpretation. With these improvements, the platform can provide more reliable and convenient testing, appropriate for low-resource settings, including those within rural communities.

Future research will now also focus on integrating concentration, extraction, detection, and analysis modules into a single device to standardize and automate wastewater monitoring to identify pathogens. When using WBE to assess virus transmission in a community, it is currently challenging to correlate the number of viruses with the actual number of cases. The decay rate of SARS-CoV-2 in wastewater, the presence of PCR inhibitors, and wastewater flow rates are all factors that may affect the efficacy of the assay results.³⁸ Further effort is thus also required to understand and improve sampling, sample handling, target detection, and result analysis. Finally, we note the removal of COVID-19 restrictions and the previous lack of human-to-human contacts have led to a significant increase in influenza cases, prompting the CDC to recommend a multiplexed SARS-CoV-2 and influenza test.³⁹

In conclusion, the proposed paper microfluidic device as a sentinel sensor for rapid detection of pathogens in water enables sample-to-answer analysis in <1.5 h. Although the paper-based platform only provides a semi-quantitative result, it serves as a rapid, cost-effective, and easy-to-use tool for community wastewater analysis, reducing the burden of pandemic surveillance on centralized and/or diagnostic departments. Results can be read using a hand-held UV torch or collected by a mobile phone camera, allowing for data to be collated and stored.

Our platform was able to detect viruses at <20 copies RNA per reaction sample. Compared to gold standard PCR assay, our portable platform provides similar or higher specificity and sensitivity for pathogen detection at a much lower cost, providing a rapid, high-resolution dataset. Importantly, the methods could be readily deployed in low-resource community settings, for example, low- and middle-income countries to support the establishment of early warning systems. Such a technology has the potential for rapid surveillance of pathogens in water and latrines with the prospect of more effective community control of endemic diseases.

EXPERIMENTAL PROCEDURES

Preparation of template DNA and RNA

Six sets of LAMP primers were selected for ORF1ab,⁴⁰ S,⁴¹ and N⁴² genes on SARS-CoV-2, as well as H1N1,⁴³ H7N9,⁴⁴ and influenza B⁴⁵ genotypes. For initial optimization experiments, we designed 6 DNA fragments containing target sequences and primers with a T7 RNA polymerase promoter (Figure 2). Details of these LAMP primers and PCR primers for *in vitro* transcription are provided in Table S3 and Table S4. Intact viral RNA was also extracted from samples using a RNeasy Mini Kit.

For *in vitro* transcribed RNA template preparation, the target sequence was first PCR-amplified with PCR primers containing the T7 RNA polymerase promoter. The PCR reaction was performed in a total volume of 20 μ L, comprising 10 μ L PCR Master Mix, 0.8 μ L PCR primers (0.4 μ M), 1 μ L EvaGreen, 7.2 μ L ddH₂O, and 1 μ L DNA fragment (5 ng μ L⁻¹). The PCR conditions consisted of an initial denaturation step at 95°C for 7 min, followed by 40 cycles, with denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 1 min 30 s. The PCR amplicons were analyzed on 1.5% agarose gel stained with SYBR Safe DNA gel stain and then extracted and purified from agarose gel using a Zymoclean Gel DNA Recovery Kit to form an *in vitro* transcription template. Thereafter, the purified PCR amplicons were incubated at 37°C for 2 h to synthesize artificial RNA using a HiScribe T7 High Yield RNA Synthesis Kit. Next, the synthesized RNA was treated with DNase I to remove the DNA template and purified using a Monarch RNA Cleanup Kit. Finally, the purified RNA was stored at -80°C for further use.

For quantification of the template nucleic acid, the copy number of each template nucleic acid was determined by their molecular weight. A Qubit 4 Fluorometer with Qubit 1X dsDNA HS Assay Kit or Qubit RNA HS Assay Kit (Thermo Fisher Scientific, UK) was used to measure the concentration of the template DNA or RNA, and the DNA/RNA Copy Number Calculator (<http://endmemo.com/bio/dnacopynum.php>) was then used to calculate the copy number. The target sequence detected by LAMP was first amplified by real-time PCR with LAMP outer primers to confirm the accuracy of target detection. Subsequently, 10-fold serial dilutions of template nucleic acid were performed for sensitivity testing and to produce a standard curve.

RT-qPCR assay

The real-time RT-qPCR assay was performed on a Quant Studio 3 Real-Time PCR System. The qPCR primer sets for ORF1ab,⁴⁶ S,⁴⁷ and N⁴⁸ genes, as well as H1N1,⁴⁸ H7N9,⁴⁹ and influenza B⁵⁰ genotypes, porcine reproductive and respiratory syndrome virus (PRRSV),⁵¹ and mitochondrial DNA (mtDNA)⁵² are detailed in Table S5. mtDNA was chosen as the target of the internal positive control, while ddH₂O was added to the internal negative control. All RT-qPCR reactions were performed in triplicate in a total volume of 20 μ L, including 6 μ L TaqMan Fast Virus 1-Step RT-PCR Master Mix, 1.25 μ L PCR primers, 7.75 μ L ddH₂O, and 5 μ L sample.

The RT-qPCR conditions consisted of an RT step at 50°C for 5 min, an initial denaturation step at 95°C for 20 s, followed by 50 cycles of denaturation at 95°C for 15 s, annealing at 55°C for 30 s, and an extension at 72°C for 30 s.

RT-LAMP assay

The real-time RT-LAMP assay was performed using a thermocycler. The LAMP primer sets for ORF1ab,⁴⁰ S,⁴¹ N,⁴² H1N1,⁴³ H7N9,⁴⁴ FluB,⁴⁵ PRRSV,⁵³ and mtDNA⁵⁴ are detailed in Table S3. Our assays were designed based upon the well-established target biomarker genes, including H1N1 HA gene (GenBank: FJ984401.1), H7N9 HA gene (GenBank: MW298209.1), FluB NS1 gene (GenBank: MZ951664.1), PRRSV N gene (GenBank: M96262), and mtDNA gene (GenBank: AY972053). All RT-LAMP reactions were performed in triplicate in a total volume of 20 μ L, including 10 μ L LAMP Master Mix, 3 μ L LAMP primers (0.1 μ M F3/B3, 0.8 μ M FIP/BIP, and 0.6 μ M LF/LB), 2 μ L calcein (25 μ M), 1 μ L MnCl₂ (500 μ M), 1 μ L Gsp 2.0, 0.2 μ L reverse transcriptase, 0.8 μ L ddH₂O, and 2 μ L sample. RT-LAMP assay was performed at a constant reaction temperature of 63°C for 60 min. Threshold time ($T_{\downarrow}$) is defined as the time corresponding to 10% of the maximum fluorescence intensity and is a function of target concentration. The T_t of the real-time LAMP assay is analogous to the T_c of the real-time PCR assay.

In addition, the RT-LAMP assay was performed in the digital dry bath. Calcein together with Mn²⁺ was used as a fluorescent metal indicator.⁵⁵ As the RT-LAMP reaction proceeds, Mn²⁺ and newly formed P₂O₇⁴⁻ combined to form a Mn₂P₂O₇ precipitate, which leads to calcein with fluorescent signal. The negative reaction solution remained light yellow when observed under sunlight, while the positive reaction solution appeared light green. Results can also be read by a hand-held UV torch or digitally collected using a mobile phone camera. The negative reaction solution showed no fluorescence when illuminated using a UV torch, while the positive reaction solution showed obvious fluorescence.

Design, characterization, and optimization of paper microfluidic device with high-throughput detection

The paper device was first characterized with geometries optimized using a model pathogen, PRRSV, as a surrogate; see Figure S1. Subsequently, the design of the paper microfluidic device for the detection of three targets was defined and is shown in Figure 1A. The device contains three components, including a filter paper-based microfluidic device with wax-printed microfluidic channels, a plastic device sealed with a single-sided optical film, and one glass-fiber circular disc (4 mm in diameter) for absorbing nucleic acids from the sample. For the five reaction chambers in the plastic device, N represents the internal negative control, T1, T2, and T3 represent the targets, and P represents the internal positive control.

The unfolded paper device comprised a sample preparation zone and a detection zone and was mounted to a plastic plate to show the detection results. The paper unit for each panel had a footprint of 3 cm $\times$ 3 cm (3 $\times$ 24 cm when unfolded). The paper device was fabricated on Whatman Grade 1 Qualitative Filter Paper in three steps (Figure 1C). The structure of the device was designed in the software CorelDRAW, with factors such as operation convenience being taken into consideration during the design process. Microfluidic channels were created on the paper device by printing hydrophobic wax on the filter paper, defining the flow pathways. Finally, the patterned filter paper was baked on a hot plate at 130°C for 5 min to melt the printed wax into the porous structure of the filter paper (preventing lateral flow by capillarity). The wax-penetrated filter paper was cut into individual devices for subsequent LAMP experiments.

Paper microfluidic device for pathogen detection

This work demonstrated a paper-based platform for multiplexed detection of SARS-CoV-2 and influenza viruses in wastewater. [Figure S9](#) illustrates the workflow of the platform for virus detection in wastewater, including wastewater concentration, paper-based nucleic acid extraction, device-based LAMP reaction, and signal readout. In this case, a volume of wastewater was first filtered through syringe filters (0.45 μm and 0.025 μm), followed by the addition of lysis buffer (RNeasy Mini Kit), after which the filtrate was incubated at room temperature for 10 min.⁵⁶

The paper device, described above, was assembled with a glass fiber within a 4-mm hole punched into the printed panel. The lysate was introduced onto the glass fiber, where nucleic acids were adsorbed (the excess liquid was absorbed by the hydrophilic disc present on the third panel of the device). Any debris, including cell membrane residues, were rinsed off using 25 μL of washing buffer (70% ethanol). The paper device was then folded for elution, accompanied by close contact between glass fiber and hydrophilic channels on the device. After adding 30 μL of elution buffer (ddH₂O) to the glass fiber, the nucleic acids were released from the glass fiber and transferred to the reaction chamber for LAMP assay. Circular spots were punched and transferred to the reaction chambers of the plastic plate. LAMP reagents were subsequently added to the reaction chambers. An optical adhesive film was later used to cover the plastic plate to avoid reagent evaporation during isothermal amplification in a digital dry bath. Finally, the results were read using a hand-held UV torch and the fluorescence signal was captured using a mobile phone camera.

The whole sample-to-answer process took <1.5 h and only required basic experimental equipment, such as a digital bath and consumables (flasks and syringes). In contrast, the workflow for the reference methods, performed on wastewater samples, and involving ultrafiltration concentration, kit-based RNA extraction and RT-qPCR assay took $\sim$ 4 h. With the modification of operating procedures, process workflow, and experimental reagents (e.g., change the pore size of the syringe filter or LAMP primers), this paper-based platform can be readily extended to detect other pathogens in various environmental water samples, establishing new avenues for low-cost, rapid, and user-friendly multiplexed detection of pathogens. It is noteworthy that community-based testing uses wastewater samples from a population and that results are not subject to biases that may be introduced when sampling from individuals.

SARS-CoV-2 detection in hotels

Autosamplers were used for wastewater sampling in the four quarantine hotels around London Heathrow Airport in the summer of 2021. Sampling personnel were clothed in standard personal protective equipment used for wastewater sampling, such as protective clothing, safety glasses, face masks, and gloves. An autosampler was used to take a water sample into the instrument, which was then fed into a sampling bottle via the instrument's dispensing system. The temperature of water samples was kept constant using a thermostat system. The autosampler was also equipped with a transmitter to receive external signals such as those from a flow meter.

The autosampler was programmed to collect composite wastewater samples (50 mL of wastewater sample every 30 min) over a 24-h period, i.e., the samples that flowed through the sampling point over a day. An aliquot of each sample collected by the autosampler was transferred to a 1-L plastic bottle, stored in a thermostatic bag filled with ice packs for sample transport, and then transferred to the hotel basement for sample analysis, shown in [Figure S10](#). The wastewater samples were collected daily from four locations near the London Heathrow Airport from Monday to Friday (July 12–17, 2021).

The process for on-site SARS-CoV-2 detection is presented in the section paper microfluidic device for pathogen detection. A syringe-based membrane filtration system was designed to enrich pathogen samples from wastewater, allowing for the subsequent extraction, purification, amplification, and detection. The analytical reagents and paper-based kits were transported to the testing hotel from Cranfield University.

Data analysis

One-way ANOVA is one testing method in statistics, which can be used to test whether there are significant differences between multiple sets of data. One-way ANOVA is commonly used to test the impact of a single dependent variable on an independent variable. When the calculated p value >0.05 , the observed inter-group differences arise randomly, and there is no statistically significant difference between the two groups, while when the p value <0.05 , there is a statistically significant difference between the two samples.

RESOURCE AVAILABILITY

Lead contact

Further information should be directed to and will be fulfilled by the lead contact, Zhugen Yang (email: Zhugen.Yang@cranfield.ac.uk).

Materials availability

This study did not generate new unique materials.

Data and code availability

Data collected are described in the main text and supplemental information, and all the data generated in this study will be made available upon reasonable request to the [lead contact](#). Data supporting this study are available from CORD. Access to the data is subject to approval and a data-sharing agreement due to Cranfield University undertaking a spin-out for scaling up this technology.

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AUTHOR CONTRIBUTIONS

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript. Y.P. and Z.Y. conceived the study and designed experiments. Y.P. performed experiments with the supervision of Z.Y. and J.C. Y.P. and Z.Y. wrote the manuscript with input from all co-authors. Z.Y. supervised and acquired funding for the study. J.C. and B.W. both provided support for technical development and edited the manuscript. All authors took part in interpretation of results and preparation of materials.

DECLARATION OF INTERESTS

Z.Y. at Cranfield University filed a UK GB2598551A in 2020 (granted in 2024: GB2598551B) and filed a PCT patent PCT/GB2021/052136 in 2021 based on the research findings of this article.

SUPPLEMENTAL INFORMATION

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