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Portable Biosensor Combining CRISPR/Cas12a and Loop-Mediated Isothermal Amplification for Antibiotic Resistance Gene *ermB* in Wastewater

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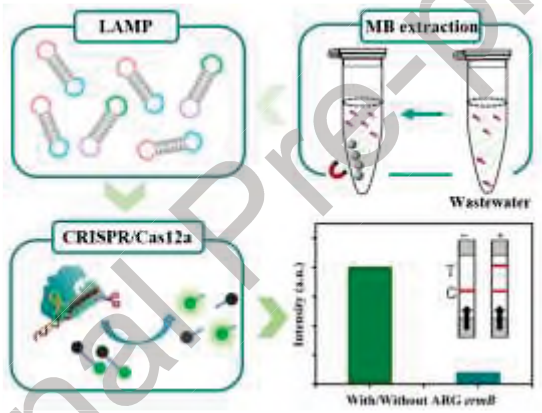
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Graphical abstract

Abstract:

Wastewater is among the main sources of antibiotic resistance genes (ARGs) in the environment, but effective methods to quickly assess ARGs on-site in wastewater are lacking. Here, using the typical ARG *ermB* as the target, we report a portable biosensor combining CRISPR/Cas12a and loop-mediated isothermal amplification (LAMP) for the detection of ARGs. Six primers of LAMP and the crRNA of CRISPR/Cas12a were first designed to be preamplification with LAMP and lead Cas12a to recognize the ermB via base pairing. Due to the trans-cleavage activity of CRISPR/Cas12a after amplicon recognition, ssDNA probes modified with reporter molecules were used to implement a visual assay with lateral flow test strips and fluorescence. After a simple nucleic acid extraction with magnetic beads, the constructed biosensor possesses

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excellent sensitivity and selectivity as low as 2.75×10^3 copies/μL using fluorescence and later flow strips in wastewater. We further evaluated the community-wide prevalence of ermB in wastewater influent and found high mass loads of ermB during different months. This user-friendly and low-cost biosensor is applicable for rapid on-site ARG detection, providing a potential point-of-use method for rapid assessments of ARG abundance in wastewater from large city areas with many wastewater treatment plants and in resource-limited rural areas.

Keywords:

Antibiotic resistance gene, CRISPR/Cas12a, loop-mediated isothermal amplification, biosensor, wastewater

Environmental Implication:

This paper reports a novel portable biosensor for antibiotic resistance genes (ARGs) based on CRISPR/Cas12a and loop-mediated isothermal amplification for fluorescence and lateral flow assays in wastewater, providing a potential point-of-use tool for wastewater surveillance.

1. Introduction

Antibiotic resistance genes (ARGs) are widespread contaminants that pose a serious threat to the environment and public health [1-3]. In particular, ARGs in wastewater are an important source of ARGs in the environment [4]. Therefore, monitoring and assessing ARG abundance in wastewater is important for regulating the entry of ARGs into the environment. To date, the main methods for detecting ARGs include gene sequencing and fluorescence quantitative PCR (qPCR). The former can obtain specific sequence information and relative gene abundance, while the latter exhibits high sensitivity and specificity and can obtain quantitative data. However, the methods are suitable only for small-scale laboratory testing due to complicated pretreatment processes, high testing costs, and reliance on precision equipment and professionals [5]. In addition, in both methods, samples must be collected and transported to a laboratory for nucleic acid testing. Wastewater containing ARGs is essentially a biological sample with poor stability; thus, information obtained from the samples is often inaccurate due to changes during sample transportation and storage. As a consequence, novel

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analytical tools are needed to facilitate rapid and on-site analyses of wastewater by personnel with less specialized training and with minimal sample processing.

Biosensors hold promise to overcome the drawbacks of conventional analytical methods. A biosensor is a small device with a biological receptor that generates a signal (electrochemical, optical, etc.) in the presence of a target [6]. Biosensors have great potential for rapid and on-site detection of targets in environmental samples due to the ease of miniaturization and the ability to measure complex matrices with minimal sample preparation [7]. In the past few decades, biosensors have been developed to measure widely varied targets, such as heavy metals [8], organic pollutants [9], biomarkers [10] and even microorganisms [11, 12] , in different environmental matrices. For wastewater analysis and surveillance, Yang et al. proposed the use of sewage sensors as rapid and inexpensive alternatives to classical analytical methods for the detection of sewage biomarkers [7]. Recently, the use of sewage biosensors for the rapid detection of a range of targets, such as rapid monitoring of community-wide drug consumption and pathogens for early warning of infectious disease outbreaks, has been reported [13, 14]. Building upon these reported studies and cases, we believe that the ability of biosensors to rapidly monitor wastewater reduces testing costs and minimizes uncertainty caused by the low stability of certain targets.

Recently, as a biological recognition element for biosensing, clustered regularly interspaced short palindromic repeats (CRISPR), an emerging technology, have attracted increasing attention because they nonspecifically cleave nontarget nucleic acids after the specific recognition of the target sequence, ultimately allowing sequence detection [15, 16]. Due to its strong specificity, excellent signal transduction capabilities, and good compatibility, this approach has already been confirmed to be an attractive method for the monitoring of microorganisms, such as bacteria and viruses [17], based on nucleic acids present in the environment at low concentrations [18]. Currently, there are three main categories of nucleic acid detection based on CRISPR/Cas systems, i.e., Cas9, Cas12/Cas14, and Cas13 [14, 15, 19, 20].

CRISPR/Cas12a is among the most widely used systems of the CRISPR toolbox in the field of biosensing detection due to its strong specificity, excellent signal

transduction capabilities, and good compatibility [21]. The detection process is based on the specific recognition of CRISPR/Cas 12 protein and sequences of guide RNA (gRNA) , followed by visual methods, such as fluorescence [15, 22]. In particular, researchers have identified a unique attribute of Cas12 that is suitable for another application beyond genome editing: nonspecific cleavage of single-stranded DNA (ssDNA). This ability has been transformed into a powerful tool for DNA diagnosis by detecting small amounts of DNA from sources such as bacteria and cancer cells [23]. Currently, the CRISPR/Cas12a-based detection method is widely applied in clinical testing, medical diagnosis and food safety [24, 25]. For example, nucleic acid detection based on the Cas12a protein can detect common viral diseases in apple trees within several hours [26]. By combining Cas12a with specific crRNA and fluorescent probes, CRISPR technology can be used to detect meat adulteration [27]. The CRISPR/Cas system also plays a role in combatting the pandemic of COVID-19. A CRISPR/Cas12a-based SARS-CoV-2 detection method was developed, providing a new option for epidemiological detection and screening [28]. Therefore, compared to conventional diagnostic methods (such as PCR or hybridization), CRISPR/Cas12 systems are very suitable for developing on-site rapid biosensing strategies because the same or higher performance is achieved with a more affordable cost [29]; as a result, CRISPR/Cas12-based biosensors will be very advantageous for on-site testing in limited-resource areas.

Although significant progress has been achieved in clinical testing, directly applying the available CRISPR/Cas12a test methods for environmental analysis remains difficult. This is because when the CRISPR/Cas12a-based biosensor is used alone to detect nucleic acids, the sensitivity is very low, which poses a great challenge for the detection of low concentrations in complicated environmental media [30, 31]. Thus, improving the sensitivity of CRISPR/Cas12a-based biosensors is essential for their environmental application. Two main methods are used to improve the sensitivity of CRISPR/Cas12a-based detection. The first method is to optimize the CRISPR/Cas12a system, such as by introducing coupled RNA and modifying the probe [32]. The more common method is to combine the CRISPR/Cas detection system with the isothermal amplification of nucleic acid, such as loop-mediated isothermal

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amplification (LAMP) and recombinase polymerase amplification, first amplifying the nucleic acid concentration and then using the CRISPR/Cas system for detection [33, 34]. Isothermal amplification of nucleic acids could overcome the inherent difficulties encountered during PCR, i.e., performing target amplification without thermal cycling, and offer potential for point-of-use applications [35]. For example, Zhang et al. combined CRISPR/Cas12a with LAMP to construct a fully automated CRISPR-LAMP platform, which achieved highly sensitive and specific detection of SARS-CoV-2 variants [36]. Therefore, there is hope that CRISPR/Cas 12a with nucleic acid isothermal amplification could quantitatively test samples at low concentrations in environmental media, such as wastewater.

Among nucleic acid isothermal amplification methods, loop-mediated isothermal amplification (LAMP) has become a widely used isothermal amplification method and has emerged as a promising technique for the amplification of a wide spectrum of targets due to its simple instrumentation and excellent specificity [37]. LAMP uses six specific primers in the first stage and four primers during the amplification, elongation, and recycling steps in the process of primer annealing followed by autocycling strand displacement at elevated and constant temperatures [38]. LAMP is widely applied in the fields of clinical diagnosis, veterinary detection, food safety detection, and environmental analysis [37]. For wastewater analysis, Yang et al. reported a rapid a rapid "sample-to-answer" platform based on LAMP that can be used for the quantitative monitoring of human-specific mitochondrial DNA used in wastewater-based epidemiology [10]. Cao et al fabricated a paper device based on LAMP for measuring SARS-CoV-2 in wastewater, and the entire measurement can be completed in approximately 1 h [14]. Thus, LAMP provides a simple, rapid, specific, and cost-effective amplification method and can be applied in a point-of-use test. This result suggested that the combination of CRISPR/Cas12a and LAMP shows great potential for application in the assessment of ARG abundance, and simple and inexpensive on-site measurement of ARGs in wastewater could be achieved.

Here, we coupled LAMP with CRISPR/Cas12a to establish a portable visual platform for detecting the macrolide resistance gene *ermB*, which is read out via fluorescence

and lateral flow assays to achieve rapid and sensitive visual on-site detection. First, primer sets targeting the ermB gene and crRNA targeting the amplification region were designed, and the LAMP reaction system and CRISPR/Cas12a detection system of ermB were constructed. Then, the feasibility of the two methods was verified by the experimental group and the control group, and on this basis, the LAMP-CRISPR/Cas12a system was constructed. Next, we evaluated and aligned the detection effects of LAMP and LAMP-CRISPR/Cas12a, discussed the effect of Cas12a on the reaction, and constructed two visualization methods based on fluorescence and lateral flow devices. Finally, to further verify the reliability, this method was applied to detect ermB in wastewater influent in Guiyang. The results are expected to provide a reference for assessing the potential risk of community ARGs and the potential ecological hazard of ARGs in wastewater.

2. Experimental section

2.1. Materials and reagents

Bst 2.0 WarmStart DNA polymerase and NEBuffer 2.1 were purchased from New England Biolabs (USA). EvaGreen was purchased from Biotium (USA), SYBR Gold nucleic acid gel stain and TAE buffer were purchased from Thermo Fisher Scientific (USA), 2X GO DNA polymerase was purchased from Shanghai Sangon (China), the Zymoclean gel DNA Recovery kit was purchased from Zymoo Research (USA), and and a colorimetric-based lateral flow device was purchased from TwistDx (UK). All experiments were independently repeated three times, and the error bars represent triplicate measurements.

2.2. Design and optimization of LAMP primers and crRNA

Specific LAMP primers targeting the gene ermB (GenBank: JN607214.1) were designed online using PrimerExplorer V5 (https://primerexplorer.jp/e/). The LAMP primer set secondary structure and primer dimers were evaluated using Oligo 7 software. The ssDNA reporters used for fluorescence detection and lateral flow detection were synthesized by Shanghai Sangon (Shanghai, China). The crRNA was synthesized by

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Biolifesci (Guangzhou, China). The sequences of the LAMP primer and crRNA are listed in Table S1.

2.3. Construction of the LAMP assay

The LAMP assay was prepared using the Bst 2.0 WarmStart DNA Polymerase Assay Kit (New England Biolabs, USA) following the manufacturer's protocol. The LAMP reaction mixture had a final volume of 20 μL, containing 0.2 μM F3/B3, 0.4 μM LF/LB, 1.6 μM FIP/BIP, 8 mM Mg^{2+}, 1.4 mM dNTP Mix, 0.32 U/μL Bst 2.0 WarmStart DNA polymerase and 2 μL of DNA template, 1 μL of 20× EvaGreen, 2 μL of 10× isothermal amplification buffer, and nuclease-free water. The fluorescence was measured on an Applied Biosystems 7500 at 65°C for 40 min. The amplicons were analysed in 1.5% (w/v) agarose gels and visualized in a Gel Imaging System (Tanon, China).

2.4. Construction of the CRISPR/Cas12a-based biosensor

LAMP coupling CRISPR/Cas12a assays were performed using LAMP for the preamplification of DNA fragments and LbCas12a for the trans-cleavage of ssDNA reporters. First, the LAMP reaction was performed as described in Part 2.3 without EvaGreen. Then, 18 μL of a premix containing 100 nM Cas12a, 125 nM gRNA, and 500 nM fluorescent probe, 1× NEBuffer 2.1, was preincubated at 37°C for 10 min. Finally, 2 μL of amplicons from the LAMP preamplification step was added to the CRISPR/Cas12a reaction, and the reaction was incubated at 37°C for 60 min, as described in a previous publication[33]. The fluorescence intensity was collected using an Applied Biosystems 7500, and the signals were normalized.

2.5. Visual detection of the CRISPR/Cas12a-based biosensor

The integrated CRISPR/Cas12a-based biosensor provides visual detection of fluorescence using blue light (480 nm) or a lateral flow strip. For fluorescence-based detection, the procedure was the same as that described in Part 2.4. After the cleavage reaction of the fluorescent probe (Table S1) was completed, the result was visualized under blue light (λ=480 nm). In the presence of the targeted *ARG ermB*, the solution showed green fluorescence visible to the naked eye, while the solution was colourless without the target. The fluorescence of the solution was quantified using ImageJ.

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For lateral-flow detection, all components and procedures were consistent with fluorescence-based detection except that a lateral flow probe (Table S1) was used instead of a fluorescent probe. Then, 10 μL of the trans-cleavage product was added to 100 μL of HybriDetect Assay buffer. The lateral flow strips were placed into the solution and incubated for 2 min in an upright position. The lateral flow strips were then removed and imaged by a smartphone. ImageJ was used to extract grey data from the test lines, and the signal was normalized.

2.6. Wastewater collection and detection of ARGs in wastewater

Wastewater influent samples were collected from the Jinyang Wastewater Treatment Plant (106.6439°E, 26.5948°N), which is located in the urban area of Guiyang City, China. The treatment capacity of the WWTP is 100,000 tons/day, and the service population is 520,000. Sampling was conducted once a month from June to November 2021. Each sample was collected for 5 consecutive days over a period of 24 h using autosamplers (FC-9624) purchased from GRASP Science & Technology Co., Ltd. (Beijing, China). Once collected, wastewater samples were immediately transported to the laboratory on ice for further processing. Heavy precipitation days were avoided for sampling.

To evaluate the feasibility of detecting ARGs in real samples, the constructed CRISPR/Cas12a-based biosensor was used to measure concentrations in wastewater samples, and these samples were also examined using conventional instrumentation, namely, real-time PCR (qPCR). The detailed analytical process is as follows:

CRISPR/Cas12a-based biosensor: Wastewater samples were filtered with a 50 mL syringe (0.22 pore size membrane). Lysis buffer (1.20 g mL^{-1} of GuSCN, 0.1 M Trishydrochloride, 0.04 M EDTA, adjusted with NaOH to pH 8.0, 26 mg mL^{-1} Triton X-100) was added, followed by magnetic extraction and further analysis using the constructed CRISPR/Cas12a-based biosensor. The detection procedure was the same as in Part 2.4 and Part 2.5.

qPCR analysis: For each wastewater influent sample, the samples (100 mL) were filtered through 0.22 μm filters to trap microorganisms. Then, genomic DNA was extracted according to the instructions of the Water DNA Purification Kit purchased

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from Fangzhou Biotechnology Co., Ltd (Guangzhou, China), and a set of replicates was prepared for each sample. After extraction, DNA solutions from the same sample were mixed to reduce errors caused by single sample collection and DNA extraction. The DNA concentration was quantified by a Qubit 4.0 Fluorometer (Thermo Fisher Scientific, USA) and stored at -20°C.

The LAMP primers F3 and B3 were used for PCR analysis of the *ARG ermB*. The PCR amplification was performed in a volume of 20 μL, including 10 μL of 2X GO DNA polymerase, 0.4 μM forward primer, 0.4 μM reverse primer, and 2 μL DNA template, brought up to volume with ddH_2O.The qPCR was performed using an Applied Biosystems 7500 (Thermo Fisher Scientific, USA). The testing process was performed as follows: 95°C for 5 min, 40 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s, and a final extension at 72°C for 5 min. Finally, the target fragments were extracted using a Zymoclean DNA Gel Recovery Kit, and the DNA concentration was quantified by Qubite 4.0. This value was used for subsequent standard samples. The quantitative standard curve of the target gene was prepared by 10-fold serial dilution of PCR products.

Mass load estimation of ARGs: The mass load of ARGs in wastewater at a specific wastewater treatment plant (WWTP) was estimated by using the following equation:

$$Mass\ load = \mathrm{C} \times \mathrm{F} \times \left(\frac{100}{100 + \mathrm{stability}}\right)$$

Mass load is the ARG abundance in the wastewater treatment plant (copies/day), C is the total amount of ARGs in 1 L of wastewater influent and suspended particulate matter (copies/L), F is the daily average flow rate of wastewater inflow in 24 hours (L/day), and stability refers to the change in stability of ARGs in wastewater (%). The stability of ARG contaminants was considered to be 100% due to their environmental persistence.

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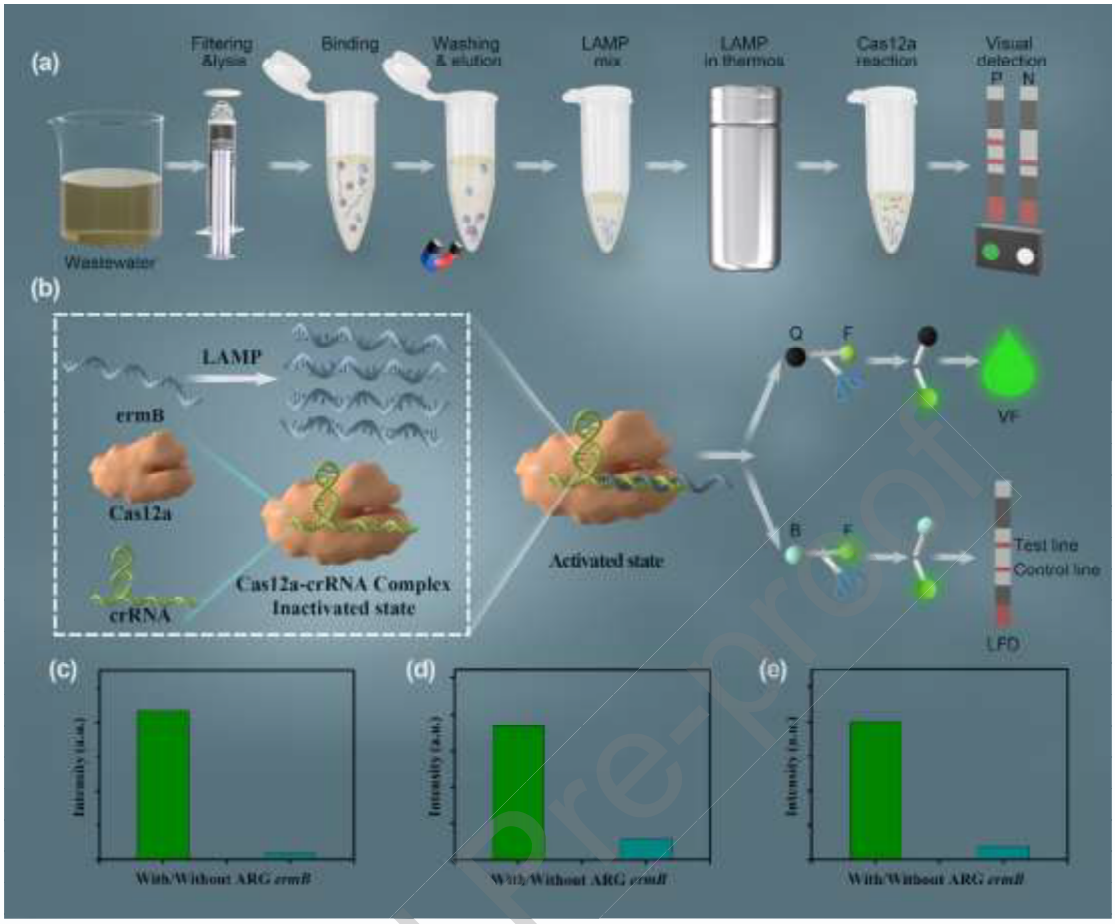


Figure 1. Detection process, mechanism and feasibility of the portable biosensor for ARG ermB in wastewater. (a) Detection process and (b) mechanism of the integrated portable CRISPR/Cas12a-based biosensor for ARG *ermB* (VF, visual fluorescence; LFD, lateral flow device); (c) feasibility of the independent CRISPR/Cas12a-based analytical method for ARG *ermB* (10 nM); (d) feasibility of LAMP for ARG *ermB* (50 pM); (e) feasibility of the integrated portable CRISPR/Cas12a-based biosensor for ARG *ermB*.

3. Results and discussion

3.1. Detection mechanism of the constructed biosensor

In this work, we constructed a CRISPR/Cas12a-based biosensor to monitor antibiotic resistance genes (ARGs) in wastewater on site. As shown in Figure 1a, the detection process of the biosensor involves the following steps: pretreatment with filtering and lysis, magnetic bead extraction, preamplification with LAMP, recognition and transduction with the CRISPR/Cas12a system, and detection with a fluorescence and lateral flow device. The pretreatment of collected wastewater, including microorganism concentration and DNA extraction with magnetic beads, was performed as described in

our previous publication [10]. After wastewater pretreatment, this assay performs isothermal amplification using LAMP for preamplification in a vacuum flask filled with hot water (using a thermometer to check temperature when required), followed by Cas12 recognition and detection of the predefined ARG ermB sequence, after which cleavage of a reporter molecule confirms detection of the ARG. We first designed LAMP primers targeting the ermB gene (Table S1). These primers amplify regions that overlap the highly conserved region sequence of the ermB gene obtained from the database of National Center for Biotechnology Information (Figure S1) but are modified to meet design requirements for LAMP.

Next, we designed Cas12 crRNA to specifically recognize and detect the ermB gene preamplified by LAMP (Figure 1b). With a segment of crRNA as a guide RNA, Cas12a can identify ermB fragments that complement the crRNA recognition segment (Figure S2). After the targeted sequence is recognized, its trans-cleavage activity for nonspecific cleavage of the ssDNA probe is activated. This trans-cleavage activity of Cas12a enables the activated Cas12a to cleave any nearby ssDNA, although these ssDNAs do not have any complementary sequences with crRNA. Using this feature, as shown in Table S1, we designed ssDNA probes modified with fluorescence and corresponding quenching agents (5'-6-FAM-TTATT-BHQ1-3') and ssDNA probes modified with fluorescence and biotin molecules (5'-6-FAM-TTATTATT-Biotin-3') for visual detection in fluorescence and lateral flow devices, respectively. In particular, the mechanism of the lateral flow device that visualizes the Cas12 detection reaction involves a FAM-biotin reporter molecule and lateral flow strips designed to capture labelled nucleic acids (Figure 1b) [15, 39]. Uncleaved reporter molecules are captured at the first detection line (control line), whereas indiscriminate Cas12 cleavage activity generates a signal at the second detection line (test line) (Figure S3).

3.2. Feasibility validation of the constructed biosensor

Using the detection mechanism mentioned above, to demonstrate the feasibility of this platform, we first finished the rapid wastewater sample pretreatment (within 10 min) by adding GuSCN lysate to a 0.22 μm filter membrane to extract sample DNA with the classic magnetic bead extraction method. We assessed the DNA recovery rate

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by testing different concentrations of DNA targets and recovery rates above 60% were obtained (see Table S2 and Figure S6).

Then, the extracted DNA template was preamplified by LAMP. We performed LAMP on a relatively conserved region in the gene ermB (Figure S1). LAMP assays were performed on a real-time fluorescence PCR instrument using different primer sets (Figure 1d and Figure S4) for the ermB gene. Among the three sets of primers, primer set L1 showed a good amplification effect in fluorescence analysis at a target concentration of 50 pM, and the signal could be observed within 20 min, while the negative control did not produce a fluorescence signal (Figure 1a and Figure S4), indicating that the LAMP assay could rapidly and specifically amplify the ermB gene and that many amplicons were produced when the target was present.

Next, crRNA targeting the amplified regions was designed to construct the CRISPR/Cas12a assay system (Figure S5). As shown in Figure 1c and Figure S5a, the fluorescence signal with ermB DNA was significantly higher than that without the target, indicating that the system can be specifically activated by ARG ermB and produce observable fluorescence.

Based on the construction of the two assays, we coupled LAMP and CRISPR/Cas12a recognition with a combination assay, and the LAMP product was added to the Cas12a-crRNA complex. As shown in Figure 1e and Figure S5b, the signal of the experimental group was significantly higher than that of the control group, indicating that the target gene was successfully detected by the constructed biosensor. This means that after the target DNA was specifically recognized under the guidance of crRNA, Cas12a trans-cleavage activity was activated, and the ssDNA probes were cleaved by nonspecific cleavage and generated observable signals.

For fluorescence-based detection, the ssDNA reporter was labelled with a fluorophore/quencher pair (FAM-BHQ). In the presence of Cas12a trans-cleavage, the ssDNA fluorescent probes were dissociated, releasing a quenching agent to restore the fluorescence signal. The results can be visually read by a fluorescent reading device or specific light source excitation (Figure 1b). Conversely, without target, the ssDNA probe was not cleaved due to the lack of activated Cas12a; thus, no fluorescent signal

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was generated. For visualization-based lateral-flow detection, a lateral flow probe was modified with FAM and biotin on each side. The colorimetric lateral flow strip usually consists of a sample pad with gold nanoparticles (AuNPs) modified with gt anti-FAM antibodies, a control line labelled with streptavidin to capture biotin, and a test line with anti-gt antibody labelled to capture gt antibody (Figure S3). Without a specific LAMP product, the ssDNA probe remains intact, and AuNPs can be immobilized to the control line due to the binding of biotin and streptavidin, resulting in red colour deposition. In contrast, the AuNPs complex is immobilized at the test line by the separation of AuNPs and biotin and binding of gt antibodies to anti-gt antibodies, forming a red band.

The experimental results mentioned above demonstrated that the CRISPR/Cas12a-based biosensor was successfully established. Next, we systematically investigated and optimized the single and combination methods to evaluate how the detection effect was affected by the combination.

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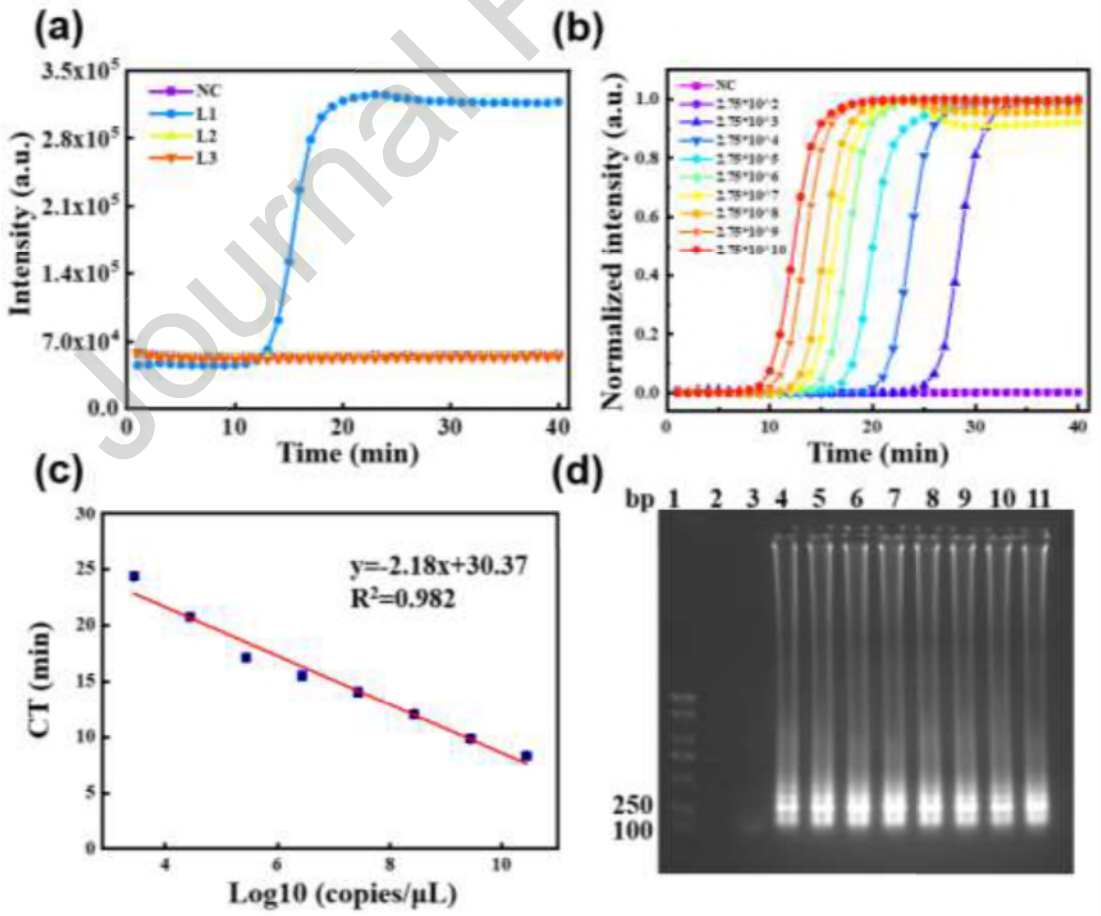


Figure 2. The LAMP system was established. (a) *ARG ermB* were amplified by three sets of LAMP primers (L1, L2 and L3), and enzyme-free sterile water was used as a negative control (NC). (b) Different concentrations of *ARG ermB* targets (10-fold gradient dilution) were detected by LAMP, and enzyme-free sterile water was used as a negative control. (c) Quantitative curves of different concentrations of *ARG ermB* and cycle threshold time, CT (defined as the time corresponding to 5% of the maximum fluorescence intensity). (d) Characterization of the LAMP product of *ARG ermB* by gel electrophoresis.

3.3. Construction of LAMP for ARG ermB

LAMP is essential for signal amplification in the crosstalk method and for initial identification of the target; in addition, the sensitivity and specificity of LAMP have an important impact on the crosstalk method. Therefore, to assess the sensitivity of LAMP when detecting the ermB gene, the target DNA fragment generated by PCR amplification was diluted in a 10-fold gradient. Enzyme-free sterile water was used instead of targeted DNA as a negative control for the blank experiment to determine whether the amplification results were reliable (checking for false-positive results). The fluorescence signal was detected by adding EvaGreen, and then the LOD of LAMP was determined. The LAMP results were further verified by agarose gel electrophoresis.

As shown in Figure 1d and Figure 2b, ARG ermB was successfully detected by fluorescence LAMP with an LOD of 2.75×10^3 copies/μL. There was a good linear relationship between DNA concentration and cycle threshold time (CT) (Figure 2c), with a linear range spanning 8 orders of magnitude up to 2.75×10^{10} copies/μL. As shown in Figure 2d, the bands in lanes 1~2 of agarose gel electrophoresis represent the DNA marker and the negative control, followed by LAMP amplification products of the DNA template from 2.75×10^2 to 2.75×10^{10} copies/μL, respectively. The results showed that the step stripe appeared from the 4-lane, indicating that ARG ermB could be detected at 2.75×10^3 copies/μL, showing a relative standard deviation of 3.2% with satisfactory stability. The results were consistent with the LOD results of the LAMP assay, indicating that the LOD of *ARG ermB* is 2.75×10^3 copies/μL.

Next, we further analyzed the fluorescence intensity of the endpoint. The sensitivity of the end-point was consistent with that of LAMP (2.75×10^3 copies/μL); however, there was no significant correlation between the target DNA concentration and the fluorescence intensity (Figure 2b) because saturation occurred when the reaction

finished and a "plateau" appeared. Therefore, the endpoint fluorescence intensity cannot be used for quantitative analysis. Considering that on-site detection is often difficult to conduct in real-time fluorescence tests, LAMP is more suitable for endpoint qualitative analysis.

However, as shown in Figure 2d, the faint bands in 3-lane indicate that primer dimers may have been generated during the amplification, as well as a corresponding fluorescent signal. Although fluorescent signals induced by dimers can be distinguished by fusion curves and electrophoresis under laboratory conditions, it is difficult to distinguish the dimer signals and specific amplified signals by the end-point detection method commonly used in field conditions. Thus, distinguishing specific amplified signals and false positive signals, such as dimers, under field conditions is of great significance for LAMP applications in the field.

3.4. Evaluation of the analytical performance of the portable biosensor

To further improve the specificity of the analytical methods, the CRISPR/Cas12a system was introduced into the assay to solve the potential false positive problem and improve sensitivity. First, eight 10-fold serial dilutions of *ARG ermB* DNA fragments were used to evaluate whether the introduction of CRISPR/Cas12a could enhance the sensitivity of LAMP. The real-time fluorescence results show that the LAMP-CRISPR/Cas12a system can detect *ARG ermB* as low as 2.75×10^2 copies/μL (Figure 3a and Figure 3b), which is an order of magnitude lower than LAMP, indicating that CRISPR/Cas12a can indeed improve the LOD. The extremely high trans-cleavage efficiency of Cas12a may confer a higher signal transduction efficiency on the coupling system [40, 41], thus converting extremely weak amplification signals into observable fluorescence signals. In addition, the negative control did not produce a signal, indicating that the LAMP coupling CRISPR/Cas12a system successfully prevents the potential false-positive problem. In conclusion, the introduction of CRISPR/Cas12a improved sensitivity and prevented false positives.

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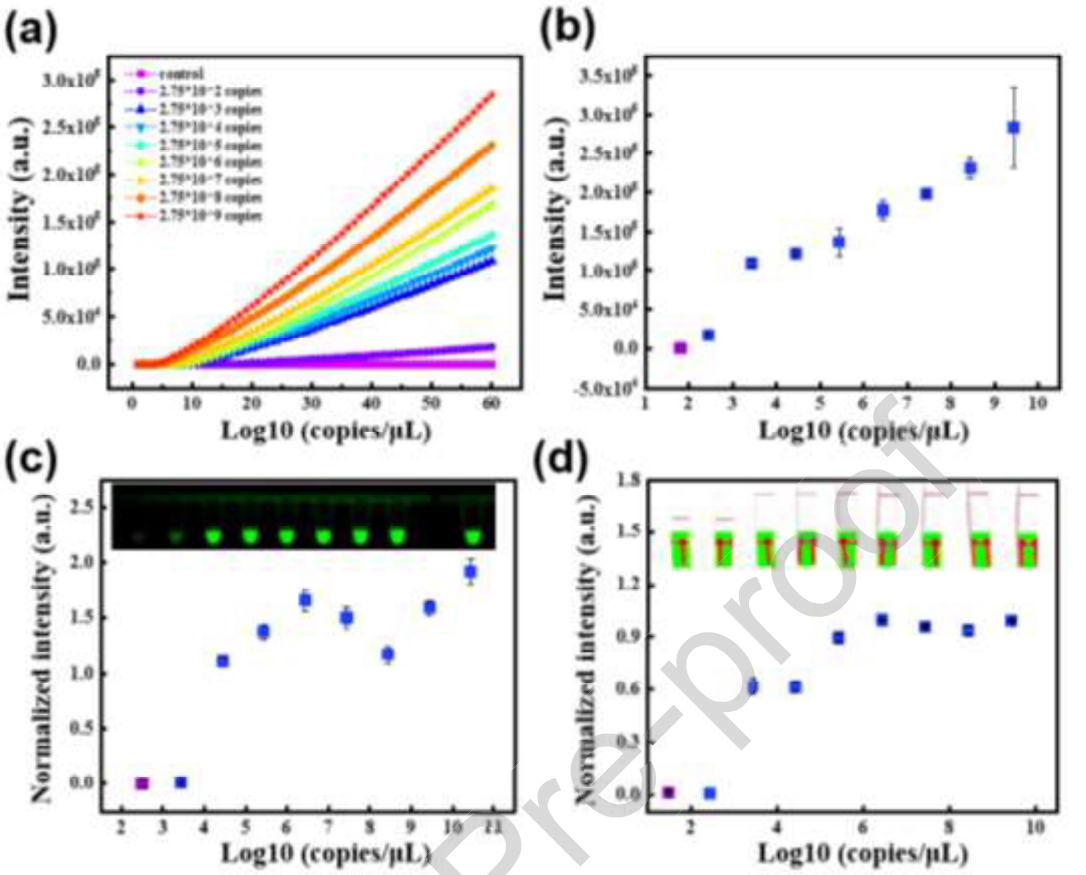


Figure 3. Evaluation of the integrated CRISPR/Cas12a-based biosensor. (a) Sensitivity evaluation of the ermB-LAMP-CRISPR/Cas12a platform by the real-time fluorescence method; (b) Fluorescence intensity of endpoints with different concentrations of the ermB gene detected by LAMP-CRISPR/Cas12a. (c) and (d) LAMP-CRISPR/Cas12 visual detection for fluorescence assay and lateral flow test strips, respectively. The fluorescence and lateral flow visual signals were quantified by ImageJ, and normalization was performed. Before the lateral flow test signal was normalized, the signal intensity was multiplied by -1. The concentration of the ARG ermB increased 10-fold from 2.75×10^{2} to 2.75×10^{9} copies/μL. Enzyme-free sterile water was used as a negative control.

Next, to address a variety of possible requirements for visualization in complicated field site conditions, a visual detection assay was performed using fluorescence and a lateral flow strip. The fluorescence assay was carried out as described previously except that the final signal was observed with blue light (480 nm) rather than a fluorescence readout device. As shown in Figure 3c, when the target concentration was higher than 2.75×10^{3} copies/μL under blue light, the solution showed visible green fluorescence; in contrast, the negative control showed no indication of fluorescence. Thus, the LOD of the constructed CRISPR/Cas12a system was 2.75×10^{3} copies/μL, consistent with the sensitivity of LAMP. It is possible that 2.75×10^{2} copies/μL was not detected because

the absolute fluorescence intensity of the system was low; thus, the fluorescence was difficult to distinguish by the naked eye. The colour data were extracted by ImageJ, and the results were in close agreement with those from the fluorescence observations.

Then, for lateral-flow strip visualization, a lateral flow probe was used instead of a fluorescent probe. As shown in Figure 3d, the test line produced darker streaks starting at 2.75×10^3 copies/μL. Although a faint streak similar to the negative control appeared on the test line at 2.75×10^2 copies/μL, this was treated as a background signal by grayscale analysis via ImageJ. The gray analysis results showed that the intensity of negative samples was 224±9.5 and that of positive samples was approximately 138~171. Therefore, the lateral flow device analysis was consistent with the sensitivity of the fluorescence assays. In addition, although visualization detection can be used for qualitative analysis of endpoints by determining the presence or absence of fluorescent or test lines, it cannot be used for absolute quantitative analysis due to the "plateau effect" of LAMP products during preamplification. As shown in Figure 3c and Figure 3d, it is difficult to distinguish the fluorescence intensity with the naked eye in fluorescence assays. In contrast, the lateral flow device showed that the red test line bands of 2.75×10^3 copies/μL and 2.75×10^4 copies/μL were significantly weaker those of the higher concentrations. The visualization sensitivity of the lateral flow device may be lower than that of the fluorescence assays [32].

To further evaluate the detection performance of the constructed biosensor for wastewater analysis, we attempted to simulate detection in 0.22 μm filtered wastewater samples. The ermB DNA fragment was added at an initial concentration of 2.75×10^9 copies/μL. The ermB DNA fragment was added to a 20 mL water sample for gradient dilution. Then, after DNA extraction and amplification, visual detection was performed. The results showed that ermB could be detected at as low as 2.75×10^3 copies/μL, which was comparable to the results of fluorescence assays (see Figure S7).

The constructed CRISPR/Cas12a visualization platform not only enables rapid nucleic acid extraction under on-site conditions but also enables qualitative and relative quantitative analysis. The detection range was from 2.75×10^3 copies/μL to 2.75×10^9 copies/μL, and the LOD was 2.75×10^3 copies/μL. To demonstrate the advantages and

disadvantages of the current method, we compared this developed analytical strategy with previously reported methods for the detection of ARGs. As shown in Table S3, although there are reports on ARGs in wastewater, they are mainly based on traditional analytical methods, such as qPCR, and there have been no reports on the point-of-use method for ARGs in wastewater. We also found that there are several point-of-use methods for detecting ARGs in other media, such as milk and tap water, and compared to these methods, the current developed method presents certain advantages. In addition, the cost for a single test using the developed method, including reagents and required consumables, is approximately 5.5 dollars, which is much lower than that of the existing nucleic acid detection equipment based on PCR technology, such as GeneXpert and FilmArray, for which the cost of a single test is 100-500 dollars [29]. Thus, the portable biosensor presented herein provides a potential low-cost method for detecting ARGs in wastewater.

3.5. Evaluation of ARG abundance at wastewater treatment plants

To verify the accuracy and reliability of the constructed CRISPR/Cas12a-based visual platform, we conducted on-site detection in raw wastewater. The samples were continuously collected from the inlet of a wastewater treatment plant in Guiyang City, China, from June to November 2021. The test results were compared with standard qPCR. Compared with qPCR (see Figure S8), fluorescence and lateral-flow strips correctly identified and distinguished raw wastewater samples from six consecutive months with a 100% detection rate. These results suggest that the constructed CRISPR/Cas12a visualization platform can be used as a valuable monitoring tool for the qualitative analysis of endpoints in wastewater analysis.

We also tried to evaluate the prevalence of the *ARG ermB* in raw wastewater collected from the wastewater treatment plant for six consecutive months (Figure 4). The absolute abundance of the *ARG ermB* in wastewater was analyzed by qPCR, and the mean daily mass load of ermB was back-calculated by the daily flow rate of wastewater. As shown in Figure 4c, the absolute abundance of the ARG ermB in the wastewater treatment plant fluctuated significantly within half a year, ranging from 7.05×10^4 copies/mL to 1.81×10^6 copies/mL. The mean concentrations of ARGs in

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wastewater from June to November were 1.06×10^6, 1.81×10^6, 9.14×10^5, 1.33×10^6, 1.32×10^6 and 7.05×10^4 copies/mL, respectively. As shown in Figure 4c and Figure 4d, the prevalence of the ARG ermB in this WWTP in half a year showed a similar trend in absolute abundance and mean mass load, as did the lateral flow test strip device (Figure 4b). However, fluorescence-based visual detection showed that there was no significant difference in the prevalence of the ARG ermB within six months; this possibly occurred because the absolute abundance of the ARG ermB was much higher than the LOD or because the sensitivity of the fluorescence assay was higher than that of the lateral flow test. These results suggest that the constructed CRISPR/Cas12a-based biosensor with a lateral flow device can potentially be used as a relative quantitative analytical tool for monitoring wastewater. The constructed CRISPR/Cas12a visualization platform enables rapid on-site detection of ARGs in wastewater, which will provide near real-time data for the effective monitoring and rapid evaluation of ARGs.

According to our results, ARGs are widely present in wastewater, which is consistent with previous reports [42]. In fact, the occurrence of ARG in wastewater has been widely reported in different countries and regions, such as China [43], the United States [44], India [45], the United Kingdom [46] and other European countries [5]. For example, Su et al detected three hundred different ARGs from thirty-two WWTPs belonging to seventeen cities in China with an average ARG concentration of 3.2×10^{11} in wastewater influent [43]. Therefore, wastewater is considered an important source of ARGs entering the environment [4].

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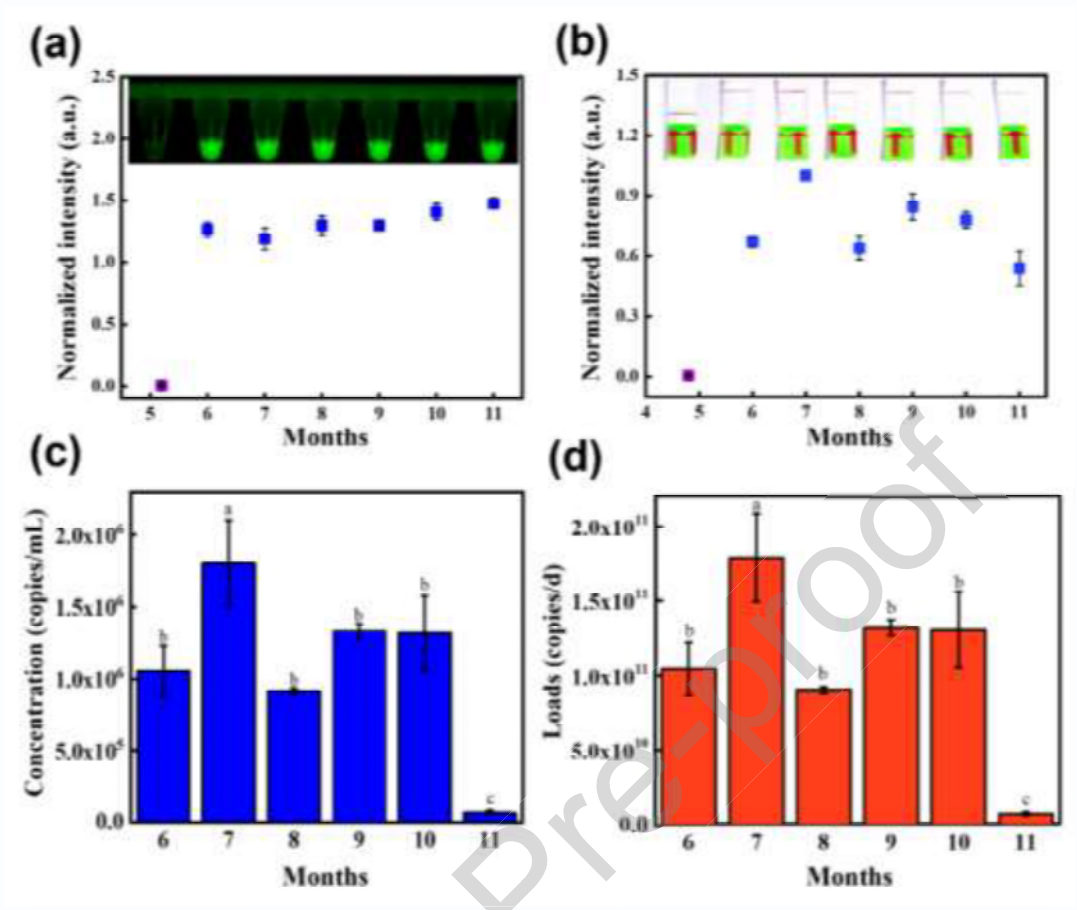


Figure 4. Community-wide evaluation of the *ARG ermB* in wastewater. Integration of the CRISPR/Cas12a-based biosensor for monitoring the *ARG ermB* in wastewater from a WWTP in Guiyang for six consecutive months from June to November 2021 with visual fluorescence (a) and lateral flow test strips (b); (c) the concentration and (d) the daily load of the *ARG ermB* in wastewater for six consecutive months in Guiyang.

There are two main sources of ARGs in wastewater. The first is the entry of external ARGs into wastewater. For example, substances such as faeces carrying ARGs enter the sewage system through the wastewater system [47]. The second is that microorganisms in wastewater can form ARGs themselves under high antibiotic stress. Thus, due to the high concentration of antibiotics entering wastewater, microorganisms such as bacteria in wastewater can develop antibiotic resistance through evolutionary adaptation, leading to gene mutations and the formation of ARGs [48]. To some extent, the emergence of the ARGs in wastewater may reveal the use of antibiotics in a community [3, 49, 50], and the results indicated that these drugs were used by people in the community during the monitoring period. For example, the highest daily loads of

the qnrS gene in wastewater in Milan, Italy, corresponded to the highest total quinolone load [5].

In addition, the results demonstrated that the mean mass load of the ARG ermB reached a maximum of 1.79×10^{11} copies/d in July and a minimum of 6.98×10^{9} copies/d in November. However, the current results demonstrated that higher mass loads of ARGs were found in wastewater influent from hot months (maximum load in August) than cold months (minimum load in November). Considering the limitations of not sampling multiple times every month and not completing a full year of data, more experiments and further validation are needed to discuss the effect of temperature on antibiotic resistance gene loading. Elder et al. think that the human population is the main driver of ARG presence in the environment because human population size is the main determinant of the magnitude of ARG burden in the environment [3].

4. Conclusions

A portable and visual CRISPR/Cas12a-based biosensor for rapid on-site detection and evaluation of ARGs in wastewater was constructed. The detection process from sampling to result is completed within 2 hours, and no sophisticated instruments are needed. Due to the high trans-cleavage efficiency and signal transduction capability of Cas12a, the method successfully overcame the false positive problem of LAMP while enabling visual detection of the ARG ermB in wastewater at less than 2.75×10^{3} copies/μL. The continuous detection and verification of the ARG ermB in wastewater influent showed that the developed detection method can be used as an effective tool for qualitative and relative quantitative analysis in wastewater. By replacing LAMP primers and the corresponding crRNA, this newly developed rapid visual method can be easily applied to other ARGs or microbial analysis in wastewater, providing an effective strategy for wastewater monitoring and wastewater-based epidemiology. Notably, to truly apply this method to wastewater analysis of ARGs on a large scale, the current method still needs to be extensively validated with more wastewater samples from different sources and types to verify the reliability of the developed method and to further integrate the current steps, such as automatic testing with a portable device, to meet the application needs of nonprofessionals in the field.

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CRediT authorship contribution statement

Kang Mao: Conceptualization; Data curation; Formal analysis; Investigation; Methodology; Visualization; Roles/Writing - original draft; and Writing - review & editing. **Hua Zhang**: Funding acquisition; Project administration; Supervision; and Writing - review & editing. **Fang Ran**: Data curation; Formal analysis; Visualization; and Writing - review & editing. **Haorui Cao**: Writing - review & editing. **Rida Feng**: Writing - review & editing. **Wei Du**: Writing - review & editing. **Xiqing Li**: Writing - review & editing. **Zhugen Yang**: Supervision; and Writing - review & editing.

Declaration of interests

√ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Highlights

- A portable biosensor for the sensitive detection of ermB in wastewater
- Higher mass loads of ermB were found in wastewater influent
- Evaluation of the community-wide prevalence of ermB during different months

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Portable biosensor combining CRISPR/Cas12a and loop-mediated isothermal amplification for antibiotic resistance gene ermB in wastewater

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