

Portable microfluidic devices for monitoring antibiotic resistance genes in wastewater

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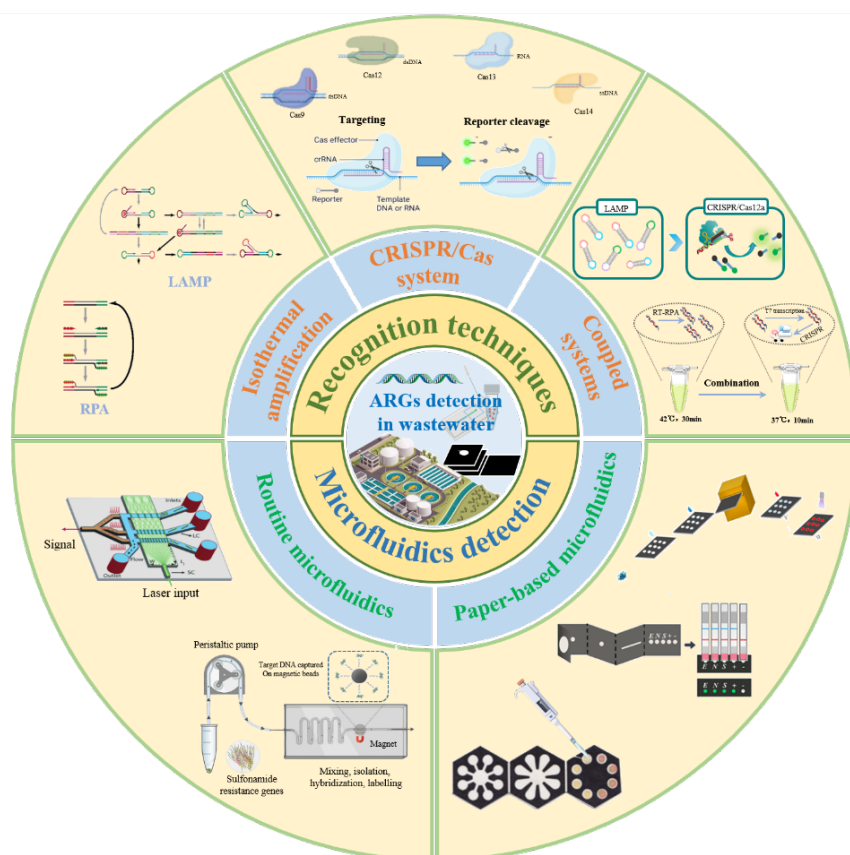
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Graphical abstract

Abstract

Antibiotic resistance genes (ARGs) pose serious threats to environmental and public health, and monitoring ARGs in wastewater is a growing need because wastewater is an important source. Microfluidic devices can integrate basic functional units involved in sample assays on a small chip, through the precise control and manipulation of micro/nanofluids in micro/nanoscale spaces, demonstrating the great potential of ARGs detection in wastewater. Here, we (1) summarize the state of the art in microfluidics for recognizing ARGs, (2) determine the strengths and weaknesses of portable microfluidic chips, and (3) assess the potential of portable microfluidic chips to detect ARGs in wastewater. Isothermal nucleic acid amplification and CRISPR/Cas are two commonly used identification elements for the microfluidic detection of ARGs. The former has better sensitivity due to amplification, but false positives due to inappropriate primer design and contamination; the latter has better specificity. The combination of the two can achieve complementarity to a certain extent. Compared with traditional microfluidic chips, low-cost and biocompatible paper-based microfluidics is a very attractive test for ARGs, whose fluid flow in paper does not require external force, but it is weaker in terms of repeatability and high-throughput detection. Due to that only a handful of portable microfluidics detect ARGs in wastewater, fabricating high-throughput microfluidic chips, developing and optimizing recognition techniques for the highly selective and sensitive identification and quantification of a wide range of ARGs in complex wastewater matrices are needed.

Keywords: Antibiotic resistance genes (ARGs), Microfluidics, Isothermal nucleic acid amplification, CRISPR/Cas, Wastewater

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1. Introduction

Antibiotic residues in the environment can cause bacteria to become resistant and lead to genetic mutations to form antibiotic resistance genes (ARGs) adapted to changes in the environment, posing risks to the environment and public health [1]. Wastewater is an important reservoir for ARGs [2, 3]. When measured ARGs from 32 wastewater treatment plants in 17 Chinese cities, approximately 300 different ARGs were detected, with an average concentration of 3.2×10^{11} copies/L [4, 5]. The main ARGs detected in wastewater are the genes resistant to macrolides (*ermB*, *ermF*), tetracyclines (*tetW*, *tetA*, *tetC*), sulfonamides (*sul1*, *sul2*), and β -lactams (*bla_{OXA}*, *bla_{TEM}*) [6, 7]. Most of the bacteria detected as harboring ARGs in wastewater belong to *Proteobacteria*, *Acidovorax*, *Hydrogenophaga*, *Aeromonas*, and *Aquabobacteria*, carried more diverse ARGs subtypes than other genera, indicating that they were the main possible hosts of ARGs [8]. The microbial community in wastewater is closely related to the distribution of ARGs [6]. For example, when the concentration of *Pseudomonas aeruginosa* which carries *vanR* and *acrB* is high in wastewater, then higher abundance of *vanR* and *acrB* is detected [6, 9]. ARGs and bacterial species in wastewater are mostly associated with human activities [10]. For example, in Poland, high concentrations of β -lactams (*bla_{OXA}*, *bla_{TEM}*, *AmpC*), macrolides, and tetracyclines (*tetA*) genes were detected due to the extensive use of β -lactams and tetracycline antibiotics [11]. ARGs in wastewater are difficult to remove completely even after treatment, and these unremoved ARGs can enter the environment through water discharge or by attaching to sludge and entering the soil as the sludge is discarded, leading to the accumulation of ARGs [5]. Thus, wastewater as an important source of ARGs can impact the level of ARGs in a region [1, 3, 12]. Moreover, ARGs can be transmitted through vertical gene transfer and horizontal gene transfer among different microorganisms and regions [1, 5]. Herein, ARGs have attracted widespread global attention as environmental and public health issues that cannot be ignored, and the detection of ARGs is essential for their identification, assessment and removal.

Polymerase chain reaction (PCR) and gene sequencing are the two main methods

used for the analysis of ARGs, and both require high-precision operations in the laboratory [3, 13]. Techniques developed based on the PCR principle include real-time PCR (qPCR), high-throughput qPCR (HT-qPCR), and emulsion paired isolation–concatenation PCR (epicPCR), which enable high-throughput detection, and high specificity and sensitivity [14-16]. Gene sequencing has undergone three generations of replacement and development, gradually evolving from short sequences to long sequences, and from single target assay to high-throughput detection, gradually eliminating the limitations of gene libraries [17]. Gene sequencing permits more accurate identification of ARGs based on their sequences and identifying information about the hosts that carry them, which is very important for deepening the knowledge of ARGs [17, 18]. However, PCR and gene sequencing often require sophisticated instruments that need to be operated by specialized personnel and have higher requirements for the detection environment in which they are located; thus, these methods are not well suited for detecting targets in the field [16, 19]. Wastewater treatment plants are usually not equipped with PCR, let alone more expensive gene sequencing instruments. In addition, as ARGs contamination increases in rural areas or areas with poor conditions, making ARGs detection from laboratory to outdoor on-site detection [20].

Recently, the development of integrated and miniaturized field analytical device has become a hotspot for ARGs analysis. Miniaturization means better portability, and integration improves the simplicity of operation, which benefits the operator in different environments [21]. Among these methods, microfluidics is considered a desirable method that promises to enable onsite detection, as many laboratory functions can be miniaturized and integrated into a single chip for the high accuracy and automation of the appropriate reaction with limited reagents, addressing the limitations of nucleic acids testing with conventional equipment [22]. The comparison between it and the conventional detection methods is shown in [Table S1](#). Especially, low-cost paper-based microfluidics are increasingly popular because of their wide range of sources as a substrate [23].

Microfluidics, which are based on new molecular detection techniques, such as

isothermal nucleic acid amplification and clustered regularly interspaced short palindromic repeats (CRISPR)-associated nuclease (Cas) systems, have received widespread attention. Isothermal nucleic acid amplification eliminates the step of repeatedly the temperature cycling for PCR by recognizing the target and rapidly amplifying the nucleic acids at a constant temperature [24]. CRISPR/Cas has shown excellent performance in detecting a wide range of targets, specifically recognizing and cleaving nucleic acids [25-27]. This combination has great complementary effects, enabling efficient target enrichment and microsample-specific identification. Integrating them into microfluidics as recognition elements simplifies complex steps and drives more accurate diagnosis of ARGs. These novel portable microfluidics have the potential to be utilized in the detection of ARGs in wastewater.

Previous reviews on microfluidics [22, 23, 28], isothermal nucleic acid amplification [29-32], and CRISPR/Cas [25, 33, 34] for detection have been published. Although these reviews have effectively summarized the (dis)advantages of these techniques, they have not explored the reliability of their application in ARGs detection. Reports on ARGs detection [5, 35, 36] are available, but most reviews are based on traditional methods, such as PCR, for ARGs detection. As we know, PCR is one of the traditional methods commonly used for nucleic acid detection to amplify specific fragments of DNA. It can also be integrated into microfluidic chips to improve its mobility and detect nucleic acids targets [37]. However, as an amplification technique that has been developed for a longer time, there have been other reviews on microfluidic PCR chips. For example, Chen et al. gave a detailed summarization of microfluidic chips for integrated PCR, including types, structure, amplification mechanism, detection and application [38]. Therefore, we don't include the microfluidic chips for integrated PCR in this work.

Herein, the main points of this review are to (1) summarize the state-of-the-art techniques for identifying ARGs in microfluidics, including isothermal nucleic acid amplification and CRISPR/Cas systems; (2) identify the strengths and weaknesses of portable microfluidics; (3) evaluate the potential of portable microfluidics for detecting ARGs in wastewater; and (4) provide recommendations for the development of portable

microfluidics to demonstrate their potential for large-scale practical applications.

2. Recognition of ARGs in portable microfluidics

2.1 Recognition of ARGs by isothermal nucleic acid amplification

According to the reaction kinetics, isothermal nucleic acid amplification can be categorized into three types: exponential amplification, linear amplification and cascade amplification [31, 32, 39]. Exponential amplification has a high amplification efficiency similar to that of PCR and mostly uses two or more primers to amplify nucleic acid templates, mainly includes loop-mediated isothermal amplification (LAMP), recombinase polymerase amplification (RPA), rolling circle amplification (RCA), and nucleic acid sequence-based amplification (NASBA). The linear amplification strategy is more convenient and excludes the interference of nonspecific amplification, which is usually triggered by a single primer for amplification, including linear strand displacement amplification (SDA), linear RCA, etc. Cascade amplification is a technique that integrates two or more amplification methods that have been developed and maintains excellent sensitivity.

Currently, isothermal nucleic acid amplification commonly used for ARGs detection are LAMP and RPA [20, 40]. Examples of isothermal nucleic acid amplification used for ARGs detection are shown in Table 1. The LAMP principle is as shown in Fig. 1A, the target sequences were complemented with four primers (F3, B3, FIP and BIP) to form a dumbbell-like structure during the LAMP reaction, a stem-and-loop structure was formed via self-priming, and finally, the amplification cycle was repeated at multiple sites, thus realizing that a small amount of the targets could be amplified in large quantities in a relatively short period, which could be as high as $1.0 \times 10^9 \sim 1.0 \times 10^{10}$ -fold [24, 29, 30, 32]. Moreover, two auxiliary loop primers (LF and LB) can be used to improve amplification performance [39].

LAMP can specifically recognize and amplify ARGs and has been widely used for various ARGs, such as *bla*, *sul*, *mcr*, and *tet* genes. LAMP has good analytical performance, can amplify DNA up to $1.0 \times 10^9 \sim 1.0 \times 10^{10}$ times in 15~60 min, and the sensitivity of detection is 10~100 times than that of PCR in several publications [32,

40]. Mu et al. [41] reported that LAMP accurately differentiated *bla_{OXA-51}* from *bla* gene variants (*bla_{OXA-23-like}*, *bla_{OXA-40-like}*, *bla_{OXA-58-like}*), with no cross-reactivity observed, indicating that the LAMP assay is highly sensitive with an LOD of 100 CFU per tube, which is 100-fold more sensitive than PCR. Sharma et al. [42] reported that the optimal temperature for LAMP was 65°C, with an LOD of 1 pg/μL (using DNA concentration), which is 10-fold higher than that of PCR. Trabasso et al. [43] designed a series of LAMP primer sets for *bla* genes and selected combinations from these primers for highly sensitive detection as low as 1.0×10^4 copies per reaction in 1 h. Li et al. [44] used a more sensitive polydiacetylene (PDA) as an instructor in the LAMP reaction for *macB* detection by the appearance of different coloured bands in the gel after electrophoresis, and the LOD was 1.34×10^{-7} ng/μL, which is at least 10-fold higher than that of PCR.

In addition to the optimization of the reaction, LAMP can usually be used in conjunction with other methods to improve the sensitivity of the assay, as well as the visualization of results, such as lateral flow biosensor (LFB) and immunomagnetic separation (IMS), which show better compatibility. LFB is a low-cost method and the results can be directly read out with the naked eyes [45]. Gong et al. [46] combined LAMP with LFB to present a LAMP-LFB method for the rapid identification of *mcr-1* in 1 h with an LOD of 6.3×10^3 CFU/mL. Li et al. [47] additionally used an IMS with high specificity and combined it with LAMP to ingeniously establish a slide-based IMS-LAMP assay for *tetL*. In this strategy, the IMS method was able to specifically isolate *tetL* within 1 h, with a capture rate of up to 80%. IMS-LAMP definitively detected the *tetL* present in pasteurized milk at 21.5 CFU/mL, suggesting that LAMP has good sensitivity for the detection of ARGs.

Usually, ARGs are not present alone in the environment, and multiple ARGs coexist; thus, the simultaneous detection of multiple ARGs is necessary to improve the detection efficiency. In this context, LAMP enables multiple ARGs to be recognized and amplified by designing different primers specific for different ARGs, resulting in a multiplex LAMP (m-LAMP) assay [48]. Gong et al. [49] used m-LAMP to simultaneously detect three *sul* genes (*sul1~sul3*) with an LOD of 0.5 pg of genomic DNA per reaction; Zhong et al. [50] also used m-LAMP to simultaneously detect five

insert the primer into the homologous site [30]. The recombinase is then disassembled, allowing the 3' end of the primer to be strand-displaced and bind to the polymerase, allowing it to extend the primer and exponentially amplify the target region on the template by repeating the process in a loop [24].

Currently, RPA is widely used in the detection of various ARGs, including *mcr*, *bla*, *van*, and *tet* genes [53]. On the one hand, the reaction substance used in RPA itself can be optimized to increase the effectiveness of the assay. For example, Warnt et al. [54] used deoxyuridine triphosphate (dUTP) to replace some of the nucleotides in the amplification reaction to achieve labelling, thereby improving the recognition ability of nucleic acids by RPA, and being able to detect *bla*_{CTX-M} less than 1.6×10^{-3} ng of genomic DNA. On the other hand, RPA can also be combined with other inspection methods to obtain highly intuitive visual results. Panpru et al. [55] combined RPA with lateral flow dipstick (LFD) strips and proposed a novel method for the rapid detection of gene *van* class within 30 min, which included *vanA* and *vanB*. This method has no cross-reactivity in multiple samples, has a 10-fold detection limit compared with that of PCR. Lu et al. [56] used the same coupling strategy and constructed an RPA-LFD method to simultaneously detect multiple ARGs, including *mcr-I*, *bla*_{NDM-I} and *tet(X4)*, which revealed that RPA-LFD can visualize the results via colour signals as low as 10 copies/ μ L, which is suitable for the quantitative measurement of multiple ARGs and meets the requirements for rapid detection. In addition to lateral flow strips, electrochemical signals are also an intuitive way to visualize the results of the assay. Butterworth et al. [57] cleverly coupled RPA with an electrochemical device, doped specially labelled thymine nucleotides into the amplified DNA strand, and detected oxacillin resistance gene in *Escherichia coli* cells by reading amperometric signals with an LOD of 319 CFU/mL.

Apart from LAMP and RPA, other isothermal nucleic acid amplification, such as RCA, have been sporadically reported (Fig. 1C) [51]. All in all, other amplification techniques are less frequently used, probably because they are relatively new and are less frequently used for ARGs alone, in addition to the complexity of the reaction process and demanding reaction conditions.

Table 1. Examples of portable microfluidic devices for ARG detection.

Classification	Recognition method	Optimization/ Assembly	ARGs	Temperature (°C)	Time (min)	Readout	Multiple detection	Dynamic range	LOD	Sample type	Quantification	Portability	Ref
nucleic acid isothermal amplification	LAMP	/	<i>bla_{OXA-51}</i> and other variants	60	>25	fluorescence	yes	$0 \sim 1 \times 10^6$ CFU/tube	1 CFU/tube	sputum	yes	yes	[41]
	LAMP	/	<i>bla_{OXA-23}</i>	65	30	fluorescence	no	1 pg/ μ L $\sim$ 100 ng/ μ L	1 pg/ μ L	bacterial strain	yes	yes	[42]
	LAMP	primer set	azole resistance gene	63	60	fluorescence	no	$1 \times 10^4 \sim 1 \times 10^7$ copies/reaction	1×10^4 copies/reaction	bacterial strain	yes	yes	[43]
	LAMP-PDA	PDA indicator	<i>macB</i>	64	40	colorimetry	no	$9.248 \times 10^{-7} \sim 9.248 \times 10^{-3}$ ng/ μ L	1.344×10^{-7} ng/ μ L	milk	yes	yes	[44]
	LAMP-LFB	LFB	<i>mcr-1</i>	63	60	lateral flow	no	36 fg/ μ L $\sim$ 3.6 ng/ μ L	360 fg/ μ L	faecal	yes	yes	[46]
	LAMP-IMS	IMS technology	<i>tetL</i>	65	50	fluorescence	no	$1.16 \times 10^0 \sim 1.16 \times 10^5$ CFU/mL	1.16×10^1 CFU/mL	milk	yes	yes	[47]

m-LAMP	multiple detection	<i>sul1, sul2, sul3</i>	64	50	electrophoresis	yes	$5 \times 10^{-2} \sim 5 \times 10^4$ pg/reaction	0.5 pg/reaction	bacterial strain	yes	yes	[49]
m-LAMP		<i>mcr-1 ~ mcr-5</i>	64	60	electrophoresis	yes	$1 \times 10^4 \sim 1 \times 10^8$ copies/ μ L	1×10^4 copies/ μ L	bacterial strain	yes	yes	[50]
RPA	/	<i>mcr-1</i>	37	30	lateral flow	no	1 fg/ μ L $\sim$ 100 ng/ μ L	100 fg/ μ L	bacterial strain	N	yes	[53]
RPA	using dUTP	<i>bla_{CTX-M}</i>	38	40	fluorescence	no	$6.4 \times 10^{-5} \sim 1$ ng	1.6×10^{-3} ng of genomic DNA	Genomic DNA	yes	yes	[54]
RPA-LF	LF	<i>vanA, vanB</i>	37	25	lateral flow	yes	$10^2 \sim 10^8$ CFU/mL	vanA: 10^5 CFU/mL; vanB: 10^6 CFU/mL	blood and faecal	yes	yes	[55]
RPA-LFD	LFD	<i>mcr-1, bla_{NDM-1}, tet(X4)</i>	37	40	lateral flow	yes	$1 \times 10^0 \sim 1 \times 10^7$ copies/ μ L	1×10^1 copies/ μ L	strains	yes	yes	[56]
RPA	electrochemical measurement	<i>mec</i>	39	40	electrochemistry	yes	/	319 CFU/mL	bacterial strain	yes	yes	[57]
RCA-LFA	LFA	<i>mecA</i>	37	30	lateral flow	yes	0 $\sim$ 1670 fM	1.3 fM	bacterial strain	yes	yes	[51]

CRISPR /Cas system	Cas9	optical DNA mappin g	<i>bla_{NDM},</i> <i>bla_{KPC}</i>	37	60	fluoresce nce	yes	/	/	plasmid	yes	yes	[58]
	Cas12a	/	<i>bla_{KPC},</i> <i>bla_{NDM},</i> <i>bla_{OXA}</i>	37	90	fluoresce nce	yes	10 pM ~ 10 nM	<10 pM	blood	yes	yes	[59]
	Cas12a	add T7 exonuc lease	<i>mecA</i>	37	60	fluoresce nce	yes	$0 \sim 1 \times 10^{12}$ ag/mL	100 ag/mL (genomic DNA)	blood	yes	yes	[60]
	Cas12a	Au- Fe ₃ O ₄ nanozy me	<i>KanaR,</i> <i>AmpR,</i> <i>CmR</i>	37	20	colorime try	yes	0 ~ 6 CFU/μL	0.06 CFU/μL	wastewa ter	yes	yes	[61]
	Cas12a	/	<i>ermB,</i> <i>sulI,</i> <i>tetW</i>	37	>35	colorime try	yes	0 ~ 20 nM	<5 nM	wastewa ter	yes	yes	[62]
Coupled system	RPA- CRISPR /Cas12a	/	<i>mcr-1</i>	37	<60	fluoresce nce	no	4.2 fg/μL ~ 4.2 ng/μL	420 fg/μL	clinical samples	yes	yes	[63]
	RPA- CRISPR /Cas12a	/	benzimi dazole ARG	37	>45	fluoresce nce	Yes	$7.82 \sim 7.82 \times 10^6$ fg/μL	7.82×10^3 fg/μL	bacterial strain	yes	yes	[64]
	LAMP- CRISPR /Cas12a	/	<i>ermB</i>	37	>60	fluoresce nce	no	$2.75 \times 10^2 \sim 2.75$ $\times 10^9$ copies/μL	2.75×10^3 copies/μL	wastewa ter	yes	yes	[65]

routine microflu idics	IEXPAR - CRISPR /Cas9 polycarb onate microflu idics	/	<i>mecA</i>	IEXPA R: 55; Cas9: 37	>70	fluoresce nce	no	100 fM ~ 1 nM	81 fM	genomic DNA	yes	yes	[66]
	LAMP		<i>mecA</i>	65	<70	fluoresce nce	no	$2 \times 10^0 \sim 2 \times 10^4$ pg/reaction	20 CFU/reaction	clinical samples	yes	yes	[67]
	glass microflu idics	LAMP ; EMA	<i>vanA</i>	/	<60	fluoresce nce; electrop horesis	no	$1 \times 10^0 \sim 1 \times 10^5$ CFU	10 CFU/reaction	genomic DNA	yes	no	[68]
	optical flow chip	light sensor	<i>bla_{KPC}</i> , <i>bla_{NDM}</i> , <i>bla_{VIM}</i>	/	<60	fluoresce nce	yes	/	3×10^6 copies/mL	blood	yes	no	[69]
	PMMA microflu idics	RPA; magnet ic bead	<i>sul1</i> and <i>sul4</i>	37	>20	electroch emistry	yes	0 ~ 10 nM/L	<i>sul1</i> : 44.2 pM/L <i>sul4</i> : 48.5 pM/L	wastewa ter	yes	yes	[70]
paper- based microflu idics	microflu idics	HT- qPCR	<i>sul1</i> , <i>int1</i> , and <i>bla_{CTX-M}</i>	therma l cycle	/	fluoresce nce; sequence	yes	/	/	wastewa ter	yes	no	[71]
	2D paper equipme nt	LAMP	<i>mecA</i> , <i>mecC</i>	64	>55	fluoresce nce	yes	<i>mecA</i> : $0 \sim 6.25 \times 10^4$ copies <i>ermC</i> : $0 \sim 1.25 \times 10^5$ copies	<i>mecA</i> : 100 copies <i>ermC</i> : 285 copies	serum	yes	yes	[72]

261
262 Compared with that of LAMP, the reaction process of RPA is more complicated,
263 but it does not require a higher reaction temperature, can react at room temperature, has
264 relatively simple primer design and is fast, which makes it suitable for detecting various
265 ARGs. The above studies have shown that isothermal nucleic acid amplification
266 techniques, mainly represented by LAMP and RPA, are advantageous for identifying
267 and detecting ARGs, and their potential to be utilized for the actual detection of ARGs
268 in the field.

269 Wastewater has multiple compositions and complex environments, and isothermal
270 nucleic acid amplification, a proven and effective method, is able to detect a multitude
271 of targets in wastewater, such as pathogens, which demonstrates its generalizability and
272 potential for the detection of ARGs in wastewater. Akter et al. [73], Alvarez-Serna et al.
273 [52] and Papamatthaiou et al. [74] designed relevant LAMP primers and used LAMP-
274 based sensors to specifically detect SARS-CoV-2 in wastewater. Of these studies, Akter
275 et al. [73] used RT-LAMP alone to accurately identify positive samples of wastewater,
276 illustrating the isothermal amplification feasibility of the assay. Building on this
277 foundation, Alvarez-Serna et al. [52] and Papamatthaiou et al. [74] introduced
278 transistors and printed circuit boards to make an easily portable device that takes a step
279 towards onsite wastewater detection as low as 0.37 ng/ μ L and 17 GC/ μ L, respectively
280 (Fig. 1D). Moreover, Martzy et al. [75] designed specific LAMP primers, which led to
281 the amplification of diagnostic gene fragments for enterococci, which in turn were used
282 for the detection of enterococci in wastewater.

283 Studies, including those described above, have shown that isothermal nucleic acid
284 amplification can be used to specifically recognize and detect different targets in
285 wastewater, including ARGs, through the design of different specific primers. However,
286 few studies are available on the direct detection of ARGs in wastewater using
287 isothermal nucleic acid amplification alone, possibly because the related technique is
288 still in the developmental stage. In addition, wastewater detection in complex matrices
289 is prone to interference and the results have not been satisfactory. And it tends to
290 identify and amplify the target and needs to be combined with other detection

techniques to achieve signal output, such as the CRISPR/Cas system for the quantitative detection of ARGs in wastewater.

Overall, isothermal nucleic acid amplification has the potential to be applied in wastewater monitoring by designing amplification primers to specifically recognize different targets in wastewater, including ARGs, and amplify them. In the field of detection, isothermal nucleic acid amplification compensates for the shortcomings of PCR in terms of its dependence on equipment and long detection period and has certain strengths in terms of detection speed, miniaturization of instruments, and visualization of results [76]. However, limitations such as a low detection throughput and false-positive results cannot be ignored, which severely restrict its use [40]. When applied in the detection of ARGs, isothermal nucleic acid amplification can be used directly for detection and improve the detection performance through the optimization of its own reactants or coupling with other techniques, but it is still not sufficient to achieve people's expectations; coupled with the complexity of wastewater composition, many types of ARGs usually exist together in wastewater, which substantially increases the difficulty of detecting ARGs in wastewater, and related research results are limited.

In this context, the highly specific recognition of CRISPR/Cas and high-throughput microfluidics provide new opportunities for the application of isothermal nucleic acid amplification to detect ARGs in wastewater, which will be discussed in detail below. In summary, although the isothermal nucleic acid amplification technique is difficult to apply directly to the identification of ARGs in wastewater alone, its unique strengths in conjunction with other techniques have great potential for detecting ARGs in wastewater.

2.2 The CRISPR/Cas system recognizes ARGs

CRISPR/Cas, with easy operation and a fast response, can accurately recognize targets and cut them and is an excellent candidate for meeting the needs of ultrasensitive nucleic acid detection, as well as being valuable in onsite testing. As shown in [Fig. 2A](#) and [Fig. 2B](#), the general steps of CRISPR/Cas-based nucleic acid detection include extracting nucleic acids from the sample, enrichment, amplification, selecting a suitable CRISPR/Cas system to localize and cut the target, and finally observing the results [2].

At present, the Cas proteins commonly used in the CRISPR/Cas-based recognition and detection of ARGs are Cas9 and Cas12 in the Class 2 system. The principle of targeted cleavage is similar between the two: Cas proteins carry primers to scan DNA sequences and recognize the protospacer adjacent motif (PAM). When the PAM is recognized, it stops moving and unwinds the upstream DNA double strand of the PAM. At this time, the primers will attempt to pair with the upstream DNA sequence. When the sequence matches, Cas proteins will cut the DNA double strand upstream of the PAM [77, 78]. The difference is that Cas9 generally carries CRISPR RNA (crRNA), whereas Cas12 carries crRNA and trans-activating crRNA. The CRISPR/Cas assay has the strengths of a low cost, low labour and high efficiency and has great potential for the specific recognition of different targets [79-82]. Examples of CRISPR/Cas-based assay used for ARGs detection are shown in [Table 1](#).

Studies have shown that CRISPR/Cas9 specifically scans its targets in a strictly PAM-dependent manner, resulting in the cleavage of the double strand of targets [33]. Among these targets are ARGs; for example, both Wu et al. [83] and Müller et al. [58] successfully used CRISPR/Cas9 to cleave plasmids containing different ARGs. Müller et al.'s study, in which Cas9 accurately recognized *bla*_{CTX-M}, *bla*_{NDM}, and *bla*_{KPC} in the same *bla* class ([Fig. 2D](#)), and Wu et al.'s study, in which Cas9 accurately cleaved a wide range of ARGs, including *sul2* and *bla*_{OXA-55}, also demonstrated the feasibility of Cas9 in recognizing and cleaving ARGs. In addition, Müller et al. [58] combined CRISPR/Cas9 with an optical DNA mapping technique, which allowed the visualization of individual DNA molecules under a fluorescence microscope, improving the accuracy of Cas9 recognition and accurately identifying carryover plasmids containing different *bla* genes.

Cas12 is the second most popular gene editing tool after Cas9, and the high specificity of its cleavage activity makes it easy to utilize in diagnostic tools such as portable microfluidics [33]. Both Geng et al. [84] and Curti et al. [59] used CRISPR/Cas12a directly for recognition and cleavage and successfully detected the *Amp* gene and *bla*_{KPC}, *bla*_{NDM} and *bla*_{OXA} genes in the *bla* genes by being guided by specifically designed crRNA primers. Curti et al. [59] reported that the LOD of

CRISPR/Cas12a-based assay can reach at least 10 pM and that pM-level targets can be detected in less than 10 min (Fig. 2C). These results show that CRISPR/Cas12a has the specificity and sensitivity for use in diagnostics, has the potential and feasibility for use in the identification and detection of ARGs, and provides a foundation for further research on the mechanism of antibiotic resistance and detection of other ARGs.

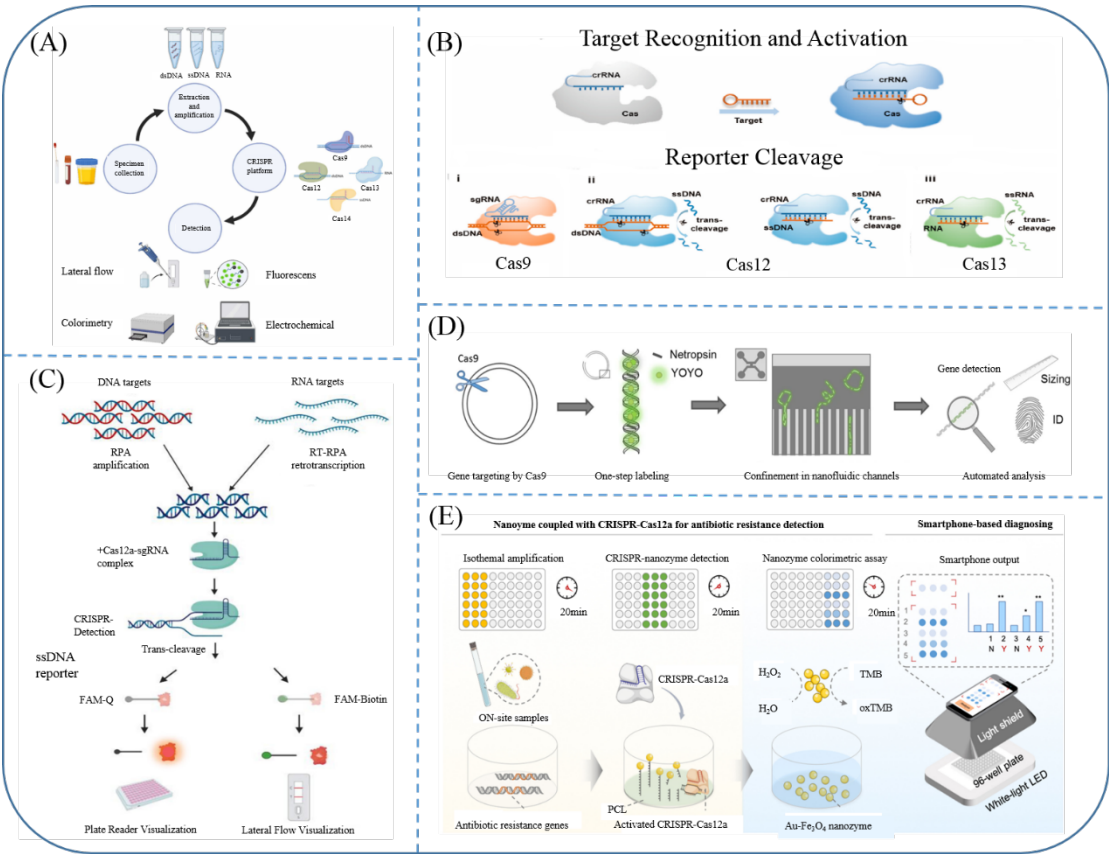


Fig. 2. Recognition and detection of ARGs via the CRISPR/Cas technique. (A) Process of the CRISPR/Cas-based microbial detection [85]. (B) Cas proteins commonly used for detection and high specificity in the CRISPR/Cas system [25]. (C) Schematic diagram of the detection process using CRISPR/Cas12a [59]. (D) CRISPR/Cas9 coupled with optical DNA mapping for the direct recognition of single plasmid molecules carrying ARGs [58]. (E) Au-Fe₃O₄ nano-enzymes coupled with CRISPR/Cas12a for the sensitive and visualizable detection of ARGs [61].

CRISPR/Cas12a has a sensitivity sufficient to detect ARGs, and in addition to optimizing the reactants of the reaction system, it can also be optimized by externally adding other reactive enzymes to optimize the detection effect. Yang et al. [60] developed a dual CRISPR/Cas12a biosensor by adding T7 exonuclease to the reaction mixture for the highly specific detection of *mecA* without a PAM with an LOD of 100 ag/mL, which is more sensitive than Cas12a alone (Fig. S2). Adding foreign reactive enzymes can also improve the ability of the CRISPR/Cas12a assay to detect ARGs.

Given the coexistence of multiple ARGs in the natural environment, Chen et al. [61] synergized the recognition ability of CRISPR/Cas12a with the addition of Au-Fe₃O₄ nano-enzymes, improving the sensitivity and, at the same time, allowing the more sensitive observation and visualization of colour change to simultaneously detect less than 0.1 CFU/μL of *KanaR*, *AmpR*, and *CmR*.

These studies mentioned above have shown that Cas9 and Cas12 in the CRISPR/Cas system can be applied to the detection of single and multiple ARGs, which is no less effective than that of PCR, and that optimization methods such as the addition of foreign enzymes can further improve the detection performance and visualization. However, few studies on ARGs exist, which may be due to the risk of off-target effects inherent in the CRISPR/Cas system. Additionally, Cas protein cleavage activity is random and sustained, which is difficult to study quantitatively, and it is limited by the concentration of the sample target. Overall, the CRISPR/Cas system is suitable for the rapid identification and onsite detection of ARGs because it is free from dependence on giant instruments compared with PCR and has high specificity for identification and cleavage compared with isothermal nucleic acid amplification. Although this approach has some shortcomings, they can be overcome in future research.

At present, although wastewater is complicated, CRISPR/Cas has been used to detect different target substances in wastewater, including viruses and pathogens [86-88], which show that CRISPR/Cas can be fully used in wastewater monitoring to detect different pollutants in wastewater with high sensitivity through primer-specific recognition, Cas protein cleavage, and signal output.

Wastewater is one of the major reservoirs of ARGs, and similar to viruses, antibiotics, and other contaminants detected in wastewater, CRISPR/Cas has the full potential to target ARGs in wastewater. Chen et al. [61] added Au-Fe₃O₄ nano-enzymes to the CRISPR/Cas12a reaction and successfully identified and accurately cleaved the target genes *KanaR*, *AmpR*, and *CmR* in wastewater containing multiple ARGs (Fig. 2E). In addition, Kasputis et al. [62] used standalone CRISPR/Cas12a and a colorimetric approach to simultaneously and specifically identify three ARGs: *ermB*, *sulI*, and *tetW*, in wastewater effluent collected from a food processing plant, with

LODs of 10, 100, and 1000 copies, respectively, which reliably detected the concentrations of a variety of ARGs at concentrations as low as 5 nM. These two studies indicate that the CRISPR/Cas system, which is mainly represented by Cas12, has more satisfactory specificity for the recognition of ARGs in wastewater than the other systems, and it can be applied to portable microfluidic field testing as the recognition element.

The CRISPR/Cas system has the potential to detect ARGs targets in complex wastewater. The designed CRISPR/Cas primers can specifically recognize the targets, Cas proteins cleave the sequences after recognition, and signal output can be visualized by fluorescence, colorimetry, electrochemistry, etc [26, 89]. Compared with PCR, CRISPR/Cas is simple, highly specific, does not rely on complex equipment or stringent laboratory conditions, and it has the same reliability and accuracy but greatly reduces the time and cost. In addition, it has the potential to detect multiple targets by different primers. However, the sensitivity of CRISPR/Cas has not yet reached a satisfactory level in practical sample. If it can be combined with isothermal nucleic acid amplification to amplify the target, the sensitivity of detection will be improved. For example, Curti et al. used CRISPR/Cas to detect ARGs with an LOD of 10 pM, while Qin et al. coupled isothermal nucleic acid amplification with CRISPR/Cas to detect ARGs with an LOD of 81 fM, which is more sensitive than CRISPR/Cas alone [59, 66].

2.3 Coupled isothermal nucleic acid amplification and CRISPR/Cas recognize ARGs

CRISPR/Cas combined with isothermal nucleic acid amplification is a novel molecular diagnostic technique developed in recent years, and their combination can overcome the limitations of the individual approaches and thus they can be better utilized in rapid onsite detection (Fig. S1). On the one hand, the CRISPR/Cas system can specifically recognize target sequences, cleavage, and detect target sequences directly, but the initial target must reach a certain concentration before its detection sensitivity can meet the detection requirements, which requires an amplification step [90]. On the other hand, isothermal nucleic acid amplification recognizes targets and amplifies them rapidly but is prone to false-positive problems, whereas CRISPR/Cas uses a reporter to detect specific targets, which reduces the likelihood of false-positive

results and achieves highly sensitive and specific detection [91]. Examples of isothermal nucleic acid amplification and CRISPR/Cas coupling for ARGs detection are shown in Table 1.

The combination of RPA and CRISPR/Cas12a is among the most commonly used strategies to identify and detect different types of ARGs. Gong et al. [63] and Hu et al. [64] used this strategy to identify and detect the *mcr-1* and *ben* genes by rapidly increasing the concentration of ARGs via the RPA and then using fluorescent signals for visualization via CRISPR/Cas12a cleavage (Fig. 3A). Gong et al. [63] used a coupled system with a detection limit of 420 fg/μL in a pure positive isolate, which can be applied for faecal sample detection, and the detection threshold of *mcr-1* was 1.6×10^3 to 6.2×10^3 CFU/mL. Hu et al. [64] used a one-pot assay in a coupled system and obtained an LOD of 7.82×10^3 fg/μL. The reactions were all completed within 60 min, without the need for precision instruments, and the operation was simple and rapid, suggesting that it can be used as an identification method for the accurate detection of ARGs in complex samples.

ARGs targets are generally DNA, and most can be cleaved by Cas12a proteins, although Cas9 can be directed to the target DNA of ARGs and site-specific cleavage can be performed under conditions through PAM and single guide RNA. Instead of adopting the conventional strategy of amplification followed by cleavage, Qin et al. [66] utilized CRISPR/Cas9 to specifically recognize *mecA* into two short fragments and triggered isothermal exponential amplification reaction (IEXPAR) for exponential amplification and detection using one of the cleaved DNA segments as the target (Fig. 3B) which could distinguish *mecA* from interfering DNA sequences and detect ARGs as low as 81 fM in real samples. These reports indicate that CRISPR/Cas combined with isothermal nucleic acid amplification is effective at identifying ARGs, which is relatively better than the pM level that is generally obtained when a single technique is used.

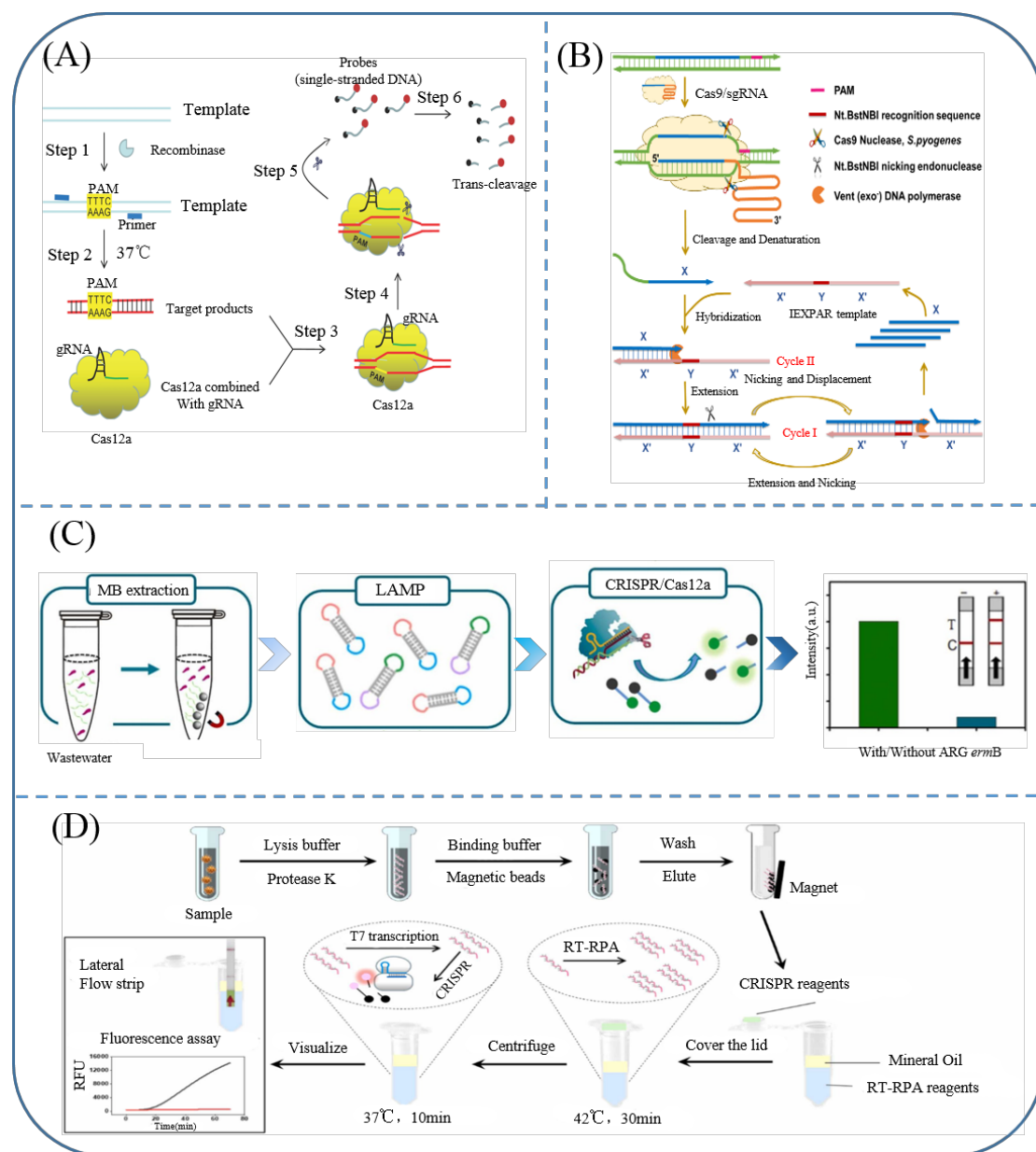


Fig. 3. Coupled nucleic acid amplification and CRISPR-based system to recognize ARGs and its application in wastewater analysis. (A) Highly sensitive and specific detection of *mcr-1* by RPA-CRISPR/Cas12a [63]. (B) CRISPR/Cas9-induced IEXPAR method for the detection of *mecA* [66]. (C) Schematic of the process of combining CRISPR/Cas12a and LAMP for *ermB* detection [65]. (D) CRISPR/Cas13a and RT-RPA-based single-tube assays for wastewater effluent detection [92].

The coupled system is suitable for the identification of many types of targets in wastewater, such as bacteria, nucleic acids, and proteins [29, 90]. Because targets can be accurately and efficiently recognized in the conjugated system by specifically designed primers and rapidly amplified to increase the initial detection concentration for subsequent CRISPR/Cas cleavage [29]. This system is suitable for detecting low concentrations of targets, such as bacteria, in wastewater (Fig. 3D) [92, 93]. Herein,

although the concentration of ARGs in wastewater is relatively low in some scenarios, related reports about the identification of ARGs in wastewater are available. As shown in Fig. 3C and Fig. S3, Mao et al reported a novel analytical method to couple CRISPR/Cas12a and LAMP for the rapid detection of *ermB* in wastewater using fluorescence and lateral flow methods [65]. The LAMP-CRISPR/Cas12a system can detect *ermB* as low as 2.75×10^2 copies/ μ L, which is an order of magnitude lower than LAMP alone (2.75×10^3 copies/ μ L). The strategy of combining the CRISPR/Cas system with isothermal nucleic acid amplifications has the obvious advantages of enriching low-abundance targets and improving the analytical performance.

However, the establishment of the coupled system indicates an increase in the number of operational steps, which increases the complexity of the detection process and the generation of error rates, and the coupled system is still in the developmental stage and is still mostly operated in the laboratory. These aspects also result in the coupled system being used less frequently to detect ARGs in wastewater on site, but it still has great potential for use, and the combination of the two technologies is expected to continue to evolve in the detection of ARGs in the future.

3. Portable microfluidics for the detection of ARGs in wastewater

Microfluidic devices are defined or described as, “A small, non-turbulent, highly ordered, portable fluid flow systems made up of several micro-channels molded or carved into a material” by Yang et al [94, 95]. It involves multidisciplinary studies the manipulation and behaviour of fluids in microliter and nanoliter volumes within micro-channels [95]. As shown in Fig. 4A and Fig. S4, microfluidic chip mainly includes elements such as sample preparation, separation, transportation, and detection [96]. Various materials have been used to fabricate microfluidics. Among these, polymeric materials, such as polydimethylsiloxane (PDMS) and polymethylmethacrylate (PMMA), are commonly used to prepare microfluidic controls due to their low price and simple fabrication [97]. Classified on the basis of control of the fluid, microfluidics can be categorized as passive and active microfluidics [98]. Passive microfluidics utilize surface hydrophobicity or capillary forces to self-propel for fluid transfer and

separation, such as paper-based microfluidic devices. It does not require an external pumping source or energy source. Active microfluidics utilize external fields such as electricity, magnetism, sound and light to drive the fluid, such as pressure microfluidics, centrifugal microfluidics, and magnetic microfluidics [23].

3.1 Routine microfluidics for the detection of ARGs

Microfluidic technique has the advantages of high integration, portability, low sample consumption and easy operation [96, 99-101]. It helps to solve the operational problems of cumbersome sample preparation and detection steps, realizes the automated integration of nucleic acid detection, improves the detection performance, and reduces the contamination caused by operational errors [28, 102]. The microfluidic devices holds huge potential for detecting ARGs.

Meng et al. [67] identified *mecA* in multiple cocci and predicted methicillin resistance by developing a LAMP-based microfluidic device, illustrating the feasibility of LAMP-based microfluidic detection of ARGs. Chang et al. [68] developed a microfluidic device for the diagnosis of *vanA* in live bacteria using ethidium monoazide (EMA) and LAMP, which showed that the device successfully detected *vanA* in "live" enterococci with high sensitivity and accuracy. Detailed examples of portable microfluidics used for ARGs detection are shown in Table 1.

ARGs usually do not exist singly in the environment; rather, multiple ARGs coexist, and simultaneous detection of multiple ARGs is necessary to improve the detection efficiency. Meena et al. [69] developed a microfluidic system based on optical signal detection for the rapid detection of *bla* genes within 1 h (Fig. 4B). In this strategy, an amplification-free approach was used for the rapid detection of ARGs; in single-channel flow, all signals in the traces were identified via a signal processing algorithm, and three channels were designed to achieve the simultaneous detection of multiple *bla* genes, including *bla_{KPC}*, *bla_{NDM}*, and *bla_{VIM}*. The results demonstrated that this multi-channel microfluidics effectively performed the high-throughput detection of ARGs.

There is a wide variety of ARGs in wastewater, while ARG in some wastewater samples are not high. For example, the average concentration of ARGs in wastewater is only 2.27×10^4 gene copies/L, as determined by Wang et al [103]. The low

concentration of ARGs in the wastewater itself can interfere with the effective detection of microfluidics. For this reason, a concentration step of ARGs prior to entering the microfluidic is usually required. In addition to this, isothermal nucleic acid amplification techniques can be used to amplify the target ARGs to further increase the concentration of ARGs, thereby increasing the feasibility of the assay as well as improving the sensitivity of the assay.

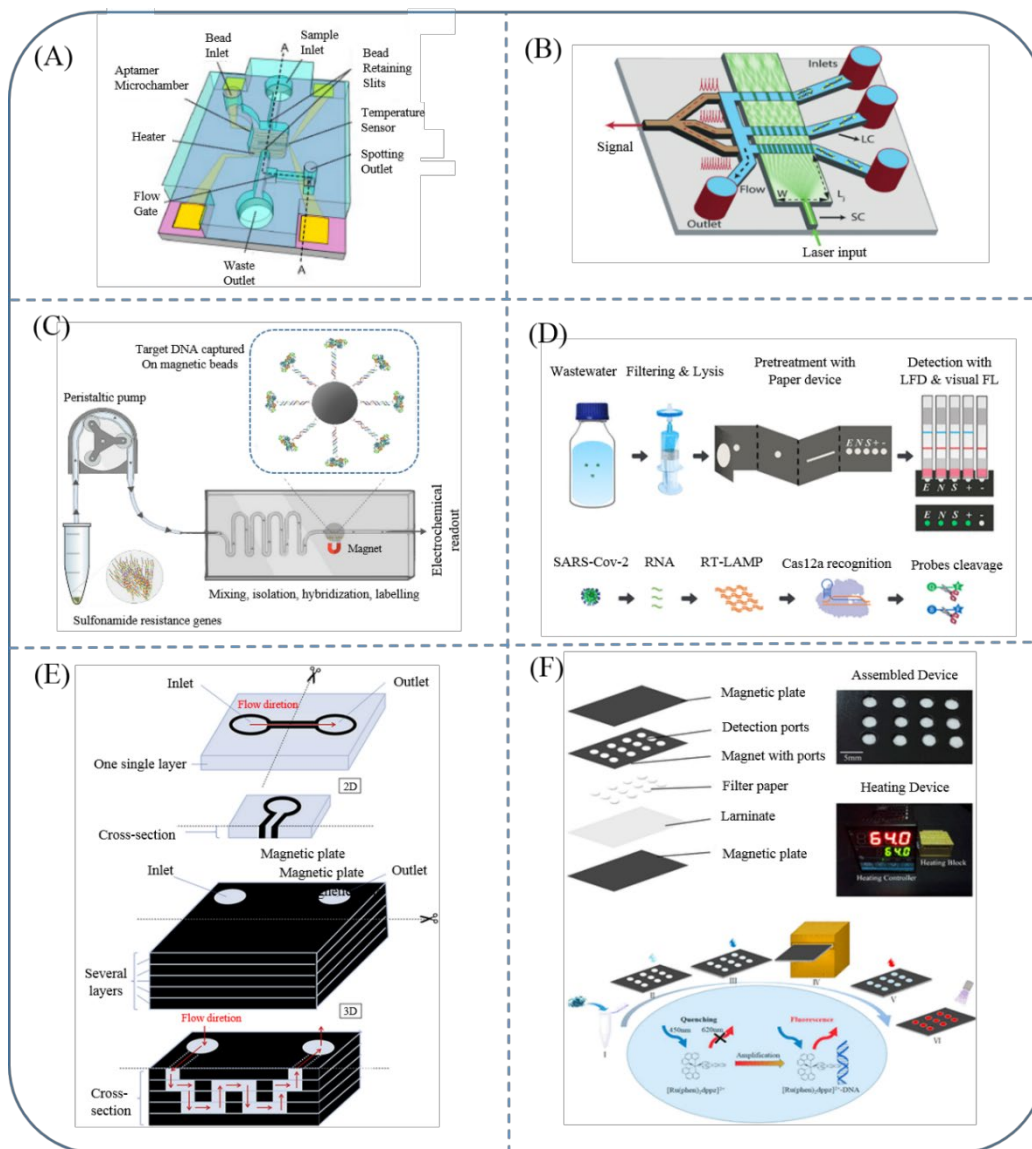


Fig. 4. Application of microfluidics in ARGs detection and wastewater analysis. (A) Schematic diagram of a conventional microfluidic sensor [96]. (B) Schematic diagram of a microfluidics used for 3-fold multiplexed detection of ARGs [69]. (C) Microfluidic-based electrochemical detection of sul genes [70]. (D) Paper-based microfluidics combining CRISPR/Cas12a and RT-LAMP for monitoring wastewater [104]. (E) The 2D and 3D structures of paper-based microfluidics [105]. (F) Integration of LAMP and the "photoswitch" molecule $[\text{Ru}(\text{phen})_2\text{dppz}]^{2+}$ for the paper-based microfluidic detection of ARGs [72].

Microfluidic devices have been used to detect ARGs in wastewater with favourable results. Sfragano et al. [70] produced a small microfluidic platform driven by microfluidic manipulation and determined *sul1* and *sul4* by electrochemical readings, showing the feasibility of the application of microfluidics to detect ARGs in wastewater (Fig. 4C). In this strategy, isothermal nucleic acid amplification was used to increase the concentration; modified magnetic beads were used to trap and separate target amplicons into micro-channels. And a multichannel peristaltic pump was used to control microfluidic exposure to each reagent. The results revealed that *sul1* and *sul4* had positive detection limits of 44.2 pmol/L and 48.5 pmol/L in wastewater, respectively. Shrestha et al. [71] coupled HT-qPCR to construct a high-throughput microfluidic quantitative PCR system for the quantification of ARGs in a wastewater treatment plant and reported that *sul1*, *int1* and *bla_{CTX-M}* were detected in all samples. These examples all show that microfluidics can be used to detect ARGs in wastewater and play an important role in researching spatial and temporal variations and their abundance. Although relatively few studies related to microfluidics exist in this area, the rapid development of microfluidics is believed to lead to more studies in the future.

Microfluidics is highly compatible for diagnostics and integrating the complex steps of the ARGs assay onto the chip to provide a portable assay [67, 68]. In particular, the integration of isothermal nucleic acid amplification in microfluidics for the detection of ARGs achieved good analytical performance, which can solve the issue of low concentration and does not require temperature cycle [106, 107]. The CRISPR-based microfluidic technique has high sensitivity and specificity and has potential for use in the detection of ARGs. The closed microfluidic device reduces the risk of contamination, and the design of multiple micro-channels achieves multiple detections with low operating difficulty and improved detection efficiency. However, problems such as complex production process, and lack of unified standards and specifications should be paid attention. Overall, existing studies show that portable microfluidic devices can be used to detect ARGs in the environment and have the potential to detect and quantify ARGs in wastewater. The integration of a series of step-by-step processes for nucleic acid detection of ARGs targets on a single chip by a highly miniaturized

microfluidic technique reduces the complexity of the operation, which helps to build an automated and efficient analytical system.

3.2 Paper-based microfluidic detection of ARGs

Paper is now considered an attractive and promising substrate material for microfluidic applications not only due to its extremely low cost and ubiquity but also due to its mechanical properties comprising flexibility, lightness, and low thickness [105]. It has a fibrous and porous structure that provides capillary action for transporting liquids without external forces; the ability to absorb and store reagents, as well as filter samples; and a high surface-to-volume ratio, which increases the amount of immobilized reagents and allows for a reduction in analytical time [105, 108-110]. Paper-based microfluidics is a miniature analytical device that utilizes a paper substrate in place of the traditional substrates of microfluidics [111, 112]. By constructing hydrophilic/hydrophobic channels and analytical units on the surface of the sample, the sample is subjected to self-driven flow and corresponding reactions on the chip for the purposes of pre-treatment, separation, purification, and detection [108, 111].

Paper-based microfluidics are passive microfluidics that utilize capillary action to generate flow and allow for the transport of liquids without the need for external forces [105, 110]. Hydrophilic/hydrophobic channels are usually created on the surface of the paper substrate so that the drop sample can flow directionally through the pre-determined channels [109, 113]. Flow can be generated in both open and closed microfluidic channels due to the surface tension of the liquid and the hydrophobicity of the substrate surface. Hydrophilic surfaces attract water and form a concave meniscus, while hydrophobic surfaces repel water and form a convex meniscus [114]. As shown in [Fig. 4E](#), based on the structure, paper-based microfluidics can be divided into 2D and 3D paper-based microfluidic devices [115]. The 2D structures are designed mainly for micro-channels on the plane, which are simple and easy to fabricate, whereas 3D structures often realize multilayer reactions in space through cascading, which enables both horizontal and vertical transport [105, 113, 116].

Although these devices have appeared relatively quickly, paper-based microfluidics are rapidly evolving in design and integration [117, 118]. Examples of

paper-based microfluidics for ARGs detection are shown in Table 1. At present, studies have shown that paper-based microfluidics can be used to detect ARGs. Li et al. [72] integrated LAMP and the "photoswitch" molecule $[\text{Ru}(\text{phen})_2\text{dppz}]^{2+}$ on a paper microfluidic device and detected *mecA* and *mecC* via fluorescence (Fig. 4F), which showed that this strategy successfully detected the target gene within 1 h, and the LOD was 100 copies for *mecA* and 285 copies for *ermC*. The above studies revealed that paper-based microfluidics has excellent assay results for the rapid detection of ARGs and is expected to be further advanced to detect multiple ARGs at the same time.

Paper-based microfluidics is also used in wastewater monitoring. As shown in Fig. 4D, for the detection of SARS-CoV-2 in wastewater, we integrated a coupled system of RT-LAMP and CRISPR/Cas12a on a portable paper-based microfluidic system to detect SARS-CoV-2 in wastewater [104]. We designed a 3D paper-based microfluidic device that integrated the lysis of microorganisms, nucleic acid purification, amplification, and CRISPR/Cas12a reaction on the device and realized liquid flow and then a reaction by folding. The results revealed this promising low-cost method was well suited for the detection of nucleic acid targets in wastewater. ARGs, like pathogens, are detected through nucleic acid testing. Therefore, this technique could be applied to the detection of ARGs in wastewater by adjusting LAMP primers and CRISPR/Cas primer.

However, there is no report that paper-based microfluidics can be used to directly detect ARGs in wastewater, possibly because immature techniques are still in the initial stage of development. Compared with routine microfluidics, the composition of paper-based microfluidics is rich and inexpensive, the materials can be degraded and regenerated as an environmentally friendly material. The paper device can also utilize its own capillary action as a driving force; however, concerns such as low sample utilization, easy leakage, and poor detection effects have also been noted. All in all, with the rapid development of paper-based microfluidic technique, various paper-based microfluidic devices could be fabricated to realize the diversified onsite detection of ARGs in wastewater.

4. Are portable microfluidics ready to detect ARGs in wastewater?

Wastewater contains various pollutants, including bacteria, viruses, heavy metals, and chemicals that can interfere with the detection of ARGs [119]. This, coupled with the low concentration of ARGs themselves in wastewater, increases the difficulty of detection. [120, 121]. In addition, wastewater influents from different sources are often different in nature, adding to the complexity of detection operations and exploration. The perfect way to face these challenges is to develop rapid and simple analytical methods that can achieve the goal of accurate detection [85].

Microfluidics has the potential to be an ideal method to detect ARGs in wastewater, with strong compatibility and a high degree of automation to integrate multiple molecular diagnostic techniques, including isothermal nucleic acid amplification and CRISPR/Cas systems, which are the novel recognition techniques. The small size of microfluidics offers the possibility of portable transportation to the field, directional movement of fluids through micro-channels, delineation of defined areas for reaction, and reading of resultant signals. Compared to the standard qPCR, portable microfluidics, which combines a novel recognition techniques, has the required sensitivity and is even superior to qPCR. Compared with sequencing techniques, microfluidics eliminates the high-precision operation of large instruments in the laboratory and is flexible and versatile for different application scenarios. Therefore, microfluidics that integrates suitable recognition techniques and with rational design has the potential to be applied to the detection of ARGs in wastewater.

Microfluidic detection of ARGs in wastewater requires sample pre-treatment and subsequent analytical steps [122]. The sample preparation step is an essential part of the process. Due to the low concentration of ARGs in wastewater, a large volume of samples (e.g. 200 mL) need to be pre-treated, including concentration, lysis, and purification, which allows for compatible use with microfluidic devices [71]. The current studies for the detection of ARGs in wastewater is mainly implemented outside the microfluidic control for some or all of the operations of the sample preparation step [71, 123]. However, this does not affect the detection of ARGs and the use of

microfluidics [124]. For example, when Shrestha et al. used PCR-based microfluidics for the detection of ARGs in wastewater, 200 mL of samples were concentrated and extracted before detection by microfluidics, and also successfully detected *sulI*, *intI*, and *bla_{CTX-M}* [71]. In addition, with the introduction of isothermal nucleic acid amplification techniques and CRISPR/Cas, ARGs can be amplified and the sensitivity can be increased, whereby the sample volume can be reduced. For example, Mao et al combined CRISPR/Cas12a and LAMP with only 10 mL of wastewater, and it did not affect the analytical performance of microfluidic detection [104].

If the sample preparation steps can be fully integrated into microfluidics, it will shorten detection time, and increase the simplicity and portability of operation. For example, Zong et al. [125] integrated the pre-treatment and subsequent detection into a centrifugal microfluidic platform for the detection of blood-borne infectious diseases, which realized a series of functions, such as separation, adsorption, washing, elution, and detection, and only required 500 μ L of samples, which was easy to operate with high-automation.

There are still some gaps between microfluidics in the detection of ARGs in wastewater and the full-step integration to microfluidics, while this does not hinder the use of microfluidics for the detection of ARGs in wastewater. For example, Shrestha et al. successfully detected *sulI*, *intI* and *bla_{CTX-M}* [71]. The research can be developed towards the full-step integration to microfluidics. It is believed that with the continuous development of microfluidics, the full-step integration to microfluidics can be realized in ARGs detection, and further commercialized products can be formed.

Commercialization is one of the important trends in the development of microfluidic technique [105]. Cepheid has successfully commercialized the combination of microfluidic technique and PCR, producing a series of commercial products represented by GeneXpert [126]. BioFire has also combined multiplex PCR with microfluidic, producing products represented by FilmArray [127]. These are specific examples of successful commercialization of microfluidics, all of which have received emergency use authorization from the U.S. Food and Drug Administration (FDA) for the detection of SARS-CoV-2 [128]. The integration of all sample

preparation and detection steps into microfluidics is one of the critical directions driving the development and successful commercialization of microfluidics. Microfluidic cartridges are an important part of the GeneXpert product and automate sample processing such as cell lysis, nucleic acid extraction, and nucleic acid purification. The qPCR reaction will then be performed in the qPCR reaction tube [126, 129]. Compared to the PCR techniques used in these commercialized products, isothermal nucleic acid amplification is free from temperature cycle, and CRISPR/Cas has high specificity and selectivity. They are new methods that have the potential to replace traditional PCR in specific scenarios, and research has been done to realize their integration into microfluidics for ARGs detection. For example, Meng et al. [67] developed a LAMP-based microfluidic device that successfully detected *mecA*. These studies could provide a solid foundation for future commercialization.

Therefore, let us return to the following question: "Are portable microfluidic devices ready to detect ARGs in wastewater?" The answer is obvious, yes, and we would like to answer it at three levels. First, choosing the right recognition method and designing the right portable microfluidics hold the ability to detect ARGs in wastewater. For example, Sfragano et al. [70] developed a small microfluidic device that can determine *sulI* and *sul4* in wastewater. Second, although few publications on microfluidic techniques for detecting ARGs in wastewater are reported, microfluidic devices are widely applied to detect various targets. We could redesign and optimize these existing microfluidic devices to enable their use for detecting ARGs in wastewater. For example, Chang et al. [130] integrated a centrifugal microfluidic device through a nylon membrane, utilizing a handheld fan and a rechargeable hand warmer as a centrifugal rotor and a reaction heat source, respectively, to rapidly detect live bacteria in wastewater. This microfluidic device can be further applied to detect ARGs in wastewater by borrowing and optimization. Third, limited by the current technique as well as the principle itself, portable microfluidics is not yet able to fully cover the application of traditional PCR or sequencing methods in wastewater analysis, such as the need for gene sequencing for the identification of unknown ARGs. However, even traditional methods have their own unique flaws or shortcomings, and herein we should

hug this novel technique with a tolerant attitude. Studies have also shown that microfluidics can be readily applied to detect ARGs in wastewater and that it is in a rapid development phase, with the potential to be perfected for a wide range of applications.

5. Conclusions and perspectives

In this work, we summarize current status of the portable microfluidic devices for monitoring antibiotic resistance genes in wastewater. The novel recognition techniques for ARGs are isothermal nucleic acid amplification, represented by LAMP and RPA, and the CRISPR/Cas system, represented by Cas9 and Cas12. The former has better sensitivity due to amplification, and the latter has better specificity. The combination of the two can achieve complementarity and improve the analytical performance to a certain extent. These identification techniques have been used in the detection of ARGs and, either alone or in conjunction with other related techniques, for the detection of single ARG or simultaneous detection of multiple ARGs. Furthermore, integrated portable microfluidics have promising applications in detecting ARGs in wastewater because of their own excellent performance. Low-cost and biocompatible paper-based microfluidics can rely on capillary action for fluid flow, but their reproducibility and high-throughput detection capabilities are weak. Nevertheless, we reasonably predict that microfluidic device will play an important role in environmental monitoring in the future and are expected to evolve towards the quantification and onsite detection of ARGs in wastewater.

Although some microfluidic devices have started to be used to detect ARGs in wastewater, the technique is still in its infancy, and most existing studies have been conducted in laboratory and have not been applied on a large scale for the onsite detection of wastewater. Due to wastewater with complex matrices, many unknown contaminants may affect the detection performance of microfluidics, and sufficient stability to detect ARGs in wastewater under different scenarios is lacking. Therefore, when microfluidics is applied to the field of ARG detection in wastewater, future research can focus on the following aspects: (1) developing more specific isothermal

nucleic acid amplification, optimizing CRISPR/Cas, and moving towards highly sensitive and selective recognition to detect ARGs; (2) integrating the detection process into microfluidics in a more complete way and manufacturing portable, high-throughput microfluidic chips (e.g., large volume samples can ultimately enter microfluidic devices directly without the need for additional concentration); (3) Developing microfluidic construction substrates that are low cost and environmentally friendly under the premise of guaranteeing detection performance; and (4) standardizing microfluidic production and detection to reach commercialization, etc. These efforts can be performed using microfluidics for the regular monitoring of ARGs in different scenarios to enable the monitoring of ARGs in wastewater for risk assessments and treatment.

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Declarations

Ethical Approval

Not applicable.

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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Data availability

No datasets were generated or analysed during the current study.

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