微生物学报
Acta Microbiologica Sinica
2024, 64(8): 2591–2609
http://journals.im.ac.cn/actamicrocn
DOI: 10.13343/j.cnki.wsxb.20240111

Special Section 专 论

抗生素抗性基因检测方法发展历程及应用现状

孙凌涛[1,2]，刘子述[1,2]，胡宝兰[1,2*]

1 浙江大学环境与资源学院，浙江 杭州 310058
2 污染环境修复与生态健康教育部重点实验室，浙江 杭州 310058

孙凌涛, 刘子述, 胡宝兰. 抗生素抗性基因检测方法发展历程及应用现状[J]. 微生物学报, 2024, 64(8): 2591-2609.
SUN Lingtao, LIU Zishu, HU Baolan. Development history and current applications of methods for detecting antibiotic resistance genes[J]. Acta Microbiologica Sinica, 2024, 64(8): 2591-2609.

摘 要：耐药性的传播已引起全球广泛关注，抗生素抗性基因(antibiotic resistance genes, ARGs)检测技术的开发是研究 ARGs 在环境-动植物-人群中迁移传播的关键。本文梳理了现有核酸检测技术的发展历程及首次应用于 ARGs 检测的时间节点，并从检测原理、应用优缺点、开发潜力等方面对各技术进行分类综述。在此基础上提出以等温扩增结合 CRISPR/Cas 技术为核心的 ARGs 原位快速检测技术的开发及应用前景展望。本综述旨在回顾各技术发展历程的基础上，为 ARGs 新检测技术的开发及应用提供参考，为耐药性传播的研究及控制提供技术支撑。

关键词：抗生素抗性基因；聚合酶链式反应；基因测序；等温扩增；原位快速检测

Development history and current applications of methods for detecting antibiotic resistance genes

SUN Lingtao[1,2], LIU Zishu[1,2], HU Baolan[1,2*]

1 College of Environmental and Resource Sciences, Zhejiang University, Hangzhou 310058, Zhejiang, China
2 Key Laboratory of Environment Remediation and Ecological Health, Ministry of Education, Hangzhou 310058, Zhejiang, China

Abstract: The spread of antibiotic resistance has aroused global concern. The development of

资助项目：国家自然科学基金(22193061, 22206166)；国家重点研发计划(2020YFC1806903)
This work was supported by the National Natural Science Foundation of China (22193061, 22206166) and the National Key Research and Development Program of China (2020YFC1806903).
*Corresponding author. E-mail: blhu@zju.edu.cn
Received: 2024-02-22; Accepted: 2024-03-22; Published online: 2024-03-28

technologies for detecting antibiotic resistance genes (ARGs) is essential for curbing the migration and spread of ARGs from the environment to plants/animals and human populations. This paper describes the development timeline of existing nucleic acid detection technologies and their first applications to the detection of ARGs and summarizes their detection principles, advantages and disadvantages, and development potential. Furthermore, this paper prospects that isothermal amplification combined with CRISPR/Cas might be the core technology for the development of *in-situ* rapid detection methods. By reviewing the development history of each technology, this paper aims to give insights into the development and applications of technologies for detecting ARGs and provide technical support for the research and control of antibiotic resistance transmission.

Keywords: antibiotic resistance genes; polymerase chain reaction; gene sequencing; isothermal amplification; *in-situ* rapid detection

抗生素广泛应用于人类和动物的疫病预防与治疗[1-2]。过量或未降解的抗生素通过污水、排泄物、废弃物等进入环境，并在养殖场、农田、污水处理设施和制药厂等生境中积累富集[3-5]。在这些生境中，抗生素与其他污染物的选择压力能够诱导产生抗生素抗性基因(antibiotic resistance genes, ARGs)，而 ARGs 进一步通过垂直基因传递(vertical gene transfer, VGT)、水平基因转移(horizontal gene transfer, HGT)等方式增殖转移，在环境-动植物-人群间迁移传播[6-9]。携带 ARGs 的宿主细菌会表达对抗生素的耐受性或抗性，放任其发展的结果是出现“超级细菌”无药可医的困境。抗生素耐药性因此成为最严峻的全球性环境问题之一，联合国环境规划署(UN Environment Programme, UNEP, https://www.unep.org/zh-hans)也呼吁给予其与三重行星危机(气候变化、生物多样性丧失、污染)同等的重视程度。

实现快速、准确的 ARGs 检测为精准防控抗生素耐药性传播的技术支撑。PCR 及相关衍生技术和基因测序技术是当前用于 ARGs 检测的主流技术[10-12]。基于 PCR 的几种技术往往更具靶向性，其中实时荧光定量 PCR (real time quantitative PCR, qPCR)更是凭借其快速、灵敏、可靠等优点成为了检测“金标准”[13-16]；而基因测序能够全面筛查同一样品中的所有已知 ARGs，并且可以获得未知的潜在 ARGs 序列，在 ARGs 的全面性分析中具有优势。然而，这些主流检测方法依赖专用仪器，仅限于实验室分析测试。一些新兴的分子诊断技术也被应用于 ARGs 的检测，例如基于等温扩增和基于成簇的规律性间隔短回文重复序列(clustered regularly interspaced short palindromic repeats, CRISPR)及相关蛋白(CRISPR-associated, Cas)系统的快速检测。然而，新兴技术受经济成本、稳定性、检出限等多方面原因限制，对复杂环境样品的检测能力尚未得到广泛认可，因此总体上仍然缺乏可靠的 ARGs 原位快速检测手段[17]。

本综述对现有核酸检测技术的发明与发展史进行了整理，同时追踪了各技术首次应用于 ARGs 检测的时间节点。采用统一的方式于 Web of Science 平台检索相关文献(表 1)，并在检索结果的参考文献中进一步筛查汇总。对各技术进行分类回顾，结合技术开发领域的发展现状，比较各类技术的检测原理和应用优缺点，以期对未来 ARGs 检测技术的发展及原位快速检测手段的开发提供参考依据。

表 1　核酸检测技术优缺点与首次应用于 ARGs 检测的成果检索词条

Table 1　Advantages and disadvantages of nucleic acid detection technologies and retrieval terms for the initial application in ARGs detection

Type	Technology	Search terms of initial application in ARGs detection	Basic principle	Sensitivity	Specificity	Detection speed	Qualitative/ quantitative	Uniformity of amplicons	Amplification efficiency
PCR-based	PCR	TS=(PCR OR “Polymerase Chain Reaction”) AND (ARG OR “Antibiotic* Resistance Gene*”)	Enzyme-catalyzed	High	High	Moderate	Qualitative	High	High
	qPCR	TS=(qPCR OR “quantitative Polymerase Chain Reaction” OR “quantitative PCR” OR real time PCR) AND (ARG OR “Antibiotic* Resistance Gene*”)	Enzyme-catalyzed, fluorescence	High	High	Moderate	Quantitative	High	High
	HT-qPCR	TS=(HT qPCR OR high throughput quantitative PCR) AND (ARG OR “Antibiotic* Resistance Gene*”)	Enzyme-catalyzed, fluorescence	High	High	Moderate	Quantitative	High	High
	dPCR	TS=(dPCR OR “digital PCR”) AND (ARG OR “Antibiotic* Resistance Gene*”)	Droplet microfluidic, enzyme-catalyzed, fluorescence	Extremely high	High	Moderate	Quantitative	High	High
Sequencing-based	Sanger method	TS=(“Sequencing” OR Sanger method OR dideoxy chain termination method) AND (ARG OR “Antibiotic* Resistance Gene*”)	Short-read, fluorescence	High		Slow	Relatively quantitative		
	Next generation	TS=(Next generation sequencing OR High throughput sequencing OR Metagenomics sequencing OR NGS) AND (ARG OR “Antibiotic* Resistance Gene*”)	Short-read, fluorescence/ chemical signal	High		Slow	Relatively quantitative		
	Third generation	TS=(Single Molecule Sequencing OR Third Generation Sequencing OR SMS OR TGS OR SMART Sequencing OR Nanopore Sequencing) AND (ARG OR “Antibiotic* Resistance Gene*”)	Long-read, fluorescence/ electrical signal	High		Slow	Relatively quantitative		

(待续)

(续表 1)

Type	Technology	Search terms of initial application in ARGs detection	Basic principle	Sensitivity	Specificity	Detection speed	Qualitative/ quantitative	Uniformity of amplicons	Amplification efficiency
Other fluorescence-based	FISH	TS="Fluorescence in situ hybridization" AND (ARG OR "Antibiotic* Resistance Gene*")	Fluorescence	Moderate	Moderate	Moderate	Qualitative/ quantitative		
	DNA microarray	TS=(DNA probe array OR DNA microarray OR gene chip) AND (ARG OR "Antibiotic* Resistance Gene*")	Fluorescence/ isotope	Moderate	Moderate	Moderate	Quantitative		
	Molecular beacon	TS=Molecular beacon AND (ARG OR "Antibiotic* Resistance Gene*")	Fluorescence	High	Moderate	Moderate	Quantitative		
Isothermal amplification and CRISPR/Cas	SDA	TS=Strand Displacement Amplification	Enzyme-catalyzed	Moderate	Moderate	Fast	Quantitative	Low-high	High
	LAMP	TS=Loop-mediated Isothermal Amplification	Enzyme-catalyzed	Moderate	High	Fast	Quantitative	Low	High
	RPA	TS=Recombinase Polymerase Amplification	Enzyme-catalyzed	Moderate	Moderate	Fast	Quantitative	High	High
	RCA	TS=Rolling Circle Amplification	Enzyme-catalyzed	Moderate	Moderate	Fast	Quantitative	Low-high	Low-high
	HDA	TS=Helicase-dependent Amplification	Enzyme-catalyzed	Moderate	Moderate	Fast	Quantitative	High	High
	CRISPR/Cas	TS=CRISPR	Cas protein cleavage	Low	High	Fast	Quantitative		

✉ actamicro@im.ac.cn, ☎ 010-64807516

1 ARGs 检测技术的阶段性发展与分类

基于文献检索结果，整理了各类检测技术的发明时间(图 1)。早在 20 世纪 60 年代末，以同位素标记的原位杂交技术(*in situ* hybridization, ISH)的发明为标志，现代核酸分子生物学检测技术兴起[18]。此后的 20 年间陆续出现了 Sanger 测序、聚合酶链式反应(polymerase chain reaction, PCR)等奠基性的技术，深远地影响了核酸检测领域的后续发展。1990–2010 年间，各类技术大量涌现，这 20 年的技术研发重点一方面是

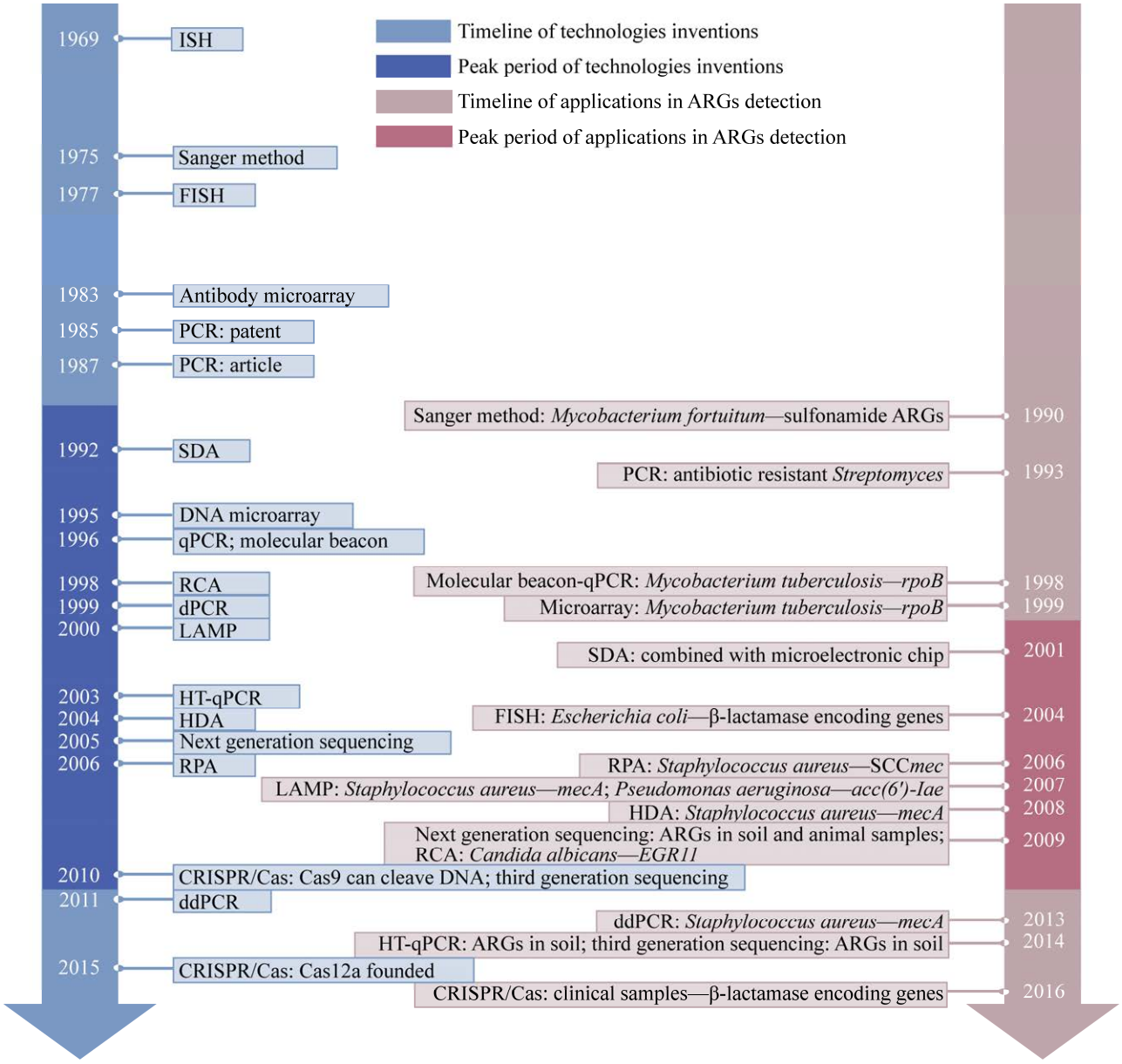

图 1 核酸检测技术发明发展及首次应用于 ARGs 检测时间轴

Figure 1 Timeline of the invention, development, and initial application in ARGs detection of nucleic acid detection technologies.

基于已有技术的优化，例如 PCR 和测序技术的深入开发；另一方面是延续 PCR 的核酸体外扩增技术路线，探寻新的引物设计策略和不同的功能酶以实现各式各样的等温扩增。

在 ARGs 检测方面，各类技术的应用始于 20 世纪 90 年代。同样以 Sanger 法和 PCR 技术为先导，分别对磺胺类 ARGs 和氨基糖苷类 ARGs 进行检测[19-20]。2000–2010 年间是 ARGs 检测技术探索最为活跃的时段，一方面相关技术多集中在 1990–2010 年被发明，经过几年的优化和改进后才被应用于各个领域；另一方面，20 世纪末至 21 世纪初学术界逐渐认识到了抗生素抗性作为全球性环境问题的严峻性，欧盟细菌耐药性监测系统(European antimicrobial resistance surveillance system, EARSS)的建立、世界卫生组织遏制抗生素耐药全球策略(WHO global strategy for containment of antimicrobial resistance)的提出、多方联合“One Health”理念的形成等一系列事件反映了其受公众关注程度，也促使相关研究成为了科研热点，推动 ARGs 检测技术的探索。这一时期检测对象也存在一定的偏好性，*mecA* (一种常见于金黄色葡萄球菌的青霉素结合蛋白编码基因，介导对 β-内酰胺的抗性)、*rpoB* 基因(一种 RNA 聚合酶，为利福平的结合位点，其突变会产生耐药性)、β-内酰胺酶(通过降解失活 β-内酰胺而提供耐药性)编码基因是最常用的检测目标，为后续新技术的应用探索提供了参考[21-23]。

通过 ARGs 检测技术的回顾，根据检测原理与应用普及程度，将涉及到的核酸检测技术分为四大类(图 2)：(1) 检测“金标准”——PCR 及衍生技术；(2) 全面性检测技术——基因测序；(3) 新兴检测技术——等温扩增和 CRISPR/Cas；(4) 其他基于荧光策略的技术。对每个类别的技术发展进行了更为详细的阐释，并介绍其原理、优缺点及应用现状(表 1)。

2 检测金标准——PCR 及衍生技术

PCR 技术衍生种类众多，在 ARGs 检测领域应用广泛。对近些年所发表文献的粗略统计显示，PCR 及其衍生技术在 90%以上的 ARGs 靶向研究中起到关键作用，在预扩增、检测、验证等流程中被频繁使用。PCR 发明于 1985 年，其发明者 Kary Banks Mullis 在 2 年后的一篇文章中正式介绍其原理：通过设定的热循环，在 DNA 聚合酶的作用下，经过变性、退火、延伸等步骤实现对靶标的指数扩增，理论上经过 35 个循环可以将靶标浓度扩增至初始浓度的 10^{10} 倍，然而实际情况下会因为反应抑制而降低扩增效率[24-26]。Webb 等[20]首次将该技术引入 ARGs 检测，他们以琼脂糖凝胶电泳为下游分析手段，定性检测了包含氨基糖苷转移酶 ARGs *aph3″* 的链霉菌(*Streptomycete*) 16S rRNA 基因的扩增子。PCR 技术无法单独做到定量检测，通常被用于提高靶标 ARGs 浓度，联用其他技术从而进一步分析[27]。为了突破这一限制，Heid 等[28]于 1996 年开发了 qPCR，该技术以荧光基团或荧光探针为表征手段，直接将扩增产物的浓度可视化。Piatek 等[29]首次利用该技术分析结核分枝杆菌(*Mycobacterium tuberculosis*)中利福平 ARGs *rpoB* 的突变。qPCR 可在 2 h 左右实现 DNA 样品中个位数 copies/μL 的 ARGs 精准定量，综合检测能力极佳，是 DNA 检测的“金标准”[30-33]。

此类技术主要向高通量和高灵敏度 2 个方向进一步发展。多重 qPCR 和高通量 qPCR (high-throughput qPCR, HT-qPCR)被开发以提高检测通量，然而针对不同基因采用相同的反应条件以及更复杂或更小的反应体系也在一定

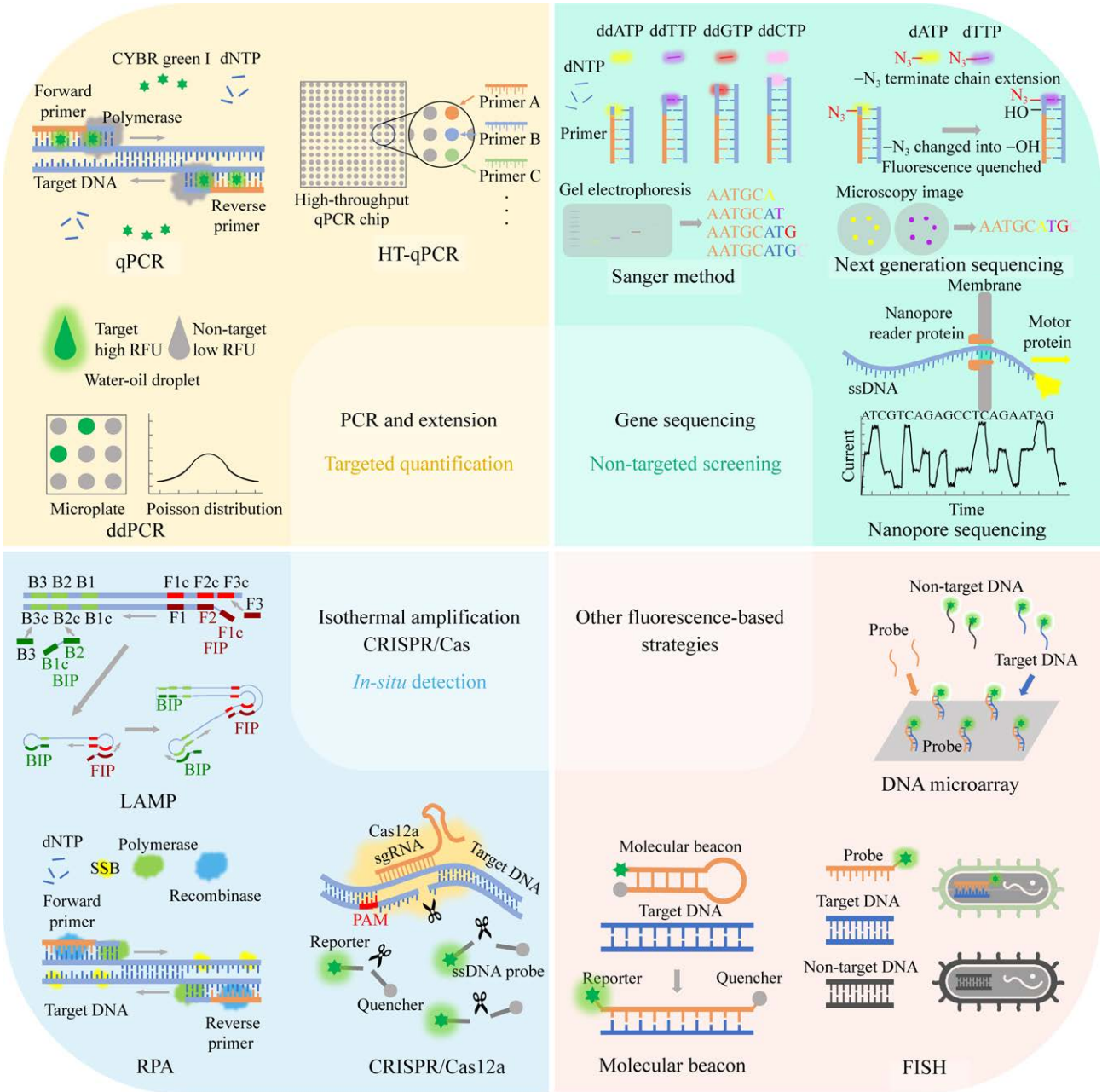

图 2　ARGs 检测技术分类及原理示意图

Figure 2　Classification of ARGs detection technologies and illustration of principles.

程度上降低了扩增效率和检测灵敏度[34]。早在 2003 年 HT-qPCR 便已问世[35]，然而直到 2014 年 Wang 等[36]才首次利用 HT-qPCR 技术检测 ARGs，该研究采用了 295 对引物，对再生水灌溉的公园土壤中氨基糖苷类、β-内酰胺类等主要 ARGs 进行了同步定量。HT-qPCR 一般只能相对 16S rRNA 基因定量，或通过 16S rRNA 基因的测定实现 ARGs 的间接绝对定量[37]。Pärnänen 等采用 HT-qPCR 对城镇污水处理厂污水中的 ARGs 进行间接绝对定量，认为

20 copies/μL 是该方法可以检测到的最低极限浓度[38]。在高灵敏度方面，2011 年发明的微滴式数字 PCR (droplet digital PCR, ddPCR)是典型代表。其基本原理是在成千上万的油包水小液滴中进行微体系 PCR 反应，每个体系可以达到纳升甚至皮升级别，由于阳性液滴数量与样本中靶标基因浓度符合泊松分布，因此基于对小液滴的阳性判读可以输出定量结果[39]。该技术 2 年后便被应用于 ARGs 检测，Kelley 等[40]利用双重 ddPCR 技术检测了 397 个临床样本中金黄色葡萄球菌(*Staphylococcus aureus*)的甲氧西林 ARGs *mecA*，其临床敏感性和特异性都与 qPCR 相当。Cavé 等[41]将 qPCR 和 ddPCR 在相同条件下进行比较，结果显示 ddPCR 的检测限比 qPCR 低一个数量级。此外，新型荧光探针开发也能够进一步提高基于 qPCR 技术对低浓度 ARGs 的检测能力。

PCR 类技术的应用主要局限于靶标的定量检测，其他功能仍有待开发。例如，宏基因测序能够同时获得样本中物种、其他基因、可移动遗传元件(mobile genetic elements, MGEs)等信息，而 PCR 类技术要想获得这些信息则需要花费成倍的时间、经济和人力成本。Xie 等[42]开发了一种基于 HT-qPCR 的毒力因子基因(virulence factor genes, VFGs)高通量检测芯片 VFG-Chip，或许可以基于此进一步实现不依赖测序的 VFGs 和 ARGs 高通量同步检测。此外，重金属抗性基因芯片与 ARGs 芯片的结合也可以为相关研究提供便利[43]。然而乳液、配对分离和串联 PCR (emulsion, paired isolation and concatenation PCR, epicPCR)则可以将 ARGs 与未培养宿主关联起来分析，进一步开展 ARGs 溯源、风险评估等工作[44]。相关手段的研发有助于 PCR 类技术在 ARGs 研究领域更多样化地应用。

3 全面性检测技术——基因测序

测序技术能够实现对样本中所有碱基的读取，这种特性使其在 ARGs 筛查方面具有明显的技术优势。最早发明的 Sanger 测序法使用了双脱氧核苷酸(dideoxy-ribonucleoside triphosphate, ddNTP)，−OH 基团的缺失使下一个脱氧核苷酸(deoxy-ribonucleoside triphosphate, dNTP)无法进一步结合，因此链延伸会在结合 ddNTP 的位置终止。反应在 4 个体系中进行，每个体系都包含 4 种 dNTP、1 种 ddNTP 以及 DNA 聚合酶，与引物结合的待测模板单链在其中延伸，在结合 ddNTP 后终止，得到长短不一的产物。由于每种 ddNTP 都被不同颜色的荧光标记，根据凝胶电泳分析结果可以推断出对应长度位置上的碱基[45-46]。早在 1990 年，Sanger 测序法就被 Martin 等[19]成功用于偶发分枝杆菌(*Mycobacterium fortuitum*)的磺胺类药物 ARGs 检测，并证实了 Tn610 转座子中包含整合酶编码基因 *orf2M* 和磺胺类 ARGs *sul3*。Sanger 法后来也被称作一代测序，得益于其精确性和经济性，目前仍有应用[47-48]。

2005 年 Roche 公司发布了第一台二代测序仪 454 GS20，标志着二代测序的诞生[49]。二代测序通过 dNTP 的可逆修饰，赋予了其可控的终止末端，能够实现边合成边测序，因此大大提高了测序通量。2009 年，Allen 等[50-51]尝试将二代测序应用于 ARGs 研究，分析了土壤中的 β-内酰胺酶基因和舞毒蛾(*Lymantria dispar*)幼虫肠道微生物群落的 ARGs，证明了二代测序在复杂环境和生物样本中 ARGs 检测的独特优势。基于二代测序的宏基因组测序可以分析包括环境、动物、反应器等多种样品中的宏基因信息，是混菌体系基因研究最常用的方法之一[52-54]。它不仅可以注释成百上千种 ARGs，还能识别上下游遗传信息并筛查 MGEs，以评估 ARGs

的传播风险[55-58]。根据 Illumina 公司(https://www.illumina.com.cn)提供的参数，旗下的各类平台二代测序耗时 4−55 h 不等，序列读长在 150−300 bp。相较于一代测序，二代测序大幅缩减了获得同等数据量情况下的检测耗时，但一代测序约 1 000 bp 的序列读长具有更高的精度，因此 2 种技术并不存在明显的优劣。

2010 年，Flusberg 等[59]发明了基于荧光信号读取的三代测序技术——PacBio SMRT (single molecule real-time)，这是一种利用甲基化修饰 dNTP 产生不同特征荧光信号从而识别序列的长读长测序手段。另一种常用的三代测序技术是 Oxford Nanopore，该技术的核心原理是基于不同碱基产生不同的电信号实现序列读取。2014 年，Wichmann 等[60]结合功能宏基因组学和 BioPac SMRT 分析了奶牛粪便中的 ARGs，大大扩展了动物肠道菌群的功能性 ARGs 清单，同时也为三代测序在该领域的应用提供了借鉴。长读长测序一方面能够大量保存基因的上下游信息，另一方面显著减少了 reads 拼接过程中出现的错误和遗漏。PacBio SMRT 的平均读长为 13.5 kb，而 Oxford Nanopore 最大读长超过 4 Mb，相较于前两代测序展现出极大的优势[61-63]。

非靶向筛查的特性赋予测序技术独特的优势。例如远超 HT-qPCR 的通量，调控基因和遗传元件的同步分析等。与其他靶向检测手段相比，测序也更适合探索新 ARGs。功能宏基因组学方法是目前新 ARGs 发掘最常用的技术之一，其核心思想是首先提取环境样品中的全部 DNA 并剪切、导入至模式菌构建文库，随后通过扩大培养、药理生化筛选等手段获得具有特定功能的菌株(如耐药菌)，最后对获得的功能菌株进行测序及基因功能分析验证[64-65]。然而，我们仍然不能忽视测序类技术的一些弊端，例如不能像 qPCR 那样做到绝对定量、单靶检测成本高以及检测耗时长等[66]。

测序技术实现了极高的检测通量和全面的非靶向筛查，但却无法兼顾节省时间和经济成本，弥补这方面的弊端有利于满足更多需求[67]。除了原理导致的反应流程缓慢，庞大的宏基因数据分析也会占用大量的时间。数据库与分析手段的选择极为重要，通过数据库的更新和算法的优化也可以节省该流程的时间，并且可以为研究者提供更有价值的信息[68]。此外，测序类技术不像 PCR 可以直接实现样品中 ARGs 的绝对定量检测，推出一种易于使用的、广泛认可的绝对定量方法也是测序技术发展的趋势之一[69-70]。

4 新兴检测技术——等温扩增和 CRISPR/Cas

常规的 PCR 和测序等由于对高温、控温仪器的依赖而局限于实验室分析，新兴的等温扩增类技术有望突破该瓶颈。等温扩增与 PCR 最大的区别在于模板链的解旋、引物的结合与延伸都可以在同一恒定温度下实现，降低了对控温仪器的依赖性[71]。由于反应在蛋白/酶的介导下直接进行，避免了反复的升温与降温程序，从而缩短了检测耗时[72]。此类技术起步相对较晚，1992 年 Walker 等[73]发明的链置换扩增技术(strand displacement amplification, SDA)是最早出现的等温扩增技术之一，其链置换原理在其他等温扩增技术中也有体现。2000–2010 年是等温扩增技术发明与应用于 ARGs 检测的高峰期，环介导等温扩增(loop-mediated isothermal amplification, LAMP)、解旋酶依赖性等温扩增(helicase-dependent isothermal amplification, HDA)、重组酶聚合酶扩增(recombinase polymerase amplification, RPA)等相继出现[74-76]。这 3 项技术在 ARGs 的首次应用集中在 2006–2008 年，而

且都选取了耐甲氧西林金黄色葡萄球菌(methicillin-resistant *Staphylococcus aureus*, MRSA)作为研究对象[76-78]。

LAMP 是最为成熟和广泛运用的等温扩增技术之一，其原理是利用 4 个不同的引物扩增原始模板。其中有 2 个特殊的引物，称作前导内引物(forward inner primer, FIP)和后随内引物(backward inner primer, BIP)，它们的 5′端片段可以与引物下游的序列互补，从而使初始扩增子形成双端茎环结构的单链，并以此为循环的起点进行扩增，循环产物为长短不一的茎环结构 DNA 混合物[74]。该技术能够在 55−70 ℃的条件下实现个位数 copies/μL 基因的 1 h 以内快速检测，相较于 PCR 具有更强的扩增能力，针对 6 段序列设计的 4 个引物虽然增加了设计难度，却也提供了较强的特异性[79]。Suea-Ngam 等[80]将 LAMP 与银纳米板结合，检测 MRSA 中的 *mecA* 并通过智能手机读取信号，在 30 min 内精准定量了个位数 copies/μL 的靶标，显示出了 LAMP 在场地快检的巨大潜力。作为一种新兴的等温扩增技术，RPA 在近几年也备受关注。在 RPA 反应中，重组酶与引物的复合体识别双链 DNA 上的同源序列并入侵、打开双螺旋结构，单链 DNA 结合蛋白负责稳定解旋后的 D 环结构，随后由 DNA 聚合酶延伸引物完成扩增[81]。Butterworth 等[82]利用直接标记的固相 RPA 检测大肠埃希氏菌(*Escherichia coli*)中的苯唑西林 ARGs，虽然在这项研究中并未优化方法，却也能在 1 h 内实现个位数 copies/μL ARGs 的检测。RPA 的优势在于快速、低温的反应特性(10−60 min, 37−42 ℃)，然而蛋白/酶的成本和保存等问题在一定程度上限制了该技术的应用[76]。Wang 等[83]通过冻干策略使试剂便于保存，但也略微损害了蛋白/酶的活性。

CRISPR/Cas 技术同样可以实现低温恒温条件下的 ARGs 检测。CRISPR/Cas 是细菌免疫系统的一部分，具有强大的基因靶向识别和编辑能力。CRISPR 是发现于原核生物基因组的特殊序列，通过编码功能性的 Cas 蛋白可以实现对外源基因的识别、整合及破坏。2010 年，Garneau 等[84]使用 CRISPR/Cas9 实现了 DNA 双链的特异性切割，该技术迅速发展为强大的基因编辑手段。在 CRISPR/Cas9 体系中，一条人工设计合成的、与靶标 DNA 互补的单导 RNA (single-guide RNA, sgRNA)和 Cas9 蛋白形成复合体，该复合体将特异性识别并结合至双链 DNA 靶标，随后在 Cas9 蛋白的顺式切割(*cis*-cleavage)活性作用下切断特定位点[85]。2016 年，Müller 等[86]结合 CRISPR/Cas9 和 DNA 光学图谱检测临床菌样品中的超广谱 β-内酰胺酶编码基因 $bla_{\text{CTX-M-15}}$ 和碳青霉烯酶编码基因 bla_{KPC}、bla_{NDM}，显示出了 CRISPR/Cas 体系在 ARGs 检测领域的巨大潜力。随着越来越多的 Cas 蛋白被发现，CRISPR/Cas 的应用领域也不断拓宽。Cas12、Cas13 等蛋白还具有额外的反式切割(*trans*-cleavage)活性。对于 Cas12，该活性表现为无差别切割附近所有的游离单链 DNA，而对于 Cas13 则切割单链 RNA[87-88]。反式切割活性只有在 sgRNA-Cas 蛋白复合物结合至靶标后才会激活，因此向反应体系中加入基于荧光共振能量转移(fluorescence resonance energy transfer, FRET)原理设计的单链荧光探针，若探针被切断，荧光信号产生，则说明样品中含有靶标 DNA，其浓度在一定范围内与荧光值成正比，由此实现基于 CRISPR/Cas 的核酸检测方法[89-90]。CRISPR/Cas 技术的检测限欠佳但特异性强，使用等温扩增技术预先扩增靶标可以降低整体的检测限[91-92]。Aggarwal 等[93]使用 HDA 结合 CRISPR/Cas12a 可以实现 1 h 检测 0.6 copies/μL 的 DNA 靶标，在低至 0.6 CFU/μL

的肺炎克雷伯氏菌(*Klebsiella pneumoniae*)中检测到碳青霉烯 ARGs bla_{KPC}。CRISPR/Cas 在 ARGs 检测领域尚未普及，但针对其他靶标开发的检测技术已有不少报道。例如，Gootenberg 等[94]基于 LAMP 和 CRISPR/Cas13a 开发的特异性高灵敏度酶报告基因解锁(specific high-sensitivity enzymatic reporter unlocking, SHERLOCK)用于多种病毒和病原菌的检测；Chen[92]基于 RPA 和 CRISPR/Cas12a 开发的 DNA 核酸内切酶靶向 CRISPR 反式报告基因(DNA endonuclease-targeted CRISPR trans reporter, DETECTR)用于人乳头瘤病毒(human papillomavirus, HPV)的检测；Hu 等[95]基于 RPA 和 CRISPR/Cas12a 开发的光活化单管法用于多种病毒的检测。

直接在现有等温扩增及 CRISPR/Cas 等技术的基础上进行二次开发和优化是实现 ARGs 原位快速检测最高效的方式之一。等温扩增技术众多且发展历史短，在检测速度、稳定性、灵敏度和特异性方面仍有较大优化空间。与探针法 qPCR 类似，等温扩增可以结合形态功能各异的探针来优化检测效果[96-97]。针对不同的应用选用不同的等温扩增技术可以更好地满足检测需求，例如在靶标序列可以满足复杂引物设计的情况下，选取 LAMP 获得较强的特异性，或在低温应用场景中采取反应温度更低的 RPA。虽然 CRISPR/Cas 也能在恒温条件下完成检测反应，其检出限(6×10^5–3×10^9 copies/μL)却无法满足大多数场景的应用[98]。等温扩增技术与 CRISPR/Cas 的结合是一种极具潜力的技术组合，靶标的扩增保证了方法的灵敏度，而 CRISPR/Cas 对靶标的识别则保证了特异性。若将等温扩增与 CRISPR/Cas 反应同步进行，有利于简化流程并缩短整体反应时间。然而，混合反应体系中组分之间的不良竞争是必须突破的技术瓶颈，引物与 sgRNA-Cas 复合物会同时竞争靶标导致整体检测效果受到影响，并且 Cas12 蛋白还会通过反式切割活性切断引物直接降低扩增效率[99]。为了缓解这种不利影响进行了诸多探索，大多以降低 CRISPR/Cas 反应活性为核心思想，例如采用无切割活性的 dCas9 (dead Cas9)蛋白，或基于次优原型间隔子相邻基序(protospacer adjacent motif, PAM)策略或光笼化的 sgRNA 对 CRISPR/Cas 反应进行抑制或沉默[85,95,98]。等温扩增和 CRISPR/Cas 在反应条件和反应速度方面有着天然的优势，通过微流控芯片、侧流试纸等商业化产品的研发和推广，这类技术有望实现 ARGs 的原位快速检测[100-101]。此外也应关注 DNA 快速提取技术的进步，例如热裂解、纸基材料等；而免去 DNA 提取直接对样品进行检测同样可以缩短分析周期，但这种方法对样品的纯度和检测技术的可靠性也提出了巨大的挑战[83,102]。

实现原位快速检测是当下 ARGs 检测最大的技术需求之一，这意味着在现有技术的基础上同时突破灵敏度和检测分析速度的瓶颈。根据样品特点、检测条件等选取合适的等温扩增方法，结合 CRISPR/Cas 反应体系构建电化学或荧光表征方法，最有望满足该需求。然而要真正实现在各类复杂环境样品中精准、稳定地检测特定 ARGs，不仅需要在反应体系和原理上不断优化与改进，还需要及时开发高效便捷的核酸提取技术和研发配套检测设备，以进一步助推新技术的应用与普及。

5 其他基于荧光策略的技术

根据本综述分类筛选方法，还有一些技术与前文所述 3 种方法的技术原理有较大差别，但都结合了荧光表征手段，因此统一归为其他基于荧光策略的技术，以下作简单介绍。

荧光原位杂交(fluorescence *in situ* hybridization,

FISH)技术发明于 1977 年，该技术通过荧光标记的探针与互补序列结合，从而将靶标可视化，其前身为 1969 年发明的同位素标记原位杂交技术[18,103]。2004 年，Zwirglmaier 等[104]开发了识别单个基因技术(recognition of individual genes FISH, RING-FISH)，利用这种改进的 FISH 对大肠埃希氏菌(*Escherichia coli*)中的 ARGs β-内酰胺酶进行了检测，灵敏度可以达到<10 copies/cell。FISH 适合对靶标基因进行定位、追踪或对微生物进行鉴定，在 ARGs 定量检测方面应用较少。

1995 年，Schena 等[105]发明了 DNA 微阵列(DNA microarray)技术，具有快速、高通量、多靶标等特点。该技术预先使用荧光基团修饰样本中的核酸，并将大量特异性探针固定在微小的芯片上，通过探针捕获互补的靶标实现特定基因的可视化。1999 年，2 个科研团队先后使用了该技术检测结核分枝杆菌(*Mycobacterium tuberculosis*)中利福平 ARGs *rpoB* 的突变，证明了该技术的 ARGs 检测应用能力[106-107]。Patterson 等[108]利用 DNA 微阵列从土壤和动物粪便样品中检测出 23 种四环素类 ARGs 和 10 种红霉素 ARGs。DNA 微阵列在检测灵敏度方面没有优势，只能检测到 10^2 copies/μL 的 ARGs，此外探针固定、荧光修饰等烦琐处理步骤也限制了该技术的普及[109]。

荧光探针是一种特殊的检测手段，可以与前文所述的许多技术相结合。荧光探针一般由特异性核酸序列、荧光基团、淬灭基团构成，通过靶向识别、结构改变或破坏而产生荧光信号。分子信标(molecular beacon, MB)是一种基于 FRET 原理设计的茎环结构荧光探针，当它与靶标杂交后，茎环结构打开，荧光基团与淬灭基团空间距离增大从而产生荧光信号[110]。分子信标最常联用的技术是 qPCR。1998 年，Piatek 等[29]利用分子信标与 qPCR 的联合方法分析结核分枝杆菌(*Mycobacterium tuberculosis*)中利福平 ARGs *rpoB* 的突变，是分子信标与 qPCR 首次在 ARGs 检测领域的应用。Singh 等[111]同样使用分子信标与 qPCR 相结合的方法检测地表水中的 β-内酰胺 ARGs，灵敏度可以达到 10 copies/μL。

6 总结与展望

综上可见，现有的常用 ARGs 检测技术尚存在优化与提升空间。检测“金标准” qPCR 具有出色的综合检测能力，可以在约 2 h 定量个位数 copies/μL 的 ARGs，但检测依赖高精密度控温变温设备。测序类技术的非靶标筛查和未知基因探索功能使其在短时间内成为 ARGs 检测的主流技术之一，不可忽视的是这类技术具有相对较高的时间和经济成本，以及在 ARGs 绝对定量方面的阻力。等温扩增类技术可以在保证个位数 copies/μL 灵敏度的基础上将时间缩短至 1 h 以内，但也一定程度上在特异性、准确性和稳定性方面作出了让步。CRISPR/Cas 少于 30 min 的反应时间使其在检测速度上具有极大的优势，其灵敏度却不够理想。虽然条件的优化可以在一定程度上弥补检测效果上的不足，但技术的突破需要依赖原理层面的改进，因此在未来的技术开发中可以侧重于新型探针的研发、功能蛋白的发掘、信号表征手段的探索等。

现有常用技术尚未同时实现 ARGs 快速且灵敏的定量检测、难以满足原位快速检测的需求，等温扩增和 CRISPR/Cas 技术最有望填补该空白。一方面，相近的反应速度和反应温度保证了技术耦合的可行性；另一方面，等温扩增提高了检测的灵敏度，CRISPR/Cas 则提供了高特异性的信号表征手段。如何实现单管同步扩增-检测是该方法进一步优化的主要方向，而其关键技术瓶颈是两种体系之间的不良竞争。研

究报道通过降低 CRISPR/Cas 反应活性[85,95,98]，以及采用空间/相分离等物理隔离方式[112-114]，可以有效提高整体的检测能力。诸多尝试的初步成功支持了等温扩增和CRISPR/Cas在原位快速检测领域的快速发展，而真正的普及应用则仍需通过实验室的优化论证以及商业化的开发生产来实现，这也是相关科研工作者以后需要重点关注的探索方向。

参考文献

[1] HUTCHINGS MI, TRUMAN AW, WILKINSON B. Antibiotics: past, present and future[J]. Current Opinion in Microbiology, 2019, 51: 72-80.

[2] RIBEIRO Da CUNHA B, FONSECA LP, CALADO CRC. Antibiotic discovery: where have we come from, where do we go?[J]. Antibiotics, 2019, 8(2): 45.

[3] LYU J, YANG LS, ZHANG L, YE BX, WANG L. Antibiotics in soil and water in China: a systematic review and source analysis[J]. Environmental Pollution, 2020, 266(Pt 1): 115147.

[4] ZHU YG, ZHAO Y, LI B, HUANG CL, ZHANG SY, YU S, CHEN YS, ZHANG T, GILLINGS MR, SU JQ. Continental-scale pollution of estuaries with antibiotic resistance genes[J]. Nature Microbiology, 2017, 2: 16270.

[5] KARKMAN A, PÄRNÄNEN K, JOAKIM LARSSON DG. Fecal pollution can explain antibiotic resistance gene abundances in anthropogenically impacted environments[J]. Nature Communications, 2019, 10: 80.

[6] SMITH WPJ, WUCHER BR, NADELL CD, FOSTER KR. Bacterial defences: mechanisms, evolution and antimicrobial resistance[J]. Nature Reviews Microbiology, 2023, 21: 519-534.

[7] MA YY, RAMONEDA J, JOHNSON DR. Timing of antibiotic administration determines the spread of plasmid-encoded antibiotic resistance during microbial range expansion[J]. Nature Communications, 2023, 14: 3530.

[8] WEI ZY, SHEN WL, FENG K, FENG YZ, HE ZL, LI Y, JIANG CY, LIU SJ, ZHU YG, DENG Y. Organic fertilizer potentiates the transfer of typical antibiotic resistance gene among special bacterial species[J]. Journal of Hazardous Materials, 2022, 435: 128985.

[9] XIE JW, JIN L, HE TT, CHEN BW, LUO XS, FENG BH, HUANG W, LI J, FU PQ, LI XD. Bacteria and antibiotic resistance genes (ARGs) in $PM_{2.5}$ from China: implications for human exposure[J]. Environmental Science & Technology, 2019, 53(2): 963-972.

[10] FITZPATRICK KJ, ROHLF HJ, SUTHERLAND TD, KOO KM, BECKETT S, OKELO WO, KEYBURN AL, MORGAN BS, DRIGO B, TRAU M, DONNER E, DJORDJEVIC SP, de BARRO PJ. Progressing antimicrobial resistance sensing technologies across human, animal, and environmental health domains[J]. ACS Sensors, 2021, 6(12): 4283-4296.

[11] VASALA A, HYTÖNEN VP, LAITINEN OH. Modern tools for rapid diagnostics of antimicrobial resistance[J]. Frontiers in Cellular and Infection Microbiology, 2020, 10: 308.

[12] BOOLCHANDANI M, D'SOUZA AW, DANTAS G. Sequencing-based methods and resources to study antimicrobial resistance[J]. Nature Reviews Genetics, 2019, 20: 356-370.

[13] HONG B, BA YB, NIU L, LOU F, ZHANG ZH, LIU HQ, PAN YJ, ZHAO Y. A comprehensive research on antibiotic resistance genes in microbiota of aquatic animals[J]. Frontiers in Microbiology, 2018, 9: 1617.

[14] LI B, YAN T. Next generation sequencing reveals limitation of qPCR methods in quantifying emerging antibiotic resistance genes (ARGs) in the environment[J]. Applied Microbiology and Biotechnology, 2021, 105(7): 2925-2936.

[15] WANG Z, HAN MZ, LI EH, LIU X, WEI HM, YANG C, LU SY, NING K. Distribution of antibiotic resistance genes in an agriculturally disturbed lake in China: their links with microbial communities, antibiotics, and water quality[J]. Journal of Hazardous Materials, 2020, 393: 122426.

[16] WU DL, ZHANG M, HE LX, ZOU HY, LIU YS, LI BB, YANG YY, LIU CX, HE LY, YING GG. Contamination profile of antibiotic resistance genes in ground water in comparison with surface water[J]. The Science of the Total Environment, 2020, 715: 136975.

[17] MAO K, ZHANG H, RAN F, CAO HR, FENG RD, DU W, LI XQ, YANG ZG. Portable biosensor combining CRISPR/Cas12a and loop-mediated isothermal amplification for antibiotic resistance gene *ermB* in wastewater[J]. Journal of Hazardous Materials, 2024, 462: 132793.

[18] PARDUE ML, GALL JG. Molecular hybridization of radioactive DNA to the DNA of cytological

preparations[J]. Proceedings of the National Academy of Sciences of the United States of America, 1969, 64(2): 600-604.

[19] MARTIN C, TIMM J, RAUZIER J, GOMEZ-LUS R, DAVIES J, GICQUEL B. Transposition of an antibiotic resistance element in mycobacteria[J]. Nature, 1990, 345: 739-743.

[20] WEBB V, DAVIES J. Antibiotic preparations contain DNA: a source of drug resistance genes?[J]. Antimicrobial Agents and Chemotherapy, 1993, 37(11): 2379-2384.

[21] ALEXANDER JAN, WORRALL LJ, HU JH, VUCKOVIC M, SATISHKUMAR N, POON R, SOBHANIFAR S, ROSELL FI, JENKINS J, CHIANG D, MOSIMANN WA, CHAMBERS HF, PAETZEL M, CHATTERJEE SS, STRYNADKA NCJ. Structural basis of broad-spectrum β-lactam resistance in *Staphylococcus aureus*[J]. Nature, 2023, 613: 375-382.

[22] RASOULY A, SHAMOVSKY Y, EPSHTEIN V, TAM K, VASILYEV N, HAO ZT, QUARTA G, PANI B, LI LT, VALLIN C, SHAMOVSKY I, KRISHNAMURTHY S, SHTILERMAN A, VANTINE S, TORRES VJ, NUDLER E. Analysing the fitness cost of antibiotic resistance to identify targets for combination antimicrobials[J]. Nature Microbiology, 2021, 6: 1410-1423.

[23] WILAMOWSKI M, SHERRELL DA, KIM Y, LAVENS A, HENNING RW, LAZARSKI K, SHIGEMOTO A, ENDRES M, MALTSEVA N, BABNIGG G, BURDETTE SC, SRAJER V, JOACHIMIAK A. Time-resolved β-lactam cleavage by L1 metallo-β-lactamase[J]. Nature Communications, 2022, 13(1): 7379.

[24] MULLIS KB. Process for amplifying nucleic acid sequences: US4683202[P]. 1987-07-28.

[25] MULLIS KB, FALOONA FA. Specific synthesis of DNA *in vitro via* a polymerase-catalyzed chain reaction[J]. Methods in Enzymology, 1987, 155: 335-350.

[26] ARYA M, SHERGILL IS, WILLIAMSON M, GOMMERSALL L, ARYA N, PATEL HRH. Basic principles of real-time quantitative PCR[J]. Expert Review of Molecular Diagnostics, 2005, 5(2): 209-219.

[27] WU YC, DONG N, CAI C, ZENG Y, LU JY, LIU CC, WANG HY, ZHANG YY, HUANG L, ZHAI WS, SHAO DY, LI RC, LIU DJ, CHEN S, ZHANG R. *Aeromonas* spp. from hospital sewage act as a reservoir of genes resistant to last-line antibiotics[J]. Drug Resistance Updates: Reviews and Commentaries in Antimicrobial and Anticancer Chemotherapy, 2023, 67: 100925.

[28] HEID CA, STEVENS J, LIVAK KJ, WILLIAMS PM. Real time quantitative PCR[J]. Genome Research, 1996, 6(10): 986-994.

[29] PIATEK AS, TYAGI S, POL AC, TELENTI A, MILLER LP, KRAMER FR, ALLAND D. Molecular beacon sequence analysis for detecting drug resistance in *Mycobacterium tuberculosis*[J]. Nature Biotechnology, 1998, 16: 359-363.

[30] ZHANG ZH, LI X, LIU H, ZAMYADI A, GUO WS, WEN HT, GAO L, NGHIEM LD, WANG QL. Advancements in detection and removal of antibiotic resistance genes in sludge digestion: a state-of-art review[J]. Bioresource Technology, 2022, 344(Pt A): 126197.

[31] CACACE D, FATTA-KASSINOS D, MANAIA CM, CYTRYN E, KREUZINGER N, RIZZO L, KARAOLIA P, SCHWARTZ T, ALEXANDER J, MERLIN C, GARELICK H, SCHMITT H, de VRIES D, SCHWERMER CU, MERIC S, OZKAL CB, PONS MN, KNEIS D, BERENDONK TU. Antibiotic resistance genes in treated wastewater and in the receiving water bodies: a pan-European survey of urban settings[J]. Water Research, 2019, 162: 320-330.

[32] PAULUS GK, HORNSTRA LM, ALYGIZAKIS N, SLOBODNIK J, THOMAIDIS N, MEDEMA G. The impact of on-site hospital wastewater treatment on the downstream communal wastewater system in terms of antibiotics and antibiotic resistance genes[J]. International Journal of Hygiene and Environmental Health, 2019, 222(4): 635-644.

[33] ROSSI F, PÉGUILHAN R, TURGEON N, VEILLETTE M, BARAY JL, DEGUILLAUME L, AMATO P, DUCHAINE C. Quantification of antibiotic resistance genes (ARGs) in clouds at a mountain site (puy de Dôme, central France)[J]. The Science of the Total Environment, 2023, 865: 161264.

[34] KEENUM I, LIGUORI K, CALARCO J, DAVIS BC, MILLIGAN E, HARWOOD VJ, PRUDEN A. A framework for standardized qPCR-targets and protocols for quantifying antibiotic resistance in surface water, recycled water and wastewater[J]. Critical Reviews in Environmental Science and Technology, 2022, 52(24): 4395-4419.

[35] BOOKOUT AL, MANGELSDORF DJ. Quantitative real-time PCR protocol for analysis of nuclear receptor

signaling pathways[J]. Nuclear Receptor Signaling, 2003, 1: e012.

[36] WANG FH, QIAO M, SU JQ, CHEN Z, ZHOU X, ZHU YG. High throughput profiling of antibiotic resistance genes in urban park soils with reclaimed water irrigation[J]. Environmental Science & Technology, 2014, 48(16): 9079-9085.

[37] TAN YL, CAO XD, CHEN SJ, AO XL, LI JL, HU KD, LIU SL, PENTTINEN P, YANG Y, YU XM, LIU AP, LIU CX, ZHAO K, ZOU LK. Antibiotic and heavy metal resistance genes in sewage sludge survive during aerobic composting[J]. The Science of the Total Environment, 2023, 866: 161386.

[38] PÄRNÄNEN KMM, NARCISO-DA-ROCHA C, KNEIS D, BERENDONK TU, CACACE D, DO TT, ELPERS C, FATTA-KASSINOS D, HENRIQUES I, JAEGER T, KARKMAN A, MARTINEZ JL, MICHAEL SG, MICHAEL-KORDATOU I, O'SULLIVAN K, RODRIGUEZ-MOZAZ S, SCHWARTZ T, SHENG HJ, SØRUM H, STEDTFELD RD, et al. Antibiotic resistance in European wastewater treatment plants mirrors the pattern of clinical antibiotic resistance prevalence[J]. Science Advances, 2019, 5(3): eaau9124.

[39] HINDSON BJ, NESS KD, MASQUELIER DA, BELGRADER P, HEREDIA NJ, MAKAREWICZ AJ, BRIGHT IJ, LUCERO MY, HIDDESSEN AL, LEGLER TC, KITANO TK, HODEL MR, PETERSEN JF, WYATT PW, STEENBLOCK ER, SHAH PH, BOUSSE LJ, TROUP CB, MELLEN JC, WITTMANN DK, et al. High-throughput droplet digital PCR system for absolute quantitation of DNA copy number[J]. Analytical Chemistry, 2011, 83(22): 8604-8610.

[40] KELLEY K, COSMAN A, BELGRADER P, CHAPMAN B, SULLIVAN DC. Detection of methicillin-resistant *Staphylococcus aureus* by a duplex droplet digital PCR assay[J]. Journal of Clinical Microbiology, 2013, 51(7): 2033-2039.

[41] CAVÉ L, BROTHIER E, ABROUK D, BOUDA PS, HIEN E, NAZARET S. Efficiency and sensitivity of the digital droplet PCR for the quantification of antibiotic resistance genes in soils and organic residues[J]. Applied Microbiology and Biotechnology, 2016, 100(24): 10597-10608.

[42] XIE ST, DING LJ, HUANG FY, ZHAO Y, AN XL, SU JQ, SUN GX, SONG YQ, ZHU YG. VFG-Chip: a high-throughput qPCR microarray for profiling virulence factor genes from the environment[J]. Environment International, 2023, 172: 107761.

[43] ZHOU M, LIU ZS, ZHANG BF, YANG JW, HU BL. Interaction between arsenic metabolism genes and arsenic leads to a lose-lose situation[J]. Environmental Pollution, 2022, 312: 119971.

[44] QI Q, GHALY TM, PENESYAN A, RAJABAL V, STACEY JA, TETU SG, GILLINGS MR. Uncovering bacterial hosts of class 1 integrons in an urban coastal aquatic environment with a single-cell fusion-polymerase chain reaction technology[J]. Environmental Science & Technology, 2023, 57(12): 4870-4879.

[45] SANGER F, COULSON AR. A rapid method for determining sequences in DNA by primed synthesis with DNA polymerase[J]. Journal of Molecular Biology, 1975, 94(3): 441-448.

[46] SANGER F, NICKLEN S, COULSON AR. DNA sequencing with chain-terminating inhibitors[J]. Proceedings of the National Academy of Sciences of the United States of America, 1977, 74(12): 5463-5467.

[47] EGLI K, WAGNER K, KELLER PM, RISCH L, RISCH M, BODMER T. Comparison of the diagnostic performance of qPCR, Sanger sequencing, and whole-genome sequencing in determining clarithromycin and levofloxacin resistance in *Helicobacter pylori*[J]. Frontiers in Cellular and Infection Microbiology, 2020, 10: 596371.

[48] GÜNERI CÖ, STINGL K, GROBBEL M, HAMMERL JA, KÜREKCI C. Different *fosA* genes were found on mobile genetic elements in *Escherichia coli* from wastewaters of hospitals and municipals in Turkey[J]. The Science of the Total Environment, 2022, 824: 153928.

[49] MARGULIES M, EGHOLM M, ALTMAN WE, ATTIYA S, BADER JS, BEMBEN LA, BERKA J, BRAVERMAN MS, CHEN YJ, CHEN ZT, DEWELL SB, DU L, FIERRO JM, GOMES XV, GODWIN BC, HE W, HELGESEN S, HO CH, IRZYK GP, JANDO SC, et al. Genome sequencing in microfabricated high-density picolitre reactors[J]. Nature, 2005, 437: 376-380.

[50] ALLEN HK, MOE LA, RODBUMRER J, GAARDER A, HANDELSMAN J. Functional metagenomics reveals diverse beta-lactamases in a remote Alaskan soil[J]. The ISME Journal, 2009, 3(2): 243-251.

[51] ALLEN HK, CLOUD-HANSEN KA, WOLINSKI JM, GUAN CH, GREENE S, LU S, BOEYINK M,

BRODERICK NA, RAFFA KF, HANDELSMAN J. Resident microbiota of the gypsy moth midgut harbors antibiotic resistance determinants[J]. DNA and Cell Biology, 2009, 28(3): 109-117.

[52] ZHU L, YUAN L, SHUAI XY, LIN ZJ, SUN YJ, ZHOU ZC, MENG LX, JU F, CHEN H. Deciphering basic and key traits of antibiotic resistome in influent and effluent of hospital wastewater treatment systems[J]. Water Research, 2023, 231: 119614.

[53] LI CY, LI XY, GUO RJ, NI W, LIU KP, LIU Z, DAI JH, XU YR, ABDURIYIM S, WU ZY, ZENG YQ, LEI BB, ZHANG YF, WANG Y, ZENG WB, ZHANG Q, CHEN CF, QIAO J, LIU C, HU SW. Expanded catalogue of metagenome-assembled genomes reveals resistome characteristics and athletic performance-associated microbes in horse[J]. Microbiome, 2023, 11(1): 7.

[54] PAN C, GUO LY, YU Y, LI WJ, XU DD, CHEN WD, ZHU L, HU BL, ZHENG P, ZHANG M. Enhanced resuscitation of anammox granular sludge by adding folate: a perspective from the growth factor[J]. Chemical Engineering Journal, 2023, 478: 147470.

[55] LIU ZS, ZHAO YX, ZHANG BF, WANG JQ, ZHU LZ, HU BL. Deterministic effect of pH on shaping soil resistome revealed by metagenomic analysis[J]. Environmental Science & Technology, 2023, 57(2): 985-996.

[56] ZHANG T, JI ZQ, LI J, YU LY. Metagenomic insights into the antibiotic resistome in freshwater and seawater from an Antarctic ice-free area[J]. Environmental Pollution, 2022, 309: 119738.

[57] ZHAO RX, FENG J, HUANG J, LI XY, LI B. Reponses of microbial community and antibiotic resistance genes to the selection pressures of ampicillin, cephalexin and chloramphenicol in activated sludge reactors[J]. The Science of the Total Environment, 2021, 755(Pt 2): 142632.

[58] TANG HM, LIU ZS, HU BL, ZHU LZ. D-ring modifications of tetracyclines determine their ability to induce resistance genes in the environment[J]. Environmental Science & Technology, 2024, 58(2): 1338-1348.

[59] FLUSBERG BA, WEBSTER DR, LEE JH, TRAVERS KJ, OLIVARES EC, CLARK TA, KORLACH J, TURNER SW. Direct detection of DNA methylation during single-molecule, real-time sequencing[J]. Nature Methods, 2010, 7: 461-465.

[60] WICHMANN F, UDIKOVIC-KOLIC N, ANDREW S, HANDELSMAN J. Diverse antibiotic resistance genes in dairy cow manure[J]. mBio, 2014, 5(2): e01017.

[61] SCHMIDT K, MWAIGWISYA S, CROSSMAN LC, DOUMITH M, MUNROE D, PIRES C, KHAN AM, WOODFORD N, SAUNDERS NJ, WAIN J, O'GRADY J, LIVERMORE DM. Identification of bacterial pathogens and antimicrobial resistance directly from clinical urines by nanopore-based metagenomic sequencing[J]. Journal of Antimicrobial Chemotherapy, 2017, 72(1): 104-114.

[62] WENGER AM, PELUSO P, ROWELL WJ, CHANG PC, HALL RJ, CONCEPCION GT, EBLER J, FUNGTAMMASAN A, KOLESNIKOV A, OLSON ND, TÖPFER A, ALONGE M, MAHMOUD M, QIAN YF, CHIN CS, PHILLIPPY AM, SCHATZ MC, MYERS G, DePRISTO MA, RUAN J, et al. Accurate circular consensus long-read sequencing improves variant detection and assembly of a human genome[J]. Nature Biotechnology, 2019, 37: 1155-1162.

[63] YANG Q, LI Y, FANG L, LEI TL, CAI H, HUA XT, ZHENG M, YU YS. A novel KPC-113 variant conferring carbapenem and ceftazidime-avibactam resistance in a multidrug-resistant *Pseudomonas aeruginosa* isolate[J]. Clinical Microbiology and Infection: the Official Publication of the European Society of Clinical Microbiology and Infectious Diseases, 2023, 29(3): 387.e7-387.e14.

[64] SUKHUM KV, DIORIO-TOTH L, DANTAS G. Genomic and metagenomic approaches for predictive surveillance of emerging pathogens and antibiotic resistance[J]. Clinical Pharmacology and Therapeutics, 2019, 106(3): 512-524.

[65] HADJADJ L, BARON SA, DIENE SM, ROLAIN JM. How to discover new antibiotic resistance genes?[J]. Expert Review of Molecular Diagnostics, 2019, 19(4): 349-362.

[66] LI N, LIU C, ZHANG ZG, LI HN, SONG TT, LIANG T, LI BX, LI LY, FENG S, SU QQ, YE J, ZHU CX. Research and technological advances regarding the study of the spread of antimicrobial resistance genes and antimicrobial-resistant bacteria related to animal husbandry[J]. International Journal of Environmental Research and Public Health, 2019, 16(24): 4896.

[67] LIU L, YANG Y, DENG Y, ZHANG T. Nanopore long-read-only metagenomics enables complete and high-quality genome reconstruction from mock and complex metagenomes[J]. Microbiome, 2022, 10(1): 209.

[68] YIN XL, ZHENG XW, LI LG, ZHANG AN, JIANG XT, ZHANG T. ARGs-OAP v3.0: antibiotic-resistance gene database curation and analysis pipeline optimization[J]. Engineering, 2023, 27: 234-241.

[69] YANG Y, DENG Y, SHI XH, LIU L, YIN XL, ZHAO WW, LI SX, YANG C, ZHANG T. QMRA of beach water by nanopore sequencing-based viability-metagenomics absolute quantification[J]. Water Research, 2023, 235: 119858.

[70] YAJIMA D, FUJITA H, HAYASHI I, SHIMA GT, SUZUKI K, TOJU H. Core species and interactions prominent in fish-associated microbiome dynamics[J]. Microbiome, 2023, 11(1): 53.

[71] YAN L, ZHOU J, ZHENG Y, GAMSON AS, ROEMBKE BT, NAKAYAMA S, SINTIM HO. Isothermal amplified detection of DNA and RNA[J]. Molecular BioSystems, 2014, 10(5): 970-1003.

[72] SUN PC, NIU K, DU HY, LI RX, CHEN JP, LU XB. Ultrasensitive rapid detection of antibiotic resistance genes by electrochemical ratiometric genosensor based on 2D monolayer Ti_3C_2@AuNPs[J]. Biosensors & Bioelectronics, 2023, 240: 115643.

[73] WALKER GT, FRAISER MS, SCHRAM JL, LITTLE MC, NADEAU JG, MALINOWSKI DP. Strand displacement amplification: an isothermal, *in vitro* DNA amplification technique[J]. Nucleic Acids Research, 1992, 20(7): 1691-1696.

[74] NOTOMI T, OKAYAMA H, MASUBUCHI H, YONEKAWA T, WATANABE K, AMINO N, HASE T. Loop-mediated isothermal amplification of DNA[J]. Nucleic Acids Research, 2000, 28(12): e63.

[75] VINCENT M, XU Y, KONG HM. Helicase-dependent isothermal DNA amplification[J]. EMBO Reports, 2004, 5(8): 795-800.

[76] PIEPENBURG O, WILLIAMS CH, STEMPLE DL, ARMES NA. DNA detection using recombination proteins[J]. PLoS Biology, 2006, 4(7): e204.

[77] MISAWA Y, YOSHIDA A, SAITO R, YOSHIDA H, OKUZUMI K, ITO N, OKADA M, MORIYA K, KOIKE K. Application of loop-mediated isothermal amplification technique to rapid and direct detection of methicillin-resistant *Staphylococcus aureus* (MRSA) in blood cultures[J]. Journal of Infection and Chemotherapy, 2007, 13(3): 134-140.

[78] GOLDMEYER J, LI HJ, McCORMAC M, COOK S, STRATTON C, LEMIEUX B, KONG HM, TANG W, TANG YW. Identification of *Staphylococcus aureus* and determination of methicillin resistance directly from positive blood cultures by isothermal amplification and a disposable detection device[J]. Journal of Clinical Microbiology, 2008, 46(4): 1534-1536.

[79] AUGUSTINE R, HASAN A, DAS S, AHMED R, MORI Y, NOTOMI T, KEVADIYA BD, THAKOR AS. Loop-mediated isothermal amplification (LAMP): a rapid, sensitive, specific, and cost-effective point-of-care test for coronaviruses in the context of COVID-19 pandemic[J]. Biology, 2020, 9(8): 182.

[80] SUEA-NGAM A, CHOOPARA I, LI SK, SCHMELCHER M, SOMBOONNA N, HOWES PD, DEMELLO AJ. *In situ* nucleic acid amplification and ultrasensitive colorimetric readout in a paper-based analytical device using silver nanoplates[J]. Advanced Healthcare Materials, 2021, 10(7): e2001755.

[81] LI J, MACDONALD J, von STETTEN F. Review: a comprehensive summary of a decade development of the recombinase polymerase amplification[J]. The Analyst, 2018, 144(1): 31-67.

[82] BUTTERWORTH A, PRATIBHA P, MARX A, CORRIGAN DK. Electrochemical detection of oxacillin resistance using direct-labeling solid-phase isothermal amplification[J]. ACS Sensors, 2021, 6(10): 3773-3780.

[83] WANG YX, CHEN H, GAO HX, WEI HJ, WANG YL, MU K, LIU LY, DAI EH, RONG Z, WANG SQ. CESSAT: a chemical additive-enhanced single-step accurate CRISPR/Cas13 testing system for field-deployable ultrasensitive detection and genotyping of SARS-CoV-2 variants of concern[J]. Biosensors & Bioelectronics, 2023, 229: 115238.

[84] GARNEAU JE, DUPUIS MÈ, VILLION M, ROMERO DA, BARRANGOU R, BOYAVAL P, FREMAUX C, HORVATH P, MAGADÁN AH, MOINEAU S. The CRISPR/Cas bacterial immune system cleaves bacteriophage and plasmid DNA[J]. Nature, 2010, 468: 67-71.

[85] LU SH, TONG XH, HAN Y, ZHANG K, ZHANG YZ, CHEN QB, DUAN JY, LEI XL, HUANG MH, QIU Y, ZHANG DY, ZHOU X, ZHANG Y, YIN H. Fast and sensitive detection of SARS-CoV-2 RNA using suboptimal protospacer adjacent motifs for Cas12a[J]. Nature Biomedical Engineering, 2022, 6: 286-297.

[86] MÜLLER V, RAJER F, FRYKHOLM K, NYBERG LK, QUADERI S, FRITZSCHE J, KRISTIANSSON E, AMBJÖRNSSON T, SANDEGREN L, WESTERLUND F. Direct identification of antibiotic resistance genes

on single plasmid molecules using CRISPR/Cas9 in combination with optical DNA mapping[J]. Scientific Reports, 2016, 6: 37938.

[87] ZETSCHE B, GOOTENBERG JS, ABUDAYYEH OO, SLAYMAKER IM, MAKAROVA KS, ESSLETZBICHLER P, VOLZ SE, JOUNG J, van der OOST J, REGEV A, KOONIN EV, ZHANG F. Cpf1 is a single RNA-guided endonuclease of a class 2 CRISPR-Cas system[J]. Cell, 2015, 163(3): 759-771.

[88] ABUDAYYEH OO, GOOTENBERG JS, KONERMANN S, JOUNG J, SLAYMAKER IM, COX DBT, SHMAKOV S, MAKAROVA KS, SEMENOVA E, MINAKHIN L, SEVERINOV K, REGEV A, LANDER ES, KOONIN EV, ZHANG F. C2c2 is a single-component programmable RNA-guided RNA-targeting CRISPR effector[J]. Science, 2016, 353(6299): aaf5573.

[89] YUAN MZ, DING RH, CHEN SY, DUAN GC. Advances in field detection based on CRISPR/Cas system[J]. ACS Synthetic Biology, 2021, 10(11): 2824-2832.

[90] AMAN R, MAHAS A, MAHFOUZ M. Nucleic acid detection using CRISPR/Cas biosensing technologies[J]. ACS Synthetic Biology, 2020, 9(6): 1226-1233.

[91] JOUNG J, LADHA A, SAITO M, KIM NG, WOOLLEY AE, SEGEL M, BARRETTO RPJ, RANU A, MACRAE RK, FAURE G, IOANNIDI EI, KRAJESKI RN, BRUNEAU R, HUANG ML W, YU XG, LI JZ, WALKER BD, HUNG DT, GRENINGER AL, JEROME KR, et al. Detection of SARS-CoV-2 with SHERLOCK one-pot testing[J]. The New England Journal of Medicine, 2020, 383(15): 1492-1494.

[92] CHEN JS, MA EB, HARRINGTON LB, Da COSTA M, TIAN XR, PALEFSKY JM, DOUDNA JA. CRISPR-Cas12a target binding unleashes indiscriminate single-stranded DNase activity[J]. Science, 2018, 360(6387): 436-439.

[93] AGGARWAL N, LIANG YM, FOO JL, LING H, HWANG IY, CHANG MW. FELICX: a robust nucleic acid detection method using flap endonuclease and CRISPR-Cas12[J]. Biosensors & Bioelectronics, 2023, 222: 115002.

[94] GOOTENBERG JS, ABUDAYYEH OO, LEE JW, ESSLETZBICHLER P, DY AJ, JOUNG J, VERDINE V, DONGHIA N, DARINGER NM, FREIJE CA, MYHRVOLD C, BHATTACHARYYA RP, LIVNY J, REGEV A, KOONIN EV, HUNG DT, SABETI PC, COLLINS JJ, ZHANG F. Nucleic acid detection with CRISPR-Cas13a/C2c2[J]. Science, 2017, 356(6336): 438-442.

[95] HU ML, LIU RH, QIU ZQ, CAO F, TIAN T, LU YX, JIANG YZ, ZHOU XM. Light-start CRISPR-Cas12a reaction with caged crRNA enables rapid and sensitive nucleic acid detection[J]. Angewandte Chemie (International Ed in English), 2023, 62(23): e202300663.

[96] GUAN ZW, SUN Y, MA CB, LEE JJ, ZHANG SC, ZHANG XJ, GUO ZJ, DU Y. Dual targets-induced specific hemin/G-quadruplex assemblies for label-free electrochemical detection capable of distinguishing *Salmonella* and its common serotype in food samples[J]. Biosensors & Bioelectronics, 2023, 236: 115438.

[97] LI JX, ZHOU JW, XIA Y, RUI YL, YANG XY, XIE GY, JIANG GM, WANG HS. Rolling circle extension-assisted loop-mediated isothermal amplification (Rol-LAMP) method for locus-specific and visible detection of RNA N6-methyladenosine[J]. Nucleic Acids Research, 2023, 51(9): e51.

[98] van der VEER HJ, van AALEN EA, MICHIELSEN CMS, HANCKMANN ETL, DECKERS J, van BORREN MMGJ, FLIPSE J, LOONEN AJM, SCHOEBER JPH, MERKX M. Glow-in-the-dark infectious disease diagnostics using CRISPR-Cas9-based split luciferase complementation[J]. ACS Central Science, 2023, 9(4): 657-667.

[99] JIAO J, LIU YQ, YANG ML, ZHENG JC, LIU CH, YE WX, SONG SW, BAI TH, SONG CH, WANG MM, SHI JL, WAN R, ZHANG KX, HAO PB, FENG JC, ZHENG XB. The engineered CRISPR-Mb2Cas12a variant enables sensitive and fast nucleic acid-based pathogens diagnostics in the field[J]. Plant Biotechnology Journal, 2023, 21(7): 1465-1478.

[100] NOURI R, JIANG YQ, POLITZA AJ, LIU TY, GREENE WH, ZHU YS, NUNEZ JJ, LIAN XJ, GUAN WH. STAMP-based digital CRISPR-Cas13a for amplification-free quantification of HIV-1 plasma viral loads[J]. ACS Nano, 2023, 17(11): 10701-10712.

[101] SHANG YT, CAI SZ, YE QH, WU QP, SHAO YN, QU XY, XIANG XR, ZHOU BQ, DING Y, CHEN MT, XUE L, ZHU HH, ZHANG JM. Quantum dot nanobeads-labelled lateral flow immunoassay strip for rapid and sensitive detection of *Salmonella typhimurium* based on strand displacement loop-mediated isothermal amplification[J]. Engineering, 2022, 19: 62-70.

[102] CHEN YX, HU YX, LU XN. Polyethersulfone-based microfluidic device integrated with DNA extraction on paper and recombinase polymerase amplification for the detection of *Salmonella enterica*[J]. ACS Sensors, 2023, 8(6): 2331-2339.

[103] RUDKIN GT, STOLLAR BD. High resolution detection of DNA-RNA hybrids *in situ* by indirect immunofluorescence[J]. Nature, 1977, 265: 472-473.

[104] ZWIRGLMAIER K, LUDWIG W, SCHLEIFER KH. Recognition of individual genes in a single bacterial cell by fluorescence *in situ* hybridization: ring-FISH[J]. Molecular Microbiology, 2004, 51(1): 89-96.

[105] SCHENA M, SHALON D, DAVIS RW, BROWN PO. Quantitative monitoring of gene expression patterns with a complementary DNA microarray[J]. Science, 1995, 270(5235): 467-470.

[106] TROESCH A, NGUYEN H, MIYADA CG, DESVARENNE S, GINGERAS TR, KAPLAN PM, CROS P, MABILAT C. *Mycobacterium* species identification and rifampin resistance testing with high-density DNA probe arrays[J]. Journal of Clinical Microbiology, 1999, 37(1): 49-55.

[107] HEAD SR, PARIKH K, ROGERS YH, BISHAI W, GOELET P, BOYCE-JACINO MT. Solid-phase sequence scanning for drug resistance detection in tuberculosis[J]. Molecular and Cellular Probes, 1999, 13(2): 81-87.

[108] PATTERSON AJ, COLANGELI R, SPIGAGLIA P, SCOTT KP. Distribution of specific tetracycline and erythromycin resistance genes in environmental samples assessed by macroarray detection[J]. Environmental Microbiology, 2007, 9(3): 703-715.

[109] ZHANG XX, ZHANG T, FANG HHP. Antibiotic resistance genes in water environment[J]. Applied Microbiology and Biotechnology, 2009, 82(3): 397-414.

[110] TYAGI S, KRAMER FR. Molecular beacons: probes that fluoresce upon hybridization[J]. Nature Biotechnology, 1996, 14: 303-308.

[111] SINGH G, VAJPAYEE P, RANI N, AMOAH ID, STENSTRÖM TA, SHANKER R. Exploring the potential reservoirs of non specific *TEM* beta lactamase (bla(*TEM*)) gene in the indo-gangetic region: a risk assessment approach to predict health hazards[J]. Journal of Hazardous Materials, 2016, 314: 121-128.

[112] FU XR, SUN JD, YE YL, ZHANG YZ, SUN XL. A rapid and ultrasensitive dual detection platform based on Cas12a for simultaneous detection of virulence and resistance genes of drug-resistant *Salmonella*[J]. Biosensors & Bioelectronics, 2022, 195: 113682.

[113] HU F, LIU YF, ZHAO SH, ZHANG ZM, LI XC, PENG NC, JIANG ZD. A one-pot CRISPR/Cas13a-based contamination-free biosensor for low-cost and rapid nucleic acid diagnostics[J]. Biosensors & Bioelectronics, 2022, 202: 113994.

[114] LIN M, YUE HH, TIAN T, XIONG EH, ZHU DB, JIANG YZ, ZHOU XM. Glycerol additive boosts 100-fold sensitivity enhancement for one-pot RPA-CRISPR/Cas12a assay[J]. Analytical Chemistry, 2022, 94(23): 8277-8284.