



Enlarging the Toolbox Against Antimicrobial Resistance: Aptamers and CRISPR-Cas

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In the post-genomic era, molecular treatments and diagnostics have been envisioned as powerful techniques to tackle the antimicrobial resistance (AMR) crisis. Among the molecular approaches, aptamers and CRISPR-Cas have gained support due to their practicality, sensibility, and flexibility to interact with a variety of extra- and intracellular targets. Those characteristics enabled the development of quick and onsite diagnostic tools as well as alternative treatments for pan-resistant bacterial infections. Even with such potential, more studies are necessary to pave the way for their successful use against AMR. In this review, we highlight those two robust techniques and encourage researchers to refine them toward AMR. Also, we describe how aptamers and CRISPR-Cas can work together with the current diagnostic and treatment toolbox.

Keywords: antimicrobial resistance, molecular diagnostic, alternative treatments, aptamer, CRISPR-Cas

ANTIBIOTIC RESISTANCE CRISIS

Despite antimicrobials' impact on modern medicine since their introduction in the first part of the 19th century (Powers, 2004; CDC, 2019), resistant bacteria quickly emerged throughout the decades. Drug resistance to all available antibiotics has been detected in clinical bacteria, threatening all advances achieved within the antibiotic era and urging for alternative treatments (CDC, 2019).

Abbreviations: ALISA, Aptamer-linked immobilized sorbent assay; AMR, Antimicrobial resistance genes; CRISPR-Cas, Clustered regularly interspaced short palindromic repeats, CRISPR-associated enzymes; crRNA, CRISPR RNA; COVID-19, Coronavirus disease 2019; DNA, Deoxyribonucleic acid; FDA, Food and Drug Administration; HIV, Human immunodeficiency virus; KPC, *Klebsiella pneumoniae* carbapenemase; LAMP, Loop-mediated isothermal amplification; MALDI-TOF MS, Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; MRSA, Methicillin-resistant *Staphylococcus aureus*; NDM, New Delhi metallo-β-lactamase; NGS, Next-generation sequencing; PAM, Protospacer adjacent motif; PBP, Penicillin-binding protein; RNA, Ribonucleic acid; RNP, Ribonucleoprotein; RT-qPCR, quantitative reverse transcription PCR; SARS, Severe acute respiratory syndrome; SARS-CoV-2, Severe acute respiratory syndrome coronavirus 2; SELEX, Systematic evolution of ligands by exponential enrichment; sgRNA, single-guide RNA; siRNA, Small interfering RNA; SNP, Single-nucleotide polymorphism; TALEN, Transcription activator-like effector nucleases; tracrRNA, trans-activating CRISPR RNA.

Bacteria have developed resistance mechanisms to avoid, disrupt, eject, or resist the currently used antimicrobials (**Box 1**). They can be intrinsically resistant to antibiotics by using structural or functional inherent bacterial features or acquire resistance via genetic mutations or by horizontal transference of genetic elements (Blair et al., 2015; Munita and Arias, 2016).

The spread of bacterial resistance mechanisms has been much faster than the development of new treatments. New investments on antimicrobial research have been discouraged due to their elevated production costs and long-term development process (Adams and Brantner, 2010). On top of that, the misuse and over-prescription of antibiotics, which stem from uncertainties in diagnosis, contribute to the antimicrobial resistance (AMR) crisis escalation (Llor and Bjerrum, 2014; Malik and Bhattacharyya, 2019). The lack of rapid diagnostic tools directly affects initial treatment decisions, which might lead to empirical treatment guided only by clinical presentation (Fischer et al., 2004; Leekha et al., 2011).

Phenotypic-based diagnostics are currently considered as gold standards in AMR assessment. The “catch-all” resistance characteristic of phenotypic tests enables the evaluation of microbial susceptibility in a relatively unbiased way (Mitsakakis et al., 2018). Although efforts have been made to provide quick phenotypic tests (~7 h) (Pancholi et al., 2018) to better guide antibiotic treatment, the most used techniques still require microorganism culture, with a turnaround time of around 18 h. This delays the availability of the AMR profiles, which might be accessible up to 72 h after sample collection (Leekha et al., 2011). Besides time-to-result limitation, phenotypic tests generally require laboratory structure (Mitsakakis et al., 2018). Therefore, quicker and accessible diagnostic tools are imperative to guide the first medical decisions regarding antimicrobial therapy prescription worldwide.

MOLECULAR APPROACHES

The search for more precise molecular diagnostic tools with quicker turnaround times has been encouraged to better guide clinical practice and public health policies. The recent global SARS-CoV-2 (severe acute respiratory syndrome coronavirus 2) outbreak has shown that in a matter of weeks, diagnostic centers have been overloaded with patients' samples and quick result release is required for viral spread control. So far, until 21 December 2020, SARS-CoV-2 virus infected 75,704,857 individuals, with 1,690,061 deaths worldwide¹. We dare to draw here a parallel between SARS-CoV-2 and the AMR crisis. Currently, AMR infections cause around 700,000 deaths per year (O'Neil, 2016). In both cases, an early diagnosis would give trustworthy information for discrimination and contention of the causative agent. With alarming death numbers, the exploration of alternative treatments and diagnostics comes into the spotlight as an attempt to revert the current scenario caused by AMR.

Different molecular tools have been employed as a diagnostic to identify infectious disease agents and their resistance profile.

¹<https://covid19.who.int/>

RT-qPCR (quantitative reverse transcription PCR) and NGS (Next-Generation Sequencing) have been currently playing a key role in the diagnostics of the SARS-CoV-2, different from what happened in the 2002 SARS outbreak (Sheridan, 2020). qPCR is also an outstanding tool for the molecular detection of antimicrobial resistance genes (ARGs) (Waseem et al., 2019) directly from patient samples such as urine, blood, and cerebrospinal fluid (Singh et al., 2017). In addition to qPCR, metagenomic, LAMP (Loop-mediated isothermal amplification), and whole genome sequencing approaches not only characterize pathogens at the species level but also detect ARGs (Zankari et al., 2012; Dekker, 2018; Ota et al., 2019).

The molecular diagnostic tools described above offer an abundant panel to recognize DNA and RNA of infectious microorganisms. Complementarily, proteomics- and metabolomics-based techniques have been gaining momentum into the clinical molecular diagnostic field, for instance, MALDI-TOF MS (Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry) (Patrinos et al., 2017). However, these tools require expensive equipment and laboratory structure, hampering their wide implementation as *in loco* diagnostic tools.

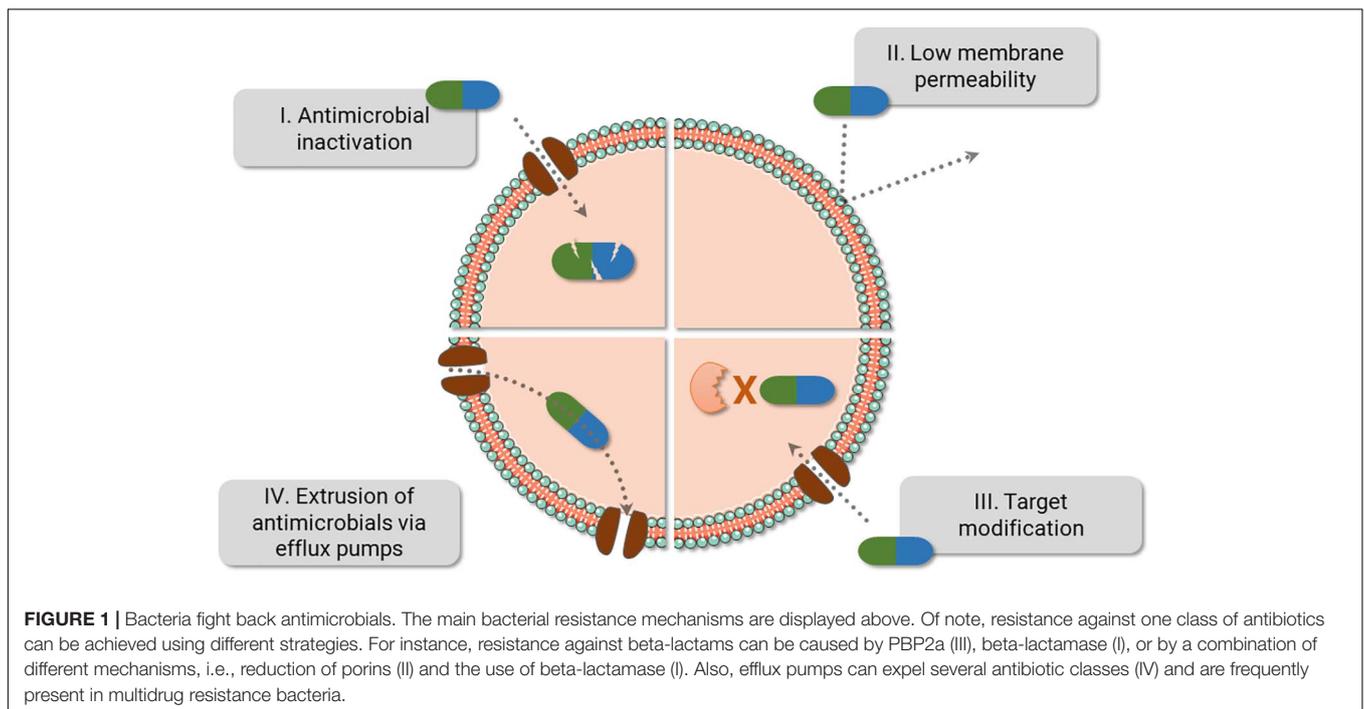
Aptamers and CRISPR-Cas (clustered repetitive interspaced short palindromic repeats, CRISPR-associated enzymes) systems have been slowly gaining support in clinical diagnosis and treatment of infectious diseases. Both can be employed as an onsite diagnostic tool with a quick turnaround time, which makes them more interesting than other methods targeting proteins or nucleic acids. Therefore, reviewing these two robust techniques attempts to encourage molecular biologist researchers to develop and refine clinical molecular tools against ARGs. Those methods do not necessarily intend to substitute the already implemented diagnostic approaches, but to stimulate their combination to circumvent antibiotic misuse. Also, their application in therapy will be reviewed to help paving the way for their use as treatments against multidrug-resistant agents.

APPLICATION OF APTAMERS INTO DETECTION AND NEUTRALIZATION OF AMR FACTORS

A substantial boost in aptamer application in research and clinical institutes has been noted worldwide (McKeague et al., 2015). Also called chemical antibodies (Toh et al., 2015), most aptamers interact with their targets in a constant equilibrium with binding affinities up to 1 pM (Ha et al., 2017). They offer a cheap large-scale production with chemical modifications, low or no immunogenicity, small size (close to 3 nm), flexibility in tridimensional structure, and great stability in different conditions of pH, temperature, and organic solvents (Yoon and Rossi, 2018). Also, aptamers interact with high sensitivity toward their targets, being able to discriminate a single amino acid mutation (Chen et al., 2015). Due to their small size, aptamers reach cavities that are often not accessible to monoclonal antibodies and, therefore, penetrate cell tissues more easily (Xiang et al., 2015). In this process, intracellular

BOX 1 | Antimicrobial resistance mechanism.

Resistance mechanisms date back to thousands of years and have been probably used to endure the presence of toxic compounds present in nature—including antimicrobials derived from different microorganisms, while they also provide alternative cellular functions (Allen et al., 2010; D'costa et al., 2011). Bacteria can evade antimicrobials via reduction of drug intracellular concentration either by low membrane permeability or through antibiotic efflux; target modification by genetic mutation or post-translational modification; and inactivation of the antibiotic by hydrolysis or its modification (Blair et al., 2015). With the introduction and constant presence of antimicrobials in medical care, agriculture and animal health, the spread of resistant microorganisms and the evolution of their defense strategies have been accelerated (Figure 1; CDC, 2019). From all resistance mechanisms, genes responsible for antibiotic inactivation and target alteration (Figure 1 I and III, respectively) are commonly spread by plasmids and phage transduction (Munita and Arias, 2016; Calero-Caceres et al., 2019). Antibiotic inactivation is a usual strategy adopted for instance against beta-lactams and aminoglycosides. Beta-lactams can be hydrolyzed by enzymes encoded by the *bla* genes (beta-lactamase genes), such as *bla_{TEM}*, *bla_{KPC}*, and *bla_{OXA}*. Aminoglycosides, by its turn, are chemically inactivated by mainly three biochemical reaction, named adenylation, acetylation, and phosphorylation catalyzed by the enzymes nucleotidyltransferases (ANT), acetyltransferases (AAC), and phosphotransferases (APH), respectively (Doi et al., 2016; Munita and Arias, 2016; Bush and Bradford, 2019). An advantage of the hydrolysis over the chemical alteration strategy is the requirement of water instead of chemical compounds as a co-substrate, which ease enzyme activity outside the cell. Target alteration can be achieved by four main strategies, affecting several antimicrobials (not limited to the examples), as follows: (i) Target protection. One of the best-studied examples involves the determinants Tet(M) and Tet(O), which confers resistance to tetracycline. They interact with the ribosome and dislodge the drug from its binding site. (ii) Mutation of the antimicrobial target site. The development of mutations in the chromosomal genes *gyrA-gyrB* and *parC-parE* codifying for DNA gyrase and topoisomerase IV, respectively, promotes resistance against fluoroquinolones. (iii) Enzymatic alteration. Macrolide resistance is acquired by *erm* genes (erythromycin ribosomal methylation), which codify enzymes responsible for 50S ribosomal subunit methylation. This alteration weakens the binding of the erythromycin to the ribosome. (iv) Replacement/bypass of the target. Beta-lactam resistance is frequently acquired by Gram-positive microorganisms via *mecA* gene. The gene encodes an exogen penicillin-binding protein (PBP2a) that has low affinity for the beta-lactams, opposite to what happens to endogen PBPs (Munita and Arias, 2016).



aptamers could be internalized by either a clathrin-dependent or -independent mechanism and co-localized in subcellular compartments, directly associated to its target, as reviewed in Yoon and Rossi (2018; Figure 2).

Single-stranded oligonucleotides can assume several secondary conformations such as hairpin, loop, pseudoknot, and G-quadruplex, which assure a unique folding for each sequence and allow interaction with specific sites (Yunn et al., 2015). DNA and RNA aptamers have similar binding characteristics, although DNA nucleotides have lower operating cost and offer greater stability than RNA, which in turn have greater versatility in their three-dimensional structures that directly affect target affinity.

Aptamer selection commonly occurs via a randomized process of systematic evolution of ligands by exponential enrichment (SELEX), firstly reported in 1990 (Ellington and Szostak, 1990; Tuerk and Gold, 1990). SELEX is based on iterative rounds of incubation of the oligonucleotide pool with the target, frequently divided into four stages: incubation, partition, recovery, and amplification. Also, aptamer–target affinity slowly increases alongside the rounds until the most specific aptamer is selected. SELEX allows greater versatility of binding conditions, which favors the adaptation of selected oligonucleotides to different cellular and non-cellular environments (McKeague et al., 2015).

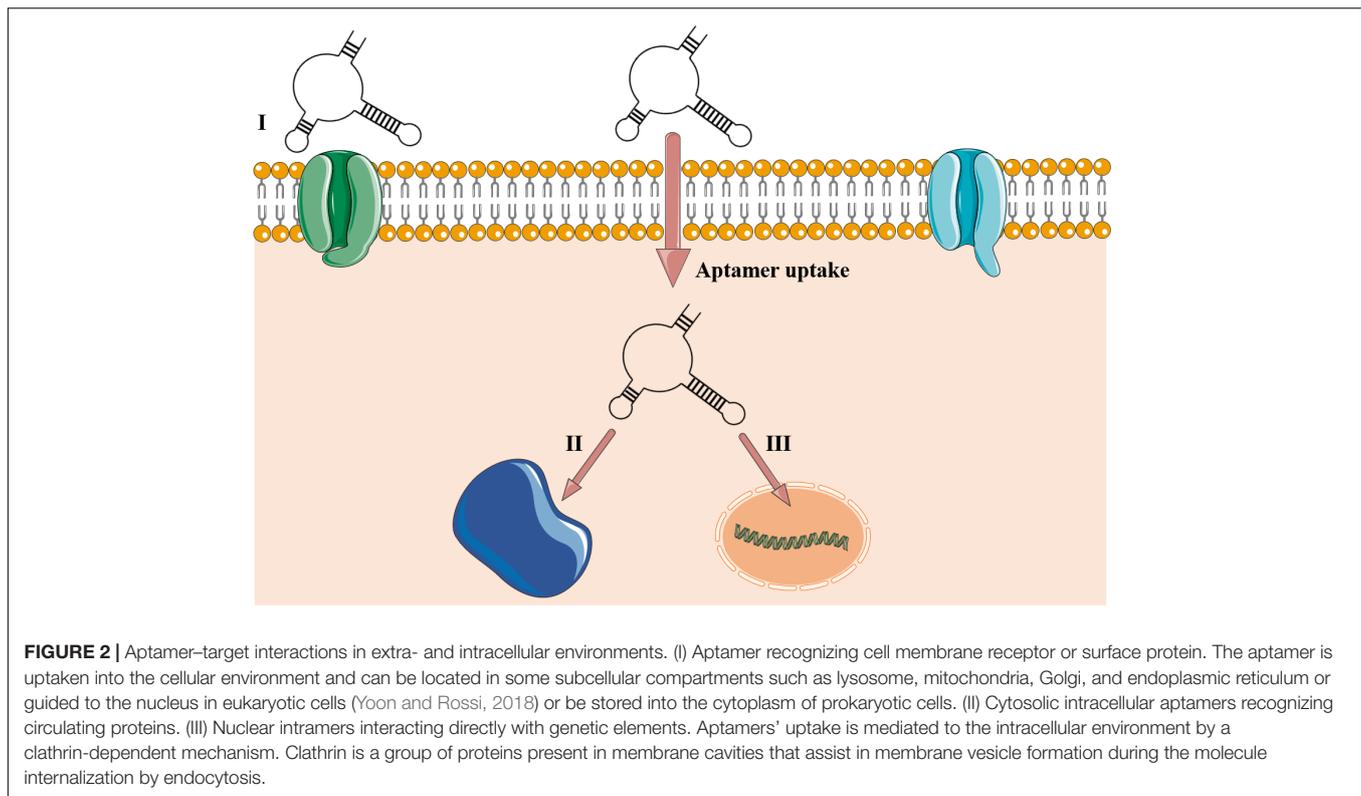


FIGURE 2 | Aptamer–target interactions in extra- and intracellular environments. (I) Aptamer recognizing cell membrane receptor or surface protein. The aptamer is taken up into the cellular environment and can be located in some subcellular compartments such as lysosome, mitochondria, Golgi, and endoplasmic reticulum or guided to the nucleus in eukaryotic cells (Yoon and Rossi, 2018) or be stored into the cytoplasm of prokaryotic cells. (II) Cytosolic intracellular aptamers recognizing genetic elements. (III) Nuclear intramers interacting directly with genetic elements. Aptamers’ uptake is mediated to the intracellular environment by a clathrin-dependent mechanism. Clathrin is a group of proteins present in membrane cavities that assist in membrane vesicle formation during the molecule internalization by endocytosis.

Aptamer selection against many biological and chemical targets has been described (Qiao et al., 2018; Dalirirad and Steckl, 2020), along with its use in drug development (Esposito et al., 2018), bioimaging (Kim et al., 2019), food inspection (Duan et al., 2016), genetic modulation (Mol et al., 2019), and as a delivery vehicle (Zhuang et al., 2020). In antiviral therapy, G-3 aptamer dually inhibits HIV-1 cell replication both by blocking virus entrance via CCR5 receptor and by delivering a siRNA that decreases HIV-1 cytoplasmic traffic (Zhou et al., 2015). Also, aptamers blocked quorum sensing and inhibited biofilm formation in *Pseudomonas aeruginosa* infections (Zhao et al., 2019). Even with many progresses, there is currently only one aptamer approved for clinical use, named pegaptanib. It is the main component of Macugen[®], released by the FDA in 2004 to treat age-related macular degeneration (Gragoudas et al., 2004). Recently, there have been 10 therapeutic aptamers at different stages of clinical trials (Kaur et al., 2018), most of them employed to inhibit protein–protein interaction or act as antagonist (Yunn et al., 2015). To date, there is no aptamer currently approved by FDA for diagnostic purposes, even though they fit the quality standards of the diagnostic industry: affordable, sensitive, specific, user-friendly, and robust.

Different diagnostic devices based on aptamers have been proposed, including Aptamer-Linked Immobilized Sorbent Assay (ALISA), dot-blot, lateral-flow strips conjugated to nanomaterials, and the promising aptamer-based sensors (Stoltenburg et al., 2016; Shin et al., 2018; Su et al., 2018; Xiong et al., 2020). Aptasensors can be conjugated to a wide diversity of reporter molecules without modification of their

activity (McKeague et al., 2015). Signal transducers commonly conjugated to aptasensors include but are not limited to colorimetric, electrochemical, and fluorescent approaches (Bai et al., 2017; Bayrac et al., 2017; Cai et al., 2019). Linking aptamer-based biosensors with nanomaterials can increase specificity and sensitivity of target binding up to 10-fold and offers a platform for rapid point-of-care diagnostic (<1 h) (Dalirirad and Steckl, 2020). Aptasensors have well-established protocols of chemical conjugation of aptamers with color or signal-transducer molecules, such as gold, silver, platinum, iron oxide nanoparticles, or carbon nanotubes and graphene oxide (Duan et al., 2016; Dehghani et al., 2018; Gao et al., 2018; Hua et al., 2018; Das et al., 2019a; Fan et al., 2020). The application of aptasensors for disease diagnosis has been tested in different samples, e.g., plasma and spiked nasal swab (Qiao et al., 2018), cultured bacteria (Maldonado et al., 2020), and urine and serum samples (Su et al., 2018). A lateral-flow paper strip conjugated with a gold nanoparticle aptamer-based sensor was developed to onsite detection of dopamine in urine samples (Dalirirad and Steckl, 2020).

When repurposed to bacteria, aptamers could recognize them by binding to antigens or cell surface receptors, or interacting with the whole cell through unknown targets (Tang et al., 2016; Song M. Y. et al., 2017; Shin et al., 2019). Although there are uncertainties concerning the mechanisms of aptamer uptake in bacteria, a report indicates that aptamers could traffic inside bacterial cells (Afrasiabi et al., 2020), similarly to what is shown in **Figure 2**. From 2016 to 2020, several papers have reported the use of aptamers applied to diagnostics of bacterial infections

(**Table 1**). Nearly all studies employed biosensors based on DNA aptamers, which indicates that for diagnostic purposes, the DNA stability overcomes the advantage of tridimensional possibilities of RNA. It is worth mentioning that only three of these studies targeted resistant bacteria or products of ARGs. First, Fan et al. (2020) made a graphene-oxide aptasensor based on peroxidase-like activity for the detection of purified PBP2a protein, encoded by the *mecA* gene. Also, Maldonado et al. (2020) employed a fast and label-free photonic pegylated aptasensor that recognizes both pure PBP2a protein and methicillin-resistant *Staphylococcus aureus* (MRSA)-infected cells in culture. Finally, Qiao et al. (2018) detected PBP2a in *S. aureus* cells collected in clinical plasma and spiked nasal swab samples infected with MRSA strains using a single bacterial lysis step. Predominantly, the diagnostic approach using aptamer has been focusing on the detection of the whole cell instead of its biological specific components, such as proteins or toxins. Therefore, due to the well-established protein-SELEX approach, there is still room for finding highly specific aptamers that bind to proteins associated with ARGs and enrich the diagnostic toolbox.

CRISPR-CAS AS A TOOL AGAINST ANTIMICROBIAL-RESISTANT PATHOGENS

CRISPR-Cas is a ribonucleoprotein (RNP) prokaryotic complex present in 50% of the bacteria and in most archaea that behaves as a prokaryotic adaptive immune system (Makarova et al., 2015). The system confers protection against mobile genetic elements, i.e., bacteriophages, plasmids, and transposons in three coordinated phases: adaptation, CRISPR RNA (crRNA) biogenesis, and interference (Hille et al., 2018). Nearly all CRISPR-Cas system counts on an ingenious mechanism to prevent self-targeting. This includes the recognition of a short sequence called protospacer adjacent motif (PAM) during adaptation and interference stages, present only in foreign nucleic acids (Mojica et al., 2009; Marraffini and Sontheimer, 2010).

Based mainly on the signature *Cas* genes, the new classification of CRISPR-Cas systems includes two different classes, six types, and 33 subtypes (Makarova et al., 2020). The most widespread class 1 CRISPR-Cas comprises types I, III, and IV. It is characterized by effector complexes with multiple Cas proteins responsible for a coordinated action from pre-crRNA processing to target cleavage. By its turn, class 2 consists of types II, V, and VI, which contains a single-protein effector module able to recognize and cleave the targeting nucleic acid (**Table 2**; Makarova et al., 2020).

Upon unraveling the CRISPR-Cas potential of gene editing in an easier, cheaper, and flexible way compared to previously established tools [for instance, TALENs (transcription activator-like effector nucleases) and Zinc-finger nucleases], the systems have been quickly repurposed to the biomedical and biotechnology fields (Chen and Gao, 2014; Liu et al., 2018; Zhang et al., 2019; Li et al., 2020). Class 2 CRISPR-Cas has been an attractive option for gene editing as a result of the

effector module's simpler architecture when compared to class 1 (Makarova et al., 2020). Target specificity and cutting activity of the nucleases can be virtually programmed to any gene of interest by means of the short-length crRNA sequence. The engineering of a single-guide RNA (sgRNA) by fusing Cas9 crRNA and tracrRNA was a benchmark for gene editing (Jinek et al., 2012), but off-target effects still hold back CRISPR-Cas full potential. Several studies have tried to overcome this drawback by employing a plethora of modifications to increase the system specificity for gene editing (Kleinstiver et al., 2016; Slaymaker et al., 2016; Chen et al., 2017; Casini et al., 2018; Lee et al., 2018). Similarly, collateral effects of some Cas enzymes, i.e., the ability to indiscriminately (*trans*-) cleave ssDNA/ssRNA unleashed by site-specific DNA/RNA (*cis*-) bound by the crRNA, may also be a limitation for gene editing. However, here, this feature has been exploited for diagnostic purposes.

The collateral effect of Cas12 and Cas13 has been used as a key step to create several diagnostic platforms, such as DETECTR, HOLMES (both using Cas12), SHERLOCK, and CARMEN-Cas13 (the last two using Cas13) (Gootenberg et al., 2017; Chen et al., 2018; Li S. Y. et al., 2018; Ackerman et al., 2020). All platforms have similar diagnostic strategies, which involve the incubation of Cas enzymes (Cas12/Cas13) along with the target nucleic acid and fluorescent ssDNA/ssRNA reporters. By detecting the target nucleic acid, the Cas enzymes *trans*-cleave the quenched-fluorescent ssDNA/ssRNA reporters inserted into the platform, generating a robust fluorescent signal from around 1-h incubation (**Figure 3**) with a good correlation with PCR-based methods (Gootenberg et al., 2017; Chen et al., 2018; Gootenberg et al., 2018). In order to achieve attomolar sensitivity, the nucleic acid detection platforms were coupled to DNA amplification steps (i.e., PCR, recombinase polymerase amplification, and loop-mediated amplification) or reverse transcriptase combined with a DNA amplification step and T7 transcription for RNA targets (Gootenberg et al., 2017; Chen et al., 2018; Gootenberg et al., 2018; Li S. Y. et al., 2018; Ackerman et al., 2020; Broughton et al., 2020). To further enhance signal sensitivity, CRISPR type III effector nuclease Csm6, responsible for non-specific RNA degradation, can be combined with Cas13 activity (Gootenberg et al., 2018).

CARMEN-Cas13 and SHERLOCK have also been explored for multiplexing assays. The first one was specifically developed for this purpose and uses droplets containing either sample or detection solution, arranged pairwise. CARMEN can test more than 4500 crRNA-target pairs on a single microfluidic chip, which represents a simultaneous detection of around 170 agents (Ackerman et al., 2020). SHERLOCK, however, explored different *trans*-cleavage ssRNA preferences of Cas13 orthologs to develop a four-channel single-reaction multiplexing (Gootenberg et al., 2018). A different multiplexing strategy is employed by FLASH, a platform that uses Cas9 to enrich low-abundance targets from complex backgrounds (including clinical specimens) before NGS (**Figure 3**; Quan et al., 2019). Both CARMEN-Cas13 and FLASH offered an important multiplexing capacity but rely on robust laboratory structure, which may impair its implementation in less developed regions. SHERLOCK, however, has demonstrated its feasibility also as a paper-based test, which

TABLE 1 | Report of literature aptamers applied in the diagnostic of bacterial infections from 2016 to 2020.

Target	Oligo	Binding affinity or LOD	References
<i>Acinetobacter baumannii</i> isolates	DNA	7.547 ± 1.353 pM	Rasoulinejad and Gargari, 2016
<i>Bacillus subtilis</i> , <i>Citrobacter freundii</i> , <i>Escherichia coli</i> , <i>Enterobacter aerogenes</i> , <i>Klebsiella pneumoniae</i> , and <i>Staphylococcus epidermidis</i> cells	DNA	9.22–38.5 nM	Song M. Y. et al., 2017
<i>Campylobacter jejuni</i> cells	DNA	100 CFU ml ⁻¹	Dehghani et al., 2018
<i>Escherichia coli</i> and <i>Staphylococcus aureus</i> pathogenic cells	DNA	100 CFU ml ⁻¹	Xu et al., 2018
<i>Escherichia coli</i> ATCC cells	DNA	11.97 ± 2.94 nM	Marton et al., 2016
<i>Escherichia coli</i> cells	DNA	3 CFU ml ⁻¹	Jin et al., 2017
<i>Escherichia coli</i> cells	DNA	0.66 CFU ml ⁻¹	Hua et al., 2018
<i>Escherichia coli</i> O157 cells	DNA	107.6 ± 67.8 pM	Amraee et al., 2017
<i>Escherichia coli</i> O157:H7 cells	DNA	1.46 × 10 ³ CFU ml ⁻¹	Yu et al., 2018
<i>Escherichia coli</i> whole cells	RNA	2 × 10 ⁴ CFU ml ⁻¹	Dua et al., 2016
<i>Escherichia coli</i> whole-cells	DNA	102 CFU ml ⁻¹	Wu et al., 2017
Glycolipid antigen of <i>Mycobacterium tuberculosis</i>	DNA	668 ± 159 nM	Tang et al., 2016
Gram-negative outer membrane vesicles	DNA	25 ng/ml	Shin et al., 2019
HspX protein in tuberculous meningitis	DNA	10 pg	Das et al., 2019b
<i>Listeria monocytogenes</i> cells	DNA	2.5 CFU ml ⁻¹	Suh et al., 2018
Methicillin-resistant <i>Staphylococcus aureus</i> strains	DNA	1.38 × 10 ³ CFU ml ⁻¹	Qiao et al., 2018
MPT64 antigen of <i>Mycobacterium tuberculosis</i>	DNA	0.2 fg ml ⁻¹	Bai et al., 2017
MPT64 antigen of <i>Mycobacterium tuberculosis</i>	DNA	100 CFU ml ⁻¹	Li N. et al., 2018
<i>Mycobacterium tuberculosis</i> cells	DNA	100 CFU ml ⁻¹	Zhang et al., 2017
<i>Mycobacterium tuberculosis</i> H37Ra cells	DNA	5.09 ± 1.43 nM	Mozioglu et al., 2016
Mycolactone in Buruli ulcer	RNA	1.59–73.0 μM	Sakyi et al., 2016
Mycoplasma-infected cells	DNA	Not informed	Liu et al., 2019
<i>Neisseria meningitidis</i> serogroup B	DNA	28.3–39.1 pM	Mirzakhani et al., 2017
PBP2a detection	DNA	20 nM	Fan et al., 2020
PBP2a in nosocomial infections	DNA	29 CFU ml ⁻¹	Maldonado et al., 2020
Protein A of <i>Staphylococcus aureus</i>	DNA	11.3 nM	Stoltenburg et al., 2016
Protein A of <i>Staphylococcus aureus</i>	DNA	10 CFU ml ⁻¹	Reich et al., 2017
<i>Pseudomonas aeruginosa</i> cells	DNA	100 CFU ml ⁻¹	Gao et al., 2018
<i>Pseudomonas aeruginosa</i> cells	DNA	60 CFU ml ⁻¹	Das et al., 2019a
<i>Salmonella enterica</i> serovar <i>typhimurium</i> in milk samples	DNA	3.37 × 10 ² CFU ml ⁻¹	Zhang et al., 2018
<i>Salmonella enteritidis</i> cells	DNA	0.309 μM	Bayrac and Oktem, 2017
<i>Salmonella enteritidis</i> cells	DNA	25 CFU ml ⁻¹	Chinnappan et al., 2017
<i>Salmonella typhimurium</i> cells	DNA	10 CFU ml ⁻¹	Duan et al., 2016
<i>Salmonella typhimurium</i> cells	DNA	123 ± 23 nM	Lavu et al., 2016
<i>Salmonella Typhimurium</i> cells	DNA	1 CFU ml ⁻¹	Ren et al., 2019
<i>Salmonella Typhimurium</i> cells	DNA	80 CFU ml ⁻¹	Wang et al., 2020
<i>Salmonella Typhimurium</i> cells	DNA	10 CFU ml ⁻¹	Appaturi et al., 2020
<i>Shigella sonnei</i> cells	DNA	15.89 ± 1.77 nM	Song M. S. et al., 2017
<i>Staphylococcus aureus</i> and <i>Escherichia coli</i> cells	DNA	10–2,000 CFU ml ⁻¹	Shen et al., 2016
<i>Staphylococcus aureus</i> cells	DNA	16 CFU ml ⁻¹	Kurt et al., 2016
<i>Staphylococcus aureus</i> cells	DNA	10 ³ CFU ml ⁻¹	Bayrac et al., 2017
<i>Staphylococcus aureus</i> cells	DNA	39 CFU ml ⁻¹	Cai et al., 2019
<i>Streptococcus pyogenes</i> cells	DNA	7 nM	Hamula et al., 2016
<i>Streptococcus pyogenes</i> serotype M3 cell	DNA	7.47 ± 1.72 pM	Alfavian et al., 2017
<i>Vibrio parahaemolyticus</i> cells	DNA	2.04e ⁻⁹ ± 0.12 M	Ahn et al., 2018
<i>Vibrio parahaemolyticus</i> cells	DNA	10 CFU ml ⁻¹	Sun et al., 2019

Papers recovered on the PUBMED NCBI website. Binding affinity and Limit of Detection (LOD) were represented by K_d and CFU counting, respectively.

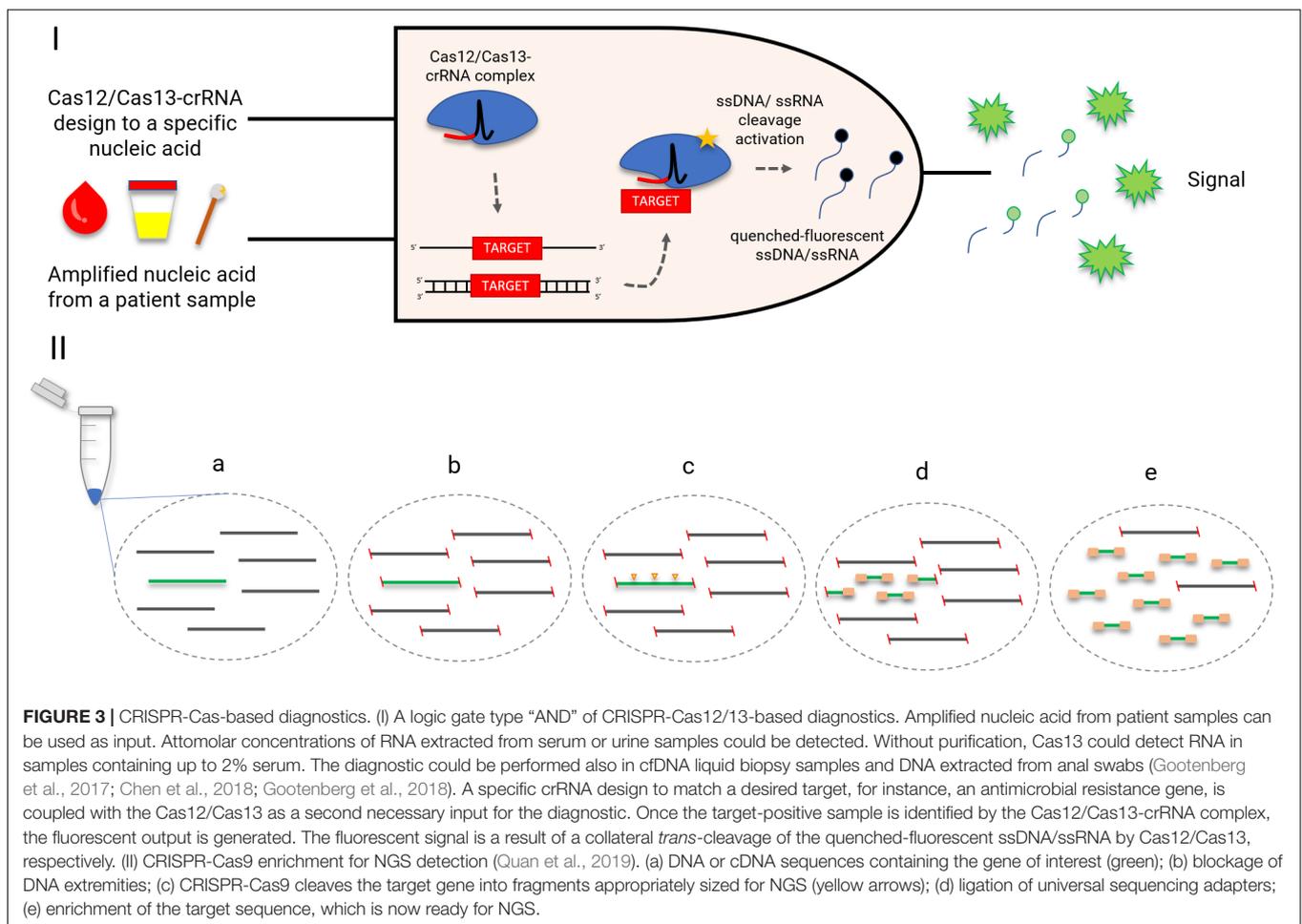
amplifies its potential to become a widely spread quick-and-cheap (\$0.61 per test) diagnostic method (Gootenberg et al., 2017). The addition of an extra step called HUDSON before SHERLOCK protocol enabled the viral detection directly from

bodily fluids, contributing to the creation of a field-deployable diagnostic platform (Myhrvold et al., 2018). Its efficiency to detect bacteria directly from patient samples, however, is yet to be defined. Also, SHERLOCK might benefit from Cas13 engineering

TABLE 2 | General features of CRISPR-Cas systems based on the most well-characterized subtypes.

Type	Multisubunity RNP complex	Single protein	Signature enzyme	Seed sequence	Most common substrates		Cleavage
					RNA	DNA	
I	X		Cas3	1–5 nt and 7–8 nt		X	Single-stranded DNA cleavage
II		X	Cas9	10–12 nt		X	Blunt double-stranded DNA break
III	X		Cas10	Not defined*	X	X	Specific and non-specific ssRNA cleavage. Double-stranded DNA break
IV	Defective CRISPR-Cas loci typically lacking the effector nuclease and the adaptation module						
V		X	Cas12	~18 nt		X	Double-stranded DNA break with staggered overhangs, non-specific ssDNA break
VI		X	Cas13	Not defined [#]	X		Specific and non-specific ssRNA cleavage

*Seed sequence: PAM-proximal sequence in which full complementarity is required for CRISPR-Cas interference. As (i) some CRISPR-Cas subtypes have promiscuous PAM sequences, (ii) the site sequence varies among the subtypes; the PAM sequences have not been displayed in this table. Gleditsch et al. (2019) review the PAM recognition strategies for all CRISPR-Cas types and engineering approaches to change the PAM recognition sequence. *Inconsistent conclusions regarding the seed region of CRISPR type III: absence of seed or its presence in the 5' end (Cao et al., 2016) or 3' end (Peng et al., 2015). #Central seed region proposed for Cas13a (Abudayyeh et al., 2016; Liu et al., 2017). Table based on Semenova et al. (2011); Chen et al. (2018), Hille et al. (2018), and Makarova et al. (2020).*



in order to increase target preference options and therefore the multiplexing panel.

When focusing on ARGs, *Klebsiella pneumoniae* carbapenemase (KPC) and New Delhi metallo- β -lactamase (NDM) were precisely detected and distinguished from five

clinical isolates of *K. pneumoniae* (Gootenberg et al., 2017). Also, HIV drug resistance mutations from 22 patient samples could be identified (Ackerman et al., 2020). As an advantage, these platforms offer a highly specific detection of single-nucleotide polymorphisms (SNPs), which can be valuable to precisely

distinguish any desired resistance gene variant (Li S. Y. et al., 2018; Myhrvold et al., 2018).

Of note, the power of the platforms to face real-world challenges has been demonstrated with the detection of SARS-CoV-2 during the COVID-19 pandemic (Ackerman et al., 2020; Broughton et al., 2020) and with the FDA emergency authorization for CRISPR SARS-CoV-2 Rapid Diagnostic using SHERLOCK platform (Guglielmi, 2020).

When employed against AMR, CRISPR-Cas9, Cas3, and Cas13 have been explored as a powerful sequence-specific antimicrobial. Cell death is an expected outcome when sgRNA is directed to genes on the chromosome or plasmids containing a toxin-antitoxin system. For vectors without toxin-antitoxin, plasmid clearance or drastic copy number reduction can be achieved when targeting plasmids up to 300 copies/cell (Bikard et al., 2014; Citorik et al., 2014; Yosef et al., 2015; Kiga et al., 2020; Tagliaferri et al., 2020). Consequently to plasmid clearance in clinical isolates, other non-targeting ARGs harbored on the target vector were also eliminated, and antibiotic reusability could be confirmed in a *Galleria mellonella* infection model (Tagliaferri et al., 2020). Several variants or sub-variants of the resistance gene can be covered with one sgRNA/crRNA directed to a conserved genetic region (Kim et al., 2016; Tagliaferri et al., 2020), while selecting a sequence from a variable region may be a strategy to achieve a narrow-spectrum effect. CRISPR-Cas-mediated interference can also be fine-tuned according to the delivery approach. CRISPR-Cas delivery can be mediated by bacteriophages, and the specificity of phage-host interactions is an advantage when the target is placed in complex environments, such as microbiota (Bikard et al., 2014; Citorik et al., 2014; Yosef et al., 2015). Alternatively, the CRISPR-Cas system can be delivered via conjugative plasmid (Citorik et al., 2014; Rodrigues et al., 2019; Ruotsalainen et al., 2019). Opposite to the phage-mediated approach, lack of specific receptors for plasmid uptake during conjugation is considered as an advantage over phage delivery, as mutations in the receptors may lead to phage resistance. On the other hand, the conjugation rate is slower when compared to transduction (Ruotsalainen et al., 2019).

DISCUSSION AND FUTURE DIRECTIONS

Even with eminent demand, little has been explored of CRISPR-Cas and aptamer potential toward treatment of bacterial infection. As for CRISPR-Cas approach, Cas9 immunogenicity must be considered (Crudele and Chamberlain, 2018), as well as the definition of the most appropriate delivery method to optimize CRISPR-Cas effect in targeting bacteria within complex microbial communities. Environmental assessments may be required to evaluate risks involved on plasmid clearance and bacterial death, which can affect the frequency of non-targeting bacterial species and non-targeting plasmids. Also, with the ordinary or induced death of the targeting bacteria, CRISPR-Cas nucleic acid will naturally be released into the environment and strategies to prevent spread and horizontal transference of CRISPR-Cas system still need to be developed.

Important limitations of *in vivo* use of aptamers stem from their susceptibility to nuclease degradation and rapid elimination due to renal filtration, but chemical adjustments to the oligonucleotide structure have contributed to decrease those shortcomings (Rohloff et al., 2014). As an FDA-approved aptamer-based therapy is already a reality, we believe that the extension of this technology to other fields, including AMR, is a matter of time.

In contrast to treatment application, CRISPR-Cas-mediated diagnostic has been recently FDA-approved for detecting SARS-CoV-2, paving the way for further applications. Its high scalability and multiplexing properties are of great value for the detection and surveillance of the wide varieties of ARGs. A limitation of this approach is the target of either DNA or RNA, which confirms the presence but not the functionality of the ARGs. Aptamers by their turn target ARG products, but in order to be used as an independent diagnostic tool, increased sensitivity to attomolar levels may be required to bacterial detection in bloodstream (Kelley, 2017). As a counterpoint, the target flexibility of CRISPR-Cas, its simplicity, and the rational design of sgRNA/crRNA can be an advantage over the more complex and randomized process of aptamer selection.

We envisioned that, in the near future, CRISPR-Cas and aptamers can be combined to treat and/or diagnose resistant bacterial infections due to their aforementioned complementary characteristics. Together, those strategies have already shown to reduce CRISPR-Cas-related off-target effects in the HEK293 cell line (Zhao et al., 2020) and to increase the delivery selectivity in liver cells (Zhuang et al., 2020), and the combination was a powerful and reliable molecular sensor able to detect nasopharyngeal carcinoma biomarkers (Li et al., 2021). Whether their combined characteristics will also be beneficial for AMR diagnostics and for treating infections caused by resistant bacteria is yet to be determined. A recent study developed a strategy to recognize surface proteins on MRSA strains by aptamer and CRISPR-Cas12a-assisted rolling circle amplification (Xu et al., 2020). Still, there is a gallery of CRISPR-Cas/aptamer combinations and target bacteria to be tested, as well as further optimizations to achieve attomolar sensitivity.

Studies employing aptamer and CRISPR-Cas for diagnostics have demonstrated their ability to provide shorter turnaround time results than the gold standard AMR phenotypic tests, which can take up to 72 h to be released. This, along with the possibility of developing paper-based diagnostics, highlights the techniques' potential to be employed as a first guidance to clinical decisions related to antimicrobial use. Altogether, the molecular approaches may offer a suitable solution to circumvent antibiotic misuse in the first antibiotic prescription, currently guided only by empirical decisions.

AUTHOR CONTRIBUTIONS

HP, TT, and TM designed and wrote the manuscript. HP and TT analyzed the data. All authors contributed to the article and approved the submitted version.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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