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Fighting nosocomial antibiotic-resistant infections through rapid and sensitive isothermal amplification-powered point-of-care (POC) diagnostics

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ABSTRACT

Point-of-care nucleic acid screening is a crucial clinical practice for addressing nosocomial infections in developed and developing countries, as well as in settings where a centralized laboratory approach encounters limitations. This rapid and accurate detection is important not only for the timely initiation of appropriate antibiotic therapy but also for resolving outbreaks and minimizing subsequent antimicrobial resistance. Current systems to diagnose nosocomial infectious diseases are mainly culture-based or PCR-based methods, with limitations of complex and time/cost-consuming procedures. Designing an integrated device that can simultaneously deliver sample preparation, nucleic acid amplification, and detection in a sensitive, specific, and timely manner remains a challenge. This review reports recent advances that may address this challenge, with particular emphasis on emerging developments that may lead to significant improvements in the point-of-care diagnosis of multidrug-resistant pathogens and new directions that can be used to guide antibiotic therapy.

1. Introduction

Nosocomial infections, also called Healthcare-Associated Infections (HAI), are acquired during the process of healthcare within 48 h of hospital admission, 3 days of discharge, or 30 days of an operation. Data on the prevalence of nosocomial infections suggest that they affect between 5% and 20% of hospital patients globally and can be manifested in different areas such as hospitals, ambulatory settings, or after discharge. These considerable numbers lead to increased financial impacts on healthcare systems. Indeed, nosocomial infections cause antimicrobial resistance increase, prolonged hospital stays, and higher mortality rates. In Europe, costs associated with nosocomial infections are estimated at approximately 7 billion € per year [1].

In the last years, the World Health Organization (WHO) is pointing out the emerging concern of Anti-Microbial Resistance (AMR) that occurs "*when bacteria, viruses, fungi, and parasites no longer respond effectively to medicines, making it harder to treat infection and increasing the risk of disease spread, severe illness, and death*" [2]. According to guidance published by the WHO, which stated that "*AMR could push 28.3 million people could be pushed into extreme poverty by 2050 due to high costs of treatment and chronic infections*", AMR threatens development with significant effects on a range of Sustainable Development Goals (SDGs) [3]:

- ✓ SDG1 (no poverty), as AMR cause an additional 28.3 million people to be pushed into extreme poverty by 2050 due to high costs of treatment and chronic infections;

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- ✓ SDG2 (zero hunger), as animals harmed by AMR affect farmers' livelihood and broader food security;
- ✓ SDG3 (good health and wellbeing), as it increases treatment costs and enhances child and infant mortality. Each year, 200,000 newborns die from drug-resistant infections;
- ✓ SDG 8 (decent work and economic growth), as AMR increases mortality and morbidity, labor supply will decline and could cause a decrease of 1–3% in global economic output by 2030, amounting to losses up to USD 3.4 trillion.

HAI etiology is based on infection source/type and responsible pathogen (e.g. bacterial, viral, or fungal, with bacteria the most common). Meta-analysis reveals that, although variations among different countries, nosocomial infections are most commonly caused by Gram-negative species (e.g. *Acinetobacter*, *Pseudomonas*, *Escherichia,* and *Klebsiella*) [4]. However, Gram-positive genera *Enterococcus* and *Staphylococcus aureus* are also highly prevalent, with the latter especially common in patients in low-risk clinical settings (i.e. non-intensive care units). Moreover, 54% of all *S. aureus* isolated are resistant to methicillin [5].

Currently used methods for infectious organisms' detection are based on prolonged, costly protocols that entails expensive instrumentations and skilled personnel: culture-based methods or Polymerase Chain Reaction (PCR). Microbiological culture requires skilled personnel and growth of a day as well as further tests are, therefore, needed to detect antibiotic resistance, which takes 1-2 additional days. Hence, patients receive broad-spectrum empirical treatment before making a specific diagnosis [6,7]. However, the excessive use of broad-spectrum antibiotics can lead to increased AMR, insufficient treatment of patients with resistant or non-targeted pathogens, more adverse drug reactions, and an increased risk of resulting opportunistic infections from the disruption of the microbiome [8–10]. PCR also places significant obstacles to a wide application in rapid diagnosis, because it requires laborious and time-consuming target identification strategies, such as gel electrophoresis or amplicon sequencing [11–13], and specific labeled identification reagents built into a real-time PCR, which are expensive. PCR methods are also sensitive to contamination [14] and inhibition by the compounds present in the template material.

The lack of clinical diagnostic tools to support antibiotic prescription, together with unfettered access, and minimal product regulation, is the cause of the epidemic continuing to increase in AMR. For these reasons, effective monitoring and screening of infectious diseases are crucial to face this emergency. In this context, biosensors have acquired paramount importance in the biomedical field as precise and powerful analytical tools able to provide fast response, continuous measurement, repeatability, accuracy, low volume amount, and the potential of integration and miniaturization. The development of a biosensor able to detect contaminant pathogens and their potential AMR could allow the identification of the most suitable treatments avoiding improper and unnecessary antibiotics in primary care. However, many drawbacks still hinder biosensor applications and commercialization, such as stability, specificity, and sensitivity. The last trends in biosensing highlighted the importance of powering the biosensing devices with vanguard technologies for the isothermal amplification of the nucleic acids with the first aim to enhance the sensitivity and specificity. To address the drawbacks of currently used methods, many isothermal amplification methods have been developed to accurately detect NAs [15–17]. Isothermal amplification methods enable nucleic acids (NAs) amplification without temperature changes with simple devices, as well as faster amplification times [18,19]. Among them, Loop-Mediated Isothermal Amplification (LAMP) has potential applications for clinical diagnosis as well as surveillance of infectious diseases in developing countries without requiring sophisticated equipment or skilled personnel, with the advantages of rapid amplification, simple operation, and easy detection. Few systems have been described in the literature exploiting other isothermal amplification assays, such as Rolling Circle Amplification (RCA), Helicase-Dependent Amplification (HAD), Nicking Enzyme-Assisted Amplification (NEAA), Multiple Cross Displacement Amplification (MCDA), Nucleic Acid Sequence-Based Amplification (NASBA), Recombinase Polymerase Amplification (RPA), among others [20,21]. Table 1 reports the main advantages and disadvantages of the currently used methods for the screening of nosocomial infections.

In this scenario, depicted in Fig. 1, this review describes the latest trends in the design of rapid and sensitive point-of-care (POC) diagnostics powered by isothermal amplification systems for the screening and monitoring of the main pathogens responsible for nosocomial infections as well as resistance-associated genes.

2. Isothermal amplification assisted biosensors for nosocomial infection pathogens

The ultimate requirement of the medical and bioclinical sectors is linked to the development of rapid and effective diagnostics for the identification of the main pathogens responsible for nosocomial infections and their antibiotic resistance-associated genes. As also stated by Căpăţînă et al. [22] "the treatment options for severe infections with this pathogen are greatly reduced". Indeed, in 2017 the WHO added the so-called ESKAPE group of pathogens to the short list of the antibiotic-resistant critical, which includes *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Klebsiella pneumonia*, *Acinetobacter baumannii*, *Enterococcus species* [23].

For this reason, new POC antimicrobial resistance tests are highly demanded to meet the challenge of high-throughput screening and antimicrobial resistance values in a multiplex approach to a broad range of antibiotics [24]. The following chapters report the latest trends in the design of POC devices integrating the isothermal amplification methods for the detection of the ESKAPE group of pathogens responsible for nosocomial infections and related antibiotic susceptibility.

2.1. *Staphylococcus aureus*

S. aureus is a Gram-positive aerobic bacterium with a spherical shape, giving rise to colonies in which the microorganisms assume a "cluster" arrangement. It is one of the most virulent pathogenic bacteria and is capable of causing various serious diseases, such as bloodstream infections, pneumonia, or bone and joint infections. Some strains of *S. aureus*, known as methicillin-resistant S. aureus (MRSA), develop resistance to beta-lactam antibiotics such as penicillin. MRSA is especially problematic in hospitals, where patients with weakened immune systems are vulnerable to infection. For this reason, monitoring the presence of this bacterium in healthcare settings is crucial to avoid infections among patients. Conventional methods for the screening of *S. aureus* are based on microbiological culture and PCR, which are time-consuming and dependent on laboratory professionals and equipment. To overcome the drawbacks of the traditional methods, isothermal amplification has been introduced to assist biosensors in monitoring *S. aureus*. Among the various methods, helicase-dependent isothermal amplification (HAD) was described by Frech et al. [25] for the amplicon detection of *S. aureus* in nasal swabs by hybridization to a chip surface. The HDA reaction consisted of a very low amount of nasal mucus lysate (5 μL) first diluted, then mixed with a commercial HDA-Mix (IsoAmp II Universal tHDA Kit, BioHelix,

Table 1
Pros and cons of analytical techniques for pathogen detection.

	PROS	CONS
Culture-based methods	Able to recognize viable cells Easy to quantitate cells in a sample High sensitivity with appropriate media	High skill level is necessary Closely related to laboratory use Time and resource intensive Relies on phenotypic biochemical characterization
Conventional PCR	Gold standard for identification in the absence of live agent Early diagnosis Widely used	Not quantitative PCR handling which can cause contamination Expensive equipment and consumables 3–4 h required for amplification Thermal cycler requirement Gel documentation system Unable to distinguish live from dead cells
Real-time PCR	Real-time monitoring of amplification Quantitative analysis Increased sensitivity due to fluorescent chemistry High throughput analysis thanks to software-based operation	Expensive equipment and consumables Use of fluorescent probes Need for high-skill personnel, reference laboratories, and financial support Unable to distinguish live from dead cells
Microfluidic-assisted amplification diagnostics	Sample- and cost-saving route Miniaturized and portable devices On-site analysis Massive production	High background noise Unable of quantitative detection Limited sensitivity Unstable and susceptible
LAMP	Thermal cycler not required Higher efficiency and sensitivity Real-time as well as quantitative Naked eye visual monitoring either through turbidity or color change by fluorescent intercalating dye (SYBR Green I)	Thermoblock required Complicated primer design DNA recovery influenced by cell lysis Closely related to laboratory use Unable to distinguish live from dead cells
RCA	Simple mechanism Temperature of reaction (30–37 °C) Tolerance to biological substances Easily detectable	Numerous rounds of amplifications RNA amplification is complex Works only with circular templates
HDA	Simple primers design No initial heating step Robust to biological substances	Expensive enzymes
NEAA	Extremely quick (15–30 min) Only two primers needed No initial heating step	High temperature (54–60 °C) Non-specific background
MCDA	Fastest method for SARS-CoV-2 detection No initial heating step Speed (40 min) Sensitivity, specificity Low cost	Complex primers design High temperature (60–67 °C)
NASBA	Specifically designed to detect RNA and in turn RNA viruses Temperature of reaction (41 °C)	Denaturation step Less efficient in amplifying RNA targets out of the range 120–250 bp Pre-heating step
RPA	Temperature of reaction (37 °C) Simple primers design Extremely quick (20 min) No initial heating step Robust to biological substances	Non-specific background
MCDA	Fastest method for SARS-CoV-2 detection No initial heating step Speed (40 min) Sensitivity, specificity Low cost	Complex primers design High temperature (60–67 °C)

Beverly, MA), and finally loaded into a LightCycler 480 Multiwell Plate 96 (Roche Diagnostics, Indianapolis, IN). This latter plate was transferred into a Light Cycler 480 instrument and incubated for 60 min using an isothermal temperature profile set to 65 °C. By a visible readout through a digital camera capture of the chip image, 70 subjects were analyzed achieving 89% sensitivity and 94% specificity. If compared to direct culture, this system proved to be much faster and more sensitive, appropriate for the identification of >100 colony-forming units per swab.

Besides HAD, most of the isothermal amplification-assisted biosensors for *S. aureus* have been developed in recent years using loop-mediated isothermal amplification. Misawa et al. [26] described the use of the LAMP technique to target the spa gene of S. aureus, which encodes for the specific protein A, and the mecA-gene, which encodes the penicillin-binding protein-2′ for methicillin resistance. This system can detect MRSA within 2 h after the blood culture signal became positive. Once obtained the amplicons, the authors observed the turbidity in the reaction tube with the naked eye and confirmed the results by electrophoresis in 3% agarose gels. This system was able to provide very satisfactory sensitivity and specificity, also compared to a duplex real-time polymerase chain reaction (Drt-PCR).

Hanaki et al. [27] also exploited a LAMP assay for targeting the femB and the mecA gene associated with methicillin resistance and identified methicillin-resistant staphylococci with 100% specificity, under the same reaction conditions within 60 min at 63 °C, highlighting the suitability of this technique to quickly identify S. aureusisolates and their antibiotic resistance.

Within even less time, Xu et al. [28] used the LAMP method for recognizing eight distinct sequences on three targets: 16S rRNA,

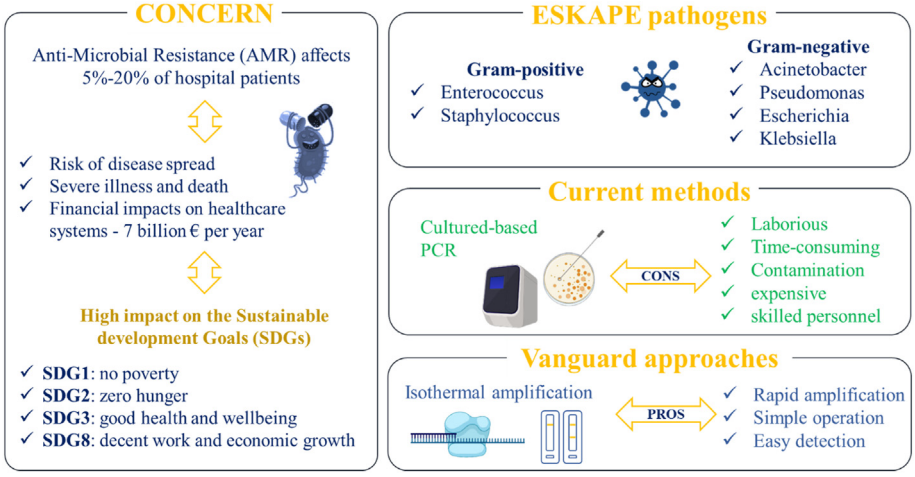

Fig. 1. The overall scenario of the current situation linked to the antimicrobial resistance concern and the need for the design of novel diagnostics.

femA and mecA of *S. aureus*. With optimal reaction conditions at 65 °C for 45 min, this assay was capable to provide detection limits of 100 fg DNA/tube and 10 CFU/reaction for 16S rRNA, 100 fg DNA/tube and 10 CFU/reaction for femA, 1 pg DNA/tube and 100 CFU/reaction for mecA, respectively. This study confirmed the LAMP method as a powerful tool for the rapid, simple, inexpensive analysis of a huge number of samples, with broad application for bacteriological detection of methicillin-resistant Staphylococcus isolates.

Su et al. [29] reported the use of a LAMP assay for targeting *orfX*, a highly conserved open reading frame in *S. aureus*. This assay, with an optimal amplification obtained under 65 °C for 45 min on a very low reactant volume (25 μl), was performed on 667 *Staphylococcus* (566 MRSA, 25 MSSA, 53 MRCNS and 23 MSCNS) strains and comparatively validated by PCR. A detection limit was determined to be 10 DNA copies and 10 CFU/reaction, as well as a high specificity, was observed.

More recent studies also confirmed the diagnostic accuracy of the LAMP method in comparison with conventional blood culture diagnostics, for the rapid diagnosis of *S. aureus*, methicillin-resistant *S. aureus* (MRSA) and *mecA/C* resistance genes [30]. High sensitivity and specificity were observed on 797 blood cultures as well as shorter time results, a crucial feature for early appropriate treatment.

All these studies [27–34] demonstrated that the LAMP method is an affordable and effective amplification method for sensitive, selective, and rapid screening and thus paving the way for the further design of integrated biosensors for point-of-care analysis. Indeed, in the last years, LAMP has been integrated into portable devices for rapid screening at the point of need.

As an example, Meng et al. [35] proved the ability of the LAMP technique to analyze and discriminate *S. aureus*, *S. epidermidis*, *S. haemolyticus*, and *S. hominis*, and predict their methicillin resistance by targeting the *mecA* and *femA* genes within 70 min using a LAMP-based microfluidic device. The authors designed and fabricated a portable instrument (dimension 35 × 34 × 18 cm - weight 8 kg) consisting of a temperature control module to provide a constant temperature of 65 °C for isothermal amplification using a Peltier heater, a detection module, and a movement module (Fig. 2a). This device was made of polycarbonate microfluidic chips containing 10 independent reaction chambers with a volume of ~4 μl, connected by long thin channels with a width of 500 μM and a depth of 200 μM. Different sets of primers were preloaded and dried at room temperature for 1 h in the corresponding reaction chambers, providing limits of detection of 20 CFU/reaction for *S. aureus*, *S. epidermidis*, *S. hominis*, and methicillin-resistant *S. aureus*, and of 200 CFU/reaction for *S. haemolyticus*. This integrated handheld device proved to be fast, accurate, and easy to use, as well as capable of providing information for the timely institution of appropriate antimicrobial therapy and a great potential for clinical applications, especially in resource-constrained settings.

The LAMP method has been integrated also into a Surface Plasmon Resonance (SPR) biosensor for the detection of methicillin-resistantS. aureus, as described by Nawattanapaiboon et al. [36]. In this study, DNA samples collected from sputum and blood hemoculture were undergone LAMP amplification for femB and mecA genes and the amplicons hybridized with ssDNA probes immobilized onto a bio-functionalized gold sensor chip surface of a multiplex array platform. This system demonstrated a good selectivity and sensitivity towards the methicillin-resistant S. aureus with a detection limit of 10 copies/μl, and, in addition, the sensor surface was able to be regenerated allowing at least five amplification cycles with a short assay time.

Variants of the LAMP method have been also described, as the multiplex loop-mediated isothermal amplification reported by Long et al. [37], which integrated the amplification assay into a nanoparticle-based lateral flow biosensor to detect *S. aureus* species including the methicillin-resistant one. The *femA* and *mecA* genes were targeted and the multiple-LAMP products obtained after incubation at 63 °C for 40 min were identified using the later flow biosensor within a total time of 80 min, providing a detection limit of 100 fg of genomic DNA template per reaction and a 100% sensitivity with no cross-reactions.

With the aim to design a portable and automated device incorporating the LAMP method, Choopara et al. [38] tried to overcome the limitations of this technique in terms of simplicity in the amplicons identification, e.g. the lack of simple color results that could hinder point-of-care detection. In detail, the authors fabricated a fluorometric paper-based biosensor loading LAMP reagents and a biotinylated primer onto a cellulose membrane together with a DNA fluorescent dye to identify the amplicons rapidly and accurately by the smartphone camera (Fig. 2b). This system was a simple paper strip able for instant detection of methicillin-resistant *S. aureus* in clinical samples as low as 10 ag (equivalent to 1 copy) within 36–43 min also in blood samples. Moreover, it provided 100% specificity and sensitivity compared to qPCR results and had 35-day stability under 25 °C storage.

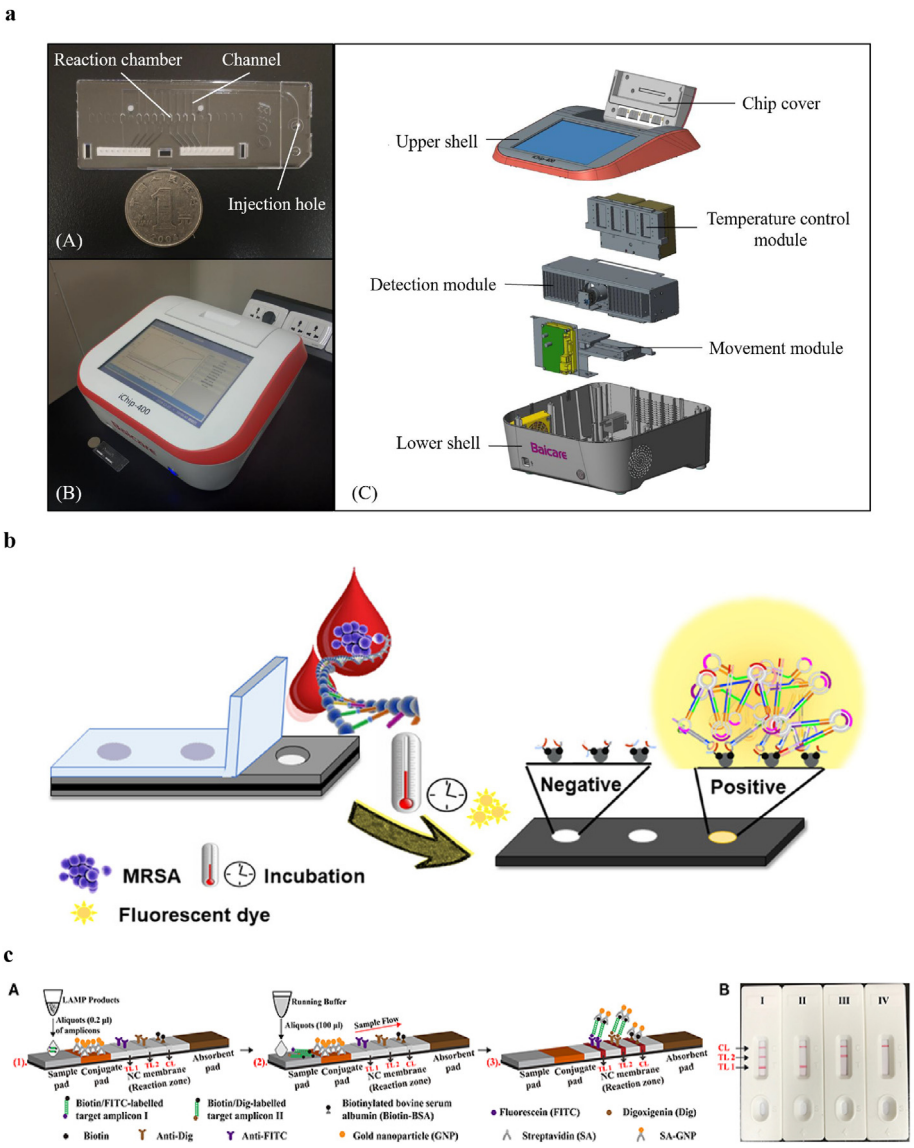

Fig. 2. a) Scheme of the LAMP-based microfluidic chip and instrument. Reprinted with permission from Meng et al. (2020). Rapid detection of *mecA* and *femA* genes by loop-mediated isothermal amplification in a microfluidic system for discrimination of different Staphylococcal species and prediction of methicillin resistance. Frontiers in microbiology, 11, 1487. Copyright 2020 Frontiers. b) Scheme of the paper-based LAMP device. Reprinted with permission from Choopara et al. (2021). Fluorometric paper-based, loop-mediated isothermal amplification devices for quantitative point-of-care detection of methicillin-resistant *Staphylococcus aureus* (MRSA). ACS sensors, 6(3), 742−751. Copyright 2021 American Chemical Society. c) Scheme of lateral flow biosensor for visualization of LAMP products. Reprinted with permission from Wang et al. (2017) Loop-Mediated Isothermal Amplification Label-Based Gold Nanoparticles Lateral Flow Biosensor for Detection of *Enterococcus faecalis* and *Staphylococcus aureus*. Front. Microbiol. 8, 192. Copyright 2017 Frontiers.

A lateral flow biosensor (LFB) was described by Wang et al. [39] for targeting the *nuc* gene of *S. aureus* using fluorescein derivative (FITC)-and digoxin-modified primers for the multiplex LAMP reaction. In detail, the authors used biotin- and FITC-/digoxin-modified primers to obtain biotin- and FITC-/digoxin-attached duplex products successively detected through biotin/streptavidin interaction (biotin on the duplex and streptavidin on the gold nanoparticle) and immunoreactions (FITC/digoxin on the duplex and anti-FITC/digoxin on the LFB test line) (Fig. 2c). Gold nanoparticle accumulation produced a red line, enabling visual pathogen detection without instrumentation, with a limit of detection as low as 250 fg of genomic templates per reaction within a total time of 75 min, with good results also in blood samples.

2.2. *Pseudomonas aeruginosa*

P. aeruginosa is a Gram-negative aerobic pathogen representing a leading cause of nosocomial infections related to ventilator-associated pneumonia in long-term care hospitals, urinary tract infections, sepsis, and skin and subcutaneous tissue infections [40,41]. The emergence of multidrug-resistant *P. aeruginosa*, especially carbapenem-resistant, is recently becoming a challenge for global health. Therefore, accurate and rapid detection methods are critical for the early control of infection. As for most pathogens, routine *P. aeruginosa* identification relies on microbiological culture, PCR, biochemical identification, and mass spectrometry, which are time-consuming (>2 days) and reliant on professionals and laboratory instrumentations. Besides the conventional approaches, many isothermal amplification methods, mainly LAMP-

based assays, have been optimized in the last years for the detection of *P. aeruginosa*, all of them demonstrating their suitability in terms of sensitivity and specificity. These assays were constituted of amplification reactions optimized under different conditions of primer and template concentration, incubation time, temperature, and so on, and the identification of the amplification products was provided using diverse fluorescent dyes or dye-labeled oligonucleotides and optical transduction [42–46].

However, very few isothermal amplification assays have been integrated in the last years into embedded devices for point-of-care analyses of *P. aeruginosa* as a nosocomial pathogen. Indeed, most of these studies highlight that the availability of portable and field-deployable tools is still limited. To address this lack of point-of-care devices, Chen et al. [47] provided an example of such integration combining the multiple loop-mediated isothermal amplification (mLAMP), targeting *ecfX*, *exoS*, and *exoU* genes, with a lateral flow nucleic acid biosensor (LFNAB) to identify the amplification products through dual immunoreactions and visual detection. In particular, a test strip (Fig. 3a) was used made of a conjugate pad, NC membranes, sample pad, and absorption pad. Anti-biotin Au-NP conjugates were sprayed on the glass fiber conjugate pad was prepared while FITC, hex, digoxin antibodies, and anti-biotin antibodies were drop cast on the NC membrane. Then, the NC membrane was covered with the conjugate pad and the absorption pad on each side. The sample pad receiving the liquid sample to be analyzed was attached to the other side of the conjugate pad. The accumulation of AuNPs due to the amplification reaction produced a visual red band, enabling *P. aeruginosa* detection without instrumentation at concentrations as low as 20 CFU/mL within 50 min.

A similar configuration based on the lateral flow approach was described by Dong et al. [48] (Fig. 3b). In this case, a set of six primers was designed to target *oprL* gene of *P. aeruginosa* by LAMP reactions conducted at 65 °C for 50 min. The amplification products were loaded on the paper strip of a lateral flow biosensor functionalized with crimson red streptavidin-coated polymer nanoparticles for visual detection. Once optimized all the analytical conditions in terms of temperature and time reaction and target genome concentration, the proposed biosensor provided very good sensitivity and selectivity as well as the capability for the analysis of clinical specimens.

More recently, Wu et al. [49] fabricated a microfluidic chip for a 10-reaction array made of polymethylmethacrylate (PMMA) and composed of the bottom layer consisting of reaction wells, a primary channel, an inlet hole and a vent hole, and a top layer consisting of waterproof and breathable membranes, a sealing gasket and a vent hole clip. A set of eight primers targeting *blaKPC*, *blaIMP*, *blaVIM*, *blaoprD2*, *blaNDM*, *blaOXA-23*, *blaOXA-48,* and *blaOXA-58* genes was designed and loaded into the reaction wells of the bottom layer. The chip was heated and the reaction mixture was cut off by the waterproof and breathable membranes. The amplification reaction was loaded into the main channel and the holes were sealed with PMMA cover. The chip was placed into the analyzer and incubated at 37 °C for 5 min, then at 65 °C, and finally at 65 °C for 40 min (Fig. 3c). The increase in the fluorescence signal in real time was exponentially correlated to the increase of nucleic acid amplicons. This system exhibited 87.5% accuracy, 97.7% sensitivity, and 78.8% specificity, proving to be very effective as it consumes minimal amounts of samples and amplification reagents, is low-cost, and requires constant temperature equipment.

A faster instrumentation was developed by Kaymaz et al. [50] based on a portable colorimetric platform for the high-throughput diagnosis of *P. aeruginosa* using LAMP assays within 30 min. This device consisted of a thermally isolated cup, low-cost electronic components, a polydimethylsiloxane sample well, and a fast-prototyped case that covers electronic components. With LAMP reactions at 65 °C and colorimetric readouts, pure genomic DNA and crude bacterial colonies were analyzed with god sensitivity and specificity, carrying out 16 or more reactions at the same time and with the potential of remote control of the operational parameters.

2.3. Enterococcus species

Enterococci are Gram-positive cocci often occurring in pairs as diplococci or short chains. Two species are common commensal organisms in the intestines of humans: *E. faecalis* (90–95%) and *E. faecium* (5–10%), while rare infection clusters occur with *E. casseliflavus*, *E. gallinarum*, and *E. raffinosus*. Clinical infections associated with Enterococcus are urinary tract infections, bacteremia, bacterial endocarditis, diverticulitis, meningitis, and spontaneous bacterial peritonitis. Ampicillin, penicillin, and vancomycin are mainly used to treat sensitive strains; however, intrinsic antibiotic resistance has been extensively documented for this bacterium towards β-lactam-based antibiotics (penicillins, cephalosporins, carbapenems), many aminoglycosides, and vancomycin. This latter is especially known in the case of vancomycin-resistant Enterococcus (VRE), in which the two most ubiquitous related genes, *vanA* and *vanB*, are responsible for the vancomycin-resistant phenotype. A few isothermal amplification assays have been developed for targeting such genes, with optimized conditions of the amplification reaction in terms of time, temperature, primers, and so on, as well as exploiting different detection of the amplification products including necked eye [51] and color change by hydroxy naphthol blue [52] or by phenolphthalein [53]. These studies represented a valid starting point for the design of more recent point-of-care devices to be used for timely, bedside diagnosis. Chang et al. [54] described the fabrication of an integrated microfluidic system to automatically identify the vancomycin-resistant gene (*vanA*) from live bacteria in clinical samples, combining LAMP assay. The microfluidic chip consisted of two silica-gel layers composed of pneumatically driven micro-pumps, micro-mixers, normally closed micro-valves, and micro-chambers for hosting samples and LAMP reagents (Fig. 4a). The sample was treated with Ethidium Monoazide Bromide (EMA) (an intercalating agent able to distinguish between live and dead bacteria) and exposed under visible light for 5 min. Then, reagents for low-temperature chemical lysis and 16S ribosomal ribonucleic acid (16S rRNA) nucleotide probe-coated magnetic beads were added into the reaction chamber. At this stage, the 16S rRNA nucleotide probe-coated magnetic beads cannot capture the EMA-intercalated double-stranded DNA (dsDNA) of dead bacteria. Therefore, there will be not any LAMP product resulting from dead bacteria. On the other hand, the 16S rRNA nucleotide probe-coated magnetic beads could successfully capture non-EMA-intercalated dsDNA in live bacteria that can be amplified by the LAMP procedure. After low-temperature chemical lysis at 37 °C for 2.5 min and magnetic beads capturing at 56 °C for 15 min, a magnet was placed underneath the microfluidic chip to collect DNA-magnetic bead complexes. Finally, LAMP reagents for *vanA* detection with fluorescent dye was pumped into the reaction chamber and the fluorescence signals resulting from LAMP products were detected by a photomultiplier. This integrated system was able to detect Enterococcus in joint fluid specimens with a detection limit of 10-colony formation units/reaction within 1 h, however, it cannot be considered a disposable point-of-care device as it requires lab setting equipment and trained personnel.

A version closer to the POC concept was described more recently by Trinh et al. [55], which fabricated a pop-up greeting card-inspired paper-based microdevice integrating DNA extraction, LAMP reaction, and pH-dependent colorimetric detection for vancomycin-resistant Enterococcus identification. In detail, a 3D

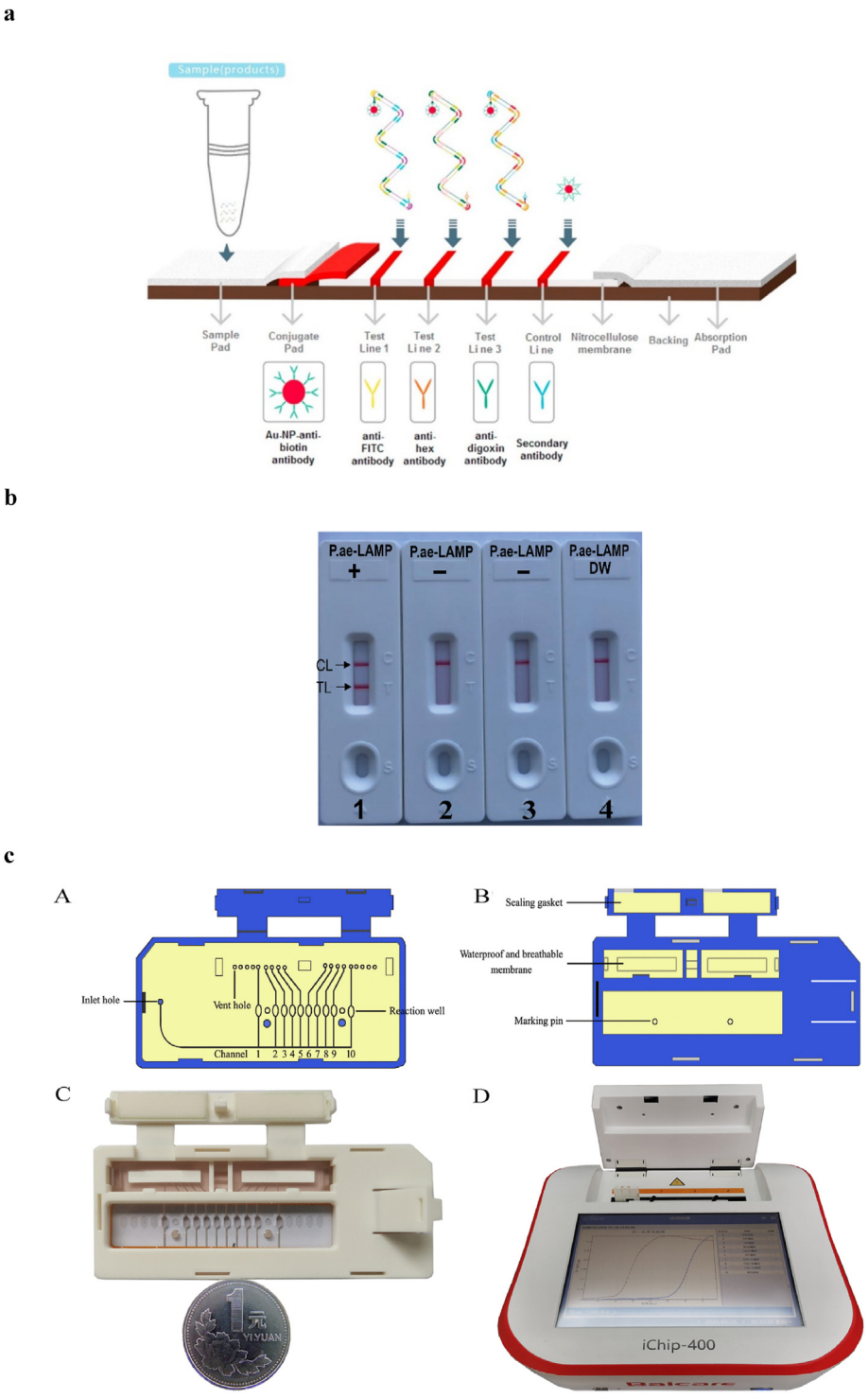

Fig. 3. a) Principle of Multiplex LAMP coupled with LFNAB. Reprinted with permission from Chen, Y., Cheng, N., Xu, Y., Huang, K., Luo, Y., & Xu, W. (2016). Point-of-care and visual detection of P. aeruginosa and its toxin genes by multiple LAMP and lateral flow nucleic acid biosensor. Biosensors and Bioelectronics, 81, 317–323. Copyright 2016 Elsevier. b) Confirmation and verification of *P. aeruginosa* LAMP products on lateral flow biosensor. Reprinted with permission from Dong et al. (2021). A Loop-mediated Isothermal Amplification With a Nanoparticle-Based Lateral Flow Biosensor Assay to Detect *Pseudomonas aeruginosa* in Endophthalmitis. Translational Vision Science & Technology, 10(14), 26-26. Copyright 2021 ARVO. c) Microfluidic chip and setup for on-chip LAMP for the parallel detection of multiple carbapenemases genes. Reprinted with permission from Wu et al. (2022). Development of microfluidic chip-based loop-mediated isothermal amplification (LAMP) method for detection of carbapenemase producing bacteria. Microbiology spectrum, 10(5), e00322-22. Copyright 2022 ASM Journals.

paper platform able was realized to fold on itself to mix samples and reagents for DNA purification and amplification (Fig. 4b). Folding the extraction zone the DNA was transferred to the chamber devoted to the LAMP reaction, performed at 65 °C for 45 min, and the amplicons were detected by color change (yellow in the absence of target DNA, colorless in the presence of amplified DNA). This disposable system was able to achieve a detection limit of 10^2 CFU/mL, promising accurate and fast point-of-care applications in healthcare settings.

Dinh et al. [56] also realized a similar paper microdevice (Fig. 4c)

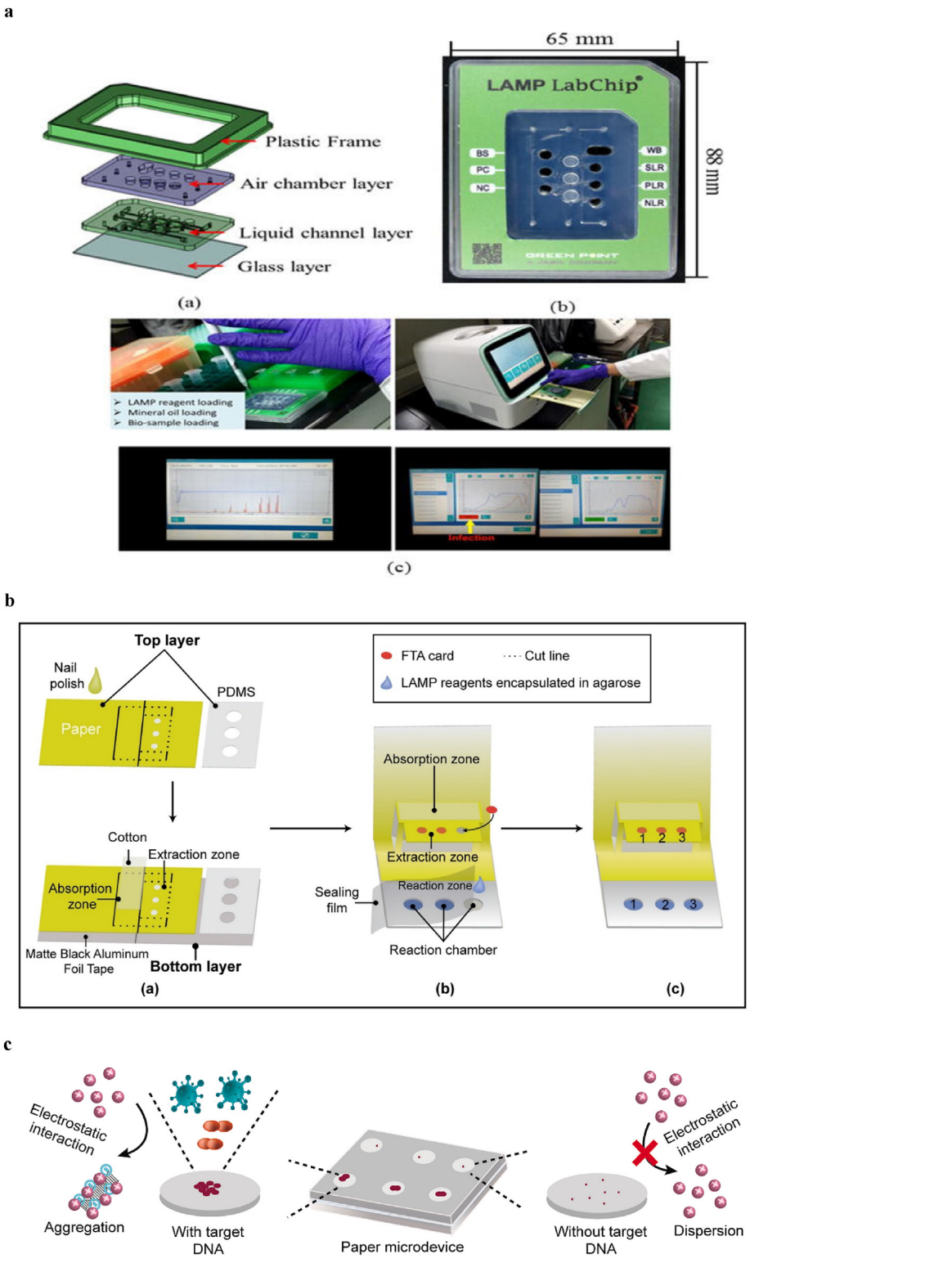

Fig. 4. a) Exploded view and a photograph of the integrated microfluidic chip. Reprinted with permission from Chang et al. (2017). Vancomycin-resistant gene identification from live bacteria on an integrated microfluidic system by using low-temperature lysis and loop-mediated isothermal amplification. Biomicrofluidics, 11(2), 024,101. Copyright 2017 AIP Publishing. b) Schematic illustrating the fabrication of the pop-up paper-based. Reprinted with permission from Trinh et al. (2021). Pop-up paper-based and fully integrated microdevice for point-of-care testing of vancomycin-resistant Enterococcus. Sensors and Actuators B: Chemical, 345, 130,362. Copyright 2021 Elsevier. c) Schematic illustration of the on-chip Safranin O-based colorimetric detection of LAMP amplicons. Reprinted with permission from Dinh and Lee (2022). Fabrication of a fully integrated paper microdevice for point-of-care testing of infectious disease using Safranin O dye coupled with loop-mediated isothermal amplification. Biosensors and Bioelectronics, 204, 114,080. Copyright 2022 Elsevier.

fully integrating DNA extraction, LAMP, and Safranin O-based colorimetric detection of *E. faecium*. The paper microdevice, composed of sample and reaction chambers, allowed foldable motions for transferring DNA from the sample to the reaction chamber. In this case, the LAMP reaction performed at 65 °C for 30 min provided the production of amplicons detected by a novel aggregation-based DNA approach using Safranin O polymerization. Precisely, safranin O felt polymerization by the addition of oxidant to form oligomers of Safranin O. Electrostatic interaction between the positively charged oligomers of Safranin O and the negatively charged DNA encompassing the LAMP amplicons resulted in aggregation with a dark red color. While, in the absence of LAMP amplicons, the safranin O oligomers were well dispersed showing their original red color. This biosensor was capable to identify *E. faecium* by the naked eye in 60 min with a limit of detection of 10^2 CFU/mL, demonstrating its potential as a rapid, fully integrated paper microdevice for controlling infectious diseases.

2.4. Klebsiella pneumoniae

K. pneumoniae is a Gram-negative bacterium that causes various infections, such as pneumonia, bloodstream infections, wound or surgical site infections, and meningitis. In healthcare settings, *K. pneumoniae* infections occur among patients under treatment for other medical conditions. Progressively, *K. pneumoniae* has developed antimicrobial resistance, particularly toward the carbapenem class of antibiotics. To face the limitations of the conventional identification methods, preliminary studies demonstrated the suitability of isothermal amplification assays for the detection of *K. pneumonia* to provide optimized conditions for the realization of POC testing. The latter, indeed, is able to furnish advantages in terms of rapidity, specificity, sensitivity, simplicity, and affordability, especially in comparison to the traditional methods for pathogen monitoring such as microscopy, disk diffusion method, biochemistry, serotype, and antibiotic dilution method. LAMP assays are the most described in the literature for the specific amplification of *K. pneumonia* targeting *ureR_1* [57], *CelB* [58], and *rcsA* [59] genes. All these studies evidenced the need for the optimization of the amplification/analytical parameters in terms of specific primer design, amplification mix, template concentration, reaction time, and temperature, useful features for further integration of such assays into POC systems.

Multiple cross displacement amplification (MCDA) has been also used [60] to target the *rcsA* gene for the genome-based identification of the pathogen, associated with a gold nanoparticle lateral flow biosensor (LFB) based on the binding of antibodies (embedded on the LFB) and haptens (labeled on the 5′ side of primers) (Fig. 5). The practical applicability of the proposed biosensor was evaluated on sputum samples (24 CFU per reaction), providing positive results for *K. pneumoniae* (30/100) in comparison with the culture method and the PCR (25/100).

2.5. Acinetobacter baumannii

A. baumannii is a Gram-negative coccobacillus that has the strength to remain present in healthcare environments for at least 30 days. It accounts for most Acinetobacter human infections in the blood, urinary tract, and lungs (pneumonia). In 2017, carbapenem-resistant Acinetobacter caused an estimated 8.500 infections in hospitalized patients and 700 estimated deaths in the United States [61]. In patients with sepsis, pneumonia, or urinary tract infections, early detection of both the pathogen and associated drug resistance is important for optimizing therapy and preventing the selection of resistance. Molecular-based techniques, such as PCR, can reduce the delivery result time with very high specificity and sensitivity; however, they are not suitable in resource-limited settings or for very fast testing. For this reason, simple, cost-effective, and rapid systems are required. Many isothermal amplification assays have demonstrated their huge potential for the early detection of significant pathogenic species and their antibiotic-resistance genes in clinical samples, including towards *A. baumannii*. We can count LAMP [62], real-time fluorescence LAMP [63], multiplex LAMP [64], and recombinase polymerase amplification (RPA) [65]. Among them, LAMP assays remain the most exploited for targeting *blaVIM-2*, *blaIMP-1*, and *blaOXA-23* [66], *blaKPC*, *blaNDM*, *blaVIM*, *blaOXA-48*, *blaOXA-23*, *blaOXA-40*, and *blaOXA-58* [67], *KPC*, *NDM*, *OXA-48*, *VIM*, *OXA-23*, *OXA-40*, and *OXA-58* [68], and *ISAba1-blaOXA-51-like* [69] genes as critical components for carbapenem resistance. The 16S-23S rRNA intergenic spacer (ITS) sequence has been also exploited for the detection of *A. baumannii* [70]. These studies have highlighted the efficacy of isothermal amplification methods for the sensitive and specific detection of *A. baumannii* also in clinical isolates, emphasizing their suitability for integration into POC testing, which the literature seems currently lacking for this pathogen.

3. Discussion and conclusions

The discovery of antibiotics has made diseases that previously could have caused death curable, thus saving millions of lives over the last century, but now our time with these drugs is running out. Antibiotics have been used so widely that many have lost the ability to defeat bacteria, and although new drugs are being developed, the rate at which superbugs develop resistance to antibiotics is dramatically high. Furthermore, the development of AMR is accelerated by the late diagnosis and inadequate treatment of HAIs.

Currently, AMR poses a global threat to health and development the WHO has declared AMR as one of the top ten threats to public health globally. According to The Lancet magazine, approximately 5 million deaths in 2019 were caused by bacterial AMR, and without urgent action, this number is expected to increase exponentially [5,6]. Furthermore, the exponential increase in antibiotic resistance

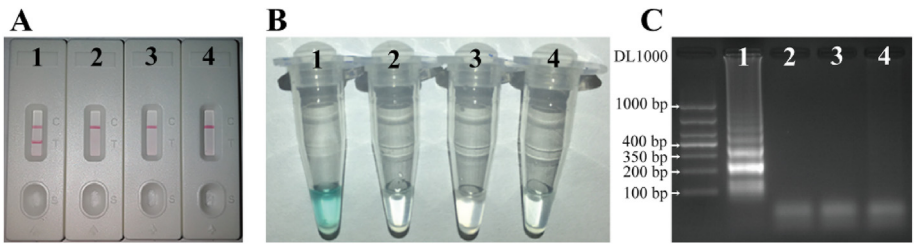

Fig. 5. The *K. pneumoniae*-MCDA-LFB assay. Reprinted with permission from Dinh and Lee (2022). Fabrication of a fully integrated paper microdevice for point-of-care testing of infectious disease using Safranin O dye coupled with loop- Niu et al. (2018). Isothermal amplification and rapid detection of *Klebsiella pneumoniae* based on the multiple cross displacement amplification (MCDA) and gold nanoparticle lateral flow biosensor (LFB). Plos one, 13(10), e0204332. Copyright 2018 Plos One.

inevitably leads to longer hospital stays with higher medical costs and more deaths. The latter accounts for half a million cases a year, kills more than road accidents, and has a major impact on state coffers: every year the Regions pay millionaire compensation to families who have suffered damage to their health.

Hospital infections (Fig. 6a) occur all over the world and concern both developed and developing countries. Research conducted by the WHO in 55 hospitals in 14 countries (Europe, Eastern Mediterranean, Southeast Asia, and Western Pacific) has shown that about 8.7% of hospitalized patients had a hospital infection. About 1.4 million people worldwide have infectious complications acquired during hospitalization. The highest frequencies of nosocomial infections have been reported in hospitals in the Eastern Mediterranean Regions and Southeast Asia (11.8% and 10.0%, respectively), with a prevalence of 7.7% and 9.0%, respectively in European and Western Pacific regions [71].

In particular, in Europe, HAIs cause each year:

- 16 million additional days of hospitalization
- 37,000 attributable deaths
- 110,000 deaths, for which infection is a contributing cause.

This results in very high costs, which have been estimated to be approximately 7 billion euros, including only direct costs [72].

The highest prevalence of nosocomial infections is recorded in the intensive care units and in the emergency and orthopedic departments and, in particular, the most frequent are those caused by surgical wounds, urinary tract infections, and lower respiratory tract infections. However, the microorganisms involved vary over time. Until the early 1980s, HAIs were mainly due to gram-negative bacteria (e.g. *E. coli* and *K. pneumoniae*). Then, due to antibiotic pressure and the greater use of plastic health devices, infections sustained by gram-positive (especially *Enterococci* and *Staphylococcus epidermidis*) and those by fungi (especially *Candida*) have increased, while those caused by Gram-negatives have decreased [73]. However, some gram-negative bacteria, such as carbapenemase-producing enterobacteria (CPE) and *Acinetobacter* spp., responsible for severe infections, have recently become very common in hospital care. Many patients assisted in intensive care and undergoing mechanical ventilation can develop severe complications caused by multi-resistant, predominantly gram-negative microorganisms. *K. pneumoniae*, *P. aeruginosa,* and *A. baumannii* represent the most worrying scenario for the patient and for the clinician who has objective difficulties in adopting timely antibiotic therapies. Fig. 6b reports the most common bacteria that cause HAI.

It is estimated that in 2050 bacterial infections will cause about 10 million deaths a year, far exceeding deaths from cancer (8.2 million), diabetes (1.5 million), or road accidents (1.2 million, with estimated costs over 100 trillion dollars: this is the forecast drawn up in a review in the “Economist” dedicated to antibiotic resistance by Jim O'Neill, the current British minister of Commerce, commissioned by the British government to analyze the problem and propose solutions that can be implemented on a global scale [74]. Already now in Europe, there are 4 million infections with antibiotic-resistant germs every year which cause over 37,000 deaths and are responsible for the significant absorption of resources which amount to around 1.5 billion euros a year. In Italy, antibiotic resistance remains among the highest in Europe and is, in most cases, above the European average. In our country, every year, from 7% to 10% of patients encounter an infection multidrug-resistant bacterial with thousands of deaths [75]. The prevention of infections contracted by patients during and after hospitalization is a strategic objective to stem the health emergency. For this reason, it requires urgent multi-sectoral action to achieve the SDGs.

Based on the above, we believe that a faster and more precise identification of the infectious agent and its sensitivity to the antibiotics currently in use is the best way to address the problem.

Traditional methods for identifying microorganisms are laborious and time-consuming, and the results are ready only after several days. With the advent of the “molecular biology age,” a large number of techniques and tools for molecular diagnostics have been developed for the detection and identification of bacteria for both clinical and research purposes. The use of genetic tools for bacterial identification began in 1985 with the invention of the polymerase chain reaction (PCR). Since then, many methods based on different principles, have been proposed. Currently, many infectious disease test kits are based on sputum and/or nasopharyngeal sampling and the use of techniques such as PCR, RT-PCR, and real-time PCR. DNA amplification techniques are very sensitive and accurate in identifying the agent that caused the infection, obviously only after selecting appropriate oligonucleotides.

However, to make molecular diagnostics suitable for low-resource settings and in-field applications, it is crucial to continuously adapt the working steps associated with DNA amplification, namely sample preparation, DNA extraction, and visualization of the results. To address these challenges, in recent years many

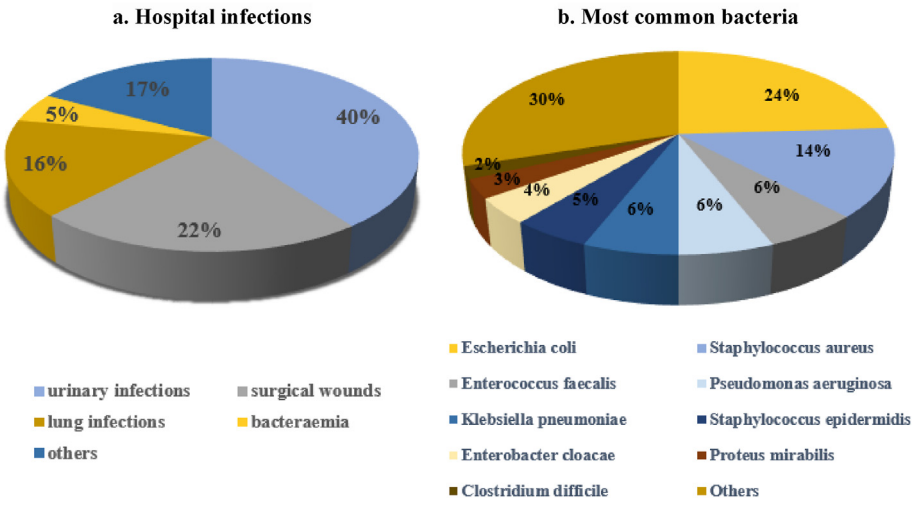

Fig. 6. a) Main nosocomial infections acquired during hospitalization. b) Most common bacteria causing HAI.

studies have been aimed at identifying new approaches such as the use of ionic liquids for the rapid isolation of nucleic acids from organisms relevant for food and water analysis or supplementation of entire analytical workflows on microfluidic chips. Indeed, many microfluidic-assisted diagnostic configurations have been described also in combination with nanomaterials to further enhance biosensing performances [76,77].

The aim of future molecular diagnostics (Fig. 7) is to design inexpensive, rapid, specific, easy to use and visualize, portable, and robust equipment that can be easily taken to the field to the desired analysis site (often referred to as "point-of-care" in clinical diagnostics). That is why the future of isothermal amplification applications will undoubtedly lie in ready-to-use cartridges combined with inexpensive portable devices for on-site analysis with the ultimate goal of moving molecular diagnostics from the lab to the field.

To allow for broad application, the reagents should be very robust and not require special storage procedures. As for the analysis time, the result should be visible within approximately 30 min to allow for a quick response to potentially unexpected or unwanted results. Finally, the methods should be easily applicable even by unskilled personnel thanks to the use of a reduced number of phases and to an intuitive manipulation that limits the sources of error. The importance of these goals was also addressed by the WHO that summarized, for the first time, these requirements in their ASSURED guidelines for point-of-care testing (Affordable, Sensitive, Specific, User-friendly, Robust and rapid, without equipment, deliverable to those who need it) [78].

One of the major achievements pursued over the past two decades to meet these requirements has been the development of isothermal DNA amplification methods. All amplification methods studied so far have in common the fact that amplification takes place at a constant temperature. This principle not only makes this technique faster than PCR due to the lack of heating and cooling steps but also eliminates the need for sophisticated equipment such as thermal cyclers, instead, a heating block or water bath is sufficient to provide the conditions necessary to conduct the analyses. Moreover, the isothermal amplification techniques have the advantages of higher stability and efficacy also at room temperature, thus avoiding the use of any instrumentation. These important features open up new application areas ranging from basic laboratories not equipped for DNA analysis to environments with limited resources. In this way, molecular diagnostics can be moved from centralized laboratories directly to sampling sites. In addition,

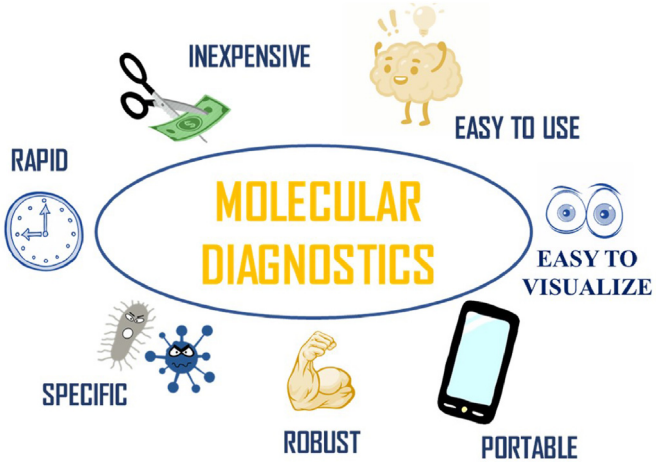

Fig. 7. Molecular diagnostics future goals.

the potential applications of isothermal amplification-assisted devices cover almost all fields of research, from clinical to environmental, food, and feed diagnostics, from the detection of plant and animal species to pathogenic microorganisms.

Further research should integrate such biosensing systems with Artificial Intelligence (AI) and Machine Learning (ML), as large datasets need to be managed for screening nosocomial infections. This will allow for speed, consistency, and capability of handling a huge amount of data, as well as for the availability of high-quality representative datasets that will support decision-making to fight antibiotic resistance. Further research should integrate such biosensing systems with artificial intelligence (AI) and machine learning (ML), as large datasets need to be managed for the screening and follow-up of nosocomial infections. This will enable speed, consistency, and the ability to handle huge amounts of data, as well as the availability of high-quality representative datasets that will support decision-making to fight antibiotic resistance.

Declaration of competing interest

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.

Data availability

No data was used for the research described in the article.

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