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Implications of direct amplification for measuring antimicrobial resistance using point-of-care devices

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Abstract

Antimicrobial resistance (AMR) is recognized as a global threat to human health. Rapid detection and characterization of AMR is a critical component of most antibiotic stewardship programs. Methods based on amplification of nucleic acids for detection of AMR are generally faster than culture-based approaches but they still require several hours to more than a day due to the need for transporting the sample to a centralized laboratory, processing of sample, and sometimes DNA purification and concentration. Nucleic acids-based point-of-care (POC) devices are capable of rapidly diagnosing antibiotic-resistant infections which may help in making timely and correct treatment decisions. However, for most POC platforms, sample processing for nucleic acids extraction and purification is also generally required prior to amplification. Direct amplification, an emerging possibility for a number of polymerases, has the potential to eliminate these steps without significantly impacting diagnostic performance. This review summarizes direct amplification methods and their implication for rapid measurement of AMR. Future research directions that may further strengthen the possibility of integrating direct amplification methods with POC devices are also summarized.

Introduction

Emergence of growing antimicrobial resistance (AMR) is now recognized as a global crisis with the accompanying dangers of inaction or weak action.¹ Steps are finally being taken to

address some of the key challenges to help sustain human health and quality of life (unimaginable before the advent of penicillin). The Antibiotic Stewardship Program by the Centers for Disease Control and Prevention (CDC) is a U.S. initiative² to reduce bacterial selective pressure while improving patient outcomes. This requires accurate and rapid diagnostics of pathogens and AMR coupled with prudent use of antibiotics. Numerous national and international surveillance networks exist, focusing on various aspects contributing to such stewardships.³ The “Core Elements of Hospital Antibiotic Stewardship Programs” recommends antibiotic “time outs” and tracking of “resistance patterns” to review and correct the empirical approach for prescribing the antibiotic in a timely manner. Policy changes that allow faster introduction of antibiotics are also being recommended.^{4–6} Among the challenges to stewardship are slow development of new antibiotics, weak control of prescription-based antibiotics, unnecessary use of these as growth promoting agents by the animal industry, costs of treatment, and the ultimate challenge to human suffering and loss of life in treating resistant organisms.

Globally there are at least a dozen resistant organisms of concern.¹ Carbapenem-resistant enterobacteriaceae (CRE; *Klebsiella pneumoniae*, *Enterobacter* species, and extra-intestinal pathogenic *Escherichia coli*), *Acinetobacter baumannii*, and *Pseudomonas aeruginosa* are among the key organisms known to cause antibiotic resistant infections. CRE infections are on the rise in US hospitals and throughout the globe.^{7–14} They are also associated with high mortality - often as high as 40–72%.^{15–18} *Klebsiella pneumoniae* carbapenemases (KPC), first reported in the US in 1996, is now the most prevalent CRE in the US.¹⁹ *E. coli* NDM-1 (New Delhi metallo- β -lactamase-1), first reported in New Delhi in 2011,²⁰ is now reported from more than 40 countries over all continents except Antarctica and South America.^{21,22} Increasing use of carbapenems is leading to the emergence of multi-drug resistant and pan-resistant CRE.²³ At present, tigecycline and colistin are among the few remaining antibiotics that continue to be effective against CRE.^{7,24,25} Even these may not persist in their efficacy without careful management.²⁶ Introduction of new antibiotics has also slowed because of market forces. Timely and accurate information about CRE will help in the management of antibiotics with improved patient outcomes.

Overall, infections due to these and many other microorganisms often result in sepsis and affect more than 750 000 people in the US alone; of which 215 000 die annually.²⁷ Survival rate for sepsis patients decreases by the hour and timely administration of correct antibiotics matters.^{28,29} If correct antibiotics are prescribed within the first hour, the survival rate could be as high as 80% but if treatment is delayed, it could be as low as 5% after 36 hours. For this reason, sepsis is treated as a medical emergency. Physicians generally prescribe broad spectrum antibiotics immediately based on other indicators and modify the course when the AMR test results become available through a combination of culture and nucleic acids-based analyses in a centralized laboratory which is generally after 1 to 4 days. At least one day is also required for sample transport.

For sepsis, the unmet need is to obtain the results in less than 1 hour, preferably within 15 min, be able to screen for multiple pathogens, and ideally at much lower concentrations (such as 1–10 colony forming units or cfu ml⁻¹). Point-of-care (POC) devices capable of measuring nucleic acids have a critical role in providing such rapid diagnosis. However,

sample processing, especially nucleic acids extraction and purification, must be integrated, automated, simplified, or eliminated for such POCs to be rapid and simple (Fig. 1). Evidence suggests that elimination of the nucleic acids extraction step is possible for certain polymerases and especially those used for isothermal amplification (e.g., Bst polymerases). For polymerase chain reaction (PCR) polymerases, only some of the more recently developed polymerases may allow direct amplification when combined with cell lysis steps. This review evaluates the potential of direct amplification for measurement of antimicrobial resistant bacteria by POC devices. The focus of direct amplification is on isothermal amplification because most POC devices are isothermal. However, this is changing and many qPCR and PCR-based POC devices are also on the horizon and some of the literature related to direct PCR may also be useful for these qPCR and PCR-based POC devices.

Rapid analysis at the best possible detection limit are key to measuring resistance in clinical settings

Because of the challenges faced at the lower detection limit, most methods do not start from blood, cerebrospinal fluid (CSF), pleural fluid, and urine – body fluids that are normally sterile. They employ blood culture or colony suspensions which take a minimum of 8 hours and more commonly 16–24 hours. This may already be too late for the majority of sepsis patients.³⁰ Most methods that claim to be rapid are actually referring to the time for “rapid confirmation of resistance - starting from positive blood culture or colonies” rather than “rapid detection of identity and resistance in normally sterile body fluids”. Due to the need for large number of cells (as high as 10^5 to 10^7 cells) to be present in a small volume of sample, most molecular methods require positive blood culture or colonies irrespective of the marker or principle used for antibiotic resistance confirmation. For example, detection and confirmation of CRE by quantitative polymerase chain reaction (qPCR) requires extracted and purified DNA starting from blood culture or colonies.³¹

Direct qPCR of lysed cells from colonies (which must first be grown) helps eliminate the DNA extraction step but lysed cell volume must be kept low (2–5 μ l) to avoid inhibition of qPCR. Thus direct qPCR may not be used for normally sterile samples. Other molecular methods are rapid but require blood culture or colonies. For example, matrix assisted laser desorption ionization – time of flight, a powerful rapid confirmation platform, can confirm a given species by comparative proteomics and the spectra can be obtained within a few minutes but the protocol requires processed mono-culture from positive blood culture.³² Similarly, NP Carba, a rapid biochemical confirmation method for carbapenemases less than 20 min, must use colonies.³³ These “rapid confirmation methods” are still quite useful compared to culture-based confirmation by antibiotic susceptibility profiles which takes an additional 1–3 days. Because of the need to use colonies or cultures, none of these methods can detect AMR within an hour starting with normally sterile samples. The challenge emanates from the very low concentration of organisms in normally sterile samples – as low as 1 cfu ml⁻¹. No culture-independent method known today can detect 1 cfu ml⁻¹ and sample concentration steps are generally required. A number of assays are closer to this goal by one to two orders of magnitude (Fig. 2). Idaho Technologies, for example, can detect 50–300 cfu ml⁻¹ of several infectious agents including *Yersinia pestis* in whole blood.³⁴

Similarly, SeptiFast from Roche can detect a set of 25 pathogens at 30 cfu ml⁻¹ in whole blood³⁵ and 30–100 cfu ml⁻¹ in synovial fluids, cardiac valves, and purulent exudates³⁶ but is currently available in Europe only. Roche cobas can detect 3–5 cfu ml⁻¹ of *Neisseria gonorrhoea* in urine.³⁷ To attain these superior detection limits, sample processing step involving cell lysis, DNA extraction and purification through the binding of magnetic glass particles, and PCR on the extracted DNA are generally integrated and often automated.³⁸ The resulting instruments are expensive, require highly trained personnel, and cost per assay is also high.

Many other FDA-cleared or commercially available assays for AMR bacteria have inferior sensitivities ranging from 100 cfu ml⁻¹^{39,40} to 100 000 cfu ml⁻¹⁴⁰ in samples that are normally known to contain much higher bacterial cell concentrations (blood culture, rectal swabs, colonies). One means of obtaining FDA clearance is that devices and assays can demonstrate substantial equivalence to another FDA-cleared device/assay. The assays provided in Fig. 2 (with the exception of Gene-Z and SeptiFast) have been cleared through the demonstration of substantial equivalence, suggesting that their diagnostic performance is similar to other more traditional and commercially-available methods.

Direct amplification studies using isothermal or PCR polymerases

Loop mediated isothermal amplification (LAMP) and PCR have the maximum number of studies documenting direct amplification. Multiple direct amplification studies with LAMP support the notion that Bst polymerase (the amplification enzyme used in LAMP) in can tolerate higher levels of organic materials in the sample matrix that are inhibitory to PCR.⁴⁴ Direct amplification studies with PCR or quantitative PCR (qPCR) have more commonly used pure culture or colonies or sample matrices that do not contain significant amount of materials inhibitory to Taq polymerase. Even though qPCR is the current gold standard for nucleic acids amplification-based diagnostics, the availability of field-deployable PCR or qPCR POC devices lags behind the availability of isothermal POC devices. In the subsections below, selected studies that have shown direct amplification with samples either using isothermal or PCR/qPCR are reviewed and their suitability for integration into POC devices is discussed.

Direct loop-mediated isothermal amplification (LAMP)

LAMP is an isothermal amplification technique that uses Bst polymerase with strand displacement activity eliminating the need for temperature cycling.⁴⁵ Its primer design approach uses three sets of primers. Since the first publication on LAMP in 2000, more than 4500 studies have been reported using various targets. Because LAMP has the potential to be rapid (as low as 7 min for a very high target concentration with efficient primers, never taking more than an hour to detect a single copy per reaction), it has been used in many nucleic acids-based POC devices. Tolerance of LAMP to substances that are inhibitory to traditional PCR was recognized early on⁴⁶ and it was suggested that when using LAMP, DNA purification step may be skipped. It is perhaps ideally suited for direct amplification because of its simplicity and studies report at least 100-fold lower inhibition with numerous sample matrices as compared to PCR.⁴⁵ Amplification of methicillin-resistant

Staphylococcus aureus was demonstrated directly with blood cultures by targeting the *MecA* gene encoding penicillin-binding protein-2 for conferring methicillin resistance (lower detection limit or LOD of 10^2 copies per reaction) and *Spa* gene encoding protein A (LOD of 10^3 copies per reaction).⁴⁷ In plasma, the direct detection of *Pseudomonas aeruginosa* was demonstrated with LOD of 2.8 ng total DNA per ml plasma.⁴⁸ In faeces, amplification of DNA from pathogens has been demonstrated with a number of targets including *Clostridium difficile* cytotoxin B,⁴⁹ *Campylobacter jejuni* and *Campylobacter coli*,⁵⁰ *E. coli*,⁵¹ and others, without DNA isolation or other extensive sample processing. The use of LAMP for direct detection of pathogens in urine has also been demonstrated targeting the *malB* gene specific to *E. coli*.⁵¹ Though PCR is typically inhibitory at urea concentrations larger than 50 mM,⁵² LAMP in this study was not inhibited. Direct amplification of targets from swab has been reported with enterovirus,⁵³ with results in concordance of 86.8% and 100% sensitivity and specificity compared to DNA extraction and PCR. An LOD of 1 cfu per reaction was demonstrated in spiked human sputum samples for carbapenem-resistant *A. baumannii* using direct amplification of OXA-51 gene (*ISAbal*).⁵⁴ Our group has extensively used direct amplification strategies including Bst polymerase and LAMP for a large number of matrices including bacterial cells spiked in blood, urine, sputum, and cerebral spinal fluid, protozoan cells in sludge, fish and veliger tissue suspended in water, crushed seeds and leaves, algae, bacterial cells in concentrated groundwater,⁴⁴ and spores. It is now well established that direct isothermal amplification using LAMP may be easily achieved by a crude lysis of the cells. Lysing agents used in the past include heat,⁵³ buffers,⁵³ or portable mechanical-based lysis approaches such as simple bead-beating⁵⁵ or portable sonication.⁵⁶

Direct amplification using qPCR

Clinical sample types for which direct qPCR has been demonstrated include whole blood,^{57–60} faeces,⁶¹ urine,⁶² buccal swab,⁶³ and cell culture.⁶⁴ Even though the same approach is relevant to PCR, due to the need for confirmation of amplification product using gel electrophoresis, direct amplification by PCR without measuring fluorescence is not relevant for the present discussion. Direct PCR amplification from blood-based samples is difficult due to inhibition by hemoglobin,⁶⁵ lactoferrin,⁶⁶ and immunoglobulin G,^{66,67} anticoagulants.^{68,69} The common qPCR inhibitory substrates found in faeces include phytic acid, metabolic products, and complex polysaccharides.^{70,71} For achieving direct amplification using PCR polymerases, reagents such as Ampdirect⁵⁷ or buffers with a higher pH are added to reduce the electrostatic interactions between proteins and genomic DNA.^{61,72} Direct PCR detection of targets in urine is particularly useful for cytomegalovirus⁷³ and *Leptospira*.⁶² In urine, PCR is more notably inhibited by urea at concentrations greater than 50 mM.⁵² A direct PCR method originally developed for plants has also been used with swabs of cheeks and skin, where clinical swabs are directly added into wells containing the buffer solution.⁷⁴ Some direct PCR-based approaches are also available commercially as part of sample collection devices (e.g. buccal swabs⁷⁵) to collect and preserve DNA. Direct PCR on cell cultures may also be useful but the time to culturing (from 24–72 h) may limit its usefulness for sepsis. Increasing the sensitivity of these tests may allow use in POC devices as it may eliminate the need for culturing.

Other isothermal approaches with limited evidence for direct amplification

Studies documenting direct amplification on other less commonly used isothermal amplification approaches^{76–78} are fewer, as expected. Because some of them use multiple enzymes or steps and lower temperatures, the complexity of these assays may also be a reason for the lack of studies reporting direct amplification.

Genome exponential amplification reaction (GEAR), for example, developed at CDC⁷⁹ is similar to LAMP in that it also uses 2–3 sets of primers and Bst polymerase with a reaction mixture incubated at 65 °C. It is expected that most of the direct amplification observed using the LAMP primer design approach, may also be possible using GEAR. This is supported by direct amplification of *E. coli* O157:H7 at 20 cfu per reaction obtained by concentrating the cells present in 100 l of water. Primers targeted the *rfbE* gene and the test was complete within 60 min.⁸⁰ Similar to LAMP, reverse transcriptase can also be integrated with GEAR.⁸¹

Recombinase polymerase amplification (RPA) exhibits rapid amplifications with the potential for relatively simplified instrumentation. Using this approach, direct detection of *Klebsiella pneumoniae* without DNA extraction was recently reported.⁸² After capturing and concentrating the pathogens present in urine samples, the cells were lysed by heating and extracts were used for the amplification of the *blaCTM-X-15* gene. The sample processing time was 10 min in addition to the 20 min amplification time normally required by RPA. An LOD of 10³ cells per ml of urine was reported. Previous studies with *Francisella tularensis* using extracted DNA as a template has shown more rapid results (10 min) with better LOD (10 to 100 copies per ml plasma prior to extraction).⁸³ RPA has also been used for the detection of methicillin-resistant *Staphylococcus aureus* (MRSA) with an LOD of less than 10 copies of DNA per reaction.⁸⁴ Besides distinguishing the methicillin-resistant from a methicillin-sensitive *S. aureus* strain, RPA was also able to ascertain the presence of three different genotypes of MRSA.

Strand displacement amplification (SDA) is another isothermal amplification approach that uses either Bst polymerase or Klenow fragment DNA polymerase.⁸⁵ It works at 37 °C and is somewhat slower, requiring a reaction time of ~120 min. Replication starts at nicks created by a strand-limited restriction endonuclease (HincII). The nicked site is ligated with each displacement step resulting in exponential amplification. Commercial platforms are also available *e.g.*, BDProbeTec™ ET System with inbuilt heating and centrifugation system that separates the DNA from the samples. A number of pathogens have been validated using *Mycobacterium tuberculosis*,⁸⁶ *Chlamydia trachomatis* and *Neisseria gonorrhoeae*.⁸⁷

Helicase-dependent amplification (HDA) uses DNA helicase to generate single stranded templates which is then amplified using DNA polymerase.⁸⁸ Direct detection of MRSA from blood cultures using HDA is known.⁸⁹ The samples required a dilution and heating step prior to the amplification of *nuc* and *mecA* genes and reaction time was around 60 min with an LOD of 50 cfu per reaction.

Smart amplification process (SMAP/SMAP2) is an isothermal amplification technique which can be used for direct detection of single nucleotide polymorphisms through its

complete suppression of background signals.⁹⁰ SMAP has the potential to determine the presence or absence of metastasis to lymph nodes using a cancer specific marker with a reaction time of only 30 min.⁹¹ Its updated version (SMAP2) has also been employed for the direct detection of many specific somatic and cancerous mutations including lung adenocarcinoma mutations.⁹² This technique has also been utilized for the detection of lamivudine resistance associated hepatitis B virus mutation with a sensitivity of 20 mutant copies per reaction.⁹³

For most PCR or isothermal approaches, primer design approach and reverse transcriptase enzyme may be integrated to accomplish mutation detection and RNA analysis. Other polymerases have some innate reverse transcription activity allowing their potential use in both amplification and reverse transcription (*e.g.*, Bst polymerase and Klenow fragment DNA polymerase).⁹⁴ Nucleic acid sequence-based amplification (NASBA), however, was developed specifically for amplification of RNA sequences. Features of NASBA include: (i) utilization of three enzymes including T7 RNA polymerase, RNase H, and AMV reverse transcriptase,^{95,96} (ii) constant isothermal temperature of 37–41 °C, (iii) reaction time of about 60–90 min, and (iv) multiplex amplification capabilities.⁹⁶ Due to its ability to quantify mRNA transcript of the target gene, it can be used as a useful tool for measuring gene expression and thus can be used as a strong predictor of resistant phenotype.⁹⁷ Differentiation of *blaKPC* variants from their pure culture is an example of such a phenotypic approach for combating with antibiotic resistance.⁹⁸ It has traditionally been used for rapid detection and quantification of different RNA viruses. Using an RNA-based NASBA, detection of parvovirus B19 DNA with an LOD of 10 genome copies per reaction has also been reported.⁹⁹ The utility and application of NASBA for direct detection and quantification of viable *Ralstonia solanacearum* cells has also been reported with an LOD of 10⁴ copies per reaction.¹⁰⁰

Required analytical sensitivity for nucleic acids-based POC devices

It is rather common to aim for the best LOD possible for any new assay. However, poor LOD alone is not an indication that the method is not valuable (many routinely used antibody assays have poor LOD). In rare cases, a method that is highly sensitive may even be less desirable because some background level of the infectious agent, not indicative of disease, may always be present (*e.g.*, nucleic acids based assay for *C. difficile*).¹⁰¹ On the other hand, there are many instances where assay LOD of 10² to 10³ cfu ml⁻¹ is sufficient to diagnose an infection and take action. This is especially true for limited resource settings where actionable diagnosis cannot be made based on non-nucleic acids-based test results which are seldom available anyway. A POC test providing viral load or genotyping data for HIV at a fraction of the cost may be extremely useful even if the treatment cut off of 50 viral particle per ml of blood is not achieved by the assay. This is because direction of response (from 50 million viral particles to say 50 000 and then to 500 viral particles convey the required information to the physician). The last bit of data *i.e.*, the point at which treatment can be stopped (50 viral particles per ml) may then be obtained by tests conducted in a centralized lab. The same logic applies for genotyping by POC which may provide information about correctness of treatment. Similarly, the situations where POC genetic testing is envisioned, a patient may get significant benefit from a less powerful test with

results available right away rather than a more powerful test available with results available much later. This point was nicely illustrated in a report by Bio-Ventures for Global Health.¹⁰² The report states that a diagnostic test with lower clinical sensitivity *e.g.*, a rapid antibody-based tests with 70% sensitivity done at the point of care may be able to treat more patients compared to a diagnostic test with higher clinical sensitivity *e.g.*, genetic tests with 90% sensitivity done in a centralized lab. This is because the rate of return of patients in countries where travel distances to seek healthcare is long, is a significant determinant of how many patients get treated. In the case of POCs, the rate of return is 100% while for centralized testing it could be much lower (say 70%). When multiplied by the sensitivity, the total number of treated patients could be 70% for POCs with less sensitive assay and 63% with centralized lab tests with more sensitive tests. Consequently, if the more sensitive genetic assays could be conducted using POC devices, the number of patients treated should be much higher (90%).

If a nucleic acids-based POC test is being developed for emergency rooms in developed nations, then clearing them for use in diagnostics through the Clinical Laboratory Improvement Amendments (CLIA) waiver process or U.S. Federal Drug Administration's 510(k) clearance is required. In certain cases, lab-developed tests (LDTs) – tests developed and used within the healthcare facility may be acceptable but regulations are changing rapidly for these as well. The analytical sensitivity of these established assays for detection of AMR and other pathogens is crucial for its clinical utility, particularly when used directly with clinical samples. At present, genetic testing directly from clinical samples using POC devices is rare. It is not surprising that out of the hundreds of tests available at the more than 225 000 CLIA-certified laboratories in the U.S., only few are for bacterial and viral pathogens (*e.g.*, for Hepatitis C, Strep A, Influenza A/B, HIV-1, *Helicobacter pylori*, and *Borrelia burgdorferi*) and none are based on nucleic acids amplification. This is because CLIA-waived assays must be simple and rugged and nucleic acids-based assays are not yet simple enough because they require sample processing and DNA/RNA extraction. Cumbersome protocols and kits for nucleic acids extraction and purification have been an integral part of traditional genetic assays. This required the samples to be shipped to a centralized lab for processing by expert molecular biologists. The analytical sensitivity for nucleic acids-based POC devices, may therefore be guided by their beneficial use case scenario in a given geographical location rather than as a competing technology with centralized screening tools. In some cases, such as quickly determine the AMR which is not always dependent on LOD, somewhat inferior instrument detection limit may not be a hindrance in the usefulness of the test.

Nucleic acids-based POC devices

Multiple reviews exist on POC devices, highlighting potential for measuring antimicrobial resistance under field conditions.^{103,104} Important features of POC devices for on-site detection include ease-of-use, cost, assay time, and ruggedness. Amplification chemistries and assays should also be sensitive and specific for clinical utility. Though POC devices are typically developed to detect a single assay, multiplexing for detection of multiple pathogens (*e.g.* sepsis) is crucial. This also allows for analysis of genes related to specific functions, as opposed to simple organism identification through 16S rRNA, for predicting virulence and

potential resistance, enabling better treatment decisions. Self-digitizing microfluidics with multiple reaction wells is one such means of multiplexing. The simplicity of using plastic⁴² or paper-based¹⁰³ materials for this purpose allows for more rugged (though still powerful) systems, while keeping equipment and consumable costs low, particularly when integrated miniaturization. Use of direct amplification with POC devices further reduces the time-to-results by potentially eliminating sample processing. Other POC devices (particularly qPCR-based devices) implement sample processing directly into these devices.

Some of the nucleic acids amplification-based POC devices exist that are commercially-available or near commercialization include Twista,¹⁰⁵ Illumigene, NucleSENSEasyQ, and Gene-Z. Twista (TwistDx, United Kingdom) uses RPA with capabilities for 8 samples per experiment while a number of devices use LAMP, detecting either fluorescence¹⁰⁶ (such as SYBR Green or SYTO82) or turbidity (*e.g.*, Illumigene,¹⁰⁷ Meridian Biosciences, United States, capable of detecting 8 assays per run). Nucle-SENSEasyQ¹⁰⁸ (BioMérieux, France) uses NASBA and can detect 8–48 assays per run. For qPCR, the options are many since it has been the gold standard but fewer can be considered POC devices. Portable, low-cost qPCR devices that integrate sample processing into the workflow and may be used for direct amplification protocols include Hunter¹⁰⁹ (Instant Labs, United States) which can detect 6 assays per run or GenePOC (GenePOC Diagnostics, United States) which can detect 94 assays per run. Open-source qPCR-POC devices are also on the horizon (*e.g.*, OpenPCR,¹¹⁰ Chai Biotechnologies, United States) which is currently available for under \$3200. It was manufactured through crowd-funding.

Direct amplification and antimicrobial resistance

It is obvious that if the sample processing step is eliminated or simplified, it will save time and equipment design and cost. Indirectly, it will also help save lives by providing timely analysis of resistance. Direct amplification is well established and approaches to enhance direct amplification are also becoming available. These include development of better polymerases, addition of nanomaterials that most likely puncture the cells, integration of sonication in microfluidic chips that may help break the cells, *etc.* Therefore, it should be possible to eliminate DNA extraction and purification steps for some of the sample matrices either entirely or partially. For a given sample matrix, it may be appropriate and beneficial to first explore if the DNA extraction and purification step is needed for the chosen amplification process and polymerase, before deciding to integrate a complex and cumbersome sample flow scheme. A simplified platform is more likely to be deployed globally for AMR surveillance – a critical component of antimicrobial resistance stewardship programs. However, some additional work summarized below needs to be done to demonstrate the value of direct amplification and integrate the overall scheme with existing and emerging POCs.

Most studies reporting direct amplification at present are carried out with limited number of samples. Validation studies with clinical specimens similar to those required for 510(k) approval for a given target may provide further confidence and also identify the limitations of direct amplification in a clinical setting. Such validation studies invariably are designed to address analytical precision and reproducibility, analytical sensitivity and specificity, clinical

sensitivity and specificity, lower limit of detection, reportable range, and accuracy among other method development parameters.

Validation of existing assays for use with direct amplification

Many primer sets already exist for detection of sepsis-causing bacteria and those that are resistant to antibiotics. It follows that the implementation of direct amplification in this area may not be difficult, since assays have already been developed and validated (Fig. 3). Primers for qPCR exist for thousands of organisms and genes and can be found in the literature and primer databases.^{111–113} Furthermore, some direct POC-based assays for pathogens including sepsis-causing organisms (*e.g.*, those described in Fig. 2) have shown detection limits as low as 5 cfu ml⁻¹ sample, though some methods require culture prior to detection. The important work that remains includes evaluation of these assays using other direct amplification protocols in more clinical matrices, particularly ensuring that sensitivity is not significantly impacted when the inherent target concentration that occurs during nucleic isolation is removed.

Primer coverage for nucleic acids-based assays

Thousands of assays are developed for single organisms relevant to AMR targeting usually a single genetic marker and using extracted DNA.^{115–120} However, when multiple pathogens or markers must be targeted, the problem becomes more complex. Even for the presumably simple case of CRE, primer and coverage issues may pose a challenge because of the diversity in AMR gene and their allelic variability. The detection of CRE, for example, may require the use of multiple genetic markers including *Klebsiella pneumoniae* carbapenemase (KPC), New Delhi metallo- β -lactamase-1 (NDM-1), oxacillinase (OXA), Verona imipenemase (VIM), imipenemase (IMP), cefotaximase (CTX-M), and extended spectrum β -lactamase (TEM, and SHV), all of which are markers of carbapenem resistance.

When designing primers, it will be important to address the allelic variability in each. Sometimes it may be easy because of similarity in sequence. For example, analysis of about 239 full-length NDM gene sequences shows that for the 810 base positions along the NDM gene, only 17 bases have some degeneracy. This indicates that universal primers are possible for NDM-1 and indeed qPCR-based methods utilize this sequence conservation for primer design to provide thorough coverage of most NDM-1 sequences. The same is true for the KPC gene. However, other antibiotic resistance gene markers may have higher allelic variability. For example, the CTX-M gene must be separated into three groups: group I (402 sequences) with 172 degenerate positions out of 930 base positions; group II (228 sequences) with 125 degenerate positions out of 930 base positions. While group III had 33 degenerate bases out of the 930 bases. What is evident is that nucleic acids-based molecular measurement of AMR will require multiple primer sets to theoretically achieve good coverage of most known sequences. For markers with high allelic variability (high diversity), the coverage may be unacceptably low highlighting the limitations of amplification-based tools compared to sequencing.

Sample concentration to enhance the lower limit of detection

When target concentration is low (*e.g.*, at levels where sample volume used does not ensure at least one cell or organism per well), sample concentration must be performed using filtration, magnetic or dielectrophoretic separation. Cartridges and approaches that can concentrate and then allow for lysis and/or direct amplification are being developed.¹²¹ We have also developed a technique in which filters were amplified directly within disposable Gene-Z chips.^{42,122} The method does not require a step to elute concentrated cells from filters and reduces loss that would occur due to DNA extraction. As such, a detection limit of 10^2 *Dehalobacter* cells per 100 ml of groundwater was observed. Others provide methods that can sort or trap the bacterial cells by other mechanisms (*e.g.*, dielectrophoresis) in order to concentrate the cells into a smaller volume starting with say 1–10 ml of blood or other body fluid. It is envisioned that over time, sample concentration schemes that need smaller sample volumes (*e.g.*, 10 ml of blood, or 50 ml of urine, 100 ml water *etc.*) will be integrated in the POC itself. This will be important to adapt direct amplification in POC devices. Sample concentration for matrices or applications where much larger sample volumes must be processed in order to obtain few cells may still use a separate concentration step (*e.g.*, hollow fibre membrane used for concentration of viruses from water).⁸⁰

Multiplexing and genotyping

The development of multiplexed approaches where multiple assays (specifically AMR) are detected simultaneously for a given sample has greater potential to reduce costs and decrease turnaround time-to-results. As of now, the most highly parallel AMR gene detection systems are based on PCR and require DNA extraction,^{123,124} but the development of POC technologies for AMR genes using isothermal direct amplification are being developed.⁴³ When using microfluidic cards with parallel reaction wells combined with LAMP, 20 AMR genes could be detected simultaneously in a single experiment.⁴³ Results were obtained with heat-lysed *S. aureus* isolates, without DNA extraction or other concentration methods. When comparing the direct isothermal amplification method with results obtained through DNA extraction and qPCR, a 98.2% agreement was observed.

Integrating cell viability and antibiotic susceptibility

Nucleic-acid based methods at the DNA level will measure both DNA from live cells as well as extracellular, if any, from dead cells of the target organism. Hence, applications where live/dead differentiation must be made and viability established may not be well served by NA-based POC devices. In these cases, a number of chemical agents including propidium monoazide¹²⁵ and ethidium monoazide¹²⁶ have been used to block the extracellular DNA from amplifying. In some instances, this blockage is partial in that amplification is delayed. One of the steps in the treatment with blocking agents is exposure to light so that the blocking agent forms a covalent bond with DNA, preventing denaturation and amplification. This is an extra step at present and could be integrated into POC devices. Similarly, the ability to carry out rapid antibiotic susceptibility testing that may take a little longer (*e.g.*, few h), in parallel to molecular tests that are available much sooner (*e.g.*, within 1 h) will be ideal. For example, one study used a short cultivation step in the absence and presence of

different antibiotics prior to padlock probe detection of the bacterial target DNA to allow a determination of susceptibility and genetic identification at a total assay time of 3.5 h.¹²⁷

Developing more robust polymerases that do not require DNA extraction or purification

The mechanism by which amplification enzymes, such as Bst polymerase, displays higher tolerance to inhibitors compared to PCR polymerases is mostly unknown. What is well established is that Bst polymerase can work in the presence of inhibitory substrates, at up to 100-fold higher concentrations, and is more successful in achieving direct amplification than Taq polymerases. Some insight can be gained by comparing the steps taken to minimize inhibition during PCR and isothermal amplification for various sample matrices. Amplification of nucleic acids by polymerases may be inhibited due to a number of factors including binding of chemical to active sites of the polymerases, alteration of DNA melting temperatures, reduced availability of Mg^{2+} required for polymerase activity, sequestering DNA from the reaction mixture, or interference with cell lysis.¹²⁸ Cellular debris, for example, is thought to interfere with PCR by causing sequestration of nucleic acids and primers due to interaction with proteins.¹²⁹ Increased primer concentration was proposed as a mechanism to alleviate some of this inhibition.⁴⁵ Urea, an inhibitor to and possible degrader of polymerases,¹²⁸ may be present up to 330 mM concentration in human urine.¹³⁰ Direct amplification is generally successful for up to 20% urine implying a tolerance of approximately 50 mM urea.⁵¹ Bst polymerase may have somewhat higher tolerance for urea compared to traditional PCR polymerases. Similarly, direct amplification in blood is inhibited by a number of components including hematin, heme, lactoferrin, and EDTA. Hematin may alter DNA melting temperature and enhance metal chelation.¹³¹ Binding of heme to DNA polymerase resulting in feedback inhibition has been known for decades¹³² and addition of bovine serum albumin is shown to reverse this inhibition.⁶⁵ Blood specimens often contain EDTA as preservative which chelates Mg^{2+} ions.⁶⁶ For isothermal polymerases to work efficiently, the available Mg^{2+} concentration must be between 4 to 8 mM.⁴⁵ Lactoferrin, another component that is present in body fluids releases Fe^{3+} ions which decreases primer specificity.¹³³ Because isothermal amplification, and especially LAMP uses 4 to 6 primers, this decrease in specificity may still not significantly impact the outcome.

Summary

The amplification approaches for detection of AMR exist, using both isothermal platforms and qPCR-based polymerases. Isothermal approaches have shown better promise for samples with higher levels of substrates inhibitory to amplification. For direct amplification, qPCR may be useful if a buffer is added to prevent or reduce inhibition during direct amplification, but results typically take longer (with a reaction time of 1–3 h; Table 1). An advantage to using qPCR for detection of ARG is the availability of published primers, as it is the gold standard for DNA amplification and thus most widely used. As there are only 2 primers required for qPCR, the issue of primer coverage is also much more easily faced. However, qPCR-based POC devices may be more costly and complex due to temperature cycling, whereas with isothermal amplification, this is not an issue. Other isothermal approaches requiring less primers, such as GEAR, RPA, SDA, SMAP/SMAP2, and NASBA,

may mitigate this issue, while also allowing the use of isothermal POC devices. Though LAMP requires 6 primers, of the isothermal approaches, LAMP has shown the most significant evidence for direct amplification, presumably due to its use of Bst polymerase, which is highly tolerant to clinical matrices. As it can also detect targets as low as a single copy in under an h, it may be best suited for direct amplification. Furthermore, the development of POC devices that use LAMP are simple, due to the isothermal temperature requirement as well as its ability to detection either fluorescence or turbidity. This would allow rapid field deployability of the assays to rapidly diagnose AMR infections. The use of direct amplification in POC systems by isothermal or PCR polymerases has the potential to rapidly diagnose AMR infections.

Future research focusing on the validation of direct amplification approaches for detection and diagnosis of AMR infections, preferably using POC platforms may further provide confidence in this emerging approach. The reduction in time-to-result will help expedite decision-making by clinicians. It may also allow better limit of detections because recovery losses during sample processing may be avoided. Studies focusing on the mechanisms and reagent conditions that allow direct amplification, primer design to provide the needed coverage of AMR, and development of more robust polymerases may also need to be explored. Finally, while the ability to detect 1 cfu per ml or 1 cfu per reaction is a good target, it is best to link such objectives to the decision-making process of the physicians, especially for goals related to prevention of antimicrobial resistance through better diagnostic tools.

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Mary Hughes received her DO from Michigan State University College of Osteopathic Medicine and is a Professor of Emergency Medicine and Chair of the Department of

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Fig. 1.
Schematic for point-of-care approaches, from sample collection to results.

Fig. 2.

Direct amplification assays for sepsis-associated organisms that have been validated using nucleic acid amplification techniques. The Y-axis notes the detection limits from different clinical samples including rectal swabs, whole blood, sputum, urine, blood cultures, synovial fluids, cardiac extracts, purulent extracts, and other matrices. Data from US FDA 510(k) submissions^{34,37,39–41} as well as publications for Gene-Z^{42,43} and SeptiFast.^{35,36}

Fig. 3.

Analysis of studies reporting primers for select sepsis-causing organisms by nucleic acid amplification method (plotted using Cir-cos¹¹⁴). Results obtained from Web of Science keyword searches.

Table 1

Summary of direct amplification approaches and the LOD achieved in some sample types

Name	Enzyme(s) required	Reaction temperature	Time to results	LOD with direct amplification
PCR, qPCR	Taq polymerase	Denaturation: ~94–96 °C Annealing: 45–60 °C Extension: 72 °C	1–3 hour	~100 cfu per ml ¹³⁴
LAMP	<i>Bst</i> polymerase	60–65 °C	60 min	10 ² copies per reaction ⁴⁷
NASBA	Reverse transcriptase; <i>T7</i> RNA polymerase; RNase H	37–41 °C	60–90 min	10 ⁴ copies per reaction ¹⁰⁰
HDA	Helicase; DNA polymerase	37; 60–65 °C	60–90 min	50 cfu per reaction ⁸⁹
GEAR	<i>Bst</i> polymerase	65 °C	60 min	20 cfu per reaction ^{79,80}
SDA	DNA polymerase; restriction enzyme	37 °C	120 min	99% sensitivity ⁸⁷
SMART-AMP/SMAP2	<i>Aac</i> DNA polymerase; Taq MutS	41 °C	30–45 min	1% mutant DNA ⁹²
RPA	<i>Bsu</i> polymerase; recombinase	37 °C	10–20 min	1000 cfu per reaction ⁸²