Point-of-care (POC) Tests for Infectious Diseases– The Next Generation!

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Abstract

Nucleic acid amplification tests (NATs) have become the cornerstone of clinical laboratories providing a same day diagnosis for a wide range of infections. Although polymerase chain reaction (PCR) has served laboratories well since its inception, PCR tests have significant disadvantages as they are labor intensive, require a thermal cycler, and are relatively slow compared with newer isothermal amplification methods. Following the introduction of isothermal amplification methods such as strand displacement amplification (SDA) and loop-mediated isothermal amplification (LAMP), several other isothermal amplification methods have been introduced, and some of these can yield positive results in as little as 5-10 minutes. First generation POC tests for bacterial and viral infections were antigen based and these tests lacked sensitivity. Laboratory-based molecular diagnostic tests using isothermal amplification have demonstrated excellent sensitivity and speed, both of which are required for POC tests for infectious diseases. Other attributes required for next generation POC tests are low cost and ease-of-use for resource poor settings including developing countries. Isothermal amplification with improved sensitivity is therefore well suited for the next generation of molecular POC tests. Since most amplification methods require nucleic acid extraction to remove inhibitors of amplification, specimen preparation is often the rate limiting step for clinical laboratories to provide rapid turn-around times. Nucleic acid purification also presents a challenge for POC tests and advances in specimen treatment will therefore be required for rapid POC tests for virus detection. The next generation POC tests will employ engineering advances in the areas of microfluidics, on-chip nucleic acid analysis, biosensors, and will facilitate the development of hand-held POC tests that can provide positive results in 20 minutes. These rapid POC tests will facilitate the detection of infectious diseases in a range of clinical and non-clinical settings including field use providing health care workers with a rapid, actionable result leading to improved patient management. The use of multiplex POC tests for detecting multiple pathogens will increase our understanding of the epidemiology of less prevalent infections, some of which may go undiagnosed in some settings.

Keywords: Point-of-care; Molecular diagnostics

Introduction

Over the past two decades, traditional testing methods used in the clinical laboratory have given way to nucleic acid amplification tests (NATs). The incorporation of molecular diagnostics into the laboratory armamentarium has improved our ability to detect low levels of viruses like HIV or Zika virus in a range of clinical specimens and has increased our accuracy of diagnosing viral infections, contributing to improved understanding of the natural history and epidemiology of viral infections. Point-of-care (POC) testing for viruses has followed in the shoes of POC testing for bacteria, transitioning from antigen to nucleic acid detection [1]. The development of POC diagnostics has allowed us to obtain real-time information on the spread of viral infections throughout a community, hospital, or on a global scale [2]. In addition, these tests can be performed using non-invasive samples such as saliva or nasopharyngeal (NP) specimens, which increases patient compliance for testing in both clinical and non-clinical settings. POC testing using patient-collected samples provides a more comprehensive analysis of emerging and re-emerging viral infections in a real-time manner with the use of mobile cell phones and GPS tracking.

Traditional testing for diagnosing viral infections has involved virus isolation in cell culture, ELISA, serology, direct fluorescent antigen (DFA) staining of nasopharyngeal (NP) specimens and shell vial culture (SVC) using a panel of monoclonal antibodies. In the early 1990s, specific

monoclonal antibodies were developed and respiratory viruses could be detected within 3 hours through DFA staining of viral antigens or within 1-2 days using SVC for more slowly growing viruses. This was far superior to the 8-10 days required for cell culture [2]. DFA staining of cells collected using NP swabs or NP aspirates (NPA) became the mainstay for many laboratories and hospitals in the 1990s; however, these approaches have now been replaced by molecular tests due to their superior speed and sensitivity. In addition, rapid antigen-based EIA tests developed in the 1980s and 1990s for point-of-care testing lacked sensitivity; the clinical sensitivities of these tests ranged from 20 to 90%, varying widely with the patient population being tested [2]. Thus, rapid EIA tests are not recommended for use in critical care settings due to their low sensitivities. NATs, especially real time PCR, multiplex PCR, and more recently isothermal amplification methods, have replaced conventional methods for detecting viruses largely because molecular tests detect 30 to 50% more positives [2-5]. The move towards isothermal amplification assays will facilitate the development of POC tests since thermal cycling is not required which will facilitate the diagnosis of infections in a wide range of settings including doctors' offices, emergency rooms, walk in clinics as well as field and home testing.

Isothermal Amplification Methods

Nucleic acid amplification plays an important role in diagnostic medicine, and is now commonly used to diagnose both bacterial and viral infections from a variety of sample types. PCR has been the cornerstone of DNA amplification since its introduction in 1983, but its reliance on thermocycling limits its applicability outside of laboratories. Hence, there has been a shift towards DNA amplification techniques that can be performed at a single temperature (isothermal amplification) such as loop mediated isothermal amplification (LAMP), strand displacement amplification (SDA), rolling circle amplification (RCA), and recombinase polymerase amplification (RPA). Below, we discuss these recent isothermal DNA amplification techniques, their mechanism, and their utility.

Loop mediated isothermal amplification (LAMP)

LAMP is currently considered one of the most powerful isothermal amplification techniques, relying on a strand-displacement polymerase combined with four to six primers [6]. These primers recognize several specific regions in the target DNA and two of the primers form loop structures to facilitate subsequent rounds of amplification producing high levels of DNA. Since the LAMP reaction is so robust, an extremely large amount of DNA is generated; accordingly, pyrophosphate ions (a byproduct of the amplification) are generated, yielding a cloudy precipitate (magnesium pyrophosphate) that can be used to determine whether amplification has occurred [7]. Using this approach, 1 to 10 copies of DNA can be amplified to 10^9 to 10^{10} copies often within 30 minutes, producing assays with excellent sensitivity and specificity [6]. In addition, multiplex LAMP assays (M-LAMP) can be developed, as has been shown for influenza A/H1, A/H3, and Influenza B, and Respiratory Syncytial Virus (RSV) A and B, with a lower limit of detection sensitivity of 1-10 genome equivalents [8,9].

Rolling circle amplification (RCA)

Rolling circle replication was first characterized as the mechanism through which viral circular genomes are replicated. Subsequently, it has been applied as both an exponential DNA amplification tool (100-fold increase in DNA) and a rapid signal amplification tool (100-fold signal amplification). In this approach, a small circular piece of DNA is primed by the target, after which a strand displacement polymerase enzyme continues around the circular DNA, displacing the complementary strand [10]. Ultimately, the synthesized DNA remains attached to the circle as more DNA is generated, generating 10^9 or more copies of the circle within 90 minutes. RCA has been applied for the detection of point mutations in human genomic DNA [11].

Strand displacement amplification (SDA)

SDA was the first isothermal amplification method described and involves restriction endonuclease nicking of a recognition site in an unmodified strand, followed by strand-displacing polymerase extension of the nick in a 5' to 3' direction, which displaces the downstream strand. The displaced strand can then act as a target for an antisense reaction, ultimately leading to exponential amplification of DNA [12]. Since its development, it has been improved using approaches such as hyperbranching and has been applied for whole genome analysis of genetic alterations [13].

Recombinase polymerase amplification (RPA)

RPA is one of the more recent isothermal DNA amplification techniques, involving a mixture of three enzymes; namely, a recombinase, a single stranded DNA-binding protein (SSB), and a strand displacing polymerase [14]. The recombinase enzyme is able to scan and target primers to their complementary sequence in the double-stranded target DNA, at which time the SSB binds and stabilizes the primer-target hybrid, allowing the strand-displacement polymerase to initiate DNA synthesis. Using this approach, DNA amplification can be achieved within 10 to 20 minutes, showing a high sensitivity and specificity. RNA amplification is also possible, as shown through the reverse transcriptase RPA (RT-RPA) assay targeting coronavirus [15]. In a recent report, Wang et al. [14] demonstrated detection of Feline herpesvirus 1 (FHV-1) within 20 minutes, at a detection level of 100 copies [14]. These reports demonstrate that RPA is a powerful tool for the rapid detection of DNA and RNA targets.

Helicase Dependent Amplification (HDA)

In HDA, DNA is replicated *in vivo* by DNA polymerase in combination with numerous accessory proteins, including DNA helicase to unwind the double-stranded DNA. The DNA helicase is included in the amplification mixture so that thermocycling is not required for amplification [16]. The single-stranded DNA intermediate for primer binding is generated by the helicase enzyme, as opposed to PCR where a heat denaturing step is required. HDA has been applied in numerous biosensors for the detection of for multiplex pathogen detection, and has promise for use in disposable POC diagnostic devices, such as for the detection of *Clostridium difficile* [17,18].

Signal Mediated Amplification of RNA Technology (SMART)

As opposed to the DNA amplification methods discussed above, SMART is based on signal amplification after formation of a three-way junction (3WJ) structure; the actual DNA or RNA target is not amplified [19]. Two oligonucleotide probes are included in the reaction, both of which have complementary sequences to the DNA or DNA target as well as a smaller region that is complementary to the other probe. The two probes are brought into proximity upon binding to their target, at which time the 3WJ is formed. Upon

formation of the 3WJ, polymerase can extend the target-specific oligonucleotide, forming a double stranded T7 promoter region resulting in constant production of RNA in the presence of target DNA, which can be detected in a real-time manner. SMART has been applied clinically to detect marine cyanophage DNA in marine and freshwater environments [19].

Point-of-Care Tests

The benefits of POC tests are significant. POC tests are performed at or near the site where a patient first encounters the health care system and provide actionable information for health care providers improving patient management. The availability of rapid results allows for timely treatment of the patient, decreasing the likelihood for disease complications or sequelae and containment of infectious disease outbreaks in a variety of environments including hospital emergency rooms (influenza) or rural settings (Ebola or Zika virus) by minimizing horizontal transmission. Accurate POC diagnosis also improves antibiotic stewardship by reducing presumptive treatment and decreasing unnecessary antibiotic usage. Molecular POC tests will have improved clinical sensitivity and specificity compared with first generation antigen tests. The employment of isothermal amplification methods in these POC tests will provide rapid and sensitive test devices capable of yielding results in a variety of settings in 20 minutes.

Lateral flow immunoassays and antigen POC tests

The first POC tests for bacterial antigens including pneumococcal polysaccharide in sera or urine from patients with lobar pneumonia were lateral flow immunoassays (LFIA). The success of these early LFIAs gave rise to other immunoassay platforms such as radioimmunoassay (RIA) and enzyme immunoassay (ELISA), which remain the dominant platforms used in clinical laboratories. However, these larger platforms are moderate to high complexity that require trained laboratory technologists and large instruments, and are not suited to POC testing. The present day LFIA platform is used for pregnancy tests, as well as diagnosing several infectious diseases [20]. In the infectious disease field, the LFIA is extremely versatile and can be used to detect both antigens and antibodies. LFIAs for antigens typically require an antibody pair, where an antibody to one analyte epitope is labeled with a reporter and a capture antibody to a second epitope is immobilized on the lateral flow strip. In this antigen-capture format, the strength of the signal at the detection line is proportional to the concentration of the antigen. This sandwich LFIA format has been used in a variety of assays including Group A streptococcal cell wall components, and to detect a variety of viral antibodies including HIV-1/2 and hepatitis C virus [21].

Many of these low complexity level tests have received CLIA waivers, making them suitable for POC use. The criteria for CLIA waived tests include ease of use and low risk of an incorrect result. This is in contrast to moderate and high complexity level tests that are typically performed at central laboratories and require knowledge to perform the test, including training and experience, the need for reagent preparation, multiple pipetting steps, the need for instruments, calibration, and independent interpretation and judgement [22]. As mentioned above, several LFIAs have been CLIA waived for the detection of HIV antibodies in either finger-stick or venipuncture whole blood specimens, as well as oral specimens. One FDA approved test for HIV antibody in oral specimens has been approved as an over-the-counter test for use with oral fluid

specimens [23]. Oral fluid specimens are more acceptable to patients due to the non-invasive specimen collection. The non-invasive specimen collection not only reduces blood exposure for health care workers, but more importantly lends itself to home use. In developing countries such as India that have limited health care resources and infrastructure, diseases such as malaria and tuberculosis are highly prevalent. The ability to detect and control these infections depends on a number of factors including the availability of sensitive diagnostic tests and their ease-of-use. Barriers to the introduction of POC tests in developing countries include incorporation of the testing into established clinical algorithms and the ability to report a result in a rapid fashion and have the result acted upon [24]. Despite these barriers, LFIAs that detect *Plasmodium spp.* antigens are among the most widely used diagnostics for malaria worldwide and these rapid malaria tests have been widely used in clinical laboratories because of their ease-of-use and round the clock availability where positive results can be confirmed the following working day. The LFIA platform has potential for detecting a wide range of viral infections and for applications in resource-poor settings, as well as for home testing including *Chlamydia trachomatis*.

Molecular POC tests

The major drawback of antigen-based POC tests is there lack of analytical and clinical sensitivity for specimens that contain low levels of antigen and the generation of false negative results. The CDC has expressed concern over the low sensitivity of rapid influenza tests compared to RT-PCR or viral culture and has stated that negative rapid test results should be interpreted with caution [25]. Similarly, the Infectious Disease Society of America has recommended that a negative rapid test for Group A Streptococcus be followed up with a culture test in children and adolescents [26]. Concern over the low clinical sensitivity of some POC tests has led to a major effort to produce POC tests with improved sensitivity. The development of POC tests that use molecular methods especially isothermal amplification is still in its infancy but there are already several approved molecular tests that are rapid, highly sensitive and CLIA waived. The first nucleic acid amplification test that was CLIA waived was the Alerei Influenza A/B test approved in January 2015, using an isothermal amplification method called Nicking Enzyme Amplification Reaction (NEAR) that detects Influenza RNA in 15 minutes. The approved sample for CLIA-waived use is a nasal swab [27]. This test has excellent clinical sensitivity and specificity compared to cell culture.

Since the approval of the Alerei Influenza A&B test, several other molecular tests have been CLIA waived including a Group A Strep (GAS) test for use on throat swabs which provides a result in 8 minutes [28]. Three tests have been CLIA waived on the Cobas Liat Platform (Roche Diagnostics), including Influenza A/B, GAS, and Influenza A/B & RSV [29,30]. The Cobas Liat System uses real-time PCR for detection of bacterial DNA targets or RT-PCR for viral RNA targets. The turn-around time for the Cobas Liat platform is 15-20 minutes for nasal and throat swabs. The Film Array® Respiratory Panel EZ assay (Bio Fire Diagnostics) has been CLIA waived for use in the U.S. and detects 11 viral and 3 bacterial pathogens providing results in approximately one hour using the Film Array platform. The Cepheid Gene Xpert Flu+RSV test is also CLIA waived, requires 1 hour for specimen processing and RT-PCR and also requires a Smart Cycler instrument. Other companies including Quidel, Qiagen, Atlas Genetics, and Gene POC are developing molecular POC platforms for clinical laboratories.

The major drawback of these first-generation molecular tests is the need for a benchtop instrument. The Alere™ i System and the Cobas Liat Platform both require specialized equipment in the form of an Alerei or Cobas Liat instrument for amplification and analysis. The Cepheid Xpert Flu+RSV test requires a Smart Cycler instrument and the Bio Fire Film Array RP EZ assay requires the Film Array platform. This equipment requirement severely limits the use of these molecular POCTs outside of normal clinical settings (doctor's office, hospital clinics, or ER), and especially for field use in developing countries for the diagnosis of emerging viral infections such as Ebola and Zika virus. The second major drawback of these assays is the need for upfront pipetting steps required for specimen processing which limits their use to trained individuals.

Discussion

The major objectives for next generation molecular POC tests include full integration providing instrument-free use with improved portability for testing in the field and in resource limited settings, together with low cost and ease of use for untrained individuals. POC test devices that use microfluidics and process small specimen and reagent volumes will be a major factor in reducing the cost of future devices. Next generation POCTs for infectious diseases will take advantage of isothermal amplification together with advances in microfluidics, on-chip nucleic acid analysis, and biosensors. These microfluidic devices will be fully integrated, instrument free, and will lyse pathogens releasing DNA and RNA, perform amplification of specific targets, and detect amplified products with electrochemical biosensors. More importantly, they can be manufactured as hand-held, single use disposable devices. The next generation of rapid and highly sensitive molecular POCTs will match the affordability and ease-of-use of traditional antigen POC tests and will have greatly improved sensitivity and specificity for use in both clinical (ER, doctor's office, walk-in clinics) non-clinical settings (field and home use) to provide an accurate diagnosis of infection in an individual and improved outbreak management. The next generation molecular POC test devices will be connected to smart phones with special apps to interpret results for the user and transmit results to a health care provider, or to a cloud database server for monitoring outbreaks as was done for the Ebola virus outbreak in West Africa. Field testing coupled with mobile phones using global positioning will allow us to link outbreaks to geographical areas to provide information on the emergence and spread of new and existing outbreaks.

Diagnostic tests used in the clinical laboratory have employed multiplex testing for the detection of multiple pathogens in a single test. Examples include the RVP° Respiratory virus panel from Luminex Molecular Diagnostics and the Bio Fire Film Array. POC tests will also be able to take advantage of multiplex testing using parallel channel microfluidics and biosensors [8,31]. Isothermal amplification like PCR also lends itself to multiplex testing for the detection and differentiation of multiple pathogens in a single specimen [8,9]. Next generation POC tests using multiplex isothermal amplification will be able to detect multiple pathogens within 20 minutes. How next generation POC tests will be integrated into various clinical algorithms and what economic benefits they will bring is a topic for debate [20,32]; however, more infections will be detected using these sensitive POC tests when applied in a variety of settings including the home, the workplace, and remote rural areas. The migration of testing out of the laboratory and into various non-clinical settings ("taking the laboratory to the patient") will permit the detection of infectious diseases in remote and difficult to access settings that otherwise would not have been possible. This data when compiled with laboratory based surveillance may improve our understanding of the true epidemiology of emerging infections like Zika virus, avian influenza A (H10N6), MERS-CoVas well as established infections such as malaria and tuberculosis that may be under-reported.

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