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**Recombinase polymerase amplification as a promising tool in hepatitis C virus diagnosis**

Zaghloul H *et al*. RPA as a promising tool in HCV diagnosis

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**Abstract**

Hepatitis C virus (HCV) infection represents a significant health problem and represents a heavy load on some countries like Egypt in which about 20% of the total population are infected. Initial infection is usually asymptomatic and result in chronic hepatitis that give rise to complications including cirrhosis and hepatocellular carcinoma. The management of HCV infection should not only be focus on therapy, but also to screen carrier individuals in order to prevent transmission. In the present, molecular detection and quantification of HCV genome by real time polymerase chain reaction (PCR) represent the gold standard in HCV diagnosis and plays a crucial role in the management of therapeutic regimens. However, real time PCR is a complicated approach and of limited distribution. On the other hand, isothermal DNA amplification techniques have been developed and offer molecular diagnosis of infectious dieses at point-of-care. In this review we discuss recombinase polymerase amplification technique and illustrate its diagnostic value over both PCR and other isothermal amplification techniques.

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**Key words:**Hepatitis C virus; Nucleic acid testing; Polymerase chain reaction; Point-of-care; Recombinase polymerase amplification

**Core tip:** Recombinase polymerase amplification (RPA) shows many advantages over both real time polymerase chain reaction and other isothermal Amplification methods. In this review we show the importance of molecular detection methods and how isothermal amplification techniques offer molecular point-of-care diagnosis. RPA shows unique characteristics among isothermal approaches that makes it a promising tool in the molecular diagnosis. Because hepatitis C virus is an endemic viral infection, we suggest that RPA may play an important role and save much time in screening infected individuals and managing the therapeutic course.

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**HEPATITIS C VIRUS**

Hepatitis C virus (HCV) is a positive-sense single-stranded RNA virus that was first cloned in 1989 and classified as a member of the family Flaviviridae[1]. This viral infection is characterized by high replication rate. It is estimated that about 1012 virions per day are produced in a given individual[2]. In addition, its genome exhibits a high degree of sequence variation caused by its error prone RNA polymerase. However, there are 6 characterized genotypes of HCV, 52 subtypes within these genotypes[3]. Humans are the only reservoir for HCV infection; which often leads to an asymptomatic chronic state in 80% of cases with subsequent development to acute liver disease.

An estimated 2%–3% of the world’s population is living with HCV infection and each year more than 350000 die of HCV-related complications, including cirrhosis, liver failure or hepatocellular carcinoma[4].

Although hepatitis C is considered to be endemic disease worldwide, there is a high degree of geographical variation in its distribution[5-9]. The prevalence of HCV infection is low, in most European countries where it represents 0.5%–2% of the general population[10,11], Americas, Australia, and South Africa (0.2% to 0.5%)[10]. Intermediate prevalence is reported in Middle East, India and Brazil[7,10]. Egypt recorded the highest prevalence of HCV in the world with about 20% of the population[7,9].

HCV is a blood prone infection, modes of transmission that have been reported include; transfusion of contaminated blood products, organ transplantation from infected donors, intravenous drug use, sexual transmission, public shaving, acupuncture, and invasive hospital procedures with contaminated equipment[12-16]. In Egypt where the highest prevalence in the world has been recorded, the major route of HCV infection was via an antischistosomal treatment program, with more than 35 million injections given over a 20-year in the period (1960–1980)[17].

The current standard treatment for chronic hepatitis C is a combination of pegylated interferon alfa and ribavirin. Sustained Virological Response (SVR) represents the endpoint of the treatment regimen, which indicates undetectable HCV RNA 24 wk post treatment[18].

Due to the lack of a vaccine or some form of post-exposure prophylaxis, the number of infected individuals will continue to increase, and in turn HCV-related morbidity and mortality, in the absence of effective care and treatment programs. The management of hepatitis C infection should not only focus on the treatment, but also prevention of infection to reduce the reservoir of infected individuals who can transmit the virus[19,20].

**HCV DIAGNOSIS TECHNIQUES**

The current laboratory techniques used for HCV diagnosis include: (1) Serological assays(*e.g.,* the enzyme-linked immunosorbent assay, recombinant immunoblot assay, *etc.*); and (2) Molecular assays: Depends on nucleic acid testing(NAT): qualitative [*e.g.,* reverse transcriptase polymerase chain reaction (RT-PCR), TMA, *etc.*] and quantitative (*e.g.,* real time PCR, *etc.*)

***Advantages and limitation of serologic assays***

The ease of automation and cost-effectiveness made serologic assays the most practical tool in HCV diagnosis[21]. However, antibody detection exhibits many disadvantages including that; detection is limited during the early stages of infection, poor sensitivity (false negative) in hemodialysis patients, immunocompromised patients[22-25], an abundance of false-positives[26] (because recovered patients may stay anti-HCV positive for years) and variability in accuracy between deferent commercial kits.

**NAT**

NAT detect and quantify HCV RNA and are now considered the gold standard in the diagnosis of HCV infection. In this approach, HCV RNA is extracted from the sample and reverse transcribed into the complementary DNA (cDNA), which is then amplified into a large number of detectable copies by the polymerase chain reaction (PCR). Unlike antibody detection that could be positive for years after resolving infection, the presence of HCV RNA indicates active infection and it can be detected in 1-2 wk post-infection[27,28]. NAT offers accurate and sensitive diagnosis of HCV without any additional confirmatory test and can be used to diagnose individuals with acute HCV infection. In addition, NAT play a crucial role in the management of antiviral therapies by monitoring HCV RNA level. It determines the basal viral load and monitors the treatment response[29]. Till now, fully automated real-time PCR is the most promising approach in NAT as it is faster, more sensitive and is not prone to contamination.

**ADVANTAGES AND LIMITATION OF NAT**

The importance of NAT arises from its ability to detect and quantify HCV RNA and in turn detecting the active infection (in contrast to anti-HCV). In addition, it can determine the level of the virus replication. Furthermore, it plays an important role in the antiviral treatment regimens and determines whether a virological response has been occurred or not[30].

However, molecular techniques for HCV diagnosis have many limitations including that; it is of complex procedures, time consuming and technically demanding as it cannot be carried out except in a highly equipped molecular biology laboratory (high cost analytical instruments).

**ADVANCES IN HCV DIAGNOSIS**

Every day the world takes a step towards NAT which becomes more practical than it was before. The competition between the commercial products enforces the companies to produce more simple, easy to use and cheap assays. In addition to the growing dependence on NAT, the significant advances in HCV diagnosis include using point-of-care (POC) alternatives instead of the routine venous puncture. POC can use specimen matrices such as oral fluid or finger-stick blood. Most existing POC are immunoassays and are now widely used for different applications. POC represent an ideal approach for the management of hepatitis C infection as it can reaches remote areas where the high equipped molecular biology laboratories are limited and in turn shorten the time of results which extend HCV screening. For instance, the development of a molecular point-of-care assay would represent a significant improvement in the field of HCV diagnosis.

**ISOTHERMAL DNA AMPLIFICATION**

Molecular analytical techniques gain a growing interest. According to the mentioned limitation combining conventional molecular methods, especially real time PCR, there was a demand to develop a simple, sensitive and cost effective technology.

Isothermal DNA amplification is an alternative to PCR-based technique and developed for point-of-care diagnosis[31,32]. In isothermal techniques, amplification reactions are performed at a constant temperature and hence there is no need for expensive thermal cycling instrument. Major practiced isothermal amplification techniques include; nucleic acid sequence-based amplification (NASBA), loop-mediated isothermal amplification (LAMP)[33], strand-displacement amplification (SDA)[34], rolling circle amplification (RCA)[35], helicase-dependent amplification (HDA)[36] and recombinase polymerase amplification (RPA)[37]. Isothermal DNA amplification techniques are simple, rapid and cost effective with equivalent specificity and sensitivity to PCR, enabling point-of-care diagnostics without the need to high costing equipment[31,32]. However, isothermal amplification approaches differed from each other in terms of operating temperature, reaction duration, mechanism, strengths and weaknesses. Table 1 summarizes the characters of the major practiced isothermal amplification methods.

As a competition between isothermal amplification techniques to perform molecular diagnosis at point-of-care, RCA will be kicked out of the race because it is incompatible with point-of-care diagnosis. Complex primer designing and the inability to perform multiplex amplification eliminates LAMP. The need to a denaturation step and the inability to tolerate inhibitory biological components exit both NABSA and SDA. Finally, RPA beats HAD in being faster and cheaper. Table 2 shows advantages and disadvantages of the major practiced isothermal amplification techniques.

**RPA**

RPA is an isothermal DNA amplification and detection method[37]. The amplification depends on a specific combination of enzymes and proteins (recombinase, single strand binding protein, and strand displacing DNA polymerase) used at a constant temperature and yielding a result in maximum 10 min. At first, RecA coat a single-stranded DNA (primers) to form nucleoprotein filaments. These filaments can then scan targeted double-stranded DNA (dsDNA) for sequences complementary to those of coated primers. Then, the nucleoprotein filaments initiate a 5´-strand invasion at the site of homology (Figure 1) forming what is known as D-loop. The strand invasion is stabilized by single strand binding protein. After that, strand extension takes place at the free 3'-end of the nucleoprotein filaments by a strand displacing DNA polymerase to synthesize a new complementary strand. During strand extension, the new synthesized strand displaces the originally paired strand.

Real-time detection of RPA amplicons is possible *via* exo-probes (Figure 2). Development of fluorescence depends on the separation of fluorophore and quencher via Exonuclease III cleaving at an internal abasic site mimic (tetrahydrofuran, THF) of the hybridized exo-probe (Figure 2)[38,39]. Fluorescence signal can be measured in real-time via a simple point-of-care scanner.

RPA technique is not restricted for amplification of the double stranded DNA targets, but also it could be used for amplification of RNA targets, as in the case with RT-PCR. Ahmed Abd El Wahed *et* *al*[40]*,* 2013 had developed RT-RPA assay for the detection of corona virus. The assay showed rapid kinetics with equal sensitivity and specificity of the real-time RT-PCR. The author suggested the diagnostic importance of the RT-RPA assay during the Hajj for the point-of-care detection of MERS-CoV infected cases to prevent the spread of the virus. Euler *et al*[39]*,* 2012 have developed a qualitative real-time RPA assay for detection of *Francisella* *tularensis* and the assay showed results comparable to real-time PCR. In another wider study by Euler *et al*[41]*,* 2013 RPA based assays were developed for the detection of Gram-negative (*Francisella tularensis* and *Yersinia pestis*) and Gram-positive bacteria (*Bacillus anthracis*), DNA viruses (variola virus), whereas reverse transcriptase RPA (RT-RPA) assays were developed for RNA viruses including Rift Valley fever virus, Ebola virus, Sudan virus and Marburg virus. The authors found analytical sensitivity and specificity equal to PCR with no cross-detection among respective targets. Also, Ahmed *et al*[42]*,* 2014 have developed RPA based assay for the detection of *Leptospira* and the method showed fast and less sensitivity to amplification inhibitors. Another competitive character compared to PCR based protocols had been reported by Kersting *et al*[43]*,* 2014 study in which RPA have been used for multiplex detection (detection multiple targets in the same reaction) of *Neisseria gonorrhoeae*, *Salmonella enterica* and *Staphylococcus aureus*. The author concluded that the kinetic performance of RPA was faster than PCR with no loss in sensitivity and specificity[43]. In the light of the above mentioned results it is clear that, RPA show competitive results as compared with PCR as regard to sensitivity and specificity, whereas RPA exceeds PCR as regard to the reaction kinetics.

In another study, RPA showed an impressive results in which the amplification reaction was conducted under a broad range of conditions from 30-45 °C with high inhibitory concentration of known PCR inhibitors in just 15 min[44].

**ADVANTAGES AND DISADVANTAGES OF RPA**

RPA overcomes the technical difficulties posed by current molecular techniques.

At first; it demonstrates a rapid kinetics, the process begins operating the immediately when the sample is contacted to the reagents and there is no need for melting the double-stranded DNA target.

Second, it operates at a constant temperature (30-42 ºC, optimum nearly 37 ºC), which give the advantage of being an energy saving technique and cost saving (there is no need for thermal cycler).

In addition, the target can be either DNA, or RNA, making RPA suitable for the detection and diagnosis of RNA viruses, like HCV.

Furthermore, the combination of probe-based detection, RPA represents a significant advance in the development of portable and accessible nucleic acid–based tests.

Unlike PCR in which the amplification reaction is controlled by temperature, digital RPA suffers from undesired reactions because the amplification could proceeds at room temperature if the nucleic acid sample is premixed with initiation reagents prior to compartmentalization and thus increasing the target count. However, any low-temperature non-specific pre-amplification reaction can be eliminated by compartmentalization of the nucleic acid template prior to adding initiation reagents[45,46].

Another drawback of low temperature amplification results from the interaction between primers even when well-designed. These interactions can create noise that defeats the analysis. However, this drawback could be avoided by using Self Avoiding Molecular Recognition System (SAMRS)[47]. SAMRS are nucleotide analogues that can bind to natural DNA but not to other SAMRS species. Therefore, primers built from SAMRS not interfere with each other. The concept of SAMRS was introduced over a decade ago[48,49] and was exploited to fix interactions between primers in PCR and multiplex PCR[50].

**CONCLUSION**

Early diagnosis and treatment of HCV infection can reduce the risk of long-term complications and prevent further transmission as well. NAT) represent the gold standard for the diagnosis of HCV infection. Detection of HCV RNA level is an important factor in antiviral regimens especially for determination of SVR. RPA combines the advantages of serologic and Molecular techniques and overcome limitations of both. It represents a simple, accurate and cost effective diagnostic tool and can be carried out at remote areas. In turn, it could improve the management of HCV infection by screening carrier individuals and stop transmission. The demand for the development of nucleic acid based point-of-care assay is increasing alongside with increasing the number of HCV infected patients. RPA based HCV diagnosis would represent a significant advance in the management of HCV.

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**Figure 1** **Recombinase polymerase amplification technology amplifcation cycle (for details, see the text above).**



**Figure 2 Example for the exo-probe of the recombinase polymerase amplification assay.**

**Table 1 Characters of some isothermal amplification techniques[51]**

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
|  | **NASBA** | **LAMP** | **SDA** | **RCA** | **HDA** | **RPA** |
| Template | DNA, RNA | DNA1 | DNA1 | DNA1 | DNA1 | DNA1 |
| No. of primers | 2 | 4-6 | 4 | 1 | 2 | 2 |
| No. of enzymes | 3 | 1 | 2 | 2 | 2 | 2 |
| Temperature | 41 ºC | 60-65 ºC | 37 ºC | 37 ºC | 65 ºC | 30-42 ºC |
| Reaction duration | 90-120 min | 60-90 min | 120 min | 60 min | 75-90 min | 20 min |
| Denaturation step | Y | N | Y | N | N | N |
| Inhibition tolerance | N | Y | N | N | Y | Y |
| Product detection | GE, RT | GE, RT, TE | GE, RT | GE | GE, RT | RT |
| Multiplex | Y | N | Y | N | Y | Y |
| Point-of-Care | Y | Y | Y | N | Y | Y |

1RNA can be amplified after the introduction of a reverse transcription step. NASBA: Nucleic acid sequence-based amplification; LAMP: loop-mediated isothermal amplification; SDA: Strand-displacement amplification; RCA: Rolling circle amplification; HDA: Helicase-dependent amplification; RPA: Recombinase polymerase amplification; GE: Gel Electrophoreses; RT: Real Time; TE: Turbidity; Y: Yes; N: No.

**Table 2 Advantages/disadvantages of some isothermal amplification methods**

|  |  |  |
| --- | --- | --- |
| **Technique** | **Advantages** | **Disadvantages** |
| **NABSA** | Specifically designed to detect RNA and in turn RNA viruses.Power saving (41 ºC) | Denaturation stepLess efficient in Amplifying RNA targets out of the range 120–250 bp |
| **LAMP** | Highly specific (utilizes 4-6 primers spanning 6-8 distinct sequences)Tolerance to biological substancesCould be detected by a cheap turbidity-meter | Primer design is complexUnable to perform multiplex amplification |
| **SDA** | Power saving (37 ºC) | Sample prep. neededNuclease selection is complex.Inefficient in long target sequences |
| **RCA** | Power saving (37 ºC)Specific enough to allow SNP analysis | Primer is complexRNA amplification is complexWorks only with a circular nucleic acid template |
| **HDA** | Simple primer designRobust to biological substancesNo initial heating step | Expensive enzymes |
| **RPA** | Power saving (37 ºC)Simple primer designExtremely quick (20 min)No initial heating stepRobust to biological substances. |  |

NASBA: Nucleic acid sequence-based amplification; LAMP: loop-mediated isothermal amplification; SDA: Strand-displacement amplification; RCA: Rolling circle amplification; HDA: Helicase-dependent amplification; RPA: Recombinase polymerase amplification.