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Nucleic Acid-Based Approaches for Detection of Viral Hepatitis

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Context: To determining suitable nucleic acid diagnostics for individual viral hepatitis agent, an extensive search using related keywords was done in major medical library and data were collected, categorized, and summarized in different sections.
Results: Various types of molecular biology tools can be used to detect and quantify viral genomic elements and analyze the sequences. These molecular assays are proper technologies for rapidly detecting viral agents with high accuracy, high sensitivity, and high specificity. Nonetheless, the application of each diagnostic method is completely dependent on viral agent.
Conclusions: Despite rapidity, automation, accuracy, cost-effectiveness, high sensitivity, and high specificity of molecular techniques, each type of molecular technology has its own advantages and disadvantages.

*Keywords:*Nucleic Acids; Molecular Diagnostic Techniques; Hepatitis

1. Background

Nucleic acid techniques (NATs) are sensitive, rapid, and reliable diagnostics that are based on amplification of specific regions of viral genomic sequences. The sensitivity and specificity of molecular screening approaches, as qualified practical viral detection methods, have been successfully recognized. NATs have enhanced the quality and accuracy of detecting viral hepatitis agents in a short time. Viral hepatitis is known as an important human disease, which is caused by six different types of viruses, namely, hepatitis A virus (HAV), hepatitis B virus (HBV), hepatitis C virus (HCV), hepatitis D virus (HDV), hepatitis E virus (HEV), and hepatitis G virus (HGV). Nevertheless, some viral hepatitis agents such as HBV, HCV, and HEV are known as common co-infection with human immunodeficiency virus (HIV) (1-4).

2. Objectives

The purpose of this article was to review nucleic acid approaches for detection of viral hepatitis.

3. Search Strategy

In the present review article, the internet databases of Google Scholar, PubMed, and Scopus were used to introduce a wide range of accessible nucleic acid approaches for detection of viral hepatitis with their advantages and disadvantages.

4. Traditional and Molecular Diagnostics

According to different epidemiologic investigations and the importance of their achievement for supervision and detect the viral infectious diseases, the use of traditional and non-molecular clinical diagnostics is not an ideal choice because the quantity of viral particles in both clinical samples and cell cultures are avoidable problems. The limited amount of viral particles leads to a decrease in test sensitivity. In addition, the routine non-molecular diagnostic methods are time-consuming procedures, too. Several serologic tests are done through commercial kits but the results for antibodies are unreliable for detecting the majority of hepatitis viruses. Furthermore, the false positive and false negative results are significant problems with serologic tests for detecting hepatitis viruses. In recent decade, various molecular diagnostics have been successfully proved as suitable viral testing techniques because of urgent need for high sensitivity, high specificity, simplicity, accuracy, and conciseness in diagnostic tools. The main advantages of molecular diagnostics is their applications for detecting and identifying fastidious, super-contagious, and uncultivable microorganisms including hepatitis viruses; however, a particular method is recognized for each viral agent (5-8).

Despite glorious advances in molecular technologies, still finding on-time diagnostic methods for detecting infectious agents is a main problem worldwide; however, in recent years the important microbial diseases are better controlled through optimization of molecular diagnostic methods. In comparison with other microorganisms, viruses encompass only RNA or DNA as genomic properties and therefore, a low amount of viral

nucleic acid is available in clinical samples (6, 9). The main aim for utilizing nucleic acid tests is to provide rapidity and high accuracy, sensitivity, and specificity in clinical sample results. Today, viral hepatitis agents including HAV, HBV, HCV, and HEV are detectable in clinical samples such as blood and oral fluid as well as the environmental samples via molecular techniques (5, 10, 11).

Drinking water is an important edible material that must be examined for probable contamination with feces. According to different studies, HAV and HEV are well-known infectious agents of acute viral hepatitis that can be isolated from fecal-contaminated drinking water. It would lead to high mortality if diagnosis of infectious agent were delayed or inappropriate therapy was administered; therefore, the access to molecular approaches is a need for early detection. Today, the progression in bioinformatics and molecular biology and combination of them might help nucleic acid technologies to develop faster (10, 12).

5. Epidemiology

It is calculated that 500 million people are infected with viral hepatitis around the world. The global prevalence of viral hepatitis is ten-times more than that of AIDS. Moreover, there is a considerable difference among hepatitis viruses. HAV and HEV are known as agents of acute hepatitis with low mortality while HBV and HCV are the viral agents of chronic hepatitis with high mortality. According to diverse reports, a large portion of viral hepatitis is seen in Asia. Until today, 108 countries have reported viral hepatitis as an important and considerable concern of their public health (10, 11, 13).

6. Behavior of Viral Hepatitis Transmission

Personal hygiene and public health are two important factors for preventing viral hepatitis infections caused by HAV and AEV because they are transmitted through fecal contaminated foods and water while HBV and HCV are normally transmitted by infected needles and blood products. Therefore, distribution of viral hepatitis caused by HBV and HCV is high among injection drug users, those with multiple sexual partners, homosexual people, and patients with AIDS and Hemophilia (10-14).

7. Results

The molecular diagnostic methods are categorized into different groups including non-amplified nucleic acid probes (liquid-phase, solid-phase, and *in situ* hybridization [ISH]) (15), amplified nucleic acid techniques (consisting of polymerase chain reaction [PCR] (5, 9, 12, 16), real-time PCR [RT-PCR] (5, 9, 17), multiplex PCR (5, 9, 18), nested PCR (5, 9, 19), reverse transcription PCR (5, 9), loop-mediated isothermal amplification of DNA [LAMP] (5, 8, 9, 12)), and microarrays approaches, which are the best known techniques for detection and identification of hepatitis viruses with high sensitivity and reproducibility (5, 8). Automation of molecular tools has revolutionized the routine viral diagnostic methods, because it has been led to low contamination risk, rapid detection, and decrease in costs. In this literature review, we tried to focus on some nucleic acid-based molecular technologies applied for detection of hepatitis viruses.

7.1. Non-amplified Nucleic Acid Probes

Each molecular approach has its advantages and disadvantages depending on target viral agent. Hence, it is impossible to study each one apart. Probe-based technologies are performable with a large number of microorganisms. This places some limitations on probe-based techniques because the analytical sensitivity of probe-based techniques is estimated at the order 10^6 molecules. The eliminatory of time-consuming clinical viral cultures via molecular diagnostic methods has provided significant advances in nucleic acid-based viral detection. The nucleic acid probe-based approach is a suitable non-amplified nucleic acid tool for nonviable, uncultivable, or fastidious organisms such as hepatitis viruses. This technique offers a rapid and specific detection for viruses (5, 20-22).

7.1.1. In Situ Hybridization Probes

The radiolabeled nucleic acid probes are traditionally used to detect viral target sequences of DNA or RNA within intact cells or tissues (Table 1) while in new generation of ISH technique, non-isotopic hapten digoxigenin is used with even better resolution. ISH is a prompt technique for intracellular localization of hepatitis viruses. The binding stability between target sequences and probes is directly depended on temperature and salt concentration as environmental factors and G + C content and the length of the hybrid (15, 22).

Peptide-nucleic acid (PNA) is used in fluorescence ISH (FISH) as a rapid and accurate clinical diagnostic method for detecting hepatitis viruses. The PNA FISH is a highly sensitive and specific method. The probes perform qualified hybridization via high degrees of powerful affinities, fast kinetics, and specificity to target nucleic acids such as rRNA (22, 23).

7.1.1.1. Advantages and Disadvantages

FISH is an accurate and sensitive assay for detecting genomic DNA and RNA viral hepatitis such as HAV, HBV, and HCV just in homogenized tissues. This method is hampered by its low specificity. This pathobiologic method is used for detection of viral hepatocancers in human hepatocytes. The major disadvantage of this method as a solid-phase hybridization is a low availability to the target sequence of nucleic acid in cells (24).

Table 1. Some Molecular Technologies and Their Applied Targets [a]

Method	Nucleic Acid Target
ISH probes	DNA or RNA
Cycling Probe Technology	DNA
Invader Technology	DNA or RNA
Ligase Chain Reaction	DNA or RNA
bDNA	DNA or RNA
Hybrid Capture Assay	DNA
Polymerase Chain Reaction Techniques	DNA or RNA
Loop-Mediated Isothermal Amplification Technique	DNA or RNA
Microarray	DNA or RNA

[a] Abbreviations: ISH, in situ hybridization; and bDNA, branched DNA.

7.2. Amplified Nucleic Acid Techniques

The use of molecular diagnostic methods dates back to 1980s via progression in PCR. Accuracy and rapidity are the most important aims in research and clinical diagnostics. According to different studies, there are several methodologies in the field of nucleic acid amplification, which are based on probe, signal, or target (5, 23).

7.2.1. Probe Amplification Techniques

In this category of the hybridization consisting of probe and target nucleic acid sequence, several copies are constructed. The isothermal nature of probe amplification techniques is their main advantage. It is important to know that each probe amplification technology has its particular properties; therefore, each technology is applied for a particular sample diagnostics. There are different probe amplification methods. The most common techniques for hepatitis viruses detection are cycling probe technology (CPT), invader assay, and ligase chain reaction (LRC) (5, 20).

7.2.1.1. Cycling Probe Technology

CPT is an isothermal signal amplification technique that is proper for detecting low amounts of DNA as a target sequence. The principle of CPT is constructed on the activity of a thermostable enzyme of RNase H and the presence of a chimeric probe (labeled with radioisotope) of DNA-RNA-DNA with 25- to 30-kb length. CPT is a simple and linear DNA detection technique (5, 25-28).

7.2.1.1.1. Advantages and Disadvantages

CPT is a specific assay for screening antibiotic resistant types of RNA and DNA viruses. The use of this technology is limited and is not used in public clinical laboratories level. This method is time consuming, which is considered as a significant disadvantage. The lower the amounts of target DNA is, the higher concentrations of RNase H are needed. CPT can be used for detecting and identifying DNA hepatitis virus of HBV, HCV, and HEV (5, 25-28).

7.2.1.2. Invader Assay

The invader assay is a non-PCR and isothermal amplification technique (3rd Wave Technologies, Madison, WI), which is based on probe amplification technology and has been created for detecting nucleic acids of DNA and RNA with high sensitivity and accuracy. In addition, invader assay is a prompt method for scoring single nucleotide polymorphism (SNP) (5, 25, 29). HBV has different viral DNA structures including covalently closed circular DNA (cccDNA), linear double-stranded DNA, and relaxed circle DNA. In the presence of cccDNA, invader probe signals of relax circle DNA and linear DNA are detectable. Invader assay is a prompt method for detecting HBV and HCV (5, 25, 29).

7.2.1.2.1. Advantages and Disadvantages

Invader assay is known as an accurate, easy, and sensitive assay for routine genotyping of HBV and HCV in clinical serum and hepatocyte samples. The Invader assay is helpful in facing with abundance of clinical specimens; however, the least mistake in invader test set up might lead to huge failure results. The invader technology is a proper technique to detect HBV and HCV in serum specimens, intrahepatic cccDNA, and total HBV DNA. This technology holds promise in the horizon (5, 30).

7.2.1.3. Ligase Chain Reaction

LCR is a DNA amplification-based strategy that is employed as molecular diagnostics for direct detecting of various infectious agents such as HAV, HBV, and HCV. LCR is categorized as a probe amplification method in which two pairs of probes hybridized to the adjacent single stranded target DNA. The specificity and sensitivity of LCR assay are high; however, they depends on the type of microorganisms (Table 1) (5, 25, 31, 32).

7.2.1.3.1. Advantages and Disadvantages

LCR is a rapid, sensitive and specific technology for simultaneous genotyping and mutants viral agents. The use of this method is limited and is applied for detecting resistant viral hepatitis agents of HBV, HCV, HEV, and HDV in human serum samples (5, 31, 32).

7.2.1.4. Branched DNA Technology

The bDNA technology is a type of sandwich nucleic acid hybridization belonging to signal amplification molecular diagnostics, which detects the branched structured target nucleic acid. In contrast with most of the molecular diagnostic methods, no enzyme is used in this technique. The first version of bDNA system was introduced in the early 1990s (5, 33-37).

The bDNA technology is proven as a sensitive, specific, and reproducible assay in molecular viral diagnostics. The bDNA technique does not need amplifying of a target sequence. In recent investigations, the bDNA assay has been used for the quantification of HCV RNA as clinical molecular diagnostic method (5, 33, 35-38). Now, the third generation of bDNA assay commercial kit for quantitation of HCV RNA and HBV DNA is available (Table 1). The third generation of bDNA test has advanced in both sensitivity and quantification (5, 33, 35-37).

7.2.1.4.1. Advantages and Disadvantages

As a quantitative assay, bDNA technique is used in both fields of research and clinical laboratories and because of its high sensitivity, it is applied for samples with very low nucleic acids. The bDNA technique is rapid, reliable, and resistant to contamination. Therefore, this method is recommended for clinical usage in routine virology and microbiology laboratories. The bDNA assay is suitable for quantitative detection of HBV and HCV in clinical samples of blood and serum (5, 33, 35-37).

7.2.1.5. Hybrid Capture Assay

The hybrid capture assay (HCA) is categorized as signal amplification tool in which antibody capture and chemiluminescent-detective agent are exploited. The first generation of HCA was presented in the mid-1990s. The HCA is a rapid viral molecular diagnostic method in which the specific single-stranded RNA probe is directly hybridized with denatured complementary DNA (cDNA) sequence as the target nucleic acid in a clinical sample. The HCA is used for detecting and identifying HBV and HCV (Table 1) (5, 35, 39).

7.2.2. Target Amplification Technologies

Target amplification-based techniques are known as a group of enzyme-mediated molecular diagnostic methods that detect and identify the viral agents directly in samples via synthesizing many copies of target sequences, named amplicons, and matching them with oligonucleotide primers. Viral molecular diagnostics are progressed over the past two decades and are mostly employed for detecting and identifying hepatitis viruses. The target amplification tools are able to detect and quantify viral genotypes and identify drug-resistant viruses. Despite their several advances, there is an important disadvantage with target amplification techniques because of possibility of contamination. Therefore, to decrease the false positive results, a proper laboratory design, work field, and practitioners are needed (5, 16, 18, 21). Here, we argue about some of these techniques:

7.2.2.1. PCR Techniques

PCR is a best-known practical technology for replicating and amplifying a targeted sequence of DNA to thousands folds within a short time. This method was introduced in the early 1980s and progressed in the meantime. The main enzyme in PCR is DNA polymerase, which is able to copy a DNA strand. Totally, a mixture of two oligonucleotide primers, target sequence, DNA polymerase, a master mix consisting of deoxyribonucleotide triphosphates (dNTPs), KCl, $MgCl_2$, and a buffer of Tris-HCl are needed. The reaction mixture is treated by heating and cooling within a PCR machine during several cycles programmed by a predetermined protocol. N cycles are able to produce second folds of amplicons. Finally, the PCR productions should be checked by different methods such as running on the gel (5, 12, 16, 18). There are some techniques which are derived from PCR technology and known as in vitro target amplification techniques (Table 1). These techniques are as below.

7.2.2.1.1. Real-Time-PCR

RT-PCR, introduced in the late 1980s, is a rapid approach for analyzing RNA molecules and amplifying their sequence targets. The first step in RT-PCR method is to produce a single stranded DNA known as cDNA copy from target RNA via reverse transcriptase enzyme. The produced cDNA is then amplified by PCR. The RT-PCR tool is useful for detecting RNA viruses such as HAV, HCV, HDV, HEV, and HGV (5, 18, 40).

7.2.2.1.1.1. Advantages and Disadvantages

RT-PCR is a rapid approach; however, the sensitivity of this assay is variable for different samples. The RT-PCR is applied for research and clinical diagnostics. HAV, HCV, HDV, and HEV are detectable in blood and serum clinical samples via RT-PCR technology (5, 18, 40).

7.2.2.1.2. Nested PCR

Nested PCR is a derivation of conventional PCR with high sensitivity and specificity. In this technique two pairs of primers and DNA polymerase enzymes are used for amplifying the target sequence during two stages of PCR (5, 18, 19, 41).

7.2.2.1.2.1. Advantages and Disadvantages

The extended time for nested PCR is a disadvantage. It increases the risk of contamination. The nested PCR has research and clinical applications. It is used for clinical detection of hepatitis viruses of HBV, HCV, and HEV in blood and serum specimens. This assay is often used for confirming PCR test results (5, 18, 19, 41).

7.2.2.1.3. Multiplex-PCR

Multiplex PCR is a revolutionized and advanced multisystem PCR technique in which two or more target sequences of DNA and more than one set of primer with similar annealing temperatures and uncomplementarity are needed. According to different studies, multiplex PCR

as the most mature of PCR technologies is a successful microbial nucleic acid diagnostics. Multiplex PCR technique is an effective and practical molecular diagnostics for detecting viral pathogens such as HBV (5, 18, 42-45).

7.2.2.1.3.1. Advantages and Disadvantages

Multiplex PCR technique is an effective and practical molecular diagnostics for genotyping. This assay is rapid, sensitive, and cost-effective molecular diagnostics for research and clinical applications. Multiplex PCR is a suitable method to detect HBV and HCV genotypes in serum samples (5, 18, 42-45).

7.2.2.1.4. Real-Time PCR

Abundant use of PCR in routine diagnostic clinical laboratories has led to accept RT-PCR (Quantitative PCR) as a detective gold standard nucleic acid method in different fields of microbiology including virology. In the quantitative PCR technique, a set of oligonucleotides or primers are used. Each primer, which acts as a DNA polymerase substrate, is hybridized to a single stranded DNA sequence target. The RT-PCR is extremely useful for detecting viral agents such as hepatitis viruses (5, 17, 18, 42, 46-48).

7.2.2.1.4.1. Advantages and Disadvantages

Real-Time PCR is engendered to provide an improved, accurate, sensitive, specific, reproducible, and quantitative application with low risk of carry-over contamination nucleic acid detection tool. This assay has research and clinical application and might be used for different clinical samples and different type of hepatitis viruses (5, 17, 42, 46).

7.2.2.2. Loop-Mediated Isothermal Amplification

LAMP is a novel nucleic acid amplification technique in which the procedure is completed with high accuracy, efficiency, rapidity, and specificity in shorter than 60 minutes under isothermal conditions. LAMP was reported by Notomi et al. in 2000. Today, the LAMP commercial kit is available (12, 49, 50). The mechanism of LAMP can be divided in three parts: an early non-cycling phase, a cycling amplification phase, and a final elongation phase. LAMP technique is a beneficial diagnostic method for detecting viral agents such as hepatitis viruses (Table 1) (5, 12, 49-52)

7.2.2.2.1. Advantages and Disadvantages

LAMP is rapid, sensitive, specific, and multi-dimensional assays. This method is suitable for detecting and genotyping all viral hepatitis agents as research and clinical diagnostics. It is also recommended for epidemiologic and treating aims (5, 12, 49-52).

7.3. Microarray

In spite of various molecular diagnostic technologies, microarray (Biochip or lab-on-a-chip) continues to revolutionize molecular diagnostics as a powerful microminiaturized technique. The principle of microarray is based on parallel hybridization of the targets (labeled nucleic acids of RNA or DNA) and the probes (several individual nucleic acid species immobilized on a solid surface in the form of spots or features) mixtures. Progression in Southern blots led to creation of microarray (5, 7, 53-57).

7.3.1. Advantages and Disadvantages

Microarray methods are rapid, accurate, sensitive, and specific technologies in which hybridization between probes and labelled target sequences is revealed via scanner. Microarrays are able to analyze thousands of microbial genes, including viral nucleotide sequences, simultaneously. Microarray technologies are applied for detecting different types of hepatitis viruses in both research and clinical specimens. This technique is great for multi-sample diagnoses; however, it is not suitable for detecting few viral hepatitis agents in limited samples (Table 1) (5, 7, 53, 56-59).

8. Conclusions

The most important aspect of nucleic acid-based approaches in virology laboratories is to detect and diagnose viral agents rapidly, accurately, and with high sensitivity and specificity. Therefore, molecular tools support an effective management for determining of viral infectious agents. In recent years, a wide range of viral diagnostic methods has been revolutionized based on nucleic acid approaches. Nowadays, the majority of molecular diagnostic techniques have replaced traditional methods as clinical diagnostic methods; however, some of them are mostly used in research centers.

Despite rapidity, automation, accuracy, cost-effectiveness, and high sensitivity and specificity of molecular techniques in comparison with serological tests and culture methods, each type of molecular technology has its advantages and disadvantages. The application of molecular techniques depends on economic availabilities, type of sample, and type of viral genome (DNA virus or RNA virus). For example, FISH is suitable for detecting HAV, HBV, and HCV in tissues while CPT and LCR are used for antibiotic resistant screening among RNA or DNA viruses; however, CPT is used only in research fields and LCR has research and clinical applications. Invader assay and microarray are good techniques for detecting RNA and DNA in abundance of clinical samples.

The bDNA and invader assay are prompt technologies for detecting HBV and HCV in clinical and research studies. PCR techniques are proper for detecting different viral hepatitis agents and have research and clinical utilization. LAMP is a suitable detection technique for viral genotyping, especially in epidemiologic investigations. Overall, selecting an accurate workflow and trained personnel for applied methodologies in different levels of laboratories might lead to a successful molecular diagnostics.

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