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REVIEW

Multiplex qPCR for serodetection and serotyping of hepatitis viruses: A brief review

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Abstract

The present review describes the current status of multiplex quantitative real time polymerase chain reaction (qPCR) assays developed and used globally for detection and subtyping of hepatitis viruses in body fluids. Several studies have reported the use of multiplex qPCR for the detection of hepatitis viruses, including hepatitis A virus (HAV), hepatitis B virus (HBV), hepatitis C virus (HCV), hepatitis D virus (HDV), and hepatitis E virus (HEV). In addition, multiplex qPCR has also been developed for genotyping HBV, HCV, and HEV subtypes. Although a single step multiplex qPCR assay for all six hepatitis viruses, *i.e.*, A to G viruses, is not yet reported, it may be available in the near future as the technologies continue to advance. All studies use a conserved region of the viral genome as the basis of amplification and hydrolysis probes as the preferred chemistries for improved detection. Based on a standard plot prepared using varying concentrations of template and the observed threshold cycle value, it is possible to determine the linear dynamic range and to calculate an exact copy number of virus in the specimen. Advantages of multiplex qPCR assay over singleplex or other molecular techniques in samples from patients with co-infection include fast results, low cost, and a single step investigation process.

Key words: Co-infection; Viral genome; Quantitative real-time polymerase chain reaction; Genotyping techniques; Serotyping; Hepatitis viruses

Core tip: The present review describes the worldwide application and the significance of multiplex quantitative real time polymerase chain reaction (qPCR) for simultaneous detection of hepatitis viruses and their subtypes in serum. The published literature has demonstrated that the multiplex qPCR assay is a fast, easy, cost-effective, and sensitive technique for the early diagnosis of hepatitis co-infections. Use of this technique, in comparison to other diagnostic procedures, is increasing in diagnostic laboratories.

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INTRODUCTION

Viral hepatitis is a serious public health problem requiring early diagnosis and timely treatment. There are a number of hepatitis viruses that have already been characterized based on their molecular structure and named alphabetically as hepatitis viruses A, B, C, D, E, and G (HAV, HBV, HCV, HDV, HEV, and HGV), respectively. These are hepatotropic and non-cytopathic in nature and cause liver damage by immune mediated cell lysis[1]. There is an additional group of viruses that cause hepatitis but are not yet characterized. These viruses have been put under the category of non A-G hepatitis viruses. HAV infects mainly the pediatric age group, occurs both sporadically as well as in epidemics, and accounts for an estimated 1.4 million cases annually[2]. Two billion people are suspected to be infected with HBV globally, and approximately 350 million of them suffer from chronic hepatitis B infection[3]. About 25% of adults infected with HBV during childhood are reported to die from hepatocellular carcinoma (HCC) or liver cirrhosis[4]. In addition, 3-4 million people are infected with HCV each year, and a high proportion of them develop chronic HCV infection. A large population infected with HCV dies from serious liver diseases annually[5]. Similarly, reports are also available on HEV infection. In addition to individual viral infection, there are cases of co-infections reported from various parts of the world. Hepatitis A and E infections usually run a benign course of disease and resolve in due course of time without developing chronic diseases. In contrast, hepatitis B and C infections cause severe liver diseases, developing chronicity in a significant number of patients. Interestingly, hepatitis A and E infections in patients with pre-existing HBV or HCV infections lead to the development of serious diseases with a significant rise in morbidity and mortality[6].

The diagnosis of hepatitis viral infections is usually done with serological markers in blood. However, there are situations where serology loses its credibility. For example, serological markers can not differentiate between past and present infections. In addition, serological tests do not address the problem of antigenic variations in viruses, infections with different genotypes, presence of silent carriers, and absence of antibody in early phase of infection[7]. Moreover, the presence of maternal antibodies makes it impossible to detect infections in newborns[8]. In order to have an alternate system, the nucleic acid tests (NAT) based methods were developed for detecting the viral genome in serum for the diagnosis of viral hepatitis. NAT based methods have the benefit of direct examination of the infectious agent's genome in serum[9,10].

The conventional polymerase chain reaction (PCR) is one such NAT based method that has been in practice in some laboratories for the diagnosis of viral hepatitis in the last few years[11]. However, conventional PCR is a lengthy procedure with several technical and operational problems, and so, it is of limited use. In addition, each marker needs to be investigated separately by PCR, and it takes a very long time to reach a final diagnosis. Because of these limitations of conventional PCR, the use of real time PCR was supposed to be a better option for early diagnosis of viral hepatitis in both sporadic and epidemic cases. Real time PCR is one of the latest techniques frequently used for the diagnosis of various infectious diseases, including viral hepatitis. It can detect causative pathogen-related nucleic acid in body fluids in a very short time period. It can also be used to determine different molecular forms and variant molecular species of pathogens, including bacteria, viruses, and several parasites[12,13]. Real time PCR is a specific and sensitive technique and uses specific probes and primers to detect target sequences in the genome. Moreover, this technique is performed on an automated machine without the need of post PCR procedures, thus minimizing cross contamination between samples, simultaneously accelerating the analysis[14].

The recent development of molecular technologies has relayed a strong message to medical researchers to explore ways to further improve the diagnostic procedures. Those researchers working in the area of medical virology have switched from traditional approaches of virus detection in clinical samples to multiplexing for simultaneous detection of multiple pathogens in a single assay[15]. Recently, several PCR based assays coupled with oligonucleotide microarray technology have been designed to allow for the simultaneous detection and genotyping of several viruses, including blood borne pathogens[16], respiratory viruses[17], and adenoviruses[18]. These assays show a significant increase in the sensitivity of detection, reaching 10-100 copies of target RNA/DNA in a sample[19]. Given the ease of performance, short reaction time, low cost, and the ability to monitor the results on a screen, these assays have proved attractive to all diagnostic laboratories furnished with minimal essential facilities. After surveying the literature on the use of PCR based multiplex assays for detecting and genotyping hepatitis viruses, we noticed several attempts to develop multiplex real time PCR assays for hepatitis in the last few years. Here, we provide an up to date review on the development, use, and significance of multiplex qPCR in the field of viral hepatitis.

Table 1 Conserved genomic regions used as templates for amplification of hepatitis viruses in qPCR assays

Virus	Conserved region	Ref.
HAV	5′ UTR	[4,15,20,22]
HBV	S-gene	[4, 19-21]
	X-gene	[15]
HCV	5′ UTR	[4,15,19-21]
HDV	Ribozyme-1	[20]
HEV	ORF2	[15,22]
	ORF3	[20]
HGV	5′ UTR	[20]

HAV: Hepatitis A virus; HBV: Hepatitis B virus; HCV: Hepatitis C virus; HDV: Hepatitis D virus; HEV: Hepatitis E virus; UTR: Untranslated region; ORF: Open reading frame.

EXPERIMENTAL APPROACH FOR MULTIPLEX qPCR

Search for conserved regions

In order to develop a multiplex qPCR assay for multiple pathogens, the first and foremost step is to explore and locate the target region on each pathogen's genome for amplification purpose. Since variation in the genome is a dynamic process, it is necessary that a multiplex assay uses the most conserved region representing all the strains/variants for detection of the pathogen in body fluid. In the case of hepatitis viral infections, studies have reported a distinct conserved region that has been used as a target for amplification of each individual viral genome[4,20,21]. Table 1 shows the list of target regions used in various studies on multiplex qPCR assays developed for hepatitis viruses. The 5′ untranslated region (UTR) was reported to be the main target template in HAV, HCV, and HGV[20,22]. It was based on the availability of most conserved sequence in the 5′ UTR for amplification purpose. Similarly, S-gene or X-gene was used for HBV, ribozyme-1 gene for HDV, and open reading frame (ORF)-2 or ORF-3 region for HEV. Different studies have reported different sequences as templates in these selected conserved regions, though, there was very little information provided about the exact location of the sequences used.

Designing of primers and probes

After deciding which conserved region and location of the sequence were to be used as template, it is important to design the primers and probes for their use in the development of qPCR[23]. The selection of the primer is based on its specificity with the target template. At the same time, its length, melting temperature, GC content, 3′ end stability, sequence complexity, and location in the target sequence determine the length and melting temperature of the amplicon produced and the amplification efficiency of the assay[23,24]. Notably, the choice of chemistry and probe design are at the liberty of the user's interest, with numerous options available to them[24]. During selection of chemistry and probe, one needs to determine whether to quantify DNA, profile mRNA, or perform allelic discrimination assays[25].

Real-time PCR and melting curve analysis (MCA) are good techniques for quantifying nucleic acids, detecting mutations, and conducting genotyping analysis. These methods often use TaqMan probes[26], Molecular beacons[27], Sunrise primers[28], Scorpion primers[29], and Light-up probes[30]. An alternative to probe-based methods is the use of DNA intercalating dyes that bind to double-stranded DNA. These dyes include ethidium bromide[31] and SYBR Green I[32,33]. However, certain drawbacks limit the use of SYBR Green I for resolving multiplex PCR based on MCA[34]. Other alternative dyes, such as BEBO[35], YO-PRO-1[36], LC Green[37], and SYTO-9[38,39] have also been tried for use in real time PCR. Table 2 provides a brief review of various chemistries/dyes offering several options for their use in qPCR assay developed for different purposes. Studies for detecting and genotyping hepatitis viruses with qPCR have reported different sets of dyes based on choice and their availability[40]. However, most of the studies conducted have reported a frequent use of hydrolysis probes despite many options available. This information is available in the data[41-71] compiled in Table 3.

The probe-based assays (*e.g.,* TaqMan assays)[72] began to gain attention in mid-1990s with the development of quenched, fluorescent probes[73,74] and the commercialization of real-time thermal cyclers[26,75]. TaqMan (also known as Fluorogenic 5′ nuclease assay) probes contain two dyes, a reporter dye (*e.g.,* 6-FAM) at the 5′ end and an acceptor dye at 3′ end, usually tetramethyl rhodamine (TAMRA). Recently, TAMRA fluorescent acceptor quencher dye was substituted with a non-fluorescent quencher, *e.g*., Black Hole Quencher[76]. The proximity of the quencher to the reporter in an intact probe quenches the fluorescence signal of the reporter dye through fluorescence resonance energy transfer. During amplification, the 5′ to 3′ nucleolytic activity of Taq polymerase cleaves the probe between the reporter and the quencher only if the probe hybridizes to the target. The probe fragments get displaced from the target, separating the reporter dye from the quencher dye, resulting in increased emission of fluorescence. Floating TaqMan probes are quenched due to random coiling in solution, where fluorophore- and quencher-labeled ends come together[77]. In contrast, Molecular Beacon probes are oligonucleotides designed in a way to induce hairpin formation and produce the quenched state[78]. TaqMan and Molecular Beacon probes have been shown to be less effective in discriminating closely related targets, as in single nucleotide polymorphisms, drug-resistant mutants, and somatic cancer mutations[79,80]. However, molecular beacons are useful in situations where it is not possible to isolate probe-target hybrids from

Table 2 Chemistries/Dyes used in qPCR assays

S. NO.	Class	Types	Structure	Mechanism of action	Advantages	Applications
1	DNA binding dyes	Ethidium Bromide, SYBR Green, SYBR Gold, YO-PRO-1, SYTO, BEBO, BOXTO, EvaGreen	Intercalating dyes	Bind to the minor groove of dsDNA during amplification	Inexpensive Easily available	Pathogen detection Gene expression SNP detection Genotyping
2	Fluorophore labeled oligonucleotide	*Primer probes*				
		Hairpins: Scorpions, Ampliflour, LUX	Loop based oligonucleotides	Bind to target during denaturation with emission of fluorescence	Inexpensive, Prevent formation of primer dimer, Less background signals	Pathogen detection Genotyping SNP allelic discrimination Mutation detection
		Cyclicons	Cyclic structure with reporter at 3′ end and quencher at 5′ end	Reporter and quencher in close proximity with energy transfer *via* FRET quenching. Their separation results in fluorescence emission during amplification	Inexpensive Less contamination Less background signals	Pathogen detection Genotyping SNP allelic discrimination Mutation detection
		Angler	Probe with DNA sequence bound to reverse primer through a HEG linker	During annealing step, DNA polymerase does extension of 3′ end reverse primer. Later on, SYBR Gold dye intercalates in dsDNA emitting fluorescence	Highly specific	Gene expression Pathogen detection SNP detection Genotyping
		Probes				
		Hydrolysis Probes: TaqMan probes, MGB-TaqMan, Snake assay	Oligonucleotide with reporter at 5′ and quencher at 3′ end	Probe is degraded by 5′ to 3′ exonuclease activity of DNA polymerase generating fluorescence during extension	Design and synthesis easy	Microarray validation Pathogen detection SNP allelic discrimination Mutation detection
		Hybridization probes: Hybprobes, Molecular Beacon, HyBeacon, MGB Probes	A pair of oligonucleotides having reporter dye on first and quencher on second oligonucleotide	Binding to target during hybridization and annealing brings fluorophore into proximity producing fluorescence by FRET	Design and synthesis quick and easy	Microarray validation Pathogen detection Viral/Bacterial genotyping SNP allelic discrimination Mutation detection
		Nucleic acid analogues				
		PNAs, LNAs, ZNAs Non-natural bases	Intercalating/inserting dyes	Identical to conventional oligonucleotides	Resistant to nuclease and proteases activity	Discriminate between DNA and cDNA in prokaryotes

All above details were collected from report published in *Clinica Chimica Acta* 2015; **439**: 231-250[25]. SNP: Single nucleotide polymorphisms.

an excess of the hybridization probes, for example in sealed tubes or within living cells[81]. An effective probe requires a careful balancing act based on melting temperature (Tm) and, therefore, repeated design and testing are needed to develop an effective probe[82]. Available evidence suggests that the use of TaqMan probes in qPCR assays for hepatitis viruses provide a good balancing act.

Designing tools

Today, several designing tools are available to guide the design of qPCR assays and analyze resulting quantitative data. Many of them are available online, and some are provided with qPCR instruments from different manufacturers[83]. Some important tools include Primer3, Primer-BLAST, PerlPrimer, FastPCR software, IDTSciTools, and UniPrime[84-89]. In addition, some of them have programming to analyze the secondary structure of primers. MP primer is used to design primers for multiplex PCR assays[90]. The Minimum Information for Publication of qPCR Experiments (MIQE) guidelines also provide clear instructions on the steps that are important for qPCR assay design[91]. Several research companies offer help for designing primers and probes with use of their designing tools. The studies reported in this article demonstrate a liberal use of tools without any specific need or choice affecting the results.

Instruments used in multiplex qPCR assay

Various types of advanced technology-based equipment for multiplex qPCR assays with analysis of amplified products are available globally. A list of the instruments used with their brands in various studies conducted on qPCR for viral hepatitis is shown in Table 3. With increasing advances in technology, the number of filters and, accordingly, the resolution of the amplification curve during the PCR assay have also increased. Now it is possible to detect/discriminate more pathogens or allelic/mutational changes[92,93] in a

Table 3 Global status of multiplex qPCR developed for hepatitis viral infections with and without other pathogens

No.	Assay systems	Instruments used	Group of pathogens detected		Types of chemistries/ detection methods used	Ref.
			Hepatitis viruses	Other pathogens		
1	Multiplex real time PCR	Mx4000 (Stratagene)	HBV, HCV	HIV type-1, T. pallidum	TaqMan-LNA probe	[21]
2	Multiplex real time PCR	Light cycler 480 (Roche)	HEV genotypes	-	N.A.	[41]
3	Real time PCR assay	ABI 7500 (Applied Biosystems)	HAV, HBV, HCV, HDV, HEV	-	TaqMan Array card	[42]
4	Multiplex qPCR assay	Light cycler 480 (Roche)	HBV, HDV	-	TaqMan probe	[43]
5	Multiplex qPCR assay	ABI 7500 (Applied Biosystems)	HAV, HEV	-	Hydrolysis probe	[22]
6	Multiplex qRT-PCR	N.A.	HAV	Norovirus genotypes 1 and 2	TaqMan probe	[44]
7	Multiplex ligation dependent probe real time PCR	Rotor-GeneQ (Qiagen)	HBV mutants	-	TaqMan probe MLPA probe	[45]
8	Multiplex real time RT-PCR	N.A.	HEV genotypes	-	N.A.	[46]
9	Multiplex qPCR	N.A.	HBV genotypes	-	SYBR Green	[47]
10	Multiplex Real time PCR	N.A.	HAV	Norovirus, Rotavirus, Coxsackievirus	TaqMan probe	[48]
11	Multiplex Real time PCR	Light cycler 2.0 (Roche)	HAV, HBV, HCV and HEV	-	FRET probe	[15]
12	Multiplex RT-PCR	ABI 2720 (Applied Biosystems)	HCV	HIV type-1	SYBR Green I	[8]
13	Multiplex qPCR	N.A.	HAV, HEV	Entero and Adeno-viruses	N.A.	[49]
14	Multiplex Real-Time PCR Assay	CFX96 (Bio-Rad)	HAV, HBV, HCV	-	READ technology based fluorophore	[4]
15	RT PCR assay	Smart cycler Ⅱ (Cepheid)	HBV, HCV	-	TaqMan probe	[50]
16	Duplex real time PCR	ABI 7500 (Applied Biosystems)	HBV variants	-	Hydrolysis probe	[51]
17	Multiplex RT PCR	N.A.	HCV subtyping	-	Electrophoresis	[52]
18	Multiplex qPCR	N.A.	HBV genotypes	-	N.A.	[53]
19	Multiplex qPCR	N.A.	HCV	HIV type-1	SYBR Green I	[54]
20	Duplex real-time RT-PCR	ABI Prism system (Applied Biosystems)	HCV variants	-	Hydrolysis probe	[55]
21	Multiplex real time PCR	N.A.	HAV	Norovirus genotypes 1 and 2	N.A.	[56]
22	Duplex real-time qRT-PCR	ABI Prism 7000 (Applied Biosystems)	HAV	MS2 bacteriophage	MGB-TaqMan probe	[57]
23	Multiplex TaqMan RT-qPCR system	MX30005P (Stratagene)	HEV	FCV	TaqMan probe	[58]
24	Multiplex real time PCR	ABI 7300 (Applied Biosystems)	HBV genotypes	-	TaqMan probe	[59]
25	Real time PCR	N.A.	HBV genotypes	-	TaqMan probe	[60]
26	Multiplex real time PCR	Mx3005P (Stratagene)	HEV	FCV	TaqMan probe	[61]
27	Multiplex RT PCR assay	ABI Prism 7500 (Applied Biosystems)	HCV	PDV	MGB hybridization probe	[62]
28	Multiplex qPCR assay	N.A.	HBV	B19, HHV-8, EBV, CMV, VZV	N.A.	[63]
29	Multiplex qPCR	N.A.	HBV, HCV	HIV type-1	SYBR Green I	[16]
30	Multiplex Real Time PCR	ABI 7500 (Applied Biosystems)	HBV mutants	-	LNA probes with SYBR Green I	[64]
31	Microarray multiplex assay	ABI Prism 7700 (Applied Biosystems)	HBV, HCV	HIV type-1	Oligonucleotide array labeled with Cy5 and Cy3	[65]
32	Real time multiplex PCR	N.A.	HAV	Entero and Adeno-viruses	Probes labeled with FAM, R6G, ROX, Cy5	[66]
33	Multiplex real time RT-PCR	LightCycler (Roche)	HCV	HIV type-1	SYBR Green	[67]
34	Real time multiplex PCR	icycler iQ (Bio-Rad)	HCV variants	-	TaqMan probes	[68]
35	Multiplex real-time RT PCR	ABI 7000 (Applied Biosystems)	HCV genotypes	-	Primer probes	[69]
36	Multiplex real-time qPCR	Mx4000 (Stratagene)	HBV, HCV	HIV type-1	TaqMan probes	[70]
37	Automated multiplex PCR	ABI Prism 7700 (Applied Biosystems)	HBV, HCV	HIV type-1	TaqMan probes	[71]

single step multiplex assay. The choice of instrument is more a function of availability, without much difference in their analytical qualities. Multiplex qPCR assays developed for hepatitis viruses may use any brand, depending on a match between the number of component pathogens to be detected and the filters

available for detection. Other features of equipment do not seem to affect the results.

Optimization of protocol

For each pathogen used as a component in the multiplex assay, a carefully developed singleplex assay is needed. The design of primers and probes is dictated purely by the nature of the target template and clear guidelines for amplification. This exercise is followed in order to prepare a record of common amplification conditions noted in singleplex assays and for their application as such in multiplex assays. The multiplex protocol is reframed in a way to have minimum possible deviations from the working protocol of the singleplex assay. During the multiplex assay, the possibility of cross interaction/interference among different molecules is quite likely and may cause unsuccessful amplification. This interaction may or may not occur, but it has to be worked out cautiously in each multiplex assay.

There have been reports on singleplex as well as multiplex assays developed for detection of some hepatitis viruses and their genotypes[16] (Table 3). Such a study was conducted at our research center where a multiplex assay was developed for simultaneous detection of hepatitis virus A, B, C, and E[15]. These viruses are frequently prevalent in India, posing a serious problem, causing incidences of both sporadic and epidemic hepatitis from time to time[94,95]. The use of singleplex followed by the development of multiplex assay in these cases does not show many changes in the experimental protocol. This implies that the amplification protocol of individual viruses is not influenced during multiplex assays. We noted a clear amplification curve on the screen during multiplex assay that was the same exact pattern noted during singleplex assay[15].

Table 3 shows the global status of multiplex assays used for analyzing hepatitis viruses with or without other pathogens[41-71]. In all these assays, viral amplification by the simultaneous presence of other pathogenic genomes was indicated. An overall survey of the experimental designs reported in multiplex assays indicated that standard conditions of reverse transcription, denaturation, annealing, and extension temperature were followed without much deviation from the singleplex protocol.

MIQE guidelines

The guidelines published by Bustin *et al*[91] in 2009 clearly defined the terms used and steps necessary to design the experiments for developing qPCR assay. Since 2009, many published reports in the area of viral hepatitis on multiplex qPCR were found to follow these guidelines and give interpretation of results referring to terminology and definitions outlined there. The guidelines state that multiplexing expands power of qPCR analysis but needs documentation for accurate quantification of multiple targets in a single tube assay.

ASSESSMENT OF SENSITIVITY AND SPECIFICITY

Generation of standard curve

In order to generate a standard curve for each hepatitis virus, the standard control that includes the conserved region targeted for amplification/ detection is synthesized artificially and cloned into a suitable vector (*e.g.,* pUC 57)[15] using cloning kits. These standards are used as a template for standardization of amplifications. The copy number of standard plasmids is calculated using their concentration and the size of linearized plasmids. Each standard template is added to PCR mix (Tris-HCl, KCl, $MgCl_2$, four dNTPs, primers, and Taq DNA polymerase in a suitable concentration ratio), and PCR is performed under standardized conditions. For generation of the standard curve, a 10-fold serial dilution of each standard plasmid (10^1-10^8 copies/μL) is prepared and run in triplicate. At the end, data are analyzed by an automatic system that generates a standard curve[21]. The standard curves are used to quantify the amplification product and to assess the linear dynamic range using 10-fold dilution series of standard plasmid of each individual virus. One specimen standard plot is shown in Figure 1, which was prepared during development of quadruplex qPCR for hepatitis virus A, B, C, and E. Such plots are used to calculate copy number of individual template using correlation coefficient and Y-intercept value based on regression analysis.

Standard curve showing amplification plots of 10-fold serial dilution of HAV template using standard cloned plasmids. Such standard curves are generated from the amplification plots run in triplicate and show a linear dynamic range. The correlation coefficient and the slope of each standard plot are shown in the figure.

Assessment of sensitivity

Using the standard curve prepared above, now it is possible to assess the sensitivity and determine the linear dynamic range of an individual virus. Moreover, observed Ct values may be used to calculate the exact copy number of virus in an unknown specimen[96,97]. Based on the data collected from various studies, including our study[15], it has been noticed that the linear dynamic range of each individual hepatitis virus usually falls in the range 10^1-10^8 copies/μL.

Assessment of specificity

The specificity of qPCR assay is assessed by evaluating sera from healthy controls and patients with unrelated diseases negative for hepatitis markers by serology and all other NAT based techniques. Negative results from these sera and clear positive signals from serologically positive hepatitis sera demonstrate the high level of specificity of qPCR. To date, all studies on qPCR demonstrate the assay to be specific[15,22]. In

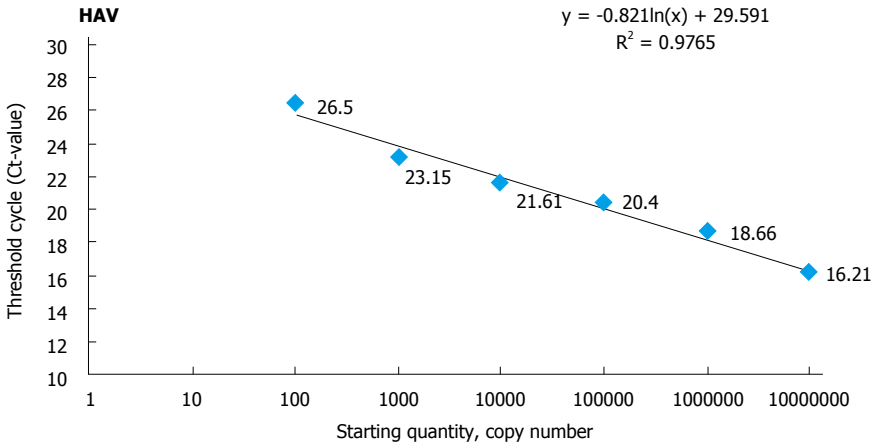

Figure 1 Standard curve showing amplification of hepatitis A virus[15]. HAV: Hepatitis A virus.

reports on viral hepatitis, qPCR assays demonstrated high specificity with a very low chance of false positive results[19,71].

MULTIPLEX qPCR IN RELATION TO OTHER ASSAYS

The multiplex qPCR assays were developed and used both for comparison as well as in combination with other molecular technologies to improve the sensitivity for detection of the viral genome[16,98]. Various other assay systems were also developed for simultaneous detection of HBV, HCV, and human immunodeficiency virus in addition to multiplex qPCR. The status of multiplex qPCR assay was assessed in comparison to other molecular techniques used for detection and genotyping of viruses, including hepatitis viruses. The other assay systems included flowcytometric microsphere based hybridization assay[99], transcription-mediated amplification (TMA)[100], and nucleic acid sequence based amplification (NASBA)[101]. Comparatively, TMA was reported to be an equally sensitive technique. However, when comparing qPCR with NASBA and TMA for the detection of hepatitis viruses, the level of sensitivity of TMA was found to be associated closely with qPCR[100]. Of course, qPCR assay was reported to be faster, more economic, and easier to perform compared to all other assays evaluated.

FUTURE AND LIMITATIONS OF MULTIPLEX qPCR

Multiplex qPCR assays are proving to be very good analytical and diagnostic procedures in medicine. Recently, these assays have been successfully used for both basic research and clinical applications[42,102]. Although the practice of doing separate assays for separate pathogens, including hepatitis viral markers, are still in place, the use of the multiplex assay is seen to be beneficial in terms of time and overall cost involved. Moreover, multiplex assays, when used for quantification of HCV- RNA, were found to resolve many problems with real time monitoring of the amplification process. In fact, in multiplex qPCR assays, real time PCR makes quantification of DNA and RNA of different organism more precisely and with better reproducibility because it depends on the threshold cycle value determined during the exponential phase of PCR rather than on end points[103]. In addition, these assays report a direct relationship between starting template copy number and the number of cycles required to get a positive signal. In this manner, real time qPCR appears to be a good option for laboratory diagnosis of viral hepatitis, both for screening as well as for the final diagnosis of suspected cases of viral hepatitis infections.

CONCLUSION

Based on the information compiled in the present review, there is an increasing trend/interest in the diagnostic area towards the development and use of multiplex qPCR assay for the simultaneous detection of hepatitis viruses or their subtypes in sera samples. Several studies have been conducted in last few years that clearly demonstrate the preferable use of qPCR over other techniques in the area of viral hepatitis. This technique has been used to detect hepatitis viruses in combination with various other viral and non-viral pathogens and reported to be a sensitive, fast, and cost-effective technique compared to other multi-step assay procedures. The use of multiplex qPCR in genotyping of hepatitis viral subtypes also provides great help in serotype detection. To date, multiplex qPCR has been successfully employed for the simultaneous detection of hepatitis virus A, B, C, D, and E and genotyping of their strains. It appears to be a good tool for screening blood donor samples in blood banks for hepatitis viruses. Moreover, a single step multiplex qPCR assay allows for an early diagnosis

and timely treatment of patients with viral hepatitis. Several studies in this field are in progress, with more important information likely to be available until the next such update is necessary.

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