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**DEVELOPMENT OF A RAPID, SENSITIVE, AND QUANTITATIVE METHOD
TO DETECT INFECTIVE HEPATITIS A VIRUS IN WATER**

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ABSTRACT

Microorganisms are responsible for more than 90% of the reported waterborne disease outbreaks in the United States; enteric viruses, such as hepatitis A virus, are identified as causing almost 10% of these. However, in 50% of the outbreaks, no causative agent is identified due to limitations in our ability to isolate and detect viruses in water samples. Historically, consumption of contaminated ground water has been the source of one-half of the reported outbreaks; in recent years, that fraction has risen to more than two-thirds. The most frequently reported source of contamination in these outbreaks is domestic sewage from septic tanks, leaking sewer lines, cesspools, etc.

As a result of the continuing waterborne disease outbreaks, and the growing fraction of them associated with consumption of ground water, the USEPA is finalizing a regulation, the Ground Water Rule, to minimize the risk of acquiring a microbial illness from ground water. This regulation will require all public water systems that use ground water as a source to assess the potential for fecal contamination of the water. One potential source of fecal contamination is the reuse of recycled water. Even tertiary treated wastewater may contain contaminants, including disease-causing microorganisms such as viruses, bacteria and parasites. Information about the numbers and types of some of these microorganisms, especially viruses, present in tertiary treated wastewater is scarce due to the limitations in the methods that are needed to detect these microorganisms in water. Current methods to detect viruses in water samples, for example, require highly specialized analytical facilities and at least two to four weeks to obtain results. In addition, the standard methods only detect one group of viruses, the enteroviruses (which includes polioviruses). From records on waterborne disease outbreaks in the U.S., it is known that the enteroviruses are not the major cause of reported viral waterborne disease; other viruses such as hepatitis A virus and caliciviruses are the public health concern. The lack of information about the presence of hepatitis A virus in water and its long survival times in water caused the USEPA to add it to the list of contaminants to be considered for regulation in drinking water.

When recycled water is used to artificially recharge groundwater, there is the potential for some microorganisms, especially viruses due to their small size, to contaminate the underlying groundwater. Although most of the microorganisms may be removed during recharge, if even a few are transported to the ground water, that is of concern, as the USEPA has determined that one virus in 10,000 liters of drinking water constitutes a public health concern.

Molecular beacons (MB) are oligonucleotide probes that become fluorescent upon hybridization and are ideal in providing real-time monitoring of target amplicons during the PCR reaction. In this project, a real-time PCR assay based on molecular beacons was developed for the rapid and specific detection of hepatitis A virus. Using the MB-based RT-PCR assay, a detection limit of 1 PFU per PCR reaction was obtained. The specificity of the MB-based PCR assay was evaluated using a variety of other enteric organisms, and only hepatitis A virus was positively identified. The method developed in this study should improve our ability to provide rapid, sensitive, and specific results for the detection and quantitation of viruses in samples from environmental waters.

INTRODUCTION

Microorganisms are responsible for more than 90% of the reported waterborne disease outbreaks in the United States (Yates and Gerba, 1998); enteric viruses, such as hepatitis A virus are identified as causing almost 10% of these. However, in 50% of the outbreaks, no causative agent is identified due to limitations in our ability to isolate and detect viruses from water samples. Historically, consumption of contaminated ground water has been the source of one-half of the reported outbreaks (Craun, 1990, 1991); in recent years, that fraction has risen to more than two-thirds (CDC, 1998). The most frequently reported source of contamination in these outbreaks is domestic sewage from septic tanks, leaking sewer lines, cesspools, etc.

As a result of the continuing waterborne disease outbreaks, and the growing fraction of them associated with consumption of ground water, the USEPA is finalizing a regulation, the Ground Water Rule (USEPA, 1998), to minimize the risk of acquiring a microbial illness from ground water. This regulation will require all public water systems that use ground water as a source to assess the potential for fecal contamination of the water. One potential source of fecal contamination is the reuse of recycled water. Even tertiary treated wastewater may contain contaminants, including disease-causing microorganisms such as viruses, bacteria and parasites (Yates and Gerba, 1998).

In response to the increasing demand for water as California's population increases, the use of alternative water sources, such as recycled domestic wastewater, has expanded. As the use of recycled water increases, care must be taken to ensure that this practice does not have negative impacts on the environment or on public health. Even tertiary treated wastewater may contain contaminants, including disease-causing microorganisms such as viruses, bacteria and parasites (Yates and Gerba, 1998). Information about the numbers and types of some of these microorganisms, especially viruses, present in tertiary treated wastewater is scarce due to the limitations in the methods that are needed to detect these microorganisms in water. Current methods to detect viruses in water samples, for example, require highly specialized analytical facilities and at least two to four weeks to obtain results. In addition, the standard methods (USEPA, 1996) only detect one group of viruses, the enteroviruses (which includes polioviruses). From records on waterborne disease outbreaks in the U.S., it is known that the enteroviruses are not the major cause of viral waterborne disease; other viruses such as hepatitis A virus and caliciviruses are the public health concern. The lack of information about the presence of hepatitis A virus in water caused the USEPA to add it to the list of contaminants to be considered for regulation in drinking water (USEPA, 1997).

When recycled water is used to artificially recharge ground water, there is the potential for some microorganisms, especially viruses due to their small size, to contaminate the underlying groundwater. Although most of the microorganisms maybe removed during recharge, if even a few are transported to the ground water, that is of concern, as the USEPA has determined that one virus in 10,000 liters of drinking water constitutes a public health concern (USEPA, 1987). Although there is currently no requirement that ground waters be treated to remove disease-causing viruses prior to distribution for consumption, the USEPA is in the process of developing the Ground

Water Rule that will require all public water systems to assess the potential for fecal contamination of their source waters. Thus, in areas where recharge of recycled water occurs, the water supplier will have to demonstrate that fecal contamination (e.g., viruses) are not reaching the groundwater. If this cannot be demonstrated, the water will have to be treated prior to distribution. At this time, 45% of the community and 80% of the non-community public ground water systems do not disinfect their ground water prior to distribution (USEPA, 1998).

As stated above, in one-half of the reported waterborne disease outbreaks that occur in the United States, the microbial agent is not identified. This is due to the fact that the methods used for routine analysis of environmental water samples for infective human enteric viruses have focused on the enteroviruses (i.e., polioviruses, coxsackieviruses, and echoviruses). Limited work has been done to assess the occurrence of other infective enteric viruses such as rotaviruses and hepatitis in drinking water due to the fastidious nature of these viruses.

Hepatitis A virus has been the most frequently identified virus in reported waterborne disease outbreaks during the past 25 years (Yates and Gerba, 1998). However, a lack of standardized methods that can be routinely performed to detect and quantify infective hepatitis A virus has limited the amount of information available on the occurrence of these viruses in drinking water and other environmental samples. Thus, an assessment of the risk of hepatitis A virus infection as a result of exposure to drinking water impacted by artificial recharge of recycled water is not possible at this time. In addition to being identified as a causative agent of waterborne disease, hepatitis A virus has been found to be one of the most environmentally-resistant viruses studied (Sobsey *et al.*, 1986). Its survival in water, and ground water specifically, is longer than for other enteric viruses. For example, it was recently reported that hepatitis A virus was found in ground water two years after a contamination event occurred (Cromeans, 1998).

Cell culture, immunological assays, and molecular nucleic acid amplification assays, such as polymerase chain reaction (PCR), are the current methods used to detect viruses in environmental samples (Enriquez *et al.*, 1993, Reynolds *et al.*, 1995, Schwab *et al.*, 1995). Each of these methods is time consuming, costly, or susceptible to the presence of compounds that inhibit the sensitivity of the assay. Cell culture has traditionally been used to detect enteroviruses in environmental samples. An advantage of this method is that it is specific for infective virus particles. However, the method is time consuming and only viruses that produce visible signs of infection in the cell line being used (*i.e.*, cytopathic effects (CPE)) are detectable. Thus, a negative cell culture result does not necessarily imply the absence of infective virus particles in a sample.

The advent of molecular biology has led to the development of new tools for pathogen detection based on specific recognition of nucleic acid sequences. In particular, amplification of nucleic acid using the polymerase chain reaction (PCR) or reverse transcription-PCR ((RT-PCR) for the detection of mRNA) is the current preferred method, and has the best test sensitivity. The advantages of PCR include greater sensitivity, rapid analysis of many samples, relatively low cost, simultaneous detection of multiple pathogens, and the ability to discriminate between species and strains if suitable primers are selected. DNA extracted from organisms within a sample is amplified, run on a gel, and hybridized with probes specific for the DNA sequence amplified. The

sensitivity of detection varies, depending on the effectiveness of recovery from samples and the degree of inhibition from sample impurities. From the standpoint of assessing public health risk, one of the major drawbacks of PCR is that the method does not differentiate between infective and non-infective microorganisms. This problem has been addressed to a certain extent by the development of immunologic-based methods that depend on the specificity and affinity of a given antibody for an antigen on the pathogen surface. One commonly used technique is immunomagnetic separation (IMS), which uses antibody-coated magnetic beads to “capture” the specific organisms of interest from the sample (Padmanabhan *et al.*, 1988). Because, theoretically, the viral capsid must be intact in order for it to bind to the antibody, the IMS-PCR method is more specific for the detection of infective virus particles than direct PCR. However, determination of the number of infective virus particles in a sample still cannot be readily performed using this technique.

Recently, a new technique known as molecular beacons has been reported for the construction of probes that are useful for real-time detection of nucleic acids (Tyagi and Kramer, 1996). These probes are based on single-stranded nucleic acid molecules that possess a stem-and-loop structure (Figure 1). The loop portion contains a sequence complementary to a target, and the stem is formed by annealing of two complementary arm sequences not related to the target. A fluorescent moiety is attached to the end of one arm and a non-fluorescent quenching moiety is attached to the other end. No fluorescence is produced when the two arms are in close proximity, due to the quenching action. When the probe encounters a single-strand target, it forms a hybrid with the target, undergoing a spontaneous conformational change that forces the arm sequences apart and causes fluorescence to occur. The interaction of molecular beacons with their targets is extraordinarily specific. No increase in fluorescence is observed even in the presence of a target strand that contains only a single nucleotide mismatch (Tyagi and Kramer, 1996). Because of the enhanced specificity, molecular beacons can be formulated for identifying closely related allelic variants.

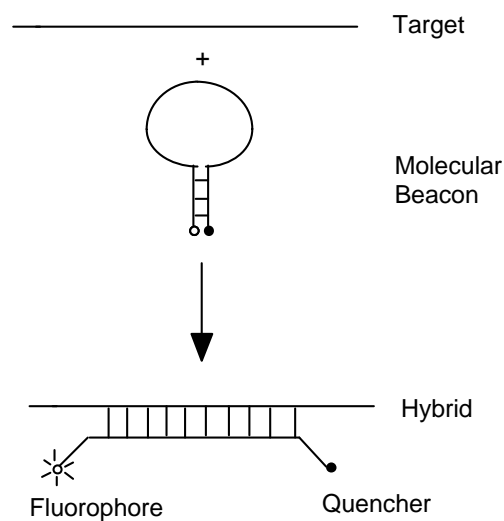


Figure 1. Principle of operation of molecular beacons.

When present in sufficient concentration, molecular beacons interact rapidly with target strands, generating fluorescence in the process. Since unhybridized beacons do not have to be separated, they can be included in the PCR reaction, permitting the progress of the reaction to be followed in real time. Gene detection is specific and sensitive, and can be carried out in a sealed tube. The very fast kinetics in renaturation makes the molecular beacons very desirable upon cyclical increases and decreases in temperature during PCR. In real-time PCR the beacons, as well as the primers, hybridize to complimentary sequences during the annealing stage. Data are collected at each annealing stage, when the beacons are hybridized to the target. When the temperature is increased during the extension stage, the beacons dissociated from the templates and do not interfere with polymerization. As the reaction progresses, the intensity of the fluorescent signal at each cycle gives an indication of the amount of perfectly complimentary fragments synthesized (Tyagi *et al*, 1998). In the absence of perfectly complimentary nucleic acid sequences the beacons will remain closed and fluorescence will not be detected.

Analysis of fluorescence data recorded at each annealing stage gives a clear profile of the amplification process. The number of amplification cycles needed before a significant increase in fluorescence from target-beacon hybrids is detected (critical cycle) can be used to quantify the initial number of template molecules in the reaction. Tyagi *et al*. (1998) found the critical cycle to be inversely proportional to the logarithm of the initial number of target molecules. This data can be used to formulate a standard quantification curve for each detection system.

Molecular beacon assays are simple and fast. Reagents are mixed in one step and reactions are carried in closed tubes, thus preventing contamination. Data are recorded during each cycle and results are automatically analyzed immediately after the reaction is completed, usually 2-3 hours. Due to their high specificity and high sensitivity, molecular beacons can be effectively incorporated into real-time PCR assays and provide a quick and accurate method for detection of specific nucleic acid sequences in homogeneous solutions. We envision that the speed and sensitivity of virus detection based on the PCR-assay method can be greatly enhanced with the application of molecular beacons.

We propose to combine IMS-RT-PCR with molecular beacons to increase the sensitivity and specificity of analysis of environmental water samples for infectious hepatitis A virus. This technology should improve our ability to provide quick and efficient results for the detection and quantitation of infective hepatitis A virus in samples from drinking water and other environmental samples.

RESEARCH OBJECTIVES

The purpose of this research is to improve on the current analytical methods to achieve rapid, quantitative detection of hepatitis A viruses in water. This methodology will facilitate the ability to monitor water for the presence of this virus. The information obtained from this monitoring will enable an assessment of the potential public health impacts of reuse of recycled water, and may provide information for the Ground Water Rule. The specific objectives of this research are to:

- Develop a molecular beacon-based procedure for the real-time detection of hepatitis A virus in water
- Improve the efficiency and quantitation capabilities of RT-PCR with the use of molecular beacons.
- Test the specificity of the newly-developed method by assessing its capability to detect other enteric pathogens.

METHODS AND PROCEDURES

Primer design

A primer set was developed in order to amplify a highly conserved region of the VP3 capsid region of the Hepatitis A virus. The forward primer (HAV6 5'-AAT GTT TAT CTT TCA GCA ATT AA-3') and the reverse primer (HAV6073 5'-CTC CAG AAT CAT CTC CAA C-3') were designed to amplify a 91-base pair (bp) fragment.

Molecular Beacon design

A molecular beacon (MB) was designed to complementarily target the internal region of the PCR amplicon. The MB (5'-6-FAM-CGC TAT TTT GCT CCT CTT TAT CAT GCT ATG G ATAGCG-DABCYL-3') was purchased from the Midland Certified Reagent company (Midland, MI) and contains a 25-bp probe moiety, which is flanked by two 6-bp arms (underlined).

Thermal Denaturing Profile

To determine the optimal recording temperature for the real-time RT-PCR assay, the thermal denaturing profiles of the beacon in the presence or absence of a perfectly complementary oligo were investigated using a BioRad iCycler. Figure 1 shows the fluorescence variation as a function of temperature. The profile confirms that, at low temperatures, the arms of the MB form a hairpin structure, therefore inhibiting fluorescence. A suitable annealing temperature could be chosen so that the MB will remain anneal to the amplicon and the resulting fluorescence should be significantly above the background fluorescence. A temperature ranging from 55-65°C was determined to be suitable.

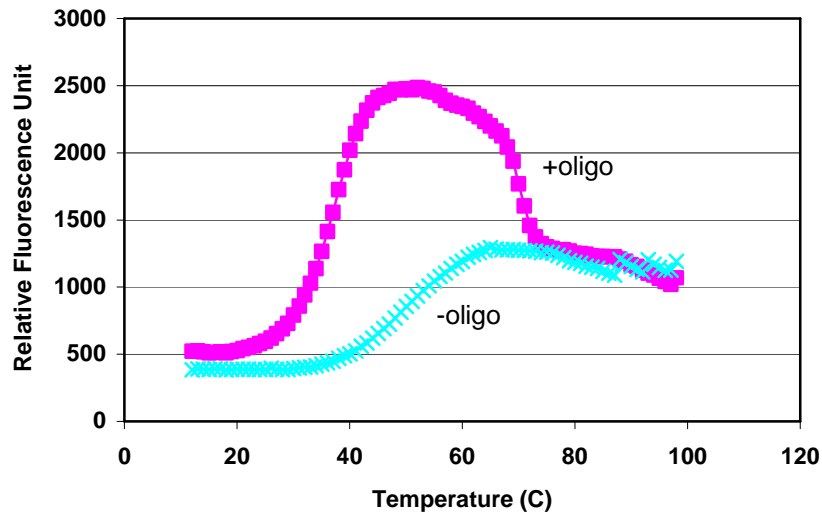


Figure 1. Thermal denaturing profile of Hepatitis A virus molecular beacon.

Real-time detection by 2-Step RT-PCR

A 2-step RT-PCR assay was developed for the detection of HAV. The samples were initially held at 95°C for 5 min. The reverse transcription was performed under the following conditions: 25°C for 10 min, 42°C for 60 min, and 100°C for 5 min. PCR was performed as follows: 95°C for 5 min then followed by 40 cycles of denaturation at 95°C for 1 min, annealing at 55°C for 1 min, extension at 72°C for 1 min, and one cycle at 100°C for 5 min. As shown in Figure 2, only reactions containing HAV RNA were detected by the molecular beacons. For samples that were not amplified (NAC) or with no templates (NTC), no increase in fluorescence was observed.

As a confirmation of this observation, the PCR samples were analyzed on a 2.5% agarose gel. The results were consistent with the real-time RT-PCR. The 91bp amplicons were detected only in the presence of HAV templates.

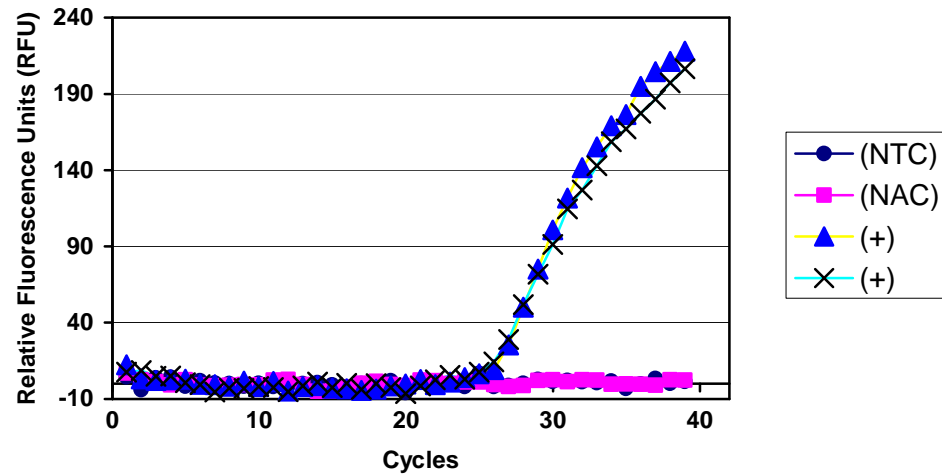


Figure 2. 2-Step RT-PCR and the molecular beacon specificity.

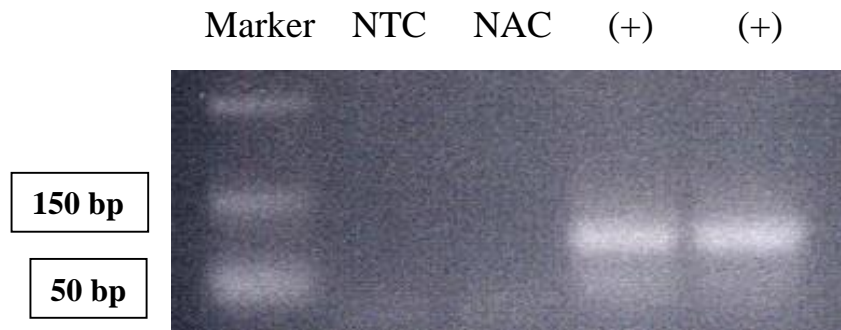


Figure 3. Detection of PCR amplicons on a 2.5% agarose gel.

Specificity of the Molecular Beacon

There are other viruses that can be amplified using the same RT-PCR protocol. These include Enteroviruses, which are also commonly found in contaminated water and are known to cause diseases such as aseptic meningitis, myocarditis, and encephalitis. Two such viruses are Echovirus 11 and Coxsackie virus B6. To investigate if the MB would detect these two viruses, RT-PCR assays were conducted with Echovirus 11 and Coxsackie virus B6 as the templates.

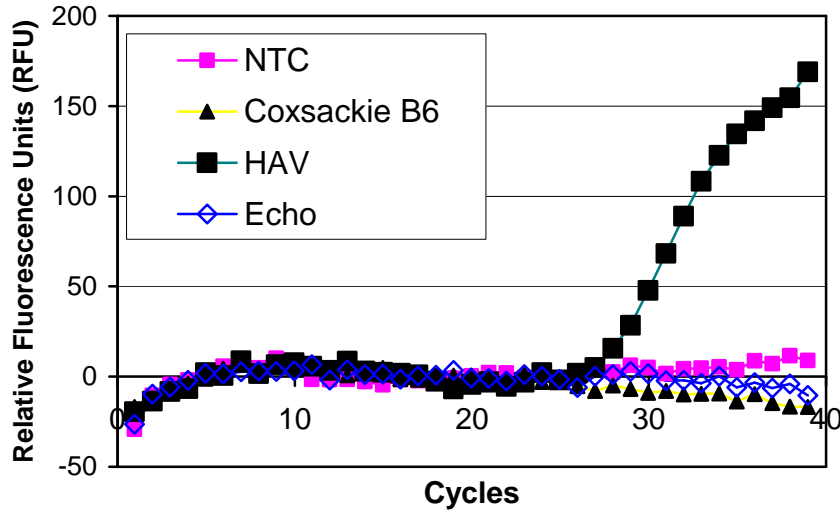


Figure 4. Demonstration of MB specificity.

As shown in Figure 4, the MB is specific to only HAV. No increase in fluorescence was detected with either Echo or Coxsackie as the templates. More importantly, even though amplicons were detected with the Echovirus 11 and Coxsackie virus B6 (Figure 5), they were not detected by the MB, again confirming its superior specificity.

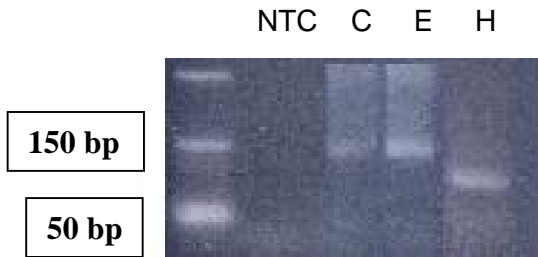


Figure 5. (A) Verification of amplification with a 2.5% agarose gel. E: Echovirus 11 C: Coxsackie virus B6 and H: HAV.

Re-design of Molecular Beacon

Several months into the second year of the project, there was a change in some of the reagents that had to be made due to a change in the manufacturers. At this point, we were unable to regain the activity of the beacon, despite repeated efforts to examine a

variety of combinations and permutations of reagents and experimental conditions. Therefore, the molecular beacon had to be totally redesigned.

A set of primers was designed to amplify a 125 bp highly conserved 5'-noncoding region (NCR) in the HAV genome. From the alignment of known genomes of different hepatitis A strains, a molecular beacon was designed to specifically recognize a 20 bp sequence of the amplicon. Figure 6 shows the thermal denaturation profile for the newly designed beacon.

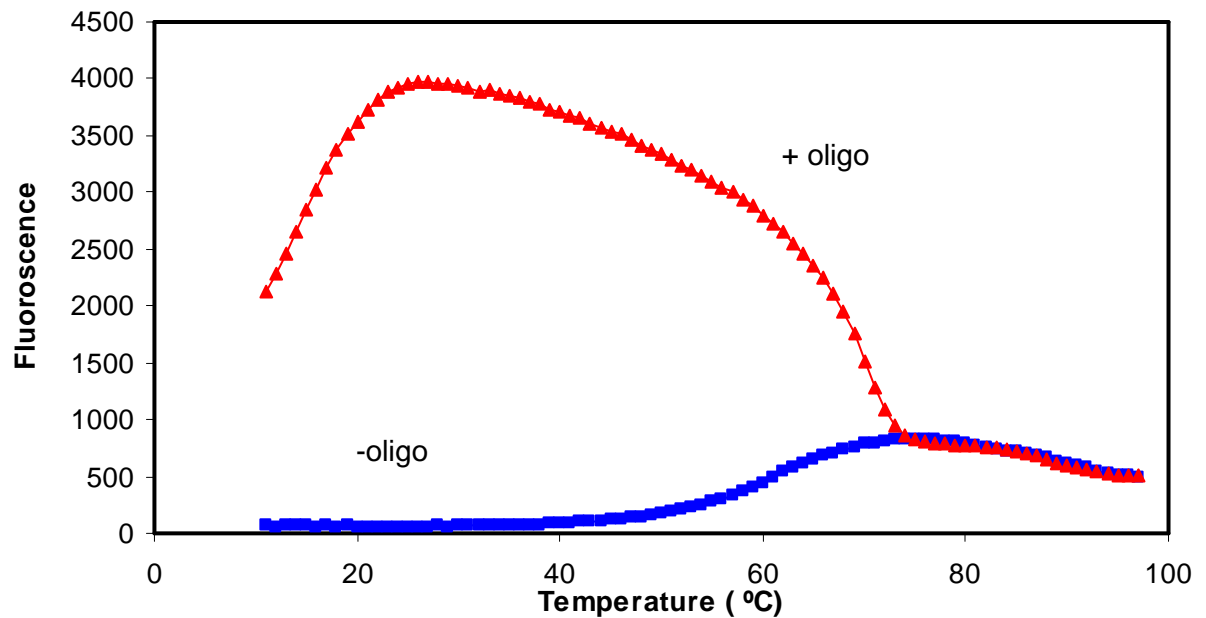


Figure 6. Thermal denaturation profile of HAV molecular beacon. The fluorescence intensity of the molecular beacon in the presence or absence of an excess amount of complementary oligo was monitored at different temperatures. The optimal working temperature for HAV molecular beacon was selected to be (56°C).

The sensitivity of the molecular beacon was examined using serial ten-fold dilutions of hepatitis A virus stock solutions. As shown in Figure 7, the beacon was able to detect a single virus particle.

The specificity of the new molecular beacon was performed using a variety of enteric microorganisms and indicators. The results of this analysis are presented in Table 1. The molecular beacon system proved to be very specific for only hepatitis A viruses; none of the other tested microorganisms were detected using the probe.

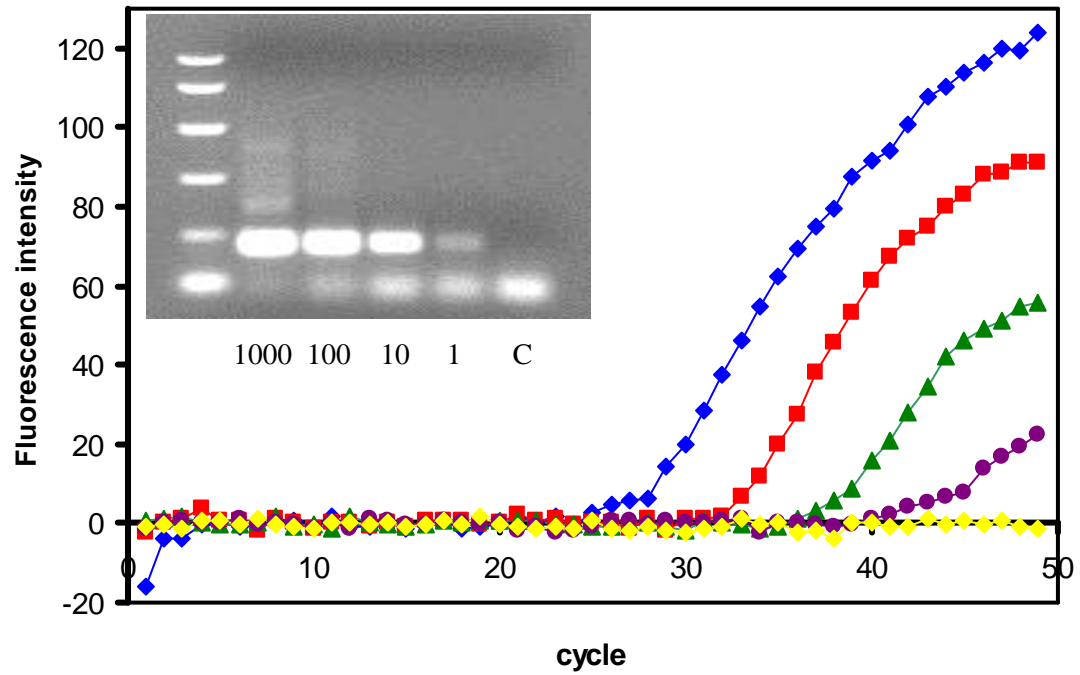


Figure 7. Real-time detection of 10-fold serial dilutions of viral RNA of Hepatitis A by MB-RT-PCR assay. (♦) represents 1000 PFU, (■) 100 PFU, (▲) 10 PFU, (●) 1 PFU, (◆) Control.

Table 1. Specificity of Hepatitis A Virus Molecular Beacon

Microorganism	Strain	Detection	Source
Hepatitis A virus	HM175	yes	ATCC ¹
Hepatitis A virus	GA76	yes	CDC ²
Coxsackievirus B1 VR-28	Conn-5	no	ATCC
Coxsackievirus B3 VR-30	Nancy	no	ATCC
Coxsackievirus B6 VR-155	Schmitt	no	ATCC
Echovirus 11 VR-41	Gregory	no	ATCC
Echovirus 17 VR-1058	CHHE-29	no	ATCC
Echovirus 19 VR-1060	Burke	no	ATCC
Human parechovirus type 1	Valencia	no	LACSD ³
Adenovirus 2	Sewage	no	LACSD
Adenovirus 15	Sewage	no	LACSD
Poliovirus 1	LScAb	no	ATCC
Rotavirus	Wa	no	ATCC
MS2	15597-B1	no	ATCC
phiX174	13706 –B1	no	ATCC

¹ATCC – American Type Culture Collection, Rockville, MD

²CDC – Hepatitis Branch, National Center for Infectious Disease, Centers for Disease Control and Prevention, Atlanta, GA

³LACSD – County Sanitation Districts of Los Angeles County, Whittier, CA

CONCLUSIONS

The results of this study have demonstrated that method for the rapid detection of hepatitis A virus can be developed. This molecular beacon-based PCR method is extremely sensitive, as it can detect as few as one virus particle. It is also very specific for hepatitis A virus, as it was not able to detect a number of other common enteric pathogens and indicator microorganisms that might be found in environmental waters. Considering the greatly reduced time required to obtain results using this method, it holds great promise for application to water quality monitoring for viruses.

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