Journal of Medical Microbiology and Infectious Diseases

JoMMID
ISSN: 2345-5349
eISSN: 2345-5330

Comparison of PCR with Serology for Detecting Acute Hepatitis A Virus Infection

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ARTICLE INFO

Original Article

Keywords: Molecular diagnostic testing, Serological tests, Hepatitis A viruses

Received: 06 Feb. 2021
Received in revised form: 10 May. 2021
Accepted: 08 May. 2021
DOI: 10.52547/JoMMID.9.2.82

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ABSTRACT

Introduction: Early detection of acute Hepatitis A virus infection (HAV) allows adopting proper treatment measures, rapid recovery, and avoiding side effects. This study compares PCR assay with serology for diagnosing acute HAV infection. **Methods:** Twenty samples from patients presenting clinical symptoms of acute hepatitis were tested for anti-HAV IgM antibodies. Genomic RNA was extracted from IgM-positive samples, cDNA was synthesized and examined for genomic HAV using a specific HAV real-time detection kit and a nested PCR. **Results:** Among 20 sera, 14 were positive for anti-HAV IgM antibodies. The specific real-time PCR and nested PCR showed agreement, and both detected HAV genetic material in 3 out of 14 samples. **Conclusion:** High levels of anti-HAV IgM antibodies do not necessarily indicate acute HAV infection in people presenting clinical symptoms of the disease. Measuring IgM antibody levels alongside molecular detection of virus genome by DNA-based methods assay can lead to an accurate, timely, and reliable diagnosis of active HAV infection.

INTRODUCTION

Hepatitis A Virus (HAV) infection is the leading cause of acute hepatitis and affects millions of people worldwide [1, 2]. Regarding the considerable number of asymptomatic and unreported cases, the true prevalence of hepatitis A virus infection in different geographical areas in Iran is not evident; however, systematic studies and pooled analysis similarly showed that 62.24% of Iranians were positive for HAV infection [3, 4]. Improved drinking water, hygiene, and sanitation systems in most Eastern Mediterranean (EMR) and Middle Eastern (ME) countries protect susceptible people during childhood from HAV infections and decrease the disease prevalence. Hence, adults remain highly susceptible to HAV infection at older ages due to the lack of anti-HAV IgG antibodies [5]. The oral-fecal route is the primary route of HAV transmission, i.e., ingesting water or food contaminated with feces of an infected person [2].

Acute hepatitis A disease includes four stages, incubation period (15-45 days), prodromal symptoms (1-7 days), jaundice (2-6 weeks), and convalescence (up to 6 weeks). The clinical symptoms are strongly age-dependent [6, 7]. HAV infections in childhood are subclinical and sometimes unrecognizable, so that in children ≤ six years, 70% of infections are asymptomatic, while older individuals present more severe and apparent symptoms [8]. In adults, the clinical symptoms of hepatitis A range from mild, including fever, restlessness, weakness, fatigue, anorexia, diarrhea, nausea, abdominal discomfort, muscle aches, joint pain, headache, dark urine, and jaundice to severe acute liver failure and death [8-10]. HAV infection is usually self-limiting, and is clinically resolved within a few weeks. However, a small proportion of infections result in fulminant hepatitis with liver failure, which may be fatal unless managed emergently by liver transplantation [11]. The clinical symptoms of HAV last up to eight weeks after onset [12]. In the absence of clinical symptoms during the incubation period, the most infectious disease stage, the infected individuals have the highest virus shedding in feces [6]. During the viremia, patients have

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an active infection and can transmit the virus to healthy people. The active infection can only be diagnosed at this stage by amplifying the virus genome using molecular assays [10]. Hepatitis A virus infection is usually acute, less chronic, and self-limiting, and commonly all the symptoms do not appear [2, 13]. HAV infection results in increased liver enzymes such as alanine transferase (ALT) and aspartate transaminase (AST) during the prodromal phase, while bilirubin levels remain normal [14]. However, during the jaundice period (Icteric Phase), bilirubin level increases to 5-20 mg/dl [6]. The HAV gradually triggers the immune system, and the disease symptoms appear two to four weeks after the onset of infection [15].

The HAV consists of a non-enveloped icosahedral capsid of around 30 nm in diameter containing a positive ssRNA genome molecule of 7.5 kb with multifaceted symmetry containing four polypeptides [16]. The major viral capsid polypeptides VP1, VP2, VP3, and VP4 (essential for virion assembly and not detectable in mature viruses) are antigenic and immunogenic [17-20] and lead to the production of anti-HAV IgM and IgG antibodies in the host.

Anti-HAV IgM antibodies can be detected in the early clinical phase and remain for 4-6 months. Detection of elevated anti-HAV IgM antibodies is the gold standard for diagnosing acute HAV infection. About two weeks following the disease onset, anti-HAV IgG antibodies become detectable in the patient's blood [21]. These antibodies remain for the rest of the life and protect the recovered person against reinfections [21]. In seroepidemiological studies, anti-HAV IgG antibodies indicate previous and inactive infections [21]. HAV occurs in the infected people's blood and feces two weeks before the onset of clinical symptoms [15]. Diagnosis of the disease is commonly through detecting IgG and IgM anti-HAV antibodies in the patient's blood [22], and fecal samples are rarely used to diagnose acute hepatitis A [6].

Here, we deployed three methods for diagnosing HAV infection in the blood, including serology for detecting anti-HAV IgM antibodies and Real-time PCR and nested PCR for amplifying the virus genome. We compared the results to determine the best, most accurate, and cheaper approach for diagnosing active HAV infection.

MATERIALS AND METHODS

Patients and clinical specimens. Peripheral blood samples were collected from 20 individuals admitted to the Pars Hospital Laboratory in Tehran, Iran, with clinical symptoms of acute hepatitis. After obtaining the informed consent from all adult participants, the parents, or legal guardians of minors, 5 ml of peripheral blood was collected from each patient. The sera were separated and sent to the Hepatitis, AIDS, and Bloodborne Viruses Laboratory of Pasteur Institute of Iran with cold storage. All samples were tested for hepatitis B and hepatitis C infection using two commercial kits (Anti-HBs Kit, Architect, Italy) and (HCV Ag Kit, Architect, Italy) upon receipt. The exclusion criteria were hepatitis B and C positivity. At this stage, none of the patients were positive for hepatitis B or C.

Ethics approval. The Ethics Committee of Pasteur Institute of Iran approved this study (code: IR.PII.REC.1396.41). All procedures in this study were performed according to the ethical principles of the Pasteur Institute of Iran, including obtaining written informed consent from all participants.

Serological Diagnosis of HAV. The sera from patients were tested for anti-HAV IgM antibodies using a commercial ELISA kit (HAV Ab-IgM Kit, Architect, Italy). The sensitivity and specificity of the anti-HAV IgM kit were 98.6% and 99.0%, respectively.

RNA extraction. The anti-HAV IgM antibodies positive sera were subjected to RNA extraction using a viral RNA extraction kit (QIAamp® Viral RNA Kit, Qiagen, Germany) according to the manufacturer's instructions. The content and purity of extracted RNA samples were measured using a bioanalyzer Thermo Scientific Nanodrop (Fisher Scientific, USA).

Real-time PCR. All RNA specimens were screened for genomic HAV using a real-time PCR commercial kit (AmpliSens HAV-FRT PCR, InterLabService, Russia) according to the manufacturer's instructions.

Nested-PCR. The genomic RNA of all anti-HAV IgM-positive sera was converted to cDNA using the Quantinova^{TM} Reverse Transcription synthesis kit (Qiagen, Germany) as described by the manufacturer. Detection of HAV was performed by nested PCR using primers complementary to the N terminus VP1 regions of the virus genome fragment [18]. The primers in the first and second stages amplify 310 bp and 244 bp fragments of the HAV genome, respectively. Nested-PCR is of higher sensitivity than conventional PCR and detects virus genome at much lower viral loads in the blood.

In the first stage, 20 µl reactions contained 10 µl of PCR 2X master mix (Biotechrabbit, Germany), 0.5 pmol/µl of each primer, 2.5 ng/µl of cDNA, and 6 µl of nuclease-free double distilled water (ddH_2O). The PCR reaction was performed in a thermal cycler PeQlab (Avantor, UK). The amplification program included an initial denaturation step for 2 min at 95°C followed by 35 cycles of denaturation for 30 sec at 95°C, annealing for 30 sec at 53.5°C, an extension for 30 sec at 72°C, and a final extension step for 10 min at 72°C.

In the second stage, the reactions contained 10 µl of PCR 2X master mix (Biotechrabbit, Germany), 0.5 pmol/µl of each primer, 2 µl of the first stage amplicon as a template, 2 µl of DMSO, and 4 µl of nuclease-free ddH_2O. The amplification program began with an initial denaturation for 2 min at 95°C followed by 35 cycles of denaturation for 30 sec at 95°C, annealing for 30 sec at

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55°C, and extension for 30 sec at 72°C and a final extension step for 10 min at 72°C. Finally, 3 µl of PCR products from both nested PCR rounds were run on 1.5% agarose gel along with a 100-3Kb ladder and stained with Safe Stein.

RESULTS

Clinical manifestations and serology. Based on the clinical manifestations of acute liver infection, i.e., abdominal pain, diarrhea, nausea, vomiting, and anorexia, 20 individuals were included in the study. Serology detected anti-HAV IgM antibodies in 14 out of 20 individuals. The other six might have had other medical conditions, ,i. e., biliary colic, acute pancreatitis, sarcoidosis, and Kaposi lymphoma, demonstrating acute clinical symptoms of liver disease due to liver cell involvement.

PCR assays. The Real-time PCR detected HAV RNA in 3 out of 14 IgM positive individuals, while the other 11 individuals were negative for HAV RNA despite having IgM antibodies (Table 1). The nested PCR assay showed agreement with the real-time PCR method and detected genomic HAV in the same three samples (Fig. 1). The reason for not visualizing the amplicon in the first step of the nested PCR was the low viral load in the blood samples.

Table 1. Data of HAV-infected patients, and serology and PCR results.

Patient No.	Age	Clinical Signs	ELISA IgM (u/ml)	RT-PCR (C*t*)	Nested PCR
1	56	+	1.6	-	-
2	26	+	6.5	-	-
3	20	+	6.4	+ (24)	+
4	47	+	3.6	-	-
5	19	+	1.8	-	-
6	30	+	5.9	+ (25)	+
7	15	+	5.5	+ (28)	+
8	35	+	5.99	-	-
9	24	+	6.22	-	-
10	34	+	3.96	-	-
11	11	+	4.05	-	-
12	16	+	4.77	-	-
13	17	+	5.7	-	-
14	27	+	2.39	-	-

Ct, threshold

DISCUSSION

Prompt and reliable diagnosis of acute hepatitis A virus infection is essential for initiating treatment and preventing transmission to healthy individuals. At present, the diagnosis of acute HAV infection is through detecting anti-HAV IgM antibodies [23]. Detecting anti-HAV IgM antibodies is a relatively inexpensive and fast approach, but high levels of anti-HAV IgM in the serum of people recovered from HAV infection can lead to misinterpretation [24]. IgM antibodies are usually detectable in the sera of the recovered patients for up to 6 months after the virus clearance [23], but in some patients, they may last up to a year after recovery and can be detected with sensitive and unique methods, e.g., radioimmunoassay (RIA) [25, 26]. Misinterpreting of results leads to misdiagnosis of the acute HAV infection. As a result, the recovered individuals who do not have symptoms consistent with acute hepatitis A are treated with antivirals due to high IgM levels [24]. Antibody cross-reactions in people with autoimmune diseases, acute and chronic infections, and cases where activate polyclonal B lymphocytes interact with the anti-HAV IgM antibody result in false-positive results [27, 28]. Therefore, for accurate and reliable diagnosis of acute HAV infection, the measurement of anti-HAV IgM is not definitive and molecular detection of virus genome is required to confirm the infection [24]. In the present study, we detected hepatitis A virus genomic RNA in the patients with anti-HAV IgM antibodies using a real-time PCR and a nested PCR assay. Our results showed that of 20 patients with clinical symptoms of acute HAV infection, 14 were positive for anti-HAV antibodies, while only three (21.5%) of these patients had virus genetic material in blood. These three patients had an active infection and could transmit the virus to healthy people. Despite having high anti-HAV antibody levels, the other 11 cases were not considered active patients and pose no risk of virus transmition to healthy individuals. Here, our results showed that high levels of anti-HAV antibodies without detecting the virus genetic material do not necessarily confirm active infection.

Our results are consistent with the results of previous research, which showed that false-positive or misinterpretation results of anti-HAV IgM serology could lead to misdiagnosis or premature closure of diagnostic procedures. HAV nucleic acid tests can be used more broadly during the diagnostic workup to confirm acute hepatitis A, especially in patients positive

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for anti-HAV IgM antibodies and moderate or low signal-to-cut-off ratio (S/CO) ratios [24].

During the incubation period of HAV infection, which lasts about two weeks, the average virus shedding in the blood reaches 10^5 particles/ml [29-31], and the rate of virus shedding in the feces is 10^9 particles/ml or gr [32, 33]. During this period, the IgM anti-HAV antibodies titer is very low and undetectable, while the rate of the virus in the blood and feces is at its highest level. During this period, the anti-HAV IgM antibody might turn negative while the patient is at the peak of transmitting the virus to healthy individuals. Molecular tests for detecting the virus genome in the patients' sera or stool assist in early diagnosis and preventing further transmission of the virus to healthy individuals. It seems that in the first month, post-exposure, the molecular diagnosis will be much more accurate and reliable than the serology for diagnosing acute infection.

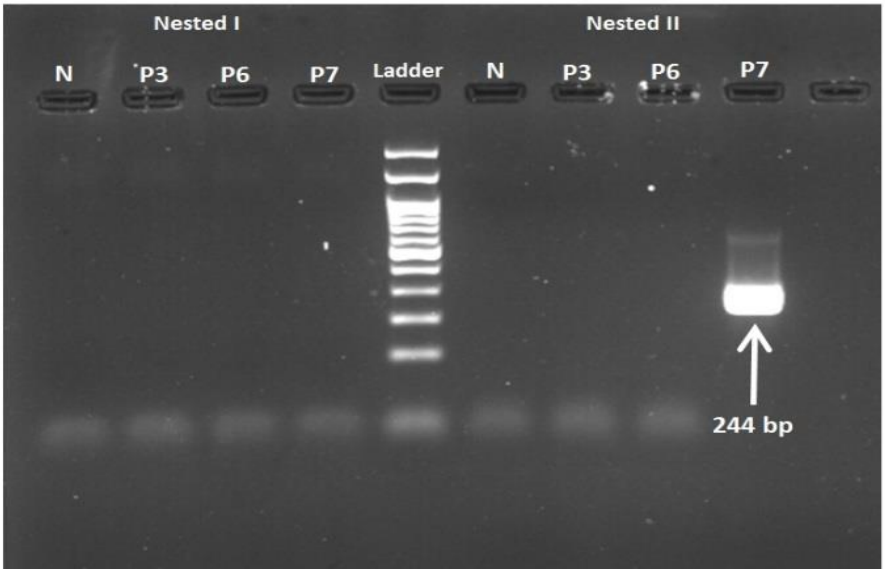


Fig. 1. Nested PCR amplification of hepatitis A virus genome fragment. From left to right, N, negative control; lanes P3, P6, and P7, nested PCR first stage; lane 5, 100bp DNA ladder; N, negative control; P3, P6, and P7 (positive), nested PCR second stage.

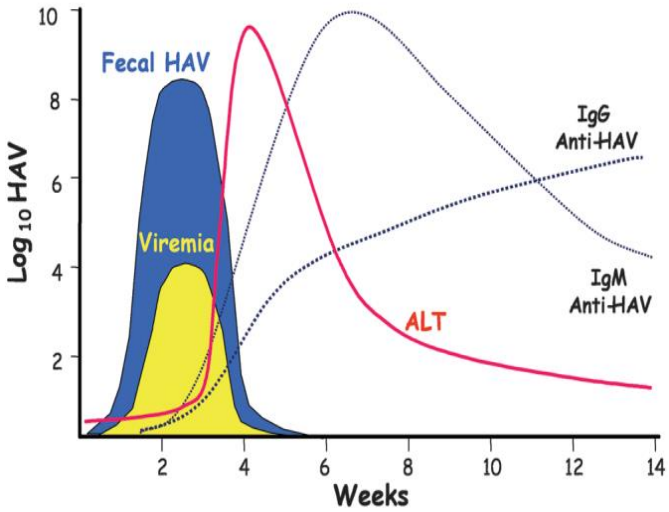


Fig. 2. An overview of the immunological and biochemical reactions to the hepatitis A virus. Viremia (yellow area), virus in feces (blue area), alanine aminotransferase (ALT) in serum (red line), anti-HAV IgM, and IgG antibodies (blue lines). With permission from Stanley M. Lemon [32].

Finally, the results of our research showed that measuring anti-HAV IgM antibody levels in people suspected of hepatitis A alone is not a reliable and accurate method for a definitive diagnosis of the acute infection. Molecular detection of the virus genome in the suspected people alongside measuring the anti-HAV IgM antibodies level significantly increases the chances of accurate, timely, and reliable diagnosis of active HAV infection. In our study, the real-time PCR and nested

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[DOI: 10.52547/JoMMID.9.2.82]

PCR showed the same sensitivity, and since the latter is of a lower cost, we recommended it for detecting the HAV genome.

ACKNOWLEDGMENTS

This article was part of the Ph.D. thesis project of Hossein Heydari in Cellular and Molecular Biology, under the supervision of Dr. Mohammad Reza Aghasadeghi, in Pasteur Institute of Iran. This project was funded by the Pasteur Institute of Iran (Grant No. 1357). We extend our gratitude to the staff of the Hepatitis, AIDS and Bloodborne viruses Laboratory, Pasteur Institute of Iran, for their technical assistance.

CONFLICT OF INTEREST

The authors have no conflicts of interest to declare.

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Cite this article:
Heydari H, Majd A, Hamidi-Fard M, Bahramali G, Aghasadeghi MR. Comparison of PCR with Serology for Detecting Acute Hepatitis A Virus Infection. J Med Microbiol Infect Dis, 2021; 9 (2): 82-87. DOI: 10.52547/JoMMID.9.2.82.

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