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Hepatitis A and E seropositivity and nucleic acid detection among chemical bombardment survivors in Iraqi Kurdistan Region

Salih Ahmed Hama[1], Shawnm Ahmed Aziz[2]

1University of Sulaimani/ College of Science/ Department of Biology (salih.hama@univsul.edu.iq)
1Komar University for Science and Technology (KUST)/ College of Sciences/ Department of Medical Laboratory Sciences (salih.hama@komar.edu.iq)
2Sulaimani Polytechnic University/ Sulaimani Technical Institute/ Department of Nursing

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Abstract

The current study was carried out to determine the percentage rates of anti-hepatitis A and E (HAV and HEV) IgG and IgM seropositivity and RNA among chemical bombarded survivors in different regions in Iraqi Kurdistan. Blood samples were collected randomly from 92 chemical bombed exposures and 45 non-exposures -controls- from July to November 2013. ELISA and conventional, nested PCR techniques were followed to detect anti-hepatitis A and E IgG, IgM and RNA respectively. Lymphocyte counting also was done for all tested exposures and controls. It was observed that the percentage rates of hepatitis A seropositivity were higher than hepatitis E. All tested exposures (100%) were seropositive for anti- HAV IgG, whereas 61.79% were positive for anti-HEV IgG. Similarly, anti-HAV IgM seropositivity was higher (8.9%) than that of anti- HEV (1.089%). Current results revealed that there were significant differences between exposures and controls regarding anti-HAV and anti-HEV IgG (p= 0.0001 and 0.0002), whereas no significant differences were observed between the two tested groups concerning anti-HAV and anti-HEV IgM seropositivity (p= 0.621 and 0.56). Moreover, significant differences were found among anti-HAV IgG and IgM as well as anti-HEV IgG and IgM seropositivity among exposures themselves (p= 0.000 and 0.0055) respectively. The percentage rate of hepatitis A RNA positivity was 15.68%, whereas no positive results were seen for HEV. Geographical distribution of exposures was appeared to be significantly effective on all obtained results (seropositivity and RNA detection) ($p < 0.05$). It was noticed that lymphocytes were significantly different between HAV-seropositive and seronegative exposures ($p < 0.05$). A high percentage rate of exposures with positive results for anti-HAV, anti-HEV, and PCR positive results, were suffering from lymphopenia. The highest lymphocyte abnormalities were among exposures with anti-HAV IgM followed by anti-HAV IgG then anti-HEV IgG seropositive exposures.

Introduction

Iraqi Kurdistan Region was exposed to the chemical bombardment in the 1980s during the Iran-Iraq war. In 1987 and 1988 several cities and villages were targeted by Sadam's regime, and different types of chemical warfare were used against innocent people [1]. Various health complaints were found among survivors who were exposed to chemical gases, such as respiratory, eye, dermatological, and immunologic complaints [2]. Using chemical weapons extensively by Iraqi army against Kurdish villages and cities such as Sheikh Wasan and Balisan valley, during April 1987 and in Halabjah on 16th March 1988, suggested that as many as 2.9% of the Kurdish population has been exposed to chemical weapons at a different level [3]. Among the common health problems due to chemical exposure are immunologic defects which predispose

the survivors to a wide range of infections including bacterial, fungal, opportunistic and viral infections. Defects in cell-mediated immunity among exposures [2] can increase the risk of viral infections. Viruses with easy transmission routes like fecal-oral like Hepatitis A and E (HAV, HEV) may be at the top of viral infection list among such patients, especially these viruses may share the same routes and mechanisms of spread, and they can cause similarly severe epidemics [4].

Hepatitis A virus (HAV) belongs to the genus Hepatovirus and is a member of the Picornaviridae family [5]. It is non-enveloped, with a positive single-stranded RNA, and non-segmented genome. The hepatitis E virus (HEV) belongs to the genus Hepevirus, a member of the family Hepeviridae [6]. They are the etiological agents of an acute self-limiting hepatitis and are the primary causes of enterically transmitted hepatitis worldwide [7]. Hepatomegaly, jaundice, fever, anorexia, nausea and abdominal pain may be the common symptoms. HAV is asymptomatic disease in children, although it can cause severe symptoms in young people mainly in older adults, and may lead to fulminant hepatitis at a rate of 1.8% in individuals over 50 years old. Among immunocompromised patient and pregnant women, the mortality rates are relatively higher [8]. It is believed that most of the waterborne outbreaks of hepatitis in endemic regions were caused by HEV [9] as the World Health Organization (WHO) estimated that one-third of the world's population had been infected with HEV. Low sanitary standards contribute to the frequent spread of Hepatitis A and E [10]. Unfortunately, some of these risk factors can be found in chemically bombarded areas, due to the massive destructions done by Iraqi army attacks, this may explain the high rate of entrically transmitted viral infections. Moreover, defects in the immune system of most of the survivors could be another risk factor. It was previously reported that the risk groups for HAV and HEV infection included those with liver disease, the elderly and the immunocompromised individuals [11].

The current study was aimed to investigate seropositivity percentages among chemical exposure survivors and RNA detection of HAV and HEV as well as studying the relation of geographical distribution and total lymphocyte counts to the positive results.

Materials and methods:

Between July and September 2013, serum separator tubes (SST) and EDTA tubes were used to collect 7-10 ml of venous blood from 137 volunteers included 92 chemical exposure survivors and 45 health non-exposures as controls. Different chemically bombed areas by Sadam's regime in 1987 and 1988 were selected included; Goptapa, Sewsenan, Shanaxse, Shex Wasan, Balisan and Halabja. Both sexes were included. Using a questionnaire form, different information was collected from each patient included their age, gender, time of exposure to the chemical warfare, past medical history of jaundice, previous surgical operations, blood transfusions, medications and recent travel abroad.

The collected sera samples were divided into two small screw-capped tubes. The first aliquot collected sera were screened for the detection of anti-HAV and anti-HEV IgG and IgM antibodies using ELISA kits (DRG Diagnostics, Germany). The other part of the collected sera was tested for HAV and HEV RNA by conventional and nested PCR respectively. Total lymphocyte counting for all tested cases were determine using automated equipment.

Primer sets			
Name	**Primer sequence 5´ - 3´**	**Supplier**	**Reference**
HEV1 (F*)	5'-AATTATGCC(T)CAGTAC(T)CGG(A)GTTG-3'	OLIGO MACROGEN	Yan *et al.*,2008 [12]
HEV2 (R**)	5'-CCCTTA(G)TCC(T)TGCTGA(C)GCATTCTC-3'		
HEV3 (F*)	5'-GTT(A)ATGCTT(C)TGCATA(T)CATGGCT-3'		
HEV4 (R**)	5'-AGCCGACGAAATCAATTCTGTC-3'		
Housekeeping gene β-actin: (S***)	5'-GTCGTACCACTGGCATTGTG-3'		
Housekeeping gene β-actin (AS****)	5'-CATCTCTTGCTCGAAGTCC-3		

* [forward primer], ** [reverse primer], *** [sense primer], **** [antisense primer]

Results

The current study showed that the percentage rates of the anti-HAV (100%) and anti-HEV IgG (61.7%) seropositivity was higher than that for anti-HAV (9.89%) and anti HEV (1.08%) IgM (Fig. 1). It was concluded that there were significant differences between exposures and controls regarding anti-HAV and anti-HEV IgG (p= 0.0001 and 0.0002) respectively, whereas no noticeable differences were observed regarding anti-HAV and anti-HEV IgM (p= 0.621 and 0.56) respectively. It was also concluded that there was a significant difference between anti-HAV IgG and anti-HEV IgG (p = 0.000), as well as between anti-HAV IgM and anti-HEV IgM results from exposures themselves (p = 0.0055). The percentage rates of RNA detection for HAV and HEV among both exposures and controls also were varied. It was appeared that the percentage rates of HAV RNA positivity were 15.68% among exposures with no positive results among controls. No positive results were seen for HEV RNA neither among exposures not among controls. (Figs. 1, 2, 3 and 4). When the results analyzed statistically, it was noticed that there were significant differences between HAV RNA for both exposures and controls (p = 0.000).

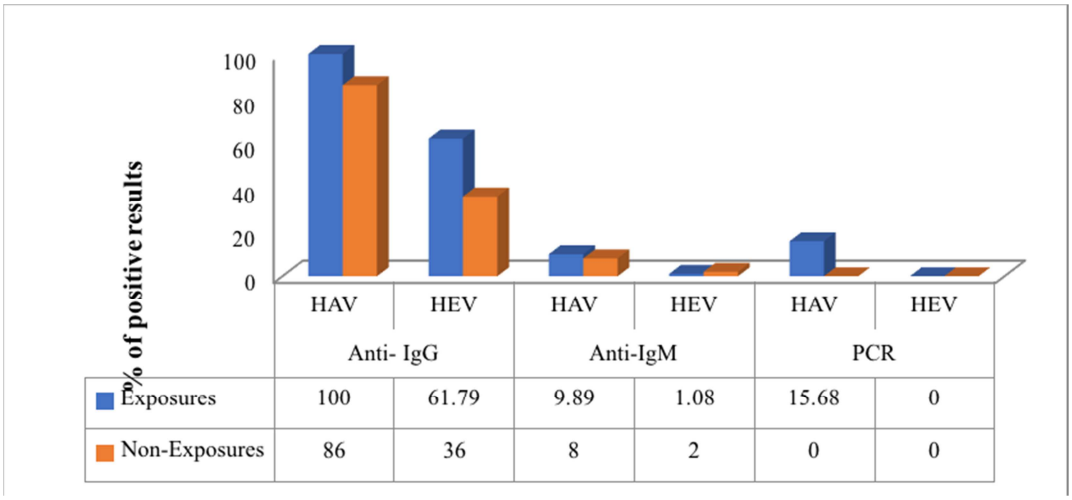


Figure-1- Percentage rates of anti-IgG, anti-IgM and PCR for both HAV and HEV

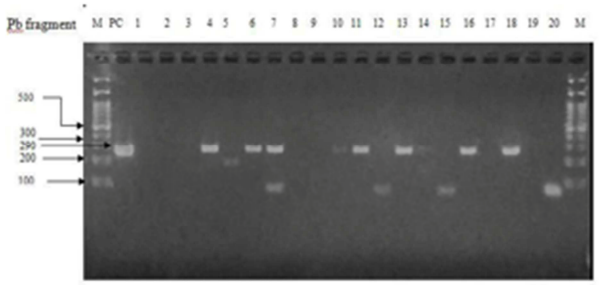


Figure-2- Agarose gel electrophoresis showing the RT-PCR of HAV (M=DNA marker; PC = positive control -290bps-

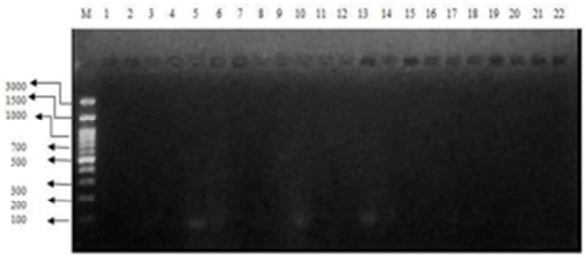


Figure-3- Agarose gel electrophoresis of the first round RT-PCR showed no positive results (M=DNA marker; 1-21 = tested samples

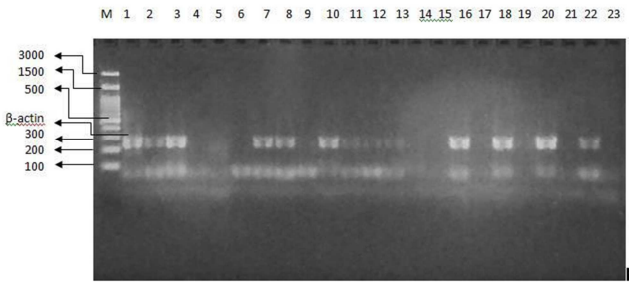
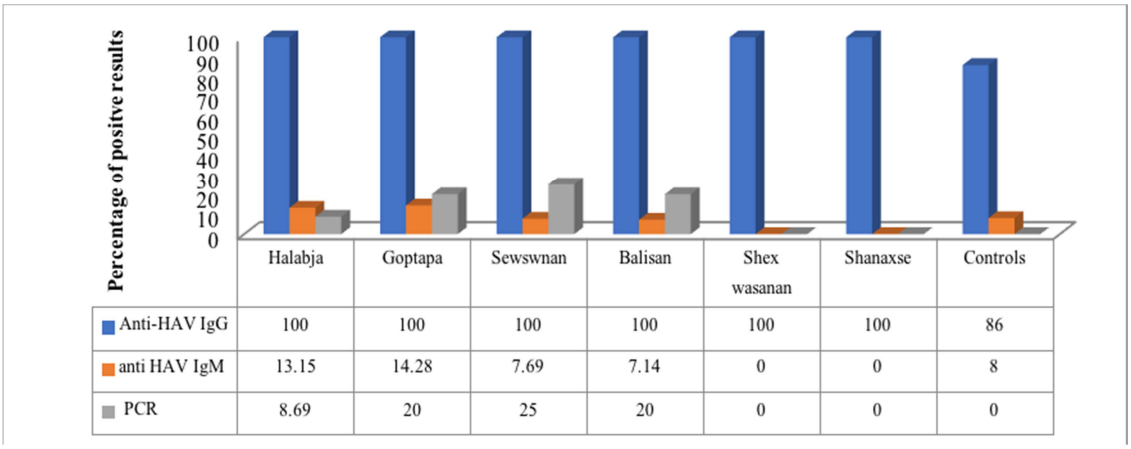


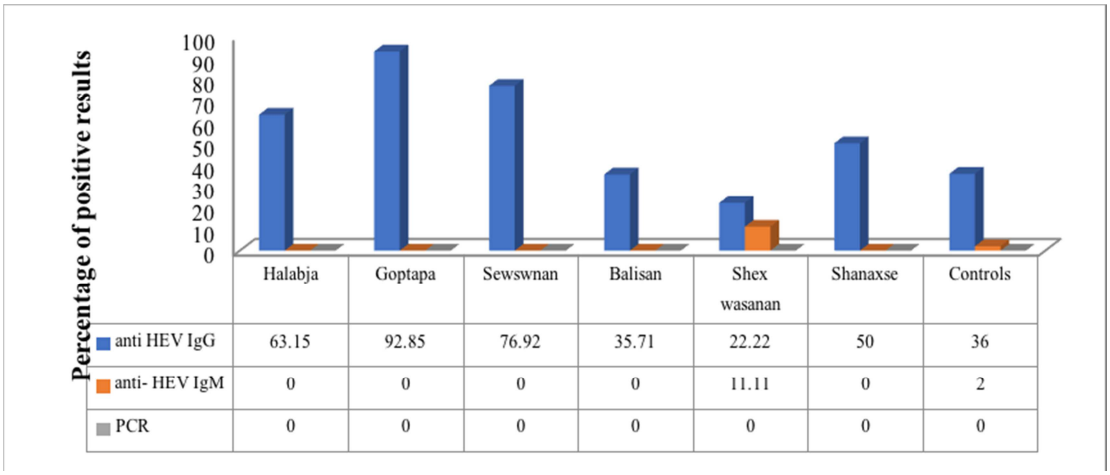
Figure-4- Agarose gel electrophoresis of the second round RT-PCR using second set of primers & β- actin housekeeping gene 313 bps, showing no HEV amplified products genes.

The percentage rates of seropositivity for both HAV and HEV and PCR results were different among exposures from the studied areas. The highest percentage rates of anti-HAV IgM were recorded among exposures in Goptapa (14.28%) followed by Halabja (13.15%), then Sewsenan (7.69%) and Balisan (7.14%). No anti-HAV IgM seropositive was seen in the other tested areas. Regarding PCR results for HAV, the higher percentage rates were among exposures in Swesenan (25%) (Fig. 5). When the obtained results analyzed statistically, it was appeared that anti-IgM seropositivity for HAV was significantly different among exposures from the studied areas (p= 0.000), which means that the geographic distribution of exposures has significant effects on the observed results.



	Halabja	Goptapa	Sewswnan	Balisan	Shex wasanan	Shanaxse	Controls
Anti-HAV IgG	100	100	100	100	100	100	86
anti HAV IgM	13.15	14.28	7.69	7.14	0	0	8
PCR	8.69	20	25	20	0	0	0

Figure-5- Seropositivity and RNA detection of HAV among different groups of chemical exposures and controls



	Halabja	Goptapa	Sewswnan	Balisan	Shex wasanan	Shanaxse	Controls
anti HEV IgG	63.15	92.85	76.92	35.71	22.22	50	36
anti- HEV IgM	0	0	0	0	11.11	0	2
PCR	0	0	0	0	0	0	0

Figure-6- Seropositivity and RNA detection of HEV among different groups of chemical exposures and controls

The highest rates of anti-HEV IgG were recorded in Goptapa (92%), while the lowest rate was in Shex Wasan 22.22% (Fig. 6). Anti- HEV IgM seropositivity was seen only among exposures from Shex Wasanan (11.1%) which was significantly different (p = 0.000) from exposures in other studied areas. Statistical analysis indicated that geographic distribution of exposures was significantly effective on anti-HEV IgM seropositivity.

Results of the current study showed that there were interactions of seropositivity and RNA detection results for both studied viruses. Anti-HAV IgG and HAV RNA positive results interaction was seen among 8.9% of the studied exposures, whereas coninfection with HAV and HEV were seen among 10.9% of exposures which were positive for anti-HEV IgG and HAV RNA at the same time (Fig. 7).

Total lymphocyte counting showed that the vast majority of exposures in all studied areas were suffering from low lymphocyte counts (lymphopenia). It was found that a relatively high percentage rates of exposures with HAV and HEV seropositivity, as well as those who showed PCR positive results, were suffering from

lymphocyte number (count) abnormalities (Either higher or lower than the normal range. The higher percentage of lymphocyte abnormalities were among exposures with anti-HAV IgM seropositive results followed by those who were anti-HAV IgG seropositive and then exposures with anti-HEV IgG positive results (Fig. 8).

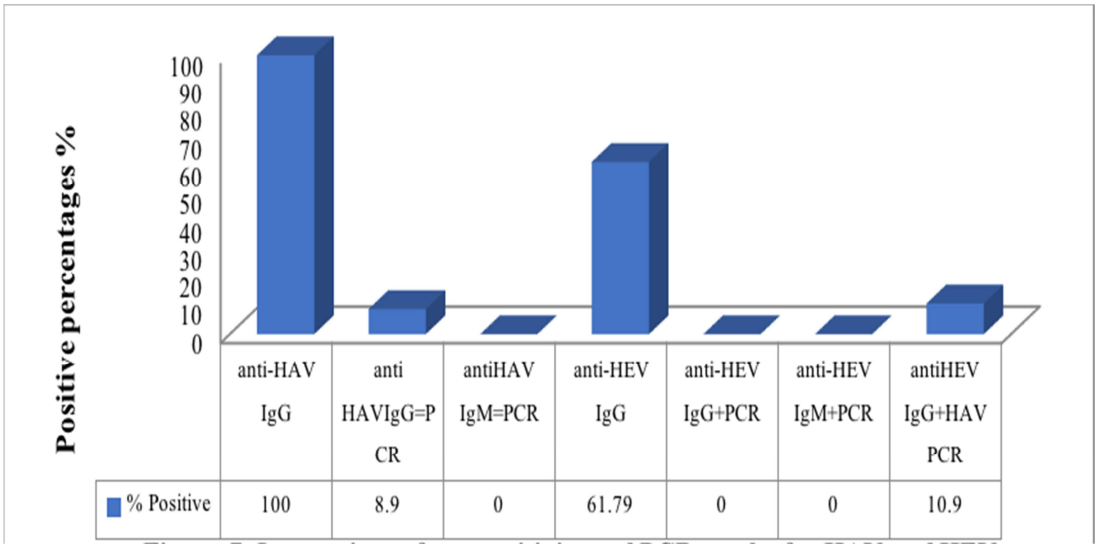


Figure-7- Interactions of seropositivity and RNA results for HAV and HEV among exposures

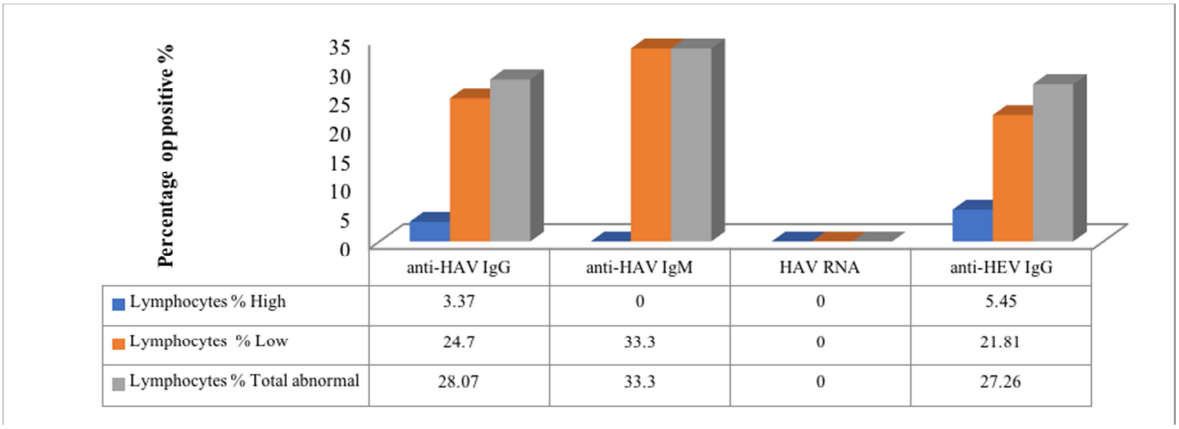


Figure-8- Lymphocyte abnormalities among HAV and HEV PCR and seropositive exposures

Discussions

The higher seropositivity rates of hepatitis A virus, especially anti-HAV IgG are due to the previous HAV infections moreover different risk factors including water sanitation and supply may be behind the higher positive results. Unfortunately, the areus under the study have no perfect water supply with international standards, due to the massive distraction in the past by the Sadam's regime, the situation that may predispose the inhabitants to fecal-orally transmitted viral infections including both HAV and HEV. The defects in the immune system of exposures [2] also may be another risk factor that explain the elevated anti-HAV and anti-HEV seropositivity among exposures. Moreover, the psychological problems due to the chemical bombing massacre and long-term effects of chemicals as well may explain the high susceptibility of exposures to HAV and HEV infections in the past or nowadays. In countries with poor sanitary conditions, HAV infection is highly endemic. Household crowding, poor levels of sanitation, and inadequate water supplies contribute to the propagation of infection within communities in countries such as Africa, Asia and Central and South America. Most individuals become infected within the first few years of life and are asymptomatic [13]. The current results were agreed with their observations. The lower rates of anti-HAV and anti-HEV IgM seropositivity indicated that most of the exposures have no acute hepatitis A and E infections. This indicates positive levels of the hygienic education of the inhabitants although the water-supply projects changed to better. It was appeared in a study done in Iraq that the percentage rates of anti-HAV IgG seropositivity were significantly higher than that of

Hepatitis E [14]. Our observations were agreed with these results. Results of the current study concluded that the percentage rates of anti-HAV IgM were higher than that of HEV, which was similar to conclusions reported in previous studies done by Turkey and his colleagues in 2011[14]. Another study done in Baghdad-Iraq reported that the percentage rates of anti-HAV IgM was relatively higher than that of anti-HEV IgM [15]. The current results were agreed with their conclusions. Studies on HAV prevalence in Adults of EMRO countries showed that 100% of residents of the Nile Delta was anti-HAV-seropositive [16]. Moreover, in a relatively newer published article on Iranian general population, 86% of the studied population anti-HAV seropositive [17], similarities can be observed between the current obtained results and conclusions recorded by the above investigators. Our observations about anti-HEV IgM was not agreed to the results obtained by other researchers in India who noticed higher seropositive rates (18.8%) [18].

In a study conducted in 2008 in Isfahan province, it was indicated that about 10% of the population had positive results for anti-HAV [19], while previous investigations reported that the rates were higher for about three times among studied population in Mazandaran province [20]. The results of the current study were diagreed with their observations. Other investigators in Saudi Arabia reported higher percentage rates of anti-HAV seropositivity, it was indicated that about 70% were anti-HAV seropositive [21]; whereas, about 90% anti-HAV seropositivity was observed among a group of healthy populations of Eastern Saudi in a proceeding study done in 2000 [22]. A study done in Lebanon, showed that about 80% of the population were anti-HAV IgG seropositive [23]. Whereas about 90% of Syrians showed to be anti-HAV seropositive [24]. Unlike these finding lower rates of anti-HAV seropositivity were recorded in Kuwait (about 30%) were appeared to be seropositive [25], which was relatively lower than observations reported in the current study.

In Turkey, it was reported that the seropositivity of anti-HAV was related to the age of the studied groups, the rate increases with increasing with age [26]. Our conclusions were in agreement with these observations. Investigators in 2010 reported that the anti-HEV IgM reaches the peak in the symptomatic period and then decline to baseline within three to six months of illness. This may explain the lowere rates of lower anti-IgM seropositivity. Whereas serum anti-HEV IgG levels continued to rise during the symptomatic phase and became detectable in the convalescent phase for two years [27, 28]. Current observations were parallel to these findings. Low PCR positive results indicated that the vast majority of exposures have no active HAV or HEV infections. Sometime production of anti-RNA antibodies may impair detection of viral nucleic acid. If the PCR was negative and the anti-IgM does not reach the detectable level in early infection stage, could lead to misdiagnosing, although in some cases with early viremia before seroconversion PCR gives positive results. Instead, positive results of nucleic acid would be more helpful for early diagnosis [29]. Performing of real-time PCR may be the gold standard here for avoiding false positive or false negative results, especially these viral infections are infectious and easily can be transmitted by fecal-oral routes, as RT-PCR can detect the minimum amount of the viral nucleic acid and minimize contamination [30]. For detection of HEV RNA nested PCR was depended because this is a modification of reverse transcription PCR, which aims to eliminate unspecific amplification in the first-round RT-PCR due to the unexpected primer binding site, while RT-PCR it is a process where RNA is reverse transcribed into DNA and subsequent exponential amplification of the resultant DNA [31]. It was concluded that detection of HEV RNA in serum or stool using nested or real-time PCR is the most sensitive and definitive diagnostic test; however, the viremic period is short and detection of HEV RNA within the proper time of diagnosis in the clinical setting is not easy, while fecal shedding of virus may last longer with a high viral titer compared to viremia in the blood [32]. Some researchers reported that Hepatitis E viremia could be detected before the onset of liver abnormality, which was accompanied by the humoral immune reaction. Moreover, since there is no robust system to grow HEV in culture, there are some limitations in the field of HEV study [33]. Other factors may be behind the negative results of HEV especially the set of RT-PCR primers used here belong to one genotype whereas different genotypes are found which are genetically heterogeneous on the nucleotide level [32, 34]. HEV RNA was usually detected in early acute infection of patients with hepatitis E infection and tended to decline to an undetectable level during the disease progressing, since anti-HEV IgM might not reach the detectable level in early infection stage, false-negative records may occur.

Abnormal lymphocyte counts among exposure are due to the long-term effects of chemical warfare used in bombing the studied areas [2]. The current results were agreed with observations reported in a far past by other investigators [35] who observed lymphocytopenia among chemical exposure survivors. Moreover, changes in lymphocyte numbers in the current study were agreed with conclusions reported by other investigators in the past [2]. In the past, it was reported that the effect of SM on the leukocytes in the circulating blood of humans and found severe toxic effects causing leukopenia [36]. He also noted that lymphocytes were the first to disappear, followed by granulocytes, which were severely affected. Observations reported by others indicated that mustard was leukocytic toxin acting on the bone marrow with myelotoxicity causing leukopenia, pancytopenia, aplastic anemia or hypoplastic bone marrow in experimental animals [37].

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