

Acute Hepatitis E. virus infection in Egypt

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Abstract

Hepatitis E virus (HEV) is largely responsible for water borne epidemics in many developing countries. The principle mode of HEV transmission is the fecal oral route in epidemic and sporadic forms with a high case fatality ratio in pregnant women. Serum samples from 50 healthy subjects and from 435 acute viral hepatitis patients, 4-75 years old, were screened for markers of acute viral hepatitis. These included (HB_sAg, anti-HBc (IgM), anti-HDV (IgM), HAV (IgM), anti-HCV (IgG), and anti-HEV (IgG), and (IgM) tests by enzyme-linked immunoassays (EIA).

Furthermore isolation of HEV from peripheral blood lymphocytes and from stools belonging to anti-HEV IgG-positive patients was attempted by inoculation of HepG₂ and Vero cell line cultures. The inoculated cell cultures were examined after immunoperoxidase staining for the detection of HEV antigen. Plasma, lymphocytes and stool samples from anti-HEV IgM positive patients were examined for HEV RNA by PCR.

Anti-HEV IgG was found in 144/435 (33%) of these acute hepatitis patients. Anti-HEV (IgM) was detected in 8/52 (15.4%) out of 52 chosen from the 144 sera that were anti-HEV IgG positive cases.

HEV was isolated in HepG₂ from 32.6% of lymphocyte and from 34.9% of stools from patients positive for anti-HEV (IgG). While it was isolated from 71.4% of lymphocytes and from 100% of stools from patients positive for anti-HEV (IgM). In Vero cell cultures there was no HEV isolation from stools but HEV was isolated from 50% of lymphocytes. HEV RNA was detected by PCR in 85.7% of stools, 62.5% of plasma, and in 37.5% of lymphocyte samples belonging to anti-HEV IgM positive cases. Analysis of these diagnostic tests indicated that virus isolation from peripheral blood lymphocytes and stools by inoculation of HepG₂ cell cultures is more sensitive than virus-RNA detection by PCR

Introduction

Hepatitis E virus (HEV) causes an enterically transmitted hepatotropic infection spreads by the fecal oral route usually through fecally polluted water. Acute viral hepatitis develops after an incubation period of 8-10 weeks. Clinical attack rates are the highest among young adults. Asymptomatic and anicteric infections occur but chronic HEV infection is not recorded. Acute HEV hepatitis may be particularly severe among pregnant women,

with maternal mortality rates reaching as high as 25 % as opposed to 0.07-0.6% in the general population (Mishra and Seef, 1992). Abortion with evidence of fetal HEV infection followed acute maternal infection (Abdel Wahab *et al.* (1996); Abou El Kheir *et al.* (2004) under publication).

In humans and in experimental virus infection of animals viral excretion in stools began approximately 1 week prior to the onset of illness and persisted for nearly 2

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weeks, while viremia was detected late in the incubation period and during acute hepatitis by amplifying virus RNA by a polymerase chain reaction (PCR).

Immunoglobulin M antibody specific to HEV (anti-HEV) inconsistently appears early during clinical illness but disappears rapidly over a few months. (Bryan *et al.*, 1994). Immunoglobulin G anti HEV appears a few days later than IgM and persists for at least a few years (Khuroo *et al.*, 1993).

Our aim in the present study is to assess the best diagnostic criterion of HEV infection in Egyptian hepatitis patients.

Materials and Methods

Subjects

Four hundred and thirty five (435) acute hepatitis patients, 285 males and 150 females from 4 to 75 years old were admitted to Abbassia fever Hospital between July 1997 and July 1998. All patients had febrile jaundice. Fifty healthy adults were recruited as control group.

Samples

From all the patients and controls about 10 ml blood sample was taken in two tubes one with anti-coagulant and the other without anti-coagulant. Stools samples were also taken and stored at -30°C until the samples were processed for virus isolation and for PCR.

Methods

Serum samples of all patients and controls were tested for liver enzyme function and for detection of the following viral hepatitis markers by commercial EIA kits (ABBOTT) which included anti-HAV (IgM), HB_sAg, anti-HB_c (IgM), anti-HDV (IgG) for HB_sAg positive samples, anti-HCV (IgG), and anti-HEV (IgG). Anti-HEV (IgM) were tested by a modification of the anti-HEV (IgG) kit (El-Zimaity *et al.*, 1993).

Stools were prepared for viral RNA extraction according to Chomczynski and Sacchi (1987) method. Peripheral blood lymphocytes (p.bl.ly.) were separated by ficol-hypaque density gradient centrifugation. When sera were positive for anti-HEV (IgG) further investigation was carried out for virus isolation by inoculation of correspondent p.bl.ly. and stools extracts into cultures of Vero and of HepG₂ cell lines. Because HEV did not produce cytopathic effects (CPE) in HepG₂ or Vero cells further tests were done for detection of intracellular HEV antigens by an indirect immunoperoxidase staining.

Also the relevant plasma, (p.bl.ly.) and stools extracts from patients who had anti-HEV IgG were tested for the presence of HEV RNA by polymerase chain reaction (PCR). (Turkoglu, *et al.*, 1996).

Results

Clinical Picture

All patients had jaundice, fever and tender right hypochondrium. Hepatomegaly was found in (31%) of the patients and splenomegaly in (7.7%). One male patient had ascitis. Liver enzyme functions were 2.5 to 5 times the normal for Egyptians. Bilirubin was elevated up to 26 mg/dl in some cases. One female 20 years old patient who was 4 months pregnant had an abortion 6 hours earlier to hospital admission. She was comatosed. Her admission laboratory findings were: bilirubin 10.5 mg/dl, AST 208, and ALT 180 she had mild degree anaemia and her serum sample was anti-HEV IgG positive and anti-HEV IgM negative by EIA. She recovered without further complication.

Serological Markers of Hepatitis Viruses

Table (1) shows the serological markers basis for diagnosis. Acute hepatitis B was diagnosed in 16% of cases, acute hepatitis A in 11% of cases, hepatitis C in 31% of cases, IgG anti hepatitis E in 33% of cases, and IgM anti HEV in 8/52 (55%) none A, B, C, E in 15% of cases. While co-

infection with more than one hepatitis virus was detected in 9% of cases and HBV co-infection with HDV in 8%. There was HBsAg alone in 3% was possible chronic carrier state or early HBV infection.

For comparison, a serological marker of non-ceitric non-febrile HBV infection possible chronic carriage of HBsAg was detected in 1/50 (2%). Anti-HCV igG was detected in 3/50 (6%) of the sera from control subjects (Table 2) which represents non-symptomating chronic HCV infection in the population.

Co-infection with Hepatitis Viruses

In this study 39 out of 435 serum samples were positive for markers of more than one hepatitis virus, the most frequent of these co-infections was 20/39 (51.3%) IgG anti-HCV and IgG anti-HEV (Table 3):

Age Related Frequency of Hepatitis Virus Infection

Our results (Table 4) show that acute hepatitis B virus affected mainly young adults [21-30 years 24/70 (34%)] while hepatitis A virus affected mainly children [0-10 year, 25/48 (52 %)]. But an acute HAV infection at 11-30 years of age [23/48(47.9%)] reflects a change of herd immunity to HAV. Anti-HCV IgG positive patients were spread over the ages with a maximum mainly in age group (41-50 years) 66 / 135 (48 %) suggesting existence of a risk factor(s) for HCV infection 40-60 years ago.

HEV infected young adults [(21-30 years). 54/144 (37.5%)] more than other groups, yet HEV infection is spread over age groups up to 61-70 years. This may be explained by re-infections due to short-lived immunity to a single serotype or re-infection with serogroups that do not share cross-protection (Table 4).

Hepatitis E virus isolation in HepG₂ cell cultures

HEV was isolated in HepG₂ cell culture from 15/46 (32.6%) p.bl.ly. and

from 15/43 (34.9%) stools of anti-HEV IgG and IgM positive patients. (Fig. 1, and Fig. 2a, 2b). HEV was isolated from 10/39 (25.6%) p.bl.ly. and from 8/36 (22.2%) stools of anti-HEV IgG positive patients. HEV was isolated from 5/7 (71.4%) p.bl.ly. and from 7/7 (100%) stools of anti-HEV IgM positive patients. Thus, virus isolation in HepG₂ is an efficient diagnostic test.

Hepatitis E virus isolation in Vero cell cultures

HEV was isolated from 4/8 (50%) p.bl.ly. samples but not from stools samples though the patients were anti-HEV IgM positive (Fig. 3a, 3b).

Comparison of diagnostic tests for HEV infection

Fig (4) shows that PCR test detected HEV-RNA more in plasma samples than in stools, or in p.bl.ly. in that order Using PCR HEV RNA was detected in 5/8 (62.5%) plasma samples, in 3/8 (37.5%) p.bl.ly. samples and in 6/7 (85.7%) stool samples (patients were anti-HEV IgM positive). Thus when the patient is IgM anti HEV positive the best diagnostic test is HEV isolation in HepG₂ cell cultures using stools and p.bl.ly. PCR ranked second to virus isolation in HepG₂ in this situation. When the patient was IgM plus IgG anti HEV positive the chances of HEV isolation is HepG₂ is equal whether PBL or stools is used while PCR does not offer an advantage in HEV diagnosis. Using cell culture HEV was isolated from 15/46 (32.6%) p.bl.ly. samples and from 15/43 (34.9%) stools samples cultured in HepG₂ cell line (all patients were anti-HEV IgG positive), and from 5/7 (71.4%) p.bl.ly. and from 7/7 (100%) stools when patients were anti HEV IgM positive. Using VERO cell line HEV was isolated from 4/8 (50%) p.bl.ly. samples and could not be isolated from any stool samples (all patients were anti-HEV IgM positive). Using EIA anti-HEV IgM was detected in 8/52 (15.4%) serum samples positive for anti-HEV IgG.

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Table (1): Acute Clinical Hepatitis: Serological Markers of Hepatitis Viruses

Hepatitis virus	Positive	Negative	Total	Percent
HBsAg and anti-HBc IgM	70	365	435	16%
Anti-HDV-Ag +HBsAg	7	76	83	8%
HBsAg	13	422	435	3%
Anti-HAV IgM	48	387	435	11%
Anti-HCV IgG	135	300	435	31%
Anti-HEV IgG	144	291	435	33%
Anti-HEV IgM	8	44	52	15.4%
Non A-E	66	--	435	15%
Multiple markers of hepatitis viruses	39	396	435	9%

Table (2): Control group: Markers of hepatitis viruses

[Hepatitis virus	Positive	Negative	Total	Percent
HBsAg	1	49	50	2%
Anti-HBc IgG	0	50	50	0%
Anti-HAV IgM	0	50	50	0%
Anti-HCV IgG	3	47	50	6%
Anti-HAV IgM	0	50	50	0%

Table (3): Markers Of Hepatitis Viruses Co-Infection in Egyptian Hepatitis Patients

Markers of co-infection with hepatitis viruses in 39 out of 435 patients	Sera	Percent
Anti-HCV and anti-HEV IgG	20	51.3%
HBs Ag and anti-HEV IgG	5	12.8%
HBs Ag, anti-HCV IgG and anti-HEV IgG	2	5.1%
HBs Ag, anti-HBc IgG and anti-HEV IgG	1	2.6%
Anti-HBc IgM and anti-HEV IgG	1	2.6%
Anti-HAV IgM and anti-HEV IgG	2	5.1%
HBsAg and anti-HBc IgM and anti-HCV IgG	1	2.6%
Anti-HBc IgM and anti-HCV IgG	1	2.6%

Table (4): Distribution of Hepatitis Viruses Markers in Relation to Hepatitis Patient Age

Positive hepatitis marker	0:10Y	11: 20Y	21:30Y	31: 40Y	41: 50Y	51:60Y	61:70Y	Total
HBsAg and anti-HBc IgM	5	16	24	11	9	3	2	70
HB _s Ag	1	3	4	3	1	1	--	13
Anti-HAV IgM	25	15	8	--	--	--	--	48
Anti-HCV IgG	--	2	16	25	66	21	5	135
Anti-HEV IgG	3	33	54	18	18	12	6	144

Fig. (1) HEV Isolation from Stools and Peripheral Blood Lymphocytes in HepG₂ Cell Culture: Correlation with Anti-HEV IgG and/or IgM Reactive Sera.

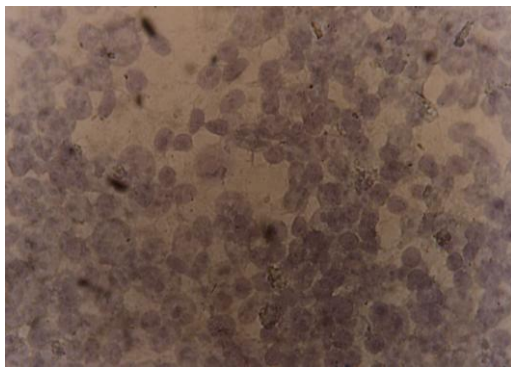
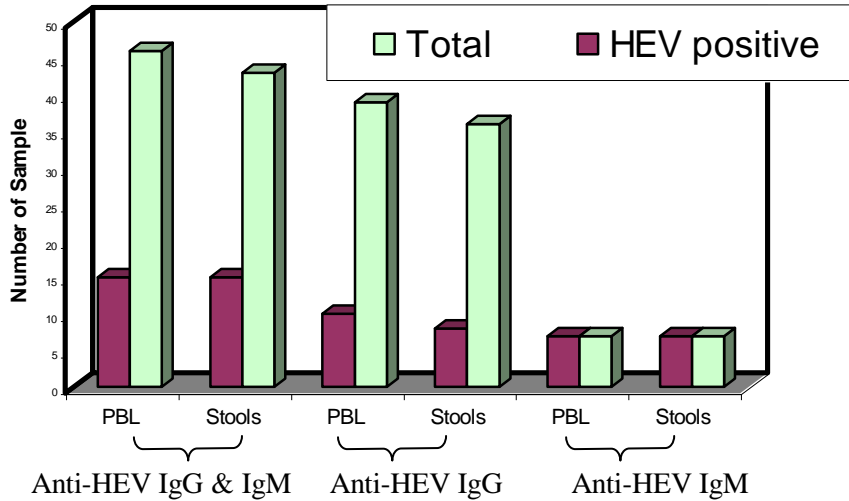


Fig. (2a): HepG₂ cell culture negative for HEV antigen by indirect immunoper-oxidase. (40 x 10).

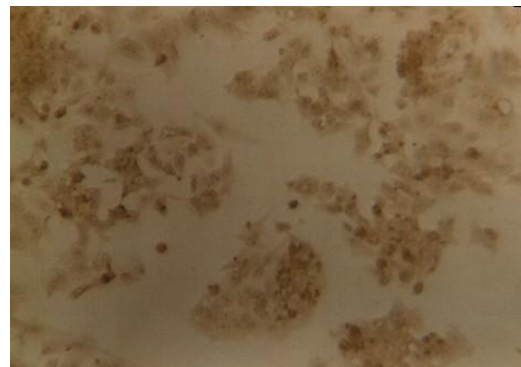


Fig. (2b): HepG₂ cell culture stained by indirect immunoperoxidase. The dark brown positive reaction for HEV antigen is located both in cytoplasm and in nuclei (25 x 10).



Fig. (3a): Control VERO cell culture negative for HEV antigen stained by

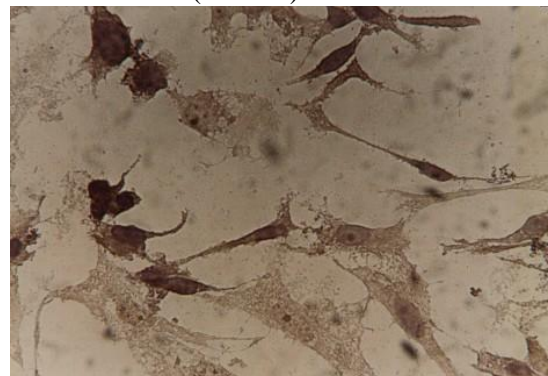


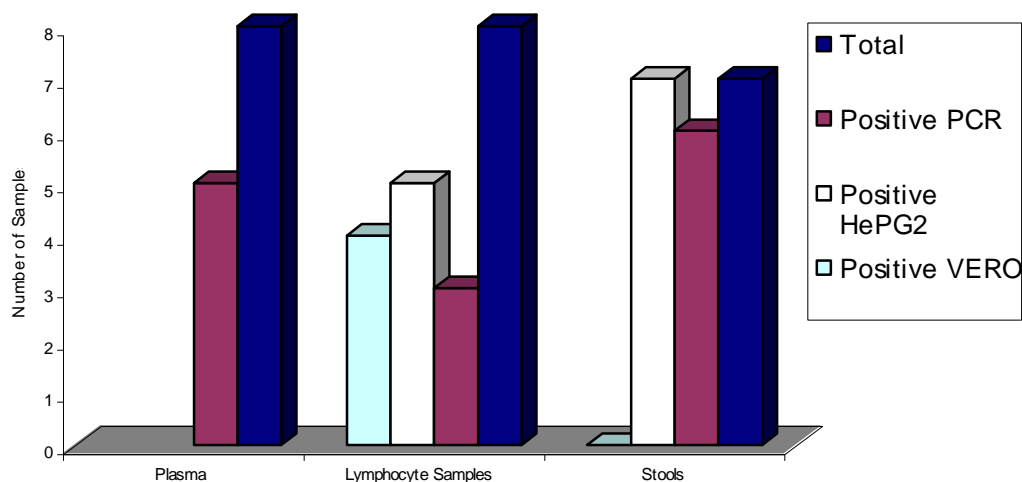
Fig. (3b): VERO cell culture showing dark brown positive reaction of HEV

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indirect immunoperoxidase staining
(25 x 10).

antigen located in both cytoplasm and
nuclei (40 x 10).

Fig. (4): Clinical hepatitis: Diagnostic Profile of anti-HEV IgM Positive Patients.



Discussion

In the present study anti-HEV IgG was detected in 144 out of 435 (33%) of sporadic hospitalized hepatitis patients. These results reinforce previous studies, which indicated that HEV is a common cause of acute sporadic childhood hepatitis in Egypt. [Goldsmith *et al.* (1992), El-Zimaity *et al.* (1993), Kamel *et al.* (1994), Gomatos *et al.* (1996) Abdel Wahab *et al.* (1996), and Divizia *et al.* (1999)] as well as adulthood hepatitis (Abdel Wahab *et al.* (1996) and Abo El kair *et al.* 2004 (under publication).

Because the appearance of IgM anti-HEV immune response is not predictable EIA was used for detection of anti-HEV IgM in the sera of (52) anti-HEV IgG positive cases. Only 8/52 (15.4%) were found to be positive for anti-HEV IgM (Table 1) which were considered as acute HEV infection. Stools and lymphocytes of these (8) cases were inoculated in HepG₂ and in VERO cell lines for HEV isolation and for detection of HEV antigens by immunoperoxidase staining.

The question whether IgM antibody heralds clearance of HEV viraemia was raised. Therefore, sera, Lymphocytes and

stools of these (8) cases were examined by PCR test to detect the presence of HEV RNA. HEV RNA was detected in 5/8 (62.5%) of sera, in 3/8 (37.5%) of lymphocytes and in 6/7 (85.74%) of stools (Fig. 3 & 4). Although the number of samples tested is small, yet, it is clear that there is ongoing viraemia and virus excretion in stools. This is in agreement of other studies which detected HEV RNA in many HEV hepatitis acute phase stools and sera by PCR (Ray *et al.*, 1991), McCaustland *et al.* (1991), Turkoglu *et al.* (1996), Divizia *et al.* (1999), Bussion *et al.* (2000) and Aggarwall *et al.* (2000)). Our finding of low recovery of HEV-RNA from lymphocytes (Fig. 4) distinguishes it from other enteroviruses.

An overall investigation for serological markers of other hepatotropic viruses (HBV, HCV and HAV) infections by EIA technique revealed HBsAg in 5/52 (9.6%) and anti-HBc (IgM) in (1/52) (1.9%) of anti-HEV IgG positive cases which may indicate acute HBV infection on top of past infection of HEV. Likewise, anti-HCV IgG was detected in 14/52 (26.9%) of these anti-HEV IgG positive

samples. These two results are indicators of the frequent exposure to hepatotropic viruses in Egypt. Meanwhile, there were 33/52 (63.5%) sera positive for anti-HEV IgG alone. This was considered pure HEV infection. Detection of multiple re-activities to hepatitis viruses markers in a serum indicated that among Egyptian patients suffering from acute hepatitis there may be an ongoing one or more hepatotropic virus activity (Table 3). But there is a possibility of an induction of polyclonal anti-body reactivity related to HCV chronic infection. There were many trials to culture HEV (Kazachkov *et al.* (1992) Meng *et al.* (1996) Jameel *et al.* (1996) Dzagurov *et al.* (1997) Divizia *et al.* (1999) Huang *et al.* (1999) and Panda *et al.* (2000). In the current study inoculation of stools and of p.b.l.y. in HepG₂ provided evidence that HEV replicated in these experimentally infected cells. Viral antigens were detected by an immunoperoxidase staining in 15/43 (34.9 %) HepG₂ inoculated with stools samples and in 15/46 (32.6%) HepG₂ inoculated with lymphocytes from anti-HEV IgG positive patients. Previously Jameel *et al.* (1996) and Panada *et al.* (2000) results indicated that HepG₂ supported HEV replication. As shown in our study: 5/7 (71.4%) of HEV isolations were detected by immunostaining of HepG₂ cells inoculated with lymphocytes, and 3/8 (37.5%) of these lymphocytes were positive for HEV RNA by PCR. So when lymphocytes of anti-HEV IgM positive cases were used as a sample of choice, HEV inoculation in HepG₂ cells gave a higher percentage of positive cases than HEV RNA detection by PCR. Vero cell cultures were less efficient than HepG₂ for HEV propagation in our experience. Further work to improve Vero cell receptivity for HEV is needed.

The use of stools for HEV diagnosis in our experience was rewarding. Virus isolation by inoculation of HepG₂ cells diagnosed 7/7 (100 %), while HEV RNA was detected in 6/7 (85.7%) using PCR. Failure to detect HEV-RNA in 1/7 stools sample may be related to a low virus load. Putting in consideration that by the time hepatitis patients in Egypt are hospitalized

they are in the early convalescence phase. Therefore some re-considerations of the markers of virus diagnosis should be involved.

Our results suggest that stools are the sample of choice for the diagnosis of acute HEV infection. The most sensitive diagnostic test was HEV isolation in HepG₂ cell line followed by immunoperoxidase staining of cytoplasmic HEV antigens and by HEV RNA detection by PCR. We would like to stress that HEV replication in HepG₂ cells enhances viral antigen and or RNA concentration with improved viral diagnosis. Serological diagnosis alone did not document HEV etiology because while IgG anti HEV was detected in 44/435 (33%) there were markers of HBV or HAV or HCV viruses. After etiologic shuffling there was only 33/435 (7.58%) single IgG anti HEV marker and 8/435 (1.84%) IgM anti HEV single marker. In our hands screening for IgG anti-HEV should be combined with HepG₂ cell culture for virus isolation from stools. For fast diagnosis, though not highly sensitive PCR of stools is rewarding though costly to be done as routine.

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العدوى بفيروس التهاب الكبدى الوبائى الحاد أى فى مصر *فاتن عثمان ، **ايمان ابو شادى ، **كوكا سعد الدين ، **زينب الرشيدى * مستشفى حميات العباسية - وزارة الصحة ، ** قسم الميكروبيولوجى - كلية الطب - جامعة الازهر (بنات)

يعتبر فيروس التهاب الكبدى الوبائى (أى) هو المسئول عن العديد من الاوبئة التى انتشرت فى كثير من البلاد النامية والطريقة الأساسية لانتقال العدوى بهذا الفيروس عن طريق الفم ويظهر فى شكل وبائى أو حالات فردية وترتفع نسبة الوفيات فى السيدات الحوامل المصابات بهذا المرض. والغرض من هذه الدراسة هو تقييم الطرق المختلفة لتشخيص هذا المرض واختيار أنسبها للكشف عن التهاب الكبدى الفيروسى (أى)

وقد تم فحص خمسين عينة من الأشخاص الأصحاء كمجموعة مقارنة و 435 مريض بالتهاب الكبدى الوبائى الحاد من مستشفى حميات العباسية وهذا الفحص يشمل :-
تحديد وظائف الكبد

الكشف عن المثبر لفيروس التهاب الكبدى الوبائى (ب) والجلوبلينات المناعية أم للفيروس الكبدى (ب) والفيروس الكبدى (د) والفيروس الكبدى (أ) والجلوبلينات المناعية ج للفيروس الكبدى (سى) والجلوبلينات المناعية أم و ج لفيروس التهاب الكبدى الوبائى (أى) بطريقة التقييم المناعى الانزيمى .
وقد تم كذلك فصل الفيروس (أى) من خلايا الليمفوسيت و عينات البراز للمرضى الايجابيين لجلوبلينات التهاب الكبدى الوبائى (أى) ج فى خلايا كبدية حية وخلايا كلوية حية. وقد تم فحص الخلايا بطريقة صبغة الانزيم المناعى لمعرفة وجود المثبر للفيروس (أى)
وتم فحص عينات المصل وخلايا الليمفوسيت و عينات البراز لوجود الحمض النووى للفيروس بطريقة التفاعل الانزيمى المتسلسل

وقد وجد فى هذه الدراسة أن الجلوبلينات المناعية (ج) المضادة للفيروس (أى) كانت ايجابية فى 435 / 144 بنسبة (33%) وأن الجلوبلينات المناعية (أم) المضادة للفيروس أى كانت (15.4%) فى 52 مريض مختار من ضمن 144 مريض الايجابيين للجلوبلينات (ج)
وتم فصل الفيروس أى فى خلايا الكبد الحية من خلايا الليمفوسيت بنسبة 32.6% ومن عينات البراز بنسبة 34.9% بالنسبة للمرضى الايجابيين لجلوبلينات الفيروس (أى) ج بينما تم فصله من المرضى الايجابيين لجلوبلينات الفيروس (أى) أم من خلايا الليمفوسيت بنسبة 71.4% ومن عينات البراز بنسبة 100% .
أما باستخدام الخلايا الكلوية الحية فقد كانت النسبة فى خلايا الليمفوسيت 50% ولم نستطع فصله من عينات البراز

وقد كانت نسبة تواجدها الحامض النووى للفيروس (أى) فى عينات البراز 85.7% وفى عينات المصل 62.5% وفى خلايا الليمفوسيت 37.5% وذلك بالنسبة للمرضى الايجابيين لجلوبلينات الفيروس (أى) أم
وبتحليل هذه الاختبارات التشخيصية وجد أن فصل الفيروس من خلايا الليمفوسيت و عينات البراز بحقنها فى خلايا كبدية حية أكثر حساسية من الكشف عن وجود الحمض النووى للفيروس بطريقة التفاعل الانزيمى المتسلسل.

وأن باستخدام طريقة التقييم المناعى الانزيمى يمكن إعطاء نتائج سريعة ودقيقة لحالات التهاب الكبدى الوبائى الفيروسى (أى) ويمكن فحص عدد كبير من العينات فى وقت قصير.