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Seroprevalence of anti-HEV(IgG) in apparently healthy adults in Yangon

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In Myanmar, hepatitis A, B, C and E are endemic, with the disease occurring subclinically or clinically in sporadic or epidemic forms. Hepatitis E is a small, non-enveloped RNA virus that is leading cause of non-enterically-transmitted viral hepatitis worldwide. The prevalence of antibody to HEV in suspected or documented endemic region has been lower than expected (3% to 26%) and this antibody in nonendemic region has been much higher than anticipated (1% to 3%). The aim of this study was to evaluate the anti-HEV IgG seropositivity in adult study population in Yangon. A total of 84 serum samples of adults (15-60 years) comprising of 43 males and 41 females were tested for anti-HEV IgG using the recombinant baculovirus expressed Viral Like Protein (VLP) of HEV antigen ORF2 by the ELISA (Enzyme Linked Immunosorbent Assay) test. Twenty-four subjects (28.57%) comprising of 16 males and 8 females were found to be positive for anti-HEV IgG. The prevalence of HEV was variable according to age group. The highest prevalence was between 20-30 years and 30-40 years age groups. Anti-HEV positivity rate was 37.2% in male and 19.52% in female subjects. In other studies, the prevalence of anti-HEV remained constant at 10-40% in young adults with no difference between males and females. The presence of anti-HEV IgG is the evidence of past infection. The use of baculovirus expressed ORF2 apparently had a sensitive and specific rate of detection to anti-HEV. Recombinant protein test detected more than 90% of the undiluted known positive sera. Anti-HEV was associated with partial protection from hepatitis E. Anti-HEV has been found to persist as little as few months to 14 years. Recombinant protein assays can detect anti-HEV in human serum obtained 13 years after infection.

INTRODUCTION

In Myanmar, hepatitis A, B, C and E are endemic, with the disease occurring subclinically or clinically in sporadic or epidemic forms. Hepatitis E is a small, non-enveloped RNA virus that is leading cause of non-enterically-transmitted viral hepatitis worldwide [1]. It is transmitted mainly by the faecal-oral route and large epidemics due to this virus are often associated with contaminated water [2]. It is a positive strand RNA virus (7.5 Kb) and grouped with Caliciviridae, a family of small round-structure virus. There are two strains of hepatitis E known as old world isolate (Myanmar) and new world isolate (Mexico). These strains are 90.5% identical in genetic sequence [3]. All HEV strains have same serotype [4]. It was first recognized in 1980 and 32-34 nm viral like particles were visualized by immunoelectron microscopy

(IEM) in 1983 [5]. Its genome consists of 3 Open Reading Frames (ORFs) such as ORF1-5079 nucleo-tides (nonstructural protein), ORF2-1980 nucleotides (structural protein) and ORF3-369 nucleotides (highly immunogenic protein of unknown function). Antigens derived from ORF2 and expressed in *Escherichia coli* or insect cells have also been used for detection of anti-HEV by Western blot and the entire ORF2 has been expressed in insect cells and a part of the expressed antigen spontaneously forms virus like particles used in ELISA test for anti-HEV. Recombinant protein-based tests have higher sensitivity than the prior assays, detecting 90-95% of anti-HEV in acute hepatitis cases in endemic area [6]. The common age group was highest in young adults (15-40 years) and attack rates were higher for males than for females [4]. The rising titre of anti-HEV IgG is also diagnostic and exists for a long time. Anti-HEV IgG peak starts between 2-4 weeks after onset of hepatitis and 100% of young adults are still positive 20 months after onset of hepatitis and some adults are still positive for 14 years after this infection. The prevalence of anti-HEV in suspected or documented endemic region has been lower than expected 3% - 26%. This antibody in non-endemic region has been much higher than anticipated 1%-3% [4]. In Myanmar, epidemic HEV infection occurred in Mandalay (1976-1977), Moulmein (1978) and Yangon (1982) which was spread by the fecal-oral route and with sporadic cases occurring all over Myanmar [7]. Hepatitis E infection is commonly transmitted from infected mothers to their babies with significant perinatal morbidity and mortality. All infants had anti-HEV IgG in their birth serum samples and five infants had HEV RNA in their serum at birth by polymerase chain reaction [8].

The objective of the present study is to determine the anti-HEV IgG positivity in apparently healthy adults aged 15-60 years who are living in Yangon and to evaluate the seroprevalence of anti-HEV IgG in different age groups and gender.

MATERIALS AND METHODS

Anti-HEV IgG was detected in human serum samples by Enzyme Linked Immunosorbent Assay (ELISA). Hepatitis E viral like particles (HEV-VLP) the structural protein, with its terminus truncated (amino acid residues 112 to 660 of ORF2) was expressed in insect Tn5 cells by a baculovirus. The efficient expression of N-terminally truncated HEV capsid protein was derived from a Myanmar strain isolated in 1986 [9].

Construction of recombinant transfer vectors

In 1986, acute-phase stool specimens from patients with Non A, Non B hepatitis in Myanmar were pooled and injected into Rhesus monkeys (*Macaca mulatta*) via the intravenous route. Bile which contained large number of HEV virions were collected from these monkeys and the total RNA was extracted by using the RNA zol (Biotex Laboratories) which contained large numbers of HEV virions. The poly (A)-containing RNA was purified with Oligotex–dT 30 (Super-Roche Diagnostic System Tokyo, Japan).

Ampilification of the entire ORF2 by polymerase chain reaction (PCR) was carried out with specific primers HEV D2 (5′-CAACAGAAAGAAGGGGGGCACAA-3′) and HEV U2 (5′-CAACAGAAAGAAG-GG GGGCAC AA-3′). The product of PCR were cloned into a TA cloning plasmid vector PCR II (Invitrogen) to generate pHEV 5134/7161 which was digested with restriction enzymes NruI & XbaI and resultant 2-kb fragment was ligated with a transfer vector, pVL1393 (Pharmingen) which was digested with Sma I & Xbal to produce plasmid vector pVL 5147/7126. DNA fragments with deletion of the N-terminal 111 amino acid encoded by ORF2 were amplified by PCR with HEV D13 (5′-AAGGATCCATGGCGGTCGCTCCAGCC CATGACACCCCGCCAGT-3′) and HEV U14(5′-GGTCTAGACTATAACTCCCGA-GTTTTACCCACCTTCATCTT-3′) primers

with cDNA as a template. The fragments flanked by restriction enzymes site Bam HII and XbaI were ligated into transfer vector pVL 1393 to yield plasmid vector pVL 5480/7126 (Fig.1). Tn5 cells were infected with recombinant baculoviruses at multiplicity of infection (MOI) of 10 and incubated in Ex-cell 405 media (JRH Biosciences, Lenexa, Kans) for 5-7 days at 26.5°C until an extensive cytopathic effect appeared.

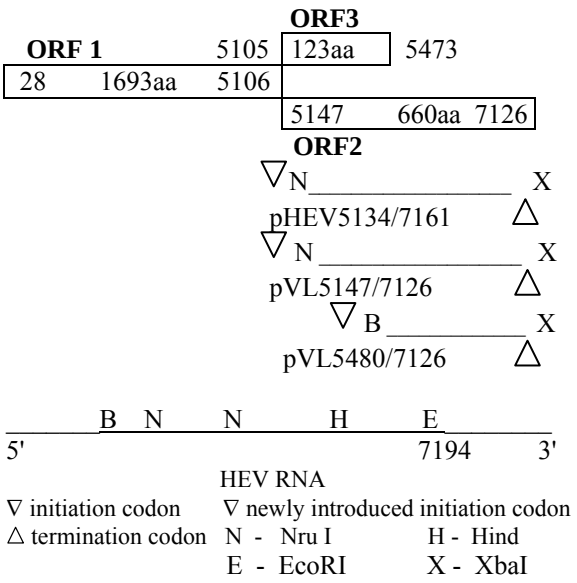

Fig. 1. Genome organization of HEV and schematic diagram of two baculovirus transfer vectors

Purification of Viral Like Proteins (VLPs) of HEV antigen

The culture medium was harvested and centrifuged at 1000 g for 15 minutes and further centrifuged at 10,000 g for 30 mins to remove progency baculoviruses. The VLPs in the supernatant were concentrated by 8% polyethylene glycol 6000 in the presence of 0.4M NaCl at 0ºC overnight and followed by centrifugation with 10,000 g for 30 min. The VLPs were resuspended in phosphate buffer saline (PBS) pH 7.5, layered onto 1ml each of 10%, 20% sucrose solution and centrifuged at 100,000g for 2hr at 4ºC. A white band was collected between two layers of sucrose and diluted with 10mM PBS, pH 7.2 and recovered by centrifugation at 100,000g for 2hr. The yield of the purified VLPs was amounted to 1 mg/10^7 of Tn5 cells. Further purification was done by $CSCL_2$ equilibrium density gradient centrifugation. These VLPs, 23.7 nm, small round empty, viron-like particles were observed under electron microscope (EM) and used as coating antigens for antibody of HEV-ELISA assay test for antibody (Fig. 2). The protein concentration was measured with a protein assay kit (Bio-Rad Laborities, Richmond, Calif.) using Bovine Serum Albumin (BSA) as standard. The VLPs (50KDa) protein was determined by sodium dodesyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) method. The purified VLPs protein (1µg/ml) was used to detect anti-HEV IgG and IgM by Enzyme Linked Immunosorbent Assay (ELISA) method. A total of 84 serum samples of apparently healthy adults with 15-60 years age group, comprising of 43 males and 41 females, were tested for anti-HEV IgG using the recombinant baculovirus expressed VLP (Viral Like Protein) of HEV antigen ORF2 by ELISA test.

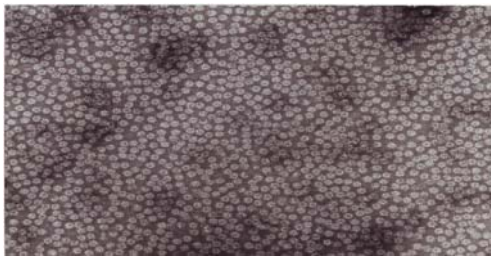

Fig. 2. Purified Viral Like Proteins (VLPs) of hepatitis E virus antigen as observed by electron microscope

Detection of anti-HEV IgG by ELISA test

Ten microliter of recombinant baculovirus expressed VLP of HEV ORF2 antigen was mixed with 10 ml of Coating Buffer (Carbonate-Bicarbonate Buffer, Sigma, pH 9.6). Each well of the polystyrene plate (Immulon 2, Dynatech) was coated with 100µl (1µg/ml) of HEV ORF2 antigen and incubated at 4ºC overnight. Plate was blocked with 5% skim milk with phosphate buffer saline and tween 20 (PBS-T) and incubated in 37ºC incubator for two hours. One-hundred microlitre of each serum sample (1:100 dilution of 1% skim milk with PBS-T) were placed into a well of ELISA plate and incubated at 37ºC for one hour. Three known positive control serum

and distilled water (negative control) were used as control. After washing the plate with PBS-T for 3 times, 2µl of horseradish peroxidase (HRP) conjugated goat anti-human IgG (1:5000 dilution in 1% skim milk with PBS-T) was added into each well and incubated at 37ºC for one hour. After washing the plate with PBS-T for 6 times, 100 µl of substrate (5 mg of OPD, 12.5 ml of phosphate citrate buffer, 5µl of hydrogen peroxide) was added into each well and incubated at room temperature for 30 min and then 50 µl of 4N H_2SO_4 was added for stopping the reaction. The optical density (O.D) was read by ELISA reader at 492 nm wave length. The cut-off value was determined as 0.5 and positive control showed two times higher than the cut-off value (>1.0) and negative control showed lower than the cut-off (0.5).

RESULTS

Twenty-four subjects (28.57%) comprising of 16 males and 8 females were found to be positive for anti-HEV IgG. The highest prevalence was between 30-40 years age group of male healthy subjects. In female subjects, 50-60 years age group had the highest prevalence of anti-HEV. The highest prevalence of age group was 20-29 years in other studies. In this study, the anti-HEV IgG positivity rate was 37.2% in males and 19.52% in females in normal healthy subject. Anti-HEV IgG was not detected in 60 cases. Anti-HEV IgG was absent in 50-60 years age group among males and under 20 years and 40-50 years groups among females in this study (Table 1).

Table 1. Anti-HEV IgG positive cases in apparently healthy adults in Yangon

Age groups (yrs)	Male			Female		
	Total no. of cases	No. of positive cases	%	Total no. of cases	No. of positive cases	%
<20	11	6	54.54	2	0	0
20-30	21	5	23.80	23	4	17.39
30-40	7	4	57.14	12	3	25.00
40-50	3	1	33.33	1	0	0
50-60	1	0	0	3	1	33.33
	43	16	37.20	41	8	19.52

Table 2. Anti-HEV IgG negative cases in apparently healthy adults in Yangon

Age groups (yrs)	Male			Female		
	Total no. of cases	No. of negative cases	%	Total no. of cases	No. of negative cases	%
<20	11	5	45.45	2	2	100
20-30	21	16	76.19	23	19	82.6
30-40	7	3	42.85	12	9	75
40-50	3	2	66.66	1	1	100
50-60	1	1	100.00	3	2	66.66
	43	27	62.79	41	33	80.48

DISCUSSION

The prevalence of hepatitis E virus infection in general population differs widely in many countries (1.5% in UK, 1.8% in Netherlands, 7.4% in Chile and 16.5% in Hong Kong) and varied according to geographical regions of the world [10]. In endemic countries, anti-HEV IgG was detected in 20% of population [11]. In this study, the seroprevalence of anti-HEV was found to be 28.57% in all apparently healthy subjects although the sample size was not enough for statistical evaluation for age and gender preponderance. The prevalence in males was nearly twice as high as females. Findings from other studies showed the prevalence of anti-HEV has remained constant at 10-40% in young adults with no difference between males and females [2].

In this study, the highest prevalence of anti HEV was seen in 30-40 years age group and the prevalence was not different between male and female subjects. The use of baculovirus expressed ORF2 antigen apparently had a very sensitive and specific rate of detection to anti-HEV. IgG antibodies to ORF3 do not persist as long as antibodies to ORF2, because of different response of the immune system to the different HEV proteins [12]. Recombinant protein-based (Immunodominant epitopes of ORFs 2&3) test has demonstrated increased sensitivity compared to Western blot and Enzyme immunoassays (EIAs) and detecting anti-HEV(IgG) in 90-95% of HEV infective

cases in endemic region [6]. IgG response begins to develop shortly after the IgM response and its titres increased throughout the acute into convalescent phase, remaining high for 1-4.5 years after the acute phase of disease. The presence of anti-HEV IgG is evident of past infection of hepatitis E virus [13]. Recombinant protein assays could detect anti-HEV IgG in human serum obtained after 13 years of infection [6]. The prevalence of anti-HEV IgG was associated with partial protection from hepatitis E. The cleaved protein from ORF2 spontaneously forms Viral Like Protein (VLP) and appears to be more immunogenic than the full length of ORF2 product and it is also basis for a highly immunogenic candidate vaccine. Naturally acquired anti-HEV IgG correlates with the protection in humans in epidemics of hepatitis E infection [3].

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