Aus dem Zentrum der Hygiene
der Johann Wolfgang Goethe-Universität
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Institut für Medizinische Virologie
Direktor: Prof. Dr. Hans Wilhelm Doerr

Hepatitis E - Virus (HEV): Comparative evaluation of IgG antibody assays in a low-endemicity setting

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Dekan:	Prof. Dr. med. J. Pfeilschifter
Referent:	Prof. Dr. med. H. W. Doerr
Korreferent:	Prof. Dr. med. S. Zielen
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Now to God be the glory, for all the things he has done!

Blessed are those who dwell in your house, oh God: for they will ever praise You. Blessed is the man whose strength is you. (Psalm 84:4-5)

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1 Introduction and Background

1.1 Historical Notes

Hepatitis E (HEV), the causative agent of what has been referred to as enterically transmitted non-A, non-B hepatitis or ´waterborne hepatitis´, is a major cause of epidemic and acute sporadic hepatitis in developing countries (Khuroo et al., 1980; Bradley et al, 1990; Mushawar et al., 1997).The disease was first recognized as distinct, clinical entity in the 1980´s when the sera from persons affected during a large waterborne epidemic of viral hepatitis during 1956-56 in New Delhi, India and another epidemics in Kashmir were found to lack serological markers of acute hepatitis A and B .(Viswanathan et al., 1957;Khuroo et al., 1980). The occurrence of the first recorded epidemic of hepatitis E as late as 1955 and the frequency of this disease suggest that hepatitis E is a new, emerging infectious disease. The first proof of the existence of a new viral hepatitis agent was obtained in 1983, when virus-like particles were detected by immune electron microscopy in the faeces collected from a volunteer who was infected with faecal material from patients with suspected enterically transmitted non-A, non-Bhepatitis. (Balayan et al., 1983). Outbreaks of HEV infections have occurred in a number of countries in Africa, Asia as well as in Mexico, and are usually associated with warm weather and poor sanitation leading to faecal contamination of drinking water. Sporadic cases can also occur in areas where HEV is endemic, usually during periods between major outbreaks. Sporadic cases have been also reported in areas not considered to be endemic for HEV. Many of these can be associated with travellers returning from visits to areas where HEV is endemic. There have been reports, however, of HEV-associated hepatitis among individuals with no history of travel to regions endemic for HEV (Zaijer et al., 1993; Zannetti et al., 1994). It was generally believed that such occurrences are rare in developed nations.

The genome of the agent was originally identified and characterized using cynomoglus macaques infected experimentally with human faeces obtained from patients during an outbreak in Burma (Reyes et al.,1990). A second isolate was similarly identified from an outbreak that had occurred in Mexico. These first two prototype isolates of HEV were similar in overall genomic organization but still distinct from each other having 76% identity over the

entire genome comprised of three discontinuous open- reading frames (ORF´s) (Huang et al., 1992). In the years immediately after the identification of the Burmese and Mexican prototype viruses, a number of additional isolates were identified from Pakistan, India and China.. Interestingly, all of these isolates were related very closely to the original isolate from Burma having a greater than 93% nucleotide identity across the genomes. At that time, the genotypic distribution of HEV consisted of a group of Burmese-like and the lone Mexican isolate.

The first indication that additional genetic diversity may occur in regions non-endemic for HEV was the identification of a distinct endogenous variant identified in an acute hepatitis patient from United States (US) who reported no recent travel history to endemic regions (Schlauder et al., 1996; Kwo et al.,1997). Additional sequencing of the full-length genome of this and the isolate confirmed that a new group of HEV did exist that was distinct from the Burmese-like and Mexican isolates (Schlauder et al., 1998; Erker et al., 1999).

The discovery of the human US strains of HEV led to the identification of a number of additional isolates from other non-endemic regions using primers based on the sequence from the first human US isolate. Patients from Italy and Greece were identified who were infected with variants of HEV that were quite distinct from the original isolates from Burma and Mexico, as well as the those from US (Psichogiou et al., 1995; Schlauder et al., 1999; Zanetti et al., 1999). These variants were related most closely to the US isolates, however, they were not subtypes of the US group. In fact, they were found to represent three new groups of HEV.

Although there appeared to be a geographical distribution of genotypes up to this time (fig.1), the identification of the two distinct isolates from Greece indicated that there could be significant diversity between strains from the same region. Recently, isolates from patients from Argentina and Austria have been identified and are distinct from each other as well as all previously identified isolates (Worms et al., 1998; Schlauder et al., 2000). Most recently , two isolates from patients from Spain have been identified and were reported to be related most closely to one of the Greek isolates (Pina et al., 2000). Extensive diversity has also be reported between HEV strains from a number of patients from China and Taiwan that are distinct from the original Chinese isolates, which are closely related to the Burmese isolates (Wang et al. 2002; Norder et al., 2002).

The possibility of zoonotic infections from pigs to humans was initially indicated after the experimental infection of pigs with human strain of HEV (Balayan et al.,1990) The identification of antibodies to HEV in domestics farm animals in Thailand, and the identification of HEV RNA and HEV-like sequences in swine from China, New Zealand, Thailand, and the US, support this possibility (Clayson et al., 1996; Meng et al., 1997; Garkawenko et al., 2000; Haqshenas et al., 2002). In addition, HEV RNA very similar to the isolates identified in patients from Spain was also detected in swine slaughterhouse sewage (Pina et al., 2000). The observation that the Chinese and US isolates are almost closely related to the human viruses from those regions suggests that human and swine HEV may co-exist in several different geographical regions. These results were highly suggestive that HEV infection could be a zoonotic, although no direct evidence has yet been demonstrated (Haqshenas et al., 2002; Norder et al., 2002).

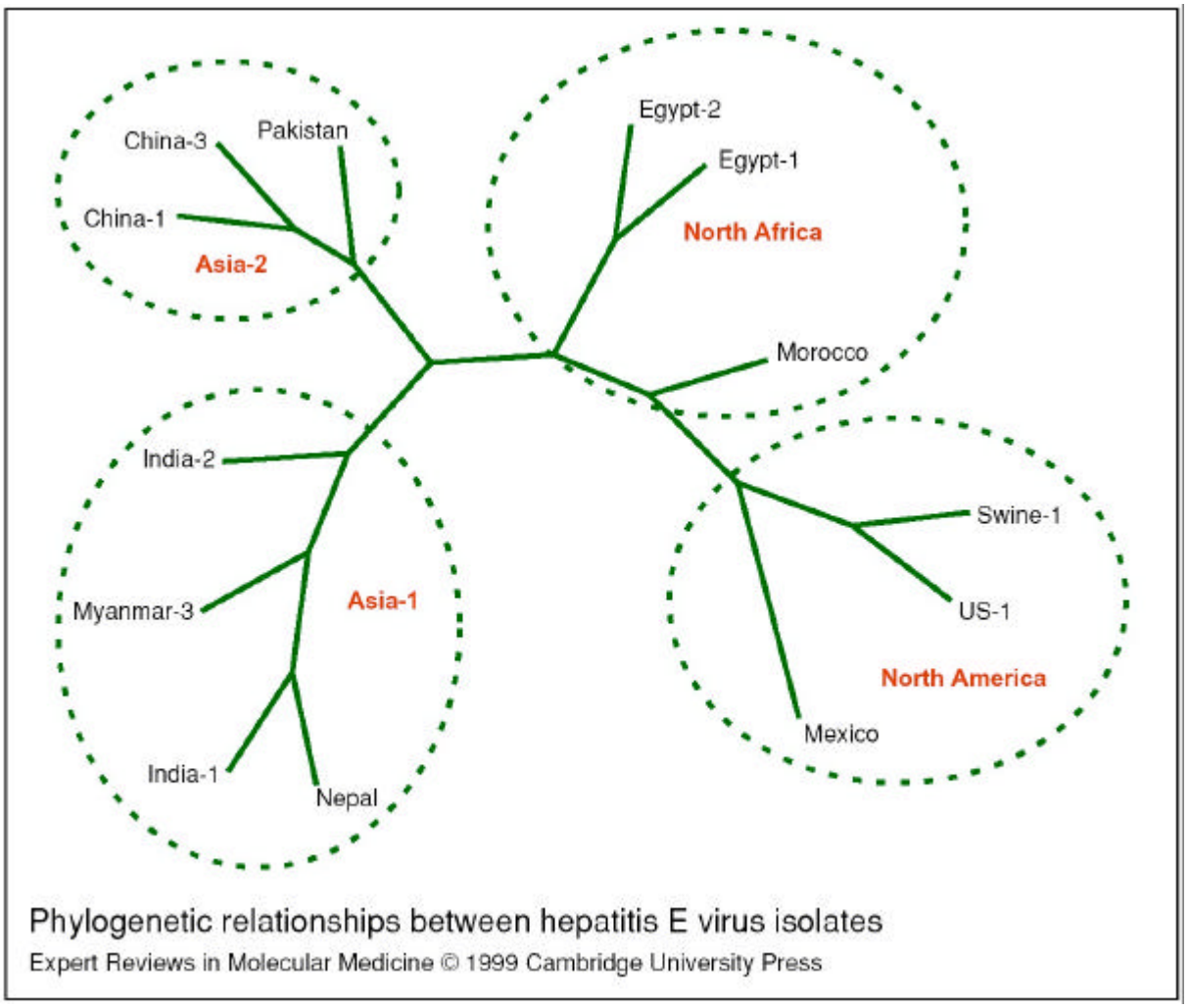


Figure 1. Phylogentic relationships between hepatitis E virus isolates Nucleotide sequences encoding the structural regions (Open Reading Frame ORF2 and ORF3 of selected hepatitis E virus (HEV) isolates from different geographic locales were subjected to phlylogenetic analysis by S. Jameel Expert Reviews in Molecular Medicine 1999; Cambridge University Press.

1.2 The Genome of the Hepatitis E Virus (HEV)

After successful transmission of Virus like particles (VLP´s) to non human primates and by using bile (Bradley et al., 1994) as source of VLP´s for cloning of the viral genome, the virus was finally characterized and named hepatitis E virus. The cloning of HEV was followed by the sequencing of its genome. Sequencing of four distinct isolates of HEV including those from Burma, Mexico, Pakistan and China (Yarbough et al., 1991) has already been completed. A partial HEV genome sequence has been identified in a strain from Kirgizstan and Uzbekistan (Yarbough et al., 1991).

Molecular analysis of the HEV genome has shown that it is a positive single-stranded RNA of a about 7.5kb in length with short 5´- and 3´-noncoding regions which contain three separate open reading frames (ORF´s) and very small variations among isolates (Purdy et al., 1993).

Minor sequence divergence was noted between Asian and Mexican isolates. The main features of the HEV genome are as follow;

The putative non structural genes at end of the genome.ORF1 is located at the 5´end and the structural genes are located at the 3´end of the genome (Fig 2). ORF1 is located at the 5´end (5kb), it begins 28 nt away from the 5´end, extends 5.079 bp , ends at the 5,107 nt and encodes for non structural proteins. The ORF2 is located at the 3´end, begins at 5,147 nt, extends 1,980 bp, terminates 65 bp upstream of the poly (A) tail and encodes for structural proteins. The ORF3, the smallest one, is located at the end of ORF1, encompasses 369 bp overlaps the first ORF1 at its 5´end by 1 nt, significantly overlaps ORF2 and encodes a protein of an unknown function(s).

The 5´untranslated region (28bp) possibly plays a role in transcription of the virus. Analysis of the nucleotide sequence of the Burmese isolate reveals the presence of consensus sequences in ORF1 (putative non structural gene) encoding;

a) An RNA-dependent RNA polymerase located at the extreme carboxy-terminal portion of the ORF1 polyprotein.

b) An RNA helicase (seen in all other geographically distinct isolates).

c) A methyl transferase, located at the amino-terminal of the polyproteins, suggesting that HEV is capped.

d) An Ý ´domain of unknown function.

e) A putative papain-like cystein protease domain.

f) An ´X´ domain of unknown function, acting as a flexible hinge (Bradley et al., 1991).

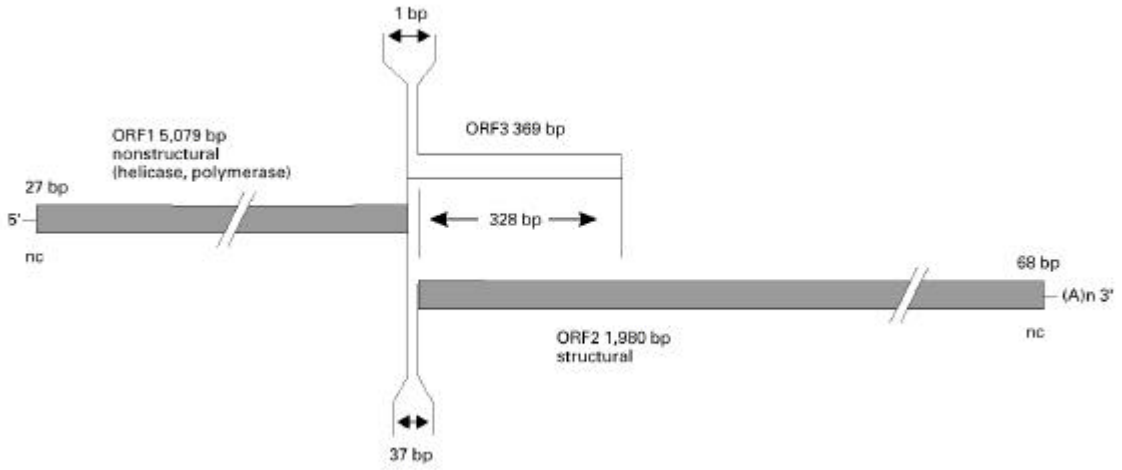


Figure 2: The genetic structure of HEV Virus (Yarbough et al, 1999)

ORF2 encodes major structural proteins comprising a glycosylated protein (660aa) used in transport, major immunological epitopes and a polypeptide with a high percentage of basic amino acids (Jamell et al., 1996). ORF3 encodes a protein (123aa) with an immunoreactive epitope at the carboxy –terminus and a transmembrane sequence at the amino-terminus.

HEV appears to be substantially different from picorna viruses including HAV (Bradley et al., 1991). Based on hybridisation experiments, IEM and immuneflourescence blocking studies, it was indicated that a single major agent is responsible of ET-NANBH cases worldwide (Bradley et al., 1991). A comparison of parts of the sequences of the ORF2 regions of HEV cDNA demonstrated that the nucleotide sequence homology is 95.7% among the four Chinese strains of HEV. The amino acid sequence identity ranged from 95.4 to 98.5% The homology of the nucleotide and the amino acid sequence of the ORF2 regions between the four Chinese HEV strains and the Burma strain (92.3 to 94.5% and 96.2 to 97.7%, respectively) was much higher than the Mexico strain (79.3 to 80.2% and 88.8 to 93.9%, respectively). Thus, all four Chinese strains showed a higher degree of nucleotide sequence homology compared to the Burma and Mexico strain, suggesting that HEV subtypes may be associated with different geographical location (Yarbough et al., 1999).

1.3 Genetic Heterogeniety

The genome sequence of HEV seems to relatively stable. The nucleotide identity of strains isolated in a single outbreak is high and serial passages in cynomolgus monkeys do not results in a genetic drift. Substantial genetic diversity one isolate in comparison to others from the same outbreak probably represents the sporadic cases of hepatitis E (Arankalle et al, 1999).

The genome of strains isolated from geographically distinct locations is generally more diverse. The full-length overall nucleic acid identity of two prototype strains Myanmar and Mexico. However, the deduced amino acid identity is 84%, 93% and 87% for ORF1, ORF 2 and ORF 3, respectively (Takahahi et al, 2003). Most of the nucleotide substations are conservative and only hypervariable sequences of ORF1 result in substantial aminoacids diversity. No evidence has been found that serological heterogeneity results from the genetic diversity, thus HEV seems to exist as a single serotype.

As present, no consensus exists on genotype classification of HEV and the same holds true for antigenic classification. Currently, the detected HEV strains are genetically characterized in laboratories on the basis of ORF1 and 2 as well as ORF 3 regions. In addition, some research workers started to antibodies produced by recombinant-expressed capsid proteins (Wang et al., 2001). Genetically distinct strains may turn out to have serologically surface-exposed epitopes. Strains isolated in endemic regions can be classified into genotype (strain from Asia and Africa). Genotype II would be the Mexican-type group. Besides strains from Mexico, this group from Nigeria (Schlauder et al, 2001). Strains detected in cases of acute HEV infection in the US, in parts of Europe (Italy, Greece, Spain) and Argentina show a relatively high degree of diversity (> 20%) to strains from endemic areas, and would genotype III strains. Genotype IV consist of recently detected Chinese isolates. Sequences of ORF 1 fragments indicates that Taiwanese isolates may be a part of this group (Shlauder et al, 2001).

In addition, novel strains have been isolated in pigs (Purcell et al, 2000). Some swine HEV isolated showed a close genetic relatedness between the genotype III humans strains and swine strains isolated in the same area (Hsieh et al, 1999). Both the human and the swine isolates , can cross species barriers. This was demonstrated by infecting primates, rats and swine with human HEV isolates and primates with human isolates (Takahashi et al 2003, Meng et al, 1998).

1.4 Cell Culture

The replication strategy of the HEV is poorly understood. Several strategies for experimental propagation and production of HEV to study the molecular biology and facilitate vaccine development have been published, but their reproducibility and feasibility need confirmation (Clemente-Casares et al, 2003).

A recent in vivo cell culture experiment used in vivo infected highly differentiated primate liver cells for the in vitro replication of HEV in a serum-free medium supplemented with growth factors and hormones. A strand-specific RT-PCR technique was used for monitoring.(Worm et al , 2002) Both positive strand and negative-strand HEV RNA were detected in cellular RNA of the culture cells and the positive-strand HEV RNA was detected in the culture medium (indicating shedding of virus-like particles into the culture medium).No cytopathic effects were observed. Thereafter, using the identical culture system, primary hepatocytes were infected with non-inactivated tissue culture-derived viruses and replication of REV RNA was demonstrated in this model. A neutralizing anti-HEV antibody directed against the ORF-2 encoded putative capsid protein blocked the infection of the liver cells (Yarbough et al, 1999).

New Chinese strains of HEV have been isolated and cultivated in an in vitro cell culture using continuous cell lines derived from the lung, kidney, or liver. Recently, a Chinese HEV isolate was successfully cultivated in a A549 cells (human lung carcinoma cells) under the conditions of a relatively high concentration of MgCl2 (30 mM), a pH of 7.2, and a short (< 6 months) preservation time of propagated strains. Cytopathic effects (cell rounding and mono layer destruction) were visible at day 2 post-inoculation and could be neutralized by specific acute-phase antibodies to HEV (Huang et al, 1999).

1.5 Stages of Replication

Because evidence for propagation of HEV in cell culture is quite limited(Huang et al . 1992), and because HEV is not closely related to any other well- characterized virus, little is known about its strategy for replication. The mechanism of attachment, entry, and uncoating of HEV is unknown but assumed that the virus attaches to receptor sites on the hepatocytes and possibly cells in the intestine. After uncoating, the 7,5-kb genome is probably translated via cellular mechanisms that recognize capped RNA. Cleavage of translated ORF-1 is likely achieved by cellular proteases. The motif of a papain-like protease has been detected in the sequence ORF-1 (Koonin et al., 1993), but it is not known whether it is functional. Replicative intermediate negative-stranded RNA is probably synthesized by the viral polymerase, as are subsequent strands of positive sense full-length (genomic and messenger) RNA, as well as at least two subgenomic and co-terminal messenger RNAs, 3,7 and 2,0-kb in size, respectively(Tam et al.,1991). Nothing is known about assembly and transport. The gene products of both ORF-2 and ORF-3 contain hydrophobic signal-like sequences at their 5 ´ends but it is not known whether they have a function (Reyes GR et al.,1991). Nothing is known about release of the virus from infected cells.

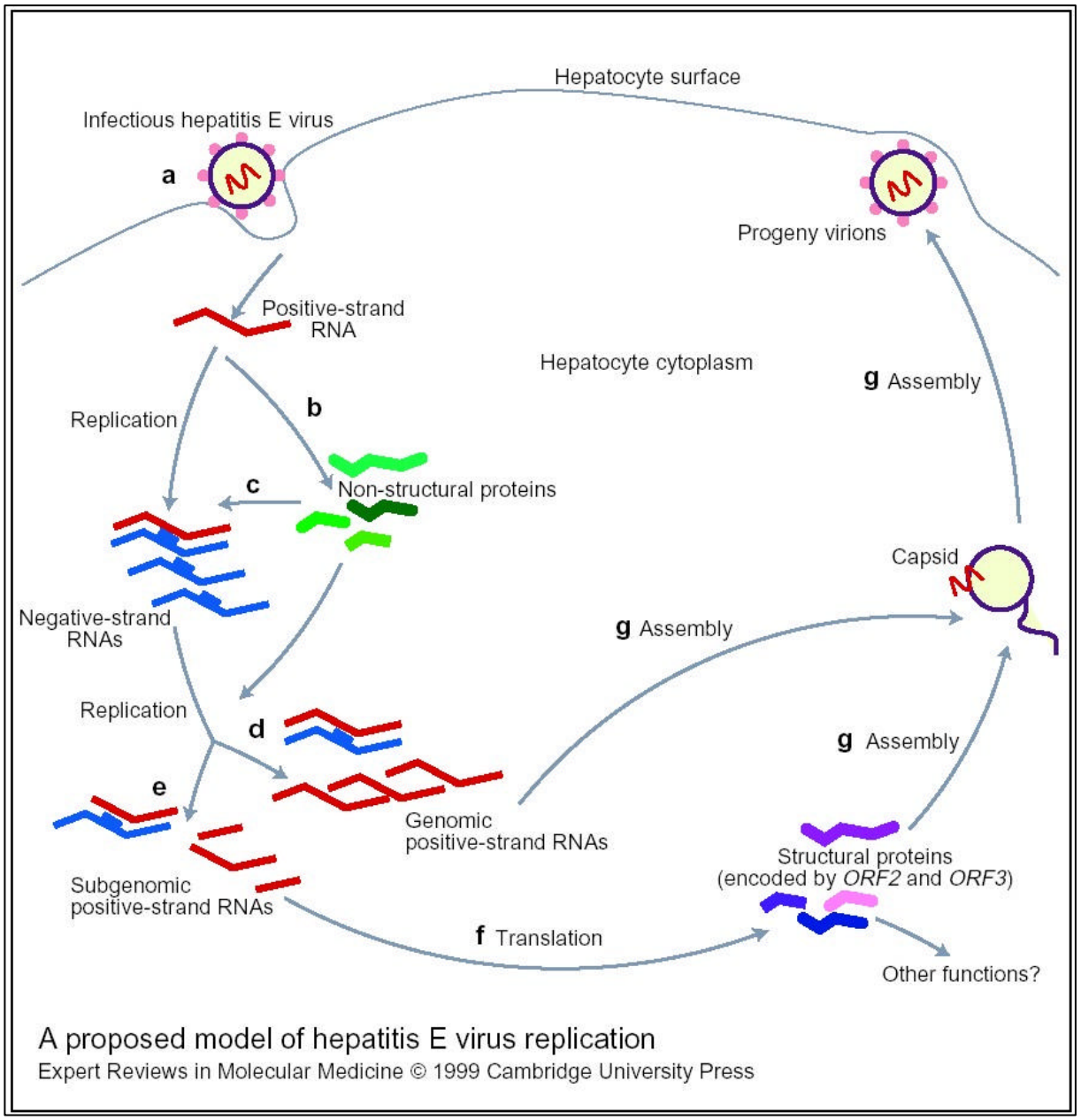


Figure 4. A proposed model of hepatitis E virus replication.

This model is based on domain homologies between hepatitis E virus (HEV) and other positive-stranded RNA viruses. (a) Following attachment to an as-yet-uncharacterised receptor on the surface of hepatocytes, HEV is internalised and uncoated in the cytoplasm by unknown mechanism. (b) The genomic positive-strand RNA is translated into nsP, the

non structural polyprotein encoded by ORF1, which can be processed into individual functional units that might include methyltransferase, protease, helicase and replicase activities. (c) The replicase so generated can use the positive-strand RNA as templates to synthesise the negative strand replicative intermediates. Because of homology to alphaviral junction sequence (shown as boxed region on the negative-strand RNA), it is proposed the two classes of positive-strand RNA species, genomic (d) and subgenomic(e), are synthesised from the negative-strand RNA intermediates. (f) The genomic RNAs are translated into pORF2 viral structural proteins encoded by ORF2 (and possibly pORF3 encoded by ORF3).

1.6 Pathogenesis and Pathology

Because serologic and molecular tests for HEV were developed only recently, the pathogenesis of hepatitis E is poorly understood. However, some speculative conclusions can be drawn. Entry of the virus into the host is believed to be primarily by the oral route via contaminated water. The site of primary replication has not been identified but is presumed to be intestinal tract. It is not clear how the virus reaches the liver but it is presumably via the portal vein. It replicates in the cytoplasm of hepatocytes (Bradley et al 1991) and is released into the bile and the blood by mechanisms that are not understood. Based upon limited studies of oral infection in a volunteer, viremia was first detected by PCR 22 days after exposure and over a week before onset of disease on 30 days (Chauhan et al., 1992). Virus particles were found by IEM in the faeces by 34 days after exposure .Liver enzyme values peaked on day 46. Anti-HEV was first detected on day 41 and was still detectable 2 years later. Similarly, in another, earlier volunteer study, HEV was detected in faeces by IEM on the day 28, and liver enzyme levels peaked on a approximately day 4 .(Balayan et al 1983). The individual volunteers in each of the studies recovered completely. As in hepatitis A, specific IgM and IgG immune responses occur early in the disease, usually by onset of clinical illness. IgM anti-HEV disappears after several months; IgG anti-HEV persists , but at a relatively rapidly decreasing levels shortly after infection (half-life of IgG anti-HEV;< 6 months) (Bryan et al.,1994). Later, the decay of anti-HEV is less rapid but it is still considerably more rapid than that of anti-HAV. Nevertheless, anti-HEV can be detected for as long as 14 years after infection (Khuroo et al., 1993). It is not known whether such antibody eventually disappears or remains at low but detectable levels. Anti-HEV of the IgA class has also been detected in the serum of naturally infected individuals (Dawson GJ et al 1990). The significant of such antibody is not known. Virus found in the faeces of the individuals is presumed to be primary source of infectious virus in the environment. Because large quantities of HEV have been found in the bile of experimentally infected primates, it is assumed the bulk of virus in the intestinal tract originates in the liver (Reyes et al., 1990). In this respect, hepatitis E is similar to hepatitis A. The severity of HEV infections is, on average somewhat greater than the severity of HAV infections. Mortality of hepatitis E has varied in different reports bit has been as high as 1%, compared to 0,2% for hepatitis A (Purcell et al., 1989). More

important, however, is the of hepatitis E in pregnancy increases with each succeeding trimester and my reach 20%. In contrast, none of the other four recognized hepatitis viruses causes such severe hepatitis in pregnancy (Mishra et. al 1992). The reason for the excessive mortality of hepatitis E in pregnancy is unknown. Fulminant NANB hepatitis occurring in non-endemic areas not to be caused by HEV (Kuwada et al.,1994). Histological changes in the liver of patients with hepatitis are thought by some to be characteristic (Purcell et al.,1982). The changes include focal necrosis with minimal infiltration and no localization to a particular zone of the lobule. Modest inflammation consisting predominantly of Kupffer cells and the polymorphnuclear leukocytes is seen and the focal lesions resembles drug-associated toxic hepatitis. Cholestatic hepatitis is often present is often, characterized by ballooning hepatocytes, cytoplasmic cholestasis , and the focal cytolytic necrosis. An unusual “pseudoglandular” alteration of the hepatocyte plates has been recognized in some epidemics. The pathogenesis of fulminant hepatitis E of pregnancy was though by the late Hans Popper to be a manifestation of endotoxin – mediate release of toxic cytokines as the result of damage to Kupffer cells, the main protection of hepatocytes from toxins generated in the intestinal tract (Purcell et al.,1982). The discrepancy between the time of appearance of viral replication in the liver and histopathologic and biological evidence of hepatitis suggest that HEV is not cytopathic and that the pathogenesis of hepatitis E is immunologically mediated, but there is no direct evidence for or against this hypothesis(Soe et al.1989) . It is not known whether HEV causes sequelae or extrahepatic manifestation of infections. None has been recognized , except for excess fetal wastage, which has been reported in some (Malkani et.al.l1992), but not all , studies of pregnant females with fulminant hepatitis E. Premature deliveries with a high infant mortality (33%) have also been reported (Song D-Y et al., 1991).

1.7 Epidemiology

Figure: 3 Geographic distribution of the Hepatitis E Virus

Hepatitis E virus infection is endemic in Southeast and Central Asia. Several outbreaks of hepatitis E have been observed in the Middle East, northern and western parts of Africa and North America (Mexico), Fig: 3) (Krawczynski K. Hepatology 1993;17 923-41).

In other parts of the world, HEV infection is infrequent and restricted predominately to persons who have travelled to disease-endemic areas. Hepatitis E outbreaks are large, affect several hundred to several thousand persons, and vary from short-lived, single-peaked outbreaks to prolonged, multimodal epidemics lasting for more than a year .(Vishwanathan R. Indian J. Med. 1957;45:1-29). During these outbreaks, overall attack rates range fro 1 to 15%, being higher among adults (3 to 30%) than among children (0,2 to 10%) (Khuroo et al,. 1980). The lower attack of children may reflect a higher frequency of anicteric and / or subclinical HEV infection in this age group. Males are usually more frequently affected. The outbreaks are characterized by a particularly high rate and mortality(as high as 25%) among pregnant women (Naik SR Bull. WHO 1992; 70;597-604) In areas where hepatitis E outbreaks occur, HEV account for a substantial proportion of acute sporadic hepatitis in both children and adults. In India, HEV infection accounts for 50 to 70% of all sporadic viral hepatitis (Khuroo et al., 1993;) Demographic and clinical features of patterns with sporadic hepatitis E resembles of those patients with epidemic hepatitis E Sporadic hepatitis E has been observed in several countries where outbreaks

have been not reported, including Egypt, Hong Kong, Senegal and Turkey. In non-endemic regions, the disease accounts for fewer than 1% of reported cases of acute viral hepatitis; these hepatitis E cases are almost associated with travel to endemic-regions (CDC Weekly Rep.1993;42;1-4), although some cases have been reported among persons with no history of such travel (Dawson et al.,1994). In the United States, all cases of HEV infection have been related to disease-endemic countries until recently, when HEV infection was reported in a patient without such a travel history (Dawson GJ et al., 1998). Molecular study have shown that the patient was infected with the novel HEV isolate (HEV US-1).

1.8 Transmission, Routes of Spread and Reservoirs

The faecal-oral route is the predominant mode of transmission of epidemic HEV infection. Most reported epidemics have been shown to be related to consumption of faecally contaminated drinking water. (Khuroo et al., 1980). The outbreaks frequently follow heavy rains and floods, when water sources have become contaminated. (Bradley et al.,1984). Some epidemics have occurred in hot summer months , when the reduction of water flow in rivers and streams may contribute to an high risk of infection (Corwin A et al.,1995). In Indian subcontinent, China Indonesia and Central Asia republics of the former Soviet Union, a pattern of recurrent epidemics has been observed, this is probably related to continuous existence of conditions that that allow faecal contamination of water. During hepatitis E outbreaks, person to person transmission of HEV appears to be distinctly uncommon (Aggrawal et al.,1994;). Secondary attack rates among household members of hepatitis E cases are only 0,7-2,2%; in contrast, 50-75%, of susceptible household contact of hepatitis A cases known to become infected (Anderson et al., 1982). Even when such multiple cases occur among members of a family, such occurrence is related to exposure to a common source of contaminated water rather than person to person spread (Naik et al., 1994). The mode of transmission responsible for sporadic HEV infection is unclear. Water contamination appears to be responsible for most cases in this setting.It is however, plausible that food and fomites, and even person to person may play a role. Nosocomial spread of HEV was presumed to be responsible for acute hepatitis in three health- care workers in South Africa who had treated a patient with fulminant hepatic failure due to HEV infection (Robson SC et al.,1992).

The reason of transmission patterns of hepatitis A and HEV are presently unclear but may relate to difference in viral titres in stools of infected persons, number of viral particles required to cause disease, or viability of these viruses in the environment. Vertical transmission of HEV infection from mother to infants is known to occur. One study, six of eight babies born to mothers who had either acute uncomplicated hepatitis or fulminant hepatic failure from HEV infection in the third trimester of pregnancy were found to have evidence of HEV infection (Khuroo et al 1.,995). Of these six babies, five had HEV-RNA in their cord blood ; another one baby had serological evidence of HEV infection acquired

before birth. There is no evidence of sexual transmission of HEV, although in a report from Italy , 20% of homosexual men had anti-HEV antibodies as compared with only 3% of intravenous drug users (Psichogiou et al.,1996), thus suggesting faecal-oral transmission of HEV. There is no evidence serological for transmission of HEV by transfusion of blood or blood products, however, serological tests used by Thomas and colleagues (Thomas et al, 1997) have reported that anti-HEV is detected in about 21% of blood donors and 23% of injection drug users from Baltimore, Maryland. In Northern California, about 1.2 to 1.4% of blood donors are found to be anti-HEV positive. Similar results have been reported in other industrialized countries including the Netherlands, Italy Sweden, Germany, Greece, England, Finland, Spain and Taiwan (Peng, CT et al, 2002). However, it should be remembered that the specificity and the sensitivity of these ´´ in house´´ anti-HEV serological assays are unknown, though some anti-HEV assays validated with serial serum samples from animals experimentally infected with different HEV strains (Xiang-Jin et al 2002). Furthermore, anti-HEV antibody prevalence rates among patients with haemophilia and thalassaemia and intravenous drug users are similar to those for general population (Schulman S JMV 1995;46;153-6). Presumably, an environment reservoir of HEV exist in disease-endemic areas that is responsible for recurrent epidemics. Laboratory investigations have shown, however, that HEV is labile virus when exposed to high concentration of salt, freeze-thawing and pelleting (Bradleyet al.,.1992). Another potential reservoir for persistence of HEV during interepidemic periods in disease-endemic areas may be in the form of serial transmission among susceptible individuals who have sporadic or subclinical hepatitis E. Recent data suggest that the hepatitis E may be zoonotic disease. Hepatitis E virus- RNA has been detected in faeces of domestic swine in Nepal and anti-HEV antibodies have been detected in sera of pigs, cattle, sheep and rodent in disease-endemic areas .(Clayson et al.,.1995). Pigs and lambs have been shown to develop transaminasaemia, histological changes in the liver and viral excretion in faeces after experimental HEV infection (Usmanov et al., .1994) In addition, a novel of strain of HEV, a swine HEV has recently been identified and shown to be ubiquitous in the USA. Antibodies to swine HEV cross- reacted with human capsid proteins from human strains of HEV. (Pilar, C et al 2003). Swine in USA become infected by swine HEV at about two to three months of age. Although the infection is subclinical, microscopic evidence of hepatitis has been found in naturally infected swine (Xian-Jin et al, 2002).HEV

infection in swine is likely to be common worldwide. Recently the prevalence of anti-HEV IgG in swine was assessed in two countries where HEV is endemic (China and Taiwan) and the two countries where it is non-endemic (Canada and Korea) (Tsutomu, N et al 2003). It was found that swine herds in all four countries contain many swine that are seropositive for HEV, suggesting that HEV is enzootic in swine regardless of whether HEV is endemic in the respective human population (Masaharu, T et al, 2003). As refer to above, a US isolate of HEV and swine HEV have been shown to be phylogenetically related and to posses cross species infectively, leading to further support to the zoonotic hypothesis (Meng et al, 1998).

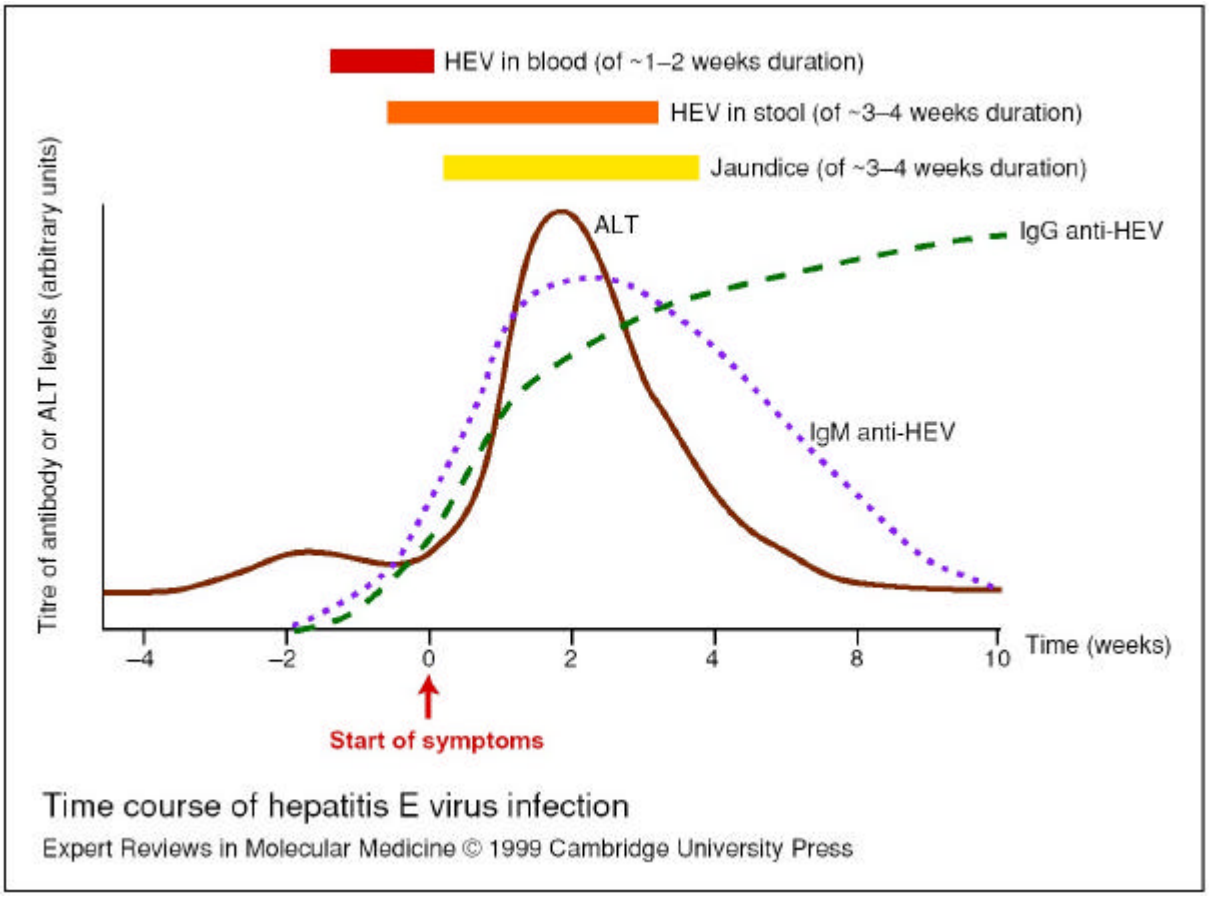


Figure 4. Time of course of hepatitis E virus infection.

Biochemical markers (e.g serum ALT levels) and symptomatic markers (e.g. jaundice) of viral hepatitis are correlated with detection of HEV RNA by TR_PCR in the bloodstream, or shedding of virus in stools, and the immune response is measured as anti-HEV IgM or IgG levels, detected by enzyme immunoassay on serum samples. Four to eight weeks after exposure to HEV, there is a rise in ALT and the appearance of jaundice.. Immediately prior to the onset of clinical symptoms, HEV can be detected in the bloodstream, but continues to be shed in stools for 3-4 weeks. At the onset of clinical symptoms, HEV is lost from bloodstream, but continues to be shed in stools. Anti-HEV IgM and IgG titires continue to increase in the asymptomatic phase.

The anti-HEV IgM titire peaks during the symptomatic phase and declines thereafter to baseline within 3-6 months of symptomatic disease. The anti-HEV IgG remains detectable for 2-13 years as determined in various studies (Modified from the viral hepatitis slide set published by the US Centres for Control, Atlanta, GA, USA).

1.9 Hepatitis E in Pregnancy

One distinctive clinical feature of hepatitis E, compared with other forms of viral hepatitis, is its increased incident and severity in pregnant women (Khuroo MS et al1994), which results in up to 20% mortality. By contrast, none of the other hepatitis viruses causes such severe hepatitis in pregnancy. Though the mechanism(s) is not known, a hypothesis has been put forward to explain the pathogenesis of fulminant hepatitis E in pregnancy (Purcell RH et al Allan Liss Press N.Y USA). This suggests that the liver sinusoidal cells, particularly the Kupffer cells, are damaged by HEV, which diminishes the ability of these cells to protect hepatocytes against endotoxins that originate from Gram-negative bacteria found in the intestinal tract. Hepatocytes can be injured directly by endotoxins or indirectly intravasal coagulation affecting the liver and kidneys. In pregnant women, a high incidence of disseminated intravascular coagulation associated with hepatitis E is well recognised. However, in experimental HEV infection of pregnant monkeys, no increased mortality has been observed, casting doubt on whether this is a good model for this aspect of human hepatitis E. Liver histology of patients with hepatitis E reveals portal triaditis, chlolestasis, lobular inflammation and degeneration of the liver to varying degrees, which are suggestive of acute viral hepatitis. However, nearly half of the patients have distinctive morphological changes designated as cholestatic viral hepatitis. The discrepancy between the time of appearance of viral replication in the liver with the histopathological and biochemical changes suggest that HEV might not be directly cytopathic and its pathogenesis might be immunologically mediated. However, there is no direct evidence for, or against, this hypothesis (Purcell Rh et al 1998). It is not known whether HEV causes other sequelae or extrahepatic manifestations. None has been recognised apart from the increased incidence of miscarriage, which has been reported in some, but not all, studies on fulminant hepatitis E during pregnancy.

1.10 Diagnosis and Seroprevelance Data

The prevalence of anti-HEV in healthy subjects has been studied in various population worldwide to measure the extent of exposure to HEV. It has been found that anti-HEV antibodies are present in persons living in all geographical areas. In disease-endemic areas of Asia and Africa, the prevalence rates among healthy populations are much higher than those in non-endemic areas. In most disease-endemic areas, anti-HEV has been detected in as many as 5% of children less than 10 years of age, and this ratio increases to 10-40% among adults older than 25 years of age (Moeckli et al., 1992). These findings suggest that HEV infection, unlike that of enterically transmitted agents, is not frequent among young children in developing countries. However, in a recent report from India anti-HEV were detected in more than 60% of children below the age of 5 years (Aggarwal et al.,1997). The differences between disease-endemic may be related to varying epidemiological condition in different geographical areas, differences in diagnostic techniques used, or both. In developed countries 1-5% of population have anti-HEV antibodies (Yarbough et al.,1992). This range appears to be relatively high compared with the low rate of clinically evident hepatitis E disease in these areas. However, in a recent study that used two different serological test to estimate the prevalence of anti-HEV, concordance between the two test was 27% (Favorov MO et al. 1997). Enzyme immunoassays currently used in various laboratories were directed compared using a panel of coded samples. Seroprevalence studies indicates that HEV EIA´s based on large antigens expressed from ORF2 or capsid-like particles are superior to those based on short sequences of ORF2 or antigenic epitopes of the ORF3 in detecting convalescent-phase anti-HEV (Worm et al 2002). It should be noted however, that the overall concordance of different tests for assessing the seroprevalence of anti-HEV in a non-diseased population is low. Pair-wise comparison of twelve test set show a concordance in blood donor sera raging from 41% to 94% (mean 68%), and a concordance among reactive sera from 0% to 89% (mean, 32%) (Mast et al, 1998). EIAs based on synthetic peptides could be not detected the convalescent-phase anti-HEV reliably. They are used mainly to confirm positive test results from EIA based on recombinant proteins and exclude non-specific anti-HEV in a acute-phase serum (Takashi et al, 2003). The specificity and sensitivity of these tests for detecting convalescence-phase IgG have not

been precisely established, which limits the reliability of the results from seroepidemiological studies. Our study showed that their sensitivity rates varied widely (from 17-100%), and concordance rates among reactive serum specimens range from none to 89% (median 32%) (Purcell et al Hepatology 1998). The assays compared in this study used recombinant HEV proteins, which differ in length, part of the genome with which they correlate, and the geographical strain of HEV to which they correspond. Thus, it remains unclear whether the anti-HEV seroreactivity in non-endemic areas reflects subclinical and /or anicteric HEV infection, serological test , or a combination of all these factors.

1.11 Therapeutic Approaches to Hepatitis E Infection

No therapeutic compounds against hepatitis E are currently available; the only treatments are supportive in nature. Possible drug targets include the HEV Pr and RdRP enzymes on which even the basic biochemical information is not yet available. Such information will be critical for developing assays to screen libraries of natural or synthetic molecules to search for compounds with anti-HEV activity. The HEV RNA 5´and 3´ ends appear to interact with viral and cellular proteins and are crucial for its replication; strategies designed to block these interactions, for example with anti-sense oligonucleotides, ribozymes or small molecules, might be of therapeutic value. No information is available as to whether any of these approaches are currently being employed. HEV, or ET-NANBH virus as it was called then, was recognised as a distinct entity in the early 1980s. It took almost 10 years to develop suitable animal models and obtain epidemiological information. This directly led to cloning of viral genome in 1990 (Reyes et al., 1990) and designation as HEV. Along with the molecular cloning of the HCV genome a year earlier, this heralded a new age in molecular virology, in which the genomes were cloned without first isolating or propagating the virus in culture .The initial cloning of the genome of the Burma isolate of HEV (Reyes et.al.,1990) led to the subsequent cloning of genomes from other geographically distinct isolates and development of a diagnostic test. These developments have further increased our knowledge of the epidemiology of hepatitis E. However, the pace of research on hepatitis E has been slow for a number of reasons. Hepatitis E is not a significant health problem in countries have technological capabilities and funds to carry out front-line biological research. Furthermore, HEV causes an acute, self-limiting infection with no associated chronicity, unlike HBV and HCV. However, new findings that hepatitis E might have zoonotic reservoir and indigenous pockets of infection in industrialised nation, and the identification of risk groups of displaying high mortality such as pregnant women, are likely to provide impetus to hepatitis E research.

This would be especially welcome in the direction of anti-HEV vaccines and therapeutics. Limited information available on the molecular biology of HEV shows to be interesting, virus that might serve as a good model to study virus- host interactions at the molecular level (Shahid et.al.,1999). Perhaps this will also stimulate research.

1.12 Immunoprophylaxis

No products are currently available to prevent hepatitis E. Passive immunization using immuneglobulins prepared from plasma collected from HEV-infected persons in non-HEV-endemic areas is not effective in preventing clinical disease during hepatitis E outbreaks, and the efficacy of the immuneglobulins prepared from plasma collected in HEV-endemic areas is also unclear (Shahid et al., 1999).

In studies with prototype anti-HEV vaccines in animals, vaccine-induced antibody could attenuate HEV infection but did not prevent virus excretion in the stools of infected immunised animals. For viral pathogens that are difficult to culture and therefore not easily amenable to the development of live attenuated strains, a promising approach is to develop subunit vaccines. A subunit vaccine consist of a part of the virus, typically a protein capable of generating a protective immune response in immunised persons. Recombinant DNA technology is now routinely used to generate large amounts of purified viral proteins to be used as subunit vaccines. For HEV, the most promising subunit vaccine candidate so far appears to be the ORF-2-encoded protein when expressed in insect cells using recombinant baculoviruses. Two of such candidates were developed simultaneously at the National Institutes of Health(Bethesda, MD USA) at the Genlabs Technologies (Redwood City, CA, USA) (Zhang Y et al..1997).

After a pilot-scale production at the Smith Kline Beecham (Belgium), both vaccine undergone feasibility testing in experimental animals and have shown promise (Fricker .et. al.1999). The NIH vaccine candidate has also been subjected to a Phase I trial in US volunteers and has been shown to be safe and immunogenic. A similar trial of this candidate vaccine in Nepal, an area endemic for hepatitis E has been started in last year (results unpublish). Immunogenicity and efficacy data should be available sometime in the near future and will help answer many question still remaining. One question yet unanswered concerns the number of epitopes on the Virion. Only neutralisation epitope has been thus identified thus far (Emerson et al.,2000 ; Meng et al, 2001): it maps between amino acids 578 and 607 0f the capsid protein (Emerson et al, 2003). Virus mixed in vitro with either of two monoclonal antibodies that recognised this epitope was unable subsequently to infect macaques. This epitope should be present in each of the

baculovirus-expressed candidate vaccine. However, this epitope should not be in the shorter protein that forms VLPs but includes only amino acids 112-578. Interestingly, vaccination with this shorter protein did not prevent rhesus macaques from hepatitis following a high dose challenge although the virus load appeared to be reduced (Emerson et al, 2002). At this time, there is no good explanation as why vaccination with either the 112-607 or 112-578 protein reduced virus shedding to a similar extent whereas only the larger protein afforded protection from hepatitis.(Emerson et al, 2003). Another unanswered question has a more practical aspect. It concerns the duration of immunity induced vaccination. Recently, the products of N-terminally truncated ORF-2 were shown to form empty virus-like particles (VPLs) (Li TC et al 1997). These VLPs retain native virus epitopes and appear to be good vaccine candidate(He J. et al !997). Alternative strategies for developing anti-HEV vaccines are also being tried in research laboratories. A naked DNA immunisation approach in which ORF-2 was injected as an expression plasmid directly into muscles resulted in moderate anti-ORF-2 titres in mice (He J et al 1997;) within days of ORF-2 plasmid DNA injection, the subsequent injection of genes encoding either of the immunomodulatory cytokines interleukin 2(IL-2) or granulocyte-monocyte colony-stimulating factor GM-CSF) resulted ain higher anti-pORF-2 titres in mice (Shahid et.al.,1999).

1.13 Prevention

Any improvements in water quality and sanitation will be helpful in preventing transmission of HEV since most of the outbreaks described can be attributed to contaminated water supplies. Improvement should be wherever possible. Although passive protection has been demonstrated in animals (Emerson et al, 2003), when administration of immuneglobuline has been tried in humans it has met with disappointed results. Such preparations are particularly of little value if they are produced in countries where HEV is not endemic and there is little immunity. Arankalle et al, 1998 and Khuroo et al, 1996 looked at the efficacy on an Indian preparation of immune serum globulin.

1.14 Objective

To evaluate the suitability of currently available HEV IgG antibody assays for use in low-endemicity areas. Sera referred for routine antibody screening, mostly from long-term expatriates (development aid workers and family members). Samples reactive by Abbott HEV antibody EIA since April 1998 were included in the panel.

Paradoxon: HEV antibody seroprevalence rates

1) in endemic areas lower than expected (compare with Hepatitis A)

2) but in non-endemic areas higher than expected, in the absence of a consisitence clinical or travel history (e.g in blood donors).

2 Methods and Materials

2.1 Methods

Sera were selected on the basis of al least borderline reactivity in the routinely used Abbot EIA. Most were tested as part of routine screening of long-term expatriates in endemic countries. The following assays (recombinant anitigens) were used : Abbott EIA, Genelabs ELISA, Mikrogen recomBlot and a Prototype DSL ELISA.

HEV ANTIGENS EMPLOYED
Abbot EIA:
SG-3 327aa): C-terminus of ORF2
8-5 (123aa): full ORF3
Burma strain

GENELABS ELISA:
Type-common epitopes of ORF2 and ORF3
Mexico and Burma strains

MIKROGEN RECOMBLOT
N-terminal, C-terminal, middle portion of ORF2, full ORF3
Madras isolate

DIAGNOSTIC SYSTEMS LABORATRIES ELISA
ORF2
ORF3
Burma strain

2.2 Materials

ABBOTT EIA

Biological Principle of the procedure.

In the Abbott HEV EIA, human serum or plasma is diluted in a spicemen diluent and incubated with a polystrene bead coated with recombinant HEV proteins representing sequences from Open Reading Frame (ORF2) and ORF3 of the Burmese strain of HEV. If antibody is present in the sample, immunoglobulins in patient sample are fixed to the coated bead. After removing the unbound materials and washing of the bead, human immunoglobins remaining bound to the solid phase are detected by incubating the bead-antigen-antibody complex with a solution containing horseradish peroxidase labeled goat antibodies directed against human immunoglobulins

Unbound enzyme conjugate is then removed and the beads are washed. Next, o-Phenylendiamine (OPD) solution containing hydrogen peroxide is added to the bead and, after incubation, a yellow-orange colour develops in proportion to the amount of anti-HEV which bound to the bead.

Specimens with absorbance values less than the Cut-off Value are considered initially reactive by the ABBOT HEV EIA and then repeated in duplicate .Specimens with absorbance values within ± 10% of the Cut-off Value are considered to be in the grey zone and should be retested in duplicate to confirm the initial results.

2.3 Test Principles:

2.3.1 Diagnostics Systems Laboratries ELISA

Hepatitis E kit is based on the ELISA technique (Enzyme linked Immunosorbent Assay). In the assay, Negative, Positive Controls and unknowns are incubated in microtitration wells coated with recombinant derived HEV antigen of ORF2 and ORF3. After incubation and washing, the wells are treated with the conjugate, composed of anti-human IgG-monoclonal antibodies labelled with peroxidase. After a second incubation and washing step, the wells are incubated with the substrate tetramethylbenzidine (TMB). An acidic

stopping solution is then added and the degree of enzymatic turnover of the substrate is determined by wavelength absorbance measurement at 450 nm. The absorbance measured is directly proportional to concentration to the anti-HEV antibodies present.

ASSAY PROCEDURE

All specimens are reagents must be allowed to reach room temperature (25°C) before use. Serum Samples, Control Positive and Control Negative should be assayed in duplicate.

Mark the microtitration strips to be used. Dilute the serum samples 1:101 distributing 10 µl of serum into 1 ml of Assay Buffer. Pipette 100 µl of each of each diluted serum sample ready to use positive control, negative control serum to the appropriate wells. Leave one for the blank, and performed using 100 µl of the substrate mixture. Cover the wells with protective film and incubate for 45 minutes at 37 °C. Aspirate and wash each well four times for 30 seconds with Washing Solution using an automatic microplate washer or manually using dispenser (e.g. Multipette Ependorf).

Blot and dry by inverting plate on absorbent material. Add 100 µl of Enzyme Labelled 2^{nd}. Antibodies into each well. And cover the wells with protective film and incubate for 45 minutes at 7°C. Aspirate and wash each well four times for 30 seconds with Washing Solution using an automatic microplate or wash manually using dispenser (e.g. Multipette Eppendorf). Blot and dry by inverting plate on the absorbent material. Add 100 µl of TMB Chromogen .

Solution to each well using a dispenser And then incubate for 10 minutes at room temperature and avoid exposure to direct sunlight. Add 100 µl of Stopping Solution to each well using a dispenser. Read the absorbance of the solution in the wells within 30 minutes, using a microplates reader set to 450nm. If wavelength correction is available, set the instrument to dual wavelength measurement at 450 nm with background wavelength correction set at 600 or 620 nm.

2.4 Genelabs ELISA

2.4.1 Chemical and Biological Principles of the Procedure

The wells of the polystrene microplate strips are coated with three recombinant HEV antigens which correspond to the structural regions of the Hepatitis E Virus. Humans serum or plasma, diluted in diluent buffer, are incubated in these coated wells. HEV specific antibodies , if present, will bind to the solid phase HEV antigens. The wells are thoroughly washed to remove unbound materials and a mouse monoclonal anti-human IgM labelled with horseradish peroxidase is added to the wells . this labelled antibody will bind to any antigen-antibody complexes previously formed and excess unbound labelled antibodies are removed by washing a substrate solution containing hydrogen peroxide and o-Phenylenediamine Dihydrochloride (OPD.2HCL) is then added to each well.

The presence of specific antibodies is indicated bye the presence of a yellow-orange colour after substrate addition. Reaction is terminated bye addition of sulpheric acid. The intensity of the colour measured spectrophotometrically at 492nm and is proportional to the amount of antibodies present in the specimen.

2.4.2 Assay Procedure

Remove microplate from the aluminium bag and shake specimen and control

vials before use.. Then fill a reagent reservoir with Diluent. .By using a multi- channel pipettor, add 200ul of Diluent to all wells. Wells A1 and B1 are ´BLANKS´. Add an additional 10ul of diluent to these wells.

Then add 10ul of specimen to the assigned well, starting at well H1. This will give a final specimen dilution of 1:21. Add 10ul of Non-reactive Control to wells C1, D1 and E1.Add 10ul to wells F1 andG1 and mix thoroughly by tapping gently on all sides of microplate, taking care to keep the plate flat on the bench-top. Carefully cover microplate with a plate cover provided to prevent evaporation during incubation. Incubate for 30 minutes at 37°C.Prepare Working Conjugate as describe in the Preparation of Reagents prior to washing to washing the microplates. Then wash the microplate with dilutet buffer. Blot dry

by inverting the microplate and tapping firmly onto absorbent paper. Add all residual plate wash buffer should be blotted dry. Colour formation can be inhibited during the substrate incubation by residual plate buffer. Fill a reagent reservoir with the Working Conjugate using a multi channel pippetor , add 100ul of Working Conjugate to all wells and apply another cover. Incubate the microplate for 30 minutes at 37°C. Prepare Working Substrate Solution as according to instructions in the Preparation of Reagents. Shield the substrate from light.

Excess solution should be discarded after use. After that, remove and discard the plate cover. Fill reagent reservoir with Working Substrate Solution. Using a multi channel pippettor, add ^00ul of Working Substrate Solution to each well. Incubate for 15 minutes in the dark at room temperature. Then using a multi channel pipettor, add 50ul of Stop Solution to each well. and mix gently by tapping the plate.

Determine and Absorbance for each well at 294 nm . If a dual filter instrument is used, the reference wavelength should be 620 nm.

2.5 Mikrogen Recomblot

TEST PRINCIPLE:

The purified, recombinant antigens used are separated according to their molecular weight by using SDS polyacrylamide gel eletrophoresis. The HEV proteins are then transferred electrophoretically to a nitrocellulose membrane (Western Blotting). The membrane is subsequently incubated with a protein solution to block free binding sites, washed, cut into strips, and then into tubes.

To detect, HEV-specific antibodies, the strips are incubated with the diluted serum sample. During incubation, the antibodies bind to the antigens fixed on the strips are incubated ($2^{nd)}$ with anti- human-IgG or anti-human-IgM coupled to horseradisch. Specifically bound antibodies are detected by a peroxidase- catalyzed colour reaction. Dark bands appear on the test strip, showing the presence of reactivity against one of the HEV proteins. As the reaction control, a band of anti-human immunoglobulin, which must show a reaction with every serum, is applied at the top end of the strip below the number.

2.5.1 Test Procedure

2.5.2 General Aspects.

The reproducibility of Western blot results depends to a great extent on the uniform washing of the strips. Therefore, the washing frequencies described.

First Incubation:

Before use, all reagents must be brought to room temperature (18-25°C) for about 30 minutes. The test is also carried out at room temperature. Spin reagents and control sera in the small reaction tubes prior to use.. One well in an incubation tray is required for each test. 2 ml of ready-to-use wash/dilution buffer are pipetted into each wells. With the help of plastic tweezers, one test strip is carefully dipped into each of the wells. filled with wash buffer. The number of the strip should point upwards.

Addition of Sample:

IgG test procedure: in each incubation batch 33 µl of an undiluted sample (human serum or plasma) or the corresponding weak positive control are pipetted into the designated well (1:60).

IgM test procedure: in each incubation batch 33 µl of an undiluted sample (human serum or plasma) are pipetted into the designated well (dilution 1:60). (weak positive control not available)

In each experimental run, the corresponding weak positive control should be included, irrespective of the number of sera to be tested.

The incubation tray is covered with the plastic lid and incubated with gentle

shaking for 2 hours at room temperature. The incubation temperature should be between 18 and 25°C. It is very important that no contamination occurs between neighbouring wells, possible giving an in correct positive results.

Washing:

After incubation, the plastic lids are carefully removed from the incubation trays. The diluted serum is carefully withdrawn from each well, preferably with a withdrawal device that has an attached desinfection trap.2 ml of ready-to-use wash buffer are subsequently added to each well and incubated on the shaker with gentle shaking for 5 minutes. The wash buffer is withdrawn after incubation. The washing steps is repeated altogether four times.

Second Incubation:

After washing the strips, 2 ml of the correspondingly prepared conjugate are added to each well and incubated with gentle shaking for one hour at room temperature. The incubation tray should be covered during this process. The conjugate solution are withdrawn from wells and the strips are washed and again.

Third Incubation:

2 ml of ready-to -use substrate solution are added to each wells and incubated with gentle shaking for 10-15 minutes at the room temperature.

Stop the reaction:

After withdrawal of the substrate solution, the strips are washed three times with deionised water. Using tweezers, the strips are carefully taken out of the water and between absorbent paper for 2 hours to dry. Subsequently, the strips can be stuck onto the attached evaluation sheet and the result recorded.

2.6 Assay Procedure

Dilution of specimen

A dispense of 10ul of each control or specimen into the bottom of an individual test tube, and a dispense of 200ul of specimen diluent to each test tube. containing sample. and then ensure adequate mixing by a gently tapping..10ul of each diluted control or specimen will be transferred into appropriate well of reaction tray. Add a dispense 200ul of specimen diluent to appropriate tray containing control or specimen and after that a adequate mixture. by tapping.

First Incubation:

Carefully add one bead to each well containing diluted control or specimen and apply cover seal. After that, gently tap the tray to cover beads and remove any trapped air bubbles. Then incubate at 40°C for 65minutes in a Water Bath.. After that, remove and discard the cover seal. Aspirate the liquid and wash each bead three times for a total rinse volume of 12 to 18 ml of distilled or deionised water.

Second Incubation:

Pipette 200ul of diluted conjugate into each well containing a beads. Apply new cover seal and gently tap the tray to cover the beads and remove any trapped air bubbles. Then incubate it at 40°C for 65 minutes in a Water Bath.

Remove and discard cover seal and aspirate the liquid and wash each bead as first incubation.

Colour Development

Then immediate transfer of the beads to properly identified assay tube. Pipette 300ul of freshly prepared OPD substrate solution into two empty tubes (substrate blanks) and then into each tube containing a bead. Cover and

incubate at room temperature for 30 minutes and add 1ml of 1N Sulfuric Acid in each tube.

Reading:

Blank spectrophotometry with a substrate blank at 429nm. Determine absorbance of controls and specimens at 492nm within two hours after addition of acid.

2.7 Test Interpretation

ABBOTT EIA:
Cut-off = mean NC + (0.45* mean PC)
Grey zone = cut-off +- 10%
If sample O.D. > cut-off……. Repeat in duplicate

GENELABS ELISA:
Cut-off = mean NC + 0.5
Grey zone not define
If sample O.D. > cut-off……. Repeat in duplicate

DIAGNOSTIC SYSTEM LABORATRIES ELISA:
Cut-off = mean NC +0.1
Sample O.D.> cut-off……. Positive
Sample O.D.< cut-off but > cut-off -10%…… borderline
Sample O.D. < cut-off - 10%…….negative

MIKROGEN RECOMBLOT:

reaction control

O 2 - N

O 2 - C

O 2 - M

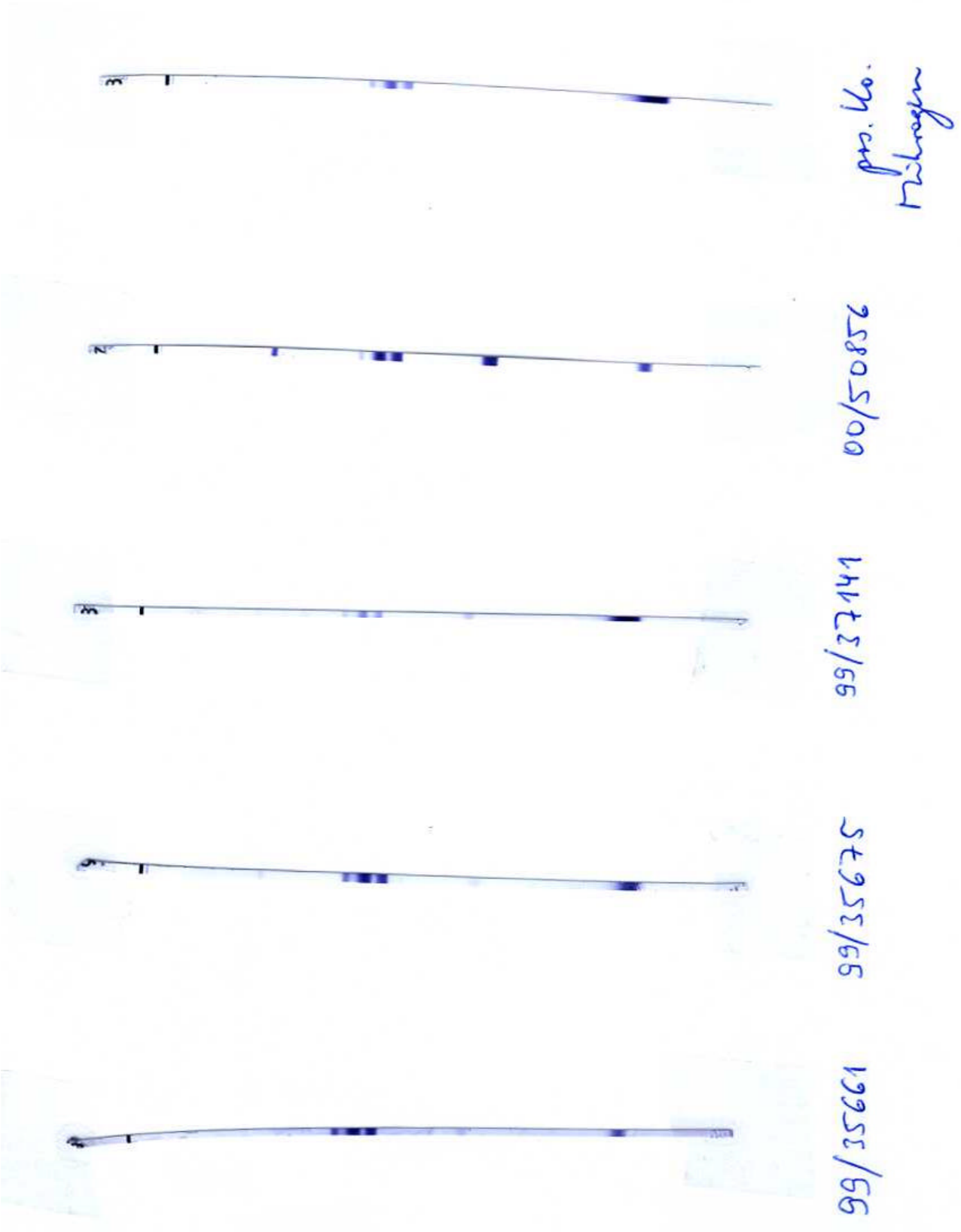
O 3

´´Scoring´´ system:
uppermost band of triple band O2-C with weak- positive control serum : +;

no band visible	-	0 point
very weak band	+-	1 point
weak band	+	2 points
strong band	++	3 points
maximum score :		12 points

Interpretation :

4-12 points : positive
3 points ´´questionable´´
0-2 points negative


pro. Ko.
Nikogyn
00/508856
99/37141
99/35675
99/35661

RESULTS.

Table 1: Results Sample I

Assay	positive	borderline	negative	total
Abbott	59	22	0*	81
Genelabs	22	0	45	67
Mikrogen	36	5	8	49
dsl	43	4	14	61

Table 2: Samples tested by all 4 assays

Assay	positive	Borderline	negative	total
Abbott	32	12	0*	44
Genelabs	20	0**	24	44
Mikrogen	31	5	8	44
dsl	33	2	9	44

* selection criterion ** not defined

Our experience in Frankfurt am Main
over the past four years (starting 1 Jan 1998)

	all	male	female	unkn	% positive
total tested	1184	716	423	45	100
positive	57	28	29	0	4.8
borderline	40	25	13	2	3.4
negative	1087	663	381	43	91.8

•Concordance between assays (pairwise;
categories: positive, borderline, negative)
:

- Abbott versus Genelabs: 36.4 %
- Abbott versus Mikrogen: 56.8 %
- Abbott versus dsl: 54.6 %
- Genelabs versus Mikrogen: 61.4 %

•Disagreement between assays (pairwise;
categories: positive/negative or vice versa):

- Abbott versus Genelabs: 50.0 %
- Abbott versus Mikrogen: 9.1 %
- Abbott versus dsl: 13.6 %
- Genelabs versus Mikrogen: 27.3 %

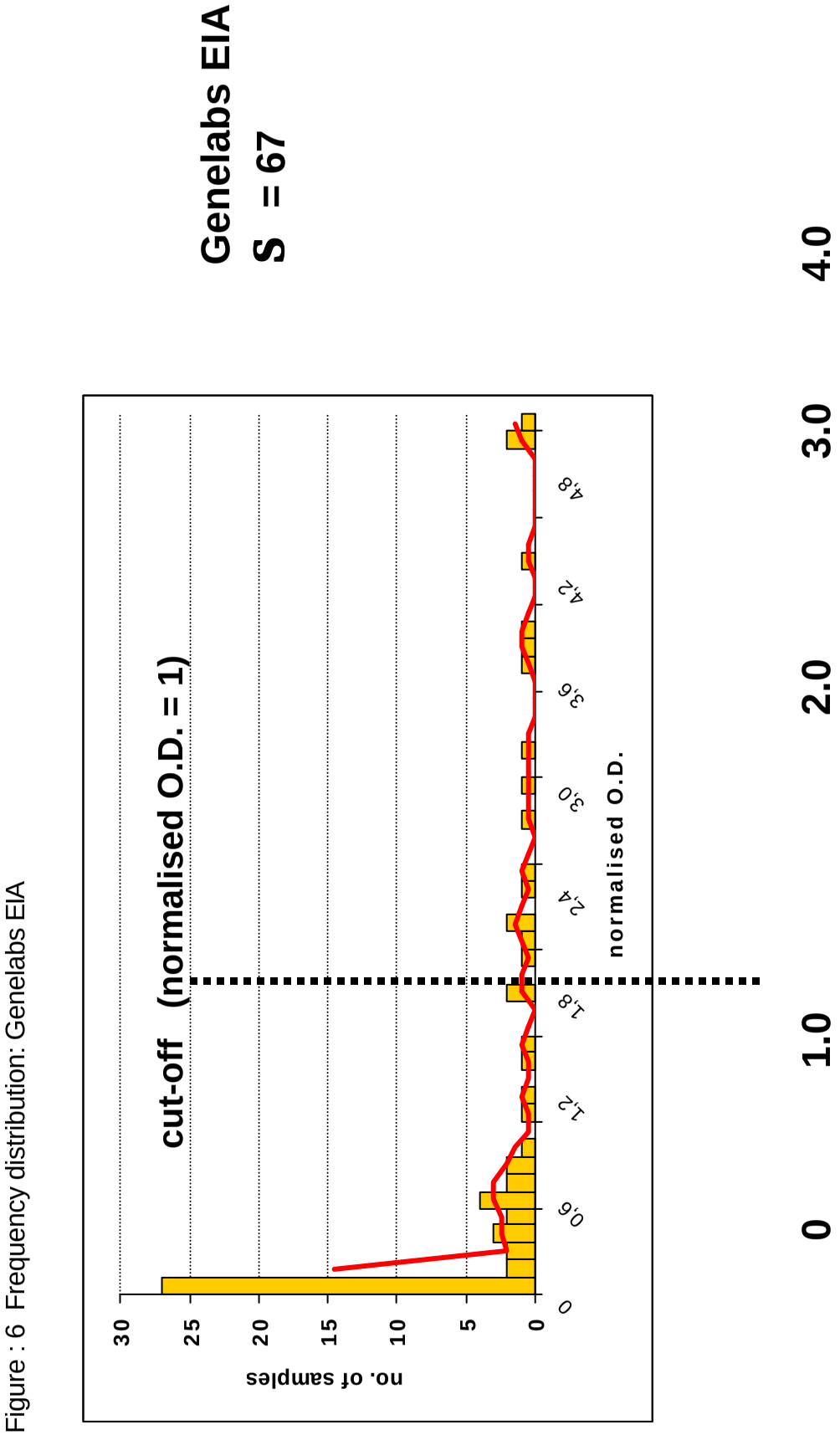
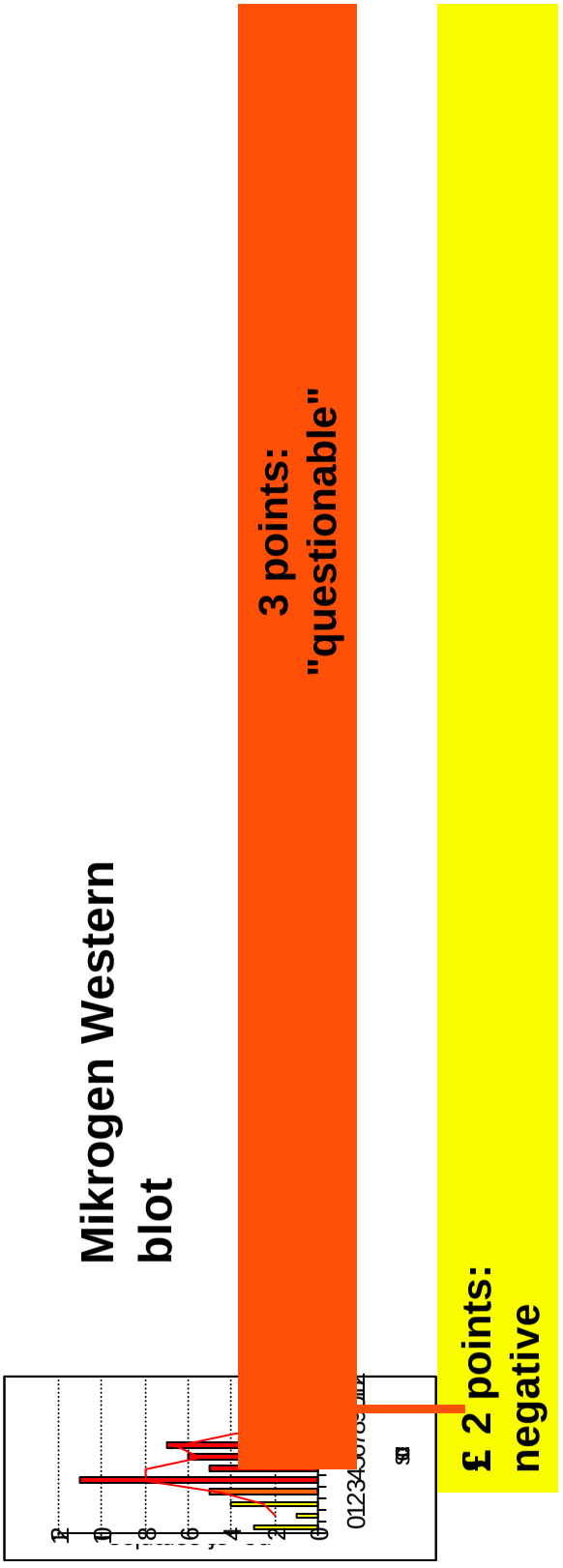
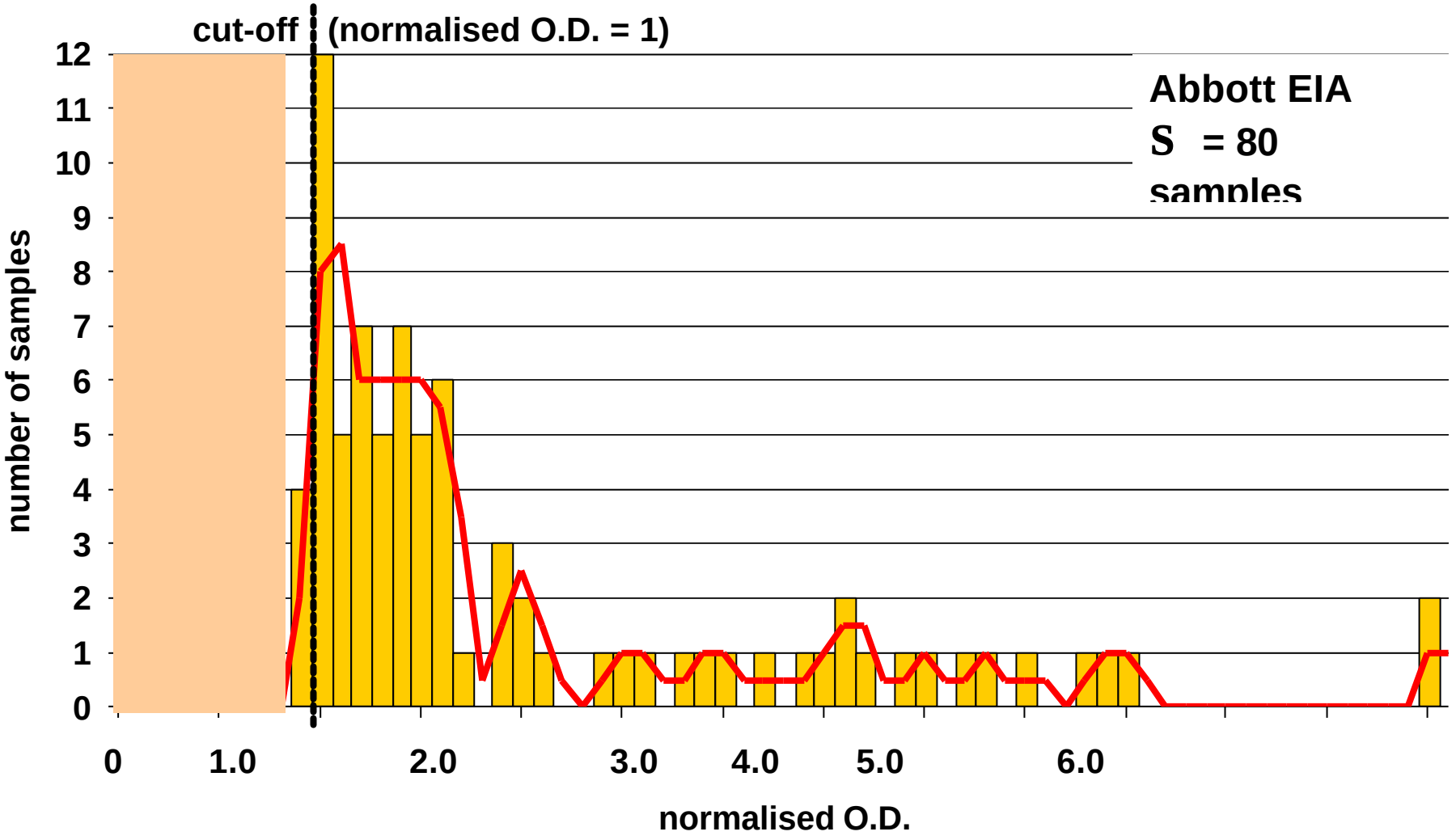


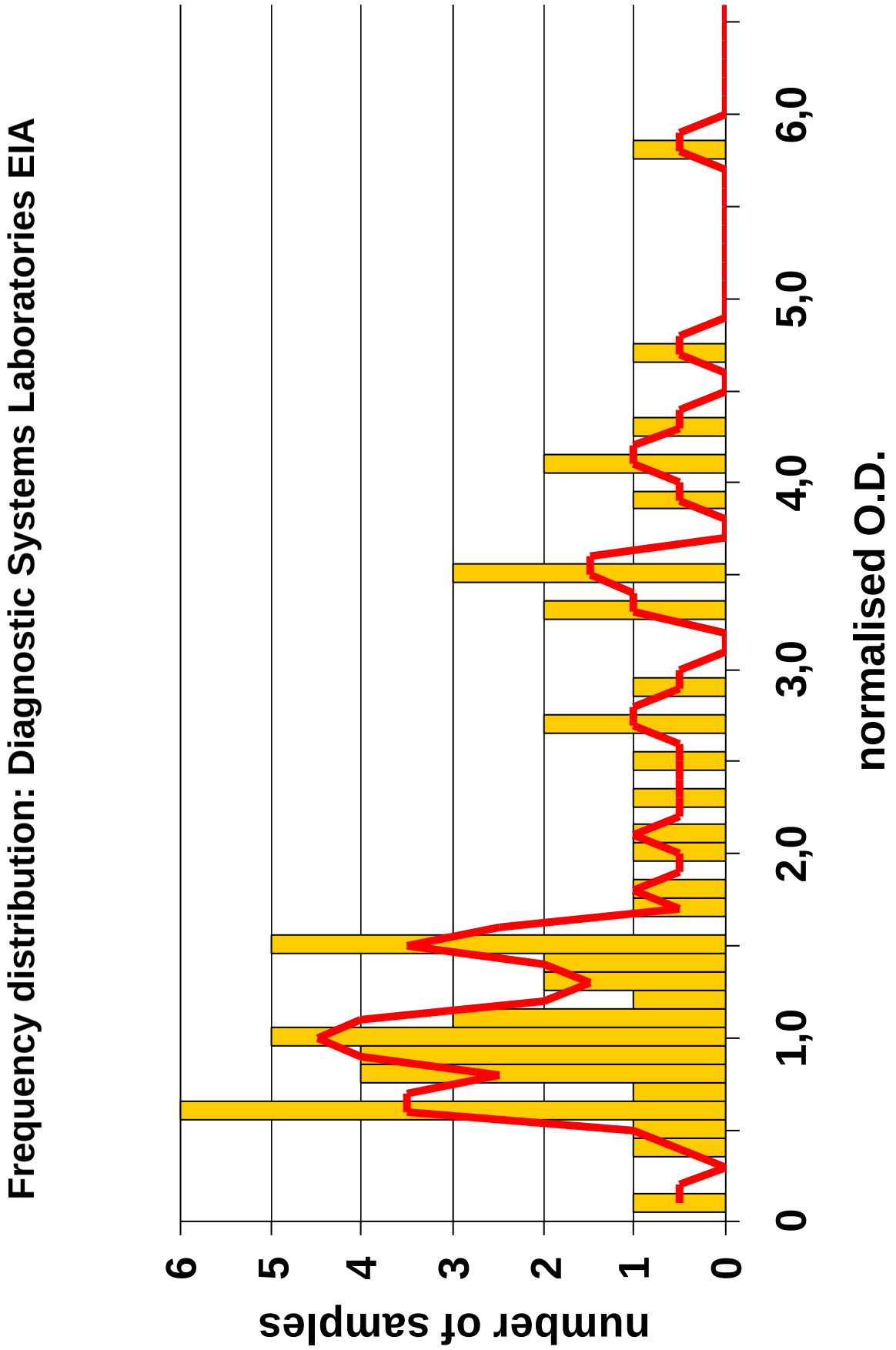
Figure : 6 Frequency distribution: Genelabs EIA



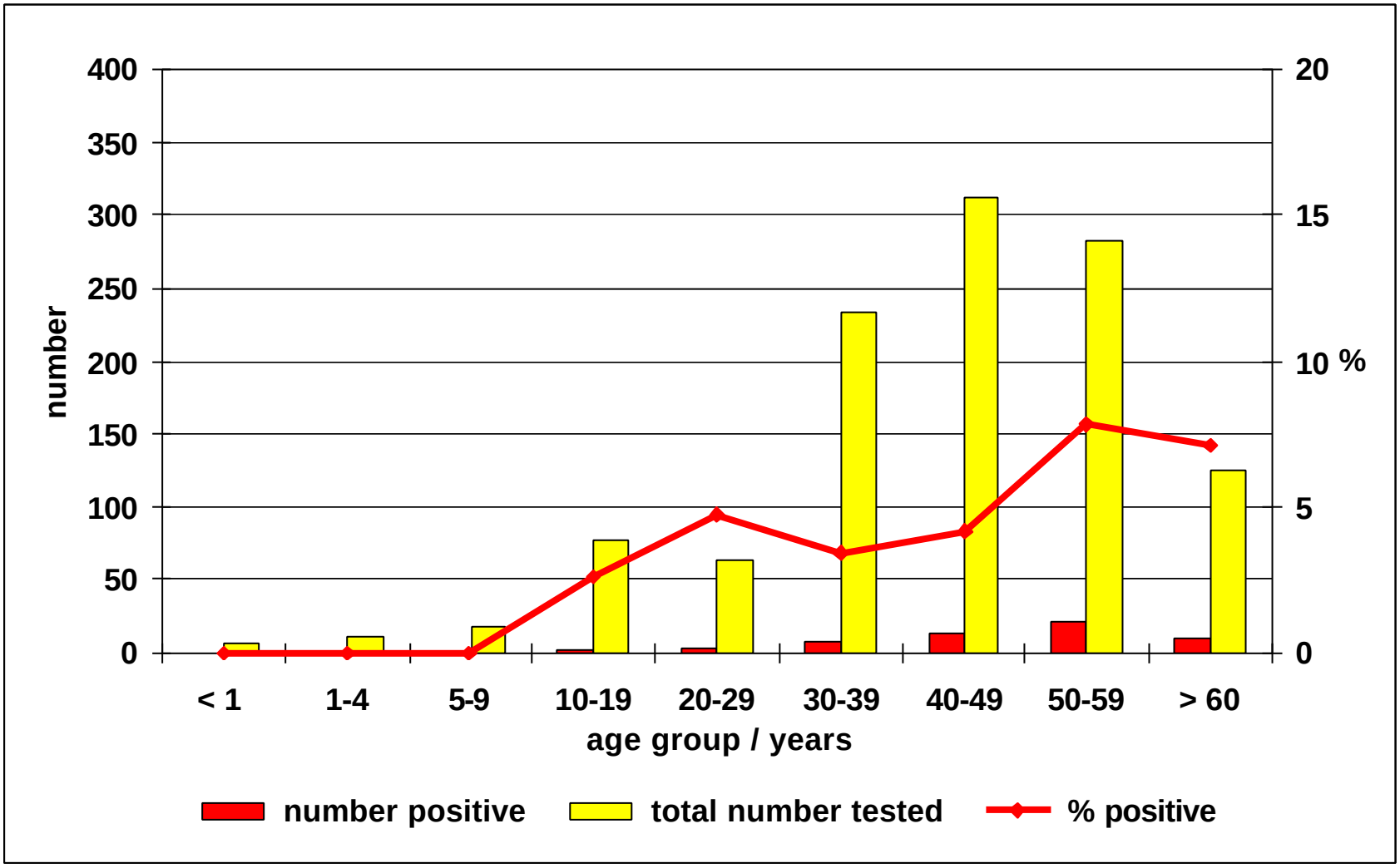
Frequency distribution: Mikrogen Western blot.
Figure:7

Frequency distribution: Abbott EIA




Frequency distribution: Diagnostic Systems Laboratories EIA
number of samples
0
1
2
3
4
5
6
0
1,0
2,0
3,0
4,0
5,0
6,0
normalised O.D.

HEV tests and test results according to age group



3 Results and Discussions

3.1 Abbott EIA Assay

. Eighty-one patients who had been determined to be positive for HEV RNA were selected in this study. The serum samples were stored at –80°C until tested for anti-HEV IgM and anti-HEV IgG using an enzyme immunoassay (Abbot GmbH Diagnostics, Wiesbaden Germany) This assay is based on two recombinant antigens (ORF2 and ORF3) derived from different open reading frame of the Burmese of HEV expressed as a CMP-2-keto-3-deoxyoctulosonic acid synthetase fusion protein in Escherischia-coli .These samples were tested in accordance with the manufacturer´s instructions, and those with absorbance less than the Cut-off (CO) value were considered negative. Samples with the absorbance(S) greater than or equal to the CO value were tentatively considered reactive and then retested in duplicate to confirm the result. Results were recorded as S/CO ratios to allow comparison of the intensity reactions of individual samples. The samples were considered reactive when S/CO was higher than 1.0.

Fifty-nine turned to be positive and twenty-two reactive. This study confirms the high prevalence of anti-HEV in countries where clinical hepatitis E is not endemic. The reason for this study could reflect a mixed source of infection. A proportion of human HEV infections probably occur through exposure in the endemic regions since some of these patients were workers in these regions. But secondary spread of HEV infections between humans is not common (2% secondary cases compared to 15% for HAV) (Skidmore et al 1995), and the majority of the study population had no travel exposure , so this could explain a minority of cases (Christensen et al., 2002). We had access to serial specimen collected over months from 81 hepatitis E patients. This specimens demonstrated that IgM antibody levels were very high soon after illness onset, declined little over several weeks, and then declined rapidly to low levels over next 4 to 6 months. This is typical of IgM responses to other acute, self-limited, systemic viral infections (Seriwatana et al.,2002).

The weeks-long duration of markedly elevated IgM levels after disease onset means that the diagnosis using relatively sensitive IgM detections methods should be successful, even if patients come to medical attention late. We began this study anticipating that an HEV IgM

test would improve serological diagnosis on hepatitis E, then based on detection of HEV-specific IgG. We confirmed that the detection of HEV IgM is the best serological test for diagnosis of hepatitis E. Yet the most interesting aspect of our work was the observation that in some cases of hepatitis E, there was a weak or absent IgM response. By combining HEV IgM and IgG test, we identified primary immune responses among the hepatitis E patients in areas of HEV IgM to- total IgG ratio.

This is an important observation because previously, some authorities have speculated that waning immunity explained why most cases of hepatitis E among adults. The fact that more than 90% of hepatitis E cases in areas of HEV endemicity occur in patients who have a primary response refutes this speculation of waning immunity, since previously exposed persons should mount an anamnestic response upon reexposure (Seriwatana et al 2002).

3.2 Genelabs ELISA

Detection of antibodies in sera of patients with Hepatitis E by use of Genelab assay.

Sixty-seven patients with clinical symptoms of acute hepatitis were diagnosed as having hepatitis E on the basis of detection of antibodies by the Genelabs assay. These 67 samples were tested by EIA based on the recombinant polypeptides from HEV genotypes

encoded by ORF2 and ORF3 of HEV genome (Genelabs Diagnostics, Singapore). The assay was performed according to the instruction of the manufacturer. The results showed that twenty-two of this samples were positive and forty-five were negative. These tests were conducted at least three times. HEV antibodies were determined by ELISA of distinct antigenic specificity. In this study we observed a wide range of sensitivity and specificity. This information implies that this assay might be unreliable for the diagnosis of HEV infection in areas where hepatitis is not endemic. However, most anti-HEV assays have not been correlated with the HEV RNA determined by reverse transcription (Lin et al., 2000). In this study, we evaluated the diagnostic value for acute hepatitis E patients and in long-term expatriates of commercial anti-HEV IgG and IgM enzyme linked immunosorbent assays (ELISA) relative to RNA detection. The prevalence of anti-HEV among the general population in Frankfurt was also re-evaluated with this assay. We found a fairly good sensitivity (70%) of the IgG anti-HEV for the diagnosis acute hepatitis E verified by HEV RNA. However, the

sensitivity (50%) of the IgM anti-HEV appeared to be less satisfactory. In previous reports, anti-HEV detection had a wide range of sensitivity and poor concordance among different assays (Mast et a.,1998).

The sensitivity of IgG anti-HEV in this study was comparable to that of previous reports (Favorov et al.,.1998) and (Meng et al ., 1999).

However, the sensitivity of IgM was relatively poor. Our study might underestimate the sensitivity of these assays, since about five of the IgG anti-HEV-negative serum samples has tested positive for IgG anti-HEV with the same kit in a previous test. These discrepant results might be due to low-titer antibodies having been destroyed by repeated freezing and thawing in the later test. There are three possibilities for low sensitivity of IgM anti-HEV in this study. The first, delayed sampling, might account for negative IgM anti-HEV in some patients. Although both HEV viremia and serum IgM anti-HEV were short-live (Koshy et al.. 1996), protracted viremia has been reported for as long as one to four months in some patients (Nanda et al.,1995) IgM anti-HEV might have declined to an undetectable level before disappearance of HEV RNA. The second possible explanation is sequence variations among different HEV genotypes. It was reported that IgM anti-HEV were detectable in a patient with HEV strain US-1 using an assay based on Burmese and Mexican strain (Ferguson et al., 2002). It is likely that IgM anti-HEV have been undetectable in some of patients infected with a same genotype HEV using the same assay based on different genotypes (Tian-Chang et al.,2000). Finally, a poor host immune response to HEV infection might also account for undetectable IgM anti-HEV in some of our patients, as a evidenced by lower IgG anti-HEV optical density values in acute hepatitis E patients who were negative for IgM anti-HEV. We observed that, antibodies status may differ with the stage of disease, and screening of a population with a significant number of individuals in a convalescent phase could give results from those present study in terms of immunoreactivity to each of the three recombinant polypetides (Younchun, W et al.,.2001). The results of our study suggest that some patients diagnose provisionally as having non-A to non B–Hepatitis in Frankfurt may in fact, have hepatitis E and a single test for anti-HEV IgG is insufficient for diagnosis. The relatively low positive rate of circulating antibodies (observed in out test IgG anti-HEV) in the populations of the endemic areas combined with the unexpectedly high prevalence of anti-HEV in non-endemic countries like Germany make the interpretation difficult. Because of lack of antibody

detection assays the significance of HEV infection, that is without doubt a serious infection in developing countries, might be underestimated in developed countries (Schlauder et al 1999).

In conclusion, the overall sensitivity and specificity of the Genlabs ELISA seemed to be the same as those shown in previous publications (Li et al, 1998) and (Mast et al, 1998), probably because both the present and the previous assays used the baculovirus –expressed capsid proteins. The serological tests with sera collected from hepatitis E patients indicated a that the circulating IgG antibody was maintained at a high level.

3.3 Mikrogen RecomBlot:

Detection of anti-HEV IgG in sera from patients with hepatitis E.

To select conditions for a sensitive diagnostic assay and to examine the dynamics of antibody responses to each fusion proteins, Western blots were conducted to detect anti-HEV IgG from sera from patients with hepatitis E. Out of forty-nine sera selected, thirty-six were positive, five were reactive and eight negative. Each sample was tested at least three times in single wells. Any sample which was positive on the either initial test was retested in duplicate. Samples in which both duplicate wells were positive were designated as a confirmed positive, all other were considered negative. A Western blot assay was developed for detection of anti-HEV with three HEV-GST fusion proteins. GST-ORF2.1 and GST-ORF2.2, which encoded by portions of ORF2

Overlapping at the entire ORF3 from a Chinese strain of HEV. This assay proved to be sensitive and specific for HEV in tests with sera from patients with different types of acute hepatitis, and to our knowledge it is the first assay described which detects long –lasting antibody reactivity in a high proportion of patients and experimentally infected animals (Zhang et al.,2002). The detection of this persistent antibody reactivity represents an important advance in the understanding of immunity to HEV infection.

Many serum specimen used in this study were previously tested for anti-HEV IgG with commercial Abbot tEIA and Genelabs ELISA. This Western blot therefore, shows utility as a diagnostic assay for HEV infection. While IgM and IgG –class antibodies were detected in this study, previous publications show a high specific IgM reactivity against the fusion proteins

in acute-phase hepatitis E sera. However, we observed that the reactivity is not consistent. The molecular cloning of HEV genome (Li et al, 1994 and Jameel et al 1999) and the expression of recombinant proteins have allowed the development of numerous diagnostic and research immunoassays. However, problems remain both with specificity of some assays particularly when applied to seroepidemiological study as observed in our study (Thomas et al 1997; Mast et al 1998). One major problem in the seroepidemiological studies of HEV infection is the variable reactivity of recombinant antigens with respect to detection of past infection (Mast et al 1998; Scholfield et al 2000). Many HEV antigens, including synthetic peptides, recombinant antigens based on ORF3, and most fragment of ORF2 when expressed in Escherichia-coli, have reactivity with acute phase sera but little or variable reactivity with convalescent sera (Mast et al 1998). For example, the ORF2 antigen 3-2(M) was reactive with IgG from 91% of HEV-infected patients from Egypt at the time of admission , but fell to between 27 and 50% at 6-12 months after admission., while analogous 3-2(M) antigen was unreactive with only 64% of the sera at admission and none after (Goldsmith et al 1992) Conversely, the 3-2(M) was unreactive with convalescent sera from patient infected in Pakistan, while the 3-2(B) antigen reactive with sera collected from patients 4.5 years after illness (Dawson el al, 1992). Thus while many patients become unreactive, other patients maintain high levels of antibody to these proteins, which makes the interpretation of reactivity difficult as shown in this study. This may also be true of the ´mosaic´antigens (Favorov et al , 1996) expressed in E. coli as represents the fusion of a number of linear peptide epitopes. As consequence of the variable , but generally low rate or reactivity with convalescent sera, these antigens have had some use for the diagnosis of acute infection in non-endemic areas by the detection of HEV-specific IgG , but prevalence of past infection in endemic areas must be underestimated .

Conversely, a second group of recombinant antigens appear to demonstrate consistent reactivity with both acute and convalescent phase sera. These antigens include HEV virus-like or subviral particles (SVP´s) formed by truncated ORF2 expressed in insect cells using the baculovirus system (Tsarevet al, 1993; Li et al, 1999) and ORF2.1 expressed in E.coli. Such antigens are logically the most suitable for seroepidemiological studies, although the titre of anti-HEV may decline rapidly following the acute phase, it appears to remain at detectable levels for many years (Favorov et al, 1996).

The observations that the presence of such antibody correlates with protection from infection during epidemics (Bryan et al,1994) and that passive immunisation is sufficient to confer immunity to HEV disease in macaques (Tsarev et al, 1994) underline the practical value of detection this log-lasting antibody to HEV. Ghabral et al,1998 have recently compared assay for HEV-specific IgG and IgM using recombinant antigens from either baculovirus-expressed SVP´s or E. coli expressed fragments of ORF3 and ORF2. Their findings demonstrate the improved sensitivity of the SVP´s ELISA for detection of past HEV infection, but the detection of very high rates of reactivity in presumed non-endemic populations in the USA using this assay (Thomas et al,1997) raise the questions regarding the specificity of the assay for IgG-anti HEV. The results of this study also have implications for the development for the development of more convenient assays for HEV antibody by other methods. First, the reactivity of the HEV fusion proteins in Western blot appears to be greater than that of native, synthetic proteins in an Abbott-EIA (Dawson et al., 1994).

3.4 Diagnostics Systems Laboratories (DSL) ELISA

Acute hepatitis E has been rarely been reported in industrialized countries, but the rate of seroprevalence of hepatitis E virus (HEV) is inappriopriately high. The sensitivity and the specificity of the assay used to test for immunoglubulin G (IgG) and IgM anti-HEV have not been well established in areas where hepatitis is not endemic (hereafter referred to as ´non-endemic areas´). Enzyme immunoassay based on recombinant proteins of HEV have been used for most prevalence studies. The recombinant proteins contain immunodominant epitoped encoded by open reading frame (ORF2) and (ORF3) of the HEV genome from Burmese strain. A wide range of sensitivity and specificity has been reported for this assay (Bhaduree et al 1998). This information implies that this assay might be unreliable for the diagnosis of HEV infection in areas where hepatitis E is not endemic especially Germany. We collected serum samples from sixty one HEV patients and to test for IgG anti-HEV by enzyme-linked immunoabsorbent assay (ELISA). Forty three were positive for IgG anti-HEV, fourteen were negative for anti-HEV and four borderline for IgG anti-HEV. IgG class were measured according to the Diagnostic Systems Laboratories (DSL) procedure.

3.5 Concordance Between Assays

3.5.1 Evaluation Of Abbott EIA Versus Genelabs ELISA

The results of serologic tests for hepatitis E have varied widely from laboratory to laboratory, making interpretation of seroepidemiologic studies difficult. This study compares serologic results with different antigens and tests developed by two laboratories (Abbott EIA and Genelabs ELISA) for their ability to diagnose hepatitis E and measure antibody prevalence in area where hepatitis E is not endemic. The concordance between both assays in specificity and sensitivity were 34,4% but 50 % disagreement. Our results does not differ from other reported publications (Purcell et al., 1998), (Aggrawal et al, 2000), (Seriwatana et al, 2002). The performance of tests for antibody to the hepatitis E virus anti-HEV is an important factor in accessing the epidemiology of hepatitis E infection. Although these assays are specific, they have limited sensitivity: anti-HEV has been detected in only 50% to 70% of patients with acute hepatitis during hepatitis E outbreaks, and anti-HEV titres decline to subdetectable levels within several months after acute detection. Several recombinant protein-based tests have demonstrated increase sensitivity compared to prior assays, detecting anti-HEV in 90% to 95% of patients with acute hepatitis during outbreaks of hepatitis E in HEV endemic areas (Mast et al, 1997).

Recently, a number of cases of hepatitis E, diagnosed on the basis of serologic testing, have been reported among persons who had no history of travel to endemic areas. (Christensen et al, 2002).

However, the interpretation of these findings is problematic because few data are available to evaluate the performance of anti-HEV assays for diagnosis of acute hepatitis E in this setting. In addition, the performance of these assays in detecting anti-HEV in persons with remote infection is unknown, and several studies have reported unexplained positive anti-HEV results among persons who do not have disease or unknown exposure to HEV (Thomas et al., 1997). We present the findings of a serum panel evaluation conducted to access the sensitivity and specificity of available tests for anti-HEV and to assess the variability in detecting anti-HEV among tests. The results of this study indicate that several of the recombinant protein assays have an adequate combination of sensitivity and specificity to perform well for this purpose. The peptide-based assays were generally much less sensitive

compared to the recombinant protein assays and are therefore likely to be non reactive in a high proportion of acute hepatitis E cases. Further comparative studies that includes testing for immunoglobulin M IgM –anti HEV would be useful to validate the performance of the recombinant protein assays for the diagnosis of hepatitis E. In addition, the performance of these assays for diagnosis of acute hepatitis E in persons who do not have a history of travel to HEV-endemic regions need to be determined.. In prior studies, HEV isolates from various geographic regions have been demonstrated to have at least one major cross-reactive epitope by a variety of serologic assays.(Favarov et al, 1994 ;Bradley et al 1998, Krawczynski et al, 2000).

However, substantial variation in detection of anti-HEV by these tests in acute and convalescent-phase has been found in sera from chimpanzees infected with HEV isolates from various geographic regions. One possible reason for this findings is differences in the geographic strain-specific antigenic domains in these tests (Scholfield et al, 2000). However, there is little variation in the RNA sequence of ORF2 among HEV isolate from various geographic regions. Moreover, some assays (Scholfield et al, 2000), did not detect the anti-HEV in chimpanzee sera even though these tests included ORF3 epitopes from the same geographic region as the chimpanzee inoculum. The seroreactivity of recombinant proteins may also vary if they are produced in different expression system or used in different tests formats (i.e.,Abbott EIA vrs Genelabs ELISA). In addition, all these assays were designed to detect human antibody, and the differences may exist in the ability of assay conjugate to detect chimpanzee antibody. However, if the assay conjugate were the reason for a test´s not detecting anti-HEV, the assay would be expected either to be non reactive in all the chimpanzee sera or to have a uniform decline in seroreactivity in chimpanzee sera compare with human sera.

Furthermore, seroprevalence studies among blood donors in some non-endemic countries have found an anti-HEV prevalence of 1% to 20%, which is relatively high compared to the low rate of clinically evident disease associated with HEV in these areas. (Paul et al, 1994 ,Yarbough et al, 1997). In one study, anti-HEV seroreactivity among persons living in non-endemic regions with increasing age and was associated with a travel to endemic regions findings that are consistent with prior HEV infection (Yarbough et al., 1997). Thus, the interpretation of seroreactivity among patients living in non-endemic regions is currently

problematic. We therefore suggest that, further studies need to determine highly discrepant results among blood donor sera. Studies are also needed to determine the significance of anti-HEV seroreactivity among persons living in non-endemic HEV areas, including the relation of seroreactivity to exposure to the recently discovered virus in pigs that is closely related to human HEV isolates (Purcell et al, 1997). Finally, improved tests are needed for use in seroprevalence studies in nonendemic regions and confirmation tests are needed to very the specificity of these assays as shown in our study.

3.5.2 Evaluation of Abbot EIA Versus Mikrogen RecomBlot

Despite advances in knowledge of the molecular of biology of HEV over the past decade, the diagnosis and seroepidemiological study of HEV infection have remained problematic. Although a large number of serological assays have been developed for HEV, (Mast el al, 1998), have demonstrated that there is a significant lack of concordance between many assays, with major problems of many assays failing to detect anti-HEV in a convalescent sera and/or against heterologous HEV strains. This was a particular problem with assays based on synthetic peptides and many recombinant proteins expressed in E. coli, however, assay based on SVP´s expressed in the baculovirus system (Mast et al 1998), and Western immunoblots based on the ORF2 protein Anderson et al, 1999), appear to have a satisfactory sensitivity for analysing convalescent sera and divergent strains. In our study, we found a concordance of 56% between Abbot EIA and Mikrogen Western blot and a disconcordance of only 9,1%. We have observed a high rate of divergent results in using both assays. The results show clearly an ELISA based on ORF2 fragment offers sensitive and specific of HEV-specific IgG in both acute and convalescent sera for divergent strains of human HEV. It is considered that the utility of this antigen is due to the efficient presentation of a highly conserved, confirmational epitope which is immunodominant in convalescent antibody response.(Anderson et al, 1999). It was observed recently that ORF2 also has optimal reactivity with convalescent sera from domestic pigs naturally infected with a swine HEV in Australia (Chandler et al, 1999). Taken together, the results suggest that the use of conserved epitopes such as ORF2 for detection of anti-HEV may be more effective than the use of multiple, strains-specific epitopes which is by definition dependent on identifying all possible strains of the virus .However, further studies will be required to establish the reactivity

of ORF2 with numerous distinct HEV genotypes which have recently been described. (Hsieh et al, 1998; Erker et al, 1999; Schlauder et al, 1999; Wang et al, 1999).

It has been shown in previous studies, that using SVP ELISAs levels of HEV-specific IgG fall by approximately 90% following the acute phase infection, but then remain stable (Tsarev et al, 1993; Bryan et al, 1994) This antibody is also thought to correlate with immunity to reinfection (Bryan et al, 1994), and it is therefore appropriate to measure the levels of specific anti-HEV rather than only the presence antibody. The above listed HEV epitope may one of reasons high divergent of these assays used in our study. In this regard, the quantitative characteristics of the IgG ELISA reported should prove very useful, and the availability of the international Reference HEV serum reported by Fergusson et al, 2002 should further assist in studies of HEV immunity. Previous reports of both Abbot EIA and Western blot assays for anti-HEV have described similar rates of detection for acute hepatitis E (Mast et al, 1998) however, the detection of past infection appears to be less efficient, with rapid decline in antibody reactivity within weeks and months.

It is believed that the IgG ELISA based on ORF2 antigen offers a reliable method for the detection and quantitation of antibodies against HEV. The results of this study also have implications for the more convenient assays for HEV antibody by other methods .The use of both assays should assist in greater understanding of HEV immunity and the epidemiology of HEV world wide.

3.5.3 Evaluation of Genelabs ELISA Versus Mikrogen RecomBlot

The ELISA reported here detects both anti-HEV-IgM and anti-HEV-IgG and it is a convenient method for the diagnosis of acute or past HEV infection. To characterize the relationship between antibody potency determined by a widely used commercial test kits. In this study, we compared the sensitivity and specificity of both assays Genelabs EIA and Mikrogen Western blot. We observed a concordance of about 61,4% and a high disagreement of about 27,3% between the assays. The commercial test employ a mixture of a recombinant HEV ORF2 and ORF3 polypeptides expressed in E. coli. The disagreement in our study have raised the question about the sensitivity and specificity of these current available test for ant-HEV as well as questions about how long anti-HEV can be detected after infection of HEV. The variable of persistence of IgM anti –HEV and IgG anti-HEV makes it difficult to accurately determine the

frequency of prior exposure and raises the possibility of re-infection after disappearance of this antibody (Zhang et al.,2002).This might allude to a high disconcordance in our study. The cloning of HEV and the availability of EIA ´sand GST fusion protein for detection for detection of anti-HEV have created a major breakthrough in our understanding of epidemiology and the clinical course of hepatitis E. However, it must be realized that the current diagnostic assays for hepatitis E are sub-optimal. False positive and false negative results may occur, as was the case in this study.

Although, there is a high degree of homology among HEV sequences from Pakistani and the Burmese isolates, the homology between Mexican and Burmese isolates is less.(Purcell et al, 1998). Thus, EIA that utilize HEV antigens from one ORF or one isolate my yield negative results. In all these studies in which multiple HEV antigens in both ORF2 and ORF3 from Burmese and Mexican isolates were used, the detection rate for individual antigens from 0 to 100% among the reactive samples. (Krawczynski et al. 1999). Comparative studies with HEV antigens from other isolates have not been conducted. However, it is obvious that multiple HEV antigens from more than one isolate have to be incorporated into anti-HEV EIA´s for the diagnosis for hepatitis E in diverse geographical areas (Mast et al, 1998). There is less information on the specificity of the current anti-HEV assays. Confirmatory tests for HEV infection has to be developed. Direct comparison between the reactivity with recombinant HEV antigens and the corresponding synthetic peptides showed that smaller proportion of healthy subjects reacted against the synthetic peptides in the IgG anti-HEV assays, suggesting that EIA using recombinant antigens may give false positive results. (Seriwatana et al, 2002). Similar comparison has to be conducted on the IgM anti-HEV EIA´s as reviewed in this study.

3.5.4 Evaluation of Abbott EIA Versus DSL ELISA

The results of serologic tests for hepatitis E virus have varied widely from laboratory to laboratory, making interpretation of seroepidemiologic studies difficult.

The present comparison of tests for anti-HEV is first to make a detailed examination of the utility of the many gene products which have been utilized for assays for anti-HEV .In our study we compared the reactivity of anti-HEV IgG in both assays Abbot EIA and DSL ELISA. We observed a concordance of 54% and a disconcordance of 13,6%. The results of this test clearly showed that tests for anti-HEV based upon expressed ORF2 were more sensitive for detecting anti-HEV than were tests based upon antigens derived from ORF3 (Ghabrah et al, 1998). This was true for both IgG anti-HEV and IgM anti-HEV. The poorer showing of ORF3-based tests may result from a combination of a less vigorous immune response to this small protein and a shorter half-life of antibodies to ORF3. We observed a wide range of sensitivity and specificity as shown in our results 54% concordance. The reasons for the discrepancy between both assays are not clear. We probably believed , however, that these assays of the IgG class antibodies often missed remote HEV infections because of their low sensitivity in areas where hepatitis E is not endemic. Thus, a reliably sensitive and specific antibody assay is needed to conduct accurate epidemiological studies. Further reason for a wide range of discrepant results might be due to low-titre antibodies having been destroyed by repeated freezing and thawing in later study. Another possible explanation for decline detectability of IgG antibodies in these assays might be sequence variations among the different HEV genotypes. Specifically, the choice and size of antigen appears to make a significant difference in the results (Nishizawa et al, 2003). Finally a poor host immune response to HEV infection might also account for undetectable IgG anti-HEV optical density values.

4 Discrepancies

These findings have raised the questions about the sensitivity and the specificity of the currently available test for anti-HEV as well as questions about how long anti-HEV can be detected after infection .For instance, Goldsmith et al 1992 found out that IgG anti-HEV could be detected for less than 6 months in about half tested children who were convalescing from hepatitis E but Bryan et al 1994 found all of 33 convalescent adults to be positive 20 months after infection and anti-HEV has been detected by Kharoo et al 1993 as long as 14 years after clinical hepatitis E in India. Many of these unexpected results and discrepancies can be ascribed to differences in assays for the anti-HEV. Specifically, the choice and the size of the antigen, appears to make a significant difference in the results. Assays for anti-HEV based on antigenic epitopes of the ORF-3 gene product detect a lower prevalence of anti-prevalence suggesting these antibodies directed against epitopes of the products of ORF-2. This problems is compounded by a greater genetic heterogeneity of ORF-3 genes, possibly leading to serologic differences among different HEV strains, and diminished sensitivity of assays based upon only one or a limited number of genetic variants of the ORF-3 product. In contrast, gene products of ORF-2 are more genetically homogeneous and measure anti-HEV that remains detectable for years. Clinical disease data suggest that hepatitis E virus (HEV) is a pathogen with a restricted geographical range. The purpose of our study was to compare hepatitis E assays:

Abbott EIA, Mikrogen Westernblot, Genelabs and DSL ELISA (a Prototype) of their sensitivity and specificity and to evaluate their abilities to diagnose hepatitis E with acute sporadic viral hepatitis and convalescent phase sera in a routine test at the Virology Department of Frankfurt University clinics between 1998-2001.

Possible explanations of discrepances can be allude to the following reasons:

- a low sensitivity of available assays?
- specifically choice and size of HEV antigen?
- duration of antibody persistence ?
- subclinical (anicteric) HEV infection ?
- a cross-reactivity with a different agent ?
- a non-specificity leading to false positivity ?
- infection with non-pathogenic HEV strain, e.g. swine HEV or zoonotic strain ?
- and a combination of any of the above?

4.1 Problems and Conclusions

The study of Hepatitis E has been an instructive story recent advancements in medical science. Substantial progress has been made in a short period of time through both epidemiological studies and basic research, which has relied on modern cloning and recombinant technology. New tools have clearly accelerated the pace of research. Within less than two decades of the discovery of HEV, the major epidemiological features of this unique pathogen have been described, serologic tests have been developed, and a vaccine has been evaluated in an initial clinical trial. (Yabough et al., 1999).

Nevertheless, there are still may mysteries about the epidemiology, pathogenesis, and immune response of HEV infection. The unknown aspects of hepatitis E could have a major impact on vaccine development and use.. However, vaccine research could be also illuminate the immunologic characteristics of this unique infectious disease. The development of an animal model of HEV infection in the laboratory would aid research efforts substantially. It is likely that a HEV vaccine will be developed that provides at least short-term protection from disease.

Who will make and buy this vaccine? Because of incidence of acute HEV infection among travellers from developed world to endemic countries has been estimated to be less than one million (Piper-Jenk et al 2000), the market for this vaccine in non endemic countries will be limited.(Shlim et al., 2000). Pregnant women living in endemic regions may derive the greatest benefit from an effective HEV vaccine.

Our study has led us to draw the following conclusions:

- we observed a high rate of divergent results using three commercially available and one ´´prototype´´HEV IgG antibody assays on sera reactive by the Abbott EIA
- This is in agreement with previous studies that used variety of different assays.
- The interpretation of HEV antibody test results in ´´low-risk´´ populations remains problematic
- A ´´grey zone´´ needs to be defined as low-level reactivity is common and mostly cannot be confirmed by additional tests but is probably non-specific.

5 Summary

Hepatitis E virus (HEV) is a positive-stranded RNA virus with a 7.2 kb genome that is capped and polyadenylated. The virus is currently unclassified : the organisation of the genome resembles that of the Caliciviridae but sequence analyses suggest that it is more closely related to the Togaviridae. HEV is an enterically transmitted virus that causes both epidemics and sporadic cases of acute hepatitis in many countries of Asia and Africa but only rarely causes disease in more industrialised countries. Initially the virus was believed to have a limited geographical distribution. However, serological studies suggest that that HEV may be endemic also in the United states and Europe even though it infrequently causes overt disease in these countries. Many different animal species worldwide recently have been shown to have antibodies to HEV suggesting that hepatitis E may be zoonotic. Although two related strains have been experimentally transmitted between species, direct transmission from animal to a human has not been documented.

Our main objective in this study is to evaluate the suitability of current available HEV antibody assays for use in low-endemicity areas such as in Germany.

Methods: We selected sera on the basis of at least borderline reactivity in the routinely used Abbot EIA. Most were tested as part of routine screening of long-term expatriates in endemic countries. The following assays (recombinant antigens : ORF2 and ORF3) were used: Abbot EIA, Genelabs ELISA, Mikrogen recomBlot and a `Prototype´ DSL-ELISA.

We observed a wide range of sensitivity (average of 56.8%) and specificity (an average of 61.4%) in these used assays. These results implies that , these assays might be unreliable for detection of HEV infection in areas where hepatitis E is not endemic. However, most anti-HEV assays have not been correlated with the HEV RNA determined by reverse transcription. Many of these unexpected results and discrepancies can be alluded to the following reasons:

I. The choice and the size of the HEV antigen.
II. Duration of the antibody persistence
III. A cross reactivity with different agent
IV. Due to geographic species
V. A low sensitivity of the available assays.
VI. And infection with non-pathogenic HEV strain. (zoonotic strain?).

We therefore suggest that, further studies will be required to improve the sensitivity and specificity of the available commercial assays on the market.

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8 Zusammenfassung

Das Hepatitis E Virus (HEV) ist ein RNA+ - Virus mit einem 72 kb Genom, das behüllt und polyadenyliert ist. Das Virus ist momentan nicht eindeutig klassifiziert. Die Organisation des Genoms ähnelt der der Caliciviridae, aber Sequenzanalysen deuten darauf hin, dass es näher mit den Togaviridae verwandt ist. HEV wird enteral übertragen und verursacht sowohl Epidemien wie auch sporadisch auftretende Fälle von akuter Hepatitis in vielen Ländern Asiens und Afrikas, aber nur vereinzelt Erkrankungen in den industrialisierten Ländern.
Anfänglich glaubte man, das Virus besitze eine nur begrenzte geographische Ausbreitung. Serologische Studien lassen hingegen vermuten, dass HEV auch in den USA und Europa endemisch sein könnte, auch wenn es nur selten ansteckende Krankheiten in diesen Ländern hervorruft. In vielen verschiedenen Tierspezies weltweit konnten HEV-Antikörper nachgewiesen werden, so dass vermutet werden kann, bei der Hepatitis E handele es sich um eine Zoonose. Obwohl zwei verwandte Stränge experimentell zwischen zwei Spezies übertragen wurden, ist bislang keine direkte Übertragung vom Tier auf den Menschen nachgewiesen.

Das Ziel dieser Studie liegt darin, die Anwendbarkeit gebräuchlicher HEV Antikörper Assays für den Gebrauch in Niedrigendemiegebieten wie Deutschland zu evaluieren.

Methoden: Wir haben Sera ausgewählt, die im routinemässig verwendeten Abbott EIA mindestens grenzwertig reagiert haben. Die meisten wurden im Rahmen des Routinescreenings von Personen nach Langzeitauslandsaufenthalten (deutsche Entwicklungshelfer) in Endemiegebieten durchgeführt.
Folgende Assays (Rekombinante Antigene ORF2 und ORF3) wurden verwendet: Abbot EIA, Genelabs ELISA, Mikrogen recomBlot und ein „Prototyp“ DSL-ELISA.

Wir beobachteten eine große Spannweite in der Sensitivität (im Durchschnitt 56,8%) und Spezifität (im Durchschnitt 61,4%) bei den verwendeten Assays. Diese Ergebnisse legen den Schluss nahe, dass diese Assays möglicherweise für den Nachweis einer HEV-Infektion in Gebieten, in denen HEV nicht endemisch ist, unzuverlässig sind. Bislang ist die Mehrzahl der HEV-Assays nicht mit dem Nachweis durch Reverse Transkription hergestellter HEV-RNA korreliert worden.
Viele dieser unerwarteten und diskrepanten Ergebnisse können den folgenden Gründen zugeschrieben werden:

I der Auswahl des HEV-Antigens und dessen Größe
II der Dauer der Antikörper-Persistenz
III der Kreuzreaktivität mit verschiedenen Agentien
IV dem geographischen Subtyp
V einer niedrigen Sensitivität der verfügbaren Assays
VI und einer Infektion mit einem nicht-pathogenen HEV Strang (zoonotische Übertragung?).

Aus diesen Gründen hielten wir weiterführende Studien mit dem Ziel, die Sensitivität und Spezifität der am Markt verfügbaren Assays zu verbessern, für sinnvoll.

9 Lebenslauf

Persönliche Daten

Name:	Mark Twum-Barima Asante
Geboren am :	30.04.1973
in:	Asiakwa/ Ghana
Familienstand :	ledig

Schulausbildung

1979 - 1985	Grundschule Brooklyn, NY
1985 - 1991	Realschule Dallas TX
1994 – 1994	Berufliches Gymnasium, Wiesbaden Abschluss: Abitur
1994 - 1995	Ausbildung als Chemisch Technischer Assistent, Kerschensteiner Schule, Wiesbaden, Abschluss: CTA

Hochschulausbildung

10/95 - 02/99	Vorklinisches Studium an der Universität Rostock
04/99 – 10/03	Klinisches Studium an der Johann-Wolfgang-Goethe Universität, Frankfurt am Main
10/02 - 9/03	Praktisches Jahr
30.10.2003	3. Staatsexamen
Seit 11/03	Arzt in Praktikum, Bethanien Krankenhaus Frankfurt a. M., Cardiologisches Centrum
Promotionsarbeit	Prof. Dr. Hans-Wilhelm Doerr, Direktor des Institutes für Virologie, Johann-Wolfgang-Goethe Universität, Frankfurt am Main
	Thema: Hepatitis E: Comparative evaluation of IgG antibody assays in a low endemicity setting
Sprachen	Englisch in Wort und Schrift (Muttersprache) fließend Deutsch in Wort und Schrift

Persönliches Interessen

Sport	Basketball, Fußball
Musik	Gospels, und Klassisch

10 Ehrenwörtliche Erklärung

Ich erkläre hiermit ehrenwörtlich, daß ich die dem Fachbereich Humanmedizin der Johann Wolfgang Goethe-Universität Frankfurt am Main zur Promotionsprüfung eingereichte Arbeit mit dem Titel:

Hepatitis E - Virus (HEV):
Comparative evaluation of IgG antibody assays in a low-endemicity setting

Unter Leitung von Herrn Professor Dr. med. H.W. Doerr mit Unterstützung durch Dr. med. Wolfgang Preiser ohne sonstige Hilfe selbst durchgeführt und bei der Abfassung der Arbeit keine anderen als die der Dissertation angeführten Hilfsmittel benutzt habe.

Ich habe bisher an keiner in- oder ausländischen Medizinischen Fakultät ein Gesuch um Zulassung zur Promotion eingereicht noch die vorliegende Arbeit als Dissertation vorgelegt.

Frankfurt an Main, den 03.08.2003

Mark Asante