Chikungunya virus belongs to family Togaviridae, genus Alphavirus. It was first recognized in 1963 in Kolkata after that it has caused several outbreaks in India. According to earlier view this virus is known to disappear for a prolonged period of time after causing epidemic. During past 3-4 decades several strains have been isolated. Recently, this virus has been isolated and antibodies have been detected from Maharashtra state.

### Chikungunya Virus

Chikungunya virus belongs to family Togaviridae, genus Alphavirus. It was first recognized in 1963 in Kolkata after that it has caused several outbreaks in India. According to earlier view this virus is known to disappear for a prolonged period of time after causing epidemic. During past 3-4 decades several strains have been isolated. Recently, this virus has been isolated and antibodies have been detected from Maharashtra state.

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#### 1. Prevalence of Chikungunya virus infection in India.

J P Thakare
D T Mourya, S Hundekar
jyothakare@hotmail.com

**Objectives**

- To carry out retrospective and prospective study to know the prevalence of CHIK virus by means MAC ELISA and by haemagglutination (HI) test.
- To study the prevalence of CHIK virus infection in DEN endemic as well as non-endemic Areas.

**Achievements**

- Mab based MAC ELISA to establish recent virus infection from human samples was standardized incorporating samples collected during CHIK virus epidemic at Barsi, 1973. Mab based antigen capture ELISA to detect virus from field-collected mosquitoes was also standardized. Mab against E, glycoprotein of African CHIK virus strain was kindly supplied by department of Virology.
- To study the activity of virus infection in population, 675 samples collected from fever cases during a period of 2000-2003 March, mainly from Maharashtra, Gujarat, Kerala and Andhra Pradesh were tested in MAC ELISA.
- The samples positive for virus specific IgM antibodies were treated with 2- Mercaptoethanol. (2ME) and tested in MAC ELISA. There was a drop in optical density readings as compared to the results obtained in samples without 2 ME treatment. The results indicated the true positivity of IgM results.
- ELISA results indicated that patients were recently infected with CHIK virus and thus supports the evidence of prevalence of virus although at a lower rate

#### Location Samples Tested / positive

<table>
<thead>
<tr>
<th>Location</th>
<th>Samples Tested / positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solapur</td>
<td>70/1</td>
</tr>
<tr>
<td>Kolhapur</td>
<td>153/5</td>
</tr>
<tr>
<td>Nagpur</td>
<td>70/3</td>
</tr>
<tr>
<td>Vidarbha</td>
<td>133/8</td>
</tr>
<tr>
<td>Kerala</td>
<td>6/0</td>
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<tr>
<td>Nanded</td>
<td>21/1</td>
</tr>
<tr>
<td>Satara</td>
<td>11/0</td>
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<tr>
<td>Pune</td>
<td>82/3</td>
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<tr>
<td>Raigad</td>
<td>14/0</td>
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<tr>
<td>Bhor</td>
<td>13/0</td>
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<tr>
<td>Vidarba</td>
<td>133/8</td>
</tr>
<tr>
<td>Total</td>
<td>865/32</td>
</tr>
</tbody>
</table>

#### Future Plans

Screening of samples for PCR based diagnosis of CHIK virus infection will be continued.
2. Establishment of diagnostic RT-PCR for Chikungunya virus

D T Mourya
P Yadav
mouryadt@vsnl.net

Objectives
To establish RT-PCR based diagnostic method for Chikungunya

Achievements
i) RT–PCR was standardized for the genus specific detection of Alphaviruses viz. CHIK, Semliki Forest virus based on conserved region within NSP1 gene. Sensitivity, was found to be ~ 1200 pfu for first PCR but~ 20 pfu for semi-nested PCR.

ii) Diagnostic RT–PCR was standardized for the Chikungunya virus specific detection based on conserved region within NSP1 gene. Sensitivity, was found to be ~ 5-10 pfu.

Results of RT-PCR carried out using Chikungunya virus specific (NSP1 region) primers on the mouse brains negative and positive for CHIK virus.

Future Plans
Samples suspected for CHIK virus infection will be screened for diagnosis by this method.

3. Studies on geographical lineages and evolutionary relationship of CHIK viruses isolated in India.

D T Mourya
P Yadav
mouryadt@vsnl.net

Objectives
To determine the possible origin of Indian strains of CHIK viruses
To determine whether virus is maintained in some occult cycle in nature or completely disappears from an area during inter-epidemic period

Achievements
RT–PCRs Data based on 257bp sequence of E1 gene showed that there were three clades. All the Indian strains fall in the Asian genotypes except the strain from Vishakhapatnam (655873) isolated in 1965, showed ~ 100% homology with Central African strains. However, based on these sequences another strain from Vishakhapatnam (655855), which was also isolated from same area in the year 1965, showed more homology with other Asian isolates from Thailand. It is interesting to note that a recently isolated strain from Yawat, Maharashtra (2000) and certain other strains from central part of the country that were isolated during 1960s and 1970s belonged to the same clade in Asian genotype.

It is surmised from the data that during the span of last 37 years several strains were introduced in this country and many of them were re-circulating in different geographical areas. It is probably maintained in occult cycle.

Phylogenetic tree based on 257 bp partial E1 gene sequence:

Future Plans
Future isolates of CHIK virus will be processed similarly to up-date the phylogenetic tree.
4. Partial Sequencing of an Indian strain of Chikungunya Virus  
(Partial nsp4 protein and complete 26S RNA)  
S N Randive  satishranive_n@yahoo.com  

Objectives  
- To obtain full sequence of structural genes of chikungunya virus  
- To determine possible origin of CHIK strains in India.  
- To develop strategies for production of a vaccine  

Achievements  
Complete nucleotide sequence of structural genes of Indian strain is determined. The homology (identity) of the nucleotide and amino acid sequences of CHIK virus (Nagpur strain-653496) were compared with other alpha viruses by constructing Phylogenetic tree. The results showed that the Indian strain of CHIK virus  (Nagpur ,653496) was very closely related (98 percent identity) to African strain L37661 which was isolated from Tanzania in 1953. Analysis also indicated that the O’ nyong-nyong virus is the closely related to CHIK virus. However amino acid analysis showed that it forms an antigenically distinct group.

Future plans  
Data will be used to predict B-cell and T-cell epitopes on E1 and E2 proteins for developing vaccine.

5. Role of gregarine parasite Ascogregarina culicis  
(Apicomplexa: Lecudinidae) in the maintenance of Chikungunya virus in vector mosquitoes  
D T Mourya  mouryadt@vsnl.net  
P Yadav, M D Gokhale, P V Barde  

Objectives  
- To determine role of gregarine parasites in the maintenance of CHIK virus  

Achievements  
Ascogregarina culicis and As. taiwanensis are common gregarine parasites of Aedes aegypti and Ae. albopictus mosquitoes respectively. These mosquito species are also known to transmit dengue and Chikungunya viruses. The sporozoites of these parasites invade the midgut epithelial cells and develop intracellularly and extracellularly in the gut to complete their life cycles. The midgut is also the primary site for virus replication in the vector mosquitoes where these parasites develop. Therefore studies were carried out with a view to determine their possible role in the vertical transmission of dengue and Chikungunya viruses from larval to adult stage and in the maintenance of viruses in the oocysts in nature. Direct vertical transmission and the vertical transmission of CHIK virus through the oocyst of the parasites were observed in the case of Ae. aegypti mosquitoes. It is surmised that As. culicis may have important role in the maintenance of CHIK virus during the inter-epidemic period.

Experimental protocol  
Ascogregarina oocysts + 1st instar larvae + virus in 5ml water  
Emerging adults held for 2-3 days before trituration  
Homogenates dried on filter papers and stored at RT for 5 days  
1st instar larvae fed on the fresh homogenates.  
One part used for the experiment  
Second part stored at 80oC & used for detection of virus by RT-PCR  
Fresh 1st instar larvae fed on dried oocysts of parasites  
Emerging adults screened for virus byMice inoculation ELISA / RT-PCR  

Future plans  
Project is concluded.
6. Experimental transmission of Chikungunya virus by Anopheles stephensi mosquitoes

P Yadav
D T Mourya
M D Gokhale

Objectives
- To determine possible role of Anopheline mosquitoes in the transmission of CHIK virus

Achievements
CHIK is considered as re-emerging viral disease and recent reports suggested that this virus has not disappeared from India but maintained at low level. Aedes aegypti mosquito has been incriminated as the principal vector. CHIK epidemics usually occur in urban situations. Anopheles stephensi is another highly endophilic and anthropophilic mosquito hence during epidemic there is very high probability of this mosquito to feed on CHIK infected patients and pickup the virus. Therefore, study was conducted to determine CHIK virus transmission capabilities of An. stephensi mosquito. Results showed that this mosquito species is capable of transmitting CHIK virus.

Future plan
Project is concluded.

7. Cloning of E1 gene of Chikungunya in bacteria and Baculovirus expression vector

P Yadav
D T Mourya

Objectives
- Cloning of E1 gene of CHIK in bacteria and Baculovirus expression vector

Achievements
cDNA was synthesized from the viral RNA using a oligonucleotide primer. PCR amplification was performed on the first strand cDNA using the poly(T) primer and a forward primer designed to anneal to genome positions 10344 to 10360, covering the carboxy-terminal portion of the E2 envelope glycoprotein gene. PCR products (1.2kb) were cloned using TOPO-TA vector and white bacterial colonies screened for plasmids containing inserts of the correct size. The clones were sequenced using the plasmid-specific T7 promoter and m13 reverse primers combined with internal, CHIK virus-specific primers (C3205, 5' GCRACAAACCCAGTAAG 3'; C3152, 5' ACTGGCTRAAAGAACGAGG 3') for confirming positive clones.

For cloning and over expression of Chikungunya surface protein, based on the complete sequence of the open reading frame of Chikungunya E1 surface protein, primers were designed that allow amplification of complete open reading frame. Restriction endonuclease sites were added to the primers to facilitate cloning of amplified product. The clones generated for the phylogenetic analysis were used for amplifying ORF using these primers. This product is being cloned in Baculovirus expression vector pBlueBacHis2 (Invitrogen) that will allow over expression as well as single step purification of expressed protein.

Future plans
Project is concluded since sensitive RT-PCR based diagnostic method was established.
Hepatitis

<table>
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1. Development of candidate vaccine for hepatitis E.

T M Deshmukh
K S Lole, VA Arankalle

Hepatitis E is endemic in India and presents in epidemic as well as sporadic forms. Several large-scale epidemics are reported every year from urban as well as rural parts of India. During epidemics, there is considerable mortality among pregnant women. In sporadic settings, fulminant hepatitis E has been observed in men and non-pregnant women. There is a need for hepatitis E vaccine.

Objectives
Development of candidate vaccines:
- Recombinant protein-based
- DNA-based
- Prime and boost approach based

Achievements
HEV structural protein (ORF2) expressed in baculovirus system was found to react with sera from epidemics (1976-2003) of hepatitis E from different parts of the country as well as sporadic cases of NANB (1978-2003). This protein remains membrane bound in Sf9 cells and is unstable. Truncated form of this protein (with N-terminal 111 amino acid deletion) was expressed in the same system. This protein is expressed in a soluble form in Sf9 cells. HEV-ORF-2 was expressed in quantities sufficient for purification.
HEV ORF2 gene was cloned into mammalian expression vector pcDNA 3.1. Transient expression of ORF2 protein is being checked in COS7 cells by transfection assays. For co-administration of cloned structural genes of HEV and GM-CSF gene in the DNA vaccine, mouse GM-CSF gene was TA-cloned. The gene was cloned into pcDNA3.1. Later in the year, Genegun to be used for animal inoculations was received.
In the absence of monkey facility, titration of challenge virus pool was not possible.
Hepatitis

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Future plans
Purification of ORF-2 protein and mice immunization with DNA and protein and assessment of immune responses will be undertaken.

2. Cloning and sequencing of the entire genome of swine HEV and expression of ORF-2 & ORF-3 proteins

LP Chobe
K S Lole, VA Arankalle
leenachobe@hotmail.com

Zoonotic spread of HEV has been shown in different countries. Our earlier studies have shown circulation of different genotypes in humans (Type I, 1976-2003) and pigs (type IV, 1985-2000). Type I HEV could not be transmitted to pigs and rats. In order to understand the role of swine HEV in causing human infections it is necessary to express and assess immunoreactivity of swine HEV proteins of diagnostic significance. Genomic characterization of swine HEV in circulation is of utmost importance.

Objectives
• To clone and sequence entire genome of swine HEV
• To express ORF-2 and ORF-3 proteins and evaluate immunoreactivity
• To use the sequence information for the generation of full-genome cDNA clone

Achievements
The entire genomic sequence of swine HEV including 3' and 5' ends was determined. The sequence is being analysed for various parameters. Swine HEV ORF2 protein was expressed in baculovirus expression system. This protein reacted very well with swine (genotype-IV) as well as human (genotype I) sera. Truncated form of this protein (N-terminal 111 amino acid deletion) is also expressed to facilitate purification.

Blood samples were collected from pigs

Fig. 10. HEV Immuno-electron micrograph
3. Generation of infectious cDNA clones for swine and human HEV and chimeric swine-human HEV clones

K S Lole
VA Arankalle
lolekavita37@yahoo.com

Though difficult, generation of infectious cDNA clones has proved useful in the study of host-virus interactions, mechanisms of viral replication. In the absence of in-vitro cell culture system generation of infectious cDNA will prove useful in basic studies. Mechanism of species specificity of HEV could be understood.

Objectives
- To generate infectious cDNA clone for swine HEV and human HEV.
- To generate chimera of swine and human HEV.

Achievements
We have generated sequence for the whole genome of Indian swine HEV. Based on this sequence, primers were designed for the construction of infectious cDNA. The swine genome was amplified in five overlapping fragments including the 5' and 3' ends. These fragments were individually TA-cloned. After confirming the sequences and the orientations, these clones were digested with restriction enzymes and ligated together to reconstitute the complete genome cDNA sequence in pGEM T-EASY vector (Promega).

Future Plan
1) To study infectivity of the swine HEV cDNA clone.
2) To generate infectious cDNA clone for human HEV. A chimeric cDNA will also be constructed and tested for infectivity.
4. Assessment of host/virus factors leading to fulminant hepatitis E and A

A.S. Tripathy anuradha.tripathy@hotmail.com

M S Chadha, V A Arankalle

Our earlier studies have clearly shown that in sporadic settings, HEV and HAV respectively are mainly responsible for fulminant hepatic failure among adults and children. High mortality among pregnant women remains the characteristic feature of HEV epidemics, though a significant proportion of such women experience subclinical infection. Risk factors for development of fulminant hepatic failure (FHF) are yet to be ascertained. Study of mechanism of FHF may lead to development of appropriate treatment protocols and identification of prognostic markers.

Objectives
To attempt to understand the mechanism(s) of fulminant hepatic failure following hepatitis E and A infections.

Achievements
Cytokine levels were measured in the supernatants of lymphocyte cultures using enzyme linked immunosorbent assay (ELISA). For hepatitis E, cases of early acute (n=3), acute (n=15), convalescent (n=10), sub-clinical (n=5) and past HEV infection (n=10) were examined to measure both the Th1 and Th2 cytokine profiles. Eight (n=8) healthy men and ten (n=10) healthy pregnant women controls were also included. Acute-resolving HEV patients had a Th1 type of cytokine profile, with significant elevation of both IFN-γ and IL-2 when compared with IL-4 and IL-10. The convalescent and sub-clinical cases did not have any distinct elevation or suppression of any specific cytokine. The normal healthy men and pregnant women had base level secretion of Th1 and Th2 cytokines.

For hepatitis A category, spontaneous release of cytokines was studied in sera of acutely infected HAV individuals (n=32). IFN-γ levels were significantly elevated (p<0.005) when compared with IL-10. Serum IL-2 levels were significantly elevated (p<0.005) when compared with IL-4. Thus, self-resolving acute hepatitis A patients showed a Th1 type of cytokine profile at the serum levels.

Future plan
With all the methodologies in place, we will now initiate examining FHF cases. Number of patients in different categories will also be enhanced. HLA status of the self-resolving and fulminant hepatitis patients will be studied.


M S Chadha mscniv@hotmail.com
C R Raut, S R Vaidya, V A Arankalle

Recent data generated in several countries suggest zoonotic spread of HEV. In developing countries with inadequate sanitation and overburdened public health infrastructures, more than 50% of sporadic cases of viral hepatitis can be attributed to HEV. The predominant mode of HEV transmission is by fecal oral route. Hence, sewage treatment plant workers, water treatment plant workers and safai kamgars from corporations may be at increased risk for HEV. To study the role of animals in the spread of hepatitis E among humans, it would be important to evaluate the risk of animal handling in exposure to this virus. Similarly, other high-risk populations need to be studied to evolve definite preventive strategies, if necessary.

Objectives
Determination of occupational risk of HEV infection among:
- animal handlers
- Sewage Treatment Plant workers
- Water Treatment Plant workers
- Safai Kamgars from Pune Municipal and Pimpri Chinchwad Municipal Corporations.
- To look for different HEV genotypes in acute cases of hepatitis among animal handlers.

Achievements
This year, 21 pig handlers from Uttamnagar, Pune were bled to determine exposure to HEV in them. Nine persons were found to be IgG anti HEV positive.

Future plan
Additional samples will be collected from animal handlers. Further work will be done in relation to other occupational risk groups.
6. Assessment of role of HCV HVR1 and host HLA status in influencing progression of hepatitis C and response to antiviral therapy.

S P Shrotri sandhyashrotri@yahoo.com
A S Tripathy, M S Chadha, V A Arankalle

Hepatitis C virus (HCV) is an important cause of chronic liver diseases worldwide. There are six major genotypes of HCV exhibiting a high degree of genetic heterogeneity. The greatest degree of heterogeneity is observed in the N terminal region of the envelope region (E2) and is known as the hypervariable region 1 (HVR1). Evolution and changes in this region have been shown to be associated with the outcome of HCV infection and success of antiviral therapy. Further, HLA alleles of the patient may influence the severity of disease and response to interferon therapy.

Objectives
To assess the role of
- HVR1 heterogeneity
- HLA status of the host
In relation to disease progression and success of antiviral therapy.

Achievements
Sample Collection :

i) Asymptomatic HCV infected Individuals :
35 blood donors identified as anti HCV positive (using second generation anti HCV testing kits) from Nagpur (n=29) and Pune (n=6) were screened for anti HCV positivity using a third generation anti HCV kit (Ortho Diagnostic). Only one confirmed positive sample was identified.

ii) Chronic HCV individuals :
A total of 8 chronic hepatitis C patients referred to NIV were investigated for HCV RNA and ALT levels. PBMC’s and plasma were separated and stored at -70°C for subsequent analysis.

HCV Genotyping :
Core region of the HCV genome was amplified and sequenced to determine the genotype on the basis of phylogenetic analysis. Genotyping of 53 HCV RNA positive samples including 8 asymptomatic carriers 4 chronic and 41 patients undergoing interferon therapy were completed. The prevalent genotypes were: 1a = 5, 1b = 9, 1c = 3, 3a = 20, 3b = 8, 3f = 1, 3g = 4, 3i = 2, 4d=1

Amplification of the HVR1 :
A total of 36 samples were amplified in HVR1 region using genotype specific primers. These included asymptomatic carriers (n=7), patients undergoing interferon therapy (n=16) and stored HCV RNA positive serum samples from chronic hepatitis C individuals (n=13). PCR product sequencing for 21 of these samples was completed.

Cloning of HVR1 PCR products for Quasispecies Analysis :
PCR products were TA cloned; 10 to 20 clones were analysed per sample. A total of 260 clones representing 17 samples (3 asymptomatic, 6 chronic and 8 patients undergoing interferon therapy) were sequenced.

HCV Quantitation:
HCV RNA was quantified in 24 samples using HCV amplicor kit (Roche diagnostics).

HLA Typing:
To carry out HLA typing approximately 15μg of genomic DNA is required per sample. DNA extraction process was standardized. Genomic DNA from 14 controls and 25 anti HCV positive individuals was isolated and stored at -70°C. HLA typing was completed for 15 HCV infected individuals using PCR-SSP method (Genovision).

Future plan
More number of samples will be included in the study.
7. Study of specific immune response and cytokine patterns in patients with HCV infection.

A S Tripathy
K S Lole, M S Chadha, V A Arankalle

Hepatitis C is a parenterally transmitted disease characterized by an unusually high frequency of persistent infection and about 20% of the infected patients may develop cirrhosis and hepatoma. Persistent infection may result from an inadequate immune response and imbalance between T-helper cytokines. More information about the immune response in HCV infection is required to understand the mechanism(s) underlying chronicity.

Objectives

To assess the role of
• cytokines
• CD4 T cell response to core antigen
• HLA status of the patients towards disease progression/recovery.

Achievements

The present study was conducted to characterize the mechanisms responsible for viral persistence in HCV infection by measuring (1) the proliferation of PBMCs in response to recombinant HCV core antigen and (2) cytokine production profile i.e. both Th1 (IL2, IFN-gamma) and Th2 (IL4, IL10) in different categories i.e. blood donors identified as anti-HCV positives during voluntary blood donations with (Group A, n=6) or without (Group B, n=4) circulating HCV RNA, chronic active hepatitis C patients (CAH, Group 3, n= 5) and therapy responders (Group 4, n=3). Anti-HCV titers were determined for all the subjects using ELISA (Ortho HCV, 3.0 ELISA test).

The results indicate that as compared to the non-patient category, CD4+ T cell activation is stronger in those with more severe liver disease. Results are depicted in table.

In conclusion, a defect in both Th1 and Th2 cytokine production and insignificant amount of cytokines released may attribute for the persistence of HCV infection. Though statistically insignificant, therapy responders had low anti-HCV titres. Overall, HCV carriers with persistently normal and abnormal ALT levels behave differently in their CD4+ Th cell responses.

<table>
<thead>
<tr>
<th>Group</th>
<th>Core response in SI *</th>
<th>Anti-HCV Titer **</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>1.4</td>
<td>20000</td>
</tr>
<tr>
<td>A</td>
<td>1.3</td>
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<tr>
<td>A</td>
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<tr>
<td>A</td>
<td>1.6</td>
<td>80000</td>
</tr>
<tr>
<td>B</td>
<td>3.9</td>
<td>10</td>
</tr>
<tr>
<td>B</td>
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<td>10</td>
</tr>
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<td>B</td>
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<td>C</td>
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<tr>
<td>D</td>
<td>3.3</td>
<td>10</td>
</tr>
<tr>
<td>D</td>
<td>3.3</td>
<td>10</td>
</tr>
</tbody>
</table>

*SI = Stimulation Index  **Group A vs B: p < 0.01  Group B vs C: p < 0.01

The bars depict the supernatant protein concentrations of IL-2, IL-10, IL-4 and IFN-γ produced in vitro by PHA, PMA+ionomycin stimulated lymphocytes of HCV infected individuals and healthy controls. When comparing the patient group with the control group, Th1 cytokines, i.e. IFN-γ and IL-2 were not changed (IFN-γ : 268 ±259 vs 131 ±115, p>0.05) (IL-2: 209± 411 vs 11.8± 14.9, p>0.05), similarly, the Th2 cytokines, i.e. IL-4 and IL-10 were not changed (IL-4: 41.8 ±69.7 vs 32± 3.1, p>0.05) (IL-10: 77.9 ±143 vs 29.1±38.4, p>0.05).

Future Plan

More patients will be enrolled in the study. Study of HLA patterns to be continued.
8. Intra familial spread of hepatitis C

M S Chadha

mscniv@hotmail.com

The extent of intra familial spread of HCV in India is not known. Immuno-compromised patient
are known to circulate HCV in high titres. For contact management, a comparison of HCV spread
among contacts of healthy carriers and immuno-compromised patients would be necessary.

Objectives
- To determine extent of HCV infection among family members of Hepatitis C patients/carriers/
imuno-compromised patients.
- To understand routes and risk factors for transmission.

Achievements
Anti-HCV positive donors from different blood banks were studied. Blood samples of these
donors were taken together with a detailed history. Fifty-two donors from Pune were screened
of which 9 were confirmed to be anti-HCV positive. Six of the above 9 were also HCV-RNA positive
(3 had elevated ALT levels). An additional 29 donor samples from Nagpur and 46 from
Aurangabad were collected. All donors from Nagpur tested negative, whereas, 4 from
Aurangabad were anti HCV positive of which 3 were HCV RNA positive. 14 family contacts
were examined of which one (brother of donor) was found positive for anti HCV and HCV RNA.

Though additional family contacts need to be investigated, the data suggests that intrafamilial
spread of HCV seems to be insignificant in our settings.

Future Plan
To investigate contacts of patients on maintenance haemodialysis.

9. Monitoring of sewage treatment plant and water treatment plant for the presence of Hepatitis A and E viruses by RT-PCR.

S R Vaidya
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V A Arankalle

Study based on the sewage samples collected during 1999-2000 by NIV demonstrated
substantial prevalence of HAV and HEV RNA in un-concentrated samples, pre and post sewage
treatment. For generating true picture of prevalence of these enteric hepatitis viruses in sewage,
it was felt necessary to generate data for three years.

Objectives
To assess the presence of HAV and HEV RNA in sewage samples.

Achievements
- During 2001-2002, 89 sewage samples were collected from inlet and outlet points of
  the sewage treatment plant.
- Screening of the samples for HAV: Nineteen (untreated and treated) samples were screened
  by RT-PCR for the presence of Hepatitis A virus, using genotype III specific primers based on
  5'-noncoding region. None of the samples were found positive.
- Screening of the samples for HEV: Eighteen sewage samples were screened for the
  presence of swine HEV (type 4) using specific primers. Out of these one was found to be
  positive by RT-PCR. 24 sewage samples (treated and untreated) were screened using
  consensus primers (genotype 1&4) based on ORF2 region. None of the samples was found
  to be positive.

Future plans
The above results seem to be due to the limitations of the primers. These samples need to be
repeated with a new set of primers. Screening of the remaining stored sewage samples for HAV
and HEV RNA will be done with the new primers.
10. Virological evaluation of commercially available household water treatment units by RT-PCR.

S R Vaidya
V A Arankalle

Several water purification systems are being widely used to get better quality of water. However, no data in relation to efficacy with respect to enteric viruses is available. Evaluation of water purification units using sensitive virological tests would be extremely useful for the consumers.

Objectives
To assess usefulness of water filtration units in elimination of enteric hepatitis viruses.

Achievements
Water purification units based on the principle of polyiodide resin technology were evaluated. HEV was spiked in distilled water and passed through respective water purification units. The filtrate water collected from each unit was concentrated using ultra-filtration based membrane cartridge. The concentrated water samples were subjected to HEV RT-PCR. Water concentrates from all the three water purification units showed absence of HEV RNA.

Future plan
This experiment needs to be repeated. Other household water purification devices need to be evaluated.

11. Genomic characterization of hepatitis A virus isolates from Pune, western India.

S D Chitambar
K S Lole, M S Joshi

Hepatitis A virus infection in India has been highly endemic. However its current status indicates changing pattern and presents the features of both developing and developed countries. Increase in the clinical disease burden in adults suggests shift from high to intermediate endemicity of hepatitis A. The disease is in complex form in fulminant hepatic failure and while in co-existence of hepatitis B and E infections. In view of this, determination of HAV strain variations and genotypes prevalent in India is important.

Objectives
- To sequence partial variable (VP1/2A) and conserved (RNA polymerase) genomic regions of HAV isolates from Pune.
- To determine the genotypes and variations in HAV strains circulating in Pune by analysis and comparison of nucleotide sequence data.

Achievements
A total of 43 clinical specimens obtained from suspected cases of viral hepatitis during 1992-2001 were tested by RT-PCR. Thirty-one of the thirty four specimens positive for anti-HAV IgM showed presence of HAV RNA. PCR products derived from twelve specimens were partially sequenced in hypervariable (VP1/2A) and conserved (RNA polymerase) regions and compared in equivalent regions with HAV strains HM175 and Nor21 representing subgenotypes IB and IIIA respectively. Nucleotide sequence data of a fragment of 168 bps from VP1/2A region indicated co-circulation of IB and IIIA subgenotypes in hepatitis A patients from Pune (Fig 19). Analysis of a fragment of 116 bps of RNA polymerase region revealed two clusters of isolates one closer to Australian (HM175) and North African (MBB) strains and the other closer to Norwegian (Nor21) strain (Fig. 20). The sequence data indicated the possibility of co-infection with IB and IIIA subgenotypes in single individual hepatitis A patients. For clarification nearly 30 primer pairs specific to variable and conserved regions of HAV genome were synthesized and attempted in combination for PCR amplification and subsequent nucleotide sequencing of purified DNA products. So far PCR products of three isolates were detected to have both the subgenotypes IB and IIIA indicating mixed infection in three hepatitis A patients (Fig. a and b).
### Future plan

Investigation of clinical specimens from hepatitis A patients will be continued further to detect HAV genotypes and strain variations.
12. Evaluation of animal derived polyclonal anti-HAV antibodies for diagnosis of hepatitis A

S D Chitambar  
M S Joshi

Currently polyclonal anti-HAV antibodies derived from human are used for the preparation of hepatitis A diagnostic reagents. Antibodies generated in animals against HAV are alternative source for such preparations. It is intended to minimize the use of variable source of anti-HAV antibodies derived from human by generating anti HAV antibodies in animals.

**Objectives**
To determine the suitability of animal derived anti-HAV antibodies for diagnosis of hepatitis A

**Achievements**
Goat (1), rabbit (1) and guinea pigs (2) were immunized via intramuscular route against HAV using 600ng, 300ng and 150ng per dose respectively. Booster doses were administered at 2-3 week intervals. Serum samples collected at different time intervals showed anti-HAV antibody titres in a range of 1:3200 - 1:6400 in blocking ELISA test.

**Future plan**
Polyclonal anti-HAV IgG will be purified to prepare capture antibodies and immunoconjugates. These will be subsequently tested in various ELISA protocols for detection of recent and past infection of hepatitis A.

13. *In vitro* studies on growth and characterization of Indian isolates of hepatitis A virus

R S Fadnis  
S D Chitambar

Currently hepatitis A vaccines derived from Australian, Costa Rican, German, British and Swiss strains of hepatitis A virus are marketed in many countries including India. Indigenous vaccine is not available as yet. A strain of HAV was therefore, isolated in buffalo green monkey (BGMK) cell line and transferred to industry. Since this strain is proposed to be employed for production of hepatitis A vaccine, it needs to be characterized.

**Objectives**
- To improve the yield of isolated HAV in tissue culture system
- To determine its physico-chemical, immunological and genomic characteristics
- To obtain alternative isolates of HAV from acute hepatitis A patients

**Achievements**
In order to determine the viral yield in one step growth condition, normal BGMK cells maintained at passage level 101 were infected with HAV derived from persistently infected BGMK cells at four different dilutions. Cell cultures were harvested 10 days post infection. S/N (Sample OD/Neg control OD) ratio as indicated by antigen capture ELISA test was directly proportional to the dose of inoculum used for infection as depicted in the figure. Rapid passaging of HAV infected BGMK cell culture is being carried out to obtain fast growing strain or to observe cytopathic effect.

**Future plan**
Various cell lines will be employed for adaptation of HAV isolates for improvement in the yield of virus.
14. Isolation, adaptation and characterization of hepatitis A virus isolates identified as Genotype III A using tissue culture system

M S Joshi
S D Chitambar

The strains of HAV employed for manufacturing of hepatitis A vaccine belong to genotype IA or IB. HAV has a single serotype, and therefore, immune response generated against currently available vaccines is likely to protect the vaccinees against infections with other HAV strains. However, vaccine using a strain with genotype III A has not been attempted so far. This kind of vaccine would be useful if current strategies of control of disease are required to be modified.

Objectives
- To adapt the strain of HAV with genotype III A for growth in cell cultures, establish persistent infection and monitor the yield of virus.
- To characterize the viral genome during adaptation of HAV strain in tissue culture system.

Achievements
Fecal samples collected from serologically confirmed anti-HAV IgM positive 10 cases of viral hepatitis were screened for the presence of HAV RNA by nested RT-PCR using primers to VP1/2A region of the genome. One of these specimens characterized as genotype III A was inoculated in normal BGMK and MRC-5 cell lines. Serial passages of cell lines were made for propagation of virus. Presence of virus is being examined by RT-PCR, ELISA and immune electron microscopy.

Future Plan
The yield of virus will be monitored by antigen capture ELISA at different passage levels. RT-PCR will be carried out on virus samples recovered from infected cells to obtain nucleotide sequence data and analyze the molecular changes that would take place during adaptation to tissue culture.

15. Follow up of Hepatitis B vaccine recipients: Evaluation of cellular and humoral response to HBsAg and their cytokine patterns

A S Tripathy
M S Chadha

It is recommended that healthcare workers at risk of occupational exposure to HBV should be vaccinated. Data on long-term follow up of vaccinees in relation to humoral and cellular responses is not available from India.

Objectives
- To investigate:
  - Humoral
  - Cellular
  - Responses to HBsAg in previously immunized laboratory personnel.

Achievement
Staff members of the NIV at high risk for Hepatitis B had been vaccinated in 1988-89. Twenty-nine vaccinees were followed up at 13 years post vaccination; 19 tested anti-HBs positive. Of the ten negatives, 8 received a booster dose of vaccine and 4 seroconverted to anti-HBs. Three of the 4 vaccinees not responding to the booster dose were non-responders after primary vaccination. The fourth had an S/N ratio of 10 after initial three doses of vaccine and was constantly anti-HBs negative, since two years post vaccination. Additional 10 laboratory workers who were 5 to 10 years post vaccination were also included in this study. Of these, eight were anti-HBs positive. The negatives were not available for boosting.

Twenty-five of the above 39 vaccinated individuals were further studied for cellular response. Of the 14 anti-HBs positive vaccinees, 12 had positive T-cell response against recombinant HBsAg (SI> 2). Two anti-HBs positive non-responders at the T-cell level need to be further investigated.

Three of the 11 anti-HBs negative individuals had proliferative T cell response against recombinant HBsAg. All anti-HBs positive vaccinated individuals had significant levels of Th1 cytokines (IL2, INF gamma) when compared with the controls. (IL2: 15.3±1.6 vs 12.8±1.3, p<0.05) (IFNgamma: 12.4±1.5 vs 18.3±1.3, p<0.01). The cellular response to HBsAg could be elicited after boosting the anti HBs negatives and the Th1 cytokine profiles became comparable with the anti-HBs positives. In conclusion, the anamnestic response to a booster dose of vaccine in vaccinated individuals without anti-HBs confirms the presence of B and T memory cells.

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Hepatitis B Virus infection causes acute hepatitis, chronic liver disease, liver cancer and liver cirrhosis which may lead to death. Estimates show that 1% of all adult deaths (15-45 years of age) in India are related to HBV infection. Liver disease due to HBV infection is considered to be fourth or fifth important cause of mortality in the most productive period of life. It is preventable by vaccination. It can even be eradicated through implementation of appropriate strategies. An Estimate of its burden is essential to develop methodology for health policies w.r.t. HBV.

Objectives
To create a baseline data to standardize the methodology for estimation of burden of disease due to HBV and extrapolate for the whole country.

Achievements
Collected data on infective hepatitis from the State bureau of Health Intelligence and Vital Statistics, Pune. This data represents Maharashtra state.

Crude DALYs were calculated using the formula given by WHO guide.
Following assumptions were made based on published reference:
10% of all the infective Hepatitis is due to HBV
Since the data is for infective hepatitis and age distribution was not given, published reference of NIV was used to derive the age distribution.
Total duration of the disease is 8 weeks. First 3 weeks 100% ill, next 3 weeks 50% ill and next 2 weeks 20% ill. No Discount rate and Age Weights were given. The Disability weights varied from 0 to 1 according to the severity of the disease in terms of % illness. Following were the results for 2001 and 2002.

<table>
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<th>Age group</th>
<th>2001 Male</th>
<th>2001 Female</th>
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<th>2002 Male</th>
<th>2002 Female</th>
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<tr>
<td>16-25</td>
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<td>0.04</td>
<td>0.13</td>
<td>0.10</td>
<td>0.05</td>
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<td>26-35</td>
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<td>0.03</td>
<td>0.11</td>
<td>0.09</td>
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<tr>
<td>Total</td>
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<td>0.36</td>
<td>0.30</td>
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</table>

Objectives
Conducted meetings with DDHS and requested to include the age of the patient along with duration and severity of the disease for better estimates. Efforts are being made to improve the quality of data for better estimates. These are rough estimates. It appears that the BoD is increased within a year.

Future Plans
Implement new data collection format through DDHS to collect more data to improve the BoD estimates.
Efforts to include data from the private hospitals.

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