Molecular characterization of hepatitis A virus from a large outbreak from Kerala, India

V.A. Arankalle, K.L. Sarada Devi*, K.S. Lole, K.T. Shenoy*, V. Verma & M. Haneephabi*

National Institute of Virology, Pune & *Trivendrum Medical College
Thiruvananthapuram, India

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Background & objectives: Hepatitis A is highly prevalent in India and mainly presents as a sporadic disease. This study investigated an outbreak of viral hepatitis at Medical College Hospital area, Kottayam, Kerala state, India during January 2005.

Methods: Blood (133), faecal (1), sewage (4), and water samples (13) were collected. Sera were tested for IgG- and IgM-anti-HAV and IgM antibodies against hepatitis E (IgM-anti-HEV). Sewage, faeces and water samples were tested for HAV RNA in nested RT-PCR and HAV RNA positive samples were further processed for RNA quantitation using Real Time PCR.

Results: Of the 1180 total cases, 540 were reported from Medical college area. Two deaths were reported among doctors. Patients from the community gave a previous history of visit to medical college hospital area. The sewage treatment plant at the campus was non-functional since 1990 and the untreated sewage was constantly overflowing and getting mixed with a canal. At the time of the study, all the water sources were superchlorinated. HAV RNA was present in the faeces of hepatitis A patient (1.36 × 10⁷ copies/ml), sewage tank (2.57 × 10³ copies/ml and the canal (<100 copies/ml). None of the 13 water samples concentrated 10,000-fold and the soil sample showed presence of HAV RNA. Phylogenetic analysis based on 5'-non-coding and P2 regions showed HAV-genotype IIIA in all samples.

Interpretations & conclusions: The aetiological agent of the present outbreak was found to be HAV. Epidemic hepatitis A (genotype-III A) is emerging in Indian adults, emphasizing the need for definite policy for control.

Key words Environmental samples - hepatitis A virus - outbreak - phylogenetic analysis

Hepatitis A is an enterically transmitted viral disease of global public health importance. Person-to-person contact transmits hepatitis A virus (HAV), generally by the faecal/oral route. Common source outbreaks can occur from faecally contaminated food and water. Among children about 90 per cent infections are subclinical whereas exposure of adults and adolescents mainly leads to clinical form.

In developing countries, hepatitis A is highly endemic and a large proportion of population acquires immunity through asymptomatic infection.
early in life. Improvement in hygienic and socio-economic conditions has resulted in a decrease in the number of natural childhood infections. As a consequence, an increase in the susceptible adults with associated increased proportion of clinical disease is noted.

HAV is a member of *Picornaviridae* family and hepatovirus genus. Different HAV isolates have been classified in 7 genotypes. Human isolates belong to genotypes I, II, III and VII while genotypes IV, V and VI represent isolates from Old World monkeys. Importantly, a single serotype exists.

Based on the serological surveys conducted in 1982, 1992 and 1998, a changing epidemiology of hepatitis A was documented by us and the possibility of hepatitis A outbreaks in near future was suspected. This was mainly because the population belonging to lower socio-economic status continues to be hyperendemic, excreting HAV in large quantities whereas a substantial pool of anti-HAV negative adolescents/adults is now present among the higher socio-economic class. In the last few years, small outbreaks of hepatitis A in children have been recorded from western India (unpublished observations). This study was carried out to investigate an extensive outbreak of hepatitis A in adults from the state of Kerala, southern India.

**Material & Methods**

**Study area:** The outbreak occurred in the Kottayam Medical College Hospital area and nearby Panchayats (smallest rural constituents) of Kottayam district situated in a southern Indian state, Kerala. According to the local health authorities, an outbreak of viral hepatitis at Medical College Hospital area, Kottayam was reported on September 17, 2004. The District level Rapid Response Team conducted a survey on September 19 and started preventive and control activities. As on January 19, 2005, a total of 1180 cases of viral hepatitis were reported from the district including 540 from the medical college area. The National Institute of Virology (NIV), Pune was informed of the outbreak on January 22, 2005 and investigations were undertaken immediately, thereafter.

**Collection of samples**

**Clinical samples:** Blood samples (n=132) were collected from the staff and students of School of Medical Education. A detailed history was recorded in a pre-set proforma. Blood and faecal samples (KOT-1) were collected from a hospitalized patient with HAV.

**Environmental samples:** About 100 ml of a sewage sample was collected from the sewage tank of 50,000 litre capacity, situated within the hospital campus (KOT-2). The sewage treatment plant was non-functional since 1990 and the sewage water was found to be overflowing. This overflowing sample (~ 100 ml) was collected about 10 meters away from the tank (KOT-3). About 200 meters away from the tank a canal was flowing and getting mixed with the sewage water. A sample was collected from this canal (~ 100 ml) (KOT-4). About 250 g of soil sample was collected from an area near to the sewage tank (KOT-5). Twenty litres each of 13 water samples were collected representing wells in the nearby areas and restaurants and as per the instruction from the health authorities, these water sources were superchlorinated. All specimens except water samples (at room temperature) were transported to the NIV on wet ice.

**Serology:** All the serum samples were screened for the presence of IgM and IgG antibodies against hepatitis A (General Biologicals, Taiwan) and E viruses.

**Preparation of samples for PCR:** Sewage and canal samples (250 µl each) were directly processed without any treatment/concentration. Water samples were initially concentrated from 20 litres to about 300 ml using membrane-based ultrafiltration technology with a membrane with 60,000 daltans.
exclusion limit. Subsequently, 300 ml was concentrated to about 2 ml in an amicon cell (Millipore) and stored at −70°C in aliquots. To 100g soil sample, 100 ml of distilled water was added and subjected to sonication (SONIFIER, BRANSON, USA). Following centrifugation at 16,000 g for 15 min, the supernatant was concentrated to 1.5 ml. 100 µl of the serum sample and 10 per cent faecal suspension in phosphate buffered saline (PBS) from the hospitalized patient were used for the detection of HAV RNA by PCR.

RNA was extracted using TRIZOL LS reagent [Life technologies (now Invitrogen), USA] according to the manufacturer’s instructions. Primers representing the conserved 5’ non coding region (5’-NCR) were used: outer forward: 5’ GGC TAC GGG TGA AAC CTC TT 3’ outer reverse 5’ CCA ATT TTG CAA CTT CATG 3’ inner forward 5’ TAA CAG CGG CGG ATA TTG GTG 3’ and inner reverse 5’ GGT CAA GGC CAC TCC CAAC 3’ (primers were obtained from Bangalore Genei, Bangalore). In addition, a 2118 bp fragment encompassing 5’ NCR, complete VP4, VP3, VP2 and partial VP1 was amplified from 3 samples. The primers used were: outer forward 5’ GGC GGG GTC AAC TCC A TG 3’ outer reverse 5’ GGC AA T CCA TGA GGA GGA TTA GA 3,’ inner forward 5’ AGC TGT AGG AGT CTA AAT TGG G 3’ and inner reverse 5’ GTC TCA GGC ACT TTC TTT GC 3’.

Sequencing— PCR products were column purified (QIA gel purification kit, Qiagen, USA) and both the strands were sequenced using Big Dye Terminator cycle sequencing Ready Reaction Kit (Applied Biosystems, USA) and an automatic sequencer (ABI PRISM 310 Genetic Analyser, Applied Biosystems, USA).

Phylogenetic analysis— The phylogenetic status of HAV-RNA positive specimens was assessed employing the software MEGA9. For analysis in MEGA, Jukes-Cantor (JC) distance was utilized employing the neighbour joining (NJ) algorithm. The reliability of different phylogenetic groupings was evaluated by using the bootstrap test (1000 bootstrap replications) available in MEGA.

Accession numbers and designations of the sequences employed for analysis in the present study were as follows: Genotype IA: X75215 (GBM), AF485328 (LY6), Genotype IB: M14707 (HM175), M20273 (MBB), Genotype IIIA: AY644337 (isolate from Germany), M66695 (GA-76, isolate from US); M34084 (PA21), AJ299464 (NOR-21), Genotype IV: M59286 (CY145), Genotype V: D00924 (AGM27), Genotype VII: AY032861 (SLF88).

Real time PCR for quantitation of HAV RNA— A 342 bp partial 5’NCR region of HAV genome was PCR amplified and cloned into pGEM-T Easy vector (Promega, Madison, USA). Clone with sense orientation was cut with Sall and run off transcription was done with T7 polymerase. Transcript was purified, serially diluted and used as RNA standard. Viral RNA was extracted from samples using QIAamp viral RNA mini kit (Qiagen, USA). Primers and probe corresponding to HAV 5’NCR as forward primer 5’ AAC AGC GGC GGA TA T TGG 3’; reverse primer 5’ AAT GCA TCC ACT GGA TGA GAG 3’ and TaqMan minor groove binder (MGB) fluorophore attached probe, VIC 5’ AAA AAC CAT TCA ACG CCG 3’NFQ, MGB; were used and real time RT-PCR assay was performed in SDS 7000 (ABI PRISM, USA). Serial dilutions of the standard RNA were used to see the linearity of the standard curve. Standard curve showed linear relationship (\(r^2=0.99\)) from \(10^2-10^{10}\) RNA copies/reaction. The detection limit of the assay with the RNA standard template was 100 copies.

**Results**

Description of the outbreak: Of the 540 cases in medical college hospital area, 170 were from the members of medical community including residents of Medical College Men’s hostel, women’s hostel, post graduate’s hostel, nurses’ hostel and house surgeon’s quarters, and 67 cases were recorded from the students of School of
Medical Education. The remaining patients represented the community including mainly local residents of three panchayats around medical college hospital campus, namely Arpookara (127), Athirampuzha (91) and Kumaranellor (85). Two adjacent panchayats, Aymanam and Ettumanoor recorded 40 and 73 hepatitis cases respectively. In majority of the cases from areas other than the Panchayats nearby medical college hospital area, a previous history of visit to medical college hospital area and consumption of food/water from unhygienic food establishments around medical college hospital campus within a period of 2-3 wk before being infected were noticed. A substantial proportion of viral hepatitis patients were care-taking relatives of the patients hospitalized for other causes. A distinct cluster with 178 cases was noted in the Panchayat of Mundaakayam. Our investigations were restricted to medical college campus.

The peak of the epidemic was in the last week of September 2004 (121 cases). From the third week of November onwards, a small number of patients (0-4 cases/week) probably reflecting sporadic secondary hepatitis A cases were reported (Fig.1).

Though piped water supply is provided by the Kerala state, use of well water was a common practice in several houses and restaurants. After recognizing outbreak of viral hepatitis in the hospital campus, the local health authorities examined the suspected water sources and identified well water samples from the nearby areas and School of Medical Education to be unsuitable for human consumption. Unfortunately, these samples were not tested for HAV. As on January 15, 2005, a total of 7990 chlorinations were carried out including repeated chlorinations of some of the water sources. The policy of daily chlorinations of proven contaminated sources and weekly chlorination of other drinking water sources was made and

Fig.1. Showing the number of hepatitis cases in the Kottayam Medical College area during September 2004 – January 2005. Arrow indicated the time point at which the investigations were undertaken.
rigorously followed throughout the district. The sewage treatment plant at the campus was non-functional since 1990, and untreated sewage was constantly overflowing from both the 50,000 litre capacity sewage tanks and spreading over a large area by way of mixing with a canal.

Of the 285 patients investigated at the Kottayam medical college hospital, 248 (87%) were anti-HAV IgM positive. Two deaths among the doctors were reported. However, the serum samples were not stored for further analysis.

Serology: Of the 132 serum samples collected from staff and students of School of Medical Education (83 males and 49 females), 52 (37.7%) were positive for IgM-anti-HAV antibodies. These included 95 individuals with a history of jaundice (41 IgM-anti-HAV positive, 43.1%) and 37 individuals without such history (11 IgM-anti-HAV positive, 29.7%) indicating subclinical hepatitis A. Thirty three of 60 (55%) and 8 of 35 (22.8%) patients giving history of jaundice < 4 and > 4 months respectively, were IgM-anti-HAV positive indicating disappearance of IgM antibodies during the later stage of the disease in a significant proportion of patients ($P < 0.001$, Table I).

Age-wise IgM-anti-HAV positivity was 17 of 40 (18-20 yr, 42.5%), 29 of 70 (21-25 yr, 41.4%), 2 of 10 (26-35 yr, 20%) and 2 of 8 (36-52 yr, 25%). Age of remaining four adults was not known and two of these were anti-HAV IgM positive. Of the 80 anti-HAV-IgM negatives, 75 were IgG-anti-HAV positive. Thus, at the end of the epidemic, only 5 of 132 individuals investigated remained susceptible for HAV. In order to assess involvement of HEV, the most common epidemic-causing virus in India, all the serum samples were screened for IgM and IgG anti-HEV antibodies and none and 2 respectively were found reactive. One of the IgG-anti-HEV reactive was IgM-anti HAV positive and the other was IgG-anti-HAV positive. The only serum sample from a hospitalized hepatitis A patient was IgM-anti-HAV positive.

**PCR, sequencing, viral load and genotype status:** The only faecal sample available from an acute hepatitis A patient and all the sewage-related environmental samples were positive for HAV RNA (Table II). HAV RNA was detected after the first-round PCR in all except the canal sample exhibiting positivity only after the second-round PCR. The viral load as estimated by real time PCR (Table II) was highest in the faecal sample ($1.36 \times 10^7$ copies/ml). In the sewage tank and the overflowing sewage collected about 10 meters away, the viral load was $2.57 \times 10^3$ and $2.65 \times 10^3$ copies/ml respectively. The viral load in the canal sample was below the detection limit of real time PCR assay (100 copies/ml). None of the 13 water samples concentrated almost 10,000-fold and the soil sample showed presence of HAV RNA.

<table>
<thead>
<tr>
<th>Number (History of jaundice in months)</th>
<th>HAV-IgM positives</th>
<th>HAV–IgM negatives</th>
<th>HAV-IgM Neg. HAV-IgG Pos.</th>
<th>HAV-IgM Neg. HAV-IgG Neg.</th>
</tr>
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<tbody>
<tr>
<td>60 (&lt;4)</td>
<td>33</td>
<td>27</td>
<td>24</td>
<td>3</td>
</tr>
<tr>
<td>35 (5-24)</td>
<td>8†*</td>
<td>27</td>
<td>26</td>
<td>1</td>
</tr>
<tr>
<td>37 (No history)</td>
<td>11</td>
<td>26</td>
<td>25</td>
<td>1</td>
</tr>
<tr>
<td>Total 132</td>
<td>52</td>
<td>80</td>
<td>75</td>
<td>5</td>
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†$P<0.001$ compared to patients with history of jaundice < 4 months

*12, 10, 8, 8, 9, 9, 7, 6 months
Fig. 2 presents phylogenetic status of HAV isolates from the present epidemic on the basis of 5'-NCR (291 nucleotides) and structural (complete VP4, VP3, VP2 and partial VP1, 1747 nucleotides) regions of HAV genome. Both regions placed the Kottayam HAV isolates in genotype-IIIA. In 5’-NCR, per cent nucleotide identity (PNI) of the Kottayam isolates was 97 (German isolate) and 98.4 (PA-21). The PNI of Kottayam isolates in the P1 region with the German isolate was 99.4. At protein level, the sequence of 582 amino acids was identical in the German and two of the Kottayam isolates. One substitution (at position 377, VP3 region) was noted for KOT-2 isolate. The accession numbers for the sequences generated during this study were DQ004690 to DQ004696.

Discussion

This is the first recorded outbreak of HAV in Indian adults. The National Institute of Virology has investigated over 100 outbreaks of waterborne hepatitis in adults in different parts of India. Of these, except one\textsuperscript{10,11}, all were attributed to HEV\textsuperscript{11,12} (unpublished observations). Surprisingly, no HEV outbreaks have been reported from the state of Kerala. In the present study, only 2 of 132 individuals examined were IgG-anti-HEV positive indicating low HEV endemicity in this region. Subsequently, we tested 42 hepatitis patients admitted to Kottayam medical college during the months of February-March 2005 and 41 were IgM-anti-HAV positive (data not shown) documenting continued HAV activity in the region.

General hygiene, especially in relation to sanitation, water supply and food preparation reflects socio-economic status and living standards and has major influence on HAV endemicity\textsuperscript{13-16}. Though no community-based serological data were available, the eruption of an outbreak of hepatitis A in adults from Kerala clearly documented that a substantial proportion of individuals were not exposed to HAV till adulthood.
Fig. 2. Phylogenetic analyses based on 5'NCR (291nt, Fig. 2a) and P1 region (1747nt, Fig. 2b) sequences of HAV isolates. The Kottayam sequences are marked in bold. Per cent bootstrap support is indicated by the values at each node.
Based on population-based serosurveys conducted in urban and rural areas of Pune, western India, during 1982, 1992 and 1998, we showed that 69 per cent (6-10 yr), 53 per cent (11-15 yr) and 15 per cent (16-25 yr) of the population belonging to higher socio-economic status was anti-HAV negative in 1998. In contrast, 94-100 per cent of those belonging to lower socio-economic status were exposed to HAV in the same age groups respectively. Based on these results, possibility of epidemics of HAV in high socio-economic status population was predicted.

The important question is if the sewage treatment plant was not functional since 1990, why similar epidemics did not occur in the earlier years. The possibility of less circulation of the virus and lack of gross contamination of drinking water sources with sewage-containing high HAV load may be an important factors.

As the investigations were undertaken at the end of the epidemic, a large number of specimens were not available for PCR and sequencing. Nevertheless, all four sequences obtained belonged to genotype IIIA. So far, genotypic data have been reported for a sporadic north Indian isolate (1990, genotype IIIA) and a day care center from Pune, western India (1995, genotype IB). All the four outbreaks of hepatitis in rural children reported during 2002-2004 were shown to be due to genotype IIIA HAV (unpublished observations). Importantly, an outbreak of hepatitis in children from an urban area during February-March 2004 from Maharashtra was also attributed to genotype IIIA (unpublished observation). Genotype IIIA therefore seems to be the predominant epidemic strain circulating in India.

Molecular characterization of 83 and 8 recent epidemic HAV isolates in Estonia and France respectively led to the demonstration of genotype IIIA as the epidemic strain in Estonia and co-circulation of sub-genotype IA, IB, and the presence of IIIA sub-genotype for the first time in France. Genotype IA was associated with recent epidemics of hepatitis A in south-America and Thailand and IA and IB in Brazil. In Estonia, predominance of genotype IA was recorded among non-outbreak isolates available from 1994 to 2000. Data for currently circulating strains in sporadic hepatitis A cases in India need to be generated. In the PI region, Kottayam isolates were 99.4 per cent identical with the recent German isolate (Genbank accession No. AY644337).

HAV RNA negativity in all the water samples tested could be due to super-chlorination. Importantly, fresh cases were also rare at this time. A study from Spain showed HAV concentration in water to be 1000-fold less than in sewage. In the present study, HAV RNA titre was $2.57 \times 10^3$ copies/ml sewage and concentration of 20 litres of water to 2 ml should have allowed detection of HAV. We therefore believe that the strategy of chlorination was effective.

Detection of HAV RNA in the unconcentrated samples from the sewage tank, overflowing sewage and the canal poses concern of HAV infection of susceptible populations if mixed with drinking water at a high viral load and supplied without appropriate doses of chlorine. Of the 132 staff and students of school of medical education exposed during this outbreak, only 3.7 per cent individuals were anti-HAV negative in January 2005 documenting universal exposure to HAV leading to clinical/subclinical hepatitis A infection in the susceptibles. To avoid another outbreak of hepatitis A in new entrants, there is an urgent need to make the sewage treatment plant efficiently functional and also to provide hepatitis A vaccine to anti-HAV negatives. Detection of HAV RNA in the contamination source well 6 months after the initial contamination emphasizes need for monitoring ground water for HAV. As far as Kottayam medical college campus and surrounding region is concerned, adequate treatment and disposal of sewage, proper chlorination of drinking water sources and supply of safe water to the users should take top priority. Cost permitting, vaccination of susceptibles should be encouraged.
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References


Reprint requests: Dr V.A. Arankalle, Deputy Director and Head, Hepatitis Division, National Institute of Virology
20-A Dr Ambedkar Road, Pune 411001, India
e-mail: varankalle@yahoo.com