Material & Methods
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The present study was conducted in the department of Pediatrics in collaboration with department of microbiology, M.L.B., Medical College Jhansi, over a period of one year.

Criteria for selection of cases

78 children upto 16 years of age of either sex who were admitted in pediatric ward of this hospital or presented in Out Patient Department, with complaints suggestive of acute viral hepatitis were included in this study. The diagnosis of acute hepatitis was made on the basis of history, clinical findings & biochemical investigations such as S. Bilirubin, Alanine transferase (ALT), and Aspartate transferase (AST).

Selection of cases

The criteria used for diagnosis of acute viral hepatitis were Recent onset of jaundice (within 3 months) in the absence of prior history of jaundice or chronic liver disease. No other cause to account for jaundice including drugs, hepatotoxins, severe infections such as falciparum malaria, which is endemic in this region.

Serum bilirubin of 2 mg% or more, with an increase in ALT, two times above the upper limit of normal (Normal range for 1 to 19 years is 5 – 45 IU/ ml, according to Nelson textbook of Pediatrics).

Acute phase sera were collected from all cases for biochemical and serological tests. Sera was analyzed for IgM class of antibodies
to hepatitis A virus (anti HAV IgM) by third generation ELISA kits from Adaltis Italia code – 07.0999, and HBsAg by Latex agglutination test from Tulip Diagnostic Ltd. Goa.

Acute viral hepatitis was defined as acute self limiting disease with serum AST elevations atleast two to five folds or clinical jaundice or both (Smedile et al 1982).

Fulminant hepatitis was diagnosed when after a typical acute onset, the patient became deeply jaundiced and developed hepatic encephalopathy within four weeks of onset of jaundice, with no past history of chronic liver disease (Trey and Burns 1966).

Hepatic encephalopathy was classified into four grades (according to Nelson textbook of Pediatrics edition 17th):

<table>
<thead>
<tr>
<th>Stage I</th>
<th>Stage II</th>
<th>Stage III</th>
<th>Stage IV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Symptoms</td>
<td>Period of lethargy, euphoria, reversal of day – night sleeping; may be alert</td>
<td>Drowsiness, inappropriate behaviour, agitation, wide mood swings, disorientation</td>
<td>Stupor but arousable, confused, incoherent speech</td>
</tr>
<tr>
<td>Signs</td>
<td>Trouble drawing figures, performing mental tasks</td>
<td>Asterixis, fetor hepaticus, incontinence</td>
<td>Asterixis, hyperreflexia, extensor reflexes, rigidity</td>
</tr>
<tr>
<td>EEG</td>
<td>Normal</td>
<td>Generalized slowing, theta waves</td>
<td>Markedly abnormal trirphasic waves</td>
</tr>
</tbody>
</table>

History and Examination

Detailed history of all patients were taken, with due emphasis on following points:- Duration of jaundice, fever, gastrointestinal symptoms such as nausea, vomiting, abdominal discomfort, coloured
urine. Extrahepatic manifestations such as pruritis, rash, arthralgia was specifically asked from the patients or their attendants. CNS manifestations in the form of altered sensorium and convulsions were also noted.

Clinical examinations of the children was done with the emphasis on following points – Pallor, temperature, icterus and liver size, (consistency, tenderness, margins, surface, upper border dullness and span was noted in all cases of liver enlargement). Spleen whether palpable or not was also noted. Detailed CNS examination was done. Complications such as hematemesis, hemorrhagic RTA, malena, ascites, hepatencephalopathy, acute renal failure encountered in children with AVH were also noted.

Investigations like hemoglobin, TLC, DLC, ESR, serum bilirubin, Alanine transferase (ALT), Aspartate transferase (AST) were done in all cases.

Sera of all patients were analyzed for IgM anti HAV antibodies by third generation ELISA kit and for HBsAg by Latex agglutination method.

IgM ELISA Kit Test :-

Principle of the assay

AntiHAV IgM test is based on the principal of Enzyme Linked Immuno Assays or (ELISA) and it is used to determine such antibodies in human sera or plasma. The microplate is activated with a monoclonal antibody to human IgM that in the first incubation specifically captures IgM from the sample. In the following step a complex pre-formed between HAV antigen and polyclonal anti HAV labelled with HRP is added. In the last step substrate HS (mixture of
3,3', 5,5' tetra methyl benzidine and H2O2 ) is added to microplate on which enzyme bound on the plate acts generating an optical signal that can be measured by an ELISA reader at 450 nm.

Total incubation time : 2 hours, 20 minutes

**Required Testing Material**

1. Microplate coated with monoclonal antihuman IgM.
2. Negative control- Vial of serum base non-reactive for antiHAV IgM.
3. Positive control- Vial of serum base reactive for antiHAV IgM.
4. Conjugate- Containing a polyclonal antibody to HAV labelled with HRP.
5. Antigen- Buffered protein solution containing HAV inactivated antigen.
6. AG/Conjugate diluent- Buffered solution containing proteins and preservatives for the dilution of conjugate and the antigen to obtain Ag/conjugate immuno complex (HAV/ antiHAV- HRP immuno complex).
7. Washing buffer.
8. Sample diluent.
9. Substrate HS containing mixture of TMB (3,3', 5,5' Tetra Methyl Benzidine) and H2O2 (Hydrogen Peroxide).
10. Stop solution- Containing 0.3 M H2SO4.
11. Pipettes- (5, 50, 100, 200, 1000 µl).
12. Dilution tubes- (5 and 15 ml).
13. Vortex.
15. Micro plate washer.
16. ELISA reader for measurement of absorbance at 450 nm.

**Specimen Collection and Storage**

Fresh sera was used for the test. The specimen was stored at 2-8°C for a period up to one week and was frozen at −20°C if longer storage was required. Usual precautions for venepuncture was observed. No special sample pretreatment was necessary.

Repeated freezing of sample was avoided.

Measures were taken to keep samples free of particles, evident haemolysis and high content of lipids.

**Assay Procedure**

- All reagents were brought to room temperature before use.
- Dilute samples 1:200 (as final dilution) with sample dilution.
- Controls were ready to use, no dilution was done in them.
- Controls and samples were dispensed according to the following table.

<table>
<thead>
<tr>
<th>Position</th>
<th>Control/Samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>A₁</td>
<td>empty for blanking.</td>
</tr>
<tr>
<td>B₁ C₁</td>
<td>100 μl (micro litre) negative control.</td>
</tr>
<tr>
<td>D₁ E₁</td>
<td>100 μl positive control.</td>
</tr>
<tr>
<td>F₁,...,H₁₂</td>
<td>100 μl sample.</td>
</tr>
</tbody>
</table>

- Incubation was done at 37°C for 60 minutes.
- Plate was washed with diluted washing buffer to remove any liquid residual on an adsorbant paper.
- 100 μl of Ag/conjugate immuno complex was dispensed into all well except A₁ (blank).
Preparation of antigen / conjugate immunocomplex: 20 minutes before the use mix equal volumes of concentrated antigen and antigen conjugate diluent, then mix on vortex. Use this solution to dilute the conjugate 1 : 20 and mix again on vortex. For preparation proceed according to following table:

<table>
<thead>
<tr>
<th>N strips</th>
<th>Antigen volume (ml)</th>
<th>Diluent volume (ml)</th>
<th>Conjugate volume (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>0.95</td>
<td>0.95</td>
<td>0.1</td>
</tr>
<tr>
<td>4</td>
<td>1.9</td>
<td>1.9</td>
<td>0.2</td>
</tr>
<tr>
<td>6</td>
<td>2.85</td>
<td>2.85</td>
<td>0.3</td>
</tr>
<tr>
<td>8</td>
<td>3.8</td>
<td>3.8</td>
<td>0.4</td>
</tr>
<tr>
<td>10</td>
<td>4.75</td>
<td>4.75</td>
<td>0.5</td>
</tr>
<tr>
<td>12</td>
<td>5.7</td>
<td>5.7</td>
<td>0.6</td>
</tr>
</tbody>
</table>

- Incubation was done at 37 °C for 60 minutes.
- The plate was washed with diluted washing buffer and 100 µl of substrate HS was dispensed into all wells (including A1).
- Incubation was done at room temperature for 20 minutes.
- The reaction was stopped by adding 100 µl of stop solution to each well.

After adding the stop solution, the colour developed on the microplate reader at 450 nm was red. (The reading should be done within 60 minutes from the stop).

Calculations of Result
Results were calculated by means of a cutoff value determined as :-
Cutoff = OD mean negative control + 0.150
The sample was considered positive if its OD 450 nm. was higher than the cutoff value.
The sample was considered negative if its OD 450 nm. was lower than the cutoff value.

Test Validity
A. OD 450 nm value of blanked well A\textsubscript{1} must be less than 0.100.
B. Mean OD 450 nm value of negative control must be less than 0.150.
C. Mean OD 450 nm value of positive control must be higher than 0.500.

This third generation ELISA kit is reported to have a sensitivity > 99.5% and specificity of 99.3%.

Detection of HBs by Latex agglutination method
Principle
Latex particles coated with anti HBs Ag antibodies will agglutinate when mixed with serum or plasma containing hepatitis B surface antigen within the detectable levels. Agglutination is absent when the HBsAg is absent or not within the detectable levels.

Required testing material
1. Reagent pack containing Latex reagent coated with anti HBsAg antibody, causative control reactive with Latex reagent, negative control non-reactive with Latex reagent.
2. Glass slide with 6 reaction circles.
3. Mixing sticks.
4. Rubber teats.
5. Sample dispensing pipettes.
6. Test tubes (10 X 75 mm).
7. Isotonic saline.
8. Stop watch.

Specimen Collection and Storage

No special preparation of the patient was required prior to sample collection by approved techniques.

The specimen was stored at 2-8°C for a period up to 24 hours.

Procedure

All reagents and samples were brought to room temperature before testing.
1. Pipette one drop of sample to be tested onto one of the reaction circles of the glass slide, using a sample dispensing pipette.
2. Prepare a 1:40 dilution (0.05 ml serum + 1.95 ml isotonic saline) of samples to be tested in isotonic saline.
3. Pipette one drop of diluted sample on the next reaction circle of the glass slide.
4. Place one drop of positive and negative control onto the remaining reaction circles of the slides (do not dilute controls).
5. Shake the Latex reagent while gently to uniformly disperse the reagent suspension. Add one drop of the Latex reagent to each of the samples and controls on the slide.
6. Mix with separate mixing sticks, spreading the mixture uniformly over the entire reaction circle.
7. Immediately start a stop watch. Rock the slide gently back and forth, observing for agglutination macroscopically at 5 minutes.

**Interpretation of the results**

1. No agglutination with diluted and neat samples was a negative test : HBsAg absent.

2. Agglutination with neat sample but no agglutination with diluted sample was a positive test result : HBsAg present (weak positive).

3. Agglutination with both neat and diluted samples was a positive test result : HBsAg present (moderate positive).

4. Agglutination with diluted sample but no agglutination with neat sample was a positive result : HBsAg present (strong positive).