Bibliography

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Mast EE, Margolis HS, Fiore AE, Brink EW, Goldstein ST, Wang SA. 2005. A comprehensive immunization strategy to eliminate transmission of hepatitis B virus infection in the United States: recommendations of the Advisory Committee on


Noguchi A, Hayashi J, Nakashima K, Hirata M, Ikematsu H & Kashiwagi S. 1994. HBsAg subtypes among HBsAg carriers in Okinawa, Japan. Evidence of an important relationship in seroconversion from HBeAg to anti-HBe. Journal of Infection 28,141-150.


virologic characteristics of hepatitis B virus genotype B having the recombination with genotype C. Gastroenterology 124:925–932.


Appendix
APPENDIX I

Grading and Staging of liver biopsy specimens (Ishak et al., 1995).

<table>
<thead>
<tr>
<th>Modified HAI grading: Necroinflammatory scores</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>A. Periportal or periseptal interface hepatitis (piecemeal necrosis)</strong></td>
<td></td>
</tr>
<tr>
<td>Absent</td>
<td>0</td>
</tr>
<tr>
<td>Mild (focal, few portal areas)</td>
<td>1</td>
</tr>
<tr>
<td>Mild/Moderate (focal, most portal areas)</td>
<td>2</td>
</tr>
<tr>
<td>Moderate (continuous around &lt;50% of tracts or septa)</td>
<td>3</td>
</tr>
<tr>
<td>Severe (continuous around &gt;50% of tracts or septa)</td>
<td>4</td>
</tr>
<tr>
<td><strong>B. Confluent necrosis</strong></td>
<td></td>
</tr>
<tr>
<td>Absent</td>
<td>0</td>
</tr>
<tr>
<td>Focal confluent necrosis</td>
<td>1</td>
</tr>
<tr>
<td>Zone 3 necrosis in some areas</td>
<td>2</td>
</tr>
<tr>
<td>Zone 3 necrosis in most areas</td>
<td>3</td>
</tr>
<tr>
<td>Zone 3 necrosis + occasional portal-central (P-C) bridging</td>
<td>4</td>
</tr>
<tr>
<td>Zone 3 necrosis + multiple P-C bridging</td>
<td>5</td>
</tr>
<tr>
<td>Panacinar or multiacinhar necrosis</td>
<td>6</td>
</tr>
<tr>
<td><strong>C. Focal (spotty) lytic necrosis, apoptosis and focal inflammation</strong></td>
<td></td>
</tr>
<tr>
<td>Absent</td>
<td>0</td>
</tr>
<tr>
<td>One focus or less per 10X objective</td>
<td>1</td>
</tr>
<tr>
<td>Two to four foci per 10X objective</td>
<td>2</td>
</tr>
<tr>
<td>Five to ten foci per 10X objective</td>
<td>3</td>
</tr>
<tr>
<td>More than ten foci per 10X objective</td>
<td>4</td>
</tr>
<tr>
<td><strong>D. Portal inflammation</strong></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>0</td>
</tr>
<tr>
<td>Mild, some or all portal areas</td>
<td>1</td>
</tr>
<tr>
<td>Moderate, some or all portal areas</td>
<td>2</td>
</tr>
<tr>
<td>Moderate/marked, all portal areas</td>
<td>3</td>
</tr>
<tr>
<td>Marked, all portal areas</td>
<td>4</td>
</tr>
<tr>
<td>Maximum possible score for grading</td>
<td>18</td>
</tr>
</tbody>
</table>
Staging: Architectural Changes, Fibrosis and Cirrhosis

<table>
<thead>
<tr>
<th>Change</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>No fibrosis</td>
<td>0</td>
</tr>
<tr>
<td>Fibrous expansion of some portal areas, with or without short fibrous septa</td>
<td>1</td>
</tr>
<tr>
<td>Fibrous expansion of most portal areas with or without short fibrous septa</td>
<td>2</td>
</tr>
<tr>
<td>Fibrous expansion of most portal areas with occasional portal to portal (P-P) bridging</td>
<td>3</td>
</tr>
<tr>
<td>Fibrous expansion of portal areas with marked bridging (P-P) as well as portal-central (P-C)</td>
<td>4</td>
</tr>
<tr>
<td>Marked bridging (P-P and / or P-C) with occasional nodules (incomplete cirrhosis)</td>
<td>5</td>
</tr>
<tr>
<td>Cirrhosis, probable or definite</td>
<td>6</td>
</tr>
<tr>
<td>Maximum possible score</td>
<td>6</td>
</tr>
</tbody>
</table>

APPENDIX II – CHEMICAL’S AND BIOCHEMICAL’S

The chemical’s and biochemical’s used in the study were of either molecular biology grade or extra pure analytical grade. Sodium chloride, Chloroform, Isoamyl alcohol, Isopropanol, Glacial acetic acid, Phenol, Sodium hydroxide (Qualigens); Agarose (Invirogen, USA); Ethidium bromide, EDTA, Tris, Boric acid, Sodium Dodecyl Sulphate, Sodium acetate (Sigma, USA); Bromophenol blue, Xylene cyanol FF (USB, Cleveland OH, USA); Proteinase K (Ambion Inc, USA); Taq DNA polymerase (Biotaq-Bioline); NH₄+ Reaction buffer, dNTP’s, MgCl₂ (Bioline); Nuclease free water, 1kb DNA ladder, 100bp DNA ladder, 50bp DNA ladder, 6X DNA loading dye (Promega, Madison, Wisconsin, USA); Analytical grade absolute alcohol (Les Alcools De Commerce Inc. Ontario); ABI PRISM Big Dye Terminator v 3.1 cycle sequencing mix (Applied Biosystems, CA, USA); Restriction Enzymes Hinf I, Tsp509I and StuI (New
England Biolabs, Ipswich, MA). All the primers used in the study were custom synthesized by Genosys, Sigma. The positive control used for the molecular work was a kind gift from Dr Kramvis, Witwatersrand University, South Africa; GFX™ PCR DNA purification kit (Amersham, UK).

**Glass wares and plastic wares**

Sterile, nuclease free and protease free glass wares (Borosil or Schot-Duran) and plastic wares (Tarson or Axygen) were used for the preparation and storage of reagent and experimental procedures, Syringes (Dispovan).

**Computer software’s**

Primer Premier v 5.0 (Premier Biosoft International, CA, USA), BioEdit v 7.0.8 (Tom Hall, Ibis Biosciences, CA, USA), Quantity One Image analysis program (Bio-Rad, Alfred Nobel Drive, Hercules, CA, USA) and MEGA 4 (Sudheer Kumar, Center for Evolutionary Functional Genomics, AZ, USA), Statistical Package for the Social Sciences version 15 (SPSS Inc., Chicago, IL).

**Histopathologic Stains and Reagents**

Gill’s II Hematoxylin stain

Eosin (5% aqueous solution)

Xylene

Isopropanol

Bluing Reagent (1.5 % Lithium carbonate in distilled water)

Acid Water (0.3 % HCl conc. in distilled water)

Ethanol (Absolute)
## REAGENT PREPARATION

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Preparation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris-HCl</td>
<td>1M Tris, (pH 8).</td>
</tr>
<tr>
<td>EDTA</td>
<td>0.5M, pH adjusted with NaOH, (pH 8.0).</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>5M in water, autoclaved.</td>
</tr>
<tr>
<td>SDS</td>
<td>20% in sterile water.</td>
</tr>
<tr>
<td>Proteinase K</td>
<td>20 mg/mL in sterile water.</td>
</tr>
<tr>
<td>Phenol (Neutral)</td>
<td>Saturated with Tris HCl (pH 8.0).</td>
</tr>
<tr>
<td>Sodium Acetate</td>
<td>3M in water, pH adjusted with Glacial acetic acid to pH 5.2, autoclaved.</td>
</tr>
<tr>
<td>Ethidium Bromide</td>
<td>10mg/mL in sterile water.</td>
</tr>
<tr>
<td>Magnesium chloride</td>
<td>1M in water, autoclaved.</td>
</tr>
<tr>
<td>Sodium hydroxide</td>
<td>2M in water, autoclaved.</td>
</tr>
<tr>
<td>Glucose</td>
<td>1M in water, autoclaved.</td>
</tr>
<tr>
<td><strong>TE Buffer</strong></td>
<td></td>
</tr>
<tr>
<td>Tris Buffer</td>
<td>0.12g.</td>
</tr>
<tr>
<td>EDTA</td>
<td>0.037g.</td>
</tr>
<tr>
<td>Volume</td>
<td>100ml.</td>
</tr>
<tr>
<td><strong>TBE (5X), 1 Litre</strong></td>
<td></td>
</tr>
<tr>
<td>Tris base</td>
<td>54g.</td>
</tr>
<tr>
<td>Boric acid</td>
<td>27.5g.</td>
</tr>
<tr>
<td>0.5M EDTA (pH 8)</td>
<td>20ml.</td>
</tr>
<tr>
<td><strong>Gel Loading Dye</strong></td>
<td></td>
</tr>
<tr>
<td>Bromophenol blue</td>
<td>0.25% (w/v).</td>
</tr>
<tr>
<td>Xylene cyanol FF</td>
<td>0.25% (w/v).</td>
</tr>
<tr>
<td>Glycerol in water</td>
<td>30% (v/v).</td>
</tr>
</tbody>
</table>
### APPENDIX III  Details of primers used in the study.

<table>
<thead>
<tr>
<th>PCR</th>
<th>Primer</th>
<th>Position</th>
<th>Sequence</th>
<th>Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>S1 PCR</td>
<td>2410F(+)</td>
<td>2410-2439</td>
<td>5'-TCAATCGCGCGTGCGAGAGATCTCTCAATC-3'</td>
<td>2150</td>
</tr>
<tr>
<td>(S1F/S1R)</td>
<td>1314R(-)</td>
<td>1314-1291</td>
<td>5'-TCCAGACCGCCTCGAGCAAAACA-3'</td>
<td></td>
</tr>
<tr>
<td>S2 PCR</td>
<td>2451F(+)</td>
<td>2451-2482</td>
<td>5'-AATGTTAGTATTCTTTGACTCATAAGGCTGGG-3'</td>
<td>2050</td>
</tr>
<tr>
<td>(S2F/S2R)</td>
<td>1280R(-)</td>
<td>1280-1254</td>
<td>5'-AGTTCCCGAGTATGAGTGCCAGGAGA-3'</td>
<td></td>
</tr>
<tr>
<td>P7 / P8</td>
<td>256F(+)</td>
<td>256-278</td>
<td>5'-GTGGTGGACTTCTCTCAATTTTC-3'</td>
<td>541</td>
</tr>
<tr>
<td></td>
<td>796R(-)</td>
<td>796-776</td>
<td>5'-CGTAWAAAGGGACTCAMGAT-3'</td>
<td></td>
</tr>
</tbody>
</table>

#### Subgenotyping

<table>
<thead>
<tr>
<th>PCR</th>
<th>Primer</th>
<th>Position</th>
<th>Sequence</th>
<th>Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>S1 PCR</td>
<td>2410F(+)</td>
<td>2410-2439</td>
<td>5'-TCAATCGCGCGTGCGAGAGATCTCTCAATC-3'</td>
<td>2150</td>
</tr>
<tr>
<td></td>
<td>1314R(-)</td>
<td>1314-1291</td>
<td>5'-TCCAGACCGCCTCGAGCAAAACA-3'</td>
<td></td>
</tr>
<tr>
<td>S22F/1192R</td>
<td>522F(+)</td>
<td>522-541</td>
<td>5'-CCTGCACGACTCTCTGCTCAAA-3'</td>
<td>671</td>
</tr>
<tr>
<td></td>
<td>1192R(-)</td>
<td>1192-1173</td>
<td>5'-CGTCAGCAAACACTTGGCAC-3'</td>
<td></td>
</tr>
</tbody>
</table>

#### Base Core Promoter/Pre Core

<table>
<thead>
<tr>
<th>PCR</th>
<th>Primer</th>
<th>Position</th>
<th>Sequence</th>
<th>Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>BCP1 PCR</td>
<td>1606F(+)</td>
<td>1606-1625</td>
<td>5'-GCATGGAGACCGCACCGTGAAC-3'</td>
<td>368</td>
</tr>
<tr>
<td>BCPF1/ BCPRI</td>
<td>1974R(-)</td>
<td>1974-1955</td>
<td>5'-GGAAAGAAGTCAGAAAGCAGCAA-3'</td>
<td></td>
</tr>
<tr>
<td>BCP2 PCR</td>
<td>1653F(+)</td>
<td>1653-1672</td>
<td>5'-CATAAGAGGACTCTCTGGACT-3'</td>
<td>307</td>
</tr>
<tr>
<td>BCPF2/ BCPR2</td>
<td>1959R(-)</td>
<td>1959-1940</td>
<td>5'-GGCAAAAAACAGAGTAAACTC-3'</td>
<td></td>
</tr>
</tbody>
</table>

#### Sequencing

<table>
<thead>
<tr>
<th>PCR</th>
<th>Primer</th>
<th>Position</th>
<th>Sequence</th>
<th>Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>BCPF(+)</td>
<td>1653-1672</td>
<td></td>
<td>5'-CATAAGAGGACTCTCTGGACT-3'</td>
<td>307</td>
</tr>
<tr>
<td>BCP(PreC)</td>
<td>1959-1940</td>
<td></td>
<td>5'-GGCAAAAAACAGAGTAAACTC-3'</td>
<td></td>
</tr>
</tbody>
</table>
APPENDIX IV - Equipments used in this study

-20°C Freezer - Bluestar
-80°C Deep Freezer - U570 Premium (New Brunswick Scientific).
Autoclave - Stericylindrical Horizontal sterilizer (Yorco Scientific)
Automated Sequencer - ABI PRISM™ ABI 3130 Genetic Analyzer, Perkin Elmer.
Centrifuges - Eppendorf Centrifuge 5417C, Eppendorf Thermomixer Comfort
Dri Bath - AccuBlock™ Digital Dry Bath Labnet (Labnet- Intl.Inc)
Heating plate - Delite, Johnson.
Horizontal electrophoresis apparatus - Broviga
Hot Air Oven - Beston.
Ice Maker - Simag- SP255
Incubator - R1-10S Bacteriological Incubator REMI
Laminar Air Flow - Alpha Linear Labline
Micropipettes - Labsystems Finnpipette (5-40 µL), (0.5-10 µL), (40-200 µL)
Microscope - Labomed Vision 2000
Cell count chamber - Neubauer hemocytometer
Microwave Oven - Sanyo
PCR Machines - Eppendorf Master cycler gradient, BioRad I Cycler
pH meters - µ pH systems 361, Systronics.
Power Packs - Power Pack 200- Bio Rad/ Broviga.
Refrigeration - Samsung
Scanner - ScanJet- 6200C, Hewlett Packard
UV Illuminator - BioRad UV gel capture
Vortexer - Cyclomixer CM 101, REMI
Water Purification - Milli Q- Plus, Millipore.
Weighing Balance - BL 600, BP221S, Sartorius.
AASLD (2009)

Chronic hepatitis B
Chronic necroinflammatory disease of the liver caused by persistent infection with hepatitis B virus. Chronic hepatitis B can be subdivided into HBeAg + and HBeAg - chronic hepatitis B.

Inactive HBsAg carrier state
Persistent HBV infection of the liver without significant ongoing necroinflammatory disease.

Resolved Hepatitis B
Previous HBV infection without further virologic, biochemical or histological evidence of active virus infection or disease.

Acute exacerbation or flare of hepatitis B
Intermittent elevations of aminotransferase activity to more than 10 times the upper limit of normal and more than twice the baseline value.

 Reactivation of hepatitis B
Reappearance of active necroinflammatory disease of the liver in a person known to have the inactive HBsAg carrier state or resolved hepatitis B.

HBeAg clearance
Loss of HBeAg in a person who was previously HBeAg positive.

HBeAg seroconversion
Loss of HBeAg and detection of anti-HBe in a person who was previously HBeAg positive and anti-HBe negative.

HBeAg reversion
Reappearance of HBeAg in a person who was previously HBeAg negative, anti-HBe positive.

Diagnostic criteria

Chronic hepatitis B
1. HBsAg positive > 6 months.
2. Serum HBV DNA > 20,000 IU/ml (10^6 copies/ml), lower values 2,000 - 20,000 IU/ml (10^4 - 10^5 copies/ml).
3. Persistent or intermittent elevation in ALT/AST levels.
4. Liver biopsy showing chronic hepatitis with moderate or severe necroinflammation.

Inactive HBsAg carrier state
1. HBsAg positive > 6 months
2. HBeAg Negative, anti-HBe Positive
3. Serum HBV DNA < 2,000 IU/ml
4. Persistently normal ALT/AST levels
5. Liver biopsy confirms absence of significant hepatitis.

Resolved Hepatitis B
1. Previous known history of acute or chronic hepatitis B or the presence of anti-HBe ± anti-HBs
2. HBsAg Negative
3. Undetectable serum HBV DNA
4. Normal ALT levels
Presentations


4. Keyter, M; Shenoy, KT; Leena KB; **Deepak G**; Soniya EV; Kew, M; Sourvinos, G; Kramvis, A. An evaluation of the relationship between hepatitis B virus genotypes, the Basal core promoter/precocore region and the 249SER p53 mutations. International meeting: The Molecular Biology of Hepatitis B Viruses, Loire Valley Tours, France. August 30-September 2, 2009