Hepatitis B Infection
Quantitative determination of HBsAg
Significance, problems and solutions

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"Natural weapons of mass destruction" on a "Richter"-scale (Weiss und McMichael NatMed 10:S70)
Why we need quantitative assays for HBsAg

• Quality control of HBsAg screening
  - Evaluation of testkits
  - Control of analytical sensitivity
  - Accurately adjusted working controls

• Monitoring of HBV infection
  - Marker for HBV gene expression
  - Surrogate marker of HBV cccDNA in liver
  - Early prognosis of acute and chronic HBV infection
  - Indication and monitoring of interferon therapy

• Establishment of reference sera and HBsAg units
Problems in the standardisation of HBsAg assays

- Heterogeneity of HBsAg particles
HBV- und subviral HBsAg particles in 1 mL blood from highly viremic carriers

$10^9$  $10^{10}$  $10^{13}$
Components of HBV and HBsAg Particles

- **virus**
- ** HBc**
- **DNA-Pol**
- **3.2kb DNA**
- **pr**
- **preS1**
- **preS2**
- **HBsAg**
- **filaments**
- **LHBs**
- **SHBs**
- **MHBs**
- **spheres**
- **length variable**
- **52 nm**
- **17-25 nm**
Problems in the standardisation of HBsAg assays

- Heterogeneity of HBsAg particles
- Heterogeneity of HBs proteins
Model of HBV and subviral HBsAg at 1983
Proteins of HBV and subviral HBsAg-Particles
Proteins of HBV and subviral HBsAg-Particles

Heermann et al. 1984
Components of HBV and HBsAg Particles

- Virus
- preS1
- preS2
- Filaments
- LHBs
- SHBs
- MHBs
- Spheres
- Variable Länge
- 52 nm
- 17-25 nm
Intracellular Life Cycle of Hepatitis B Virus

LHBs
PreS1
attachment
endocytosis

nucleus
golgi
ER
Intracellular Life Cycle of Hepatitis B Virus

LHBs
PreS1
attachment

fusion

endocytosis

release of cores
nuclear transport

uncoating of genome

S domain

nucleus

golgi

ER
Intracellular Life Cycle of Hepatitis B Virus

1. Attachment
2. Endocytosis
3. Fusion
4. Release of cores and nuclear transport
5. Uncoating of genome
6. DNA repair
7. RNA Pol II transcription factors
8. CccDNA

Steps:
- Attachment of virus to cell surface
- Endocytosis into the cell
- Fusion of viral envelope with the cell membrane
- Release of viral core and genome into the nucleus
- Uncoating of the genome
- DNA repair
- Transcription of cccDNA by RNA Pol II
Significance of HBsAg quantity in serum for the course of the infection

- **Synthesis**
  - Dependent on the amount and transcriptional activity of cccDNA in liver
  - Independent of viral genome maturation
- **Increase**
  - New infection or reactivation
- **Decrease of HBsAg in serum indicates**
  - Decrease of cccDNA in the liver and/or
  - Efficient T cell immunity
Monitoring of HBsAg positive persons

- According to German guidelines
  - HBV DNA quantitative
  - HBeAg qualitative
  - HBsAg qualitative

- More important than qualitative serology would be
  - HBsAg quantitative
**Anti-HBs**

**HBsAg**

**HBV DNA**

**PCR**

**Hepatitis**

**Anti-HBc**

**Anti-HBs**

**Acute hepatitis B resolving**
Kinetics of HBV DNA and HBsAg during acute Hepatitis B, Moscow 2002

- 52 patients, mostly i.v. drug addicts
  - Weekly samples
  - Alle except one HIV-co-infected recover
- HBV DNA
  - real-time PCR
  - Calibrated with WHO/Eurohep Standard
- HBsAg
  - Quantitative Immune electrophoresis
  - Calibration with Paul-Ehrlich Standard in $\mu$g/mL

Half life of HBV DNA (days)
Half life of HBsAg (days)
## Prognosis of acute hepatitis B

Germany, 1970ies, 370 patients, biopsy confirmed

<table>
<thead>
<tr>
<th>HBsAg concentration change within 4 weeks</th>
<th>number of cases</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>resolved</td>
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<tr>
<td>decrease &gt;60%</td>
<td>337</td>
</tr>
<tr>
<td>constant or &lt;60%</td>
<td>2</td>
</tr>
<tr>
<td>increase</td>
<td>1</td>
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</table>

The first HBsAg Standards of the Paul Ehrlich Institute (PEI)

• Positive plasma units collected in the early 1970ies
  - were screened for high HBsAg titer
  - and subtyped for HBsAg subtypes \( ad \) and \( ay \)
  - Subtypes were pooled separately and recalcified

• HBs antigen (QIE) and protein (\( \text{OD}_{280} \))
  - was quantified as described by Gerlich & Thomssen 1975
  - Adjusted to 50,000 ng HBs \( ad \) or \( ay \) protein / mL
  - Distributed to aliquotes and stored at -80 °C

• HBsAg with reactivity of
  - one nanogram HBs protein in the first PEI standards
  - is one PEI unit of HBsAg

Bonin, Gerlich, Thomssen, 1975
Problems in the standardisation of HBsAg assays

• Heterogeneity of HBsAg particles
• Heterogeneity of HBs proteins
• Diverging HBsAg units
• Diverging HBsAg standards
Three different units of HBsAg quantity

- 1975 Paul Ehrlich Institute Units (PEI-U)
  - equal to one nanogram (ng) **native** HBsAg protein*
- 1980ies WHO 1st International Standard (IU)
  - 1 IU also defined by one ng HBsAg**, pasteurised
  - One IU equalled only 0.55 PEI-U
- Various “nanogram” units (ng) from other institutions
  - Based on purified, **partially inactivated** HBsAg
- Comparison of the 3 unitages in a WHO trial showed
  - Good comparability of the PEI and IU
  - Incompatibility between PEI/IU and most “ng” units
- Official recommendation from WHO: use IU only!

* As present in highly infectious HBV carrier plasma
** As in less infectious, anti-HBe positive plasma
Problems in the standardisation of HBsAg assays

• Heterogeneity of HBsAg particles
• Heterogeneity of HBs proteins
• Diverging HBsAg units
• Diverging HBsAg standards
The WHO 2nd International HBsAg standard

- **Source material:** The Dutch Hepatitis B Vaccine lot 30
  - Purified from highly viremic HBeAg-positive plasma
    - Infectivity inactivated by heating to 102 °C for 90 sec
  - Contained in the 1980ies 66,000 PEI units/mL (Ausria)

- **Characterisation studies done in Giessen**
  - Assay of HBsAg activity in PEI-U using QIE
  - Protein composition using PAGE and silverstain
  - Western blot for preS and S proteins
  - Size exclusion chromatography
  - Density gradient centrifugation

Summary on the source material for the 2nd WHO International Standard for HBsAg

• S-HBs antigenicity was surprisingly high and stable
  - 25 years ago: 66,000 PEI units/mL by Ausria
  - today ca.: 60,000 PEI units/mL by QIE

• Successfully used in WHO trial with various immune assays

• Altered biochemical properties

• Heterogeneous particles
HBsAg quantity and the prognosis of chronic HBV infection
Different HBsAg levels in various phases of chronic HV infection

Jaroczewisz, Cornberg et al., J Hepatol 2010, Medical School Hanover
Different HBsAg levels in various phases of chronic HBV infection

Jaroczewisz, Cornberg et al., J Hepatol 2010, Medical School Hanover
Different HBsAg levels in various phases of chronic HBV infection

Jaroczewisz, Cornberg et al., J Hepatol 2010, Medical School Hanover
Different HBsAg levels in various phases of hepatitis B

Clinical significance?

H. Wedemeyer, MHH 2010
Different HBsAg levels in various phases of hepatitis B

Clinical significance?

Brunetto et al., Gastroenterology 2010: Cut-Off 1000 IU/ml

HBsAg <3500 IU/ml
No reactivations in long-term follow-up!
HBV Therapy: two concepts

• Interferon
  - Possibility for sustained viral response (SVR)
  - Limited duration 6 -12 months
  - But only in 30 % of well selected patients
  - Severe side effects
  - Not possible in advanced liver disease

• Reverse transcriptase inhibitors
  - Well tolerated, effective suppression of viremia
  - Complete cure extremely rare
  - Unlimited duration
  - Resistance
Which patient should get interferon?

Taken from H. Wedemeyer, MHH 2010
Relation between HBsAg concentration and outcome of interferon therapy

Düsseldorf, 1990ies, 96 chronic HB patients

<table>
<thead>
<tr>
<th>Response</th>
<th>yes</th>
<th>no</th>
<th>p</th>
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</thead>
<tbody>
<tr>
<td>HBeAg +</td>
<td>15 800*</td>
<td>50 000*</td>
<td>&lt;0.003</td>
</tr>
<tr>
<td>HBeAg -</td>
<td>3 800</td>
<td>4 500</td>
<td>ns</td>
</tr>
</tbody>
</table>

* ng HBsAg/mL or PEI-U/mL measured by QIE before therapy

Erhardt et al. Hepatology 2000; 31:716-725
Decrease of HBsAg* during interferon therapy


- 386 patients, HBeAg negative
- 48 weeks therapy with
  - PEG-interferon,
  - or lamivudin
  - or combination of both
- HBsAg decrease only with PEG-Ifn
  - On the average by factor 6
  - Only when complete cure occurred**
- With lamivudin alone,
  - even with persistently negative viraemia **
  - no decrease of HBsAg

*Architect, Abbott     ** < 400 copies/mL HBV DNA
48 patients with chronic hepatitis B, HBeAg-negative
- 48 weeks PEG-Ifn
- 12 (25%) SVR, <70 copies/mL
- 3 HBsAg negative
- Decrease of HBsAg only when SVR followed

Decrease

>0.5 after 12 W.: 90% NPV, 89% PPV
>1.0 after 24 W.: 97% NPV, 92% PPV
HBsAg levels after interferon therapy: SVRs vs. Relapsers

*SVRs (N=12)

NRs (N=18)

Relapsers (N=18)

*SVRs (N=12)

Moucari et al. Hepatology 2009
Intracellular Life Cycle of Hepatitis B Virus
Problems in the standardisation of HBsAg assays

- Heterogeneity of HBsAg particles
- Heterogeneity of HBs proteins
- Diverging HBsAg units
- Diverging HBsAg standards
- Different genotypes
Summary on the source material for the 2nd WHO International Standard for HBsAg

• SHBsAg surprisingly high and stable
  - 25 years ago: 66,000 PEI units/mL by Ausria
  - today ca.: 60,000 PEI units/mL by QIE
• Successfully used in WHO trial with various immune assays
• Altered biochemical properties
• Heterogeneous particles
• HBV genotype A2
  - Central European variant
  - Present in 0.9 % of HBV carriers only
• Native HBsAg of all genotypes would be preferable
HBV: one virus species
but
many genotypes A1 – I of humans
and
related genotypes of apes
Worldwide distribution of HBV Genosubtypes

From Schaefer & Gerlich, Textbook of Hepatology 2007, 825
WHO program for HBV genotype panels

- for virion-bound HBV DNA in plasma (established)
- and **native** HBsAg in plasma (in progress)
  - realised by Paul Ehrlich Institute together with Institute of Medical Virology Giessen
HBV DNA geno(sub)type / HBsAg subtype reference panel of WHO

- Collection of 215 plasmas worldwide
- Geno(sub)type determined by sequencing
  - Nomenclature A1 – H according to Norder et al. (2004)
- Selection of 16 HBsAg highly positive samples
  - from various parts of the world
  - With typical wild type sequences
HBV DNA geno(sub)type / HBsAg subtype reference panel of the WHO

- **HBs protein** amount was determined *after* purification
  - nanogram HBsAg protein (ng) per mL

- **HBs antigen reactivity** was determined *before* purification
  - in **PEI units (PU)** by in-house immune electrophoresis
    - one PU should correspond to 1 ng HBs protein
  - in **International Units (IU)**
    - by quantitative immune assay
    - Architect from Abbott
### Composition, origin and HBs protein contents of the 16 WHO panel members

<table>
<thead>
<tr>
<th>Genotype</th>
<th>HBsAg subtype</th>
<th>Origin</th>
<th>µg/ml protein</th>
<th>Notes</th>
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<tr>
<td>KG</td>
<td>D1_ayw2</td>
<td>Germany</td>
<td>93.47</td>
<td>Internal reference plasma</td>
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<tr>
<td>N4542</td>
<td>A1_adw2</td>
<td>South Africa</td>
<td>119.84</td>
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<td>Brazil</td>
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<td>N4879</td>
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<td>B1_adw2</td>
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<td>29.82</td>
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<td>N4222</td>
<td>B2_adr</td>
<td>Japan</td>
<td>80.46</td>
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<tr>
<td>N4227</td>
<td>C2_Ce_adr</td>
<td>Japan</td>
<td>84.02</td>
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<td>N4229</td>
<td>C2_Ce_adr</td>
<td>Japan</td>
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<td>N3825</td>
<td>C2_Ce_adr</td>
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<tr>
<td>N4882</td>
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<td>Germany</td>
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<td>K1146</td>
<td>H_adw4</td>
<td>Germany</td>
<td>107.88</td>
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</table>
Ratios of PEI-U or IU HBsAg to ng HBs protein in the WHO reference plasmas

<table>
<thead>
<tr>
<th>Genotype/Subtype</th>
<th>Origin</th>
<th>Start PEI-KU/ml</th>
<th>KIU/ml (Architect)</th>
<th>µg/ml protein</th>
<th>PEI-U/ng protein</th>
<th>IU/ng protein</th>
<th>IU/PEI-U</th>
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<tbody>
<tr>
<td>KG D1 ayw2</td>
<td>Germany</td>
<td>100</td>
<td>125,19</td>
<td>93,47</td>
<td>1,07</td>
<td>1,35</td>
<td>1,2519</td>
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<td>KG D1 ayw2</td>
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<td>75,3</td>
<td>131,92</td>
<td>119,84</td>
<td>0,63</td>
<td>1,10</td>
<td>1,75</td>
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<td>KG D1 ayw2</td>
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<td>94,0</td>
<td>92,69</td>
<td>0,76</td>
<td>1,01</td>
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<td>39,8</td>
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<td>29,82</td>
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<td>1,72</td>
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<td>50,1</td>
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<td>50,37</td>
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<td>KG E_ayw4</td>
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<td>24,79</td>
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<td>0,71</td>
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**MW without marked values:**

<table>
<thead>
<tr>
<th></th>
<th>PEI-U/ng protein</th>
<th>IU/ng protein</th>
<th>IU/PEI-U</th>
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<tr>
<td></td>
<td>0,90</td>
<td>1,35</td>
<td>1,73</td>
</tr>
<tr>
<td>SD</td>
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<tr>
<td>MW</td>
<td>1,08</td>
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<tr>
<td>SD</td>
<td>0,13</td>
<td>0,27</td>
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</tr>
</tbody>
</table>
Conversion factors for nanogram HBsAg protein to Paul Ehrlich Institute QIE-Units (PU)

- No outliers
- Mean value

0.90  0.20 PEI-U/ng HBs protein
range 0.62 - 1.33

- determined by QIE with “anti-a” serum generated with subsequent injection of genotype A2, D, and C2
Conversion factors for nanogram HBsAg protein to HBsAg International Units (Architect)

- One ng was between
  - 0.84 IU for genotype C2, subtype adr to
  - 3.17 IU for genotype H, subtype adw2

- Mean value
  - 1.35 ± 0.45 IU/ng
    range 0.84 - 3.17

- 1.08 ± 0.13 IU/ng
  range 0.84 - 1.18
  - if 4 outliers (B1, D3, F3, H) were excluded.
  - These reacted better with Architect than expected
Detection limits of Enzygnost HBsAg v6 with the future WHO panel

- **Picogram HBsAg protein**
  - 7.2 pg/ml for gt A2 to
  - 12.7 pg/ml for gt B2
  - mean value 9.5 ± 1.6 pg/ml.

- **Milli Paul Ehrlich Units** (in house QIE, Giessen)
  - 5.8 for gt A1 to
  - 14.6 mPU/ml for gt D1
  - mean value 8.9 ± 2.2 mPU/ml.

- **Milli International Units** (WHO, Architect)
  - 8.3 for gt A2 to
  - 18.7 mIU/ml for gt F3
  - mean value 11.6 ± 3.0 mIU/ml.
Conclusions I

Methods for HBsAg quantitation

- Reference plasmas for most HBsAg genotypes available soon
- Accurate assay of HBs proteins in SI units possible
- Significant differences between different genotypes
- HBsAg nanograms of all genotypes correlate well with „old“ PEI-antigen units
- More divergence with IU (Architect)
  - Much less without four outliers (B1, D3, G, H)
- Most consistent data obtained with ng units
Conclusions II

Medical Significance of HBsAg quantitation

• Monitoring of HBV infection
  - Marker for HBV gene expression
  - Surrogate marker of HBV cccDNA in liver
  - Early prognosis of acute HBV infection
  - Single point assay allows for prognosis of chronic HBV infection
  - Indication and monitoring of interferon therapy
Many thanks to

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Mona Saniewski
Corinna Bremer

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