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Original investigation

Molecular approaches to validate disinfectants against human hepatitis B virus

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Abstract. Disinfection is an important measure to prevent hepatitis B virus (HBV) transmission by instruments. However, virucidal testing of disinfectants against HBV is difficult, because no simple quantitative infectivity assay exists. Since molecular changes of viral epitopes and the genome may indicate virus inactivation, we measured the alteration of these constituents with 0.065% peracetic acid (PAA) for exposure times up to 1 h. Plasma of a chronic HBV carrier with 10^9 HBV genomes/ml served as viral source in the form of a 10% dilution or of a purified HB-antigen preparation. Alterations of HBV epitopes were analyzed with four monoclonal antibodies in an enzyme-linked immunosorbent assay. Changes of the HBV genomes were determined by the inability to amplify the target sequence with a quantitative real-time polymerase chain reaction, either of a short fragment (189 bp) or of the full-length (3,200 bp). The determination of the epitope and genome alteration was quantified as $\log_{10}$ reduction factor (RF) with the parallel line bioassay. Under a high protein load of 10% human plasma, PAA induced a HBV genome alteration of RF=1.5 after an exposure time of 60 min. Similar RFs were seen with the four HB epitopes. Without protein load, the alteration of these epitopes amounted to a RF of more than 3.5 within 30 min. Such inhibition of PAA activity by protein load was also seen in the virucidal tests with parvovirus. Although the RF were higher in the virucidal tests, the time-dependent dose-response curves for the epitope and genome alteration and for the infectivity inactivation followed the same inactivation kinetics. The molecular alteration and disintegration epitope and genome test may therefore be suitable to measure antiviral activity of disinfectants against HBV.

Keywords. Hepatitis B virus - Virus inactivation - Disinfection - Peracetic acid - Full-length polymerase chain reaction

Introduction

Human hepatitis B virus (HBV) infections remain a major public health problem due to (i) the highly infectious nature of HBV, (ii) the high susceptibility of the population to HBV [21], and (iii) the significant prevalence of HBV carriers in the general population [19]. Human blood plasma is the

main virus reservoir and sera from certain HBV carriers, even in dilutions of 1: 100,000,000 are still able to infect chimpanzees when given intravenously. HBV is usually introduced into the medical community by patients with unrecognized viremia.

In addition to direct inoculation from one HBV-infected person to another, transmission by medical instruments, surfaces and hands still plays an important role. Moreover, mucocutaneous contact with virus contaminated surfaces or hands, exhibits a high exposure risk to compromised patients, e.g., under immune suppression, at large operations, anti-cancer therapy, or under treatment of wounds after burns. HBV may also be transmitted if medical instruments became virus contaminated during invasive procedures and were not adequately sterilized or disinfected before further use [25, 32].

Moist heat sterilization, which is the most reliable measure for complete viral inactivation, can only be carried out on some medical instruments having the appropriate resistance against heat. Vaccination of immune-suppressed patients against HBV is not feasible and anti-HBV therapy is also not readily available. Therefore, chemical disinfection of HBV is of the utmost importance [25]. However, the activity of disinfectants against HBV is not generally guaranteed even after vigorous testing of disinfectants according to guidelines or norms.

Principally, enveloped viruses are considered to be relatively sensitive to disinfectants. However, HBV behaves differently; in some situations, it may be inactivated easily by some disinfectant measures but is very resistant in other situations [3, 29, 36]. In addition, human blood plasma protects HBV from inactivation. Therefore, validation of the virucidal action of disinfectants against HBV is essential for efficient patient's protection.

A disinfectant is active against viruses, if it reduces viral infectivity under defined conditions [8]. Generally, virucidal activity is determined on cell cultures, using quantitative tests with a panel of selected test viruses such as poliovirus, adenovirus, parvovirus and papovavirus in Europe [1, 8, 13, 30]. Testing of additional clinically important viruses is considered to be necessary to prove the specific virucidal activity of a disinfectant. One of these viruses that should be tested is HBV.

Unfortunately, no feasible infectivity assay for HBV exists to date, either in cell culture or in small animals, although several authors (e.g., [2, 11, 20, 26]) tried to adapt different cell culture systems including hepatoma cell lines (e.g., HepG2). However, the results could not be reproduced by others and a recent report lacks essential data [26].

To overcome this unfavorable situation, so-called surrogate tests were developed in Germany in the 1980s [5]. These tests measure the reduction of antigenicity [10, 28] or the morphological alteration and disintegration (AD) of the HBV viral structures instead of reduction of infectivity [35]. Some correlation between the reduction of infectivity and disintegration of electron microscopically visible viral structures was demonstrated [28, 36]. These surrogate tests, however, were abandoned recently, because of higher safety standards and improved knowledge of HBV [7]. However, it was recommended that methods to study the AD of viral structures that may be responsible for viral infectivity should be further improved.

In a first attempt, we formulated quantitative molecular alteration and disintegration tests (Molecular-ADT) for HBV, which consists of tests for the destruction of sequential epitopes (Epitope-ADT) in all three surface protein domains [33] and, even more stringent, the destruction of the viral genome in a way that it can no longer be amplified by the DNA polymerase in the PCR (Genome-ADT).

We report here results obtained with this Molecular-ADT to validate the HBV-destroying activity with a disinfectant containing peracetic acid. This disinfectant has shown its virucidal activity against poliovirus, adenovirus and vaccinia virus according to the AFNOR norm (D. Leblanc, personal communication). For comparison, we report on the virucidal activity against parvovirus

because this virus is considered to have a similar resistance to that of HBV [4].

Material and methods

Disinfectants tested

The disinfectant for instruments, further designated as RD 88[1] contains 1.3% peracetic acid and 7.5% hydrogen peroxide. It was used at a concentration of 5%, yielding a final concentration of 0.065% peracetic acid and 0.375% H_2O_2. For comparison, a control reagent with a final concentration of 0.5% peracetic acid and 2.5% H_2O_2 was used in parallel and designated as PAA. All tests were carried out at least twice as quantitative suspension tests [8] at a temperature of 20°C and reaction times of 15, 30 and 60 min.

Bovine parvovirus

Bovine parvovirus (strain Haden; kindly provided by Dr. G. Pauli, Robert Koch-Institute, Berlin) was used as test virus for virucidal disinfectants for medical instruments according to the German guideline of the Robert Koch-Institute [30] with (3 g/l bovine serum albumin) and without added protein (0.3 g/l bovine serum albumin). Parvovirus was titrated on fetal calf lung cells (FKL-107; kindly provided by Dr. W. Herbst, Giessen).

Human HBV

Plasma from a chronic HBV envelope antigen (HBeAg)-positive virus carrier with genotype D, HBV surface antigen (HBsAg) subtype ayw2 and a HBV genome titer of 6.25×10^9 genomes/ml, as calibrated against the WHO-Reference standard plasma [17, 31] was used for the Genome-ADT (one International Unit of HBV DNA corresponds to approximately five genomes). This plasma was also used as a source of HBsAg for the Epitope-ADT and contained 100 µg/ml HB protein (according to [12]).

HBsAg particles

HBsAg particles were purified by ultracentrifugation of plasma through a sucrose density gradient [15-60% (wt/wt)] at 25,000 rpm with an SW 28.38 rotor (Beckman, Munich, Germany) for 15 h. The HBsAg-containing fractions were pooled, adjusted with solid CsCl to 1.30 g/ml, and layered within a CsCl density gradient ranging from 1.16 to 1.35 g/ml at 25,000 rpm in an SW 28.38 rotor (Beckman) for 36 h. HBsAg-containing fractions were dialyzed and further purified by sedimentation in a sucrose density gradient [5-60% (wt/wt)] at 25,000 rpm with an SW 28.38 rotor for 15 h. HBsAg-rich fractions were pooled and concentrated in a Centriplus-100 ultrafiltration device (Millipore, Eschborn, Germany). Silver-stained SDS-PAGE of highly purified HBsAg showed clear bands of HBsAg without major contamination. With a protein content of 1.63 mg/ml, this preparation was used in a 1:100 dilution in PBS for the Epitope-ADT under clean conditions.

Monoclonal antibodies

Four monoclonal antibodies (mAb) corresponding to different regions of the HBV surface proteins, which should neutralize HBV, were used in the Epitope-ADT: (i) C20-02, a mouse mAb, epitope: group specific, aa 110-160 in SHBs; conformational, non-reactive in Western blot; affinity purified, concentration 1 mg/ml; (ii) MA 18/7, a mouse mAb [16], epitope: sequential, aa 31-34 (DPXF [14]) in preS1/genotype A or D, affinity purified, concentration 1 mg/ml (this antibody neutralizes binding of HBV to human hepatocytes [27]); (iii) 2-11B1, a mouse mAb [33], epitope within aa 3-12

(WNSTTFQHTLQ [33]) in preS2/genotype D, from ascites partial purified after precipitation with sodium sulfate, concentration 16 mg/ml; and (iv) 1-9C1, a mouse mAb [33], epitope includes aa 119-123 in SHBs; reactive in Western blot, better with genotype D than A [33]; from ascites, partially purified after precipitation with sodium sulfate, concentration 12 mg/ml.

Molecular-ADT test protocol

One part of HBV-containing plasma or purified HBsAg was mixed with one part of phosphate-buffered saline (PBS, pH 7.4) and with 8 parts of RD 88 of a 6.25% solution (final concentration in the test 5%). Preparations of PAA (control reagent) were prepared in the same way (final concentration of 0.5% peracetic acid). The mixtures were held at 20°C for 15, 30 and 60 min and were afterwards filtered through Sephadex G-25 either in NAP-5 or MicroSpin filter units (Pharmacia), dependent upon the sample volume (500 µl or 100 µl), to remove the disinfectant. In the case of NAP-5, this led to a twofold-diluted sample, whereas in the case of MicroSpin no further dilution took place. These eluates were used without any further treatment in the Epitope- and Genome-ADT.

Epitope-ADT

The quantitative alteration and/or disintegration of epitopes against respective mAb was monitored by ELISA. The mAb were diluted 1:500 in PBS pH 7.4 and adsorbed to microtiter plates overnight at 4°C. Afterwards the plates were washed twice with 0.25% Tween 20/PBS and three times with PBS alone to reduce unspecific reactions. The HBsAg particle suspension was diluted stepwise in PBS pH 7.4/0.1% BSA by a dilution factor of 5 to a final dilution of 1:15,625. After incubation at 37°C for 30 min, and a washing step (five times; as before) the anti-HBs-peroxidase conjugate of the test kit Enzygnost HBsAg (Dade Behring) was added at a dilution of 1:100, followed by an o-phenylenediamine /H_2O_2 substrate (Abbott). After an incubation period of another 15 min, 1 N H_2SO_4 was added and the optical density (OD) was measured at a wavelength of 492 nm. The maximum measurable OD_{492} value of the photometer was 3.0 for technical reasons.

Control of the influence of peracetic acid on the ELISA

After incubation with the disinfectant, the HBsAg particles from plasma were separated from the disinfectant by gel filtration. The reactivity of the mAb against HBsAg was not affected by residual traces of the disinfectant (data not shown).

The Epitope-ADT was carried out either under "clean conditions" according to the definition of CEN TC 216 (Disinfectants and Antiseptics) with purified HBsAg [8]. For "dirty conditions" we did not use 3 g/l bovine serum albumin but a 10% HBsAg-positive human plasma solution. As normal human plasma contains 55-80 g/l protein, a 25 times higher protein concentration was thus employed.

Fragment-DNA-ADT or Genome-ADT

The quantitative alteration and disintegration (AD) of the HBV-DNA was measured by PCR with either a short fragment (189 bp) or the full-length (3,200 bp) in five or three independent experiments. HBV DNA was extracted from 50 or 100 µl/sample with the High Pure Viral Nucleic Acid Kit according to the instructions of the manufacturer (Roche Diagnostics). The quantitative PCR of the 189-bp fragment and also of the complete viral genome was carried out with the Real Time PCR System (LightCycler) of Roche Diagnostics together with the software included using the FastStart polymerase kit for hybridization probes, the buffers included and the appropriate reaction vessels (LightCycler Capillaries; all Roche Diagnostics). All runs included HBV standard extracts with 100,000, 10,000, 1,000, 10 and 1 genomes per assay. The method was calibrated with the

international EUROHEP I Standard [17]. A 1:500 dilution of this EUROHEP I standard is now the WHO standard.

The amplification of the 189-bp fragment was performed as touchdown-PCR using primers and hybridization probes (Hyprobes) from the X region: HBV X2s [nucleotide (nt.) 1413-1436]: GAC GTC CTT TGT YTA CGT CCC GTC and HBV X2as (nt. 1601-1578): TGC AGA GGT GAA GCG AAG TGC ACA. As hybridization probe the oligonucleotides HBV 3′-FL-X (1519-1543): ACG GGG CGC ACC TCT CTT TAC GCG G-X and HBV 5′-LC-X (1545-1568): LC-Red640-CTC CCC GTC TGT GCC TTC TCA TCT GC. The reaction mixtures of 20 µl total contained 4 mM $MgCl_2$, 0.5 µM of each primer or hybridization probe and 10 µl of purified DNA extract.

The program of the LightCycler was as follows: (1) denaturation of DNA and activation of the FastStart polymerase: 95°C for 10 min, slope 20°C/s; (2) 45 cycles of amplification: 95°C for 10 s, 64°C for 15 s and 72°C for 13 s with a slope of 5°C/s. The acquisition mode for fluorescence was "single" after the 64°C step; (3) melting curve: cooling from 95°C to 50°C for 10 s with a slope of 20°C/s, and heating again to 95°C for 0 s with a slope of 0.1°C/s; (4) cooling: 40°C for 30 s with a slope of 20°C/s. The fluorescence display mode was F2/1.

For the quantitative amplification of the complete viral genome the same hybridization probes as for the 189-bp PCR were used. The sequence of the primers P1 and P2 have been published in a the report of Günther et al. [15]. The temperature program was as follows: (1) denaturation of DNA and activation of the FastStart polymerase: 95°C for 10 min, slope 20°C/s; (2) 55 cycles of amplification: 95°C for 10 s, 58°C for 20 s and 72°C for 140 s with a slope of 20°C/s. The acquisition mode for fluorescence was "single" after the 58°C step; (3) melting curve: cooling from 95°C to 50°C for 10 s with a slope of 20°C/s, and heating again to 95°C for 0 s with a slope of 0.2°C/s; (4) cooling: 40°C for 60 s with a slope of 20°C/s. The fluorescence display mode was F2/1.

Calculation of the RF factor

The extent of the AD was calculated graphically with the parallel-line-bioassay [9]. Dose-response functions were established with $x=\log_{10}$ (dilution factor) and $y=\log_{10} OD_{492}$ or $\log_{10}$ genome number. The horizontal difference between the straight and parallel parts of the dose-response curves of the untreated control and the samples treated with disinfectant equals the $\log_{10}$ RF factor. The difference has to be determined in the lower linear part of the dose-response curves (Fig. 1). Test samples that do not show any reaction in the ELISA, even undiluted, are characterized by a horizontal dose-response line. By definition, this result corresponds to a reduction larger than the last positive reference dilution.

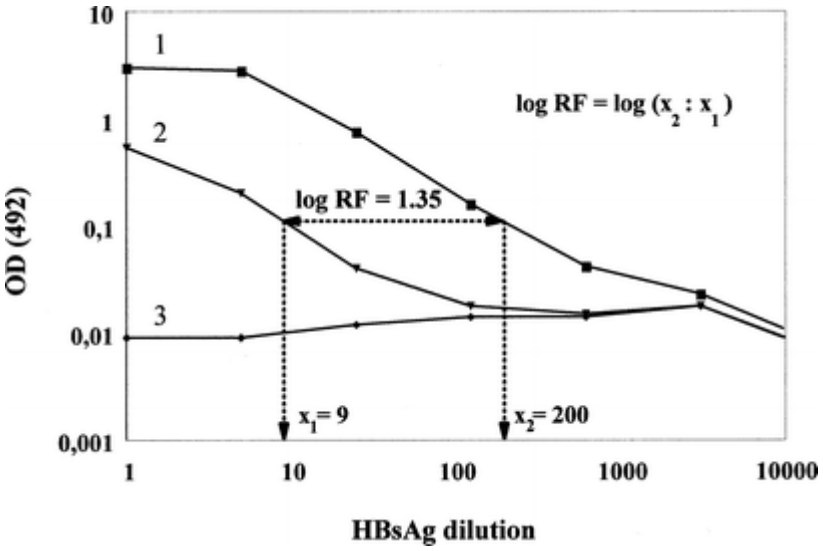

Fig. 1. Representative example for determination of RF according to the parallel line bioassay. The RF was estimated by the *horizontal distance* between *curve 1* and *2* at the same OD value (represented by the *horizontal broken line*). The $\log_{10}$ of the ratio between the corresponding dilutions (*x-axis* values; e.g., x_1=9, x_2=200) yields the RF ($\log_{10}$(200:9)=1,35). *Curve 3* does not show any reaction in the Epitope-ADT, and is characterized by a *horizontal dose-response line*. By definition, this result corresponds to a reduction larger than the last positive reference dilution (*RF* reduction factor)

Results

Virucidal activity against parvovirus

To prove the antiviral activity of RD 88, we analyzed its effect on infectivity of bovine parvovirus. Table 1 shows a 3.2$\log_{10}$ reduction of parvovirus within 15 min by 5% RD 88, when no protein was added (clean condition). This inactivation increased to 3.9$\log_{10}$ after 30 min and more than 4.0$\log_{10}$ after 60 min. With 10% human plasma (dirty condition), the inactivation was slightly inhibited and generated a reduction of 2.7$\log_{10}$ after 15 min, 3.3$\log_{10}$ after 30 min and more than 4$\log_{10}$ after 60 min.

Table 1. Inactivation of bovine parvovirus with respect to its infectivity in a quantitative suspension assay with 5% RD 88 at 20°C

Virus	Time (min)	Log10 reduction factor	
		No added protein	Plus added protein
		(Aqua bidest)	(10% plasma)
Bovine parvovirus ($\log ID_{50}$=7.1/ml)	15	3.2	2.7
	30	3.9	3.3
	60	>4.0[a]	>4.0

[a]No virus detected

Epitope-ADT under clean condition

HBsAg was used at a final concentration of 1.65 µg/ml, which allowed detection with ELISA in 1:3,125 dilution. The titration curves of purified HBsAg particles with the four mAb are shown in Fig. 2. This figure demonstrates a sigmoidal dose-response curve of the PBS-treated HBV control. There was no detectable ELISA signal for the RD 88-treated HBsAg epitopes after 15-60 min with the mAb C20-02, 2-11B1 and 1-9C1. This corresponds to a reduction of more than 1:3,125 or 3.5$\log_{10}$ or greater (or $\geq$99.97%). For the mAb MA 18/7 a faint reaction at 15 min with RD 88 was observed, representing a reduction of 3$\log_{10}$ or 99.9%. These results are summarized in Table 2. In comparison, the control reagent PAA showed a reduction of about 2$\log_{10}$ for three of the four epitopes after 15 min, but more than 3.5$\log_{10}$ or 99.97% or greater after 60 min. With the epitope 2-11B1 a reduction of more than 3.5$\log_{10}$ was already seen after 15 min.

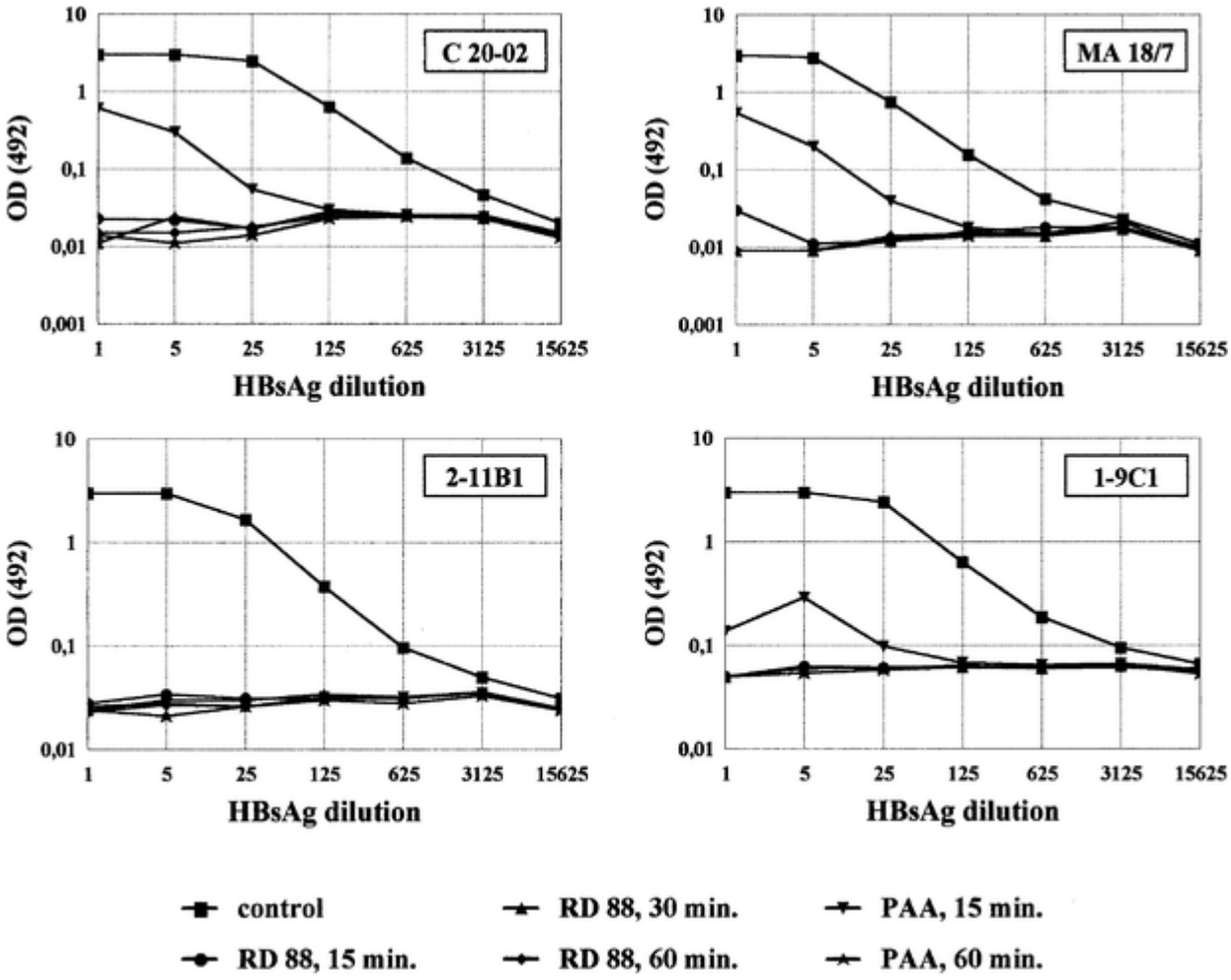

Fig. 2. Epitope-ADT under clean conditions with RD 88 and different mAb. The untreated control is represented by squares, the RD 88-treated samples by circles, triangles and rhombi. Results obtained with the control preparation are shown by *inverted triangles* and *asterisks*. The reduction (*RF*) under clean conditions for all tested mAb was 4.0 or greater for t longer than 15 min. Compared to the other mAb the epitope 2-11B1 seems to be slightly more sensitive against peracetic acid (*mAb* monoclonal antibodies)

Table 2. Results obtained with purified HBsAg (clean conditions) as well as with HBsAg in human plasma (dirty conditions) in Epitope-ADT at 20°C (*HBsAg* hepatitis B virus surface antigen)

Reagent[a]	Time (min)	Reduction of surface epitopes[b] (log_{10})			
		C 20-02	MA 18/7	2-11B1	1-9C1
Clean conditions (no added protein)					
RD 88	15	>4.0	3.0	>4.0	>4.0
	30	>4.0	>4.0	>4.0	>4.0
	60	>4.0	>4.0	>4.0	>4.0
PAA	15	1.8	1.3	>4.0	1.8
	60	>4.0	>4.0	>4.0	>4.0
Dirty conditions[c] (10% human plasma)					

RD 88	15	0.3	0.2	0.6	0.5
	30	0.6	0.5	1.0	0.8
	60	1.0	0.9	2.1	1.1
PAA	15	2.0	1.7	>4.0	1.9
	60	>4.0	>4.0	>4.0	>4.0

[a]See materials and methods

[b]Logarithmic distance between the linear parts of the control and the test kinetic (parallel-line-bioassay [9])

[c]This represents a 25-fold higher protein load according to the CEN norm [8]

Epitope-ADT under dirty condition

Figure 3 shows the results (see also Table 2) obtained with HBsAg particles in 10% plasma, i.e., under a heavy protein load. It is obvious that only a slight alteration of all tested epitopes took place after the reaction with 5% RD 88 until 60 min. The reduction of reactivity for three of the four tested epitopes did not exceed $1\log_{10}$. Epitope 2-11B1 was more sensitive to the disinfectant and its reactivity was reduced to $2.1\log_{10}$ after 60 min. PAA induced a higher AD factor, although it was also inhibited by the protein load. A reduction of $1.7\text{-}2.1\log_{10}$ was observed after 15 min for three epitopes, but $4.0\log_{10}$ or more after 60 min.

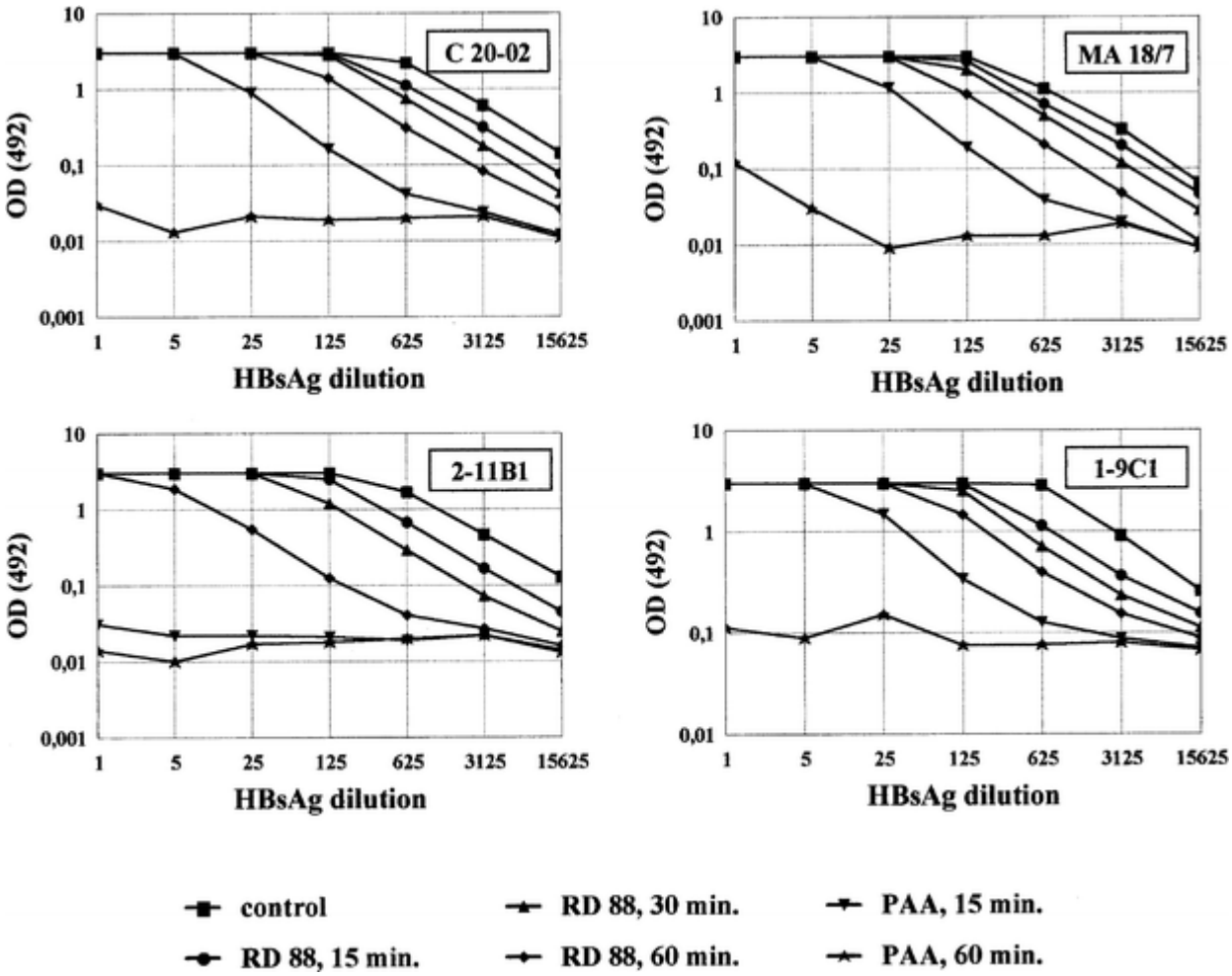

Fig. 3. Epitope-ADT under dirty conditions with RD 88 and different mAbs. Explanations see Fig. 2. The reduction (*RF*) under dirty conditions was between 0.9 and 1.1 for 60 min and less than 1.0 for t of 30 min or less. Again epitope 2-11B1 seems to be slightly more sensitive to RD 88 (and to the control reagent), because in this case the reduction is two times higher (*RF*=2.1). This sensitivity of epitope 2-11B1 could also been seen under clean conditions (Fig. 2)

Fragment-DNA-ADT

Table 3 shows the results obtained in five different tests with the 189-bp fragment PCR in a HBV suspension with 10% human plasma as protein load. The control preparation with PBS instead of RD 88 contained 1,300,000 genome equivalents (100%) per 100 µl. After reaction with 5% RD 88 the reduction was 54% after 15 min, 66% after 30 min and 74% after 60 min. PAA generated a reduction of 97% after 30 min.

Table 3. Results obtained with Fragment-DNA-ADT (189 bp) and Genome-ADT (3,200 bp) under dirty conditions (10% human plasma)

Sample	Contact time	Crossing point[a]	Genome equivalents[b]	Ratio to control[c]	Reduction[d]
	(min)	(median value ± SD)	(median value)	(%)	(%)
Fragment-DNA-ADT (189-bp PCR; *n*=5)					
Control	0	14.5 (±0.7)	1,292,160	100	-

RD 88	15	15.6 (±0.8)	592,860	45.9	54.1
	30	16.1 (±0.8)	434,760	33.6	66.4
	60	16.7 (±0.7)	318,180	24.6	75.4
PAA	30	20.0 (±0.9)	38,290	3.0	97.0
Genome-ADT (3,200-bp PCR; *n*=3)					
Control	0	25.4 (±0.3)	688,567	100	-
RD 88	15	30.4 (±0.7)	98,835	14.3	85.7
	30	30.4 (±1.4)	57,283	8.3	91.7
	60	34.4 (±0.5)	20,852	3.0	97.0
PAA	30	34.7 (±0.7)	20,347	3.0	97.0

[a]Crossing point: No. of cycle in which the amount of amplicons crosses the base line, which is comparable to a cut-off value

[b]Number of genome equivalents calculated from a calibration curve made by five parallel amplified reference samples

[c]Untreated control=100%; Ratio between sample and control (%)

[d]Antagonism from [b]; [100(%)-sample(%)]

Genome-ADT

The examination of the full-length DNA revealed much higher reduction rates. As seen in Table 3 the reduction was 86% after 15 min, 92% after 30 min and 97% after 60 min. The reduction of the control reagent amounted to 97% after 30 min.

Kinetics of antiviral activity

The time-dependent viral-inactivation kinetics induced by a disinfectant permit one to draw a more accurate conclusion about the disinfectant's activity than a one-point measurement. The AD factors of the four epitopes and the full-length 3,200 bp of the HBV genome were determined in the form of $\log_{10}$ reductions for the different contact times. Figure 4 shows significant mean $\log_{10}$ reduction values of the kinetic curves of the epitopes (RF=1.28 after 60 min) and of the full-length genome (RF=1.52 after 60 min). The kinetic curve of the parvovirus infectivity (see insertion) looks similar to the 3,200-bp HBV-DNA AD kinetic curve. Figure 4 shows clearly that the genome and the epitopes of HBV were quantitatively altered and disintegrated.

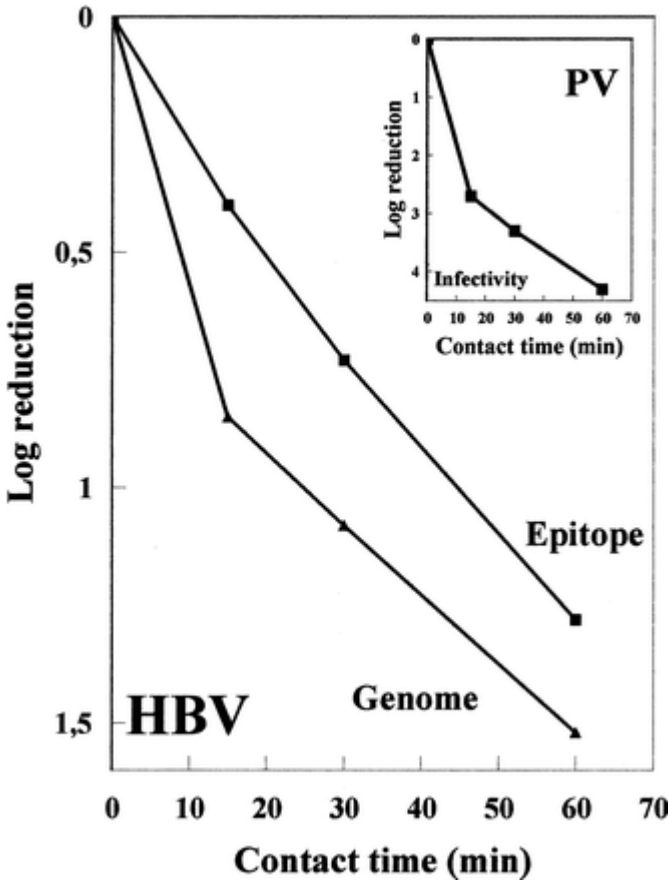

Fig. 4. Quantitative Molecular-ADT (HBV) and virucidal suspension test with parvovirus. Antiviral activity of RD 88, an instrument disinfectant with peracetic acid tested under dirty conditions at 20° C. The reduction of the HBV-epitopes as well as of the full-length HBV-genomes (3,200 bp) are presented. Additionally, the reduction of the parvovirus infectivity is shown in the *insertion*. All tests show similar characteristic inactivation kinetic curves

Discussion

Validation of inactivation procedures for HBV is an important topic in preventive medicine. The use of the chimpanzee assay (CA) is recommended as infectivity assay for the HBV safety testing of blood derivate products and hepatitis B vaccines by the U.S. Food and Drug administration (FDA) (cited [29]). However, a CA is not considered feasible for virucidal testing of disinfectants [7].

Normally, disinfectants are tested by infectivity assays in cell culture. Such infectivity systems are not yet available for HBV. Therefore, alternative approaches are necessary. One possibility could be the investigation of the viral components that are responsible for viral infectivity. From the data available, it appears that disinfectants inactivate viruses either by causing structural damages to the envelope, and/or the capsid and/or to the genome or a combination of several mechanisms [22]. Since different disinfectants may destroy different structures of the viruses to different extents, it would appear to be mandatory to determine changes of these different structural components. In earlier studies with HBV virions under the electron microscope (EM), it became obvious that the complete alteration of the viral structures and more than 80% reduction of virions under the EM paralleled inactivation of the viral infectivity in the CA [29, 34, 36].

With the mAb technique and the PCR it is possible to gain more insight into the chemical, antigenic and possible biological characteristics of the viral epitopes and the viral nucleic acid. We tried

therefore to quantitate the alteration and disintegration of different neutralizing epitopes and the genome, partially or in total, after exposure to peracetic acid for different contact times. The epitopes (Epitope-ADT) and the full-length HBV genome (Genome-ADT) were assayed by ELISA and PCR tests, respectively. The results clearly showed that a time-dependent inactivation could be established for the epitopes and the full-length genome (Fig. 4).

With the Epitope-ADT, the AD of four different HBV surface epitopes that are presumed to be involved in the process of infection could be determined with mAb specific for S, preS1 and preS2. The disinfectant RD 88 was first tested in a purified HBsAg suspension without added protein. In this test, that simulates "clean" conditions according to European norms [8], three of the four tested HBV surface epitopes were totally disintegrated after 15 min, and the fourth no longer showed a reaction after 30 min. At least the preS1 epitope of MA 18/7 is most probably involved in viral attachment to cell receptors. Therefore, substantial HBV inactivation seems to be highly probable after this time.

RD 88 was then tested in a HBV/HBsAg suspension containing 10% human plasma which represents a very heavy protein load of about 7 mg/ml compared to 0.3 mg/ml in the prEN [8]. In this experiment, all four tested HBV epitopes remained relatively unaltered for 30 min ($RF \leq 1$). This result was not surprising, because it is known from infectivity tests with peracetic acid that this reagent is strongly inhibited by organic matter as can be seen from the results of the parvovirus test. In this case 10% human plasma leads to an inhibition of RD 88 of $0.5 \log_{10}$ (Table 1).

The number of genome equivalents in a plasma sample is correlated with its infectivity [37]. A reduction of genome equivalents should be a very clear expression of viral inactivation. This reduction could be measured with the Genome-ADT that simulates a replication of viral nucleic acid by DNA polymerase in vitro. However, according to our results it is essential to include the full-length of viral genomes for validation of inactivation, since the test with the 189-bp fragment greatly underestimates the efficiency of a virucide (Table 3). The probability of a hit in the 189-bp fragment of the genome is 17 times less likely than in the entire genome of 3,200 bp. Thus, the full-length genome PCR should be more sensitive to alterations than the 189-bp PCR (cf. Fig. 5). In fact, a reduction of 85% of the HBV genome was observed already after 15 min, which exceeds the reduction seen in the 189-bp PCR by 54%. After 30 min the reduction increased to 92% and to 97% after 60 min.

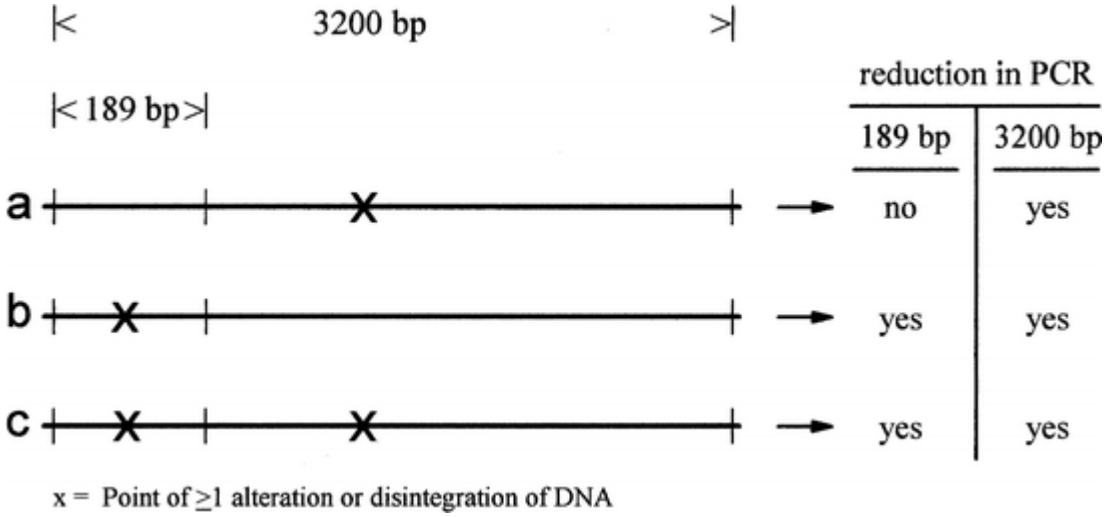

Fig. 5. Schematic drawing of AD results obtainable by PCR to demonstrate the lower sensitivity of the Fragment-DNA-ADT (189 bp) versus Genome-ADT (3,200 bp). With an equal distribution of AD and with a rate of one single hit per genome it theoretically requires 17 affected genomes to detect such an event in the conventional 189-bp PCR (1/17 genome length). So, if all genomes are

affected only once, a decrease of about 1/17 may be determined in the 189-bp PCR. In the 3,200-bp PCR every hit at any position should be effective and should lead to a complete reduction of PCR

Therefore, the examination of the viral full-length genome seems to be preferable to tests with small fragments of the genome, when the destruction of a virus needs to be validated. Some studies (e.g., with BVDV [*18*]) showed an inactivation of the viral infectivity but an insignificant destruction of the genome. Whether this lack of correlation is due to the methodological approach of using a small fragment, must be corroborated.

Peracetic acid reacts with proteins as well as with nucleic acids. With Genome-ADT, the evaluation of virus-inactivating agents that affect the viral genome such as psoralens, e.g., methylene blue or oxidizing chemicals, seem to be suitable. With the Epitope-ADT the efficacy of protein denaturing agents such as alkylating or acylating substances could be measured.

Because a direct comparison of the Molecular-ADT results with an infectivity test for HBV could not be achieved, we tested the antiviral activity against bovine parvovirus. This virus has been suggested for use in inactivation studies instead of HBV [*4*], because parvovirus has a high resistance to chemical and thermal influences. Therefore, virucidal results obtained with parvovirus may give a considerable safety margin for HBV disinfection. Why the inactivation of bovine parvovirus occurred to a greater extent than HBV epitope and genome alteration can not be explained at the moment. The Epitope-ADT, the Genome-ADT as well as parvovirus infectivity have shown similar inactivation kinetic curves (cf. Fig. 4). All follow the mathematical characteristic of catalytic growth curves [*23*]. In particular, the RF of the full-length genome seems to be a clear indicator of viral inactivation of HBV.

One alternative infectivity assay as a model for HBV was established with the duck hepatitis virus (DHBV), an avian hepadnavirus. DHBV was already used for testing disinfectants in quantitative suspension and carrier tests, either in ducklings or hepatocytes ([*6*, *24*, *28*] and G. Pauli and M. Kao, personal communication). To elucidate the relationship between infectivity and the in vitro test system, comparative tests with the DHBV model and Molecular-ADT are in preparation

This study describes for the first time the changes introduced to HBV by peracetic acid at the molecular level. If future studies show that the results of the Molecular-ADT are representative for virucidal activity, Molecular-ADT may also be of relevance for testing procedures for virus safety of blood products.

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Footnote 1

RD 88 is marketed by Septodont under the proprietary name Bioseptol, by Biodica under the proprietary name Dicagreen and by ATO Zizine under the proprietary name Peracetol.