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Methodological Approaches to Disinfection of Human Hepatitis B Virus

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Three commercial disinfectants (two quaternary formulations and one phenolic) were tested against human hepatitis B virus (HHBV). The treated virus was assayed for infectivity by the chimpanzee assay and for morphological alteration by the Morphological Alteration and Disintegration Test. The same agents were tested against duck hepatitis B virus in a duck hepatocyte infectivity assay. It is apparent that human and duck hepatitis viruses were relatively susceptible to disinfection, becoming noninfectious after ≤10 min of contact with the disinfectant. The Morphological Alteration and Disintegration Test accurately predicted activity in the two infectivity tests. The anti-human hepatitis B virus effect of the low-level quaternary ammonium germicides is a novel finding and suggests that members of the family *Hepadnaviridae* are relatively susceptible to chemical agents.

Human hepatitis B virus (HHBV) is a significant viral pathogen responsible for thousands of deaths per year. It is highly contagious, primarily being spread by needlestick injuries and exposure of mucous membranes to blood containing HHBV. It is the leading cause of cirrhosis and liver cancer and is the world's ninth largest killer. To mitigate environmental transmission of HHBV and human immunodeficiency virus type 1 (AIDS), strict regulations have been promulgated by the Occupational Safety and Health Administration (26) to protect workers from accidental infection. The most effective precaution is vaccination coupled with Centers for Disease Control and Prevention universal precautions (14). Despite the clinical importance of HHBV, no approved disinfectants are commercially available in the United States (unlike in Europe) because of a regulatory preference for an HHBV infectivity test. For most clinically relevant viruses, e.g., human herpesvirus type 1 (HHV-1) and polio, infectivity models such as cell culture, chick embryo, and suckling mice exist, which allows for the assessment of disinfectant efficacy and subsequent registration with the Environmental Protection Agency (EPA). No practical model exists for HHBV because the infectivity model requires the use of protected species (humans or chimpanzees). The cell culture model (HepG2 cells [1]) for the cultivation of HHBV is not appropriate because it has not been fully characterized according to EPA requirements with respect to resistance to drying, susceptibility to cytotoxicity, and production of relatively large amounts of infectious virions. There is a need, therefore, for an alternative to the primate model. The duck hepatocyte method is of some use (as will be discussed) but is limited because of the genetic differences between duck HBV (DHBV) and HHBV. The Morphological Alteration and Disintegration Test (MADT) is an alternative electron microscopy-based method which studies the structural integrity of the HHBV virions that remain after contact with a disinfectant. It can be used as a predictor of disinfectant efficacy when correlated with an acceptable model such as the chimpanzee assay (CA). Very few studies have been performed pertaining to HHBV disinfection and control. In an early study in which humans were tested, it was found that HHBV infectivity was destroyed by exposure to 98°C (9), and when chimpanzees were used (2, 8, 24), intermediate-to-high-level disinfectants were effective. This is the first report of the disinfection of HHBV by low-level disinfectants. Because the low-level disinfectants were tested with both the CA and the MADT, it appears that the MADT is a useful nonprimate model for the assessment of anti-HHBV activity. This report confirms the results of our earlier work (23, 24) regarding the correlation between morphological alteration of HHBV and inactivation of its infectivity in the chimpanzee. In addition, it is the first body of data to directly compare results obtained with HHBV and DHBV. We further discuss the use of these procedures in evaluating the anti-HHBV effectiveness of other disinfectants.

MATERIALS AND METHODS

Disinfectants tested. Two dual-species quaternary ammonium disinfectants, D-125 and Public Places (Microgen, Inc.), were tested along with a phenolic disinfectant, Matar. D-125 contained 25% *n*-alkyl (60% C_{14}, 30% C_{16}, 5% C_{12}, 5% C_{18}) dimethyl benzyl ammonium chlorides and 25% *n*-alkyl (68% C_{23}, 32% C_{14}) dimethyl ethylbenzyl ammonium chlorides (EPA registration no. 61178-1). Public Places (Microgen, Inc.) contained 25% *n*-alkyl (60% C_{14}, 30% C_{16}, 5% C_{12}, 5% C_{18}) dimethyl benzyl ammonium chlorides and 25% *n*-alkyl (68% C_{23}, 32% C_{14}) dimethyl ethylbenzyl ammonium chlorides (EPA registration 61178-2). The phenolic disinfectant Matar (Huntington Laboratories) contained 356 ppm *o*-benzyl-*p*-chlorophenol, 230 ppm phenylphenol, and 116 ppm *p-tert*-amylphenol (EPA registration no. 303-225). All three agents were tested by CA, MADT, and the DHBV

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TABLE 1. Validation of neutralization of residual disinfectant activity by using bacteria[a]

Disinfectant	Activity (CFU) measured:			
	Immediately (within 30 s)		At 1 h	
	S. choleraesuis ATCC 10708	*P. aeruginosa* ATCC 15442	*S. choleraesuis* ATCC 10708	*P. aeruginosa* ATCC 15422
D-125 (703 ppm)	315	296	55	231
Public Places (500 ppm)	312	281	55	264
Control	309	287	55	319
Matar	153	539	121	759
Control	113	1,375	451	891

[a] For details, see text.

hepatocyte assay. D-125 was diluted in 300 ppm Association of Official Analytical Chemists International (AOAC) synthetic hard water to a final quaternary ammonium concentration of 703 ppm, and Public Places was similarly diluted from its use concentration of 1,400 ppm to 500 ppm. Matar was prepared in 400 ppm AOAC synthetic hard water to achieve a use concentration of 702 ppm total phenolics.

CA for HBV. The CA was performed in accordance with EPA Pesticide Assessment Guideline Subdivision G: Product Performance (21) and as per a protocol submitted to the EPA (3, 4, 17). A high-titer human virus stock of HHBV containing 10^7 chimpanzee 50% infectious doses per ml in human plasma was obtained from the New York Blood Center. The pool was diluted to 10^6 chimpanzee 50% infectious doses per ml in the presence of 5% chimpanzee plasma, and then approximately 10^5 chimpanzee infectious doses (0.2 ml) was dried to a film at 37°C for 30 min on a 100-mm-diameter glass petri dish. The viral film was exposed to 2 ml of disinfectant for 10 min. After this contact time, the virus-disinfectant reaction mixture was quenched by dilution in 11 ml of chilled chimpanzee plasma.

The effectiveness of this neutralization procedure was determined by spiking HHV-1, *Salmonella choleraesuis* (ATCC 10708) and *Pseudomonas aeruginosa* (ATCC 6538) into the reaction mixture. When HHV-1 (1.0 ml) was used as the surrogate, disinfection was stopped, as in the CA, within 30 s by dilution of 1 ml of the disinfectant in 10 ml of chilled Trypticase soy broth (BBL Microbiology Systems) containing 10% sterile calf serum. Hep 2 cells were used to measure infectivity (cytopathic effect) at 37°C. Essentially, 0.1 ml of each dilution was added to each of four tissue culture wells containing monolayers of Hep 2 cells. Viral replication at virus dilutions of 10^{-2} to 10^{-7} was complete (cytopathic effect) in all four wells and in all virus dilutions with virus alone, Public Places, or Matar at day 4. When gram-negative bacteria were used as the surrogates for HHBV, disinfection was stopped as described above, except that heat-inactivated calf serum was used, ~300 CFU of bacteria was spiked into the quenched reaction mixture, and aliquots were plated on Trypticase soy agar. The viability of the bacteria was thus determined immediately after neutralization (within 30 s of inoculation) as well as 1 h subsequent to neutralization (at ambient temperature of 20 to 25°C) (Table 1).

Chimpanzees (*Pan troglodytes*) were sedated with ketamine (Aveco) and infected intravenously with the entire reaction mixture. One chimpanzee was used per formulation, and an additional chimpanzee was used as an infectivity control. The four juvenile chimpanzees used in this study were born at the Laboratory for Experimental Medicine and Surgery for Primates and maintained under hepatitis surveillance and housing conditions which met institutional standards for the care and use of laboratory animals (11, 12). These chimpanzees had no history of infection with hepatitis B or C, and were negative for serologic markers of both diseases. They had normal levels of enzyme in serum and normal liver histology at time of inoculation.

Signs of HBV infection (27, 28) were monitored for 6 months after inoculation by the following markers: HHBV surface antigen (HBsAg [Abbott AUSRIA test kit]), anti-HBsAg (Abbott AUSAB test kit), anti-HBV e antigen (anti-HBeAg) (Abbott HBe test kit), and anti-HBV core antigen (anti-HBc) (Abbott CORAB test kit). Serum levels of gamma-glutamyl transferase and alanine aminotransferase were determined by kinetic methodology at the Laboratory for Experimental Medicine and Surgery for Primates. Percutaneous liver punch biopsy tissue was examined by routine histological methods.

MADT. The commercial disinfectants discussed above were also tested against HHBV under the same experimental conditions (concentration, time, organic load) with the MADT (10) modified to EPA requirements (18, 29). In addition, Asepti-Steryl 28, an activated 2% glutaraldehyde formulation (Huntington Laboratories; EPA registration no. 63281-6-303), and Dispatch Hospital Cleaner Disinfectant with Bleach (0.55% sodium hypochlorite [Caltech Industries; EPA registration no. 56392-7]) were tested as described above, except that additional organic load was not added. The 10-min disinfection regimen was quenched with chilled plasma. HHBV was then purified from the reaction mixture of protein and disinfectant by trap and rate zonal sucrose centrifugation. Fractions were collected with a refractive index of 1.396 to 1.404 and prepared for electron microscopy.

Electron microscopy and morphometric analysis. Copper 200-mesh electron microscope grids were coated with Formvar and ionized by glow discharge just prior to use. The purified virions were adsorbed to the grid, which was then serially rinsed five times with sterile deionized water. Negative stains were prepared with 5% uranyl acetate and/or 1% phosphotungstic acid. The ultrastructural morphology of HHBV circulating forms was determined by examining negatively stained preparations at a magnification of ≥80,000. Thirty or more fields from each of 20 squares were examined. Positive staining with 2% potassium permanganate followed by 1.5% uranyl acetate was used to quantify virions. Twenty fields in each of 20 squares were counted at a magnification of ×50,000. All samples were examined with a Philips CM-10 transmission electron microscope operated at 80 kV.

Infection of primary duck hepatocytes with DHBV. The commercial disinfectants were also tested against dried

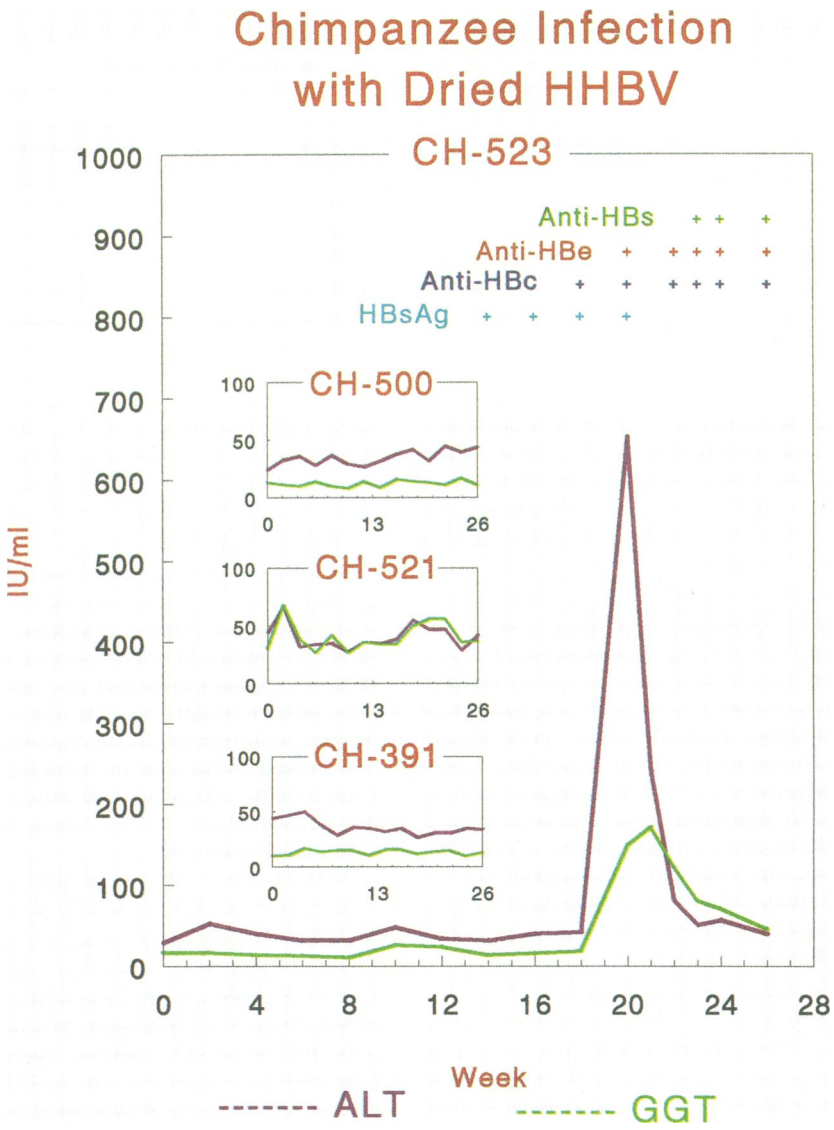

FIG. 1. Response of chimpanzees to dried HHBV and dried HHBV subjected to disinfection. Ch-523, positive control (no disinfectant); CH-500, D125; CH-521, Public Places; CH-391, Matar.

DHBV (at the same concentration, contact time, and organic load as described above), and virus infectivity was assayed with duckling hepatocyte cultures. Hepatocytes were isolated from 1- to 2-week-old Pekin ducklings. These ducklings were purchased from Metzer Farms, Gonzales, Calif., and then were tested for DHBV infection by DNA dot-blot hybridization. The source of DHBV was serum from 3- to 4-week-old congenitally infected ducklings. Hepatocytes were isolated by collagenase perfusion of the liver as previously described (20). Cells were plated at confluence in six-well tissue culture plates (Falcon) (approximately 1.5×10^6 cells were added per well) and maintained in L15 medium (GIBCO) supplemented with 15 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [pH 7.45]), 10 mM sodium bicarbonate, 300 mg of penicillin per liter, 100 mg of streptomycin, 1 mg of insulin per liter, and 10^{-5} M hydrocortisone-hemisuccinate. Cultures were incubated at 37°C, and the medium was replaced every 1 to 2 days.

DHBV-containing duck serum (0.2 ml) was applied to a 60-mm-diameter plastic tissue culture dish and incubated at 37°C for 1 h or until dried to a film. Two milliliters of an appropriate dilution of disinfectant in sterile phosphate-buffered saline (PBS) was added to the dried virus and incubated for 10 min at ambient temperature. As a control, 2 ml of PBS alone was added. After mixing, a series of 10-fold dilutions were made in L15 containing 5% fetal bovine serum. One milliliter of each dilution was added to four individual wells of cultured hepatocytes, and the virus inoculum was left on the cells for 18 h at 37°C. Virus was replaced with fresh medium, and the cells were maintained for 8 to 12 days before harvesting for DHBV DNA analysis.

Total cellular DNA was prepared from hepatocyte culture cells as previously described (20). DNA was analyzed for the presence of DHBV relaxed-circular and single-stranded DNA by Southern blot hybridization, as previously described (25). DHBV DNA was isolated from serum dilutions used for infections by addition of an equal volume of the

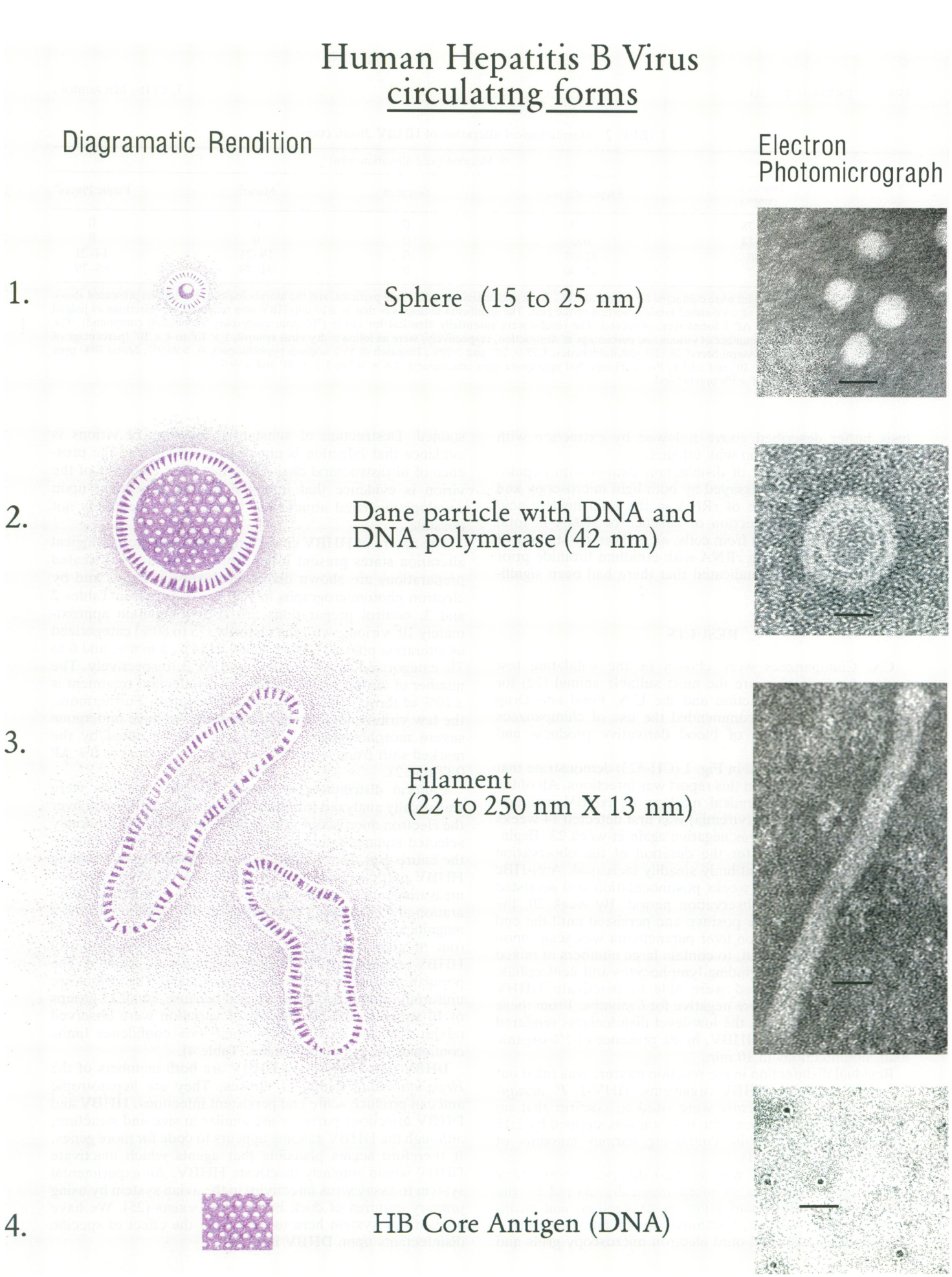

FIG. 2. Diagrammatic representation of the HHBV circulating forms and representative electron photomicrographs. The bars in panels 1 to 4 represent 28, 16.8, 25, and 35 nm, respectively.

TABLE 2. Morphological alteration of HHBV disinfection[a]

AP	% Morphological alteration with:				
	Dry virus control	Asepti-Steryl 28	Dispatch	Matar[b]	Public Places[b]
0	76–78	0.0	0	0	0
1	12–14	0.0	0	0	0
2	3–6	31–33	0	19–21	14–21
3	6–7	67–70	0	81–79	86–79

[a] Two lots of each disinfectant were contacted for 10 min at 20°C. Each grid contained $\geq 10^5$ Dane particles, and the morphological descriptions presented above are based upon observations of a validated random portion of the grid. The significant finding was that in ≤10 min HBV was rendered noninfectious as judged by the fact that no AP 0 or AP 1 forms were observed. The results were essentially identical for D-125 (703 ppm quaternary ammonium compound). The quantitative determinations (number of virions and percentage of destruction, respectively) were as follows: dry virus control, 1×10^5 to 4×10^5 (percentage of destruction not applicable); Asepti-Steryl 28 (2% glutaraldehyde), 3.75×10^3 and >99%; Dispatch (0.55% sodium hypochlorite), 0, >99.9%; Matar (800 ppm phenolic), 1.2×10^4 to 2.8×10^4 and >85%; Public Places (500 ppm quaternary ammonium), 5.6×10^3 to 7.5×10^3 and >99%.

[b] Tested in the presence of ≥5% organic soil.

lysis buffer described above followed by extraction with phenol and precipitation with ethanol.

The cytotoxic effect of disinfectant dilutions on hepatocyte monolayers was assayed by both light microscopy and the relative abundance of rRNA in the total nucleic acid preparations. The reduction or absence of rRNA in total nucleic acids extracted from cells, as assayed by staining of agarose gels containing rRNA with ethidium bromide prior to Southern transfer, indicated that there had been significant cell death.

RESULTS

CA. Chimpanzees were chosen as the validating test system because they are the most suitable animal (22) for studies of HHBV infection and the U.S. Food and Drug Administration has recommended the use of chimpanzees for the safety testing of blood derivative products and hepatitis B vaccines.

The data summarized in Fig. 1 (CH-523) demonstrate that the dried HHBV used in this report was infectious. All of the serological and biochemical markers of HHBV infection were positive. HBsAg (viremia) was first detected 14 weeks after inoculation and was negative again at week 22. Beginning at week 22 and for the duration of the observation period, the anti-HBs antibody steadily increased. Anti-HBc became positive at 18 weeks postinoculation and persisted until the end of the observation period. By week 20, the anti-HBe value became positive and persisted until the end of the study. In addition liver parenchyma was seen, upon histological examination, to contain large numbers of mixed inflammatory cells, including lymphocytes and neutrophils. The disinfectants tested were able to inactivate HHBV because all markers were negative for 6 months. From these data, we conclude that the low-level disinfectants rendered ≥99.9% of infectious HHBV, in the presence of 5% organic load, noninfectious in 10 min.

Residual disinfection in the reaction mixture was ruled out by using surrogate HHBV organisms. HHV-1, *P. aeruginosa*, and *S. choleraesuis* were used to confirm that no disinfection occurred once the reaction was stopped by 1:11 dilution in chilled media containing various amounts of organic matter (Table 1).

MADT. The MADT was used to determine how many virions were destroyed or morphologically altered by the disinfectants. After disinfection, neutralization, and purification through sucrose, fractions corresponding to HHBV were pooled, adsorbed onto electron microscopy grids and stained. Destruction of substantial numbers of virions is evidence that infection is not possible. Similarly, the presence of ultrastructural changes in the outer envelope of the virion is evidence that infection that is dependent upon envelope-mediated attachment to the host receptor is not possible.

The nature of HHBV circulating forms and morphological alteration states present in control and disinfectant-treated preparations are shown diagrammatically in Fig. 2 and by electron photomicrographs in Fig. 5. As shown in Tables 2 and 3, control preparations are seen to contain approximately 10^5 virions, with the majority (75 to 80%) categorized as alteration phase 0 (AP 0), and 9 to 14%, 3 to 6%, and 6 to 7% categorized as AP 1, AP 2, and AP 3, respectively. The number of virions present after any disinfectant treatment is ≤10% of those detected in the mock control. Furthermore, the few virions not destroyed were seen to have undergone severe morphological alteration, as can be noted by the marked shift from 76 to 78% AP 0 (intact virions) to 0% AP 0 (Table 2).

Uniform distribution of virions. The MADT data were statistically analyzed for uniformity of viral distribution over the electron microscope grid so that observation of randomly selected squares could be extrapolated with confidence to the entire grid. Determination of the variability of counting HHBV particles in random squares was made by repeated measurements of multiple squares from the same viral preparation and calculating the coefficient of variation (15). At a magnification of ×50,000, 58,345 virions were counted in 149 (out of a possible 200) squares from a dry-virus control HHBV preparation. Ten fields per square were counted. The population mean was 397 virions per square. The virions are uniformly distributed over the grid because, in all 15 groups of 10 squares, low coefficients of variation were observed (about 5%) and in all groups their 95% confidence limits contained the population mean (Table 4).

DHBV test. HHBV and DHBV are both members of the *Hepadnaviridae* family of viruses. They are hepatotropic and can produce acute and persistent infections. HHBV and DHBV infectious particles are similar in size and structure, although the HHBV genome appears to code for more genes. It therefore seems plausible that agents which inactivate DHBV would similarly inactivate HHBV. An experimental system to assay virus infectivity in the avian system by using primary cultures of duck hepatocytes exists (25). We have applied this system here to determine the effect of specific disinfectants upon DHBV infectivity.

TABLE 3. Morphological profiles of various control HHBV preparations[a]

AP	% of each AP in HHBV preparation[a]:							
	1	2	3	4	5	6	7	8
0	80.7	77.5	77.9	78.1	75.8	78.4	77.4	78.2
1	9.4	12.5	11.8	12.0	11.9	12.9	13.2	11.3
2	3.8	3.9	3.8	3.7	5.8	2.5	2.9	4.6
3	6.1	6.6	6.5	6.2	6.5	6.2	6.5	5.9

[a] The eight different viral preparations shown above were prepared and examined after negative staining. Preparations 1 and 2, wet virus (not dried); 3 to 7, dry virus; 8, dry virus treated with surfactant (0.5% $Na_2S_2O_3$). The quantitative determinations were 4.8×10^5, 1.4×10^5, 4.7×10^5, 1.2×10^5, 1.9×10^5, 8.4×10^4, 1.0×10^5, and 4.7×10^5 for preparations 1 to 8, respectively.

The effect of drying DHBV was investigated. After drying, virus was diluted in PBS before incubation with hepatocytes and the relative amount of DHBV replication in each culture was determined. Replication could still be detected at a 10^{-6} dilution of virus, which represents approximately 2.0×10^3 DHBV DNA-containing particles per ml. The Southern hybridizations show the limit of detection by this assay. It appears that 0.1 to 1.0 pg of DHBV DNA was in the hybridization standard. This corresponds to 5.1×10^4 to 5.1×10^5 DHBV DNA molecules.

Although drying the virions produced a 2-log reduction in viability (Fig. 3A), sufficient viable virions remained as required by EPA. The observed reduction in infectivity was not due to inefficient recovery of the virus particles from the petri dish (Fig. 3B). The disinfectants tested yielded 2 logs of cytotoxicity. Cytotoxicity was not present at a 1:1,000 dilution of the disinfectant-virus reaction mixture. No viral replication was detected at this dilution, which corresponded to 703, 500, 702, and 20,000 ppm for D-125, Public Places, Matar, and Asepti-Steryl 28, respectively. Accordingly, all disinfectants inactivated ≥99.9% of DHBV infectivity.

Determination of the minimum DHBV virucidal concentration. The test agents were completely effective in destroying DHBV when tested at the same concentration used in the CA and MADT. In order to determine the minimum virucidal concentration, the disinfectants were further diluted in PBS. The minimum virucidal concentrations (Fig. 4) were 354, >250 but <500, 351, and <1,250 ppm for the dual-species quaternary ammonium disinfectants D-125 and Public Places and the phenolic disinfectant Matar, respectively.

DISCUSSION

The disinfectants tested in this report were known to inactivate human herpesviruses, adenoviruses, vaccinia viruses, influenza viruses, and human immunodeficiency type 1 (AIDS) viruses. The present data extend this spectrum to HHBV and DHBV.

In the CA, all of the animals inoculated with disinfectant-treated HHBV remained negative for all serologic markers of HHBV. The positive (infectivity) control, CH-523, responded as expected. Especially noteworthy was the seroconversion to surface and core antigens, which indicated DNA replication. The serological, biochemical, and histological response of CH-523 was typical of HHBV infection in chimpanzees (Fig. 1). The premise of the MADT is that morphological integrity is required for viral infectivity. Morphological alteration of HHBV prevents its replication in chimpanzees (24) and HepG2 cells (16). Furthermore, morphological alteration induced by glutaraldehyde renders bacterial endospores nonviable (7). We report here that morphological alteration and destruction of chimpanzee-titered HHBV, produced by quaternary ammonium- and phenolic-based chemical disinfectants, also prevents HHBV infection in chimpanzees. In order to minimize the use of chimpanzees for such testing, we performed correlation experiments with the MADT and DHBV assay under the same experimental conditions used in the CA. As per EPA requirements (5), testing was performed with carriers with dried HHBV in a 10-min exposure at 20°C in the presence of 5% serum plasma. To further strengthen the argument that morphological alteration predicts subsequent loss of infectivity in an animal or cell culture model, the chemical germicides that produced alteration in the MADT assay were tested in a second infectivity assay. The duck hepatocyte infectivity assay results showed that the agents tested prevented DHBV replication because evidence of DNA replication, single-stranded DNA, was not detected. Our DHBV findings

TABLE 4. Distribution of HHBV on electron microscope grid[a]

Group (selected squares)	Distribution of HHBV:			CV	95% confidence interval (upper/lower)
	Maximum	Minimum	Mean ± SD		
Total (1–149)	439.0	361.0	396.9 ± 19.8	5.0	400.16/393.69
1 (1–10)	424.0	366.0	388.4 ± 20.0	5.1	401.04/375.76
2 (11–20)	421.0	367.0	391.9 ± 19.7	5.0	404.34/379.46
3 (21–30)	432.0	374.0	397.8 ± 20.2	5.1	410.60/385.00
4 (31–40)	412.0	374.0	389.8 ± 12.5	3.2	397.68/381.92
5 (41–50)	431.0	374.0	403.2 ± 20.7	5.1	416.28/390.12
6 (51–60)	422.0	371.0	393.0 ± 18.6	4.7	404.74/381.20
7 (61–70)	431.0	368.0	399.8 ± 22.6	5.7	414.10/385.50
8 (71–80)	429.0	374.0	403.0 ± 19.4	4.8	415.26/390.74
9 (81–90)	427.0	370.0	397.2 ± 19.0	4.8	409.22/385.18
10 (91–100)	438.0	361.0	401.8 ± 22.3	5.6	415.92/387.68
11 (101–110)	430.0	369.0	397.1 ± 22.2	5.6	411.14/383.06
12 (111–120)	425.0	372.0	393.9 ± 18.0	4.6	405.26/382.59
13 (121–130)	424.0	368.0	390.9 ± 19.6	5.0	403.32/378.46
14 (131–140)	439.0	370.0	403.0 ± 24.0	6.0	418.20/387.80
15 (141–149)	431.0	376.0	403.8 ± 19.6	4.8	416.84/390.76

[a] A total of 149 squares were counted in sets of 10. All 15 sets of squares provide low coefficient of variation (CV) values as well as 95% confidence intervals, which contain the population mean of 396.6.

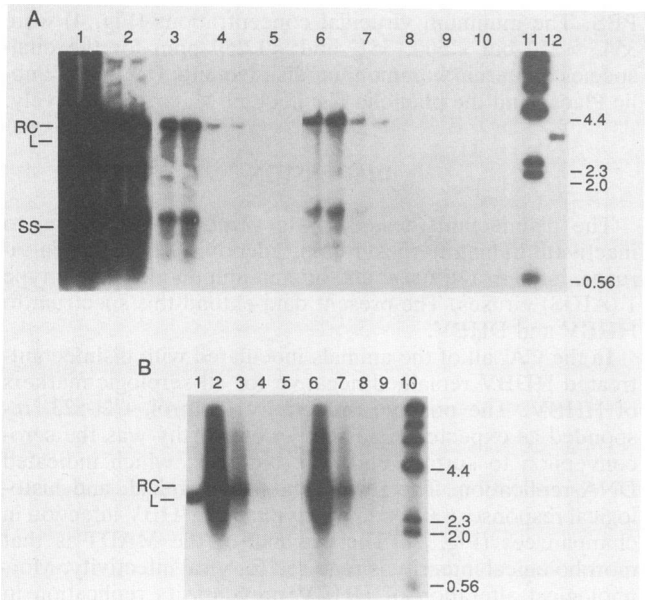

FIG. 3. (A) Effect of drying on infectivity of DHBV in vitro. Lanes 1 to 5 and 6 to 10 represent the relative amounts of DHBV DNA in duplicate cultures 8 days after infection with 2 ml of 10^{-2} to 10^{-6} dilutions of DHBV-positive duck serum, respectively. DHBV-positive duck serum (0.5 ml) was diluted in L15 medium containing 5% fetal calf serum and was used to infect primary duck hepatocyte cultures (lanes 1 to 5), or serum was first dried, reconstituted to the original volume with PBS, and then used for infections (lanes 6 to 10). Lambda *Hin*dIII markers are shown in lane 11, with sizes in kilobase pairs on the right. Lane 12 represents 10 pg of linear (L) DHBV DNA. RC, relaxed circular DNA; SS, single-stranded DNA. (B) Effect of drying and resuspension on ability to recover virus particles. Lanes 2 to 5 and 6 to 9 show the amount of DHBV DNA extracted from 100 μl of 10^{-1} to 10^{-4} dilutions, respectively, of the virus inocula described in Fig. 3A. Lanes 2 to 5 represent dilutions of untreated serum, and lanes 6 to 9 represent dried and reconstituted serum. Lane 1 represents 10 pg of cloned linear DHBV DNA, and lane 10 represents lambda *Hin*dIII markers.

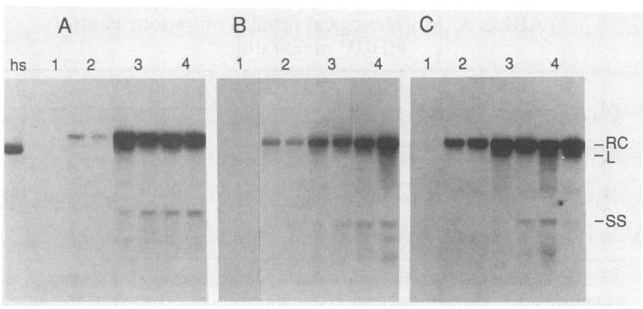

FIG. 4. Titration of inhibitory effect on DHBV infectivity of disinfectants. Each of the disinfectants was diluted in sterile PBS and then incubated with dried virus as described in Materials and Methods. Two milliliters of a 10^{-2} dilution of each virus-dilution mixture was used to infect primary duck hepatocyte cultures. Cellular DNA was harvested 8 days after infection and DHBV DNA was detected as described in the legend to Fig. 3. A, B, and C represent D-125, Public Places, and Matar, respectively. Lanes 1, 2, 3, and 4 show relative amounts of DHBV DNA in cultures infected with virus pretreated with 1:2, 1:4, 1:8, and 1:16 dilutions of D-125 and Matar or with undiluted 1:2, 1:4, and 1:8 dilutions of Public Places, respectively. Asepti-Steryl 28 abolished infectivity at each dilution, and hence these results are not shown. HS represents a hybridization of 10 pg of linear 3-kb DHBV DNA.

with glutaraldehyde are in agreement with those from the duck embryo DHBV assay (13). The duck hepatocyte procedure can be used as a companion surrogate infectivity test in conjunction with the HHBV MADT, thereby minimizing the need for primates. The DHBV findings confirm that the MADT accurately predicts loss of infectivity.

Because the quaternary and phenolic formulations were virucidal against HHBV in both infectivity assays (HHBV in the chimpanzee and DHBV in duck hepatocytes), the morphological alteration and destruction detected in the MADT electron microscopic assay accurately predicts loss of infectivity. Thus, destruction of ≥85% of HHBV virions and a threshold degree of morphological alteration (AP 2 or AP 3) as seen in the MADT (Fig. 5) appears, under the test conditions described herein, to be predictive for hepadnaviral inactivation.

The mechanism of action of the commercial disinfectants can be deduced from the MADT. In order for HHBV infection to occur, it is necessary for a protein or carbohydrate on the surface of the virus to bind to a specific receptor on the surface of the host cell. The surfactants present in the disinfectant formulations solubilize and thereby morphologically alter the virions, rendering them noninfectious because they can no longer bind to the target cell. The 42-nm spherical Dane particles and 22- to 250-nm filaments are disrupted. The virions (also referred to as Dane particles) that are not totally disintegrated appear as irregularly shaped, with damage to the core and outer envelope (Table 3). The filaments (which are not infectious) are transformed into shorter filaments and 15- to 25-nm spheres. The disruption of filaments and Dane particles is dependent on disinfectant, concentration, and contact time (data not shown). The DNA core material appears as 12- to 14-nm rings with a densely stained center. In addition to the morphological alteration reported here, our previous report (19) of the destruction of HBsAg also suggests that virus attachment to the host receptor is blocked. In sum, there is a reproducible pattern of disintegration of virions, damage to Dane particles, damage to filaments, and accumulation of 15- to 25-nm spheres, DNA core, and, finally, disintegration of spheres and DNA.

These results indicate that the 10% or fewer virions not completely disintegrated by the 10-min disinfectant treatment are not capable of infection. Furthermore, total disintegration of the virus is an alternative proof of disinfection. This report is in agreement with the conclusions of other investigators (2, 6, 23, 24). In a related experiment (24), the effectiveness of succinic dialdehyde in the chimpanzee was predicted in the MADT. The data presented in this report support the suggestions of Favero and Bond (6) that HHBV is not more resistant to chemical germicides than other lipophilic agents, e.g., HHV and human immunodeficiency virus.

Reports of HHBV replication in HepG2 cells (1, 16) grown in the presence of dexamethasone and insulin do not accommodate our need for a sensitive in vitro infectivity assay to test chemical germicides against HHBV. The HepG2 system is not appropriate because it produces relatively few morphologically intact virions and the infectivity of the putative virions to chimpanzees, humans, and HepG2 cells has not been demonstrated. Furthermore, the electron photomicrographs (16) of Dane-like virions produced in HepG2 cells do

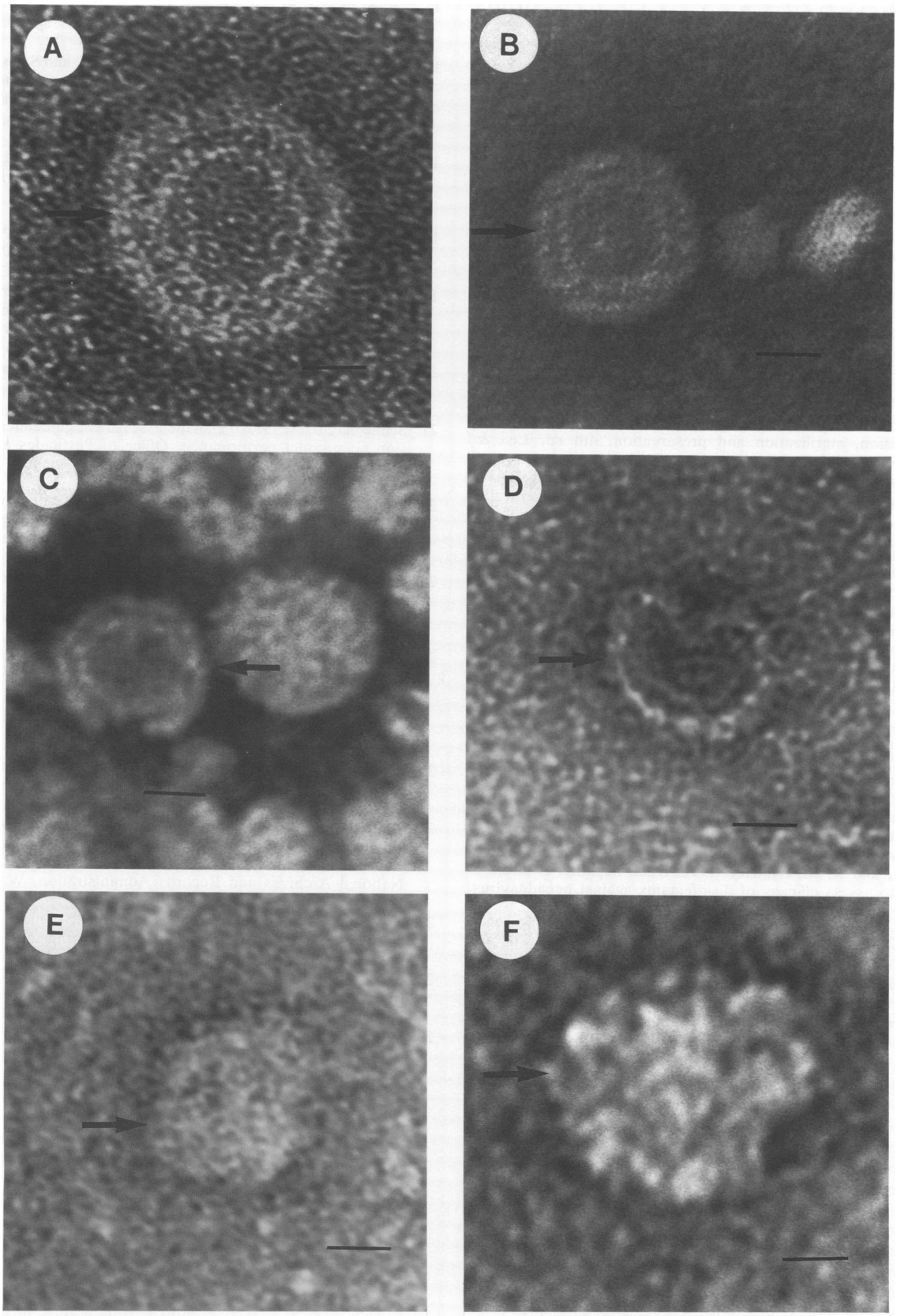

FIG. 5. (A) HHBV Dane particle AP 0. Bar, 10.7 nm. (B) HHBV Dane particle AP 0. Bar, 16.3 nm. (C) HHBV AP 1. Bar, 17.8 nm. (D) HHBV AP 1. Bar, 16.8 nm. (E) HHBV AP 2. Bar, 12.4 nm. (F) HHBV AP 3. Bar, 6.3 nm.

not appear to be 42 nm in diameter as expected for HHBV Dane particles.

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