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TOPIC HIGHLIGHT

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Is hepatitis B-virucidal validation of biocides possible with the use of surrogates?

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Abstract

The hepatitis B virus (HBV) is considered to be a major public health problem worldwide, and a significant number of reports on nosocomial outbreaks of HBV infections have been reported. Prevention of indirect HBV transmission by contaminated objects is only possible through the use of infection-control principles, including the use of chemical biocides, which are proven to render the virus non-infectious. The virucidal activity of biocides against HBV cannot be predicted; therefore, validation of the virucidal action of disinfectants against HBV is essential. However, feasible HBV infectivity assays have not yet been established. Thus, surrogate models have been proposed for testing the efficacy of biocides against HBV. Most of these assays do not correlate with HBV infectivity. Currently, the most promising and feasible assay is the use of the taxonomically related duck hepatitis B virus (DHBV), which belongs to the same *Hepadnaviridae* virus family. This paper reviews the application of DHBV, which can be propagated *in vitro* in primary duck embryonic hepatocytes, for the testing of biocides and describes why this model can be used as reliable method to evaluate disinfectants for efficacy against HBV. The susceptibility levels of important biocides, which are often used as ingredients for commercially available disinfectants, are also described.

Key words: Hepatitis B virus; Surrogate model; Duck hepatitis B virus; Disinfectants; Testing virucidal efficacy

Core tip: There is a need for disinfectants with proven virucidal activity against the hepatitis B virus (HBV). Feasible HBV infectivity assays are not available; therefore, the establishment of surrogate models for HBV infection is of high importance. This paper reviews the application of the most promising and feasible assay, the use of the duck hepatitis B virus, which can be propagated *in vitro* in primary duck embryonic hepatocytes. The paper also describes how and why this model can be used to evaluate the efficacy of disinfectants against HBV.

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WHY EVALUATE BIOCIDES FOR THEIR EFFICACY AGAINST HEPATITIS B VIRUS?

Approximately 350 million people, 5% of the total population, are chronically infected with the hepatitis B virus (HBV)[1]. Thus, the hepatitis B is considered to be a major public health problem worldwide. Furthermore,

nosocomial infections resulting from HBV, in patients during hospitalization and interventional procedures, as well as in health care workers, have been described[2,3]. Although infections attributed to transfusion of contaminated blood or blood products and transmission from infected health care workers have been reduced over the past decades by prophylactic measures, such as HBV screening of blood or vaccination of health care workers, there is still a significant number of reports about nosocomial outbreaks of HBV infections[4-7]. Common transmission pathways include the use of multi-dose vials[8], dental or biopsy equipment[9], dialysis units[10], contaminated finger-stick devices[11,12], contaminated acupuncture needles[13], reuse of syringes[14], endoscopes[15], or unsafe surgical and injection procedures[16-19]. Prevention of indirect HBV transmission by contaminated objects is only possible through the use of fundamental infection-control principles, including the use of chemical biocides[15,20], which are proven to destroy the viral infectivity. Thus, HBV must be inactivated as a result of the disinfection of instruments, surfaces, and biological materials. The use of biocides with proven microbiocidal activity against the pathogens most likely to contaminate a patients' environment has been recommended by the United States Healthcare Infection Control Practices Advisory Committee as part of their guidelines to prevent the transmission of infectious agents in health care settings[21].

In general, HBV can be inactivated by chemical biocides, such as formaldehyde, glutaraldehyde or peracetic acid, which possess broad-spectrum virucidal activity according to the norm EN 14476:2007[22]. However, like all enveloped viruses[23], HBV is thought to be relatively sensitive to biocides. A German guideline for testing the virucidal activity of chemical disinfectants in the medical field characterizes disinfectants effective against enveloped viruses as biocides with limited virucidal activity. In contrast, disinfectants effective against non-enveloped and enveloped viruses are defined as biocides with virucidal activity[24,25]. Thus, broad-spectrum biocides, of which there are few, are not required for the inactivation of HBV like other blood-borne viruses. However, validation of the virucidal action of disinfectants against HBV is essential because the virucidal activity of biocides against HBV cannot be predicted[26]. In addition, human blood plasma protects the virus from inactivation. In the literature, HBV has been described as an enveloped virus that may be difficult to inactivate[27]. The virus possesses a relatively high thermal and dry resistance. At 25 ℃ and a relative air humidity of 42%, the HBV can be infectious for more than 1 wk[28]. Therefore, the virucidal efficacy of biocides against HBV must be validated using reliable and robust laboratory methods.

METHODS FOR TESTING THE EFFICACY OF BIOCIDES AGAINST HBV

The most common methods for the evaluation of virucidal activity of biocides are infectivity assays, which measure the infectivity of viruses in cell culture systems after the virus has been exposed to the biocide in suspension[24,25]. Recently, more practice-relevant methods have been developed, testing viral infectivity after exposure to viruses dried on non-porous surfaces[29]. These procedures mimic the conditions found in actual practice. A crucial component of these assays is that the tested viruses can easily be propagated in cell cultures and the infectivity can be determined reliably by the evaluation of virus-induced cytopathic changes or other methods detecting viral antigens, which are produced during the viral replication cycle. However, the *in vitro* propagation of non-cytopathogenic HBV is difficult, especially in obtaining human liver cells. Historically, the virucidal efficacy of biocides has been stringently determined *in vivo* through the use of chimpanzee infection assays, albeit with decreased sensitivity[30-34]. Currently, animal protection and economic reasons prohibit the use of higher primates for routine tests of commercial products[35]. For *in vitro* infectivity testing, the use of the hepatoma cell line HepG2[36], which has been described in the literature[27,37], has been debated[38]. In comparison, re-differentiated HepaRG cells[39] are well accepted and reproducible as an HBV infectivity system[38]. The specificity of this HBV infection model has been determined by both the neutralization capacity of HBV envelope protein-specific antibodies and the competition with an envelope-derived peptide. However, this infectivity system has not been applied in the past for testing the hepatitis B-virucidal activity of biocides. The reasons for this are the following: HepaRG cells are very expensive, can only be used in highly HBV-specialized laboratories and require highly concentrated HBV suspensions or human sera with a high viral load. Thus far, the most promising HBV infectivity assay seems to be the use of primary hepatocyte cultures derived from Tupaias, small-squirrel-like animals living in Southeast Asia[38,40]. However, the availability of Tupaia hepatocytes is limited, thus the model is too costly for routine use. Furthermore, purified virus must contain approximately 10^9 particles/mL to demonstrate an inactivation factor of at least 10^4[38]. The virus can be obtained from human serum by sedimentation in a density gradient[41].

Thus, surrogate models have often been reported for testing the efficacy of biocides against HBV. To measure the virucidal activity of disinfectants against HBV, Hilfenhaus *et al*[42] and Thraenhart *et al*[35] validated the integrity of viral DNA using a polymerase chain reaction (PCR) technique. Several groups[43,44] have also examined HBV inactivation by measuring the enzymatic activity of the viral DNA polymerase. In addition, the destruction of HBV antigenicity and the decrease in the immunochemical reactivity of different HBV antigens, such as HBsAg, HBcAg and HBeAg, was outlined to verify the virucidal efficacy of alcohol antiseptic, formaldehyde and peracetic acid-containing disinfectants[26,45,46]. Finally, the irreversible morphological alterations of HBV particles were determined to be an indicator of HBV inactivation by

chemical biocides[43,47,48]. However, this test is subjective, and there is a qualitative but not a quantitative measurement. In conclusion, all of the abovementioned studies have shown that the results do not correlate with HBV infectivity.

DUCK HEPATITIS B VIRUS AS A SURROGATE VIRUS FOR HBV

The most promising and feasible assay for the evaluation of hepatitis B-virucidal efficacy of biocides is the use of the taxonomically related duck hepatitis B virus (DHBV) belonging to the same family-*Hepadnaviridae*-and within the genus *Avihepadnavirus*, while HBV is in the genus *Orthohepadnavirus*[49]. DHBV shares many physical properties with the closely related HBV but a sequence comparison of the two viruses indicated that there is a low nucleotide identity[49,50]. Furthermore, there are differences in the genome size (3.2 kb for *Orthohepadnaviruses* and 3.0 kb for *Avihepadnaviriruses*), and the host range of the viruses is restricted to mammals (*Orthohepadnaviruses*) or birds (*Avihepadnaviruses*). In addition, the *Avihepadnaviriuses* have larger core proteins and lack M surface protein[49]. In contrast to the *Orthohepadnaviruses*, some envelope proteins of the *Avihepadnaviruses* are not glycosylated but are phosphorylated. In addition, the proteins of the envelope are not connected by disulfide bridges and instead contain lysine side chains[51]. The model infection of DHBV in Pekin ducks has been used extensively for studying aspects of HBV infection in humans[50,52]. It has been concluded that DHBV and HBV differ primarily between the hosts they infect and the nature of the disease they produce. This has no bearing on the ability of disinfectants to abolish infectivity of the viruses[53]. Furthermore, the DHBV model has similar disinfectant inactivation kinetics to those observed in the limited studies of HBV transmission in chimpanzees[31,54]. Thus, DHBV infectivity tests have been used for testing the virucidal activity of chemical biocides against HBV in the United States and Australia[54-57] and have been proposed in Europe[58].

It is of great value that the DHBV is maintained in domestic duck flocks through vertical transmission from viremic ducks. The virus infects the developing liver *in ovo* and is not sufficiently recognized by the host immune system to produce hepatitis and liver disease or to eliminate the virus[49,59]. Thus, DHBV can be propagated *in vivo* in ducklings or *in vitro* in primary duck embryonic hepatocytes to assess viral infectivity[56,60-62]. Several authors have reported using *in vivo* DHBV assays[54,55,63-65]. To estimate DHBV infectivity, the diluted viral suspensions exposed to the biocides are injected intraperitoneally or intravenously into naïve ducklings not infected with DHBV. The ducklings are euthanized 2 wk later, and their livers are removed to be analyzed for DHBV DNA using PCR[66]. However, these *in vivo* tests conflict with ethical and legal aspects of animal protection. Therefore, the preferred method for testing the efficacy of disinfectants against DHBV is the *in vitro* assay. This protocol is in accordance with the recommendations of the United States Environmental Protection Agency[67].

Viral propagation of DHBV in duck embryonic hepatocytes is not trivial because DHBV is a non-cytopathogenic virus. This approach requires that additional tests, such as immunofluorescence[68], PCR[69] or Southern blotting[70], be used to verify the growth of the virus. Additionally, viral propagation requires a source of DHBV-free Pekin ducks, appropriate eggs for the preparation of embryonic hepatocytes, *in vitro* cultures of hepatocytes, and congenitally infected Pekin ducks as source of the virus. It is advantageous that experimental investigations on embryonated hen and duck eggs are, in general, regarded experimentally as in between *in vivo* and *in vitro* systems and do not conflict with ethical and legal aspects of animal protection[71].

IN VITRO DUCK HEPATITIS B VIRUS MODEL FOR TESTING VIRUCIDAL EFFICACY OF BIOCIDES

In Germany, an assay protocol for testing DHB-virucidal efficacy of biocides by DHBV infection of primary duck embryonic hepatocytes has been established and successfully evaluated for virucidal testing in several studies[68,72,73]. The primary duck embryonic hepatocytes were obtained from fertilized Pekin eggs and incubated for 21 d[68]; the liver tissue was harvested from several embryos[74]. A crucial step was to ensure the absence of DHBV in the source tissue using a qualitative PCR technique[68,75]. To digest the liver tissue, a solution comprised of trypsin, ethyl diamine tetraacetate solution, phosphate-buffered saline and glucose was effective. Digestion of the liver could be inhibited by the addition of fetal calf serum[69,76,77]. DHBV-negative cells were seeded in 24-well culture plates not containing collagen 1 such as CellBIND™ (Corning, Acton, United States)[74]. This step is necessary to ensure stable attachment of hepatocytes to the surface of culture vessels for successful DHBV propagation (Figure 1). The optimal growth medium can be modified according to previous reports[69,78,79]. This medium supports the maintenance of differentiating hepatocytes, which is important for the susceptibility of cells to the virus and for DHBV replication[60,80-82]. A suitable microenvironment can be achieved by coating the growth surface with Matrigel or other substrates containing extracellular matrix molecules[83,84]. Alternatively, the use of co-culture systems of hepatocytes with non-parenchymal liver cells has been described as a suitable method to maintain hepatocyte differentiation *in vitro*[85,86].

The use of hepatocytes cryopreserved by the suspension method is also suitable when freshly isolated cells from the liver of duck embryos are not available due to seasonal differences[74]. Growth medium[74] was supplemented with 10% fetal calf serum and suitable

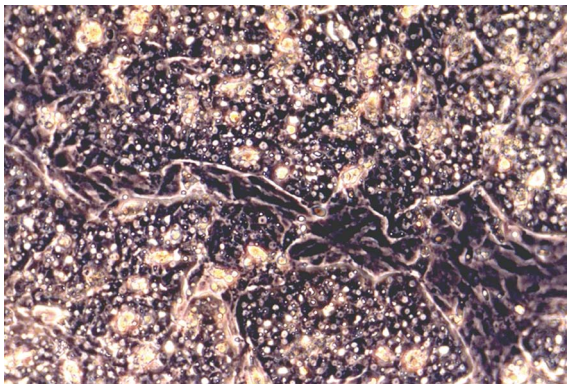

Figure 1 Primary duck embryonic hepatocytes grown in CeLLBIND™ plates at day 3 of cultivation. Monolayers of hepatocytes, which show typical polygonal morphology, are interrupted by areas of non-parenchymal cells (light microscopy, phase contrast, x 200).

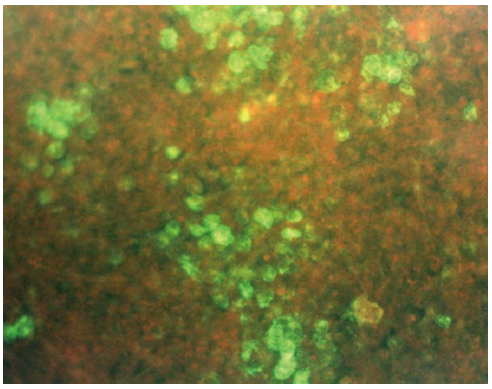

Figure 2 Detection of duck hepatitis B virus-specific surface antigen six days after inoculation of primary duck embryonic hepatocytes by indirect immunofluorescence. Polyvalent rabbit anti-DHBs (kindly provided by Dr. D. Glebe, Institute of Medical Virology, National Reference Centre for Hepatitis B and D, Justus Liebig University, Giessen, Germany) and goat anti-rabbit IgG Alexa Fluor® 488 (Life Technologies, Darmstadt, Germany) were used as antibodies (fluorescence microscopy, x 125).

cryoprotective agents, such as 10% DMSO or cryosafe-1. The freeze-thaw process does not significantly reduce the susceptibility of primary duck embryonic hepatocytes to DHBV infection, suggesting no loss of viral receptors on the cell surface.

As virus pool, DHBV-containing serum from congenitally infected ducks must be used[72,73]. Sera should contain between $10^{6.0}$ and $10^{8.0}$ tissue culture infective doses 50% of DHBV per mL, corresponding to $10^{9.0}$ and $10^{11.0}$ DHBV genomic copies. To avoid reduction of viral titers, the uninterrupted storage of aliquots at -80 ℃ is strongly recommended. One should, however, be aware that the Pekin duck is an unreliable source of the test virus, which causes difficulties for standardization. Another disadvantage is that the titers of infectious virus are often too low to detect sufficient reduction of viral titers especially when cytotoxic biocides are tested[73]. On the other hand, the DHBV prepared from the DHBV DNA-transfected hepatoma cell line D2[87] is not suitable for testing virucidal efficacy of biocides either because this virus is more sensitive than the wild type DHBV naturally occurring in the serum of Pekin ducks[72].

Virucidal tests are recommended to be carried out in accordance with national guidelines for testing the virucidal efficacy of chemical disinfectants in human medical areas[25]. At the end of the chosen exposure time, the test compounds must be immediately removed from the mixture of virus and test formulation by rapid dilution of samples or the use of sephadex-based methods[88], particularly when cytotoxic residues must be removed. However, previous experience has shown that sephadex columns can withhold the infectious virus, thus leading to inaccurate results. It is recommended that the primary duck embryonic hepatocytes are infected at day 4 of cultivation. Due to the *in vitro* method of preparing of hepatocytes, the type I interferon system is stimulated, thereby inhibiting DHBV replication during the first 2-3 d after primary cell plating[89,90]. Thus, 4 d post-infection, a high number of DHBV-infected hepatocytes are infected[68,69]. On the other hand, DHBV-negative hepatocytes lose their susceptibility to DHBV infection after the day 5 of cultivation[60,80]. This results from the dedifferentiation of hepatocytes and/or the loss of the cellular receptor for virus attachment. The presence of DMSO in the cell culture medium is also critical for this process because DMSO not only allows maintenance of viral replication but also prolongs the susceptibility of cultured hepatocytes to DHBV infection[80]. Following viral infection, the cells should be incubated for at least 6 d to achieve infection rates of approximately 40% as shown by specific fluorescence, a surrogate marker of productive viral infection[68].

Indirect immunofluorescent antigen staining has been recommended for detection of DHBV surface(s) antigen in primary duck embryonic hepatocytes to verify DHBV infection[68,72]. To this end, a polyvalent rabbit anti-DHBs antiserum that is not commercially available must be used. As shown in Figure 2, the infected hepatocytes can be easily identified because they appear in clusters[79]. A 4-$\log_{10}$ reduction of infectivity (inactivation $\geqslant$ 99.99%) is regarded as evidence of sufficient virucidal activity[25]. As the guidelines of the United States Environmental Protection Agency[56] state, an *in vitro* assay requires a demonstration of at least 3-$\log_{10}$ reduction in viral titers beyond any disinfectant dilutions that exhibit cell culture cytotoxicity. Although fluorescent analysis has a subjective output and requires experience for the analysis of results, indirect immunofluorescence can be employed for routine testing and has been applied to detect a variety of human and animal viruses[91]. An advantage of this method is that the efficacy of biocides against DHBV infection can be rigorously evaluated because production of DHBV surface proteins in hepatocytes is a late step in the viral life cycle and correlates well with the production of mature virus particles[92]. In contrast, PCR-based methods identify the presence of viral DNA, but this may not necessarily correlate with the number of infectious virus particles[93].

Table 1 Studies published in the literature to evaluate the efficacy of biocides against duck hepatitis B virus

Year	Country	Ref.	Evaluated biocides or inactivation procedures
1991	Australia[1]	Murray *et al*[54]	Glutaraldehyde; mix of glutaraldehyde, non-ionic alcohol derivate, quaternary compound and tri-ethyleneglycol surfactant
1993	United Kingdom[1]	Tsiquaye *et al*[63]	Sodium hypochlorite; sodium dichloroisocyanurate
1996	Australia[1]	Deva *et al*[94]	Glutaraldehyde
1998	United States[2]	Eble *et al*[70]	Photochemical inactivation by 8-methoxypsoralen
1999	Australia[1]	Chaufour *et al*[55]	Glutaraldehyde; ethylene oxide
1999	Australia[1]	Vickery *et al*[64]	Hydrogen peroxide
2000	Australia[1]	Vickery *et al*[95]	Glutaraldehyde
2001	United States[2]	Wagner *et al*[96]	Photoinactivation by dimethylmethylene blue
2002	United States[2]	Wang *et al*[69]	*N*-alkyl dimethyl benzyl ammonium chloride; alkyl dimethyl benzyl ammonium chloride
2004	United States[2]	Moore *et al*[97]	Ethylene oxide
2005	Australia[2]	Druce *et al*[65]	Ethylene oxide
2006	Germany[2]	Sauerbrei *et al*[72]	Peracetic acid; povidone-iodine; formaldehyde
2008	United States[2]	Roberts *et al*[98]	*Ortho*-phthalaldehyde
2012	Germany[2]	Sauerbrei *et al*[73]	Ethanol; isopropanol; peracetic acid; glutaraldehyde; formaldehyde

[1]DHBV *in vivo* test system; [2]DHBV *in vitro* assay. DHBV: Duck hepatitis B virus.

EVALUATION OF BIOCIDES USING DUCK HBV

Several study groups in Australia, the United States, the United Kingdom and Germany have used the DHBV *in vivo* test system and the DHBV *in vitro* assay to evaluate the DHB-virucidal efficacy of chemical biocides or photochemical inactivation procedures. Table 1 gives an overview of the evaluated biocides and procedures of each study group. The majority of groups waived the *in vivo* test system. Since the year 2000, DHBV *in vitro* assays have been used almost exclusively. A recent study determined the DHB-virucidal activity of the following five different chemical biocides: ethanol, isopropanol, peracetic acid, glutaraldehyde and formaldehyde, which are often ingredients present in commercially available disinfectants[73]. Testing was carried out as modified quantitative suspension test[25] in the presence of a protein load of 10% fetal calf serum. Table 2 lists the minimal concentrations and contact times to reach virucidal efficacy. This means that $\geqslant$ 40% ethanol or isopropanol, $\geqslant$ 0.05% peracetic acid and $\geqslant$ 0.1% glutardiaaldehyde within $\geqslant$ 1 min significantly inactivate infectious DHBV corresponding to a 4-$\log_{10}$ reduction in viral titers. For a 0.7% formaldehyde solution, which resulted in high hepatocytotoxicity, a longer contact of $\geqslant$ 30 min is needed. These results show that the DHBV, as an enveloped virus, is considered to be relatively sensitive to inactivation by virucides. Limited, unpublished data with HBV and Tupaia hepatocytes corroborate these findings (personal communication: D. Glebe, Institute of Medical Virology, National Reference Centre for Hepatitis B and D, Justus Liebig University, Giessen, Germany). This is also in agreement with the susceptibility of levels of HBV detected by direct chimpanzee inoculation[30]. Thus, the results presented for DHBV are likely also valid for HBV. However, it must be considered for the interpretation of the *in vitro* data obtained by the quantitative suspension test that recommendations for the application of the agents in practice can be concluded only to a limited extent. Such favourable conditions as during the homogeneous suspension are seldom to be found in practice. Thus, results of the suspension test should not be regarded as practical application in every case but they allow conclusion of the efficacy of single disinfectants and, therefore, they also allow to compare the efficacy of different disinfectants[25]. For comparison, information on stability of HBV published by the World Health Organization[99] is summarized in Table 3. These biocides or measures, including concentrations, temperatures and contact times, are recommended for clinical practice to destroy infectious HBV. In contrast, Table 2 lists the minimal concentrations and contact times for the duck hepatitis B-virucidal activity of several biocides in the quantitative suspension test in which a protein load of 10% fetal calf serum was used. When selecting the most effective method for destroying infectious HBV, it should be taken into account that the amounts of serum HBV varies considerably among HBV-infected patients[100]. Thus, there can be differences in methods according to the level of viremia in patients.

Table 2 Minimal concentrations and contact times for the duck hepatitis B virus-virucidal activity of ethanol, isopropanol, peracetic acid, glutaraldehyde and formaldehyde against duck hepatitis B virus in the presence of a protein load of 10% fetal calf serum

Biocide	Concentration (%)	Contact time (min)
Ethanol	40	1
Isopropanol	40	1
Peracetic acid	0.01	2
	0.05	1
Glutaraldehyde	0.05	2
	0.1	0.5
Formaldehyde	0.7	30

Results of quantitative suspension tests are shown[73].

Additionally, the study by Sauerbrei *et al*[73] has shown that biocides tested against DHBV are efficacious against the vaccinia virus strain Lister or the modified vaccinia Ankara strain[101], which are used in guidelines for the declaration of limited virucidal activity of biocides[25]. The testing of these viruses does not present any difficulties; therefore, it can be expected that in the absence of more direct tests, the results of DHBV, and even of

Table 3 Information on the stability of hepatitis B virus published by the World Health Organization[99]

Biocide/ measure	Concentration/ temperature	Contact time	Remarks
Sodium hypochlorite	0.25%	3 min	Antigenicity of hepatitis B surface antigen is destroyed, infectivity is probably destroyed
Sodium hypochlorite	5%	10 min	Inactivation of virus
Glutaraldehyde	2% (room temperature)	5 min	Inactivation of virus
Glutaraldehyde	2% (98 ℃)	2 min	Inactivation of virus
Formaldehyde	5%	2 min	Inactivation of virus
Isopropanol	70%	2 min	Inactivation of virus
Ethanol	80% (11 ℃)	2 min	Inactivation of virus
Autoclaving	121 ℃	20 min	Lost of infectivity
Heat sterilization	160 ℃	1 h	Lost of infectivity

the vaccinia virus or its modified Ankara strain, may be extrapolated to HBV. Therefore, the surrogate DHBV model can provide highly valuable data for the susceptibility of HBV to disinfectants.

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