Original articles

Inactivation of a hepadnavirus by electrolysed acid water

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Introduction

Cleaning and disinfection of medical devices and instruments contaminated with blood and related materials are important in the prevention of infection by microorganisms, including viruses. Glutaraldehyde (2%) was recommended for cleaning endoscopic instruments by O’Connor & Axon in 1983 and by the Working Party of the British Society of Gastroenterology (BSG) Endoscopy Committee in 1988. It has since been the most commonly used disinfectant in the world for this purpose. However, it has irritant and sensitizing properties and the Working Party of BSG has suggested ways in which exposure of healthcare personnel to glutaraldehyde can be eliminated or minimized by using alternative disinfectants or automated washer disinfectors. According to the guidelines of the BSG, alternatives to glutaraldehyde have to be at least as microbicidal as glutaraldehyde, non-irritant and compatible with both endoscope components and decontamination equipment.

Several different electrochemical solutions containing a mixture of radicals with oxidizing properties have been reported to be microbicidal and are possible alternatives to glutaraldehyde. Electrolysed acid water (EAW) has a bactericidal effect, and an endoscopic washing device using EAW has been developed in Japan. To investigate the effect of EAW on the infectivity of viruses, we treated duck hepatitis B virus (DHBV), which has similar properties to hepatitis B virus, with EAW, and determined the number of remaining infectious virus particles in a bioassay system. One-day-old Pekin ducks were inoculated with duck serum containing 10^8.5 ID₅₀ DHBV; the serum had previously been incubated with 100 volumes of EAW or ion-exchanged water at room temperature for 7 min. DHBV infection was indicated by detection of viral DNA in duck serum samples 1–8 weeks after inoculation. Treatment of serum with EAW diminished DHBV infectivity whereas treatment with ion-exchanged water did not. The virus load was estimated to have been reduced to 10^1–10^3 ID₅₀ during the first 1 min and to <10^0.5 ID₅₀ in the next 6 min of incubation when compared with the control. Thus, EAW directly inactivates DHBV and its clinical application is recommended.

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already been assessed using the DHBV model. The presence of virus can be detected in such bioassay systems with a sensitivity of 100 copies (genome-equivalent DHBV, estimated by quantification of DHBV DNA) per animal; this sensitivity is equivalent to that of the polymerase chain reaction amplification method. Detection of infectious virus is more appropriate than the detection of nucleic acids or virus antigens for determining the activity of disinfectants.

Here we present the results of an investigation into the effect of EAW on viral infectivity, in which ducks were inoculated with EAW-treated DHBV.

## Materials and methods

### Generation of EAW

EAW was generated as previously reported by the Clean-top WM-1 (Kaigen Co. Ltd, Osaka, Japan), a portable endoscope-cleaning device incorporating an electrolysis apparatus. Briefly, 10 L of tap water containing 0.05% NaCl was electrolysed at room temperature for 45 min with a current of 3 A. The anodic and cathodic sides of the solution were separated by a cationic membrane (Nation 450, Dupont, Wilmington, DE, USA); the solution on the anodic side, which contained the electrolysed products, was called EAW and used for the study. The pH, ORP and chlorine concentration were measured using a pH meter (Horiba, Kyoto, Japan), ORP meter (Horiba) and free-chlorine meter (Hach, CO, USA).

### Infection study

**Serum stock of DHBV.** A frozen stock of duck serum containing wild-type infectious DHBV (DHBV16) (22.8 ng DHBV DNA/mL, 6.6 × 10⁵ DHBV genomes/mL, 10⁻⁵.¹ ID₅₀/mL) was used as the inoculum. The ID₅₀ of this serum was calculated to be 10⁻¹.⁵ genomes by an infection study using sequentially diluted infective serum (data not shown). Infection with 10⁻⁰.⁵ ID₅₀ in 1 day old ducklings established a DHBV carrier state: none of the ducklings inoculated with 10⁻³.⁵ ID₅₀ became DHBV carriers 8 weeks after inoculation. Inocula containing 10⁻³.⁵–10⁻⁵.⁵ ID₅₀ were also used as controls in the quantitative analysis.

**Inoculum preparation.** (i) EAW-treated group. Serum containing 10⁻³.⁵ ID₅₀ DHBV (1.5 μL, 10⁷ genomes) was mixed with 100 volumes of EAW and incubated at room temperature for 7 min. The treated serum was neutralized by adding 1/10 volume (15 μL) of 10⁻× phosphate-buffered saline (PBS). In some experiments, inocula were mixed with 500 volumes of EAW or were incubated for 1 min. (ii) Ion-exchanged distilled water (IDW)-treated group. The same amount of serum was treated with IDW in the same way as with EAW.

### Quantification of DHBV DNA in serum

DNA was extracted from the serum samples and analysed by slot-blot hybridization with a ³²P-labelled DHBV DNA probe as reported previously. DHBV DNA in the samples was quantified using a BAS2000 image analyser (Fuji, Tokyo, Japan) using known amounts of cloned DHBV DNA as the control.

### Statistical analysis

Statistical analysis by the Kruskal–Wallis method was performed for the control group. The significance between control and each EAW- and IDW-treated group was analysed by the Mann–Whitney U-test.

### Results

#### Properties of EAW

The pH of the EAW was 2.15, the ORP 1153 mV and the chlorine concentration 7.15 ppm. The pH of EAW did not change during incubation with serum, but increased to 5.59 after addition of PBS. The ORP gradually declined to 620 mV during the incubation with serum and decreased to 453 mV after the addition of PBS. The chlorine concentration had decreased rapidly, to 0.3 ppm, after 1 min of incubation with serum; it did not change during the incubation or when PBS was added.

#### Infectivity of DHBV treated with EAW and IDW

The numbers of DHBV-positive ducks in the groups receiving untreated and treated inoculum are shown in the Table. The inoculum with 10⁻³.⁵ ID₅₀ of DHBV incubated with 100 volumes of EAW for 7 min caused fewer infections (none of eight ducks were infected 8 weeks after inoculation) than in the control group (all seven ducks infected).

Six ducks inoculated with the serum treated with 100 or 500 volumes of IDW for 7 min were all infected 1–8 weeks after inoculation.
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of EAW for 1 min, DHBV DNA was detected in two of five ducks 1 week after inoculation, and 3/3 were still positive for DHBV DNA after 8 weeks. Treatment with 500 volumes of EAW reduced the infectivity to 0/4 ducks even though the time of incubation was only 1 min.

**DHBV titre**

The DHBV titre of the serum after inoculation with the control and treated serum is shown in the Figure. Inoculation with $10^{5.5}\text{ ID}_{50}$ of DHBV resulted in infection in all

<table>
<thead>
<tr>
<th>Solution</th>
<th>Dilution</th>
<th>Incubation time (min)</th>
<th>Weeks after inoculation</th>
</tr>
</thead>
<tbody>
<tr>
<td>None (control)</td>
<td>–</td>
<td>–</td>
<td>1/2</td>
</tr>
<tr>
<td>EAW</td>
<td>100</td>
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</tr>
<tr>
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<td>1</td>
<td>3/4</td>
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<tr>
<td>IDW</td>
<td>100</td>
<td>7</td>
<td>3/3</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>7</td>
<td>3/3</td>
</tr>
</tbody>
</table>

Numbers of DHBV-positive ducks/numbers of ducks analysed are shown. All ducks were inoculated with $105.5\text{ ID}_{50}$ of untreated (control) or treated DHBV.

$^a$Serum samples from other ducks were not obtained at these points.

$^b$One duck died in an accident.

**Table.** Detection of DHBV DNA in the serum of ducks in control, EAW-treated and IDW-treated groups

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**Figure.** DHBV DNA in the serum of ducks after inoculation with untreated (a and b) or treated (c-f) duck serum. Inoculum contained $10^{5.5}\text{ ID}_{50}$ (10$^7$ genomes) (a, c-f) or $10^{0.5}\text{ ID}_{50}$ (10$^1$ genomes) (b) of DHBV before treatment. (a and b) Mean (●) and s.e. (bar) of DHBV DNA titre of each group. (c-f) Inoculum was incubated with 100 volumes of EAW for 7 min (c; ●, DJ1646; □, DJ1467; ◊, DJ1648; ○, DJ1649; ●, DJ1611; ▼, DJ1612; ▼, DJ1613; ○, DJ1614), 100 volumes of IDW for 7 min (d; ●, DJ1623; ■, DJ1624; ▲, DJ1625), 100 volumes of EAW for 1 min (e; ●, DJ1654; ■, DJ1655; ▲, DJ1656; ●, DJ1657; ○, DJ1658) and 500 volumes of EAW for 1 min (f; ●, DJ1659; ■, DJ1660; ▲, DJ1661; ○, DJ1662). DHBV DNA titre of individual ducks 1–8 weeks after inoculation are indicated.
ducks with high titres of viraemia (≥10 ng/mL DHBV DNA) after 1–8 weeks (Figure, panel a). Inoculation with 10^{-0.5} of ID_{50} resulted in detectable DHBV DNA ≤4 weeks after inoculation but chronic infection was not established with this amount of infectious virus (Figure, panel a). Treatment with 100 volumes of EAW for 7 min (Figure, panel c) and 500 volumes of EAW for 1 min (panel f) reduced the infection rate (Table) and the viral titre in the inoculated ducks. Ducks inoculated with the IDW-treated serum showed high-titre viraemia and the time course of infection was similar to that of the untreated control (Figure, panel d).

In the group given an inoculum treated with 100 volumes of EAW for 1 min (Figure, panel e), no DHBV was detectable after 1 week in three ducks and the titre was 0.18 and 0.08 ng/mL in the other two ducks (DJ1654 and DJ1657, respectively), much lower than in the control group (Figure, panel a).

**Statistical analysis of DHBV titre**

As previously reported, the viral titre in the early phase of infection correlated with the amount of infectious virus in the inoculum.^{24} One week after inoculation, the DHBV titres in the control group inoculated with 10^{-4.5}, 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8} and 10^{-9} ID_{50} of DHBV were 0.36, 0.07, 0.12, 0.42, 13.7, 49.2 and 39.3 ng/mL serum, respectively. The P value with Kruskal–Wallis analysis was 0.042, indicating that each group was significantly independent and that the DHBV titre after 1 week was related to the amount of infectious virus inoculated.

To confirm that EAW treatment reduced viral infectivity, we analysed the DHBV titre statistically in ducks treated with 100 volumes of EAW for 7 min and in untreated controls 1 week after inoculation. The P value (Mann–Whitney U-test) was 0.019, indicating that the infectivity of DHBV was reduced significantly by the EAW treatment. Treatment with 100 volumes of EAW for 1 min also reduced the DHBV titre significantly after 1 week (P = 0.045; treatment with 500 volumes of EAW for 1 min reduced the DHBV titre after 1 week but it was not statistically significant (P = 0.064). The reduction in DHBV titre in ducks treated with 100 or 500 volumes of IDW was not significant (P > 9.999 and P = 0.248, respectively).

To assess the level of infectious virus remaining in the inoculum treated with 100 volumes of EAW for 7 min, DHBV titres after 1 week were compared with those in control ducks inoculated with various amounts of DHBV. The P values were 0.335, 0.075, 0.045, 0.014, 0.008, 0.019 and 0.019 for the groups inoculated with 10^{-0.5}, 10^{-1.5}, 10^{-2.5}, 10^{-3.5}, 10^{-4.5} and 10^{-5.5} ID_{50} of DHBV, respectively. These results indicate that the level of infectious DHBV in the inoculum was reduced from 10^{-5.5} to ≈10^{-5.5} ID_{50} by treatment with EAW.

Five ducks were inoculated with serum that had been incubated with 100 volumes of EAW for 1 min. The titres after 1 week were significantly different from those in groups receiving 10^{-5.5}, 10^{-4.5} and 10^{-5.5} ID_{50} (P = 0.022, 0.045 and 0.045, respectively). These data indicated that the amount of infectious virus in the inoculum was <10^{-5.5} ID_{50} DHBV after 1 min of incubation.

**Discussion**

This is the first report on the direct effect of an electrochemically modified saline solution with a strong oxidizing property on hepadnavirus infectivity. DHBV infectivity was completely lost after incubation of the inoculum with 100 volumes of EAW for 7 min, and with 500 volumes for 1 min. The inactivation of the virus was irreversible after adding PBS to neutralize pH and adjusting the salt to a physiological concentration.

We quantified infectious DHBV remaining in the treated inoculum by two methods: (i) lack of DHBV infection 8 weeks after inoculation was taken as an indication that the inoculum contained less than the ID_{50} of DHBV; (ii) the DHBV titre 1 week after inoculation of treated inoculum was compared with that in controls and the significance of the difference was assessed.

Infectivity was lost after incubation of serum with 100 volumes of EAW for 7 min at room temperature and with 500 volumes of EAW for 1 min, indicating that these treatments reduced the number of infectious viruses from 10^{-5.5} ID_{50} to <10^{-5.5} ID_{50}, and to ≈10^{-0.5} ID_{50} in some preparations. It was also estimated that the infectivity was reduced to about 10^{-3} ID_{50} at the end of the first minute of treatment. As dilution of the virus with IDW did not result in any inactivation, it was concluded that EAW directly affected the virus particles and reduced their infectivity.

Electrolysis of a low concentration of NaCl in water produces Cl_{2}, HClO, H^{+}Cl^{-}, O_{2} and O_{3} at the anode; the solution at the anode site is characterized by a low pH, a high ORP and low concentrations of Cl_{2}. The bactericidal effect of EAW is explained by the low pH (<2.7),^{25} ORP exceeding 970 mV and the presence of Cl_{2}; these factors have synergic effects.^{15,26} The minimal chlorine concentration required for the virucidal effect of NaOCl and NaDCC, as assessed by in vitro DNA polymerase assay of DHBV, was 3 × 10^{7} ppm, which was equivalent to NaOCl and NaDCC solutions of about 3%. The presence of Cl_{2} is not the only reason for the virucidal effect of EAW.

Abe et al.^{13} and Morita et al.^{16} have reported that EAW-treated HBV cannot be detected by radioimmunoassay^{13} or enzyme immunoassay^{16} with HBsAb, an antibody that recognizes the tertiary structure of HBsAg on the surface of the virion, indicating that EAW affected the antigenicity of the surface antigen. Morita et al.^{16} analysed the effect on HIV-1 recombinant reverse transcriptase and HIV-1 particles in vitro and demonstrated that naked and packaged reverse transcriptase activities were reduced by EAW in a time- and concentration-dependent manner. EAW may...
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denature the protein, destroying its immunoreactivity and enzymatic function. From our results and those of Abe et al., the mechanism of actions could involve: (i) irreversible denaturation of surface proteins, including preS proteins, which are necessary for attachment of virions to the receptor on hepatocytes; or (ii) loss of function of DNA polymerase or of the viral DNA genome. Hence, EAW is expected to have virucidal effects for a wide variety of viruses, including non-enveloped viruses.

A DHBV bioassay has been used previously to assess the efficacy of glutaraldehyde, NaOCl and NaDCC as disinfectants. Because these disinfectants are irritant and toxic for intravenous injection, virions purified by ultracentrifugation or small amounts of virus were injected into young ducks. Murray et al. treated duck serum with an equal volume of 2.1% glutaraldehyde (pH 5) for 10 min at room temperature. A solution equivalent to 10^2 ID_{50} was used to inoculate 1-day-old ducks; infectivity was reduced from 100% to 60% and the DHBV titre was reduced by \geq 10^3.2. Tsiquaye & Barnard treated duck serum with NaOCl and NaDCC solutions in which the final chlorine concentration was 3.0 \times 10^3 ppm. None of the ducks inoculated with 10^{-2} ID_{50} of DHBV became infected. In our experiment, serum was treated with 100 volumes of EAW for 7 min and none of the ducks inoculated with 10^{-5} ID_{50} DHBV were infected. Thus, EAW had a strong disinfectant activity, comparable to those of glutaraldehyde, NaOCl and NaDCC.

Even though EAW appears to be virucidal, its clinical use awaits more information about: its effect on bacterial spores and mycobacteria; its stability during handling; its safety for medical personnel and the environment; the safety to patients of solution remaining on the instrument; cost–benefit analysis; and its applicability to automated washing machines.

None of the 1-day-old ducks (mean body weight 55 g) inoculated intravenously with \approx 700 \, \mu L of EAW after neutralization died because of acute toxicity. No sensitization to EAW has been reported during 2.5 years of clinical use of Cleantop WM-1 in endoscopic units in hospitals in Japan.

In this report, a virucidal effect of EAW was demonstrated in the DHBV bioassay system. EAW is an alternative to the currently used glutaraldehyde. Further investigation into the mechanisms of activity against viruses and other microorganisms is warranted.

References


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