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Duck hepatitis B virus: a model to assess efficacy of disinfectants against hepadnavirus infectivity

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SUMMARY

The efficacy of three proprietary glutaraldehyde disinfectants and their component bases was assessed using the duck hepatitis B virus (DHBV) model. Inactivation of infectivity of undiluted serum containing $10^{6\cdot8}$ ID_{50}/ml DHBV was assessed after a mixture with an equal volume of disinfectant had stood at room temperature for 10 min. A dried spill of infectious serum was simulated using sterile filter paper disks, saturated with serum containing DHBV, dried and then exposed to test disinfectant for 10 min. Residual infectivity, and hence the reduction in virus titre, was determined by inoculation of dilutions of the treated samples into 1-day-old ducklings. A greater than 3 $\log_{10}$ reduction in virus titre could be demonstrated for the disinfectants as well as for some of their component bases. Disinfectant activity varied according to the method of viral presentation but a reduction of exposure time from 10 to 2·5 min did not diminish activity. The experimental protocol permits a comparative and quantitative assessment of the efficacy of both established and new disinfectants.

INTRODUCTION

Disinfectants play an important role in the control of hepatitis B in the hospital environment. Many chemicals are used for this purpose today but none is entirely satisfactory. Ideally a disinfectant should act rapidly to kill the virus, yet be non-toxic to humans and non-corrosive to instruments as well as convenient and economic to use.

Because hepatitis B virus cannot be grown easily in cell culture there is little experimental data about its resistance to chemical and physical agents. There is, however, ample epidemiological evidence to show that hepatitis B virus in shed blood survives well in the environment at ambient temperatures and that it may cause serious nosocomial infections when dried on fomites including bath brushes [1] or even computer record cards [2]. The decontamination of fragile and expensive instruments such as endoscopes also presents problems [3]. Both dilution and the detergent effect of standard cleaning procedures will greatly reduce the amount of hepatitis B virus contaminating the instrument after use,

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but the infective dose of virus in some carriers is so high, (at least 10^6 ID_{50}/ml) that apparently clean instruments may transmit the disease. In assessing disinfectants for use in hospital, it is necessary to measure their efficacy in disinfecting objects contaminated with dried blood as well as their capacity to inactivate virus which has been highly diluted in aqueous solution.

Duck hepatitis B virus is closely related to the human virus and shares its general biological and structural properties [4]. Studies of the molecular biology [5] and pathogenesis [7–9] of DHBV infection have already extended our knowledge of human hepatitis B and there is now considerable interest in the use of DHBV for testing new antiviral drugs. The DHBV model allows for quantitation of disinfectant activity against hepadnaviruses which has not been practicable with experimental systems involving the transmission of human hepatitis B to chimpanzees [10, 11] or the use of native North American animals, such as the ground squirrel and woodchuck [12]. The ease of management and low cost of day-old ducklings permit the use of increased numbers of test animals with a great improvement in the accuracy of assays compared with protocols utilizing a single chimpanzee per test group.

Alkaline glutaraldehyde is currently recommended as the disinfectant of choice for HBV surface inactivation [10, 11, 13]. Glutaraldehyde is the most effective aldehyde disinfectant and is thought to produce cross-links of the 'correct' molecular length to interact closely with the arrangement of proteins on the outer coat of viruses [14]. Buffered alkaline glutaraldehyde is widely used in the hospital environment and the advantages claimed for its use include a broad spectrum of activity, rapid antimicrobial action, a non-corrosive nature, and relatively few problems due to chemical irritation [14]. However, the alkaline solutions suffer from disadvantages of skin sensitization and a relatively short shelf life of approximately 28 days.

Comparative analysis of disinfectants within the aldehyde class suggests that the presence of free aldehyde groups is a prerequisite for good biocidal activity and that these chemical groups may form extensive cross-links with bacterial proteins [15]. Potentiated acid glutaraldehydes, containing specific chemical additives, have been claimed to be as effective as the alkaline glutaraldehydes [16]. These acid glutaraldehydes do not need to be chemically activated, will remain active for years and result in fewer toxicity-related problems. The three potentiated glutaraldehydes, 'Aidal-2', 'Aidal-6' and 'Viradet' (Whiteley Chemicals Australia Pty Ltd, Chippendale, NSW), analysed in these experiments theoretically combined the benefits of a lower pH formulation with the known anti-microbial actions of the traditional glutaraldehydes. The lower pH substantially increases their active shelf-life by decreasing the rate of aldehyde polymerization.

Cautious extrapolation of the quantitative data about different disinfectants obtained using DHBV may add significantly to the very limited body of knowledge now available to guide recommendations about HBV disinfection in the hospital environment. With this in mind we have designed test protocols which evaluate the action of disinfection in two commonly encountered situations. First, the presence of high titre of virus in serum (as encountered when instruments are decontaminated after use), and secondly the presence of infective serum dried on paper, fabrics or other inanimate objects. In the present studies we

have compared three proprietary glutaraldehyde disinfectants with glutaraldehyde itself.

MATERIALS AND METHODS

Experimental animals

Pekin Aylesbury cross-bred ducks were obtained from a commercial supplier. The experimental work was performed in accordance with the Institute of Medical and Veterinary Science and the University of Sydney Animal Ethics Committee guidelines. All ducklings used in the experiments were obtained from DHBV negative flocks. In addition serum samples obtained immediately prior to experimental inoculations were shown to be free of DHBV DNA by dot-blot hybridization.

Disinfectants

Three disinfectants 'Aidal-2', 'Aidal-6' and 'Viradet' were compared with glutaraldehyde 2·34% pH 5. Aidal-6 and Viradet contain 2·1% glutaraldehyde in a base solution incorporating a non-ionic alcohol derivative, a quaternary compound (alkyl-[^{12}C-^{14}C]-benzyl-dimethly ammonium chloride), and a triethyleneglycol surfactant. The balance of additives, emphasising either the non-ionic (Aidal-6) or quaternary (Viradet) component, was different for each preparation. Aidal-2 contained 2·1% glutaraldehyde in a non-ionic alcohol derivative base. The acid pH of the glutaraldehyde was chosen to approximate that in the proprietary disinfectants although alkaline conditions are normally employed when it is used in practice.

Duck hepatitis B virus (DHBV)

A pool of serum from adult DHBV carrier ducks (serum no. 1) was used in experiments 1 and 2. The concentration of virions in the sample was calculated from the level of DHBV DNA detected by dot-blot hybridization. This was approximately $1{\cdot}5 \times 10^{10}$ virions/ml. A second pool of carrier duck serum (serum pool no. 2) was used in experiment 3. It contained a DHBV DNA level equivalent to approximately 8×10^{8} DHBV virions/ml.

DHBV was titrated in day-old ducklings and the ID_{50} calculated by the method of Reed and Muench [17]. Serial tenfold dilutions of the serum no. 1 were made in phosphate buffered saline (PBS) over the range 10^{-2}–10^{-8}. 100 μl aliquots of each dilution were inoculated intraperitoneally (IP) into groups of four 1-day-old ducklings. They were then bled weekly for 9 weeks and the presence of DHBV in serum samples was determined by dot-blot hybridization. At the end of the experiment the ducks were killed and serum and liver tested for the presence of DHBV DNA.

Experimental protocol

Serum containing DHBV was exposed to the test disinfectants and to samples of the constituent bases either in an undiluted form (experiment 1) or dried on filter paper disks (experiments 2 and 3). Residual infectivity was determined by inoculation of 1-day-old ducklings. The experimental details are as follows:

Serum challenge. An aliquot of 200 μl of undiluted DHBV serum pool no. 1

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containing approximately $1{\cdot}5 \times 10^{10}$ virons/ml was mixed with an equal volume of test disinfectant, disinfectant base or control phosphate buffered saline (PBS) at room temperature, RT, approximately 20 °C, for 10 min. Disinfectant activity was halted and *in vivo* toxicity eliminated by immediate 500-fold dilution of the virus/disinfectant mixture in PBS. Aliquots of 100 μl corresponding to a dose of approximately 100 ID_{50} were then injected intraperitoneally (IP) into each of five 1-day-old ducklings for each of the mixtures. A group of ten ducklings was inoculated with DHBV positive serum diluted in PBS to give approximately 100 ID_{50}.

Dried preparation of DHBV. A 200 μl aliquot of undiluted DHBV serum pool no. 1 was absorbed onto sterile filter paper disks (Whatman No. 1) and dried under vacuum for 5 h at RT. Five disks were used to test each of the disinfectants and PBS as a control. Each test disk was saturated with an excess volume of approximately 200 μl of disinfectant and each control disk with 200 μl for PBS and allowed to stand for 10 min at RT. Each group of five disks was then immersed in 150 ml of PBS for 2 h at RT followed by sonication for 2 min. Preliminary experiments determined the viral recovery rates from filter papers inoculated with DHBV-positive serum to be 50–80 % (results not shown). A 200 μl inoculum which corresponded to a dose of approximately 100 ID_{50} was then injected IP into four 1-day-old ducklings for each of the disinfectants or bases studied and into a group of eight ducklings as a PBS control.

Exposure-time evaluation. Filter paper disks inoculated with serum pool no. 2 were exposed to Aidal-2 for 2·5, 5 and 10 min periods or PBS for 5 min. Groups of six ducklings were used to determine residual infectivity following each time of exposure to the disinfectant and there were seven ducks in the PBS control.

Detection of DHBV DNA

Genome length DHBV DNA [18] subcloned into plasmid vector pACYC 184 was a gift from Dr P. Marion. Excised DHBV DNA was radiolabelled with [^{32}P]dCTP using a multiprime kit (Multiprime DNA, Amersham). Probe specific activities ranged from 0·5 to 1×10^9 disintegrations per min (dpm)/μg DHBV DNA.

The presence of DHBV DNA in serum was detected by dot-blot hybridization. A 25 μl aliquot of serum was denatured by 25 μl 1 M-NaOH and spotted onto a nylon membrane. Filters were hybridized with ^{32}P-labelled DHBV DNA, washed and autoradiographed as described previously [19].

To detect viral DNA in liver approximately 0·5 cm^3 fragments of tissue were removed immediately after the animals were killed, snap-frozen in liquid nitrogen and stored at −70 °C. Following tissue digestion the nucleic acids were extracted with phenol/chloroform and then precipitated with ethanol [20]. DNA extracts were examined as described above by dot-blot hybridization.

RESULTS

The titre of serum pool no. 1 was calculated to be $10^{6{\cdot}8}$ ID_{50}/ml. The ID_{50} for 1-day-old ducklings was found to be $1{\cdot}5 \times 10^3$ virions.

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Table 1. *Serum challenge*

	Proportion of ducks DHBV DNA positive at different intervals after inoculation (weeks)*					
	0	2	3	4	5	9
Control	0/10	10/10	10/10	10/10	10/10	10/10
Glutaraldehyde (pH 5)	0/5	0/5	1/5	3/5	3/5	3/5
Aidal-2	0/5	0/5	0/5	1/5	2/5	2/5
Aidal-2 base	0/4	4/4	4/4	4/4	4/4	4/4
Aidal-6	0/5	0/5	0/5	0/5	0/5	0/5
Aidal-6 base	0/5	5/5	5/5	5/5	5/5	5/5
Viradet	0/5	0/5	0/5	0/5	0/5	0/5
Viradet base	0/5	0/5	0/5	1/5	1/5	1/5

* An aliquot of 200 μl of serum pool no. 1 was mixed with 200 μl of test disinfectant or PBS at room temperature for 10 min prior to dilution. Each 1-day-old duck was inoculated intraperitoneally with a further dilution of the mixture to contain 100 ID_{50} of virus.

Serum challenge

Results of this experiment are shown in Table 1. DHBV DNA was detected in serum of all ten control ducks by the second week after inoculation and all remained positive throughout the experiment. All the disinfectant preparations showed some inactivation of the inoculum and with Aidal-6 and Viradet infection was completely prevented in the ducklings. The Viradet base (a predominantly quaternary compound) caused substantial inactivation of DHBV but the other two bases (predominantly non-ionic alcohol derivatives) had no effect. The mixing of Aidal 6, Viradet and to a lesser extent the Viradet base, with the undiluted serum resulted in the formation of a white coagulum, possibly a protein precipitate. This coagulum was only partly redispersed following dilution of the samples with PBS. There were no untoward effects following IP inoculation of the ducklings with this material.

The experiment was terminated at 5 weeks, because our end point titration experiments had shown that no further positives are to be expected after this time. In addition all livers were tested for DHBV DNA. The reduction of titre was calculated by reference to the end point titration. Aidal-6 and Viradet caused a reduction in titre of $\geqslant 4$ $\log_{10}$. Acid glutaraldehyde, Aidal-2 and the Viradet base caused a 3 $\log_{10}$ reduction, whereas the Aidal bases did not produce any measurable effect.

Dried preparation of DHBV

All eight control ducks were positive by 2 weeks after inoculation. The proportion of ducks which became DHBV DNA positive, at intervals after inoculation, are presented in Table 2. DHBV DNA negative ducks were killed at 8 weeks of age and the livers assayed for the presence of DHBV DNA. No additional infected ducks were found. Three of the disinfectants, conventional glutaraldehyde Aidal-2 and Aidal-6 completely prevented DHBV infection. While Viradet and both the Aidal-6 base and the Viradet base produced a 3 $\log_{10}$ reduction in titre whereas the Aidal-6 base had no effect on the infectivity of the inoculum.

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Table 2. *Dried preparation of DHBV*

	Proportion of ducks DHBV DNA positive at different intervals after inoculation (weeks)*				
	0	2	3	4	8
Control	0/8	8/8	8/8	8/8	8/8
Glutaraldehyde (pH 5)	0/4	0/4	0/4	0/4	0/4
Aidal-2	0/4	0/4	0/4	0/4	0/4
Aidal-2 base	0/3	1/3	1/3	1/3	1/3
Aidal-6	0/4	0/4	0/4	0/4	0/4
Aidal-6 base	0/4	4/4	4/4	4/4	4/4
Viradet	0/4	0/4	1/4	1/4	1/4
Viradet base	0/4	1/4	1/4	1/4	1/4
Viradet (1/3)	0/3	0/3	0/3	0/3	0/3

* Serum pool no. 1 dried on filter paper disks were saturated with the test disinfectant or PBS and allowed to react for 10 min at room temperature prior to dilution and sonication, see text for details. Each duck was inoculated intraperitoneally with approximately 100 ID_{50} of virus.

Table 3. *Effect of time of exposure to Aidal-2*

	Proportion of ducks DHBV DNA positive at different intervals after inoculation (weeks)*				
	0	2	3	4	5
Control	0/7	7/7	7/7	7/7	7/7
2·5 min	0/6	0/6	0/6	0/6	0/6
5 min	0/6	0/6	0/6	0/6	0/6
10 min	0/6	0/6	0/6	0/6	0/6

* Serum pool no. 1 dried on filter paper disks were saturated with Aidal-2 or PBS and allowed to react at room temperature for 2·5, 5 or 10 min prior to dilution and resuspension, see text for details. Each duck was inoculated with 100 ID_{50} of virus.

Effect of time of exposure

Decreasing exposure time from 10 min to 2·5 min did not diminish the efficacy of Aidal-2 (Table 3). Both the serum and liver were DHBV DNA negative in all of the test ducks when they were killed 5 weeks after inoculation, whereas all seven control ducks were seropositive by the second week and remained positive throughout the experimental period.

DISCUSSION

The main risk of nosocomial infection with hepatitis B arises from contamination of instruments or other fomites with blood or body fluids. We therefore used undiluted serum in all three experiments to simulate working conditions in hospitals as closely as possible. The use of undiluted serum provides a more realistic challenge than the suspension methods employing HBV-containing plasma diluted as much as 1/1000 [10] or partially purified HBV [21, 22] or even the carrier method of Bond [11] where a 1:10 dilution of infected plasma was employed.

We found that the main advantages of the serum suspension test was that it was

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simple to perform and the amount of virus present was known. The significance of the serum coagulum formed by the addition of some of the glutaraldehyde disinfectants needs to be resolved because the reduction of DHBV infectivity observed may be due to direct effect on the virions or a result of entrapment of infectious virions in a protein matrix.

In the second experiment, filter paper was used as a carrier to simulate dried virus on a contaminated surface. Protein precipitation was not obvious with this method. However there was some difficulty in standardizing the amount of virus released for assay after disinfectant treatment. This problem is common in all carrier systems.

The results from all the experiments show that glutaraldehyde and glutaraldehyde-derived disinfectants decrease the infectivity of DHBV. The log reduction in virus titre provides a means of comparing results obtained by different protocols and with different disinfectants. A 3 $\log_{10}$ reduction in virus titre has generally been considered to indicate effective viral inactivation [23]. However, we have shown that a 3 $\log_{10}$ reduction still allowed some ducklings to become infected. Reasonable estimates of $\log_{10}$ reduction values were calculated from these experiments but greater accuracy could be attained by using larger numbers of ducklings for each titration.

Aidal-6 and Viradet were found more effective than Aidal-2 and glutaraldehyde pH 5 against DHBV in a serum challenge, producing a greater than 4 $\log_{10}$ reduction and preventing DHBV infection. These disinfectants contained three additives (non-ionic alcohol; a quaternary compound and a surfactant) in varying amounts. Aidal-2, which lacks in a quaternary compound, performed less well against the undiluted serum achieving partial inactivation, albeit with a 3 log decrease in virus infectivity after 10 min exposure. When dried virus was used the chemical composition of the disinfectant appeared to be less crucial. Under these conditions all the disinfectants, including Aidal-2 produced a greater than 4 log reduction in virus infectivity after 10 min. This inactivation of DHBV also occurred in 5 and 2·5 min. These results support the recommended 4 min exposure time suggested by the British Society of Gastroenterologists for inactivation of HBV on endoscopes [13]. In the undiluted Viradet group one duckling became DHBV positive which is at variance with results of separate experiments using Viradet (unpublished) and we believe this may reflect an error in identification of the colour-coded leg bands.

The addition of surfactants to an acid glutaraldehyde may enhance virucidal activity to levels comparable with alkaline glutaraldehyde. Non-ionic or cationic alcohol derivatives, surfactants and quaternary compounds may all be useful. Their activity may be due to their own direct viral inactivation, enhanced exposure of the viral proteins to the disinfectant by their detergent action, or modification of the form and length of the glutaraldehyde compound to conform with the surface morphology of the virus. The Viradet base partially inactivated DHBV in both experiments whereas the bases for Aidal-2 and Aidal-6 were largely ineffective. Although all of the bases may enhance the virucidal effect of the parent glutaraldehyde compound, only Viradet base (containing quaternary compounds) showed a marked effect when used alone. The quaternary additive may disrupt the integrity of the viral particle by binding to oppositely charged

chemical groups within the viral coat and thereby also enhancing the glutaraldehyde action. The exact mode of antiviral action of glutaraldehyde is still uncertain [15]. All of the viral disks treated with glutaraldehyde-based disinfectants remained intact during the vigorous process of virus recovery and this provides support for the concept that physical fixation of proteins is a major non-specific action of glutaraldehyde.

In summary, the degree of the virucidal effect of the potentiated acid glutaraldehydes was shown to vary with the method of viral presentation and the chemical composition of the disinfectant. The experimental protocol which has been established permits an assessment of the efficacy of disinfectants on the infectivity of hepadnaviruses.

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