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Short communication

Inactivation of rabies virus

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A B S T R A C T

Rabies virus is a notifiable pathogen that must be handled in high containment facilities where national and international guidelines apply. For the effective inactivation of rabies virus, a number of reagents were tested. Virkon S (1%) solution caused more than 4 log reduction of rabies virus in culture medium supplemented with 10% foetal calf serum within 1 min. Isopropyl alcohol (70%) treatment resulted in >3 log reduction of rabies virus within 20 s when applied at a ratio of 19:1, making it a suitable agent for surface decontamination whereas 70% ethanol was ineffective. Rabies virus (from $10^{2.33}$ to 10^{3} ffu/ml) was also inactivated when cell cultures were fixed with 3% or 4% paraformaldehyde for 30 min. Regardless of inactivation procedure, when taking inactivated virus preparations out of a biological containment envelope, proof of inocuity must be demonstrated to cover any possible error/deviation from procedure.

Rabies is a non-segmented negative strand enveloped RNA virus. It is considered to be one of the most deadly pathogens known with the case fatality rate approaching 100% after the onset of clinical disease (Fooks et al., 2014; Jackson, 2014). For these reasons, the laboratories that work with lyssaviruses must comply with relevant national biocontainment and biosafety regulations (OIE, 2013). Our current local requirements dictate that all work using live virus has to be performed in Microbiological Safety Cabinets (MBSCs) in either in class I or class III mode depending on the nature of procedures undertaken. Solid waste materials (such as plastic ware) are treated with disinfectant, autoclaved and incinerated to ensure the complete destruction of infected materials. Liquid wastes are disinfected and emptied into an effluent treatment plant where the effluent is heated to 96 °C for two hours then cooled prior to release. Alongside removal of solid and liquid wastes from a high containment unit, the removal of items from MBSCs during procedures where live virus is present can also present disinfection challenges.

Virkon S (manufactured by Du Pont) is a commonly used disinfectant for all purposes in the laboratory. It contains potassium peroxymonosulfate (an oxidizing agent), sodium dodecylbenzenesulfonate, sulfamic acid, and inorganic buffers. In house data showed that rabies virus was inactivated by Virkon within 24 min at 4 °C (unpublished results). However, during standard laboratory working procedures, a 24 min contact time prohibits efficient working practices. Alongside this, the use of Virkon S can be problematic as it represents a hazard itself to operators within the laboratory: it can produce acute oral, inhalation and dermal toxicity; serious eye damage. It is toxic to aquatic life following discharge into the environment. Furthermore, Virkon S is corrosive to metal surfaces following prolonged exposure which can reduce the life span of the MBSCs (http://virkon.com/en/products-applications/disinfectants/). For these reasons, alternative disinfectants were also assessed for their suitability in the inactivation of rabies virus.

Published data indicated that rabies virus can be inactivated by a range of chemicals *i.e.* soap solutions (1%–20%), 43%–70% alcohol, quaternary ammonium disinfectants in 1:500 dilution, 5–7% iodine solution (Kaplan et al., 1966), anionic and cationic detergents, lemon juice, vinegar, hydrochloric acid, sodium carbonate (Larghi et al., 1975), glutaraldehyde (Minamoto et al., 1988), and hydrogen peroxide (Abd-Elghaffar et al., 2016). One report stated that rabies was completely inactivated within 20 min with acetone at a concentration of >30%, within 2 min with 75% ethanol, within 6 h with 0.05% formaldehyde, and within 11 min with 10% formaldehyde (Pan et al., 2011). While rabies virus could be inactivated by reagents used for the fluorescent rabies antibody test (FAT) (White and Chappell, 1982), the prescribed conditions were not practical for routine use. A recent publication reiterated the fact that acetone fixation is not suitable for complete inactivation of rabies virions, instead only causing a reversible dehydration of the virus (Jarvis et al., 2016). Considering the available informa-

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Table 1
Titration of CVS virus after treatment with 1% Virkon S.

Test conditions	Log 10 TCID50/ml ± SE	Run[a]
Neat CVS *vs* 1% Virkon S <10 s	<2.61	2
Neat CVS *vs* 1% Virkon S 1 min	0	2
Neat CVS *vs* 1% Virkon S 1 min	0	1
Neat CVS *vs* 1% Virkon S 10 min	0	1
1:40 CVS vs 1:40 Virkon S 1% 1 min	7.11 + 0.16	1
1:40 CVS vs 1:40 Virkon S 1% 10 min	7.31 + 0.16	1
Neat CVS vs water <10 s	6.41 + 0.19	2
Neat CVS vs water 1 min	6.61 + 0.17	1
Neat CVS vs water 10 min	7.01 + 0.15	1

[a] Tests were performed at two different occasions, run 1 and 2 where 50 μl and 100 μl of virus were used for the titration respectively. Ten-fold serial dilutions were performed starting from 1/81 dilution of virus and disinfectant (0 point). Due to the toxicity of Virkon, lower dilution cannot be analysed. Two out of ten wells were found to be positive when CVS was treated with Virkon S for <10 s at the 0 point. Assuming all wells in the lower dilution (8.1 fold) were positive (the worst case scenario), the amount of virus present would be $10^{2.61}$ TCID50/ml. No live virus was detected when CVS were treated with 1% Virkon S for 1 min or 10 min. Using the same assumption as above, the detection limits of the assays (the maximum amounts virus that may evade detection) were 2.71 and 2.41 log10 TCID50/ml for run 1 and run 2 respectively.

tion, the efficacy of Virkon S, 70% isopropyl alcohol and 70% ethanol was tested against rabies under laboratory conditions to determine minimal contact times and alternatives to currently accepted practises.

The Challenge Virus Standard (CVS) strain of rabies virus, that is a widely accepted representative for rabies virus for laboratory diagnostic applications was utilised as the target for inactivation in this study. Baby hamster kidney (BHK) −21A cells grown in cell culture media (G-MEM for BHK-21 from Gibco, Life Technologies) supplemented with 10% foetal calf serum (FCS) and antibiotic antimycotic solution from Sigma (A-5955) was used to culture the CVS rabies virus *in vitro*. For all titrations, BHK-21A cells were split (3×10^5/ml) and 100 μl of cells were added to all wells of a 96 well plate and incubated for 1 h at 37 °C/5% CO_2 for cells to adhere and form a monolayer.

In order to determine the minimal dilution of Virkon S that does not exhibit cytotoxicity to BHK cell monolayers, a three-fold serial dilution of Virkon S (1%), was performed in cell culture medium. The Virkon S dilutions (100 μl) were added to BHK-21A cell monolayers prepared above in four replicates and incubated for 48 h at 37 °C/5% CO_2. Each treated cell monolayer was examined by light microscopy to assess cytotoxicity. The results showed that the cytotoxicity of 1% Virkon S was completely abolished at a 1 in 81 dilution in cell culture media.

To determine if the contact time is sufficient for a disinfectant to work, the conditions that abolish the disinfectant activity must be determined. The anti-viral activity of 1% Virkon S following a 1:81 dilution in cell culture medium was tested. This was done by mixing equal volumes of CVS virus (>10^7 TCID50/ml) and 1% Virkon S following dilution of each with culture medium containing 10% FBS (1:40.5 *i.e.* 50% of the volumetric dilution that abolished the cytotoxicity of Virkon S) and left for either 1 or 10 min as the disinfectant tests detailed below (Table 1). Following incubation, ten-fold serial dilutions of the mixtures were added to the cell monolayer prepared earlier with 10 replicates at each dilution. After incubation at 37 °C/5% CO_2 for 48 h, the plates were fixed in acetone and monolayers were probed for viral antigen by staining with a fluorescein isothiocyanate (FITC) −conjugated anti-nucleocapsid (N) protein antibody following the OIE prescribed method (OIE, 2013). The titres of CVS specific antigen present were calculated based on Spearman & Karber algorithm (Aubert, 1996). The titres of virus did not decrease following treatment with the 1:81 diluted 1% Virkon S (Table 1), indicating that the dilution was sufficient to negate the anti-viral activity of Virkon S.

To assess the ability of Virkon S to inactivate rabies virus, neat virus (>10^7 TCID50/ml) was mixed with Virkon S (1%) at a 1:1 ratio and left for different time periods to enable the minimum required contact time to be determined. Three time points were chosen for assessment: less than 10 s, 1 min or 10 min. The control experiments were set up using water instead of Virkon S. Following the indicated incubation period, preparations were immediately diluted with cell culture medium containing 10% FBS (1:40.5) to inactivate the Virkon S. The treated virus samples were titrated as above and the results are shown in Table 1. The results show that after less than 10 s contact time, the viral titre decreased >3 logs. After 1 min, no virus antigen could be detected in cells. Due to the toxicity of the disinfectants to the cell, the dilution has to be made before the reaction mixtures can be added to cell culture monolayer. Therefore, in the absence of any detectable virus, it is possible the number of the viral particles in the system is simply below the detection limit of the assay (See further explanations in the note of Table 1). Nevertheless, during routine laboratory work, the amount of virus relative to the amount disinfectant is significantly lower than the conditions tested here; therefore, a 4 log reduction in virus titre should provide a sufficient safety margin.

The virus was suspended in culture medium and mixed with equal volume of Virkon (1%), which means that 0.5% of Virkon is sufficient to inactivate the virus in the presence of normal organic matters (*i.e.* culture medium and FCS) in the laboratory within a 1 min time period. This provided a margin of error because the higher concentration of Virkon (1%) is routinely employed for antiviral purposes within many laboratories. This means that Virkon S is suitable for routine surface disinfection or treating liquid or solid waste. Such a short contact time is not intended for disinfecting materials that are not easily penetrable such as large pieces of animal tissue. In such instances it is likely that a longer inactivation period is required.

Due to the limitations of Virkon S mentioned above, the suitability of isopropyl alcohol (70%) and ethanol (70%) as surface disinfectants for laboratory use was also tested. Initially, the cytotoxicity of 70% ethanol, 70% isopropyl alcohol and its commercial version, Premier Klercide 70/30 IPA (70% isopropyl alcohol blended with deionized water, supplied sterile by gamma irradiation and double bagged, http://www.ecolabcc.com/products.php?id=2&cat=5&pro=9) was assessed. As demonstrated for 1% Virkon S, the cytotoxicity of all three disinfectants was completely abolished after being diluted 1:81 with G-MEM + 10% FCS. In order to determine the contact time, the anti-viral activities of the diluted 70% isopropyl alcohol and Premier Klercide (diluted 1:81 with culture medium supplemented with 10% FCS), were tested. The results showed that their anti-viral activities were abolished (Table 2).

The suitability of these alcoholic disinfectants for fast surface cleaning was tested as below: neat CVS (2.5 μl) was mixed with 47.5 μl of 70% ethanol, 70% isopropyl alcohol or Klercide 70/30 IPA. Half of this mixture (25 μl) was then diluted with 1.9 ml of culture medium containing 10% FCS, resulting in a 1/81 dilution of the disinfectant to stop the reaction and to prevent cytotoxicity when plated out. This process took less than 20 s. The control experiments were set up using water instead of the disinfectants. The treated virus was titrated as before. No virus was detected after treatment with 70% isopropyl alcohol or Klercide 70/30 IPA. In the absence of any detectable virus, the maximum number of virus particles that can be present in the system is estimated based the dilution factors applied for eliminating the toxicity of the disinfectants (Table 2). The results demonstrated that 70% isopropyl alcohol caused more than 3 log reduction of virus titre within 20 s and the same results were obtained with Klercide 70/30 IPA. In contrast, 70% ethanol caused about only one log decrease in virus titre under the same conditions (Table 2). Early work indicates that 43–70% alcohol inactive rabies within 1–1½ minutes (Kaplan et al., 1966), however,