Inactivation kinetics of Cyprinid Herpesvirus 3

(Koi herpes virus) by Virkon ® Aquatic.

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Introduction.

The World Organisation of Animal Health (OIE) granted KHV disease 'Notifiable-Disease' status in May 2006 and while the European Union has yet to adjudicate on EU-wide control measures, the UK Government has declared its intention to give KHV 'Notifiable-Disease' status in the UK. Consequently, a consultation exercise, headed by the UK Fish Health Unit and involving all interested parties within Government, ornamental aquaculture, fisheries, anglers and hobbyists, is presently underway.

Reliable diagnostic systems for KHV are under early development; adequate epidemiological studies have yet to be undertaken and control measures have yet to be identified, accepted and implemented. There is increasingly urgent need, therefore, for effective disinfection of KHV contaminated materials, an urgency all the more pressing in the light of continued sporadic and widespread outbreaks of KHV disease.

The proprietary disinfectant, Virkon ®Aquatic has been declared effective against a variety of pathogens, including the Rhabdovirus, Infectious Haematopoitic Necrosis Virus (IHNV), Herpesviruses and Togaviruses as well as various bacterial and fungal pathogens. It is recommended that this disinfectant is used at a concentration of 1% and that surfaces are exposed to the disinfectant for 10 minutes.

We examine here the inactivation kinetics of Koi herpesvirus by Virkon ®Aquatic.

Materials and methods.

Koi Herpes Virus (KHV): Herpesviridae cyprinid herpesvirus 3 (strain F347) was originally obtained from the Centre for environment, fisheries & aquaculture science (Cefas); an agency of the UK Government Department for the Environment, Farming and Rural Affairs (DEFRA). Stocks of KHV were grown in CCB cells.

Common carp brain (CCB) cells were obtained from Dr Dieter Steinhagen, Fish Disease Research Unit and Immunology Unit, School of Veterinary Medicine, Hannover, Germany.

Tissue Culture Medium (TCM) comprised Eagle's Minimal Essential Medium (EMEM) supplemented with;

- Non Essential Amino Acids (NEAA)
- Antibiotics; Penicillin and Streptomycin
- Antimycotic; Amphotericin B
- Hepes buffer.
- Foetal bovine serum (FBS); 2% - 10% as required.
- L-Glutamine; 2mM.

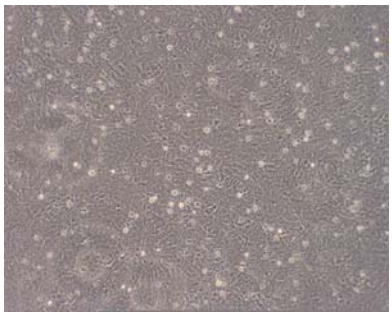
Experiments to determine the effects of Virkon ®Aquatic on CCB cell and KHV viability were routinely conducted in 96 well microtitre trays.

Titration of stock KHV

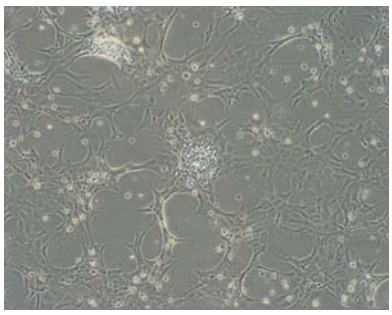
Serial dilutions of stock KHV were prepared in a microtitre tray and 100ul volumes transferred to a second microtitre tray containing confluent CCB cells (see below). Microtitre trays were incubated at 20^{o}C in a humidified atmosphere for up to three weeks or until KHV cytopathic effect (CPE) was maximal.

The '50% tissue culture infective dose' ($TCID_{50}$) is calculated as the virus dilution where 50% of the tissue culture wells exhibit viral CPE. (see below).

Subsequent experiments to determine the effects of Virkon ®Aquatic on KHV viability routinely used 10-100 $TCID_{50}$ per ml.

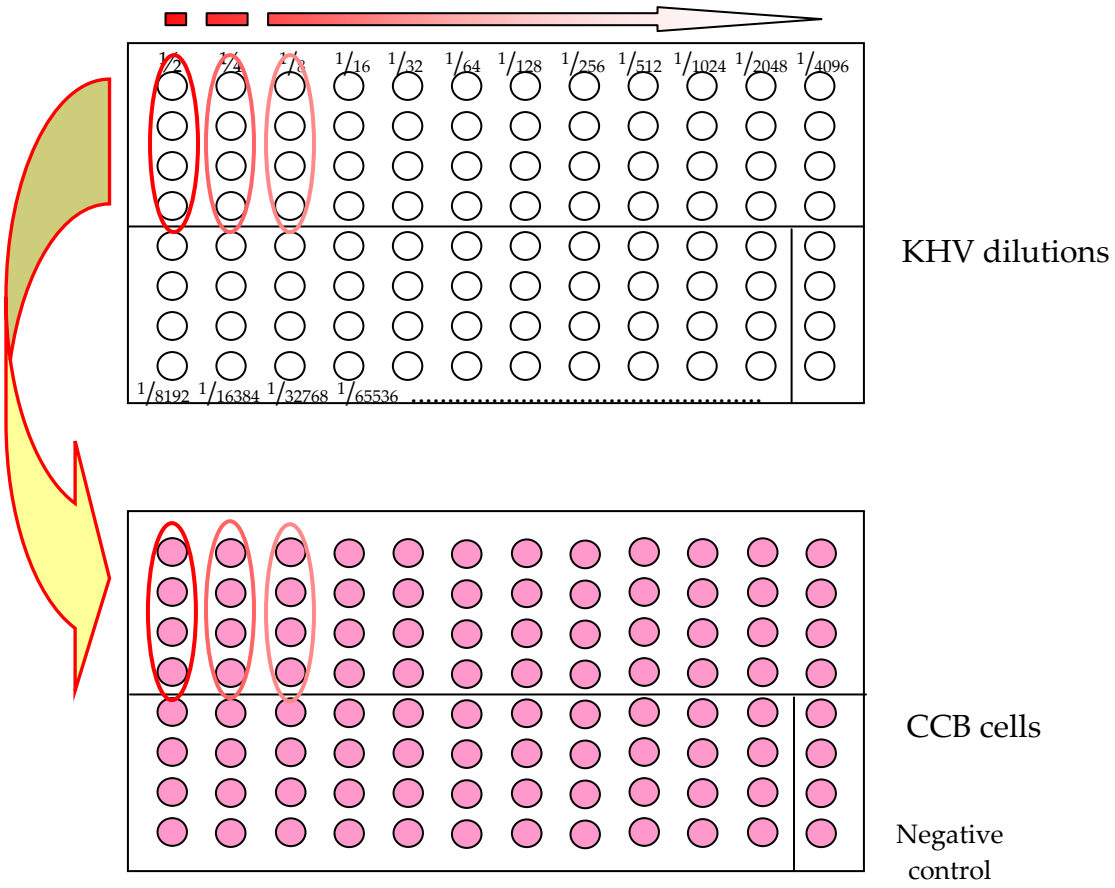


Non-infected CCB Cells



KHV infected CCB Cells

Titration of stock KHV



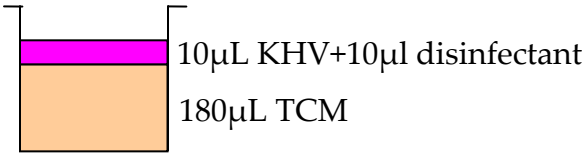
Cytotoxicity of Virkon ®Aquatic.

It was necessary to determine the cytotoxic concentrations of Virkon ®Aquatic in order that any cytotoxic effects were excluded from cultures in which the antiviral effects of the disinfectant were under investigation. Serial dilutions of disinfectant were prepared in TCM and added to confluent CCB cells in microtitre trays as described for the titration of KHV (above). Cultures were incubated at 25°C and frequently observed microscopically for changes in morphology or detachment from the culture surface etc.

There was no indication of cytotoxicity when cells were incubated with disinfectant at concentrations of 0.016% and lower; cells were morphologically normal, attached to the culture surface and there was no discolouration of the TCM. In subsequent experiments

to determine the antiviral effects of the disinfectant, [KHV + Disinfectant] 'reaction mixtures' were sufficiently diluted, prior to exposure to CCB cells, to exclude cytotoxic effects. The cells would therefore be capable of supporting replication of viable KHV.

For example;



This would impart a twenty-fold dilution on the disinfectant

However, dilutions of disinfectant in TCM were routinely applied to CCB cells, in parallel with [KHV + disinfectant] reaction-mixtures to facilitate concomitant observation and monitoring of cytotoxicity.

Kinetic inactivation of KHV by Virkon ®Aquatic

Serial dilutions of disinfectant were prepared, in replicate, in microtitre trays and KHV added to a final concentration of 10-100 $TCID_{50.}$ Reaction mixtures were incubated at 25^{o}C, sampled at time zero (T0) and duplicate microtitre trays containing confluent CCB cells inoculated with the KHV + disinfectant mixture (see below) . Replicate reaction mixtures were sampled at subsequent time points and added to cells. Cultures were incubated at 20^{o}C, observed daily for three weeks and cell morphology was recorded:

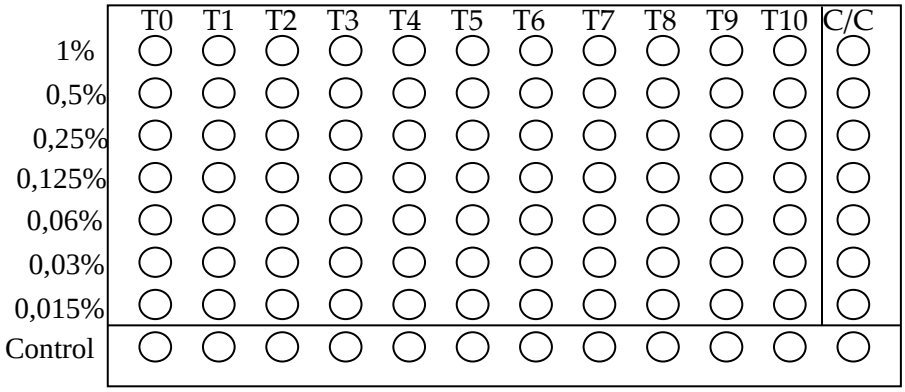
Normal cell morphology / Cytotoxic disinfectant effect / KHV cytopathology

Having excluded disinfectant cytotoxicity, the times required for each disinfectant concentration to inactivate 50-100 $TCID_{50}$ KHV per ml were recorded.

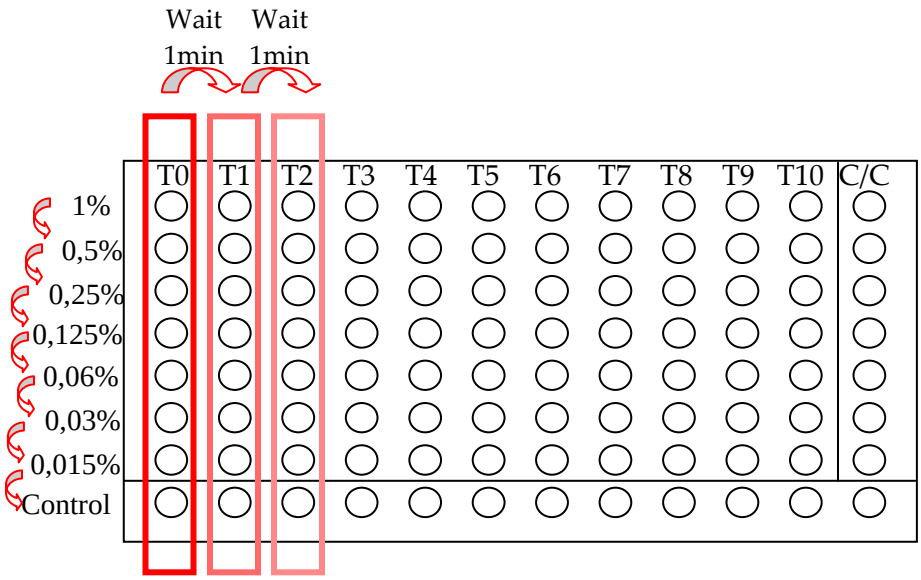
Disinfection experiments were duplicated and various disinfectant concentrations tested over different time courses.

The times required for each disinfectant concentration to inactivate 50-100 $TCID_{50}$ KHV per ml were plotted graphically. It was clear from these examples that KHV inactivation by Virkon ®Aquatic at a concentration of 0.5% was virtually instantaneous. Moreover, that at a concentration of only 0.25%, disinfection is complete within ten minutes.

[KHV + disinfectant] dilution series in replicate.

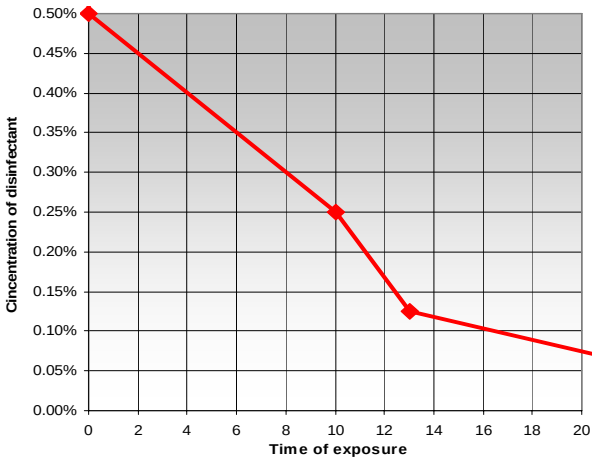


Sampled at one minute intervals and cell cultures innocculated.

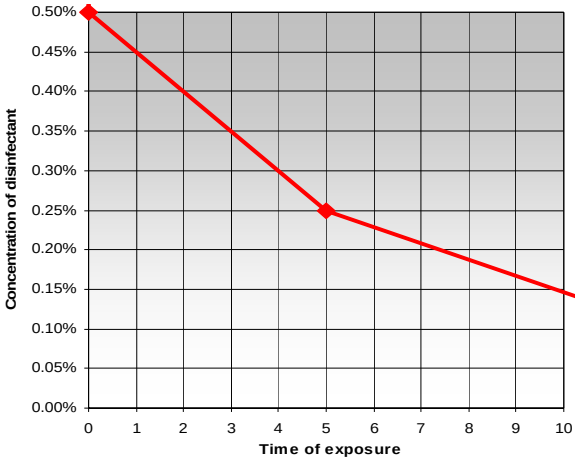


The time required for each disinfectant concentration to completely inactivate 50-100 $TCID_{50}$ KHV per ml are plotted below.

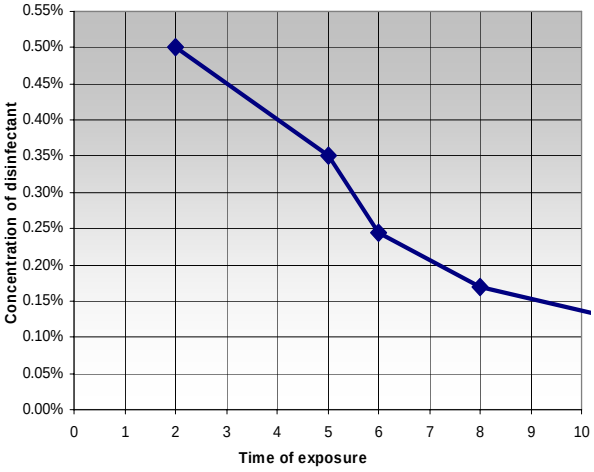
1. 0.5%- 0 % Virkon ® Aquatic;
50% dilution series
0-20 mins.



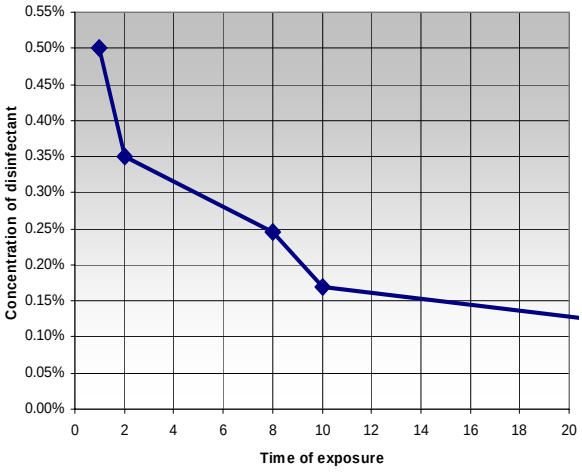
2. 0.5%-0%Virkon ® Aquatic;
50% dilution series
0-10 mins.



3. 0.5%- 0 % Virkon ® Aquatic;
30% dilution series
0-10 mins.



4. 0.5%- 0 % Virkon ® Aquatic;
30% dilution series
0-20 mins.



Conclusion.

The prescribed concentration and exposure time for disinfection of certain other viral pathogens by Virkon ®Aquatic ie 1% for ten minutes, is in excess of that required to inactive KHV under the conditions described here, where disinfection was complete within 10 minutes using Virkon ®Aquatic at a concentration of only 0.25%. At higher concentrations, disinfection was virtually instantaneous.