



FINAL REPORT

Contract ref. C2469

**Efficacy of Virkon Aquatic against IPN virus
(1:200, 30 mins, 4°C)**

Study Protocol Number: P0355

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REPORT APPROVAL -

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Study Diary: 4021

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Efficacy of Virkon Aquatic against IPN virus (1:200, 30 mins, 4°C)

1 Study Summary

The study design was an adaptation of the Defra (formerly MAFF) protocol for the *approval of disinfectants for use against Fowl pest (Newcastle disease virus, pathogenic avian influenza virus)* and the Draft British Standard prEN 14675 (*Quantitative suspension test for the evaluation of virucidal activity of chemical disinfectants and antiseptics used in veterinary field*).

Virkon Aquatic (1:200) was tested in the presence of high organic loading (1% bovine albumin and 1% yeast extract) at 4°C, for a 30 min incubation time.

The study consisted of (a) a cytotoxicity test and (b) a virus test.

- (a) The purpose of the cytotoxicity test was to determine if the disinfectant under test (Virkon Aquatic) was toxic to the CHSE-214 cells used in these investigations (Chinook salmon (*Oncorhynchus tshawytscha*) embryo cells).

In summary, Virkon Aquatic was diluted in hard water and mixed with an equal volume of Bovine Albumin (BA) + yeast extract (high organic loading). This mix was then further diluted, inoculated onto a monolayer of CHSE-214 cells and incubated at 15°C. The cells were observed for 7 days for signs of cytotoxicity. The final observations on day 7 determined the dilutions used in the viral test (b).

- (b) Virkon Aquatic (1:200) was tested for efficacy against IPNV (Sp serotype) by TCID₅₀ titration.

In summary, the disinfectant was mixed with an equal volume of virus (with high organic loading) and held at 4°C for a contact time of 30mins. This mix was then diluted in ice-cold diluent to terminate the virucidal activity of the disinfectant, inoculated onto a 96-well CHSE-214 microtitration plate and incubated at 15°C. On day 7 post-inoculation, all wells were closely examined and recorded as positive or negative for cytopathic effects. The titre was calculated from these results using the Karber formula [1931]* (See Appendix 3). Virus-only controls, with and without organic loading, ran alongside the main test (referred to as Control 1 and 2, respectively).

In compliance with the Defra protocol and prEN 14675, the disinfectant under test is considered effective if a decrease in infectivity titre of Ig 4 (TCID₅₀) is demonstrated.

* Karber, G. (1931). *Arch. J. Exper. Path. u. pharmakol.*, **162**, 480.

Results Summary

(a) Cytotoxicity test.

All cells appeared healthy throughout the 7-day observation period. No evidence of cytotoxicity was reported for Virkon Aquatic, with or without high organic loading.

This result enabled the titration in the main virus test to start with a 10^{-1} dilution.

(b) Virus test

Exposure to Virkon Aquatic at a concentration of 1:200 decreased the infectivity titre (TCID₅₀) of IPNV Fr31:75 by more than lg 4.5, clearly passing the lg 4 reduction requirement.



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2 Study Methods

2.1 Cytotoxicity Test

- 1 litre of product was prepared in WHO hard water (Appendix 1) to double the required test concentration (1:100).
- 2.5 ml product was then added to 2.5ml organic loading (2% bovine albumin + 2% yeast extract, see Appendix 2 for preparation).
- After thorough mixing, these test mixtures were held at 4°C ±1.0°C for 30 mins (vortexed every 10 mins).
- After 30 mins, 10⁻¹, 10⁻² and 10⁻³ dilutions were performed in L-15 (Leibovitz) medium (Sigma L5520) + 10% fetal bovine serum (FBS, Sigma F7524).
- 5ml was inoculated onto confluent CHSE-214 monolayers (25cm² flasks) and incubated at 15°C.
- Control flasks were inoculated in exactly the same way with 1:1 mixes of (a) organic loading (2%) + WHO hard water and (b) Virkon Aquatic + L-15.
- All flasks were examined daily for signs of cytotoxicity for a period of 7 days.

2.2 IPNV Isolate Details

Isolate	Fr 31:75 (French reference isolate). Sp serotype.
Culture	P5, CHSE-214 cells, 10 ⁻³ dilution (L-15), 15°C.
Storage	Original stock stored at -80°C. Test virus stored at 4°C after harvest (≤48 hours before test).

2.3 Virus Test

- Triplicate 1-litre aliquots of product were prepared in WHO hard water (Appendix 1) to double the required test concentration (1:100).
- Test mixtures were prepared as summarised below:

TEST

1.0 ml virus + 24.0 ml organic loading (2.1% BA + 2.1% yeast extract, Appendix 2)

2.5 ml of above mixture + 2.5 ml Virkon Aquatic (preparation as above)

CONTROL 1 (virus + yeast, no disinfectant)

1.0 ml virus + 24.0 ml organic loading (2.1% as in TEST)

2.5 ml of above mixture + 2.5 ml WHO hard water

CONTROL 2 (virus only)

1.0 ml virus + 24.0 ml (L-15, no FBS)

2.5 ml of above + 2.5 ml WHO hard water

- The above test mixtures were vortexed thoroughly and held at $4\pm 1.0^{\circ}\text{C}$ for 30 minutes (vortexing at 10 min intervals).
- After 30 mins, 1/5 dilutions were performed on ice in L-15 (Leibovitz) medium (Sigma L5520) + 10% fetal bovine serum (FBS, Sigma F7524).
- 100 μl aliquots were inoculated onto the top row of 96-well microtitration plates containing CHSE-214 monolayers and 100 μl L-15 (+10% FBS) per well (6 replicate wells for each dilution).
- 20 μl of the 10^{-1} dilution were then transferred to the next row of cells (containing 180 μl L-15), mixed, and so on down the plate to 10^{-8} .
- A representative number of wells (≥ 6) received L-15 (10% FBS) only, as a control.
- All plates were incubated at 15°C .
- On Day 7 each well was recorded as + or – for cytopathic effects (CPE) and the TCID_{50} was calculated using the formula described by Karber [1931]* (see Appendix 3).

Fail/Pass criteria: To determine the drop in infectivity titre, the average titre of the TEST mix is subtracted from the average titre of CONTROL 1. If this value is $\geq 10^4$ the disinfectant passes, if $< 10^4$ the disinfectant fails.

2.4 Dilution-Neutralisation control

A further control was performed to ensure dilution in serum rich diluent (on ice) terminated the virucidal activity of the disinfectant.

* Karber, G. (1931). *Arch. J. Exper. Path. u. pharmakol.*, **162**, 480.

In summary, the product was diluted in L-15 (+ 10% FBS), mixed with virus and titrated on a 96-well CHSE-214 microtitration plate as in the main virus test. A control ran alongside, in which the disinfectant was substituted with WHO hard water.

- 2.5ml organic loading (2%, see Appendix 2) was mixed with 2.5ml Virkon Aquatic (1:100, prepared in section 2.3).
- After vortexing this mix was diluted 1/5 in L-15 (+ 10% FBS) on ice.
- 1ml of the neutralised disinfectant was then added to 1ml Fr 31:75 (in L-15 + 10% FBS).
- After vortexing thoroughly, the pH was checked and the mix held at $4 \pm 1.0^{\circ}\text{C}$ for 30 minutes, vortexing at 10 min intervals.
- 20 μl of the 10^{-1} dilution were then transferred to the top row of 96-well CHSE-214 plates containing 180 μl L-15 (+10% FBS) per well (6 replicate wells for each dilution).
- 20 μl of this 10^{-2} dilution were then transferred to the next row of cells (containing 180 μl L-15), mixed, and so on down the plate to 10^{-9} .
- All plates were incubated at 15°C .
- On Day 7 each well was recorded as + or – for CPE and the TCID₅₀ was calculated using the formula described by Karber (see Appendix 3 for formula).

3 Results & Conclusions

3.1 Cytotoxicity Test

All CHSE-214 monolayers retained a healthy appearance throughout the 7-day observation period. No cytotoxicity was evident with Virkon Aquatic 1:200, even in the presence of high organic loading (1% bovine albumin + 1% yeast extract) and at the lowest inoculation dilution (10^{-1}).

3.2 Virus Test

Titres (TCID₅₀) for the virus test are tabulated below.

	Titre (10^x TCID ₅₀)			Average (10^x TCID ₅₀)	Infectivity drop 10^x TCID ₅₀ (Con 1 – Test)
	1	2	3		
Control 1 (No Virkon)	5.33	6.00	5.17	5.50	
Control 2 (Virus only)	5.50			5.50	
Test	<1*	<1 *	<1*	<1*	>4.50

*No CPE at any dilution. The viral titre was below the detection limit of the assay (ie. <10 TCID₅₀)

A 30 min exposure to Virkon Aquatic at a concentration of 1:200 decreased the infectivity titre (TCID₅₀) of IPNV Fr31:75 by more than lg 4.5, clearly passing the lg 4 reduction requirement of this test.

3.3 Dilution-Neutralisation Control

Titres (TCID₅₀) for the dilution-neutralisation control are tabulated below.

	Titre (10 ^x TCID ₅₀)			Average (10 ^x TCID ₅₀)
	1	2	3	
Control (WHO water)	6.00	5.83	6.00	5.94
Virkon 1:200	5.83	6.00	6.00	5.94

The comparable Virkon and Control TCID₅₀ values confirm that the dilution-neutralisation step used in the viral test (see 2.3) was effective at neutralising the virucidal activity of Virkon Aquatic.

4 Storage of Records

Validated copies of raw data are attached.

A copy of the final report and raw data will also be archived at CEFAS.



Appendices

Appendix 1: WHO Hard Water

Prepare by adding 0.304g CaCl₂ and 0.139g MgCl₂ to 1 litre distilled water. Mix well and autoclave to sterilise.

Appendix 2: Organic Loading

For a 2.0% preparation:

- Prepare a 4.0% solution of yeast (Fisher Y-0200-48) in WHO hard water and autoclave.
- Dilute autoclaved yeast solution 1:1 in L-15 (no FBS).
- Prepare 2% solution of bovine albumin (fraction V, Fisher A/1278/46) in autoclaved yeast solution.
- Sterilise by membrane filtration (0.22µm) and store at 4°C pending use.

Final test concentration must be 1% bovine albumin + 1% yeast extract.

Appendix 3: Calculation of TCID₅₀ (Karber, 1931)

- $\text{Log TCID}_{50} = L - d (s - 0.5)$
- L = log dilution of strongest concentration
- d = difference between log dilutions
- s = sum of proportional positive