Virucidal Efficacy Assay – corrected report

Sponsor:	AIONX Antimicrobial Technologies
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Viruses:	Mouse norovirus (CW1, USU #v2504)
	Influenza A/California/07/09 (H1N1, USU #v2927)

Introduction:

Testing was performed to determine if the test system would inactivate virus when exposed at various time intervals and amperage settings. Virus solutions were placed on the test surface for specified times then surviving infectious virus was quantified by standard $CCID_{50}$ assays and compared with untreated controls.

Procedures:

A specified volume of virus solution was placed on the active area of the test coupon (Figure 1). The untreated virus control aliquot was placed on the black plastic coupon outside of the treatment area. The system was turned on and the amp meter reading monitored throughout the exposure time. After the specified time interval, a sample was removed and immediately diluted 1/10 in test solution to stop antiviral activity. Samples were then titered immediately or held at -80 °C and titered later. This was repeated in triplicate for each set of parameters for the initials runs (Table 1-2) and replicate numbers varied for the final run (Table 3).

The equipment was assembled and experiments overseen by a Sponsor representative, Thomas Fuller. Initial runs with influenza and norovirus were with virus stocks suspended in MEM solution. An additional run was performed three days later with the norovirus stock first diluted 1/100 in USP water for injection (WFI) to see if better results were obtained without ions from the viral growth medium present.

Controls: To see if metal ions or other bi-products generated in the test procedure were toxic to cells, plates were observed on day-1 post infection (before viral replication could caused CPE) and no CPE was observed. A neutralization control was performed to ensure that virus inactivation did no continue after the exposure time, and that residual sample in the titer assay plates did not inhibit growth and detection of surviving virus. This was done by adding each sample to titer test plates then spiking each test well with low numbers of virus, incubating, and verifying that viral CPE was observed.

Media and cells. The test medium for influenza virus was MEM with 10 IU/mL trypsin, 1 μg/mL EDTA and 50 μg/mL gentamicin. Test medium for mouse norovirus was DMEM with 2% FBS and 50 μg/mL gentamicin. Cells for titer assays were RAW 264.7 cells for mouse norovirus and MDCK cells for influenza.

Virus quantitation. Surviving virus from each sample was quantified by standard $CCID_{50}$ end-point dilution assays. Samples were serially dilute 1/10 in test medium, then 100 μL of each dilution were plated into quadruplicate wells of 96-well plates containing subconfluent monolayers of cells. Plates were incubated at 37 ± 2°C with 5% CO_2 for 4 days for norovirus and 6 days for flu. Each well was then scored for presence or absence of virus. The $CCID_{50}$ values were calculated using the Reed-Muench (1948) equation. Three independent replicates of each sample were tested, and the average and standard deviation were calculated and compared with the MEM control. Where three replicates were tested, results were compared with untreated controls by a 2-tailed students t-test (MS-Excel for Mac).

Results:

Table 1 shows results for influenza A/CA/07/09 (H1N1) virus at various exposure times and amperages. Differences were not significant in any of the 60 amp trials. With 120 amps for 60 minutes a slight reduction in virus was observed (P = 0.03). A few additional samples were exposed until the virus aliquot was fully dried and the meter read 0 amps, then eluted in test medium and titered; these showed viable virus present in each case.

Table 2 shows results for murine norovirus CW1 suspended in MEM solution. At 60 amps less than 1 log_{10} reduction was observed after both 20 and 60 minutes. At 120 amps, an average of 2.2 log_{10} reduction was seen, which was significant (P = 0.04 by t-test) but results were sporadic. One sample was processed until fully dry then extracted; it showed a 1.0 log reduction compared with the untreated virus control (data not shown in table), which was likely because the exposed droplet dried more thoroughly than the unexposed control droplet.

Table 3 shows virucidal results for murine norovirus CW1 after the virus was diluted 1/100 in WFI. The water appeared to bubble during processing, and the solution was cloudy by the end of the exposure time. The 60 minute exposure at 60 amps appears to have reduced virus by an average of 2.5 log_{10}. The 90 min. and 120 min. exposure times at 60 amps had >0.8 log reduction, and could possibly cause full virus inactivation, but this could not be shown due to the toxicity of the test solution after exposure making the limit of detection <2.7 log CCID50/.1 mL. Table 3 experiments did not have sufficient replicates for statistical evaluation. Samples exposed for 180 minutes until fully dry showed no important virucidal effect, but were not considered valid since the virus controls were almost fully inactivated by desiccation.

Virus controls and sterile media controls were as expected in each case. Neutralization controls showed that virus was detected in the presence of the test sample on cell plates. Toxicity readings before viral CPE was present indicated that the test solution was not toxic to cells except as noted in Table 3.

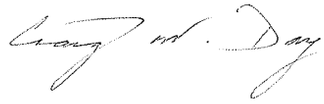

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Table 1. Influenza virus titers ($CCID_{50}$) after exposure to the AIONX test system

Time (min)	amps	Test volume (µL)	CNTL volume (µL)	Test sample $CCID_{50}$/0.1 mL	Virus Control $CCID_{50}$/0.1 mL	Log_{10} Reduction
60	60	600	600	6.3	6.5	0.2
60	60	500	500	5.7	6.0	0.3
60	60	500	500	6.7	6.7	0.0
			Average	6.2	6.4	0.2
20	60	600	200	6.0	6.5	0.5
20	60	500	200	5.7	6.7	1.0
20	60	500	500	6.5	6.5	0.0
			Average	6.1	6.6	0.5
60	120	600	200	5.5	6.5	1.0
60	120	500	200	6.0	7.3	1.3
60	120	500	500	6.0	6.7	0.7
			Average	5.8	6.8	1.0*
20	60	100	100	5.7	6.5	0.8
5	60	120	120	6.0	6.7	0.7
5	120	120	nt	6.5	nt	0.1

*P = <0.05 compared with untreated virus control by students t-test.

Table 2. Murine norovirus (CW1) titers ($CCID_{50}$) after exposure to the AIONX test system

Time (min)	amps	Test volume (µL)	CNTL volume (µL)	Test sample $CCID_{50}$/0.1 mL	Virus Control $CCID_{50}$/0.1 mL	Log_{10} Reduction
60	60	600	600	5.7	6.3	0.7
60	60	500	500	5.3	6.3	1.0
60	60	500	500	6.0	6.7	0.7
			Average	5.7	6.4	0.8*
20	60	600	200	6.0	6.5	0.5
20	60	500	200	6.0	6.0	0.0
20	60	500	500	6.0	6.7	0.7
			Average	6.0	6.4	0.4
60	120	600	200	4.7	6.3	1.7
60	120	500	200	2.7	6.5	3.8
60	120	500	500	5.0	6.0	1.0
			Average	4.1	6.3	2.2*

*P = <0.05 compared with untreated virus control by students t-test.

Table 3. Murine norovirus (CW1) titers ($CCID_{50}$) after virus stock diluted 1/100 in WFI then exposed to the AIONX test system. (Corrected)

Time (min)	amps	Test volume (µL)	CNTL volume (µL)	Test sample $CCID_{50}$/0.1 mL	Virus Control $CCID_{50}$/0.1 mL	Log_{10} Reduction
30	60	200	200	4.5	4.5	0.0
60	60	200	200	1.7	4.5	2.8
60	60	200	200	<1.7*	4.0	>2.3
90	60	200	200	<2.7*	3.5	>0.8
120	60	200	200	<2.7*	3.7	>1.0
180-until dry	60	100	100	<1.7*	1.7	0
180-until dry	60	100	100	<1.7*	1.0	0
180-until dry	60	100	100	<1.7*	1.33	0

*Limit of detection (LOD) because ions in the sample liquid (blueish color) were toxic to cell monolayer at the top 1 or 2 dilutions.

Note 1. Table 3 was re-issued after testing the toxicity of the test solution. It appears that the virus reported in the 60, 90 and 120 minute assays was actually due to toxicity of the test solution containing the bluish substance (presumed to be copper and other heavy metal ions) after exposure. Because the test solution was toxic to cells used to look for virus, the 1/10 and 1/100 dilution of the test solution appeared to have virus in it. This was verified with multiple replicate assays. This was important to show that virus titers were not necessarily higher after 120 minutes exposure than after 60 minutes exposure.
Note 2: There was no blue pigment noted in the solution when MEM was used, as in Table 1 and Table 2. Toxicity would not matter there anyway because virus titers were well above the LOD.
Note 3: No bluish color noted when WFI was used without 1% MEM solution added; still some toxicity was seen in 1/10 dilution (top row), so LOD would be <1.7 $CCID_{50}$/100 µL. This did not affect the result, but is noted here for interest, that some toxic ions are released when purified water is used.

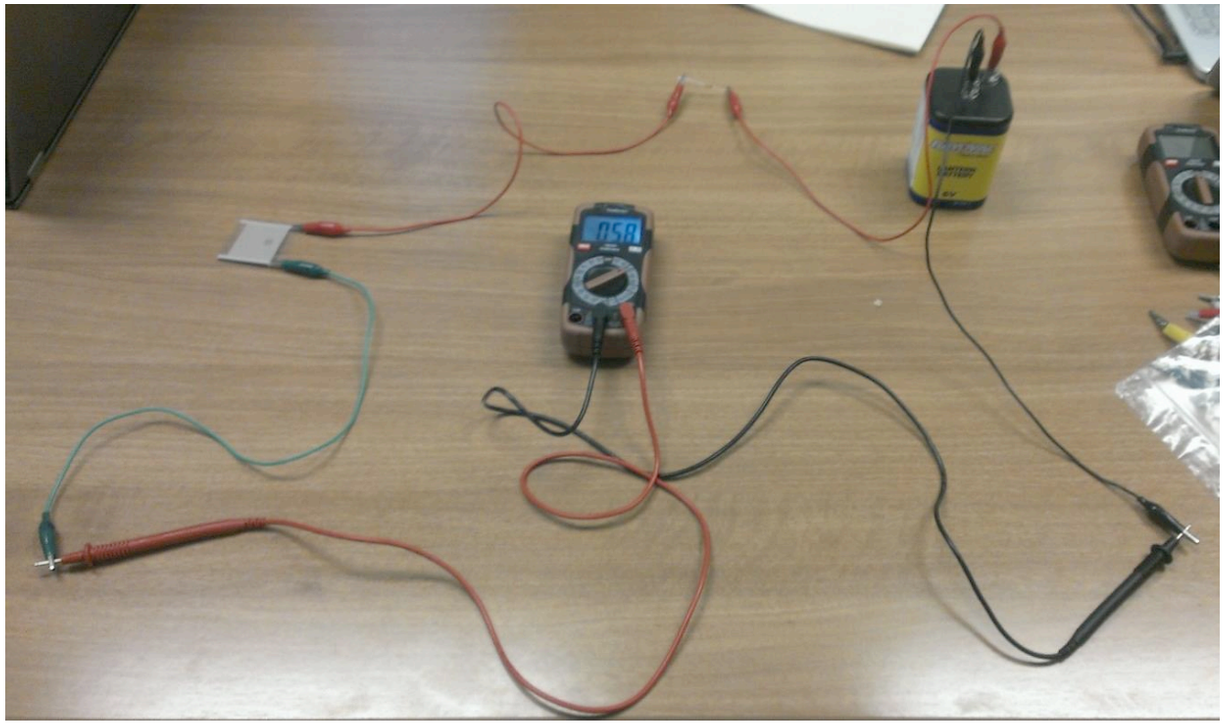

Figure 1. Test set-up by AIONX, except in this experiment test coupon was on a black plastic material with a larger surface area outside of the active, printed, charged area.