Assessment of the virucidal activity of the product Virkon S diluted at 1:500 against African Swine Fever Virus

Sponsor:

Antec International Ltd (a company of the LANXESS group)

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Date: September 11th, 2019

1.-Analyses requested

Evaluation of the virucidal activity of the disinfectant solution (DS) Virkon S to inactivate African Swine Fever Virus (ASFV) in culture medium.

2.-Name of the product tested

Virkon S (Batch: 1806BA0040)

Internal reference: 19CISA056

Provided by: LANXESS Deutschland GmbH

3. - Storage conditions

Room temperature

4. - Experimental conditions

a) DS concentration: 1:500 dilution of Virkon S in sterile hard water
b) Temperature: 10ºC
c) Time of product DS/ASFV exposure: 5 and 10 minutes
d) Interfering substance: Foetal bovine serum (FBS) (5% final concentration)
e) Viral strain used: ASFV strain Ba71V, adapted to grow in cultured VERO cells.
f) Titer of ASFV stock: $3.2x10^6$ $TCID_{50}$/mL

5. – Procedure

a) Cytotoxicity induced by the DS (Virkon S)

For determining DS-induced cytotoxicity, five 1 mL dilutions (including 1/500 dilution) of the disinfectant (Virkon S) were prepared in cell culture medium supplemented with 5% of heat-inactivated FBS**.** 200µl of each dilution was added in duplicate on microplate wells containing monolayers of VERO cells ($14x10^3$ cells/well). Morphological changes in cells were observed under microscope from 2h to 5 days after the addition of the disinfectant dilution. At day 5, cells were collected, and cytotoxicity was determined using a commercial cytotoxicity assay kit (Pierce LDH Thermo Scientific 88953). The kit specifically detects Lactate dehydrogenase (LDH), a cytosolic enzyme present in cells. Plasma membrane damage releases LDH into the cell culture media, which can be quantified by spectrophotometry. The amount of LDH released into the medium is proportional to cytotoxicity.

Cell structural changes, as well as LDH release, were compared to those observed in control VERO cells maintained at the same period without DS dilution.

In these conditions no cytotoxic effect (Table 1) of Virkon S at 1:500 dilution was observed by both microscopic morphological changes and LDH release.

b) Virucidal activity induced by DS (Virkon S) against ASFV

The incubation mixtures, containing 100 µL of ASFV ($3.2x10^5$ $TCID_{50}$) + 100 µL of the DS (diluted in sterile hard water), at the final concentration of 1:500 in the presence of the interfering substance (5% FBS), were incubated at 10ºC for either 5 or 10 minutes. After each incubation time, 100 µL of each of the incubation mixtures were added to 9.9 mL of DMEM + FBS (5%), named Solution A. After that, Serial 1:10 dilutions were prepared from Solution A in ice-cold DMEM and 100 µL of dilution was added in six-fold wells to microtiter plate wells containing VERO cells.

Microtiter plates were incubated for 5 days at 37ºC in an incubator with humidified atmosphere of 5% CO_2, and the viral infectivity was determined following a standard Reed and Muench $TCID_{50}$ virus titration method. Additionally, any cytopathic effect (CPE) was determined by microscopic examination, and results were recorded (scores from 0 to 4). Values of virus inactivation were calculated from differences of virus titres before and after incubation with the respective DS.

Table 1. *CPE results of the virucidal test*

				Dilution (-log)				
Product	**Concentration**	**Soiling**	**Contact time**	**3**	**4**	**5**	**6**	**7**
Control 5 min	n.a.	5% FBS	5 min	444444	444404	404000	030200	000000
Control 10 min	n.a.	5% FBS	10 min	444444	404004	403000	000000	000000
Virkon S	1:500	5% FBS	5 min	000000	000000	000000	000000	000000
Virkon S	1:500	5% FBS	10 min	000000	000000	000000	000000	000000

1 to 4 = Degree of CPE in six cell culture unites (M96 wells)
0 = no observable CPE

6. - Results

a) Controls

- Negative control: Six wells of the microtiter plate containing monolayers of VERO cells, were incubated with tenfold dilutions of Solution A, but without addition of ASFV. The CPE score was 0 in all of them (see Table 1).
- Positive control: Six wells of the microtiter plate, containing monolayers of VERO cells, were incubated with tenfold dilutions of Solution A, but without addition of DS. The CPE score was 4 in all of them at the maximal ASFV concentration (see Table 2).

Table 2. *Viral titres in Positive Control wells, determined by the Reed & Muench method*

Dilution (-log10)	Result	% infected
3	444444	100
4	444404	90
5	404000	44.4
6	030200	18.18
7	000000	0
ASFV control 5 min, 10ºC TCID50/ml		6.2 log

Dilution (-log10)	Result	% infected
3	444444	100
4	404004	62.5
5	403000	22.2
6	000000	0
7	000000	0
ASFV control 10 min, 10ºC TCID50/ml		5.6 log

b) Virucidal activity of DS product determined by virus titration

After 5 or 10 min of incubation at 10ºC, the DS (Virkon S, Batch# 1806BA0040) at dilution 1:500 completely inhibits ASFV replication in VERO cells (Table 3).

Table 3. *Titer reduction of ASFV after incubation with Virkon S (1:500 dilution) in a suspension test (contact time: 5 or 10 minutes, temperature: 10ºC)*

Product	Concentration	Contact Time Min	Soiling	Level of cytotoxicity	Lg TCID 50 after contact time	>4 log $_{10}$ reduction after contact time
Virkon S	1:500	5	5 % FBS	0	0	6.2
Virkon S	1:500	10	5 % FBS	0	0	5.6

c) Virucidal activity of DS product, determined by Real-time PCR

The capacity of Virkon S to inhibit ASFV replication was confirmed by Real-time PCR. For this, total DNA was extracted from VERO cells incubated with solution A described in 4.a. For DNA extraction, QIAamp DNA Mini kit (Qiagen 50951306) was used, following the procedures recommended by the manufacturer. The quantitative detection of ASFV DNA in such samples was carried out by Real-Time PCR using the virotype ASFV PCR kit (Qiagen re.281905), which includes two specific primers for ASFV DNA yielding FAM^{TM} fluorescence.

In a Real-time PCR assay, a positive reaction is detected by accumulation of a fluorescent signal. The Ct (cycle threshold) is defined as the number of cycles required for the fluorescent signal to cross the threshold. Ct levels are inversely proportional to the amount of target nucleic acid in the sample. For the assay to be valid, the Positive Control kit must give a Ct<35. The Negative Control must give no signal. Results are shown on Table 4.

Table 4

Samples tested	Ct	
Cells infected with ASFV incubated with Virkon S 1:500, 5' 10ºC	**38.13**	ASFV negative
Cells infected with ASFV incubated with Virkon S 1:500 10' 10ºC	**No Ct**	ASFV negative
Cells infected with ASFV without DS,5' 10ºC	16.21	ASFV positive
Cells infected with ASFV without DS,10' 10ºC	17.76	ASFV positive
Uninfected Cells	No Ct	ASFV negative
Negative control provided by the kit	No Ct	ASFV positive
Positive control provided by the kit	34.3	ASFV positive

7. - Conclusion

According to the European standard EN14675, we can conclude that:

The disinfectant Virkon S (Batch# 1806BA0040) possesses virucidal activity for the referenced strain of African swine fever virus (Ba71V) at a 1:500 (one part of test product plus 500 parts of diluent) dilution at the conditions described in the present report (5 or 10 min of exposure at 10ºC, in presence of 5 % FBS, diluted in hard water), since it reduces > 4 log10 of ASFV cytopathic effect, as required by the standard EN14675.

Valdeolmos, 11th of September 2019

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Firmado por ARIAS NEIRA Mª LUISA - DNI 00683346Q el día 25/09/2019 con un certificado emitido por AC Administración Pública