

ZOONO Z-71™ MICROBE SHIELD

Testing the efficacy of a disinfectant against African Swine Fever Virus

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Introduction

In order to pass the test, a disinfectant should show a minimum of a 4 log¹⁰ reduction in titer after 30 min at 10°C (obligatory test conditions NEN EN 14675 norm).³² The disinfectant Zoono Z-71™ Microbe Shield showed a strong effect on the titer of ASFv in low soiling conditions at 4°C during 5- and 30-min. incubation. The maximum reduction was ≥5.00 log¹⁰, see Table 4.

Materials and methods

Materials (as described in the test protocol)

- 1) Test virus: The Netherlands '86 ASFv isolate grown on Porcine Alveolar Macrophages (PAMs)
- 2) Test cell: PAMs
- 3) Test medium for cell culture: Roswell Park Memorial Institute (RPMI) supplemented with 5% Fetal Calf Serum (FCS) and 1% antibiotics
- 4) The diluent for disinfectant and virus: hard water according to NEDerlandse Norm English (NEN-EN) 14675 containing 5% FCS
- 5) Medium for neutralizing disinfectant: RPMI 1640 supplemented with 10% FCS and 1% antibiotics

Methods (as described in the test protocol)

- 1) Preparation of virus
 - a. The virus titer used was about 10⁷ Median Tissue Culture Infectious Dose (TCID₅₀)/ml (+/- 0.5 log) being able to determine a 4 log¹⁰ reduction. The virus was diluted as follows: 1 ml ASFv + 19 ml hard water containing 5% FCS
- 2) Preparation of the disinfectant dilution
 - a. Prepared the disinfectant to the requested dilution rates that need to be tested
 - b. The disinfectant was diluted with hard water containing 5% FCS and were kept at 4°C. The concentrations of the disinfectant including the various test conditions used, were summarized in Table 1
 - c. Cytotoxic effects were evaluated in the Immune Peroxidase Monolayer Assay (IPMA) assay

- 3) Preparation of cells for IPMA assay
 - a. Primary cells were prepared from porcine lungs and stored in liquid nitrogen. When needed, cells were thawed and seeded in 96-well plates. These plates were used in the IPMA assay
- 4) Test procedure
 - a. A sample of the product diluted with hard water containing 5% FCS was added to a test suspension of virus: 2.5 ml of virus suspension was mixed with 2.5 ml of disinfectant at the requested dilution, and is maintained in a water bath at 4°C ± 1°C for 30 min ± 10 s. During the latter incubation time every 10 min the mixture is mixed.
 - b. At the end of the contact time, part of the mixture was taken and diluted tenfold in ice-cold medium to overcome the virucidal activity. These samples were directly diluted in six serial tenfold dilutions in cold medium (so the 6 dilutions to be tested are 10E-2 up to 10E-7). The dilutions were tested immediately or stored at -70°C.
- 5) IPMA assay (end point titration)
 - a. (After thawing) 100 µl of each dilution was inoculated (in 8-fold) into separate wells of a 96-well plate and 100 µl PAM cells were added. The plates were incubated at 37°C in a humidified incubator with 5% CO₂ for four days. After four days of incubation, the plates were washed, dried and frozen. Subsequently, the cells were fixed, plates were washed again and stained using ASF- Histidine (HIS), Mouse-anti-Swine IgG/ HorseRadish PerOxidase (HRPO) conjugate and 3-Amino-9-EthylCarbazole (AEC) (IPMA protocol).
 - b. Plates were read microscopically and judged for the presence of virus. Titers were calculated according to Spearman-Kärber.

Test Evaluation

- 1) To test the titer of the virus used, a hard water control was included, which means that hard water was used instead of a disinfectant.
- 2) Two positive controls as disinfectants, NaOH 1% and 2%, were included in the test. The reduction after 30 minutes of the positive controls should be within +/- 3sd of the mean valid for these controls for a valid test. Our past experience showed that formaldehyde 0.7% was toxic for PAMs and was therefore replaced by NaOH 1% and 2%.
- 3) The reduction in ASFv titer, induced by each dilution of the disinfectant, was calculated by subtracting the ASF virus titer, measured in the mix with disinfectant, from the titer measured in the water control.
- 4) A minimum of a 4 log¹⁰ reduction after 30 minutes at 10°C is needed for a disinfectant to pass the test. In the current study, these conditions were not included.

Note: this effectivity test is built upon a biological system containing living cells and challenging virus. The outcome of the test is therefore dependent on the effect of the disinfectant(s) on the virus as well as on the cells. The difference between the viral titer obtained from cells exposed to the disinfectant at a non-cytotoxic concentration and the viral titer obtained from cells non-exposed to the disinfectant should be lower than 1 log₁₀ according to the NEN-EN 14675 norm. It is our view that a treatment of cells at a non-cytotoxic concentration of the disinfectant should by definition yield the same titer as at non-exposed cells, otherwise it is toxic. Therefore, it does not make any sense scientifically to test this issue. Unquestionably, we are aware of the possible effect of the disinfectant on either cells or virus. In case the cells are affected by the disinfectants tested, no conclusive data can be generated relating to the effect of the disinfectant on the virus applied according to the NEN-EN 14675 norm. The NEN-EN 14675 norm does not define a differentiation between these two effects.

Table 1: Concentrations of disinfectant(s) and corresponding test conditions

Temp	4°C						10°C					
	none		low (5% FCS)		high		none		low (5% FCS)		high	
Soiling	5'	30'	5'	30'	5'	30'	5'	30'	5'	30'	5'	30'
Time												
Controls												
Water			x	x								
1% NaOH			x	x								
2% NaOH			x	x								
Disinfectant(s)												
Zoono Z-71™ Microbe Shield X1.25			x	x								
Zoono Z-71™ Microbe Shield X2.50			x	x								
Zoono Z-71™ Microbe Shield X3.75			x	x								

Results

Controls

The hard water controls with 5% FCS as soiling agent at 4°C for 5-and-30 minutes showed titers with values satisfactory for the test. The reduction observed with the two positive reduction controls were within reach of validity of the test performed. See Table 2 for the log¹⁰ values of all controls included.

Table 2: log₁₀ values of controls

Temp	4°C						10°C					
	none		low (5% FCS)		high		none		low (5% FCS)		high	
Soiling	5'	30'	5'	30'	5'	30'	5'	30'	5'	30'	5'	30'
Time												
Controls												
Water			7.50	7.00								
1% NaOH			3.38	2.75								
2% NaOH			2.88	2.75								

Disinfectant(s)

The result of the effect of the disinfectant Zoono Z-71™ Microbe Shield on ASFv in low soiling conditions is displayed in Table 3. No cytotoxicity was observed in PAMs using Zoono Z-71™ Microbe Shield, see also Appendix 1: Raw data.

Table 3: log₁₀ values of disinfectant(s)

Temp	4°C						10°C					
	none		low (5% FCS)		high		none		low (5% FCS)		high	
Soiling	5'	30'	5'	30'	5'	30'	5'	30'	5'	30'	5'	30'
Time												
Disinfectant(s)												
Zoono Z-71™ Microbe Shield X1.25			≤2.50	≤2.50								
Zoono Z-71™ Microbe Shield X2.50			2.75	≤2.50								
Zoono Z-71™ Microbe Shield X3.75			≤2.50	≤2.50								

Conclusions

In order to pass the test, a disinfectant should show a minimum of a 4 log¹⁰ reduction in titre after 30 min at 10°C (obligatory test conditions NEN-EN 14675 norm).32

The disinfectant Zoono Z-71™ Microbe Shield showed a strong effect on the titre of ASFv in low soiling conditions at 4°C during 5-and 30-min. incubation. The maximum reduction was ≥5.00 log¹⁰, see Table 4.

Table 4: reduction values of disinfectant(s)

Temp	4°C						10°C					
	none		low (5% FCS)		high		none		low (5% FCS)		high	
Soiling	5'	30'	5'	30'	5'	30'	5'	30'	5'	30'	5'	30'
Time												
Disinfectant(s)												
ZOONO MICROBE SHIELD-Z71 X1.25			≥5.00	≥4.50								
ZOONO MICROBE SHIELD-Z71 X2.50			4.75	≥4.50								
ZOONO MICROBE SHIELD-Z71 X3.75			≥5.00	≥4.50								

Appendix 1: Raw data

Order: 2019 ASFv Hoseo-2													
Water							Water						
Temp: 4°C							Temp: 4°C						
Soiling: low (5% FCS)							Soiling: low (5% FCS)						
Time (min): 5							Time (min): 30						
TCID50/ml: 7.50							TCID50/ml: 7.00						
	10 ⁻²	10 ⁻³	10 ⁻⁴	10 ⁻⁵	10 ⁻⁶	10 ⁻⁷	10 ⁻²	10 ⁻³	10 ⁻⁴	10 ⁻⁵	10 ⁻⁶	10 ⁻⁷	
A	pos	pos	pos	pos	neg	neg	pos	pos	pos	pos	pos	neg	A
B	pos	pos	pos	pos	pos	pos	pos	pos	pos	pos	neg	neg	B
C	pos	pos	pos	pos	pos	neg	pos	pos	pos	pos	neg	neg	C
D	pos	pos	pos	pos	pos	neg	pos	pos	pos	pos	pos	neg	D
E	pos	pos	pos	pos	pos	neg	pos	pos	pos	pos	pos	neg	E
F	pos	pos	pos	pos	pos	neg	pos	pos	pos	pos	pos	neg	F
G	pos	pos	pos	pos	pos	neg	pos	pos	pos	pos	pos	neg	G
H	pos	pos	pos	pos	pos	neg	pos	pos	pos	pos	pos	neg	H

Order: 2019 ASFv Hoseo-2													
1% NaOH							1% NaOH						
Temp: 4°C							Temp: 4°C						
Soiling: low (5% FCS)							Soiling: low (5% FCS)						
Time (min): 5							Time (min): 30						
TCID50/ml: 3.38							TCID50/ml: 2.75						
	10 ⁻²	10 ⁻³	10 ⁻⁴	10 ⁻⁵	10 ⁻⁶	10 ⁻⁷	10 ⁻²	10 ⁻³	10 ⁻⁴	10 ⁻⁵	10 ⁻⁶	10 ⁻⁷	
A	pos	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	A
B	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	B
C	pos	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	C
D	pos	neg	neg	neg	neg	neg	pos	neg	neg	neg	neg	neg	D
E	pos	neg	neg	neg	neg	neg	pos	neg	neg	neg	neg	neg	E
F	pos	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	F
G	pos	neg	neg	neg	neg	neg	neg	neg	pos	neg	neg	neg	G
H	pos	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	H

Order: 2019 ASFv Hoseo-2													
2% NaOH							2% NaOH						
Temp: 4°C							Temp: 4°C						
Soiling: low (5% FCS)							Soiling: low (5% FCS)						
Time (min): 5							Time (min): 30						
TCID50/ml: 3.25							TCID50/ml: 3.00						
	10 ⁻²	10 ⁻³	10 ⁻⁴	10 ⁻⁵	10 ⁻⁶	10 ⁻⁷	10 ⁻²	10 ⁻³	10 ⁻⁴	10 ⁻⁵	10 ⁻⁶	10 ⁻⁷	
A	neg	pos	neg	pos	neg	neg	neg	neg	neg	neg	neg	neg	A
B	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	B
C	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	C
D	pos	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	D
E	neg	pos	pos	neg	neg	neg	neg	pos	neg	neg	neg	neg	E
F	neg	neg	pos	neg	neg	neg	neg	pos	neg	neg	neg	neg	F
G	neg	pos	neg	neg	neg	neg	neg	pos	neg	neg	neg	neg	G
H	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	H

Order: 2019 ASFv Hoseo-2													
Zoono Z-71™ Microbe Shield X1.25							Zoono Z-71™ Microbe Shield X1.25						
Temp: 4°C							Temp: 4°C						
Soiling: low (5% FCS)							Soiling: low (5% FCS)						
Time (min): 5							Time (min): 30						
TCID50/ml: ≤2.50							TCID50/ml: ≤2.50						
	10 ⁻²	10 ⁻³	10 ⁻⁴	10 ⁻⁵	10 ⁻⁶	10 ⁻⁷	10 ⁻²	10 ⁻³	10 ⁻⁴	10 ⁻⁵	10 ⁻⁶	10 ⁻⁷	
A	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	A
B	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	B
C	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	C
D	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	D
E	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	E
F	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	F
G	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	G
H	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	H

Order: 2019 ASFv Hoseo-2													
Zoono Z-71™ Microbe Shield X2.50							Zoono Z-71™ Microbe Shield X2.50						
Temp: 4°C							Temp: 4°C						
Soiling: low (5% FCS)							Soiling: low (5% FCS)						
Time (min): 5							Time (min): 30						
TCID50/ml: 2.75							TCID50/ml: ≤2.50						
	10 ⁻²	10 ⁻³	10 ⁻⁴	10 ⁻⁵	10 ⁻⁶	10 ⁻⁷	10 ⁻²	10 ⁻³	10 ⁻⁴	10 ⁻⁵	10 ⁻⁶	10 ⁻⁷	
A	pos	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	A
B	neg	pos	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	B
C	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	C
D	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	D
E	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	E
F	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	F
G	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	G
H	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	H

Order: 2019 ASFv Hoseo-2													
Zoono Z-71™ Microbe Shield X3.75							Zoono Z-71™ Microbe Shield X3.75						
Temp: 4°C							Temp: 4°C						
Soiling: low (5% FCS)							Soiling: low (5% FCS)						
Time (min): 5							Time (min): 30						
TCID50/ml: ≤2.50							TCID50/ml: ≤2.50						
	10 ⁻²	10 ⁻³	10 ⁻⁴	10 ⁻⁵	10 ⁻⁶	10 ⁻⁷	10 ⁻²	10 ⁻³	10 ⁻⁴	10 ⁻⁵	10 ⁻⁶	10 ⁻⁷	
A	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	A
B	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	B
C	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	C
D	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	D
E	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	E
F	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	F
G	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	G
H	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	H

*Cytotoxicity