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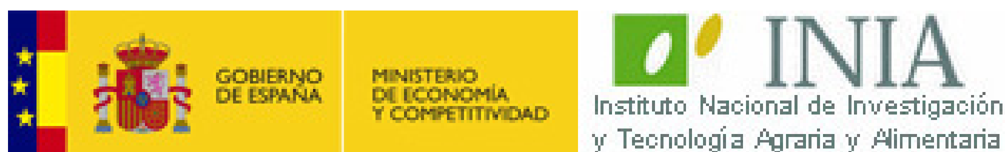

Evaluation of Virocid Disinfectant (CID LINES, Innovative Hygiene Solutions) Efficiency at manufactures recommended concentration of 0,25% Against African Swine Fever Virus

Performed by the

EUROPEAN UNION REFERENCE LABORATORY FOR AFRICAN SWINE FEVER (URL)

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CONTENTS

1. **Objectives** ……………………………………… 03

2. **Materials and Methods** ……………………………………… 03

   2.1. **Viral strain and cell cultures.** ……………………………………………. .. 03

   2.2. **Cytotoxicity control of the disinfectant in Vero culture cells…...** 03

   2.3. **Virucidal activity of the Virocid disinfectant against ASFV using the effective working dilution of 0.25%.** ……………. 04

   2.4. **Determination of the "in vitro" virucidal activity using the effective working dilution of 0.25%.** ……………………….. 04

3. **Results** ………………………………………. 05

4. **Conclusion** ……………………………………….. 05

1. Objectives.

The aim of this study was to test the **Virocid disinfectant** *(CID LINES, Innovative* Hygiene Solutions) in order to evaluate the **virucidal activity "in vitro" against African Swine Fever Virus (ASFV)**, in presence and in absence of interfering substance solution (inactivated Fetal Bovine Serum, iFBS) to simulate the organic soiling that can reduce the virucidal activity of disinfectants at the commercial dilution recommended by manufactures. **The effective working dilution of the Virocid disinfectant was established by manufactures in 0.25%.**

2. Materials and Methods.

2.1. Viral strain and cell cultures.

VERO (African green monkey kidney) cells were obtained from the American Type Culture Collection (ATCC) and grown in Dulbecco's modified Eagle's Medium (DMEM) supplemented with 10% serum fetal bovine (SFB). The VERO cell culture-adapted ASFV Spanish strain BA71V belonging to p72-genotype I was used in the study. Titer of ASFV isolate was estimated by end point dilution in VERO cells using the method of Reed & Muench (1938) and expressed as 50% tissue culture infectious doses per ml ($TCID_{50}$/ml).

2.2. Citotoxicity control of the disinfectant against Vero culture cells.

To determine the cytotoxicity, in a previous study performed in 2011, ten-fold dilutions of the disinfectant (from 10-1 to10-21) were prepared in cell culture medium supplemented with 2% of iSFB, in a final volume of 500µl and added per duplicate on Vero cells monolayers in 48 cell culture plates. The range included the effective working dilution of 0.5% or 1:200 recommended by manufactures. The morphological changes in the VERO cells were observed in the microscope since 24 to 72 hours after the addition of the Virocid. The structural changes were compared with those in control VERO cells without disinfectant maintained with culture medium supplemented with 2% of iSFB.

2.3. ***Virucidal activity of the Virocid disinfectant against ASFV in presence or absence of interfering substance solution (iFBS) using the effective working dilution of 0.25%.***

The method employed to establish the virucidal activity follows the norm ***UNE-EN 14675-2007*** *"Quantitative suspension test for the evaluation of virucidal activity of chemical disinfectants and antiseptics used in the veterinary area"* with minor modifications as is described as follows:

- Viral suspension dilutions. Two-fold dilutions from 1/20 to 1/1280 of ASFV Ba71V isolate with an initial titer of $2,1x10^{12}$ $TCDI_{50}$/ml were prepared in cell culture medium supplemented with 2% of iSFB.
- The suspension test was performed in sterile H_2O or interfering substance (iSFB) using each of the viral dilutions and Virocid disinfectant **at final concentration of 0,25% recommended by manufactures**. After mixing all the components, the tubes were incubated for 30 min at 10ºC ± 1ºC.
- After the incubation time, 48 monolayers VERO cells-culture plates at 80% confluence were inoculated by duplicate with each suspension. ASFV infected cells, without disinfectant, and cell control wells, without virus, were included in the assay.
- The cytopatic effect in VERO cells was observed in the microscope against the control cells, from 24 to 96 hours post inoculation.

2.4. ***Determination of the "in vitro" virucidal activity using the effective working dilution of 0.25%.***

The capacity of the Virocid disinfectant against ASFV was initially established after 24, 48, 72 and 96 hours post inoculation by direct staining of the infected VERO cells fixed with 70% of Methanol and 30% of Acetone cold solution using the **Immunoperoxidase Technique (IPT).** Briefly, the infected cells were incubated with a positive ASF reference serum sample diluted at 1/80 and the Horseradish protein A peroxidase as secondary antibody (1/5000) was used to detect the complexes antigen-antibody formed in the cytoplasm of ASFV infected -VERO cells. The reaction was developed by means the substrate solution (3 aminoetilcarbazol-dimetilformamide diluted in Acetate buffer). The virucidal capacity was finally estimated by counting the number of the red stained cells against viral control wells.

In order to confirm the capacity of the Virocid disinfectant to inhibit the ASFV viral replication, 48 monolayers VERO cells-culture plates 80% confluent were inoculated by duplicate with each suspension as described in 2.3. section. Inoculated VERO cells were incubated in humidified atmosphere containing 5% CO_2 at 37°C for 72 hours and subjected to one freeze-thaw cycle. The culture supernatants were centrifuged for 5 min at 650 g to clear it of cell debris and were used as an inoculum for a subsequent passage on VERO cells to a maximum of three blind passages. Total DNA was extracted from 200 μl of each supernatant obtained from VERO cells inoculated with either the ASFV-Virucid-treated or untreated and recovered in a final volume of 50 μl using a High Pure Viral Nucleic Acid kit (Roche) following the manufacturer's instructions. The World Organization for Animal Health (OIE) prescribed-real time PCR (King *et* al., 2003) for diagnosing ASF was employed to confirm the ASFV replication. The described ASFV real-time PCR method uses a primer set and a specific TaqMan probe directed to a highly conserved region of the viral genome, VP72, which ensure the detection of a wide range of ASFV isolates. The primers amplify a DNA fragment of 250 bp, from nucleotide position 2041 to 2290 of the complete VP72 gene sequence of the reference strain BA71V (GenBank accession no. M34142). TaqMan probe employed for amplified product detection is labelled with a reporter at 5' end [6-carboxy-fluorescein (FAM)] and a quencher at 3' end [6-carboxy-tetramethyl-rhodamine (TAMRA)].

3. Results.

Prior to evaluating the virucidal activity of the Virocid CID Lines disinfectant at the recommended concentration of 0.25%, the **cytotoxicity on the VERO cells** was determined in the previous study performed in 2011. **The maximum Virocid dilution considered not cytotoxic for VERO cells was established in 10^{-8}**. This dilution was selected and subsequently employed to determine the virucidal activity onto VERO cells monolyers.

The evaluation of the **virucidal activity against ASFV**, with the effective working dilution of 0.25% or 1:400 recommended by manufactures, **showed a total reduction of specific cytopatic effect at 24, 48 and 72 hours post inoculation** as it was demonstrated by the Immunoperoxidase technique. The **total reduction of specific cytopatic effect** was observed even **using the lowest ASFV viral dilution** (1/20) which corresponds to a **viral titer of 10^{11}**

TCDI$_{50}$/ml. This 100% reduction of the specific cytopatic effect was showed in any cases, in absence and in presence of interfering substance solution (iFBS).

To confirm the inhibitory effect of the Virocid disinfectant on ASFV replication, total DNA extracted from VERO cells inoculated in three subsequent passages with the Virocid-treated or untreated- ASFV was analyzed by real-time PCR. As it showed in the Figure 1, in the cells inoculated with untreated ASFV virus using the lowest viral dilution (1/20), the CT values for each of the three passages were decreased indicating active replication of the virus in the cells following the viral inoculation. In contrast, **in the cells inoculated with the Virocid-treated ASFV a total reduction in the amount of ASFV DNA (Ct>40) was observed by real-time PCR after three passages showing complete virus inactivation**. So, based on phenotypic (CPE) and molecular data, the virus infectivity was completely absent after the treatment of ASFV with 0,25% concentration of the Virocid disinfectant, as confirmed by the absence of CPE and the absence of viral genomic DNA in three subsequent cell culture passages.

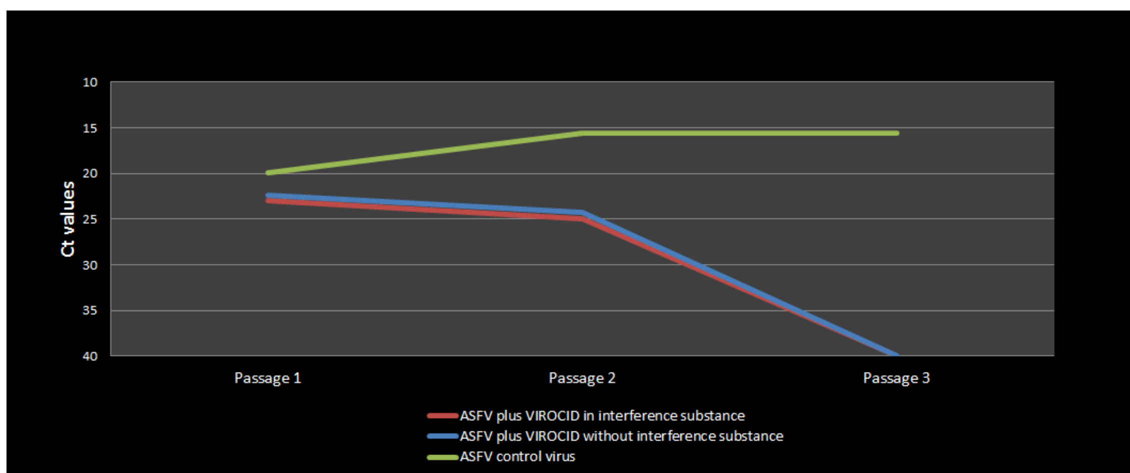

Figure 1; *Real time PCR results on infected VERO cells using untreated ASFV (control virus), Virocid-treated ASFV with interference substance (iSFB) and Virocid-treated ASFV without interference substance (H_2O). The results are expressed in Ct values after three consecutive passages on VERO cells.*

Conclusion.

The "in vitro" evaluation of the **Virocid disinfectant CID LINES at 0.25% dilution was able to reduce in a 100% the specific cytopathic effect** induced by the African swine fever virus (ASFV) Ba71V isolate on Vero cells, and thus **to avoid ASFV replication in these cells even at high virus concentration up to 10^{11} TCID50/ml,**

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In Valdeolmos, Madrid, Spain at 30 June 2012