Effectiveness of Xtreme Bio in Deactivating Porcine Circovirus 2 (PCV-2)

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Project Title: Evaluation of *in vitro* antiviral effect of the disinfectant XTREME BIO on PCV2

Project Objectives:

The objectives of this project was to evaluate the *in vitro* antiviral effect of XTREME BIO on porcine circovirus 2 [PCV-2].

Procedures to Achieve Objectives:

Materials

Liquid compounds from Midwest Ag Solutions, LLC: Xtreme Bio (1 gallon).

Virus: A PCV2d isolate (USA/NC24897/2016, P6) that grew efficiently in PK-15 cells.

Cells: Porcine kidney (PK-15) cells (ATCC CCL-33).

Cell culture maintenance medium and inoculation medium: MEM supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 0.05 mg/ml gentamicin, 10 unit/ml penicillin, 10 µg/ml streptomycin, and 0.25 µg/ml amphotericin.

D-glucosamine: 300 mM D-glucosamine.

PCV2 FA conjugate: VMRD CJ-F-PCV2-10ML.

Culture plates, serological pipette, tips and other supplies.

Methods

1. Test cytotoxicity of the product Xtreme Bio in PK-15 cells.

The recommended dilution of Xtreme Bio is 0.5 oz/gallon (1:256) on clean surface and 2 oz/gallon (1:64) in rough surface. (1 gallon = 128 oz).

In this study, the product Xtreme Bio was serially diluted in autoclaved distilled water: 1:16, 1:32, 1:64, 1:128, 1:256, and 1:512.

1) Prepare PK-15 cells in 96-well plates one day before the cytotoxicity testing.
2) The serially diluted product was inoculated in triplicate onto sub-monolayer of PK-15 cells seeded the previous day in a 96-well plate (100 µl of product per well). Controls included autoclaved water for inoculation. After 1 h incubation at 37°C, the inoculum was removed and 100 µl of fresh medium per well was added.
3) After incubation at 37°C for 24-48 h, the cells were scraped and harvested into 1 ml culture medium. Determine the cell density and percentage of live and dead cells using trypan blue staining and hemocytometer or ViCell XR (Beckman Coulter).
4) The 50% cytotoxic concentration (CC_{50}) were defined as the product dilution that caused reduction of 50% viability compared to the water-inoculated cells.

2. Treatment of PCV2 with the product Xtreme Bio (Table 1)

1) The product Xtreme Bio was serially diluted in autoclaved distilled water: 1:16, 1:32, 1:64, 1:128, 1:256, and 1:512.
2) The serially diluted Xtreme Bio product (300 µl) was mixed with the equal volume of PCV2d isolate (300 µl, 10^4-5 TCID50/ml) in 5 ml sterile snap-cap tubes (triplicate for each group). Control groups include water + virus, and water + medium. In total, 8 groups with 3 replicates for each group (treatments #1-24).
3) After incubation at room temperature (20-25°C) for 30 min, each sample was titrated for PCV2 in PK-15 cells. Briefly, serial 10-fold dilutions of each sample (10^0, 10^-1, 10^-2 and 10^-3) were prepared from each mixture and then inoculated into the sub-monolayers of PK-15 cells grown in 96-well plates (100 µl per well, 2 wells per dilution). Incubate at 37°C for 1 h. Discard the supernatant (inoculum). Add fresh medium (100 µl per well).
4) The plates were incubated at 37°C in 5% CO2 incubator for 24 h. Check if any cytotoxicity occurs. Add 50 µl of 300 mM D-glucosamine to each well of 96-well plates and incubate (with plates covered in dark) at room temperature for approximately 15-20 min. Decant D-glucosamine. Wash plate wells with 100 µl MEM/10% FBS and decant the medium. Add 100 µl MEM/10% FBS to each well.
5) Incubate plates for additional 24h. Check cells condition. Transfer the supernatants to new 96-well plates and save the supernatants. Rinse the cell plates once with PBS and fix the plates with 80% cold acetone for at least 10 min. Allow the fixed plates to dry. Add 50 µl of PCV2 FA conjugate from VMRD. Incubate at 37°C for about 1 h. Decant conjugate and wash plates with PBS for 3 times, about 5 min per time. Read plates under fluorescence microscope.
6) For each treatment (#1-24), pick one appropriate dilution (10^-1) of the supernatants for quantitative PCV2 PCR (pool duplicate wells of supernatant at that dilution).
7) Data analysis.

Table 1. Experimental design of evaluating antiviral effect of Xtreme Bio on PCV2

The mixture of PCV2 and Xtreme BioShield were serially diluted (10^0, 10^{-1}, 10^{-2} and 10^{-3}) for titration in PK-15 cells. Triplicate for each group.
The supernatants from the 10^{-1} dilutions collected from 48 h post-inoculation into PK-15 cells were tested by quantitative PCV2 PCR.

Treatment	PCV2 PCR Ct value				PCV2 genomci copies/ml				Percentage of genomic copies reduction
	Replicate 1	Replicate 2	Replicate 3	Mean	Replicate 1	Replicate 2	Replicate 3	Mean	
PCV2 + water	20.2	19.5	19.8	19.8	1.16E+08	1.81E+08	1.48E+08	1.48E+08	0.00%
PCV2 + Xtreme BioShield 1:16	27.0	26.2	26.0	26.4	1.33E+06	2.32E+06	2.60E+06	2.08E+06	98.60%
PCV2 + Xtreme BioShiel 1:32	24.5	24.4	26.2	25.0	7.01E+06	7.35E+06	2.30E+06	5.55E+06	96.26%
PCV2 + Xtreme BioShiel 1:64	22.8	23.3	22.5	22.9	2.15E+07	1.48E+07	2.63E+07	2.09E+07	85.93%
PCV2 + Xtreme BioShiel 1:128	22.9	22.6	21.9	22.5	1.97E+07	2.38E+07	3.80E+07	2.71E+07	81.71%
PCV2 + Xtreme BioShiel 1:256	21.0	20.1	21.8	21.0	6.61E+07	1.20E+08	3.91E+07	7.51E+07	49.39%
PCV2 + Xtreme BioShiel 1:512	21.2	20.6	20.7	20.8	5.90E+07	9.11E+07	8.49E+07	7.83E+07	47.19%
Medium + water	34.1	32.5	>37						

Percentage of genomic copies reduction = (untreated PCV2 genomic copies - treated PCV2 genomic copies) / (untreated PCV2 genomic copies) X 100%

Results:

1. **Cytotoxicity of the product Xtreme Bio in PK-15 cells.**
   The product Xtreme Bio at all of the tested dilutions 1:16, 1:32, 1:64, 1:128, 1:256, and 1:512 led to detachment of PK-15 cells. It appears that Xtreme Bio at these dilutions was cytotoxic to PK-15 cells.

2. **Evaluation of *in vitro* antiviral effect of the product Xtreme Bio on PCV2**
   The product Xtreme Bio was serially diluted in autoclaved distilled water: 1:16, 1:32, 1:64, 1:128, 1:256, and 1:512. Then the serially diluted Xtreme Bio product (300 µl) was mixed with the equal volume of PCV2d isolate (300 µl) and incubated at room temperature (20-25°C) for 30 min followed by titration in PK-15 cells.

   The records of cell conditions and PCV2 immunofluorescence staining results are shown in the attached Excel spreadsheet 'CPE and FA'. For the negative control (virus-negative culture medium treated with water), all of the wells were PCV2 FA staining negative. For the positive control (virus treated with water), all of the wells inoculated with 10^0, 10^{-1}, 10^{-2}, and 10^{-3} dilutions of the virus + water mixture had positive FA staining and the virus titer in this group was $10^{4.5}$ TCID50/ml. When PCV2 was treated with Xtreme Bio at 1:16, 1:32, and 1:64 dilutions, the 10^0 and 10^{-1} dilutions of the mixture led to cell detachment whereas the 10^{-2} and 10^{-3} dilutions of the mixture were PCV2 FA staining negative; thus no virus titer can be calculated. When PCV2 was treated with Xtreme Bio at 1:128, 1:256, and 1:512 dilutions, the 10^0 dilutions of the mixture led to cell detachment, the 10^{-1} dilutions of the mixture were FA staining positive and the 10^{-2} and 10^{-3} dilutions of the mixture were PCV2 FA staining negative; this gave rise to virus titer of $10^{2.5}$ TCID50/ml. Thus, compared to the untreated virus, treatment with the Xtreme Bio at dilutions of 1:128, 1:256 and 1:512 resulted in 2 log10 reduction of the infectious virus titer.

For each treatment, the supernatants from the 10^{-1} dilutions (triplicate for each treatment) of the mixture after 48 h inoculation in PK-15 cells were tested by a quantitative PCV2 PCR. The results are summarized in the Excel spreadsheet 'PCR'. Compared to the untreated PCV2 group, the treatment with Xtreme Bio at 1:16, 1:32, 1:64, 1:128, 1:256 and 1:512 dilutions resulted in 98.60%, 96.26%, 85.93%, 81.71%, 49.39%, and 47.19% reduction of genomic copies, respectively.

Conclusion:

Under the conditions of this study, compared to the untreated PCV2, treatment with the Xtreme Bio at dilutions of 1:128, 1:256 and 1:512 resulted in 2 log10 reduction of the infectious virus titer. Compared to the untreated PCV2 group, the treatment with Xtreme Bio at 1:16, 1:32, 1:64, 1:128, 1:256 and 1:512 dilutions resulted in 98.60%, 96.26%, 85.93%, 81.71%, 49.39%, and 47.19% reduction of genomic copies, respectively.