

Title: Determination of the importance of porcine parvovirus type 2 (PPV2) in porcine circovirus associated disease (PCVAD) outbreaks by elimination of PPV2 through vaccination at weaning – **NPB #15-144**

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Industry Summary

Despite widespread porcine circovirus type 2 (PCV2) vaccination in the growing pig population, occurrence of porcine circovirus associated disease (PCVAD) can be observed particularly in larger production systems. Further investigations into these apparent vaccine failure cases often indicate the presence of porcine parvovirus type 2 (PPV2). The effect of PPV2 on growing pigs is unknown. The objective of this study is to determine the importance of PPV2 by producing a baculovirus-based subunit vaccine, test the vaccine in pigs, and if successful attempt to eliminate PPV2 in a portion of pigs via vaccination at weaning on one of two PCVAD farms. A PPV2 subunit vaccine was successfully produced in the baculovirus vector. When tested under experimental condition in pigs, vaccinated pigs did not develop detectable antibodies under the study conditions by 42 days post vaccination. Unfortunately, attempts to improve the vaccine by increasing the protein concentration, changing the adjuvant, adjusting the ELISA assay, and outsourcing the PPV2 protein expression were all unsuccessful indicating that an entirely new approach to building a PPV2 vaccine is needed.

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Scientific Abstract

Despite widespread porcine circovirus type 2 (PCV2) vaccination in the growing pig population, occurrence of porcine circovirus associated disease (PCVAD) can be observed particularly in larger production systems. Further investigations into these apparent vaccine failure cases often indicate the presence of porcine parvovirus type 2 (PPV2). The effect of PPV2 on growing pigs is unknown. The objective of this study is to determine the importance of PPV2 by producing a baculovirus-based subunit vaccine, test the vaccine in pigs, and if successful attempt to eliminate PPV2 in a portion of pigs via vaccination at weaning on one of two PCVAD

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farms. A PPV2 subunit vaccine was successfully produced in the baculovirus vector. When tested under experimental condition in pigs, vaccinated pigs did not develop detectable antibodies under the study conditions by 42 days post vaccination. Unfortunately, attempts to improve the vaccine by increasing the protein concentration, changing the adjuvant, adjusting the ELISA assay, and outsourcing the PPV2 protein expression were all unsuccessful indicating that an entirely new approach to building a PPV2 vaccine is needed.

Introduction

During a porcine circovirus type 2 (PCV2) vaccine failure investigation in two different swine production systems we identified PCV2d and a novel porcine parvovirus (PPV) designated PPV type 2 (PPV2) (Opriessnig et al., 2013). Similar to circoviruses, PPVs are small, non-enveloped DNA viruses. The best known parvovirus in pigs is PPV1 which is also known as classical PPV and can be associated with reproductive failure in breeding herds. Under experimental conditions, PPV1 has also been shown to enhance PCV2 replication. Similar to PCV2, PPV1 has a strong cellular tropism for mitotically active tissues like lymph nodes or heart muscle (Allan et al., 2000a; Oraveerakul et al., 1993). The first experimental study demonstrating the potentiating effect of another pathogen on PCV2 was conducted in 1999 (Ellis et al., 1999) with PPV1 and PCV2. Several groups have since demonstrated that pigs dually-inoculated with PCV2 and PPV1 develop more severe disease and lesions than pigs infected with PCV2 alone (Allan et al., 2000b; Hasslung et al., 2005; Opriessnig et al., 2004; Ostanello et al., 2005).

Less information is available for other recognized parvoviruses in pigs including PPV2. In a study conducted in China, a peak in PPV2 viremia was detected 2–3 weeks prior to onset of severe respiratory disease associated with PRRSV and PCV2; however, a definitive relationship between presence and level of PPV2 DNA and severity of the outbreak was not confirmed (Wang et al., 2010). In a Hungarian study investigating randomly collected samples, PPV2 was detected in 5.4% (6/111) of serum samples investigated (Cságola et al., 2012). In our US study, the prevalence of PPV2 was determined to be 43.1% (31/72) in production system A and 70% (42/60) in production system B with an overall PPV2 prevalence of 55.3% (73/132) (Opriessnig et al., 2013). There was a difference in prevalence and PPV2 viral load between the farms. Of note, the prevalence of PPV2 was higher in the younger age groups in Farm B where PCVAD was also observed at an earlier age (Opriessnig et al., 2013). Finally, in a retrospective study using a total of 586 serum samples and 164 lung homogenates collected from 1996 to 2013 in the USA and Canada, all samples were tested for PPV1, PPV2 and PCV2 (Opriessnig et al., 2014). Overall, PPV2 had the highest prevalence rates in sera (35.2%) and tissues (42.7%). Concurrent infection of PCV2 and PPV occurred in 14.3% (84/586) of the serum samples and in 49.4% (81/164) of the tissue samples. Moreover, the prevalence of PPV1 or PPV2 DNA was significantly higher in tissues containing high amounts of PCV2 DNA compared to non-PCVAD cases (Opriessnig et al., 2014).

Objective

The objective of this study is to determine the importance of porcine parvovirus type 2 (PPV2) by producing a baculovirus-based vaccine, test the vaccine in pigs and if successful, attempt to eliminate PPV2 in a portion of pigs via vaccination at weaning on one of two PCVAD farms.

Materials and Methods

The experimental protocols were approved by the Iowa State University Institutional Animal Care and Use Committee and the Iowa State University Biosafety Committee. The Center for Veterinary Biologics was contacted and consulted on necessary permits to use the experimental vaccine in farmed pigs.

The study was separated into three parts

- 1) PPV2 vaccine production
- 2) Vaccine assessment under experimental conditions
- 3) Vaccine assessment under field conditions.

Part A. PPV2 vaccine production

For the PPV2 vaccine production, the Bac-to-Bac Baculovirus expression system was selected to express VP2 of PPV2. The selected PPV2 region was 1734 pb in length. A brief outline of this system is presented in Fig. 1.

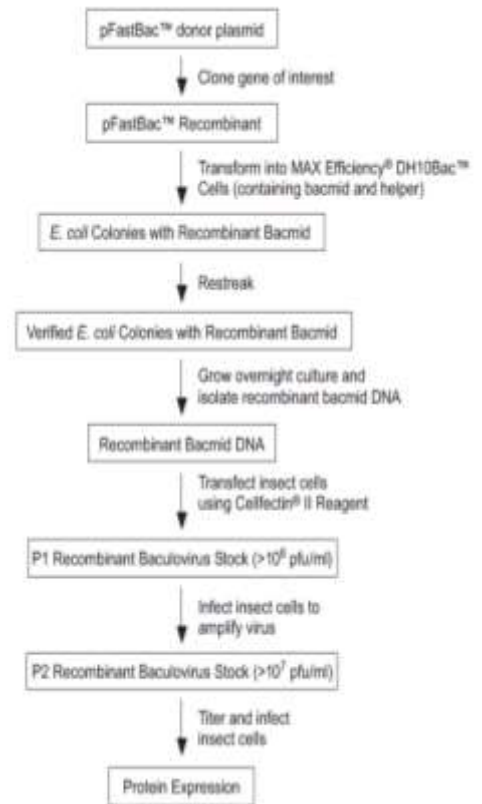
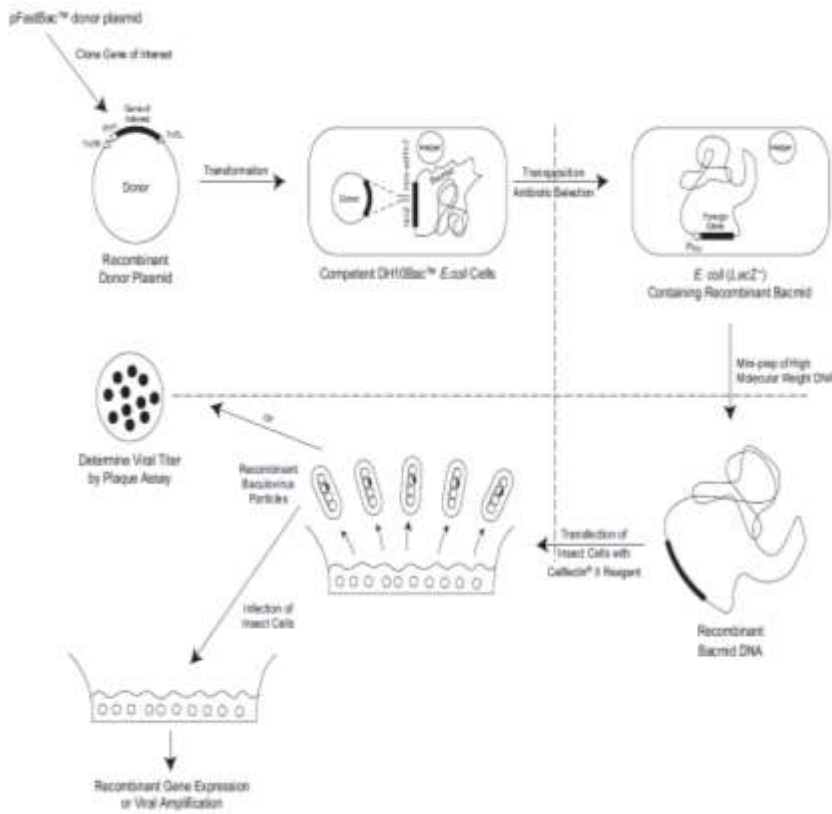


Fig. 1. Steps used for protein expression in the Bac-to-Bac Baculovirus expression system.

Initially the PPV2 VP2 gene was inserted into the pFastBac1 vector and the resulting recombinant plasmid was designated as pFastBac1-PPV2-VP2. The recombinant plasmid was then transformed into DH10Bac competent cells, and recombinant bacmid was obtained. Transfection efficiency of the P1 stock was checked by adding X-glu.

Part B. Vaccine assessment under experimental conditions

A herd with sows with low PPV2 antibody titers was identified in March 2016 and PPV2 negative pigs were selected in April 2016 and a pilot study was conducted at Iowa State University. In brief, a breeding herd previously confirmed to have PPV2 negative dams was screened and piglets were obtained from selected PPV2 negative litters. Twelve 2 week old piglets from that farm were purchased and transported to the Iowa State University research facility. The pigs were separated into groups and rooms of two pigs each as outlined in Table 1.

Table 1: Experimental design for the pilot vaccine study

Group	PPV2 dose	Adjuvant
1 (n=2)	Low (10 µg)	VaXliant® Enable S1
2 (n=2)	Medium (50 µg)	VaXliant® Enable S1
3 (n=2)	High (100 µg)	VaXliant® Enable S1
4 (n=2)	None	VaXliant® Enable S1

Based on information available in the literature a dose of 5-10µg of a baculovirus expressed purified protein is sufficient to immunize a pig. We decided to test three different doses: a low dose (10 µg), a medium dose (50 µg) and a high dose (100 µg). Vaccination was scheduled to be done intramuscularly twice two weeks apart. After consulting with the Center for Veterinary Biologics, we selected a commercially available USDA approved adjuvant (VaXliant® Enable S1).

Upon arrival in the research facility, blood was collected from the pigs and tested with an in house PPV2 ELISA. In brief, purified PPV2 VP2 protein expressed in an *E. coli* vector (50 ng per well) was coated on ELISA plates (Nunc® MaxiSorp™; Sigma-Aldrich, Roskilde, Denmark) at 4°C overnight. After three washes with phosphate-buffered saline with 0.05% Tween 20 (PBST), the plates were blocked with 1% bovine serum albumin for 2 h at 22°C and then incubated with serum diluted 1:100 in PBS containing 10% goat serum at 37°C for 1 h. Following three washing steps, the plates were incubated with a 1:10 000 diluted peroxidase-conjugated goat anti-swine IgG (Jackson ImmunoResearch; West Grove, PA, USA) for 1 h at 37°C. The peroxidase reaction was visualized using tetramethylbenzidine–hydrogen peroxide (TMB) (KPL, Gaithersburg, MD, USA) as the substrate for 10 min at room temperature and stopped using 50 µl of 2 N H2SO4. Optical densities (ODs) were measured at 405 nm by a Multiskan Ascent 96/384 plate reader (MTX Lab Systems, Vienna, VA, USA). Positive and negative controls were included on each plate. The serum antibody response was presented as sample-to-positive (S/P) ratios calculated as follows: $S/P \text{ ratio} = (\text{sample OD} - \text{negative control mean OD}) / (\text{positive control mean OD} - \text{negative control mean OD})$. Samples with an S/P ratio higher or equal to 0.3 were considered positive. Upon testing the selected pigs were found to be PPV2 antibody negative.

The vaccine preparation was done shortly before vaccination. In brief, the adjuvant was brought to room temperature and mixed with PBS. Then the pre-diluted protein was added, the vaccine was inverted several times and the pigs were vaccinated immediately and within the 30 min period of time as advised by the adjuvant manufacturer. Each pig was vaccinated with 1 ml of the vaccine preparation intramuscularly into the right neck area. Vaccination was repeated 2 weeks later. The pigs were monitored for a total of 42 days. Blood was collected every week and tested for presence of PPV2 antibodies. All pigs were euthanized by intravenous pentobarbital sodium overdose (Fatal Plus®, Vortech Pharmaceuticals, LTD, Dearborn, MI, USA) and necropsied on day 42 after first vaccination.

Part B. Vaccine assessment under field conditions

The Center for Veterinary Biologics in Ames, Iowa was contacted and information on how to conduct controlled field studies using experimental products was obtained. Based on guidance of the Center for Veterinary Biologics, an appropriate adjuvant for the field study was selected that would allow the pigs to go slaughter after study termination.

Results

A. Vaccine production

The obtained recombinant plasmid and bacmid were verified by gel electrophoresis (Fig. 2) and sequencing.

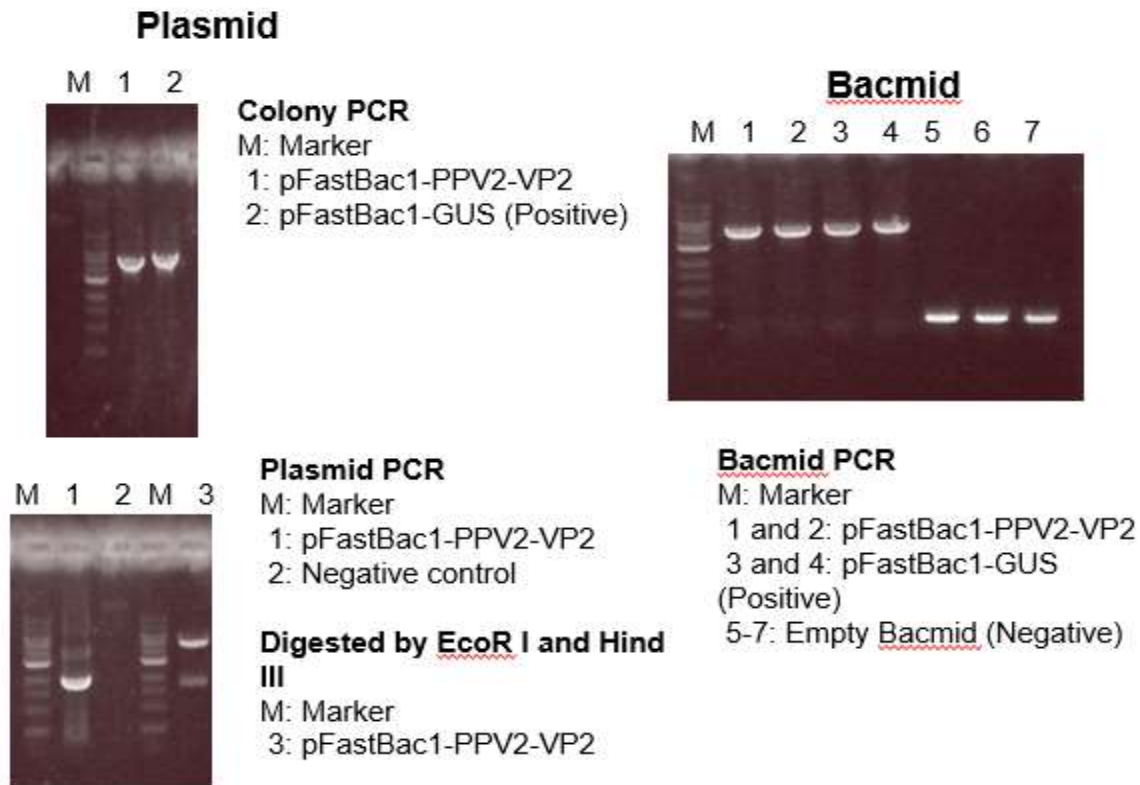
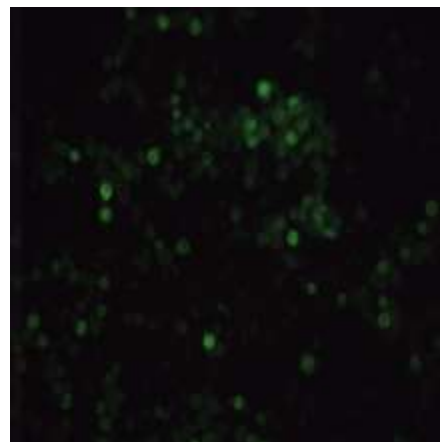


Fig. 2. Verification of the recombinant plasmid and bacmid by gel electrophoresis.

The final titer of the pFastBac1-PPV2-VP2 P1 stock was 3×10^6 pfu/ml (determined by a viral plaque assay). Sequencing confirmation of the obtained protein confirmed that the protein was correct. Subsequently insect cells were infected with the P1 stock. The purified and stored at -80°C until

Fig. 3. Confirmation of PPV2 VP2 expression (green staining) by an immunofluorescence assay (IFA).



baculoviral assay) (Fig. confirmed infected usage. staining) by

B. Vaccine assessment in experimental pigs

After experimental vaccination of pigs with the baculovirus derived PPV2 VP2, no antibody response was detected in any of the pigs. The ELISA results were repeated several times using different plates and protein propagation but the results remained negative. An example is given in Table 2.

Table 2: PPV2 ELISA OD values from pigs experimentally vaccinated against PPV2 at different days post vaccination. Selected pigs were kept two additional weeks until D56.

Pig	Treatment	D -3	D7	D14	D21	D28	D35	D42	D49	D56
41	VaXliant 0	0.164	0.161	0.145	0.132	0.176	0.145	0.153		
45	VaXliant 0	0.167	0.163	0.147	0.143	0.19	0.208	0.15		
42	VaXliant 10	0.146	0.199	0.198	0.182	0.298	0.198	0.199		
60	VaXliant 10	0.14	0.107	0.102	0.104	0.149	0.191	0.113		
53	VaXliant 100	0.142	0.123	0.112	0.105	0.134	0.148	0.133	0.181	0.135
59	VaXliant 100	0.132	0.138	0.114	0.113	0.14	0.17	0.115	0.168	0.151
54	VaXliant 50	0.114	0.105	0.134	0.087	0.13	0.186	0.124	0.212	0.189
56	VaXliant 50	0.117	0.115	0.138	0.158	0.2	0.218	0.14	0.183	0.18

NEG control	0.158			0.172	0.208	0.173	0.145	0.257	0.227
NEG control	0.161			0.169	0.197	0.163	0.152	0.257	0.229
POS control	0.764			0.779	0.868	0.561	0.616	0.618	0.611
POS control	0.718			0.737	0.773	0.521	0.737	0.629	0.596

Troubleshooting

Protein concentration too low: The dose of the protein was increased. A new set of pigs was vaccinated with 500 µg of the protein on days 0, 14 and 21. We obtained similar results as before. PPV2 seroconversion was not observed by 42 days post vaccination (data not shown).

Protein degradation somewhere in the process: Protein was produced and injected into a set of two pigs directly after protein production. An antibody response against PPV2 was not detected for the 42-day duration of the study (data not shown).

Adjuvant issue: As a next step a new set of eight pigs was identified, purchased, brought to the research facility at Iowa State University and vaccinated by using a different adjuvant (Montanide ISA 15A VG, Seppic, Pueaux, France). The results of this follow-up study were similar to previous findings and no seroconversion to PPV2 was observed (data not shown).

ELISA is not working or suitable to detect the antibodies against PPV2: The ELISA was extensively double-checked and several new batches of protein coated on new plates were tested. In addition, different serum dilution ranging from 1:20 to 1:1000 were tested. The results were similar in each run. To assess reactivity of the baculovirus protein with the ELISA, rabbit-anti-PPV2 antiserum (obtained by injecting rabbits with *E. coli* expressed PPV2) was used and correctly identified the baculovirus protein in a Western blot indicating that antibodies directed against the baculovirus PPV2 are identified by the ELISA assay.

Baculovirus production is not correct in our research facility: We identified a University associated US protein facility specialized in baculovirus protein propagation and sent our construct to them to propagate a large amount of protein for the field study. After 4 months, the company indicated they failed to produce the protein (they only obtained protein fragments).

C. Vaccine testing under field conditions

This portion was not initiated as there was no experimental evidence that the experimental PPV2 vaccine was working. In addition, funds were depleted by trouble-shooting the PPV2 vaccine in different set of pigs and by having a new batch of protein produced by a commercial company

Discussion

To investigate the importance of PPV2 on the outcome of PCV2 infection we proposed to develop a PPV2 subunit vaccine in the baculovirus vector and to test it in pigs. We were successful in obtaining the baculovirus expressed PPV2 VP2-protein; however, subsequent testing of the protein in pigs indicated that the protein was not immunogenic. Unfortunately, attempts to improve the vaccine by increasing the protein concentration, changing the adjuvant, adjusting the ELISA assay, and outsourcing the PPV2 protein expression were all unsuccessful indicating that an entirely new approach to building a PPV2 vaccine is needed.

References:

- Allan GM, McNeilly F, Meehan BM, Ellis JA, Connor TJ, McNair I, Krakowka S, Kennedy S. A sequential study of experimental infection of pigs with porcine circovirus and porcine parvovirus: immunostaining of cryostat sections and virus isolation. *J Vet Med B Infect Dis Vet Public Health*. 2000a Mar;47(2):81-94.
- Allan GM, McNeilly F, Ellis J, Krakowka S, Meehan B, McNair I, Walker I, Kennedy S. Experimental infection of colostrum deprived piglets with porcine circovirus 2 (PCV2) and porcine reproductive and respiratory syndrome virus (PRRSV) potentiates PCV2 replication. *Arch Virol*. 2000b;145(11):2421-9.
- Csárgola A, Lőrincz M, Cadar D, Tombácz K, Biksi I, Tuboly T. Detection, prevalence and analysis of emerging porcine parvovirus infections. *Arch Virol*. 2012 Jun;157(6):1003-10.
- Ellis J, Krakowka S, Lairmore M, Haines D, Bratanich A, Clark E, Allan G, Konoby C, Hassard L, Meehan B, Martin K, Harding J, Kennedy S, McNeilly F. Reproduction of lesions of postweaning multisystemic wasting syndrome in gnotobiotic piglets. *J Vet Diagn Invest*. 1999 Jan;11(1):3-14.
- Hasslung F, Wallgren P, Ladekjaer-Hansen AS, Bøtner A, Nielsen J, Wattrang E, Allan GM, McNeilly F, Ellis J, Timmusk S, Belák K, Segall T, Melin L, Berg M, Fossum C. Experimental reproduction of postweaning multisystemic wasting syndrome (PMWS) in pigs in Sweden and Denmark with a Swedish isolate of porcine circovirus type 2. *Vet Microbiol*. 2005 Mar 20;106(1-2):49-60.
- Opriessnig T, Fenaux M, Yu S, Evans RB, Cavanaugh D, Gallup JM, Pallares FJ, Thacker EL, Lager KM, Meng XJ, Halbur PG. Effect of porcine parvovirus vaccination on the development of PMWS in segregated early weaned pigs coinfecting with type 2 porcine circovirus and porcine parvovirus. *Vet Microbiol*. 2004 Mar 5;98(3-4):209-20.
- Opriessnig T, Xiao CT, Gerber PF, Halbur PG. Identification of recently described porcine parvoviruses in archived North American samples from 1996 and association with porcine circovirus associated disease. *Vet Microbiol*. 2014 Sep 17;173(1-2):9-16.
- Opriessnig T, Xiao CT, Gerber PF, Halbur PG. Emergence of a novel mutant PCV2b variant associated with clinical PCVAD in two vaccinated pig farms in the U.S. concurrently infected with PPV2. *Vet Microbiol*. 2013 Apr 12;163(1-2):177-83.
- Oraveerakul K, Choi CS, Molitor TW. Tissue tropisms of porcine parvovirus in swine. *Arch Virol*. 1993;130(3-4):377-89.
- Ostanello F, Caprioli A, Di Francesco A, Battilani M, Sala G, Sarli G, Mandrioli L, McNeilly F, Allan GM, Prosperi S. Experimental infection of 3-week-old conventional colostrum-fed pigs with porcine circovirus type 2 and porcine parvovirus. *Vet Microbiol*. 2005 Jul 1;108(3-4):179-86.
- Wang F, Wei Y, Zhu C, Huang X, Xu Y, Yu L, Yu X. Novel parvovirus sublineage in the family of Parvoviridae. *Virus Genes*. 2010 Oct;41(2):305-8.