

MPB Final Research Grant Report

- I. Project title:** Evaluation of autogenous inactivated tissue vaccine for protection from porcine circovirus-associated diseases (PCVAD) in commercial swine farms

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II. Abstract

Post-weaning multi-systemic wasting syndrome (PMWS) has been a major emerging disease problem for the US swine industry since the beginning of 2006. Porcine circovirus type 2 (PCV2) has been identified as the major cause for PMWS. In mid 2006, commercial PCV2 vaccine was just available on the market but only in limited quantity, and swine farmers hoped to have an alternative way to prevent from the economically important disease. In our preliminary study, the use of an autogenous inactivated tissue vaccine (TV) that was prepared using infected tissue antigen from PMWS affected pigs resulted in marked reduction of the mortality in growing pigs. Therefore, objectives of our research were to evaluate the safety and the efficacy of the autogenous inactivated TV. There was no detectable live virus when PCV2-infected cell monolayers were treated with formalin at concentrations of 0.2% or 0.4% for 1 hour. PCV2 was not isolated using PK-15 cell culture from the TV antigen that had been formalized at the final concentration of 0.2% for 48 hours. The virus was also not detected from colostrum-deprived pigs that had been inoculated with the formalized tissue antigen. The results suggest that there was a complete inactivation of the tissue antigen after formalization. For the efficacy trials in a Minnesota farm with clinical PMWS, mortality of 3 groups of grow-finish pigs with no vaccination was 9.4 % (136/1443 pigs), while mortality of 2 groups pigs inoculated with TV was 2.5% (27/1075 pigs). In trials of 3 Korean farms with TV, nursery mortality was 5.0%, 11.8% and 3.7% in vaccinated groups, while the mortality was 11.2%, 21.9% and 14.5% in non-vaccinated groups. No detrimental effect was reported following injection with this TV in the pigs. In an examination of virucidal effect with 9 commercial disinfectants, 1% Virkon S, Clorox (1:21.3), and 3% sodium hydroxide showed complete reduction of PCV2 infectivity in 10 minutes. The results of these studies indicate that the autogenous inactivated TV can be prepared any time inexpensively using infected tissues from PMWS affected pigs, and can be safely and effectively used for prevention of PMWS in commercial farms. In addition, we have identified Virkon S, Clorox and sodium hydroxide as effective disinfectants against PCV2.

III Introduction

Porcine circovirus type 2 (PCV2) is a very small, circular and single-strand DNA virus, and has been classified in family *Circoviridae*. The virus has been associated with several disease syndromes in pigs including post-weaning multi-systemic wasting syndrome (PMWS), porcine dermatitis and nephropathy syndrome, reproductive failure, and porcine respiratory disease complex. All of these syndromes together are now named porcine circovirus-associated disease (PCVAD).

Although PMWS was subclinical until the mid 2005, it is now considered one of the most economically significant swine diseases in the United States. Swine farms with clinical PMWS have high mortality among growing pigs. Recently, prevention of clinical PMWS has been successful through the use of different commercial PCV2 vaccines. However, PCV2 commercial vaccine was just developed and only limited quantity of the vaccine was available during the initiation of this project. Also, the vaccine is relatively expensive for swine farmers. It has been proposed that a tissue vaccine (TV) could be inexpensively prepared and used it alternatively on swine farms to prevent from the economic losses from PCVAD. Preparation of the TV has been based on using inactivated PCV2 antigen from tissues of PMWS affected pigs.

IV. Objectives

In the proposal, two objectives were designed; 1) to evaluate the safety of the autogenous inactivated TV, and 2) to evaluate the efficacy of the inactivated autogenous TV in commercial swine farms. Additionally, virucidal effect of different commercial disinfectants against PCV2 was evaluated because the use of effective disinfectants with proper bio-security practices is an important step in controlling PMWS and preventing PCV2 infection in swine farms.

V. Procedures

Experimental design for objectives 1 and 2

Formalin has been used as the most common inactivating agent in the preparation of different vaccines. In the first experiment, effect of formalin for inactivation of PCV2 *in vitro* was examined. PK-15 cell and MARC-145 culture monolayers in 24-well microplates were infected with PCV2 and PRRS virus, respectively. After removing the medium, formalin at different concentrations (0.4 ml per well) was flooded on each infected cell monolayer. The monolayers were incubated at room temperature for 1 hour, formalin was removed, and the formalized monlayers were washed with phosphate buffered saline (PBS, pH 7.2). Then, the cells were scraped, pooled and examined for the presence of live PCV2 by virus isolation. In the second experiment, formalin at a final concentration of 0.2% was added to PCV2-infected tissue extract antigen, and formalization was carried out for 48 hours at room temperature with continuous stirring. Virus isolation using PK-15 cell culture and bio-assay using colostrum-deprived (CD) pigs were performed to detect the presence of residual live PCV2 in the formalized tissue antigen.

For the efficacy test of the TV, we performed a field trial in a Minnesota farm where severe clinical PMWS was experiencing. For each batch of pigs at weaning, TV (1 ml) was

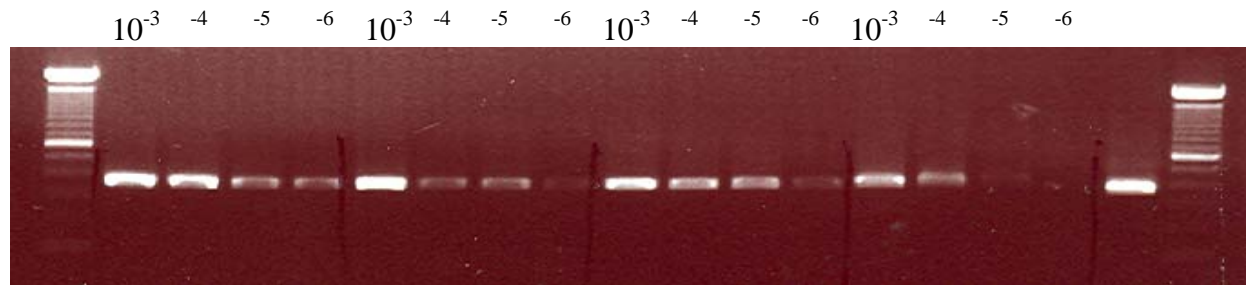
injected intramuscularly and the second dose was injected 2 weeks after the first vaccination. Clinical signs were evaluated and mortality data were obtained by attending veterinarians. During the field trials, we had difficulty in finding of the trial farms, and farm owners did not allow a group of pigs without vaccination to serve as controls. Therefore, similar experiments on the safety and efficacy of the TV were carried out in 3 farms in South Korea.

Preparation of the autogenous inactivated TV vaccine

A standard protocol for preparation of autogenous inactivated TV for PMWS is described as below.

1. Select pigs with severe PMWS (early grower pigs with high mortality exhibiting clinical signs of wasting, prominent back bone, heavy breathing & mild diarrhea) and typical gross lesions (heavy, wet and interlobular edematous lung, swollen and red lymph nodes) from the affected farms. Lungs with inflammatory lesions including abscess should not be collected, and the gross lesions should be differentiated from porcine respiratory disease complex consisting of demarcated purple lesion representing mycoplasma or pasteurella infection. Affected tissues including lung, spleen and some lymph nodes are collected from each pig and pooled them from 3-5 pigs.
2. Weigh the pooled tissues and blend finely (<1 mm in diameters) under sterile condition using a commercial blender.
3. Add cell culture medium (minimal essential medium) to the tissue homogenate (1,000 ml of medium to 1,000 grams of tissue pool)
4. Blend briefly for 1 minute, and frozen and thawed two times to release antigens into the medium. Between freezing and thawing steps, the suspension will be blended for about 1 minute.
5. Centrifuged at 3,000 – 10,000 rpm for 20-30 minutes and collect the supernatant. The supernatant will be filtered through 2-3 layers of gauze (PCV2 DNA concentrations measured by PCR are shown in Fig. 1. Note – Obvious band should be visible at dilution of 10^{-4} or higher). Typically one Kg of the tissue pool has been made 4 liters of PCV2 antigen containing 10^{9-10} copies/ml measured by a quantitative PCR.
6. Add cell culture medium to be a total volume of 4,000 ml.
7. Add formalin (37%) to the supernatant at a final concentration of 0.2% (8 ml in 4,000 ml tissue antigen) and stirred for 48 hours at room temperature.
8. For the final vaccine, 4,000 ml of the formalized tissue antigen and 1,000 ml of sterile Alhydrogel adjuvant (Accurate chemical & scientific Co., Westbury, NY) are mixed and stirred at room temperature for 2-3 hours.
9. These will be filled in sterile vaccine bottles and stored at 4 C until use.

Fig. 1. Concentration of PCV2 DNA in each dilution of tissue antigens from 4 different pigs with clinical PMWS



Pig 1 Pig 2 Pig 3 Pig 4
 Note: PCV2 antigen titer for pig 1 - $>10^6$, pig 2 - 10^6 , pig 3 - 10^6 , and pig 4 - 10^5

PCV2 isolation and infectivity test

PCV2 isolation and viral infectivity titers were carried out using PK-15 cells by routine methods in our laboratory, and the results were read by immunofluorescence antibody (IFA) test. Briefly for infectivity test, 0.1 ml of 10-fold serial viral dilutions was dispensed into a 96-well microtitration plate, and 0.1 ml of PK-15 cells ($1-2 \times 10^5$ cells/ml) was added to each well. One day later, the cell culture in each well was treated with 50 μ l of 300-mM D-glucosamine. The cell monolayer was cultured for 2 more days and fixed with 50% acetone in ethanol. The IFA test was performed on the cell monolayer by reacting it with a PCV-2 reference positive pig serum for 1 hour at 37°C, followed by three washes with PBS. The monolayer was then reacted with rabbit anti-swine IgG conjugated with fluorescein isothiocyanate for 1 hour, washed 3 times with PBS, and examined under a fluorescent microscope.

Evaluation of virucidal effect on commercial disinfectants against PCV2

Disinfectants tested in this study included 3% sodium hydroxide, a mixture of potassium peroxymonosulfate and sodium chloride (Virkon S 1:100 – Antec International, Sudbury, Suffolk, UK), 2 phenolic compounds (1-Stroke environ 1:256 – Steris Corporation, Road Mentor, OH; Tek-Trol 1:256 – Bio-Tek Industries Inc., Atlanta, GA), a quarternary ammonium compound (Roccal D Plus 1:256 – Pfizer Animal Health, Peapack, NJ), a formaldehyde and quarternary ammonium compound (DC&R 1:128 – Neogen Co., Lexington, KY), a quarternary ammonium and glutaraldehyde compound (Synergize 1:256, Preserve International, Reno, NV), chlorhexidine (Novalsan 1:48 – Fort Dodge Labs, Fort Dodge, IA), and sodium hypochloride (Clorox Bleach 1:21.3– Clorox company, Oakland, CA). Each disinfectant was prepared in water at twice the manufacturer’s recommended dilution, and equal volumes of the disinfectant and PCV2 were mixed. The virus-disinfectant mixtures were incubated at room temperature for different lengths of time. After incubation, the mixtures were centrifuged through a detoxification column. Detoxification of the virus-disinfectants was performed using a Sephadex LH-20 bead column. The detoxified virus tested for infectivity titer as described above.

VI. Results

There was no detectable virus when PCV2 or PRSSV-infected cell monolayers were treated with formalin at concentrations 0.2% or 0.4% for 1 hour. However, PCV2 was detected after the treatment with 0.05% or 0.1% for 1 hour on the infected cell monolayer, while PRSSV was not (Table 1). Presence of residual PCV2 in the tissue homogenate antigen after formalization was also examined by virus isolation using PK-15 cells and bioassay using CD pigs. PCV2 was not isolated from the formalized tissue antigens. One-day old CD pigs were inoculated intraperitoneally with tissue antigen before or after formalization, and the pigs were fed artificially for 7 days. After 7 days post inoculation, PCV2 was not isolated from pigs inoculated with the antigen after formalization, while the virus was readily isolated from pigs inoculated with the antigen before formalization (Table 2). Using the PCR assay, the results were similar to those of virus isolation but there appears to have some residual viral DNA in the sera of pigs inoculated with antigen after formalization.

The efficacy of an autogenous inactivated TV along with a commercial PCV2 vaccine was evaluated in a commercial swine farm, and the results are shown in Table 3. The TV was prepared using the tissues from PMWS affected pigs and used the vaccine in the farm that the tissues were originated. The vaccine was inoculated to the pigs at 3 and 5 weeks of age, and mortality was examined during the first 6 weeks in grow/finish barns, because it was understood that mortality due to PMWS occurs most commonly during 10-16 weeks of age. A commercial vaccine was just available during the experiment, and half of the pigs in groups 4 and 5 received a commercial PCV2 vaccine. As shown in Table 3, mortality in the groups 1-3 with no vaccination was 9.4% (136/1443 pigs), pigs in groups 4 and 5 that had been vaccinated either commercial or tissue vaccine was 1.8% (19/1064 pigs), and groups 6 and 7 that had been vaccinated with only tissue vaccine was 2.5% (27/1075 pigs). These results suggest that the TV was highly effective in reducing mortality on PMWS farms. No detrimental effect was reported following injection with this TV in the pigs.

Table 1. Effects of formalin treatment with virus-infected monolayers on viral survivability

Formalin concentration (%)	Infected monolayer with	
	PRRS virus	PCV2
0.4	-	-
0.2	-	-
0.1	-	+
0.05	+	+
None (PBS)	+	+

- = virus was not isolated; + = virus was isolated

Table 2. PCV2 detection in serum of the colostrum-deprived pigs following intraperitoneal injection of tissue antigen before (B) or after (A) formalization

No. of pigs	Injection	Viremia in pigs day post inoculation (dpi)			
		0 dpi		7 dpi	
		VI	PCR	VI	PCR
B1	Tissue antigen	-	-	Died at 2 nd day	
B2	before formalization	-	-	+	+++
B3	5 ml ip at birth	-	-	+	+++
B4		-	-	+	+++
A1	Tissue antigen	-	nt	-	±
A2	after formalization	-	nt	-	±
A3	5 ml ip at birth	-	-	-	±
A4		-	-	-	±
C1	None	-	nt	-	nt

* VI = virus isolation; nt = not tested; ± PCR positive suspected

Table 3. Mortality during first 6 weeks in grow/finish barns of the pigs following inoculation with an autogenous tissue vaccine or a commercial PCV2 vaccine at 3 and 5 weeks of age

Group	Vaccine	No. of pigs	Unthrifty pigs during		Deaths during first 6 wks in G/F
			2 wks in G/F	4 wks in G/F	
1	None	480	20	15	40 (8.3%)
2	None	503	30	20	62 (12.3%)
3	None	460	12	20	34 (7.4%)
4	TV/CV*	476	0	0	6 (1.2%)
5	TV/CV*	588	3	3	13 (2.2%)
6	TV	545	2	2	6 (1.1%)
7	TV	530	2	0	21 (3.9%)

* Half of the pigs in each T/C group were inoculated with tissue vaccine (TV) and the remaining half were with a PCV2 commercial vaccine (CV).

The efficacy trials were conducted in 3 different farms in S. Korea with vaccinated and unvaccinated control groups within the same population. The autogenous tissue vaccine was prepared using the same protocol in a local laboratory. Farm H was 300-sow farrow-to-finish operation, and TV was inoculated at 2 and 4 weeks of age. Farm C1 was 550-sow farrow-to-finish, and TV was inoculated at 1 and 3 weeks of age. Farm C2 was 600-sow farrow-to-finish, and one group was inoculated with TV at 2 and 4 weeks of age and another group with convalescent serum at 1 and 3 weeks of age (5 ml each intraperitoneally). A comparison was made for mortality during the nursery period between TV and serum groups. The vaccine efficacy was evaluated only for the nursery period because mortality associated with PMWS was

most commonly observed in nursery pigs. Numbers of pigs in each experimental group and mortality during the nursery period in farms H, C1 and C2 are summarized in Table 4. In farms H and C1, marked reduction in the mortality was observed in the vaccinated groups. In farm C2, mortality of TC vaccinated and the serum injected groups were 3.7% (36 of 927) and 14.5% (136 of 940), respectively.

Table 4. Comparison of nursery mortality of pigs following vaccination with tissue vaccine in 3 Korean farms

Farm H	Group 1		2		3		Total	
	Vac	None	Vac	None	Vac	None	Vac	None
No. of pigs	125	785	253	247	248	234	626	1266
Dead	6	86	13	32	12	24	31	142
Mortality %	4.8	10.9	5.1	12.9	4.8	10.2	5.0	11.2

Farm C1	Group 1		2		3		Total	
	Vac	None	Vac	None	Vac	None	Vac	None
No. of pigs	nt	964	443	314	296	436	739	1714
Dead	nt	240	64	80	23	55	87	375
Mortality %	nt	24.9	14.4	25.5	7.8	12.6	11.8	21.9

Farm C2	Group 1		2		3		4	
	Vac	Serum	Vac	Serum	Vac	Serum	Vac	Serum
No. of pigs	275	126	258	244	187	337	207	233
Dead	16	30	10	32	5	45	5	29
Mortality %	5.8	23.8	3.9	13.1	2.7	13.4	2.4	12.4

Vac – Tissue antigen vaccinated; Serum – convalescent serum (5 ml/pig) was inoculated

The reduction of PCV2 infectivity titers following treatment with each disinfectant and incubated at room temperature is shown in Fig 2. For virus-disinfectant mixtures of 1% Virkon S, Clorox (1:21.3), and 3% sodium hydroxide complete reduction in PCV2 infectivity occurred in 10 minutes. Roccal-D and Synergize caused substantial reduction of PCV2 infectivity when the incubation time was increased to 30 minutes. DC&R reduced the infectivity from $10^{5.0}$ to $10^{2.5}$ TCID₅₀/ml after 24 hours of incubation time. The remaining disinfectants reduced infectivity only slightly. In another experiment using Virkon-S, Clorox, Roccal-D and Synergize at 5 times weaker (1:5 dilution of the manufacturer's recommended dilution) with 12 hours of incubation time, PCV-2 was reduced from $10^{4.5}$ TCID₅₀/ml to $10^{2.25}$, $10^{1.75}$, $10^{3.5}$ and $10^{3.75}$ TCID₅₀/ml, respectively. For PRRSV ($10^{5.5}$ TCID₅₀/ml), all of the disinfectants caused no detectable virus after the virus-disinfectant mixtures were incubated at room temperature for 10 minutes.

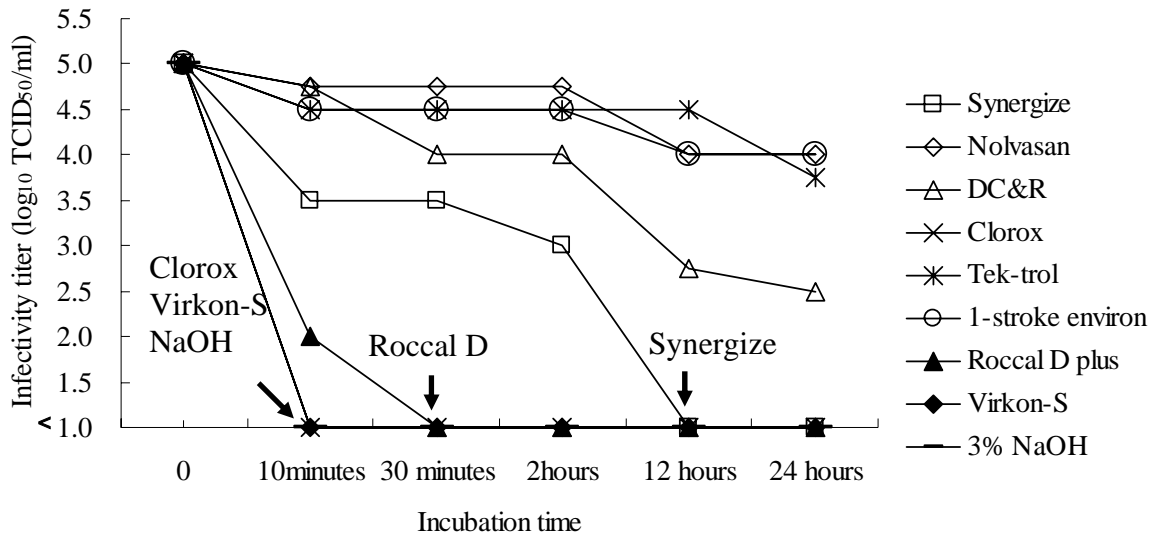


Fig. 2. Reduction of PCV2 infectivity following treatment with different disinfectants at recommended concentration. Infectivity titers were measured after each of 8 different disinfectants at two times concentration of the manufacturer's recommended dilution or 6% sodium hydroxide was mixed with an equal volume of PCV2, and the mixture incubated at room temperature for different periods of time.