RESEARCH REPORT
SWINE HEALTH

Title: PCV-2 viral DNA(s) are Infectious for Gnotobiotic Swine – **NPB #02-132**

Investigator: Steven Krakowka, DVM, PhD, Professor

Institution: College of Veterinary Medicine, The Ohio State University

Date Received: January 23, 2004

Abstract: Porcine circovirus (PCV)-2 is the cause of postweaning multisystemic wasting syndrome (PMWS). The infectious virion consists of a circularized negative sense single stranded DNA encapsidated by a single virus-coded structural (nucleocapsid) protein). Virologic dogma contends that the only infectious form of the virus is the encapsidated virion (circularized ssDNA and nucleocapsid protein). In these experiments, we have shown that, contrary to "dogma", nonencapsidated circularized Pcv-2 viral ssDNA and possibly the replicative intermediate double stranded (dsDNA) is infectious for gnotobiotic swine. The potential importance of this observation lies in the possibility that direct infectivity of viral DNAs in the presence of passive or active immunity (to nucleocapsid and thus the virion) can be accomplished in swine. Additional work must be done but the data show that viral DNAs alone are capable of disease production in swine.

Introduction: Porcine circovirus (PCV)-2 is the cause of postweaning multisystemic wasting syndrome (PMWS). With National Pork Board (NPB) support, we have shown that PCV-2 infection alone will not produce PMWS in gnotobiotic piglets. When combined with co-infection by other viruses such as porcine parvovirus (PPV) or immunostimulated with an oil-based macrophage-targeted adjuvant (KLH emulsified in incomplete Freunds adjuvant), PMWS is produced. Our studies have identified cells of histiocytic lineage may be initial cellular sites of viral infection. More importantly, we and others have shown that macrophages also accumulate infectious virus by the process of phagocytosis. In these cells, the internalized virus is not destroyed but rather progressively accumulates in these cells thereby adding to the tissue burden of infectious virus. These cells thus serve as continuous sources of infectious virus to other cell types within infected swine and are also ultimately sources of infectious virus to the environment and thus to uninfected swine housed with these infected animals.

The form of the circovirus genome is unique among the eukaryotic DNA viruses. The infectious virion is composed of circularized negative sense single stranded (ss) DNA, encapsidated by a single structural (nucleocapsid) protein. The intracellular double stranded (ds) replicative form (dsDNA) is produced from DNA virion template by PCV-2 Rep and host cell DNA polymerases. As in other viral systems, virion components are produced in large amounts within infected cells. Infectious virus is assembled in cell nuclei and cytoplasm and is released into the environment. For other nonenveloped DNA viruses, this is accomplished by cytolysis, an event which also releases unassembled viral proteins, DNA species and fragments of each.

Comparison of titers of infectious virus and DNA copy number in infected tissues demonstrate that viral ssDNA is produced in many-fold excess the number of complete virions. Copy numbers exceeding 1015 DNA copies per one-tenth gram of tissue are observed. In viremic PMWS serum, copy numbers in excess of 1012 per ml have been found. These findings suggest that free viral DNA, devoid of structural protein, may be infectious for adjacent cells or macrophages engaged in the process of tissue debridement and perhaps even to the environment. In a pilot study, we have shown that viral DNA alone is infectious for gnotobiotic swine. In this NPB-funded grant proposal, we wished to confirm that viral DNA species, devoid of encapsidation with the viral protein, is infectious for swine.

These findings, if confirmed will identify a novel form of infectivity for viral diseases in general and PCV-2 virus specifically and ultimately may explain the finding that piglets can become infected with PCV-2, even when they are “protected” by high levels of maternal origin anti-PCV-2 antibodies. In conducting this experiment, a major issue of concern is the stability of the viral DNAs. While the ssDNAs are predicted to possess very stable secondary structures, they are also likely sensitive to endogenous (DNA) nuclease degradation. Any nuclease activity which nicks the unencapsidated covalently closed circular ssDNA would render the resultant linearized DNA noninfectious. In contrast, the replicative DNA form (dsDNA) molecule is predicted to be less stable that the ssDNA form but not as sensitive to nuclease actions. Our previous work indicates that the ssDNA form of PCV-2 predominates in infected PMWS tissues and it is very likely that this form will predominate (along with encapsidated ssDNA in virions) in viremic serum and thus will be infectious when tested in gnotobiotic swine. Infectivity for the dsDNA form is expected to be low, since this form is covalently bound together. In swine, this form is produced intracellularly during the DNA replication cycle and opportunities for exposure of the dsDNA form to adjacent susceptible cells is expected to be limited.

The significance of the infectious DNA experiments, if successful, would not only be the demonstration of this fact but would also provide a molecular (and unconventional) explanation for viral persistence and spread of infectious materials within an “immune” host. In practical terms, this would provide a ready explanation for the observation that suckling piglets, presumably “protected” from infection by encapsidated conventional virus by acquisition of maternal-origin antibodies are frequently virus-positive and become infected within the first several weeks of life. The other explanation for early post-natal disease (in utero infection), while remotely possible in some cases, is not supported by survey data for presence of virus or antibodies in pre-colostral piglet sera nor by current epidemiologic data. Thus, DNA infectivity in this disease process may play a crucial role in the spread of this virus both within and between young swine in the face of conventional protection provided by maternal antibodies to nucleocapsid or by conventional vaccination processes.

Experimental Objective(s) The overall objective of this experiment is to determine if nonencapsidated single stranded (ss) and/or double stranded (ds) PCV-2 viral DNAs (both absent nucleocapsid protein) are infectious for gnotobiotic swine. The ssDNA form predominates in viremic plasma and is in the form which is encapsidated by nucleocapsid protein to make the traditional infectious virion. The dsDNA form is the intermediate or replicative DNA form of the virus and is the stable form of DNA produced during the viral replication cycle as the viral DNA is replicated in cells. The latter DNA form (dsDNA) is that which is used for transfection experiments.

Materials and Methods:

Viral DNAs With both forms of viral DNA, a significant impediment to determining infectivity of these in swine is the strong possibility that either or both will be inactivated by ubiquitous porcine viral DNAses, enzymes which hydrolyse free DNA. The double stranded DNA form is expected to be relatively resistant to the actions of DNAses and is thus a "control" for the ssDNA form. The ssDNA form, while chemically stable, is likely to be very susceptible to DNAses. For preparation of viral DNAs, molecularly cloned PCV-2 (Stoon 1010/Imp) genotype was used to generate infectious virus pools (2-3 passages on PK15 cells) by transfection of cloned DNA into PK15 cell monolayers. PCV 2 ssDNA and dsDNAs for inoculation were prepared from infected PK15 monolayers by a combination of low molecular weight DNA extraction and nuclease digestions by our colleagues in Northern Ireland (B Meehan, PhD). A total of 1015 DNA copies per 10 ul were recovered from each of these preparations. After substantial regulatory (APHIS and USDA) delays relating to the import of PCV-2 DNAs into the USA, these DNAs were received at OSU and the in vivo infectivity and challenge experiment was conducted.

Gnotobiotic Swine The modified closed Caesarian derivation surgical procedure used to derive litters of piglets (n=12/litter). Pregnant sows (and hence the piglets) were purchased from a local commercial pork producer who is free of PMWS. This operation is a closed specific-pathogen-free facility under intensive management. Sows are variably PCV-2 seropositive but piglets derived from this operation are seronegative indicating that transplacental transmission of PCV-2 in this herd does not occur. The date-mated sow is restrained in a squeeze chute, tranquilized with ketamine and epidural administration of 2.0% (v/v) lidocaine is given to effect anesthesia from the sternum caudally. Hindquarters are elevated and, after preparation of the surgical field, a ventral midline incision is made. The gravid uterus is exteriorized, severed from its abdominal attachments and entered into a transfer tank filled with disinfectant. The sow is immediately euthanatized by electrocution and exsanguination. Within the isolation unit, piglets are removed from the uterus, stimulated to breathe and, after resection of umbilici, are transferred into sterile pen-tub isolation units containing 6 separate partitions. Groups of similarly inoculated piglets can be maintained together until 7-8 weeks of age in separate self-contained isolation units in individual cubicles. Each isolation unit is equipped with a separate air exchange system. Blood samples for serum and hemograms were collected weekly or more frequently as circumstances dictate by jugular venipuncture from sedated animals. Piglets are fed a liquid sow milk replacement diet four times daily. Piglets raised in this fashion can be maintained as gnotobiotes for 45-60 days of age. Size, space limitations and changing nutritional requirements preclude longer time intervals in the germfree isolation units.

Route of Infection with DNAs To circumvent the possible problem of DNAse inactivation, two routes of infection with each form of DNA were used. A subset of gnotobiotic swine were directly injected with DNA forms into the left superficial inguinal lymph node. These regional lymph nodes were then subjected to local immunostimulation by a left hip injection of KLH/ICFA. A second subset of gnotobiotic swine were immunostimulated by injections of KLH/ICFA (both axilla and both hips) and each viral DNA form was then injected by a systemic (intraperitoneal) route.

Results: The summary results for the infectivity of viral DNAs are presented in Table 1. While preliminary, the data show that the ssDNA form, absent encapsidation by nucleocapsid protein is, in fact, infectious for gnotobiotic swine. For clarity, results are described by route (local versus systemic) routes of infection.

Table 1 A summary of the infectivity of PCV-2 viral DNAs for gnotobiotic swine.

Piglet No and Group	M/F	wt (gms)	Gross Findings L IngLN	LNs	Bron LN	Liver	Lung	Conclusions (disease status)
Local Infectivity: Left inguinal lymph node injection with PCV-2 ssDNA								
03-3158	M	1750	2*	1-2	3	-	-	subclinical PCV-2 infection
03-3159	M	2660	2	1	2	tan	-	self-limiting PCV-2 infection
Systemic Infectivity: Intraperitoneal injection with PCV-2 ssDNA								
03-3151	F	2590	2	2	3	ascites, pale tan	interstitial pneumonia	pre-clinical PMWS
03-3153	M	2780	2	2	3	-	interstitial pneumonia	pre-clinical PMWS/sub-clinical PCV-2 infection
03-3152	M	3230	2	2	3	-	-	contact control with sub-clinical PCV-2 infection
Local Infectivity: Left inguinal lymph node injection with PCV-2 dsDNA								
03-3156	F	2470	1	-	-	-	-	no evidence for PCV-2 infection
03-3157	M	3010	1	-	-	-	-	no evidence for PCV-2 infection
Systemic Infectivity: Intraperitoneal injection with PCV-2 dsDNA								
03-3154	M	2690	1	1-2	1	pale	-	possible self-limiting infection
03-3155	M	2900	2	1-2	2	-	-	possible self-limiting infection

* Scores determined on a subjective scale as negative (-) or not different from normal to 3 markedly different from the normal.

Local route of infection The left inguinal lymphadenopathy seen in piglets injected with either ds- or ssDNAs directly into that lymph node are attributable to immunostimulation with KLH/ICFA. However, the generalized lymphadenopathy (particularly of bronchial lymph nodes) in the ssDNA infection group is striking and suggestive of subclinical but active PCV-2 infection. In contrast, neither generalized

lymphadenopathy nor any other evidence of PCV-2 infection was seen in swine inoculated with dsDNA.

Systemic (intraperitoneal) route of infection Both KLH/ICFA-immunostimulated piglets inoculated with ssDNA of PCV-2 developed pre-clinical PMWS-like disease. That is, both demonstrated generalized lymphadenopathy (greater than that seen in uninfected piglets immunostimulated with KLH/ICFA), hepatic disease and interstitial pneumonia. Particularly striking was bronchial lymphadenopathy and interstitial pneumonia. The latter is extremely uncommon in PCV-2-infected gnotobiotes. Of interest also was the apparent subclinical infection of a co-housed contact control. In contrast, evidence for PCV-2 infection in the two gnotobiotes systemically inoculated with dsDNA was scanty.

Serology, histopathology immunohistochemistry and quantitative virus isolation These assays are ongoing in these PCV-2-DNA-inoculated swine. As we have shown in many previous studies these assays will confirm and extend the findings outlined above.

Discussion: From previous work, we know that high amounts of nonencapsidated viral DNA are produced in infected PMWS tissues. In fact, greater than 1015 DNA copies per 0.10 gm of tissue are produced. Both the ss- and dsDNA forms circulate in viremic plasma (>1010 copies per ml) and we have shown by PCR that viral DNA is shed from all excretions and secretions. This large amount of foreign DNA is unprecedented in viral infectious diseases and suggests that, in the case of PCV-2, an additional, novel and potentially important source of infectious material not only to the environment but also to adjacent uninfected cells is DNA alone. That DNA can infect cells both in vitro and in vivo is amply demonstrated by the fact that cell transfection experiments and even DNA-based vaccines are now "hot topics" in biology. However, the natural infectivity of viral DNAs has not yet been investigated and assessed. This is potentially important in that viral DNA is, in theory, infectious in the presence of passive or active immunity (eg. neutralizing antibodies which bind to nucleocapsid protein but not the DNAs).

The experiment described herein was deceptively difficult to accomplish. Preparation and quantitation of viral DNAs was a larger experimental hurdle than expected, particularly since it was decided early on to utilize viral DNA preparations from very low passage cloned Stoon 1010 virus to minimize any chance of using viral DNAs from a high passed PCV-2 which may have mutated from field form as a result of prolonged in vitro passage in PK15 cells. Once the transfection experiments with cloned Stoon 1010 (field PCV-2) was accomplished, methods for extraction of the two forms of DNA, separate from each other and free of viral nucleocapsid protein were developed. Lastly, these DNAs were prepared in Northern Ireland and sent to OSU for in vivo testing. This last hurdle (approval from APHIS and the USDA) took almost 1 year to accomplish. Not only has security since 9/11 been increased, but also the fact that the UK (including Northern Ireland) was in the midst of a foot and mouth disease outbreak) further delayed shipment of materials.

Our data show that at least one (ssDNA) and possibly both viral DNA species is infectious and pathogenic in gnotobiotic swine. The next logical step is assessment of the biological significance of this phenomenon to determine the relevance of infectious DNA for disease production under natural conditions. The most direct and biologically relevant approach to the question of biological significance is to determine if piglets, passively protected from PCV-2 infection by maternal antibody can be infected with

PCV-2 DNAs. Piglets raised under natural husbandry conditions, acquire virtually all maternal immunity by colostrum absorption from the gut within the first 24-48 hrs of life. Epidemiologic evidence suggests that the nursery environment is heavily contaminated with PCV-2 which is highly resistant to environmental and chemical disinfection. It is believed that maternal antibody neutralizes infectivity of virions via binding to one or more of the nucleocapsid epitopes. In contrast, unencapsidated DNAs should not be affected by these colostrum-origin antibodies. In spite of maternal-origin passive protection, many piglets acquire infection during this time interval although infection is not expressed as PMWS until after the decline of maternal antibodies at 6-8 weeks of age. This history suggests that infectious DNA may be of biologic relevance in transmitting (and hence establishing) infection in passively-protected neonatal swine. This hypothesis can be readily tested under gnotobiotic conditions since the neonates are both PCV-2-antibody- and viremia-negative at derivation and there is no opportunity for post-natal exposure to the agent or to colostrum. In these animals, passive immune protection can be simulated by parenteral administration of maternal-origin serum. Thus, we are in the position to directly test the hypothesis that in vivo infectivity of PCV-2 DNAs in the presence of neutralizing antibodies can be accomplished. Future work with viral DNAs will be directed toward testing this experimental hypothesis. There are three possible outcomes envisioned for these experiments.

1. If our hypothesis that the viral DNA(s) alone are infectious for swine, then some/all piglets given passive maternal-origin“protective”antibodies and inoculated with viral DNAs will develop PCV-2 associated disease or at the least, will be subclinically infected (untreated subgroups) with PCV-2. Recovered infectious virus and demonstration of viral materials in tissues will be proof of this supposition. There is the possibility that passively administered antibodies will limit the spread of infectious progeny virions in piglets since it is assumed that maternally acquired immunity “protects” piglets for several weeks of age. However, temporary protection is not the same as prevention of infection and clinically “protected” piglets are still virus-positive in the piglet nursery environment. Moreover, persistent infection in the presence of active immune responses is characteristic of disease in subclinically infected swine and we would therefore expect to recover infectious virus from target tissues and demonstrate viral materials in tissues in spite of the presence of these circulating antibodies of either maternal or piglet origin.

2. Of course if infection is not established, then our hypothesis is negated and we will tentatively conclude that while DNA is infectious, this source of infectivity is not likely biologically significant for disease production under natural conditions.

3. There is a third possibility. It is possible that maternal immune serum contains antibodies which bind PCV-2 DNA and “neutralize” infectivity of the DNAs. However, our Belfast colleagues have examined PMWS sera for anti-DNA antibodies; to date, none have been found.

Lay Interpretation: Porcine circovirus type 2 (PCV-2) infection is now recognized to be a common subclinical infection of swine of all ages. Disease expression as postweaning multisystemic wasting syndrome (PMWS) is seen in piglets in the post-weaning stage of development and is a reflection of systemic upregulation of the virus infection in the neonatal and immediate post-weaning period. Gravid sows are seropositive for PCV-2 and they transmit maternal “protective” antibodies to their off-spring by colostrum. While this antibody is assumed to be protective, careful study of

the disease process in the field indicate that piglets acquire the infection very early in life, within several days to several weeks after farrowing. The experiments described herein have demonstrated that the nonencapsidated closed circular form of PCV-2 viral DNA is infectious when inoculated into PCV-2-susceptible gnotobiotic swine. The potential importance of this observation relates to the strong possibility that suckling piglets may acquire the infection from the environment even though they have high levels of maternal origin virus neutralizing antibodies. While much work remains to be done, these experiments highlight the distinct possibility that infection of piglets by this unconventional means permits the establishment of viral infection within them even though they are seropositive through colostral (maternal) immune mechanisms.

Manuscripts and publications supported wholely or in part by the NPB

Clark, E. G., Ellis, J. Allan, G and Krakowka, S.: Postweaning Multisystemic Wasting Syndrome in Swine. In: Infectious Diseases of Africa with special reference to southern Africa, J. A. W. Coetzer, editor-in-chief, in press, 2004.

Krakowka, S., Ellis, J. A., McNeilly, F., Meehan, B., Rings, D. M., McCullough, K., Botner, A.,Nauwynck, H., Charreyere, C., Allan, G.: The Pathogenesis of PCV-2-associated Postweaning Multisystemic Wasting Syndrome. Fourth Intl Symposium, Emerging and Re-emerging Pig Diseases, Rome, It., June 29-July 3, p143-148, 2003.

Krakowka, S., Ellis, J., McNeilly, F., Meehan, B., Oglesbee, M., Alldinger, S., Allan, G.: Features of Cell Degeneration and Death in Hepatic Failure and Systemic Lymphoid Depletion Characteristic of PCV-2-Associated Postweaning Multisystemic Wasting Disease (PMWS). Vet Pathol, in press, 2004.

Krakowka, S, Ellis, J., McNeilly, F, Meehan, B., Waldner, C., Allan, G.: Features of Experimental Porcine Circovirus (PCV)-2 Disease: Correlations Between Histopathologic Lesions, Amount and Distribution of Virus to Clinical Outcome. J Vet Diag Invest, submitted for publication, 2004.

Krakowka, S., Ellis, J., McNeilly, F., Meehan, B., McCullough, K., Charreyre, C., Botner, A., and Allan, G.: Type 2 Porcine Circovirus Diseases (PCVD) in Swine. Symposium on Swine Infectious Diseases, Pulawy (Warsaw) Poland, June 8-10, 2004.