2007
International Porcine Reproductive and Respiratory Syndrome (PRRS) Symposium

PRRS and PRRSV- Related Diseases: Prevention and Control Strategies

Chicago, Illinois
November 30 - December 1, 2007

International PRRS Symposium (IPRRSS)
Chicago, Illinois
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PROGRAM and SCHEDULE OF ACTIVITIES

FRIDAY NOVEMBER 30, 2007

14:00	Registrants check in (Pre-registration required. No walk-ins accepted) Poster set-up in rooms F, G, H (refer to poster numbers in proceedings) All lectures in Chicago Ballroom E
14:15	NC-229 "Porcine Reproductive and Respiratory Disease: Methods for the integrated control, prevention and elimination of PRRS in United States Swine Herds" - Station Representatives meeting

OPENING SESSION
Co-Chairs: R (Bob) Rowland and P Zaabel

16:00	Welcome. R Rowland, Chair - 2007 International PRRS Symposium
16:10	Keynote Presentation: *Epidemiological studies: Can they help us improve our PRRS eradication efforts?* (#1). MCM de Jong, Wageningen University, The Netherlands
17:15	Reception and cash bar
17:15	Poster Session 1 (authors of odd-numbered posters at their posters until 18:00)

SATURDAY DECEMBER 1, 2006

PRRS ECOLOGY
Co-Chairs: R Morrison and J Zimmerman

8:00	Keynote Presentation: *PRRS in China* (#2). G-Z Tong, Harbin Veterinary Research Institute, China
8:50	*Network analysis of the role of pig movements in area spread of PRRSV* (#3). W Spencer, University of Minnesota
9:10	*Introduction and eradication of PRRSV in Sweden* (#15). U Carlsson, National Veterinary Institute, Uppsala, Sweden
9:30	*PRRSV - a role in the "High Fever" swine disease in China?* (#14). F Leung, University of Hong Kong
9:50	Coffee break, Poster Session 2 (authors of even-numbered posters at their posters)

VIRAL GENOME AND HETEROGENEITY
Co-Chairs: K Lager and J Christopher-Hennings

10:50	*Evolution of PRRSV genomes associated with failed sow protection* (#35). J Abrahante, University of Minnesota
11:20	*PRRSV non-structural protein 2: Potential functions in viral replication and pathogenesis* (#33). Y Fang, South Dakota State University
11:40	*Attenuation of PRRSV by chimera construction* (#29). K Faaberg, USDA, ARS, National Animal Disease Center

Noon Luncheon - buffet available in poster area

IMMUNOLOGY AND VACCINES
Co-Chairs: L Enjuanes and D Yoo

13:00 *Swine immunity and resistance to persistent PRRSV infection* (#49). J Lunney, BARC, ARS, USDA

13:20 *Certain PRRSV proteins inhibit INF-β promoter activation* (#59). L Beura, University of Nebraska, Lincoln

13:40 *Serum neutralization of PRRSV infection of porcine alveolar macrophages* (#48). J Li, University of Minnesota

14:00 *Impact of modified-live PRRSV vaccination for control of PRRS in nursery pigs* (#40). R Philips, Boehringer Ingelheim Vetmedica, Inc.

14:20 Break – refreshments in poster area

PRRS-RELATED DISEASES
Co-Chairs: E Thacker and JK Lunney

14:40 Keynote Presentation: *PRRS-related disease: PCVAD* (#67). XJ Meng, Virginia Polytechnic Institute and State University

15:30 *PRRS and research on PRRS in Vietnam* (#74). LT To, Department of Animal Health, Hanoi, Vietnam

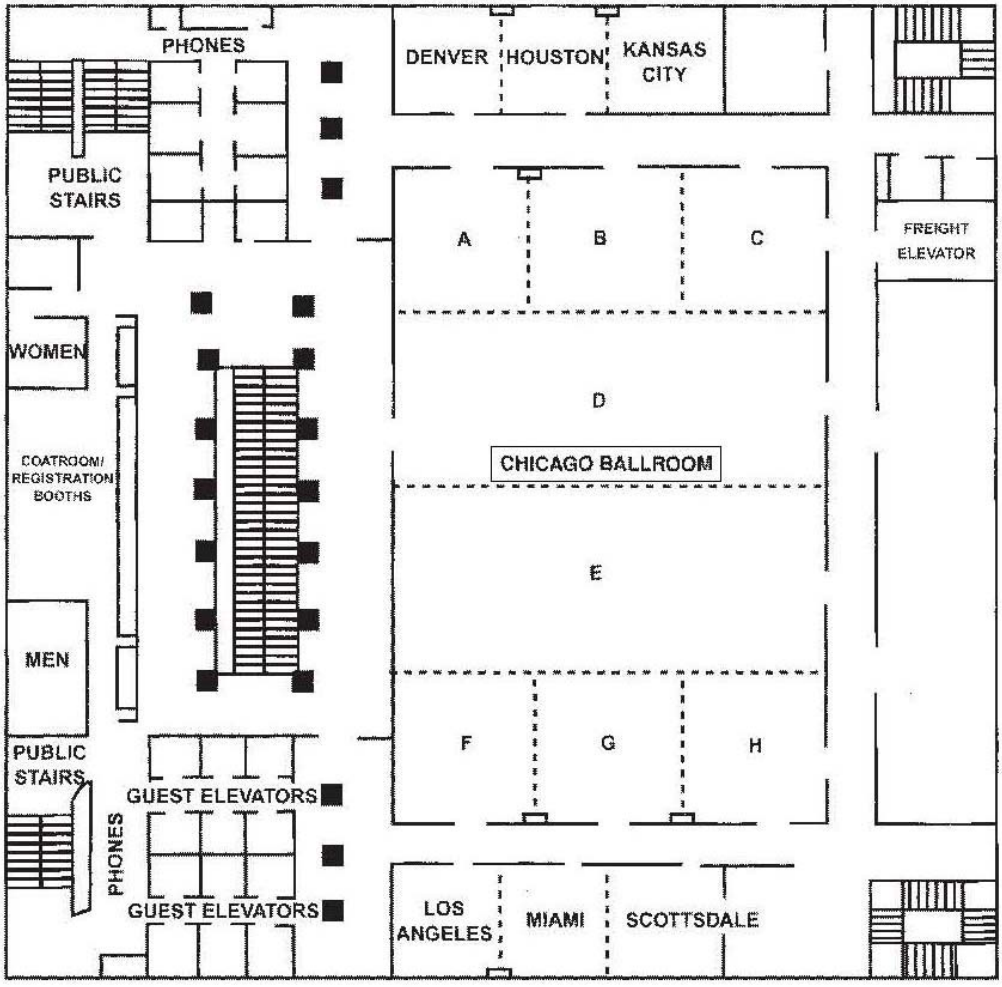
15:50 *Collagenous lectin gene polymorphisms associated with the severity of pneumonia in pigs infected with PRRSV* (#76). MA Hayes, University of Guelph

16:10 *Potential of PRRSV as a dual vaccine vector for PRRS and PCV2* (#68). D Yoo, University of Illinois at Champaign-Urbana

16:30 Adjourn. Please pick up your posters after the meeting.
(Note: rooms are reserved for another function at 17:00)

LOCATION INFORMATION

Chicago Ballroom (5th Floor)
Chicago Marriott Downtown Magnificent Mile
540 North Michigan Avenue
Chicago, Illinois 60611



ABSTRACTS

Poster No.

SECTION 1: PRRS ECOLOGY

EPIDEMIOLOGICAL STUDIES: CAN THEY HELP TO IMPROVE PRRSV ERADICATION EFFORTS?

MCM de Jong. Department of Quantitative Veterinary Epidemiology, Wageningen University

Infectious disease epidemiology develops from being solely descriptive and hypothesis generating to more in-depth study of the dynamics of infections. When we study the dynamics of infections we quantify transmission under different circumstances. These measurements lead to a better understanding of the characteristics of the virus, of the host-virus interaction and of the contact between the hosts. Methods that can be used to quantify transmission will be discussed and illustrated with applications to the control of PRRSV.

The main issues for PRRSV control being the persistence in individual hosts, vaccination, transmission between farms, eradication from farms, certification, and wild-life reservoirs. How can epidemiology help to identify the key questions regarding these issues and help to design studies?

PRRS IN CHINA

G-Z Tong*, Z-J Tian, Y-J Zhou, X-F Hao, T-Q An, T-C Wei, H-J Qiu, X-H Cai. Division of Swine Infectious Diseases, National Key Laboratory of Veterinary Biotechnology, Harbin Veterinary Research Institute, CAAS, Harbin 150001, China

Previously, pigs were raised in China mainly by farmers, but in recent years there have been an increasing number of intensive integrated operations coincident with the development of the swine industry. With the increased scale of farms and feeding density, as well as continuous entry of new litters, the morbidity and mortality of swine respiratory and reproductive diseases have also risen and the etiology has become more complicated.

PRRS, a previously unrecognized disease, was first reported in USA in the late 1980s. The disease spread rapidly worldwide in following years. In 1995, the first outbreak of a PRRS-like disease was reported near Beijing. The etiology was determined soon after, as several PRRS viruses were isolated from the aborted fetuses. Within a few years of this outbreak, many pig farms in most provinces of China became endemically infected. PRRS is now one of the severest threats to the swine industry in China. In 2006, a highly pathogenic infectious disease appeared in pigs in the central region of China. This disease was characterized by prolonged high-fever (>41°C), anorexia, red discoloration of the ears and body, desiccant excrement in early days, and loose bowels in later periods. In the following months, the disease spread quickly to the neighboring regions - the most densely populated major pig-producing provinces in China. The morbidity was 50-100% and the mortality rate reached 20-100% in some outbreaks.

An extensive survey was conducted to determine whether PRRSV was the cause. Over 70 samples (lung, kidney, spleen, liver, lymph nodes, brain and serum) collected from different swine farms in 15 provinces were found to be PRRSV-positive by RT-PCR, based on open reading frame (ORF) 5 and NSP2. In clinical samples, PRRSV was found to be the dominant virus. Experimental infection of pigs with one PRRSV isolate demonstrated that the disease could be reproduced with clinical signs virtually identical to what we saw in the field. The mortality caused by the novel PRRSV isolate in experimental infection differed, depending on the age of pigs, almost 100% for those under 60-day-old and 0% for those over 6-month-old. The morbidity caused by the virus was 100% for all age pigs.

Four out of the 60 isolates were full-length genomic sequenced. Comparative analysis revealed that these PRRSVs were of the NA-type and two deletions in the NSP2 region; one amino acid deletion at position 483, and 29 amino acids deletion at positions 535-563. Further sequencing of the NSP2 gene and ORF5 gene of all the 60 isolates indicated that these PRRSV isolates from different geographical areas shared a common feature of two non-continuous deletions of 30 amino acids in the NSP2 region. These PRRSV isolates were similar from each other with the sequence homology of 98.2%-100%, while their homology with previously described classical PRRSVs was only 69.8-86.2%. The GP5 sequences of new isolates from different geographical areas were highly homologous (98.5%-100.0%) with each other, and centralized into a single clade in the unrooted phylogenetic tree.

NETWORK ANALYSIS OF THE ROLE OF PIG MOVEMENTS IN AREA SPREAD OF PRRSV

SR Wayne*, P Davies. College of Veterinary Medicine, University of Minnesota

Knowledge of swine populations (location and disease status) is necessary for effective regional disease eradication. Swine populations, however, are dynamic, with animals frequently moving between premises, particularly in multiple site systems. The goals of this study are to use methods of social network analysis to describe and analyze pig movement patterns and to assess the potential impact of pig movement on PRRS spread in an area. Farms enrolled in the Rice and Stevens County PRRS Project were surveyed to gather animal movement information. Farm capacity, farm type, movement frequency, animals per movement, and type of animal moved were collected for all farms in the study areas. Data were entered into a relational database, were processed and presented for analysis with network analysis software (UCINET and NetDraw). Visualization of pig movement patterns was accomplished with GIS software (ArcGIS and Flow-Mapper: Flow Data Model Tools). Geographic coordinates for each location were also used to calculate inter-farm distances. Preliminary analyses indicate a high prevalence of multi-site production in both study regions, a high prevalence of outside-of-county relationships, a highly skewed distribution of travel distances, and high second-degree connectivity amongst farms through markets. These observations indicate that regional PRRS control efforts may be complicated by the high frequency of pig movements among and within regions; and regional movement patterns are likely to be important determinants of the feasibility and success of control efforts.

USE OF A PRODUCTION REGION MODEL TO EVALUATE PRRSV TRANSMISSION AND BIOSECURITY

SA Dee*, AN Pitkin, J Deen. Swine Disease Eradication Center, University of Minnesota

Area spread of PRRSV is the re-introduction of an unrelated variant of the virus via an unknown route. The inability to prevent PRRSV area spread has crippled large-scale eradication efforts. Therefore, the purpose of this study was to develop a model of a swine production region that was endemically infected with PRRSV and assess various routes of spread, the efficacy of different levels of biosecurity and to observe meteorological conditions related to airborne transmission. The model consisted of 4 facilities, the source population (experimentally inoculated with PRRSV MN-184), the high level (air filtration) biosecurity facility, the medium level (no filtration) facility and the low level (spread control) facility. Every day for 1 year, samples (air, swabs, insects and swine sera) were collected in order to track viral movements between sites, along with 18 meteorological variables. Samples were tested by PCR and meteorological data were analyzed by multivariate logistic regression, principal component analysis and factor analysis to identify variables significantly related to the presence of PRRSV in air (AIRPOSMLF). Over the course the study, PRRSV transport and transmission were not observed in the high level facility, while a 31% infection rate was observed in the medium level facility with air as the primary route. In contrast, the low level facility became infected 66% of the time via air, fomites/personnel and insects. Analysis of meteorological data indicated that wind direction, humidity, UV index and barometric pressure significantly influenced AIRPOSMLF and that clusters of related variables could be used to describe “high risk” and “low risk” atmospheric conditions for the presence of PRRSV in air. These results verify previously reported routes of transmission of PRRSV, demonstrate the ability to prevent area spread of PRRSV and describe a set of meteorological risk factors and conditions capable of influencing the risk of PRRSV in aerosols. It is hoped that this information will enhance the success of large-scale PRRS eradication efforts.

REPLICATION OF PRRSV IN LABORATORY RODENTS

P Rosenfeld[1,] D Yoo[1,2]. [1]Department of Pathobiology, University of Guelph; [2]Department of Pathobiology, University of Illinois at Urbana-Champaign

PRRSV is widespread worldwide, and PRRSV-negative pigs are often difficult to find for the study of PRRSV in vivo. To determine if a small animal model could be developed for PRRSV, three species of laboratory rodents were examined for their susceptibility to the virus. No virus replication was detected in BALB/c or SCID (severe combined immunodeficiency) mice after intra-peritoneal inoculation. Moderate replication of PRRSV was detected in primary cell culture of cotton rat lungs in vitro, but no viral replication was detected following intra-nasal or intra-peritoneal inoculation. By intra-tracheal inoculation, replicating virus was detected in the lungs of cotton rats but only for 1 day post-inoculation. This study indicates that PRRSV replication in rodent species is inefficient and suggests that the presence of a rodent reservoir for PRRSV in the wild is unlikely.

PRRSV ELISA USING ORAL FLUID SAMPLES: A POTENTIAL SURVEILLANCE TOOL?

J Prickett[1], J Johnson[1], M Ameri[1,] M Roof[2], K Klinge[2], J Zimmerman[1]. [1]College of Veterinary Medicine, Iowa State University; [2]Boehringer Ingelheim Vetmedica, Inc., Ames, Iowa

Introduction In human medicine, a variety of infections may be surveyed by detecting antibodies and/or pathogens in oral fluid samples: HIV, *H. pylori*, mumps, measles, rubella, and hepatitis A, B, and C. The diagnostic application of oral fluids has been sparse in veterinary diagnostic medicine, but has been applied to the detection of *E. coli* O157:H7 and salmonella in cattle and the diagnosis of feline leukemia virus in cats. In swine, oral fluids from PRRSV-infected pigs were previously shown to contain PCR-detectable levels of virus. In the present study, we report adaptation of a commercial ELISA to the detection of anti-PRRSV antibodies in oral fluids.

Research has shown that human oral fluids contain 10^{-2} to 10^{-3} the concentration of antibodies in serum. Cameron and Carman (2005) assert that most serum antibody assays can be adapted to detect antibodies in oral fluids by modifying assay protocols and/or testing conditions. Therefore, the objective of this research was to adapt a commercial PRRSV ELISA to the detection of anti-PRRSV antibodies in swine oral fluid samples.

Experimental Design Three research barns, each with 2 pens of 10 pigs, were used in this study. PRRSV ELISA and qRT-PCR were performed on serum samples to confirm that pigs were free of PRRSV infection prior to inoculation. Negative control animals were housed in one barn; PRRSV-inoculated animals in 2 barns. Post-inoculation, oral fluid (one sample per pen) and serum samples (10 samples per pen) were collected weekly for 63 DPI.

To optimize the PRRSV ELISA, oral fluid samples were tested under an array of operating conditions. Specifically, reagent and sample dilutions, heat inactivation of samples, incubation time, and incubation temperature were systematically varied to determine their effect on S/P response. The data were evaluated by receiver operator characteristic curve (ROC) analysis to determine the conditions that optimized diagnostic sensitivity and specificity.

Results and Discussion Among the changes to the manufacturer's protocol that most markedly improved discrimination between oral fluid samples from negative control vs PRRSV-inoculated pens were the following: 1) heat inactivation of samples (30 m at 56°C), 2) 1:3 dilution of samples, 3) overnight incubation of samples at 4°C, 4) 1:4 dilution of positive control (kit control), 5) change in S/P cutoff. Based on results to date, an S/P of 0.25 corresponded to a Se of 84% and Sp of 100%. Further improvements in diagnostic performance are expected, but the current results suggest that surveillance of nursery-grow-finish pigs using an oral fluid PRRSV ELISA is feasible.

PRRSV VERTICAL TRANSMISSION DYNAMICS IN AN ENDEMICALLY INFECTED SOW-HERD

JP Cano, S Dee, A Rovira, C Muñoz-Zanzi, R Morrison. College of Veterinary Medicine, University of Minnesota

After a PRRS outbreak, breeding herd production parameters may return to levels similar to those prior to the infection, but endemically infected herds continue "leaking" viremic piglets to the nurseries. The "traditional sampling protocol" used by swine practitioners to detect PRRSV shedding from the sow-herd is to sample 20 or 30 piglets at weaning, pool samples in groups of 5, and test them by PCR. However, in the chronic stages of the infection, the prevalence may be very low; thereby reducing the probability of detecting infected piglets. A commercial herd of 1500 sows was recently infected with PRRSV. No reproductive clinical signs had been reported for 3 months, but PCR-positive piglets continued to be weaned. The owners and the veterinarian decided to inject serum containing live PRRSV in an attempt to control the disease. This scenario gave us the opportunity to evaluate the incidence of PRRSV PCR-positive piglets and litters at birth and at weaning in an infected sow-herd, and to identify specific attributes present in PCR-positive piglets.

Serum samples were collected from every piglet of 38 litters at birth and at weaning, 4 wks after the exposure. The procedure was repeated 12 wks after serum injection. Serum samples were collected from sows before serum injection and after farrowing and were tested by PRRSV ELISA. A sample size of 38 litters from a farrowing group (n=60) was estimated based on a desired confidence interval of 95%, expected prevalence of 6.3% (1 positive pool / 4 tested pools), and 1.66 % (1 positive / 60 litters) as the lowest possible prevalence (Epi Info Version 6, Stone Mountain, GA). The proportion of viremic litters and piglets was compared using Fisher's Exact Test (Statistix® 8, Tallahassee, FL). The association between specific predictor attributes and the detection of PCR-positive piglets was evaluated by logistic regression analysis at individual and litter level.

Prior to serum injection, all of 120 sows were ELISA positive; 10% were seronegative 4 wks after exposure, and 56% were seronegative 8 wks later. The mean ELISA S/P ratio did not change 4 wks after serum injection ($p = 0.205$) but it significantly decreased 12 wks after exposure ($p = 0.001$). The detection of PRRSV was significantly lower 12 weeks after serum injection. A significant increase in the incidence of PCR-positive litters and piglets at weaning was observed during both sampling periods ($p \leq 0.05$) Of PCR-positive litters (12 wks PI), 70% had ≤2 PCR-positive piglets per litter at weaning. Only 20% of the piglets tested as PCR-positive at birth became negative by weaning.

The "traditional sampling protocol" may not detect the low prevalence determined in this study. In most litters, only a small proportion of the piglets were PCR-positive. Sampling piglets at weaning is recommended because the increase in incidence of infection and the observed prolonged viremia. Increasing sample size and targeting specific individuals may help to improve the sensitivity of monitoring protocols. While the results were significant, further studies are needed to evaluate the effect of PRRSV isolate, herd size, and control method and to better understand the dynamics of infection and to improve monitoring protocols for infected sow-herds.

THE REPLICATION OF PRRSV IN ASSOCIATION WITH CD163.

JB. Patton[1]*, RR Rowland[1], D Yoo[2], KO Chang[1]. [1]Department of Diagnostic Medicine and Pathobiology, Kansas State University; [2]Department of Pathobiology, University of Illinois at Urbana-Champaign

Porcine reproductive and respiratory syndrome virus (PRRSV) has a specific cell tropism with differentiated macrophages such as porcine alveolar macrophages (PAM). However, the precise mechanism of cell tropism and information of specific receptor(s) of PRRSV are not understood well. Several putative cell receptor(s) for PRRSV have been reported, and those include CD163 which expresses exclusively on monocytes/macrophages including PAM. CD163 is a member of scavenger receptor cystein-rich superfamily and expressed on most subpopulations of macrophages. In this study, first, we demonstrated that a pig kidney cell line, LLC-PK expressing CD163 allowed the replication of PRRSV, confirming CD163 as a receptor. We further analyzed the expression of CD163 on PAM and macrophages derived from CD14+ peripheral blood monocytes (Mac-CD14) in the correlation with PRRSV replication. By flow cytometry analysis, we discovered that over 80% of PAM or differentiated Mac-CD14 expressed CD163, and the levels of population expressing CD163 were well correlated with the replication of PRRSV. Because the expression of CD163 on monocytes/macrophages can be modulated by various agents or cytokines both in vitro and in vivo, we examined the effects of phorbol ester, lipopolysaccharide (LPS) and IL10 on the expression of CD163 and the replication of PRRSV in PAM and Mac-CD14. We found that pre-treatment of PAM or Mac-CD14 with phorbol ester or LPS resulted in a decreased expression of CD163 with a reduction of PRRSV replication. On the contrary, the incubation of Mac-CD14 with IL10 up-regulated the expression of CD163 with an increased susceptibility of PRRSV infection. These data indicate that the expression of CD163 on macrophages in different microenvironments in vivo may determine the replication of PRRSV and the virus pathogenicity.

DIAGNOSTIC PERFORMANCE OF ASSAYS FOR THE DETECTION OF ANTI-PRRSV ANTIBODIES IN MUSCLE TRANSUDATE ("MEAT JUICE") SAMPLES

RM Molina*[1], W Chittick[2], EA Nelson[3], J Christopher-Hennings[3], RRR Rowland[4], JJ Zimmerman[1]. [1]Veterinary Diagnostic Laboratory, Iowa State University; [2]Boehringer Ingelheim Vetmedica, Inc., Ames, Iowa.; [3]Department of Veterinary Science, South Dakota State University; [4]Department of Diagnostic Medicine and Pathobiology, Kansas State University

The purpose of this study was to compare the diagnostic performance of ELISA, IFA and FFN for muscle transudate ("meat juice") and serum using samples of known PRRSV infection status. Serum and muscle (longissimus dorsi) samples were collected from randomly selected animals euthanized at approximately 14 day intervals from 28 to 202 days post inoculation (DPI). Muscle transudate samples were run on a commercial PRRS ELISA at 1:2, 1:5, 1:10, 1:20, 1:40 and IFA at five dilutions (1:2, 1:5, 1:10, 1:20, 1:40). Attempts to assay muscle transudate samples for neutralizing antibodies using modified fluorescent focus neutralization (FFN) assay were unsuccessful. For the ELISA, the estimated diagnostic sensitivity and specificity of muscle transudate samples at the ROC-optimized cut-offs were >95% and 100%, respectively, for all dilutions tested. For IFA, the diagnostic sensitivity and sensitivity of muscle transudate was estimated at 63.3% and 100% at a cut-off ≥1:4. Overall, these data justify the use of muscle transudate samples collected at slaughter for PRRSV surveillance. However, better differentiation of the ELISA S/P response between negative and positive samples would be desirable before such a program were implemented.

FAILURE TO TRANSMIT PRRSV VIA CONSUMPTION OF PCR-POSITIVE MEAT

RM Molina*[1], W Chittick[2], EA Nelson[3], J Christopher-Hennings[3], R Hesse[4], RRR Rowland[4], JJ Zimmerman[1]. [1]Veterinary Diagnostic Laboratory, Iowa State University; [2]Boehringer Ingelheim Vetmedica, Inc., Ames, Iowa; [3]Department of Veterinary Science, South Dakota State University; [4]Department of Diagnostic Medicine and Pathobiology, Kansas State University

The objective of this study was to evaluate the potential for oral transmission of PRRSV via consumption of qRT-PCR-positive muscle tissue from animals of known PRRSV status. Samples were collected from animals euthanized at ~2-week intervals between 28 to 202 days post inoculation (DPI). Serum, lymphoid tissue, and longissimus dorsi muscle samples were assayed by qRT-PCR and virus isolation. By qRT-PCR: 7 (7.7%) positive among 91 serum samples, 58 (64.3%) positive of 84 lymphoid tissues samples, and 13 (14.3%) positive of 91 muscle samples. Virus isolation was successful only prior to DPI 70. Swine infectivity studies were performed by feeding 13 2-week-old PRRSV-naive pigs (recipient pigs) a minimum of 100 grams of qRT-PCR-positive meat. Thereafter, recipients were monitored for evidence of PRRSV viremia by qRT-PCR using serum samples collected 0, 7 and 14 days post-feeding. None of the 13 recipient pigs showed evidence of PRRSV infection. These findings suggested: 1) the qRT-PCR assay may have detected non-infectious virus; 2) transmission via consumption of pork may require a higher exposure dose than was present in the samples; or 3) both factors may have played a role in the outcome.

PRRSV N PROTEIN INTERACTS WITH HOST PROTEIN INHIBITOR OF ACTIVATED STAT 1 (PIAS1).

VD Rilington [1], C Song[2], D Yoo[2], HC Liu[1*] [1]Department of Animal Science, North Carolina State University; [2]Department of Pathobiology, University of Illinois at Urbana-Champaign

The PRRSV nucleocapsid (N) protein, which contains a nuclear translocation signal, participates in viral transcription. N protein may also play additional roles in PRRS viral pathogenesis via specific interactions with host proteins. To further discern the role of N protein in pathogenesis, we have employed a yeast two-hybrid system to screen for host-encoded proteins that specifically interact with N protein. The cDNA libraries that we screened were constructed using cDNA isolated from uninfected, PRRSV VR2332-infected, and PMA-stimulated macrophages. The cDNA inserts in these libraries ranged from 0.6 kb to 3.5 kb. Upon screening approximately 10^7 yeast clones that were propagated in conditional growth media, we identified protein inhibitor of activated STAT1 (PIAS1) as an interacting partner for N protein. This finding was confirmed in a parallel yeast two-hybrid study using a primate CV1 cell line cDNA library. A GST-pull down assay further confirmed the specificity of the N-PIAS1 interaction. A member of PIAS family has been shown to function as a transcriptional repressor of NF-kB. PRRSV has previously been shown to activate the NF-kB pathway. Activation of the NF-kB pathway may thus be a biologically significant aspect of PRRSV pathogenesis. Our results lead us to hypothesize that N protein may assist in activating the NF-kB pathway by sequestering PIAS1.

FEASIBILITY OF POOLED-SAMPLE TESTING FOR THE DETECTION OF PRRSV ANTIBODIES ON SERUM SAMPLES BY ELISA

A Rovira[1]*, JP Cano[1], C Muñoz-Zanzi[2]. [1]College of Veterinary Medicine; [2]School of Public Health, University of Minnesota

PRRSV surveillance in negative sow herds is usually performed by testing for the presence of antibodies against PRRSV in serum with a commercial ELISA test. However, the performance of this procedure, in terms of its sensitivity, is limited by budget constraints that determine the sample size. In addition, the use of a diagnostic test with relatively low specificity results in a large number of samples testing false positive, which need to be tested with a confirmatory test. Pooled-sample testing has proved to be an effective strategy to increase the sensitivity and specificity of some monitoring protocols for low-prevalence diseases without increasing its cost. Therefore, a study was conducted to evaluate the feasibility of pooling serum samples for detection of PRRSV antibodies by ELISA.

The effect of pool size on the sensitivity and specificity of the ELISA test was evaluated by testing 113 true positive samples and 100 false positive samples, respectively, diluted in negative sera. All samples were tested undiluted and diluted 1:2, 1:4, 1:6, 1:8 and 1:10 in negative sera to simulate the effect of pooling in pool sizes from 2 to 10. The results were evaluated at 3 different cut-off values of 0.4, 0.3, and 0.2. Furthermore, the obtained sensitivity and specificity estimates were used to calculate the herd sensitivity and herd specificity of surveillance protocols in different scenarios.

The results showed that pooling serum samples to detect PRRSV antibodies resulted in a decrease in sensitivity and an increase in specificity, compared to testing individual samples, while the reduction of the s/p cut-off value recommended by the manufacturer (0.4) had the opposite effect. Sensitivity estimates ranged from 0.92 (undiluted sample, cut-off 0.2) to 0.42 (pools of 10, cut-off 0.4). Specificity estimates ranged from 0.952 (undiluted sample, cut-off 0.2) to 0.999 (pools of 10, cut-off 0.4). At the herd level, most of the protocols evaluated were superior to the standard protocol (undiluted samples, cut-off 0.4).

We describe an approach that can increase the herd sensitivity of a surveillance protocol for breeding herds, while maintaining high herd specificity and low testing costs. This can be achieved by sampling a larger number of animals and running the samples in pools. Therefore, the conventional monitoring protocols based on ELISA on individual samples could be improved by using pooled-sample testing.

We thank Dr Dudley, Dr Torremorell and Dr Goyal for providing diagnostic samples for this study. This project was funded by the National Pork Board grant 05-163.

COMPARATIVE INFECTION EFFICIENCY OF PRRSV FIELD ISOLATES ON MA-104 CELLS AND PORCINE ALVEOLAR MACROPHAGES

M Fuentes de Abin[1], G Spronk[2], J Abrahante[1], MP Murtaugh[1*] [1]Department of Veterinary and Biomedical Sciences, University of Minnesota; [2]Pipestone Veterinary Clinic, Pipestone, Minnesota

The purpose of this study was to determine if PPAM is a more sensitive tool to isolate field strains PRRSV than MA104. To compare the sensitivity of both cells, PPAM and MA 104 were inoculated with fifty sera samples from pig farms chronically affected with PRRSv; The PRRSV-VR2332 strain was used as a control positive. We found that out of 50 sera samples tested 20 (40%) PRRSV field strains were isolated by one passage on PPAM; after three blind passes only one (2%) field strain grew in MA104. VR2332 grew better in MA104 reaching a titer of 10^7 $TCDI_{50}$/ml in 72 h, when PPAM were infected with VR 2332 (m.o.i. 2), the highest titer obtain was 10^2 $TCDI_{50}$/ml. Additionally, when PRRSV VR-2332 was used as a control positive, it did not replicate on PPAM. To evaluate PRRSV infectivity on cells, the cyto-pathogenic effect, immunofluorescence and RT-PCR were used. In this study we found than PPAM are more sensitive to isolate PRRSV field strain than MA104 cells and that VR 2332 PRRSV strain grew better in continuous cell line, in this case MA104.

PRRSV - A ROLE IN THE "HIGH FEVER" SWINE DISEASE IN CHINA?

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A large-scale outbreak of an unknown porcine disease associated with high fever emerged in mainland China from mid 2006 to early 2007. Recent study has reported that the causative agent for this disease outbreak is probably a mutated strain of PRRSV. In Hong Kong, a similar high fever disease outbreak also occurred in early 2007. Lung tissues were collected from deceased animals showing fever and PRRS symptoms and RT-PCR was performed amplifying ORF5 and nsp2 genes for genetic analysis. Phylogenetic analysis of ORF5 nucleotide sequences revealed that the strain (P385) isolated from this Hong Kong outbreak was different from those isolated in mainland China (JXA1, HUB2 and HEB1), with 80.1 to 80.8% nucleotide identity. On the other hand, these mainland-isolates showed high homology to the samples collected locally in typical PRRSV-infected animals in 2004 to 2005 (98.9 to 99.1 % similarity). Identical amino acid sequences were observed in the hypervariable region I and II suggesting that the outbreak of the high fever disease may not be directly related to the variations in this major neutralizing epitope. Besides, study on the nsp2 protein indicated that the P385 strain contains two discontinuous deletions with the sizes of 125 and 5 amino acids. When compared with the VR-2332 strain, these deletions occurred in positions 315-440 and 483-487 respectively, which were downstream to the cysteine protease (PL2) domain of the nsp2 protein. The P385 strain was inoculated into MARC-145 cells in parallel with strains possessed similar ORF5 sequence but no deletion in nsp2. Preliminary result showed that such discontinuous deletions did not confer a high virulence in vitro than those of the wild type PRRSV. Present results suggest that such deletion may not necessarily contribute to the increase in PRRSV virulence leading to the outbreak of the high fever disease. Further characterization and in vivo studies are to be carried out to clarify the role of PRRSV and its nsp2 protein in this epidemic. In summary, deletion occurred in nsp2 of PRRSV may not completely account for the outbreak of the high fever disease. Some other factors, for example, co-infection with PCV2, may probably be involved in exerting selective pressure to the virus and affecting the pathogenic process.

INTRODUCTION AND ERADICATION OF PRRS IN SWEDEN

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Porcine Respiratory and Reproductive Syndrome (PRRS) has been diagnosed for the first time in Sweden. Antibody positive samples were found through routine sampling within the national PRRS serosurveillance program which has been conducted since 1994.

The first herd was suspected for PRRS on July 5 2007 and confirmed positive on July 6. Within a day intensified sampling was initiated in a number of contact herds. Another seven herds, all situated in the south of Sweden, were declared infected within a time frame of ten days. On the infected premises the with-in herd prevalence varied between 43 and 100 %. Clinical symptoms had not been observed; however, during the follow up investigations suboptimal reproduction figures were noted.

The tracing of infected herds by collection of blood samples was mainly focused on risk contacts, such as contacts with infected herds, herds located in nearby areas and breeding herds. As a selective measure blood sampling was carried out at three major slaughter houses from the affected area. The results indicated a restricted spread of the infection and the eradication measures, which engaged several authorities and the branch, continued. Two months after the outbreak 432 herd samplings had been carried out representing 358 herds. Efforts to certify freedom from PRRS at a national level have been intensified. Serum from all herds slaughtering more than 300 pigs per year (n = approximately 1000) and a selected number of smaller herds will be collected at slaughter during this autumn. So far, half of the estimated herds have been tested, all with negative results.

Serological examinations were carried out using IDEXX ELISA. Positive samples were also tested with IPMA. If single animals showed positive results both in the ELISA and the IPMA, they were followed up by a herd test. None of these follow up investigations have revealed any new infected herds.

Nucleic acid from the PRRSV has been demonstrated from five of the infected herds and subsequent sequencing has been carried out (S1-gene, approx 500 bp) from 15 different isolates. A pattern of two clusters has been identified which corresponds with geography and contacts between affected herds. Differences among the Swedish isolates were found in twelve positions, whereas four sequences from the Gene Bank closest to the Swedish isolates differed at 39 positions. Extended sequencing is under way.

The measures implemented included slaughtering/killing, cleaning, disinfection and a vacancy period of three weeks before repopulation could occur. Since July 16 no further positive cases have been found and the eradication efforts look promising. Further epidemiological studies as well as additional sequencing data might throw light on the source of the infection.

RE-ASSESSMENT OF THE ECONOMIC IMPACT OF PRRS ON SWINE PRODUCTION IN THE USA IN THE CONTEXT OF RISING FEED COSTS

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The largest cost component in pork production is feed. It is traditionally stated that 70% of the cost of raising pigs in the US is feed, with corn as the primary ingredient. Recent developments have seen an increase in the cost of pork production due to higher feed prices related to demands from alternative uses for corn, e.g., ethanol. Additionally, other grain prices rise in association with corn as acreage shifts create shortfalls of grains for other competing uses. Previously, the impacts of PRRSV on production efficiencies and cost of production were evaluated in light of historic average market prices of both feed and hogs. Logically, the costs associated with mortality, morbidity, and treatment will rise as the costs of all farm inputs rise. Thus, the intent of this study was to re-evaluate the cost of production losses associated with PRRS as feed prices increase.

In a previous study[1] based on $2.25/bu corn ($0.088/kg), the economic effect of PRRS in the breeding and farrowing phase was calculated to be $74.16 per litter on affected farms with $45.00 derived from a reduction in the number of pigs weaned per litter and $29.16 from reduced farrowing rate. In the nursery phase, the cost was estimated to be $6.01 per head with $3.58 due to increased mortality, $1.17 due to reduced feed conversion, and $1.26 due to reduced average daily gain. In the finishing phase, the cost was estimated to be $7.67 per head on affected farms: increased mortality was $3.23, reduced feed conversion was $3.00 and reduced average daily gain was $1.44. Aggregated across the national herd, the impact of PRRS was estimated at $561.89 million, with $45.39 million in the sow herd, $229.64 million in the nursery herd and $286.86 million in the finisher.

Under increasing grain prices, for every $0.50/bu ($0.020/kg) increase in corn price, the cost of PRRS on the US pork industry increases by approximately $18.45 million per year. The sow herd impact is affected less by rising corn prices, as a higher portion of the production cost is fixed. The greater impact is felt in the nursery and grow-finish herds, where larger quantities of grain constitute production cost. In the PRRS-affected nursery, for every $0.50/bu increase, cost per pig marketed increases by $0.072. In the affected finisher, costs rise by $0.405 per head marketed. At the highest corn price utilized in the analysis ($5.00/bu), the cost of PRRS to U.S. producers rises to $664.01 million.

From these results it can be concluded that as feed prices rise, the value of veterinary services or improved health care also rises. Regardless of the cause for increased feed cost, it is imperative to continue efforts in disease management. Given that the economic impact is exacerbated at times of heightened input costs, it is imperative to continue efforts at disease control and prevention.

[1]Neumann EJ, Kliebenstein JB, Johnson CD, et al. 2005. Assessment of the economic impact of porcine reproductive and respiratory syndrome on swine production in the United States. J Am Vet Med Assoc 227:385-392.

SECTION 2: VIRAL GENOME AND HETEROGENEITY

QUASISPECIES EVOLUTION OF PRRSV DURING AN ACUTE INFECTION RESULTING FROM DIRECT *IN VIVO* TRANSFECTION OF PIGS WITH RNA TRANSCRIPTS FROM AN INFECTIOUS CDNA CLONE OF PRRSV

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The recent construction of PRRSV infectious cDNA clones represents a major milestone in PRRSV research. However, the inherited instability of PRRSV genome, the requirement of cell culture propagation, and poor virus recovery have limited the usefulness of PRRSV reverse genetics system for *in vivo* studies. We recently developed a unique strategy of infecting pigs with PRSV infectious cDNA clone by bypassing the traditional cell culture step required for *in vivo* studies. To demonstrate the utility of this unique approach, we evaluated quasispecies evolution of PRRSV during an acute infection resulting from *in vivo* transfection of pigs with homogeneous RNA transcripts from a PRRSV infectious cDNA clone. Four pigs were inoculated directly into the lymphoid tissues with RNA transcripts from an infectious clone pFL12 (a gift of Drs. Pattnaik and Osorio, Univ of Nebraska-Lincoln) and two pigs were inoculated with PBS as controls. The inoculated pigs developed an acute infection, and viremia was detectable at 7 and 14 dpi. Based upon sequence analyses of the entire ORF5 gene from 30 individual cDNA clones of each positive serum at dpi 7 and 14, quasispecies populations were identified in each pig as early as 7 dpi. The sequences from the 4 pigs at dpi 7 had 0.8% nt and 1.5% aa sequence variation with each other, whereas a 1.7% nt and 3.5% aa sequence variation with each other were identified from the 2 positive pigs at 14 dpi. The mutations in the quasispecies population appeared to be randomly distributed throughout the ORF5 gene and there were no specific regions with higher mutation rates during the acute infection. Previous studies of PRRSV quasispecies began with a virus stock that had been propagated in cell culture, thus increasing the likelihood of unfactored *in vitro* mutations. This unique *in viv*o transfection approach allowed us to bypass the cell culture steps and thus more accurately analyze quasispecies evolution in pigs. This is the first report on quasispecies evolution in pigs using an infectious clone of PRRSV.

HIGH THROUGHPUT ISOLATION AND DETECTION OF NORTH AMERICAN AND EUROPEAN PRRSV BY QUANTITATIVE REVERSE-TRANSCRIPTASE PCR (QRT-PCR)

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PRRSV (Porcine Reproductive and Respiratory Syndrome Virus) is a single-stranded, plus-sense RNA virus in order Nidovirales of the Arteriviridae family. PRRSV is one of the most costly infectious diseases for US swine producers with estimated annual losses nearing $600 million dollars. There are two main antigenic subtypes of PRRSV: North American strain (aka ATCC VR-2332) was first identified by Boehringer Ingelhiem Animal Health Inc., and the European strain (aka Lelystad strain). Infected animals can suffer from both reproductive and respiratory problems. Reproductive failure is characterized by abortions, stillbirths, and infertility. Respiratory disease is often associated with secondary infections.

We have developed an integrated workflow consisting of high throughput sample preparation, nucleic acid purification, and qRT-PCR for concurrent detection of North American and European PRRSV. The entire isolation/detection procedure can be completed in a little over 2 hours. Using Ambion MagMAX™ technology, a rapid high throughput magnetic bead-based viral RNA isolation method, viral RNA can be isolated from diverse sample matrices including serum, plasma, lung, and tonsil tissue. The RNA isolation procedure can be preformed manually using multi-channel pipettes or by automation using KingFisher Magnetic Particle Processor. The purified nucleic acid is analyzed using the Ambion AgPath-ID NA/EU PRRSV Reagent Kit on an Applied Biosystems 7500 Real-Time PCR System. The AgPath-ID NA/EU PRRSV assay can concurrently detect both the American and European strains of PRRSV thus allowing PRRSV subtyping. As few as 40 copies of PRRSV RNA can be consistently detected using the assay. An internal control RNA, XenoRNA containing unique nucleotide sequences, is also provided in the kit to monitor nucleic acid purification efficiency, detect presence of reaction inhibitors, and assess functionality of reagents and reaction preparations. The XenoRNA ensures that false negative results due to the presence of PCR inhibitors or user error are minimized, if not eliminated.

The performance of the workflow was evaluated using more than 100 field samples of known PRRSV status by RT-PCR. RNA was isolated from swine serum, tonsil, and lung tissues using MagMAX™ technology and the purified RNA was used for qRT-PCR using the AgPath-ID chemistry on the Applied Biosystems 7500 Real-Time PCR System. The results showed 98% concordance with secondary laboratory PCR results. Furthermore, subtyping of North American and European strains were 100% concordant demonstrating that this method provides an economical and rapid solution for PRRSV detection and identification. (For research use only, not for use in diagnostic procedures. Not licensed by the USDA.)

CRYO-ELECTRON MICROSCOPY OF PRRSV VIRIONS

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Very little structural information is currently available for PRRSV or any other arterivirus. The overall objective of our study is to provide a structural framework for understanding PRRSV infection, immunity, assembly and pathogenesis by a combination of cryo-electron microscopy (cryo-EM) and X-ray crystallographic structure determination.

The PRRSV virion consists of a lipid envelope that contains several envelope proteins, GP2—GP5, E and M, surrounding a nucleocapsid core composed of protein N that encapsidates the RNA genome. The most abundant envelope proteins GP5 and M are the main constituents of the viral envelope while GP2, GP4, E(2b) and possibly GP3 are minor components that may be important in host interactions and tissue tropism.

PRRSV isolate SD-23983 was grown in MARC-145 cells and harvested by centrifugation from the culture supernatant at 36 hr post infection. The virus was concentrated to $>10^9$ pfu/ml, prepared for cryo-EM by rapid plunging into liquid ethane, and observed in an FEI Tecnai F20 microscope at a nominal magnification of 65,000×.

The virions appear as round or egg-shaped particles with an average diameter of 58 nm. A few larger particles (diameter >70 nm) are also seen. The lipid bilayer of the envelope is clearly visible, and the particles display a smooth outer surface with only a few protruding features, presumably the envelope proteins. The virions contain an internal, hollow core of 39 nm average diameter, which is separated from the envelope by a 2–3 nm gap.

Since the virus particles are individually different, like those of e.g. influenza or HIV, reconstruction techniques employing averaging procedures are not available. We plan to use tomographic reconstruction to determine the three-dimensional structure of individual PRRSV virions.

EMERGENCE OF A HIGHLY PATHOGENIC PRRSV IN MID-EAST CHINA

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Pig herds in the mid-east of China have experienced recent outbreaks of a severe form of PRRS characterised by high fever and morbidity and mortality in pigs of different ages. Eighty one herds were diagnosed with PRRSV infection from June-December 2006 by clinical signs, pathobiology and reverse transcriptase polymerase chain reaction (RT-PCR) and 20 strains of PRRSV were isolated from 20 pig herds in different provinces. Nucleotide and reduced amino acid of ORF5 of six isolates showed 99.5-99.8% and 99-100% identity with each other, but only 89.4% nucleotide and 88.6% amino acid sequence identities with VR-2332, the prototypic North American isolate. To confirm the virulence and pathogenicity of the isolated virus, the biological characteristics, serological traits and Nsp2 gene sequence of one isolate designated SY0608 were determined and animals were experimentally inoculated. The results showed that the isolate caused 100% morbidity and 25-50% mortality in 30, 65, and 105 day old pigs and birth of stillborn and weak piglets in inoculated sows. Nsp2 of SY0608 was 2850 base pairs, containing two discontinuous deletions, with the size of 1 and 29 amino acids corresponding to strain VR-2332 positions 480 and 531-559, respectively. In addition, the Nsp2 gene shared only 79.4% nucleotide and 74.9% amino acid sequence identities with VR2332. These findings demonstrate that a new highly pathogenic Northern American type PRRSV had spread widely in the mid-east of China. This poses serious challenges for the control and possible eradication of PRRS in China.

MOLECULAR EPIDEMIOLOGY OF GERMAN AND EUROPEAN PRRSV ISOLATES

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The first outbreaks of PRRS in Europe were observed in 1990. In the meantime the disease has become endemic in Germany and in most other European countries. For control of the disease, two modified live vaccines (MLV) containing an NA- or an EU-type virus strain, respectively, and two inactivated vaccines are being used in Germany. In this study, PRRS field strains from diagnostic samples obtained between 2004 and 2007 were subjected to genetic typing using the sequences of the ORF5. The samples were from pig herds located in Lower Saxony, the region with the highest pig density in Germany. The aims of the study were 1) to determine the influence of the MLV on the PRRSV isolates circulating in the field, and 2) to determine if the EU-type field isolates from this region form distinct genetic clusters that allow to differentiate them from field isolates occurring in other European countries.

Phylogenetic analysis showed that of the 226 isolates analysed, 96 (43%) were closely related to the NA-type vaccine virus strain, and 130 (57%) were EU-type isolates. Of these, 15 were related to the EU-type MLV vaccine strain, which belongs to the Lelystad virus cluster. When isolates from other European countries were included in the phylogenetic analysis, most of the isolates from Lower Saxony were in an own cluster. The remaining isolates clustered together with European isolates from other countries, thus confirming previous findings. In conclusion, 1.) it is conceivable that due to the introduction of the MLV the occurrence of NA-type virus in the field is increasing. 2.) The results indicate that in a certain geographic region, the PRRSV field isolates circulating are closely related and form separate clusters. Due to import of pigs from other countries, new isolates are being introduced.

EVALUATION OF THE ADIA TEC® REAL TIME RT-PCR KIT FOR THE SIMULTANEOUS DETECTION OF NORTH AMERICAN AND EUROPEAN PRRSV

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Porcine reproductive and respiratory syndrome (PRRS) is one of the most economically important diseases of swine. Thus, there is a considerable interest worldwide in strategies for PRRS monitoring and control. However, detection of its etiologic agent, PRRSV, is a diagnostic challenge due to the high genetic variation between the European and the U.S. genotypes and within both genotypes as well as the propensity for swine to develop persistent infection in which virus is difficult to detect.

The AnDiaTec® PRRSV RT-PCR Kit is a completely automatable diagnostic test using a proprietary 2-step lysis buffer to extract viral RNA from whole blood and serum samples into the supernatant that is then used directly in the real time RT-PCR assay. European and U.S. genotype strains are detected in a single reaction and additionally an internal control (IC) proves the performance of the reverse transcription and amplification reaction steps.

The detection limit of the kit was determined to be 100 viral particles in 1 mL whole blood. The specificity of the test was confirmed with a panel of nonrelated swine viruses that all tested negative. The broad reactivity of the kit was first determined using 116 well characterized EU and NA genotype isolates (molecular sequence information was available for relevant parts of the genome) and 30 samples from ring trial that was performed in Germany in 2005, which all tested positive.

Blood samples (n = 3,189)(EDTA-Blood and serum) were tested in a following evaluation study. The study comprised pre-tested samples (samples from 19 governmental laboratories in Europe tested by in house RT-PCR and virus isolation) but also parallel examination of previously untested samples. All (n = 1493) pre-tested positive samples (100%) were confirmed positive, while additional 6 samples were tested positive only with the AnDiaTec system. Additional analysis and molecular characterization revealed a true-positive PRRSV result in the AnDiaTec test for these samples. The experiments proved that the newly developed test is sensitive, specific, ease-to-use and fast and thus, allows the standardized large-scale routine PRRSV screening of swine samples.

PROTEOLYTIC PROCESSING OF PRRSV NSP2 REPLICASE PROTEIN

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One critical step in PRRSV replication is the proteolytic processing of the ORF1 polyprotein (replicase). The replicase polyprotein is generally believed to be processed to generate at least 12 smaller nonstructural proteins (nsps) involved in replication by virus-encoded proteinases. PRRSV nsp2 has been predicted to be the largest viral replicase protein and contains multiple domains: an N-terminal cysteine proteinase (PL2) domain, a middle hypervariable region and C-terminal putative transmembrane domain. In this study, we investigated the proteolytic processing of nsp2 in PRRSV-infected MARC-145 cells. To facilitate analysis of nsp2 processing, a *c-myc* epitope was inserted into the nsp2 middle region of a Type 2 strain VR-2332 infectious clone. Recombinant virus was recovered and shown to have a growth rate similar to parental virus. Immunoprecipitation and western blot analysis using a panel of antibodies revealed that several nsp2 associated processing products could be detected. Nsp2 proteins corresponding to sizes with and without the predicted transmembrane domain were observed. A pulse-chase assay suggested that these nsp2 products appeared rapidly, possessed an extended life-span and a low turnover rate. Further deletion mutants were used to map the relative positions of the cleavage sites. Finally, mass spectrometry and other biochemical approaches were used to locate the nsp2 cleavage sites. The finding of different PRRSV nsp2 isoforms may suggest that they have different roles in the virus life cycle.

GENOMIC CHARACTERIZATION OF A HIGHLY PATHOGENIC PRRSV ISOLATED FROM SWINE HIGH FEVER

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During summer 2006, a swine epidemic disease emerged in south of china include Anhui province, Jiangxi province, Zhejiang province, Hunan province, Hubei province, Jiangxi province etc, then the disease spread to all the china mainland. High fever, red skin, and severe respiratory disease are major clinic symptoms, as well as acute death. Interstitial pneumonia, carnification, haemorrhage in lung are the features of anatomy symptom. To our surprise incidence of disease in most pig farms was 50-80%, and incidence fatality rate was 50-80% of this disease. But to our regret the result of therapy with antibiotics wasn't satisfactory. It is testified by some experiences that PRRSV is foremost cause of swine high fever in south china.

Full-length genomes of recently emerged virulent isolates of PRRSV were sequenced and compared to other PRRSV strains. The results revealed that this isolate (named PRRSV HuN), of North American lineage, represented the PRRSV genomes sequenced to date with a nucleotide length of 15□328 bases. Genetic analysis demonstrated that the isolates were shared approximately 88.82% and 57.93% nucleotide identity with prototype North American reference strain VR-2332 and European reference strain Lelystad, respectively. Three quite variable regions were identified, corresponding to putative nsp2□ORF3 and ORF5. Nsp2, the most variable region, shared only 66–75% amino acid similarity to other sequenced North American-like PRRSV nsp2 proteins. Further study revealed that the nsp2 protein of the MN184 isolates contained three discontinuous deletions when compared to strain VR-2332 nsp2 protein, with the sizes of 1 and 29 amino acids corresponding to strain VR-2332 positions 479 and 531–559, respectively.

The NSP9 was the invariable area, and the NSP2 is the variable area in non-structure proteins; the GP6 was the invariable area, and the ORF5 is the variable area in structure proteins. And alignment the 5'UTR of some virus isolated from swine high fever with reference virus, 12nt start motif, a string of 8, 9 and 11 wholly invariable nucleotides and two CTCCC boxes were found as same as other PRRSVs. And the NSP2 is the most variable area, this was 30AA deletion; the PRRSV have some invariable areas in 5'UTR;the fifth nucleotide in 3'UTR upstream of poly (A) is U but others are A.

And 13 pigs were challenged with the PRRSV, in the end, 12 pigs died between 21 days-45 days. So this PRRSV is a high pathogenic virus strain.

DYNAMIC TRAFFICKING OF THE PRRSV NUCLEOCAPSID PROTEIN

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An emerging paradigm is that many positive strand RNA viruses, of which PRRSV is a member, target a dynamic sub-nuclear structure called the nucleolus to facilitate virus replication and usurp host cell function. In the case of PRRSV, the virus targets the nucleocapsid (N) protein between the cytoplasm and the nucleolus and has been shown and/or predicted to contain various localisation signals. Given the role of trafficking in virus biology a thorough understanding of this process can lead to the development of novel recombinant viruses deficient in N protein trafficking. Live cell confocal microscopy was coupled to dynamic imaging analysis using fluorescence recovery after photo-bleaching (FRAP) and fluorescence loss in photo-bleaching (FLIP) to study trafficking of differently fluorescent labelled N protein. Comparison with a diverse selection of cellular and viral proteins, which also localise to the nucleolus, indicated that N protein shared trafficking characteristics very similar to those proteins involved in the movement of RNA between the nucleus and the cytoplasm.

GENETIC DIVERSITY AND SEROLOGY OF PRRSV IN TAIWAN

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To investigate the genetic diversity and serological prevalence of PRRSV in Taiwan, 630 blood samples were collected from 21 pig farms from July to August in 2005. Serological tests of PRRSV antibodies were performed by using IDEXX HerdChek PRRS ELISA Kits. Virus isolation was confirmed by indirect immunofluorescent antibody test using SDOW17 monoclonal antibody and genetic variation performed by RT-PCR and sequencing was analyzed by DNAStar software. Serological results showed all farms were PRRS positive with prevalence of 40.48%(85/210), 83.81%(176/210) and 95.24%(200/210) at ages of 6, 10, and 16 week old pigs respectively. Forty-two viruses were isolated and confirmed to be all belong to the North American-type. Divergence of ORF 1b, 5 and 7 were 7.7-11.4%, 11.3-14.8%, and 5.4-9.1% respectively at nucleotide level and were 0-0.7%, 12-17% and 4.8-5.6% at amino acid level. Phylogenetic analyses of these 42 ORF5 fragments indicated that Taiwan PRRSVs formed at least 5 major genetic clusters. In conclusion, PRRSV was widely prevailed in Taiwan pig farms and revealed distinct genetic diversity at the time of the study.

FULL-LENGTH SEQUENCES OF FIVE TAIWAN PRRSV ISOLATES

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From our previous study, there were at least 5 major clusters of PRRSVs developed based on phylogenetic analyses of ORF 5 fragments of 42 Taiwan PRRSV isolates collected in 2005. For further realizing the evolution situation and estimating the PRRSV diversity at the full genome level, we took 5 isolates respectively from these clusters and processed the complete genome sequencing by a set of overlapping cDNA clones. All five isolates belonged to North American type, and the sizes of 5'UTR, ORF 1b, structural genes and 3'UTR were 190, 4374, 3181 and 150-1 nucleotides, respectively. In comparison with VR-2332, the divergence of Nsp9-12, ORF2a, 2b, 3, 4, 5, 6 and 7 ranged from 9.0-15.9%, 0.5-7.3%, 0.5-1.8%, 0.1-11.7%,0-9.5%, 2.2-14.3%, 0.2-6.7% and 0.3-6.9%, respectively. The amino acid differences are compatible to nucleotide level and are randomly distributed throughout the genome. It is of interest to note that the diversified genes seen not only at the envelope gene (ORF 5), but also at Nsp9-10 encoding the RdR polymerase. These results have led us to conclude that Taiwan PRRSV has evolved into a distinct tree different from the NA type.

EVOLUTIONARY BEHAVIORS OF THE PRRSV LINEAGE ASSOCIATED WITH THE RECENT PRRS OUTBREAK IN CHINA

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Unparalleled large scale outbreaks in swine caused by highly pathogenic PRRS viruses were reported in China during the summer of 2006. We have reanalyzed the available ORF5 sequences in Genbank, along with a number of viruses sequenced in our laboratory; a lineage of viruses that shared a close monophyly with the 2006 outbreak viruses was identified. Using a relaxed Bayesian molecular clock model, we estimated the date of the most recently common ancestor of this lineage appeared in a median of 1997, almost 10 years before the outbreak. We sequenced the full genome two viruses of this lineage sampled in 2003. We found that the proposed hallmark of pathogenicity, i.e. deletion in nsp2, was absent in these viruses, suggesting this deletion may have happened recently. Population analysis using Bayesian coalescent techniques suggests this lineage is undergoing a transition of constant population to an exponential population growth. Such transition is estimated to start at around 2006, which is consistent with the reported outbreak in China. Taken together, this study provides insights to the possible role of the deletion in nsp2 in the PRRSV outbreaks in China from an evolutionary perspective.

ATTENUATION OF PRRSV BY CHIMERA CONSTRUCTION

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Two genetically distinct infectious recombinant virus clones (pMLV, constructed from Ingelvac® PRRS MLV and pMN184, constructed from virulent strain MN184) were developed to study attenuation of contemporary PRRSV. Two reciprocal chimeric clones (pMLVORF1/MN184 and pMN184ORF1/MLV) were then constructed. *In vitro* studies demonstrated that the rescued chimeric viruses possessed intermediate growth properties compared to recombinant rMLV and rMN184. Swine inoculation with rMN184 and rMLV verified that these viruses fully mimicked the respective parent virus. Earlier and higher antibody responses were detected in animals infected with rMN184 and the chimeras in contrast to those infected with rMLV. However, chimeric virus treatment groups showed much less severe pathogenesis when compared to the rMN184 group. These data suggested that genetic aspects of Ingelvac® PRRS MLV 5'UTR/ORF1 replicase region and/or the structural proteins/3'UTR can serve to attenuate virulent strain MN184. The data also indicated that designer PRRSV vaccines could be developed, keeping the known 5'UTR/replicase region of an early vaccine strain such as Ingelvac® PRRS MLV intact, but replacing the structural protein/3'UTR domain with that of an emerging virulent virus.

The recent "fatal" Chinese isolates may represent one emerging virus genotype. The largest nsp2 deletion (87 bases) noted in these newest strains encompass the second largest of three discontinuous deletions previously described in virulent strain MN184. However, the Chinese isolates are still more similar to the RFLP142 genomes (~94% ORF5 nucleotide identity). Other novel amino acid islands were seen throughout the ORF1 polyprotein (particularly nsp1b and nsp2) and in structural proteins GP2, GP3, GP5 and N. A one base deletion in the 3'UTR was also noted. A rapid vaccine for these isolates may be completed by the exchange of the ORF1 region with that of a recombinant attenuated strain.

MUTATIONAL ANALYSIS OF PRRSV NON-STRUCTURAL PROTEIN 11 ENDORIBONUCLEASE NENDOU AND THE VIRUS REPLICATION

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The arterivirus 1a/1b polyprotein contains a domain of NendoU endoribonuclease in the non-structural protein (nsp) 11 . This domain is highly conserved among nidoviruses including coronavirues and roniviruses. NendoU is a nidovirus-specific homolog of XendoU which is associated with small nucleolar (sno) RNA processing in *Xenopus* amphibians. The NendoU activity has recently been demonstrated for several coronaviruses and equine arterivirus. To investigate the biological role of NendoU for PRRSV replication, 7 highly conserved amino acid residues in the NendoU domain of nsp11 were substituted with Ala, and using an infectious cDNA clone, respective NendoU mutants were constructed. Marc-145 or BHK-21 cells were transfected with mutated genomic RNA, and their replication was examined by immunofluorescence for nsp2/3 and nucleocapsid proteins and plaque assays. In the first cycle of replication, all mutants were viable, including mutations at two putative catalytic His residues in subdomain A and at three highly conserved residues in subdomain B. The NendoU mutations however produced decreased titers of virus and reduced plaque sizes, depending on the mutated residues. Our study suggests that PRRSV NendoU in nsp11 is involved in the viral replication and life cycle with yet defined function.

THE ROLE OF CD163 FOR PRRSV INFECTION

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Porcine reproductive and respiratory syndrome virus (PRRSV) displays a restricted cell tropism. In vitro, the virus replicates only in porcine primary alveolar macrophages (PAMs) and a specific line of African green monkey kidney cells. Cell tropism is largely determined by the presence or absence of a specific receptor, and CD163, a macrophage-specific cysteine-rich scavenger receptor, has recently been described as a potential receptor for PRRSV. To investigate PRRSV tropism and permissiveness, porcine (p) CD163 cDNA was cloned from PAMs and expressed by transient transfection in three different lines of PRRSV non-permissive cells; CRFK feline kidney, Dulac porcine kidney, and LLC-PK porcine kidney cells. These cells are negative for CD163 and not susceptible for PRRSV infection. When transfected with pCD163 and infected with PRRSV, the pCD163-expressing cells became permissive for PRRSV infection and produced the virus. To further confirm this finding, stable cell lines were established to express pCD163. In the stably expressing cells, PRRSV replication was evident. Macrophages differentiated from porcine monocytes were developed as an immortalized cell line. When these cells were transfected to over-express pCD163, they became susceptible for PRRSV. Our data show that pCD163 alone is sufficient to convert PRRSV non-permissive cells to a permissive status and support that pCD163 may be the solely required cellular receptor for PRRSV.

GP3 IS A STRUCTURAL COMPONENT OF THE PRRSV TYPE II (US) VIRION

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The biological role of the minor envelope glycoprotein 3 (GP3) of PRRSV is still unclear and remains a matter of controversy. This highly glycosylated envelope protein was originally reported as being a non-structural protein of the type II PRRSV (US) strains. According to this notion, the GP3 would not be incorporated to the virion, however its high in vivo immunogenicity could be explained by its ability to be secreted from PRRSV-infected cells. However, in other studies involving PRRSV type I (Euro) strains, GP3 was shown to be incorporated in virions, with such incorporation being essential for assembly and viral infectivity. A structural role for GP3 was also suggested by recent findings reporting the ability of GP3 to induce PRRSV-neutralizing antibodies in the case of a PRRSV type II strain.

We used two 15aa peptides corresponding to 2 different immunodominant epitopes of Gp3 (PRRSV FL12) to generate a rabbit mono-specific antiserum to this protein. The specificity of this anti-GP3 antiserum was confirmed by Western blot on PRRSV-infected cells, radio-immune precipitation (RIP) both on BHK-21 cells transfected with a vector expressing GP3 and on MARC-145 cells infected with FL12 PRRSV, as well as by confocal microscopy on PRRSV- infected MARC-145 cells.

In order to determine if GP3 is a structural component of the virion, S35-labelled PRRSV FL12 virions were purified. Radiolabeled virus was pelleted through a 30% sucrose cushion, followed by a second round of purification on a sucrose gradient (20-60%) for 24 hrs. Virions were detected in gradient fractions 8-11, by radioactive counts and by viral infectivity GP3 was detected in gradient fractions containing purified virions by RIP with anti-GP3 antiserum. GP3 was less abundant in purified virions than other known structural proteins GP5 and M. Further evidence of the presence of GP3 at the level of PRRSV FL12 envelope was obtained by immuno-gold staining of the envelope of purified PRRSV and PRRSV present in the supernatant of infected cells with anti-GP3 antiserum.

We conclude that GP3 is a structural component of the PRRSV FL12 strain virion.

PRRSV NON-STRUCTURAL PROTEIN 2: POTENTIAL FUNCTIONS IN VIRAL REPLICATION AND PATHOGENESIS

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The PRRSV nsp2 is the largest viral protein of PRRSV. The N-terminal region encodes a putative cysteine protease, involved in the proteolytic processing of the ORF1ab polyprotein. In this study, we generated a panel of monoclonal antibodies (mAb) specific to the nsp2. Using this panel of mAbs, the nsp2 protein was detected in virus-infected cells. Protein sequence results showed that the N- and C-terminal cleavage sites are consistent with the previous prediction, and the cleaved mature nsp2 contains AA 386 to 1446 of the ORF1a protein. Using a reverse genetic system, we demonstrated that mutations in the cleavage sites or putative catalytic sites abolished the virus replication. Besides its potential role in viral replication, our studies showed that PRRSV nsp2 elicits strong humoral antibody responses. Previous studies identified a cluster of B-cell epitopes on the PRRSV nsp2. Our analysis revealed that most of the epitopes associate with the regions showing hypervariability where deletions and insertions were identified, and certain deletion regions also mapped to potential T-cell epitopes. To further determine the potential role of different epitope regions in viral pathogenesis, we constructed several nsp2 B-cell epitope deletion mutant viruses based on a type 1 PRRSV cDNA infectious clone backbone. The virus growth characteristics were analyzed in porcine alveolar macrophages and MARC-145 cells. All of the mutant viruses had a one-log reduction in titer compared to the original cloned viruses, except the ΔES3 mutant, which replicated at a level comparable to the parental virus. Current research is directed to determine the *in vivo* pathogenic properties of these nsp2 deletion mutants.

IDENTIFICATION AND CHARACTERIZATION OF PRRSV ORF1A-ENCODED REPLICASE PROTEINS

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The PRRSV ORF1a-encoded replicase polyprotein is predicted to be proteolytically processed into nine non-structural proteins (nsps), nsp1α, nsp1β, and nsp2 to nsp8, including functional domains of papain-like cysteine proteases, a cysteine protease and a serine protease. In this study, monoclonal and polyclonal antibodies were generated against six of these proteins, including nsp1α, nsp1β, nsp2, nsp4, nsp7 and nsp8. Using this panel of antibodies, we identified and characterized ORF1a-encoded nsps in PRRSV infected MARC-145 cells. Immunoprecipitation and Western blot analysis of the virus-infected cell lysates detected nsp1α, nsp1β, nsp2, nsp4, and proteolytic processing intermediates containing nsp7 and nsp8. Protein microsequencing analysis identified the amino- and carboxyl-terminal sequences of nsp2, and revealed the association of nsp2 with nsp3. In immunofluorescence assays, the majority of these proteins can be detected as early as 6 hours post-infection. At early times of infection, nsp1β, nsp2, nsp4, nsp7 and nsp8 localized at the perinuclear region as distinct punctate foci. Nsp1β and nsp4 partially localized to the nucleus at later times of the viral replication cycle. The nsp1α showed a distinct cytoplasmic distribution pattern different from the other nsps. Dual-labeling studies suggested that PRRSV nsp1β, nsp2, nsp4, nsp7 and nsp8 may initially form the viral replication complex and associate with viral RNA replication. However, co-localization studies did not provide strong evidence of these replicase protein associations with organelle-specific marker proteins, including marker proteins of endoplasmic reticulum, autophagasome, lysosome and mitochondria. Taken together, our results confirmed the expression and processing of PRRSV ORF1a-encoded replicase proteins, and demonstrated the sub-cellular localization patterns of these replicase proteins. Furthermore, this study produced the first panel of antibodies against PRRSV nsps, which provides important tools for PRRSV replication and pathogenesis studies and diagnostic test development.

EVOLUTION OF PRRSV GENOMES ASSOCIATED WITH FAILED SOW PROTECTION

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To prevent PRRS in pigs, common-sense practices involving herd exposure to on-farm isolates that is expected to provide complete, homologous immunity are not completely successful and rebreaks with significant reproductive disease still occur. Viruses recovered from affected sows and piglets often appear to be directly related to the ancestral immunizing virus based on ORF 5. However, ORF 5, at 600 bases in length, is only 4% of the viral genome. In order to better understand why PRRS outbreaks continue to occur in sow herds that are "protected" in the face of apparently solid homologous immunity, and to use this information to improve prevention protocols, it is essential to obtain a complete genetic description of the virus and link it with better information about virulence and immunological cross-reactivity in pregnant sows. Thus, we developed methods for whole genome PRRSV sequencing. This set of reagents and protocols allowed us to sequence and assemble 24 whole PRRSV genomes. Among these, a total of 6 pairs of field isolates, representing "before" viruses used for gilt inoculation, and "after" viruses recovered from sick piglets or sows, were grown on alveolar macrophages, sequenced and assembled via phredPhrap. Phylogenetic comparison analyses of the 6 pairs identified genomic regions that were undergoing positive selection (dN/dS >1) thus suggesting these areas might play a role in re-breaks. In addition, we observed a striking bias in the type of base changes, indicating that host factors may be driving the high mutational rate observed in PRRSV strains. In addition, we observed an unusually high transition/transversion ratio and small deletions, mostly in nsp2. We also found genomic regions depleted of a single nucleotide which might be related to conserved viral RNA secondary structures since low similarity at the amino acid level was observed. Finally, nucleotide similarity percentages based on ORF5 or whole genome revealed a high degree of correlation in most cases, thus validating the use of ORF5 for genetic comparisons. We expect these results will provide a better understanding of PRRSV persistence and re-breaks through the identification of unique and conserved regions of the viral genome.

PRRSV DIVERSITY IN BRITAIN: IMPLICATIONS FOR DIAGNOSTIC APPROACHES

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This research aims to characterise the increasing diversity of strains of PRRSV recently associated with disease in pigs in Britain.

To date, over 110 isolates have been characterised by analysing the genetic sequence of ORF7, ORF5 and ORF3 from PRRSV RNA from tissue samples obtained from recent (2004-2007) clinical cases of the disease. These were then compared with corresponding data from 16 strains isolated in Britain between 1991 and 1995, and with published data from other countries. All isolates were found to be of the European genotype. Several isolates were found with high similarity to the vaccine strain used in Britain. The similarity of ORF7 nucleotide sequence ranged from 100% to 96.9% for 1990s isolates, and from 100% to 88.6% for the recent isolates. For ORF5, these values were 100% to 96.5% for the 1990s, and 100% to 86.8% for the recent isolates. Similarly for ORF3, the range was 100% to 95.4% for the 1990s versus 100% to 84.4% for recent isolates.

The potential effects of the observed increase in diversity on diagnostic methods used were investigated. A panel of twelve monoclonal antibodies was used to evaluate the variation of particular epitopes found on the virus. The cross-reactivity of several field sera with a panel of nine defined virus isolates was compared in an immunoperoxidase monolayer assay. Finally, the primer and probe sequences of a proposed real-time RT-PCR test were analysed against the available sequence data. Only a single monoclonal antibody reacted with all the virus isolates, and considerable variation was found in the antibody titres of field sera when using different PRRS isolates in the IPMA. The PCR primers and probe were redesigned because of mismatches found in the existing sequences, and the test subsequently validated.

These findings demonstrate a significant increase in the diversity of PRRSv strains circulating in British herds between the 1990s and 2007, with consequences for current diagnostic methods used in Britain.

SECTION 3: IMMUNOLOGY AND VACCINES

DEVELOPMENT OF A PRRS VACCINE BY A REVERSE GENETIC SYSTEM

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Porcine reproductive and respiratory syndrome virus (PRRSV) infection has become epidemic worldwide since it was identified in early 1990. It is the most economically significant disease affecting the swine industry. Developing an effective and safe vaccine against PRRS is an approach to control and eliminate PRRSV infection. Here, we introduce a live vaccine against PRRS developed via a reverse genetic system. An infectious clone of PRRSV has been constructed and used to develop chimeric PRRSV clones, ptkPRRSV3 and ptkPRSV6, from the virulent wild- type of PRRSV isolates MN-184 and PTK-2005. The two chimeras were tested for safety in PRRS-naïve pigs. Results showed that 4 out of 5 the pigs in both PRRSV-inoculated and control (non-vaccinated) groups had lung lesion scores of zero and the remaining pig in each group scored less than 0.2 %. Compared with the wild-type of MN-184 or PTK-2005 that caused normally 50–70 % lung lesions, the chimeras have been attenuated and avirulent to PRRS-naïve pigs. The immunogenicity of PRRSV chimeras (ptkPRRSV3 and ptkPRRSV5) was tested in PRRS-naïve, 3-4 week-old pigs. At 5 weeks post vaccination, the vaccinated pigs were challenged with heterologous and homologous virulent strains of PRRSV, MN-184 and NADC-20. The results showed the lung lesion scores were significantly reduced in the vaccinated pigs compared to non-vaccinated control: 1.07 % vs. 61 % for heterologous challenge (NADC-20) and 3.15 % vs. 75.25 % for homologous challenge (MN-184). ELISA test data showed that all of the vaccinated pigs converted to PRRSV-specific antibody positive 14 days after vaccination, whereas viral genome detection in serum by real-time PCR was negative in all pigs at 42 days post vaccination. In addition, vaccinated pigs lost less body weight compared with challenge controls after PRRSV challenge. Finally, the study identified the potential genomic region encoding PRRSV virulence factors and a follow up experiment is examining this region.

PURIFICATION OF THE MAJOR ENVELOP PROTEIN GP5 OF PRRSV FROM NATIVE VIRIONS

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Porcine reproductive and respiratory syndrome virus (PRRSV) is the cause of an economically important swine disease that has been devastating the global swine industry since the early 1990's. The current PRRSV vaccines are not very effective largely due to heterogeneic nature of the virus. The major envelope protein, GP5, exposes outside the virion, induces neutralizing antibodies, and thus is a primary target for developing a subunit vaccine. In this study, we report a process for purification of GP5 protein from native virions of PRRSV propagated in MARC-145 cells. PRRSV virions were first purified and concentrated through sucrose cushion ultracentrifugation. GP5 protein was subsequently solubilized with Triton X-100 detergent for further processing. Cation exchange chromatography (CEX) was utilized for partial fractionation of GP5, although the viral nucleocapsid protein (N) was a major impurity in CEX elution fractions. During a second chromatographic step, hydrophobic interaction chromatography (HIC) further purified GP5 protein by means of a two-stage elution scheme. Pure GP5 protein was eluted from the HIC resin in the second HIC elution stage by Triton X-100 displacement; however the protein is present as a homodimeric/tetrameric aggregate. This process may be useful in PRRSV subunit vaccine development.

TGEV-BASED VECTORS TO PROTECT AGAINST PRRS

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Current vaccines to protect against PRRSV only afford partial protection against homologous viral strains. A set of coronavirus-derived vectors, based on transmissible gastroenteritis virus (TGEV) has been engineered. These vectors stably express high levels of heterologous genes, are potent interferon-α inducers, and present antigens in mucosal surfaces, providing both secretory and systemic immunity. A recombinant TGEV vector (rTGEV) based on an infectious cDNA clone of the virus genome was generated, expressing PRRSV GP5 and M proteins. These proteins are considered as the main inducers of neutralizing antibodies and cellular immune response, respectively. The constructed vector stably expressed PRRSV antigens in more than 80% of the TGEV infected cells, being a significant advance over rTGEVs expressing GP5 alone. Moreover, the expression levels were maintained after inoculation of piglets, as tested after recovering virus from tissues. Only low levels of neutralizing antibodies were produced after rTGEV inoculation, similarly to what occurs with PRRSV infection. This could be due to a steric hindrance of the neutralizing epitope within GP5, caused by the glycosylation sites present close to. Alternatively, the presence of an immunodominant (decoy) epitope close to the neutralizing epitope in GP5 could be deleterious for a strong neutralizing immune response. Therefore, a set of rTGEV vectors expressing mutants in GP5 glycosylation pattern, or lacking the decoy epitope, and M proteins were generated, and their immunogenicity is being tested in pigs. The objective is to improve Gp5 and M protein immunogenicity, allowing an earlier and more efficacious immune response against PRRSV.

IMPACT OF MODIFIED-LIVE PRRSV VACCINATION FOR CONTROL OF PRRS IN NURSERY PIGS

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Objectives

This study evaluated the impact of a modified-live PRRSV vaccine (Ingelvac® PRRS ATP, Boehringer Ingelheim Vetmedica, Inc., St Joseph, MO) for control of naturally occurring PRRSv infection in nursery pigs.

Materials and Methods

The study was conducted in a large commercial swine production system utilizing three-site pig flow. The breeding herds had a mixed PRRS status including negative and positive breeding herds. Weaned pigs from PRRS-positive breeding herds were flowed to PRRS-positive nurseries where clinical PRRS episodes consistently occurred. Weaned pigs from PRRS-negative breeding herds were flowed to separate PRRS-negative nursery facilities which achieved production targets. PRRS vaccination was implemented in 3-week old weaned pigs at entry to PRRS-positive nurseries. Performance of PRRS vaccinated/PRRS-positive nursery sites was compared to historical pre-PRRS vaccination performance at these same sites and also to PRRS-negative nursery sites. All nursery flows were later vaccinated.

Results

PRRS vaccination at entry to PRRS-positive nurseries reduced nursery mortality from >9% pre-vaccination to <3% post-vaccination. Vaccinated PRRS-positive nursery sites performed equally to PRRS-negative nursery sites. After all nursery flows were vaccinated, mortality rates were maintained at <3%.

Conclusions

Vaccination of nursery pigs in PRRS-positive nurseries reduced mortality and improved performance to levels comparable to that of non-vaccinated pigs reared in PRRS-negative nurseries. Subsequent implementation of vaccination across all nursery flows maintained targeted nursery performance while providing the added flexibility of commingling flows.

CONTROLLING PRRSV AND OTHER CO-INFECTIONS TO REDUCE THE IMPACT OF PCVAD IN PIGS

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Objectives

Porcine reproductive and respiratory syndrome virus (PRRSV) is the most common co-infection in cases of Porcine Circovirus Associated Disease (PCVAD). This paper describes the use of multiple vaccines to control several infectious components of PCVAD.

Materials and Methods

A three-site production system with finishing mortality rates averaging 5.2% observed average mortality rates increase to 13.9% after PCVAD was diagnosed. A piglet vaccination program targeted to control the PRRSV and salmonella co-infections was initiated. Piglets were vaccinated at weaning (~21 days of age) with a modified live PRRS vaccine (Ingelvac® PRRS MLV, Boehringer Ingelheim Vetmedica, St. Joseph, MO), and an avirulent live salmonella vaccine (Enterisol® SC-54, BIVI). Piglet PCV2 vaccination (Ingelvac® CircoFLEX™, BIVI) was implemented at 6 weeks of age upon vaccine availability. Statistical process control methods were used to assess the impact on finishing mortality.

Results

Average mortality rate decreased significantly from 13.9% (range=7-21%) during the PCVAD period to 8.3% (range=2.4-20.7%; high mortality spikes during this period were specifically due to swine influenza outbreaks) following initiation of PRRS and salmonella vaccination. When PCV2 piglet vaccination began, average % finishing mortality decreased significantly further to 3.5%.

Conclusions

Controlling PRRS and salmonella co-infections with vaccination significantly reduced mortality in the absence of PCV2 vaccination. Controlling PCV2 infection directly with PCV2 vaccination decreased mortality significantly further to levels at or below the pre-PCVAD period.

MODIFIED-LIVE PRRSV VACCINE FOR CONTROL OF PRRS IN A LARGE CONTINUOUS FLOW FINISH SITE

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Objectives

This field study evaluated the impact of a modified-live PRRS virus (PRRSv) vaccine (Ingelvac® PRRS ATP, Boehringer Ingelheim Vetmedica, Inc., St Joseph, MO) for control of PRRSV in a large, endemically infected continuous flow finishing site.

Materials and Methods

The study was conducted in a large, three-site commercial production system. The PRRSV status was classified as: *Site-1* Breeding Herds: PRRSV-negative; *Site-2* Nurseries: PRRSV-negative; *Site-3* Finisher: PRRS active. The finisher site was comprised of 16x1000-head barns representing 16 weeks of continuous flow production. Pigs were uniformly PRRS ELISA-negative at 10 weeks of age (nursery exit) but 90% tested positive by 14 weeks of age. A protocol of mass vaccination of the PRRS-positive finishing site followed by vaccination of incoming pigs 2 days post-entry to the site was implemented. Statistical Process Control methods were used to assess cull and mortality rates.

Results

A significant reduction in total cull and mortality rates were observed following PRRSV vaccination. The average combined mortality and cull rate decreased from 11.36% (range=5.58-19.68%) pre-vaccination to 5.75% (range=3.76-9.43%) post-vaccination.

Conclusions

Mass vaccination of an endemically PRRSV-infected finishing population followed by vaccination of PRRS-negative pigs at finishing entry significantly improved biologic performance of this large finishing site. We hypothesize that this is due to reduced PRRSv transmission following mass vaccination of the site, thereby allowing incoming vaccinates a greater opportunity for onset of vaccinal immunity prior to exposure.

TOLL-LIKE RECEPTOR 3 ACTIVATION DECREASES PRRSV INFECTION

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Porcine reproductive and respiratory syndrome virus (PRRSV) initiates infection in pulmonary alveolar macrophages (PAMs), elicits weak immune responses, and establishes a persistent infection. To understand the role of ssRNA and dsRNA intermediates in eliciting host immunity, we sought to determine if Toll-like receptors (TLRs), particularly those that respond to viral molecular patterns, are involved in PRRSV infection. Activation of TLR3 in PAMs with dsRNA, increased gene expression for TLR3 and interferon-β and suppressed PRRSV infectivity. In contrast, treating PAMs with a synthetic ligand for TLR7 did not influence PRRSV infectivity. To investigate activation and signaling parameters, full-length cDNAs of porcine TLR3 and TLR7 were identified and various constructs were used in cell transfection studies. When HEK293 cells that overexpressed porcine TLR3 were stimulated with dsRNA a robust calcium influx was induced. Moreover, ligand activation of porcine TLR3 expressed in MARC-145 cells elicited an antiviral response to PRRSV. Conversely, transfection of PAMs with small-interfering RNA targeting porcine TLR3 resulted in 80% suppression of TLR3 mRNA expression and an increase in PRRSV infectivity. These data provide fundamental genetic and molecular information for porcine TLR3 and TLR7, and implicate TLR3 involvement in PRRSV infection.

IDENTIFICATION OF GLYCOPROTEIN 5 T CELL EPITOPES OF TWO PRRSV STRAINS

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An *ex vivo* study utilizing peripheral blood mononuclear cells (PBMCs) from pigs infected with attenuated and wild type PRRSV strains NADC-9 and NVSL-14 was conducted to identify immunodominant T cell epitopes of glycoprotein 5 (GP5) of these two strains. Forty two PRRS naïve age-matched out-bred pigs were split into 7 groups of 6 pigs each. Separate groups were either vaccinated at 4 weeks age with the attenuated strain of one kind and 4 weeks later challenged with the autogenous or a heterogenous wild type strain, or non-vaccinated and later challenged with either wild type strain. A non-infected group served as a control. Based on the GP5 sequence of attenuated and wild type PRRSV strains NADC-9 and NVSL-14, 15-mer peptides with 11 amino acid overlaps were synthesized. PBMC samples collected from all pigs prior to challenge and 2 weeks post challenge were exposed to peptides or intact viruses and tested for IFN-γ response using the ELIspot assay. Of the 100 peptides tested, four peptides, one of which is conserved across the strains, were noted to elicit a significant IFN-γ response (20-40 spots per million PBMCs); identifying these as immunodominant GP5 T cell epitopes for the responding pigs. Responding T cell subsets were identified by performing ELIspots subsequent to antibody and complement mediated depletion of different subsets. Some pigs responded to intact virus but not to GP5 based peptides indicating that other viral proteins may provide immunodominant T cell epitopes. Further investigations to establish the entire repertoire of PRRSv T cell epitopes would need to consider variation in viral strains and the SLA haplotype of pigs.

EFFECTIVENESS STUDY OF PRRS BOOSTER VACCINATION IN SOWS

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In Russia, PRRS was reported for the first time in 1993 (Mischenko V.A. et al., 1994). Vaccination of reproductive animals and weaned piglets has been carried out for PRRS prevention in Russia since 1997. Nowadays, there are two manufacturers of vaccines against PRRS in Russia (FGI "ARRIAH", Vladimir and NARVAC R&D, Moscow). Preparations of foreign producers are not registered.

Two PRRS vaccination protocols, conventional administration and administration including additional (booster) immunization in the period of 60-70 days of pregnancy, were compared.

The traditional administration was as follows: at first sows and gilts were double vaccinated 3 weeks before the service (insemination) by the FGI "ARRIAH" inactivated vaccine with a 2-4 week interval. Subsequently, sows were vaccinated once 3 weeks before the insemination.

The new administration under testing included double immunization before the insemination and additional (booster) vaccination in the period of 60-70 days of the pregnancy. Then, sows were vaccinated once 3 weeks before the insemination and at 60-70 days of the pregnancy.

Results of comparative tests of two administrations on large PRRS-positive farm (3,758 sows) are as follows:
- before immunization (223 sows): abortions - in 5.4% of sows, stillborns – 15.7% of sows, repeat insemination – 23% of sows, number of live piglets per sow per farrow – 8, mortality level in suckling piglets – 9.7%.
- traditional administration (345 sows): abortions - in 2.0% of sows, stillborns – 8.8% of sows, repeat insemination – 16.6% of sows, number of live piglets per sow per farrow – 8.5, mortality level in suckling piglets – 5.5%.
- with booster vaccination (482 sows): abortions - in 0.5% of sows, stillborns – 1.9% of sows, repeat insemination – 11.2% of sows, number of live piglets per sow per farrow – 9.1, mortality level in suckling piglets – 3.7%.

Thus, the testing showed positive effect of the booster vaccination against PRRS in the period of 60-70 days of pregnancy.

IDENTIFICATION OF IMMUNODOMINANT EPITOPES CONSERVED AMONG NORTH AMERICAN ISOLATES IN THE C-TERMINAL OF PRRSV

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The GP5 gene of PRRSV CH-1a isolate was divided into four overlapping segments (GP5-P1, GP5-P2, GP5-P3 and GP5-P4), cloned into pGEX-6p-1 vector respectively and expressed in *E. coli.* The expression products and 15 anti-GP5 MAbs of PRRSV CH-1a strain previously developed by us (Zhou et al, 2005) were used for indirect ELISA. The results demonstrated that 13 MAbs only recognized the products of GP5-P4 and 2 MAbs reacted with the products of GP5-P3 and GP5-P4.In order to precisely locate the antigenic epitopes, a series of shorter peptides were expressed and analyzed with the 15 MAbs by ELISA. The results revealed that 2 MAbs were recognized specifically by peptide 151-156aa(R^{151}LYRWR^{156}), 4 MAbs were recognized by peptide 165-180aa(K^{165}VEVEGHLIDLKRVVL^{180}), and 9 MAbs were recognized by peptide 196-200aa(Q^{196}WGRL^{200}). The alignment of amino acids revealed that these epitopes were highly conservative among all the North American PRRSV isolates. The epitope GP5EP7 at C-terminal of GP5 was precisely analyzed, and we concluded that the core sequence of this epitope was composed by 5 amino acids (^{96}QWGRL^{200}). This core sequence had a high homology among 34 North American types PRRSV strain, only one amino acid was variant (L^{200} to P^{200}). The mutational GP5EP7 (L^{200} to P^{200}) was expressed and the indirect ELISA revealed that 4 out of 9 MAbs recognized both GP5EP7 (L^{200}) and GP5EP7 (P^{200}) and the remained 5 MAbs only recognized GP5EP7 (L^{200}). Fusion polypeptides (containing L^{200}, P^{200}, 151-156 and 165-180aa, respectively) were analyzed by Western blot using antisera from pigs infected with PRRSV. The results revealed that these fusion polypeptides were all recognized by positive antisera and GP5EP7 (P^{200}) reacted stronger than GP5EP7 (L^{200}). This indicates that the 5 amino acids (196-200aa) at C-terminus are probably the dominant epitopes at C-terminus of PRRSV.

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GENE EXPRESSION PROFILING OF PRRSV-INFECTED PORCINE ALVEOLAR MACROPHAGES

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Identifying specific pathways that associate with variation in PRRSV replication and macrophage function can lead to novel gene targets for the control of PRRSV infection. Serial Analysis of Gene Expression (SAGE) is a powerful technique that allows a detailed and profound quantitative and qualitative knowledge of gene expression profile, with out previous knowledge of the sequence of analyzed genes. Total cellular RNA was prepared from *in vitro* mock-infected and PRRSV strain VR-2332-infected porcine alveolar macrophages (PAM) at 0, 6, 12, 16 and 24 hours after infection, and subjected to SAGE analysis to obtain >100,000 tags per time point. Identified unique mRNA tags were analyzed for their identity and relative abundance. Tags were mapped to transcripts and genes by exact regular expression matching to sequences in GenBank, Harvard Gene Index, Pig Expression Database (Japan), and the USMARC EST databases. Tag abundance was corrected for sequencing error using R and sagenhaft. Relative abundance was calculated based upon the number of times a tag is represented in a given SAGE library. Examination of the SAGE data indicated that there were changes in gene expression occurring in the PRRSV-infected PAM. More than 400 unique tags with significantly altered expression levels were identified ($p<0.01$ with Bonferroni correction). The validity and kinetics of expression of SAGE identified genes were evaluated using real-time RT-PCR. Control genes that do not respond to PRRSV infection were also included in the analysis as internal controls. Differential expression of the most abundantly expressed tag, corresponding to the ferritin light chain gene, was confirmed by real-time RT-PCR. The increase in the ferritin transcript abundance, as well as a cytochrome p450 and thioredoxin, was found in cell culture. The pro-inflammatory cytokines IL-1α and CCL4 (MIP1β) declined in transcript abundance in accordance with the findings of Lopez-Fuertes et al. (2000). The transcripts encoding RNA helicase RHIV-1 and Mx1 were induced between 0 and 10 h post-PRRSV-infection in accordance with Zhang et al. (1999).

SERUM NEUTRALIZATION OF PRRSV INFECTION OF PORCINE ALVEOLAR MACROPHAGES

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Neutralizing antibody response is a major contributor to the host humoral immune response against viral infection, working by binding free virions in serum, thus preventing virus entry into target cells. But the role of serum neutralizing response against PRRSV is unresolved. The lack of a consistent method to measure PRRSV-neutralizing activity in the naturally permissive host cells further compounds the problem. The conventional method to measure PRRSV-neutralizing activity is performed on MARC-145 cells (i.e. fluorescent focus neutralization assay), which is derived from MA-104, a green monkey kidney epithelial-like cell line. And this is a totally different cell type from the naturally PRRSV-permissive cells, porcine alveolar macrophages (PAMs). These two types of cells utilize diverse mechanisms for PRRSV infection according to current literatures. Heparin is reported to work as an attachment factor for PRRSV entry into MARC-145 cells, while heparin sulfate, a closely related but different molecule, is working the same for PAMs. Vimentin and CD151 are reported to play significant roles in helping PRRSV attach and be internalized into MARC-145 cells, whereas sialoadhesin and CD163 for PAMs. The diverse molecules used in PRRSV infection might also explain why some European PRRSV strains can not infect MARC-145 cells. To build up a better platform for analyzing serum neutralizing activity, we developed a PAM-based ELISA method that was sensitive enough to detect any reduction of PRRSV nucleocapsid protein amount in PAMs inoculated with diluted serum mixture with proper virus amount. Using this PAM-ELISA method, we were able to describe the diverse serum neutralizing activity dilution pattern from different individual pigs, and analyze the presence of PRRSV-neutralizing activity in serum partions either separated by affinity chromatography or ammonium sulfate precipitation. Finally, we tried to use this method to test the serum cross neutralizing activity.

SWINE IMMUNITY AND RESISTANCE TO PERSISTENT PRRSV INFECTION

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Infection with PRRSV elicits a weak immune response that is not fully protective and that results in persistent infection in a subset of pigs. Despite substantial research efforts the exact components of a protective anti-PRRSV immune response are still not known, particularly as it effects on persistence. We hypothesized that the intensity and timing of the early cytokine responses to PRRSV infection might be a predictor of final PRRSV burden and persistence. We tested serum samples collected as part of the "Big Pig" project, an extensive analysis of virus replication and immunity in a population of 109 pigs (and 60 control pigs) sampled for up to 203 days post-PRRSV infection (dpi). We compared pigs that apparently cleared the viral infection from serum and tissues by the first 28 dpi, the Non-Persistent (NP) pigs, to Persistent (P) pigs that even at 150 dpi have evidence of long term persistent PRRSV infection. Sera, collected over the course of the PRRSV infection, were tested for relevant cytokine induction in response to infection from the groups of P, NP and C pigs. Statistical analyses indicated that innate cytokines were increased earliest. Serum interleukin-1beta (IL-1b) levels were elevated in all pigs prior to infection and these levels continued to decrease for the C pigs. The infected pigs exhibited continuing high IL-1b to 14 dpi. When IL-8 was examined at 7 dpi and 14 dpi NP pigs had higher concentrations of IL-8 than P pigs. This increased IL-8 was followed in time by an increased level of interferon-gamma (IFNg) at 28 dpi in NP pigs. P pigs only showed peak serum IFNg at 42 dpi. There appeared to be no changes in serum IL-10 associated with infection. In summary, serum cytokine protein levels indicate that NP pigs appeared to have earlier and higher serum innate cytokine, IL-8, followed by T helper 1, IFNg, levels than the P pigs. This immune cytokine trend correlated with NP pigs having lower serum and tissue viral loads; this might indicate that the NP immune response was more effective than that for P pigs and possibly enabled the NP pigs to prevent PRRSV infections to become persistent. These data will help predict protective anti-PRRSV responses and to identify novel regulatory pathways that would stimulate PRRSV immunity.

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RAPID AND HIGH LEVEL EXPRESSION OF PRRSV PROTEINS

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The reliable and robust identification of PRRSV and the capability to respond to new and emerging variants is essential in containing and combating the disease. Recombinant protein based ELISA rather than ELISA based on purified virus or infected cell extracts is an excellent way of detecting virus infection. They are safe (no live virus), reproducible (as single protein preparation and purification can be subject to quality control) and have the promise of being specific. This latter point being important for distinguishing between field strains and vaccine isolates. One of the bottlenecks in producing proteins for ELISA, especially when considering an outbreak of a rapid new and emerging strains of (any) virus is the time taken for cloning and optimisation of protein production. Recently developed high level *in vitro* expression systems were used to demonstrate that PRRSV GP5 and nucleocapsid proteins, as proof of principal, could be produced via this route. Various post-translational modifications which could also be incorporated may play an essential role in successful and optimal virus detection.

INHIBITION OF PRRSV REPLICATION BY PEPTIDE-CONJUGATED MORPHOLINO OLIGOMERS.

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PRRS has led to heavy economic losses in the swine industry. In spite of vigorous tries, the current strategies and vaccines to control the disease are incapable to accomplish the goal. Hence alternative strategies are needed. Peptide-conjugated phosphorodiamidate morpholino oligomers (PPMO) have been reported to effectively interfere with the replication of several viruses in vitro and in vivo. PPMO are analogs of single-stranded DNA oligomers that contain a modified backbone and confer highly specific binding to RNA and resistance to nucleases. In our previous study, we designed anti-PRRSV PPMO against PRRSV genomic sequences and found five PPMO inhibiting PRRSV replication in cell culture. We continued our study on antisense PPMO and present the progress. Treatment of porcine alveolar macrophages (PAM) with PPMO 5UP2, 5HP, and 6P1+7P1 reduced PRRSV replication and protected the cells from cytopathic effect. Treatment of PAM with 5UP2 protected PAM from death for at least seven days after PRRSV inoculation. Immunofluorescence assay using an anti-PRRSV monoclonal antibody demonstrated that the PPMO inhibited PRRSV replication by significantly reducing the number of PRRSV-positive cells. Virus yields in PAM were reduced after the PPMO treatment. 5UP2 and 5HP inhibited replication of ten PRRSV strains of North America genotype. Cell viability assays detected no cytotoxicity of these PPMO within the concentration-range of this study for either CRL11171 cells or PAM. PPMO 5UP1 was synthesized as conjugates with modified backbone or different peptides. Treatment of PAM with these different versions of 5UP1 demonstrated that 5UP1 conjugated with peptide $(RXR)_4XB$ was the most effective in inhibition of PRRSV replication. Combination of 5UP1 with other PPMO demonstrated enhancement inhibitory effect in suppression of PRRSV replication in CRL11171 cells. These results suggest potential applications for the novel PPMO as antivirals to control PRRSV infection.

AMERICAN, BUT NOT EUROPEAN, GENOTYPE OF PRRSV-INFECTED DENDRITIC CELLS INDUCES T_{REG} ($FOXP3^+CD25^+$) CELLS

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In this work we evaluated whether PRRSV-infected dendritic cells (DCs) induced Treg cells *in vitro*.

Two American and five European strains of PRRSV were analysed. DCs were generated by culturing adherent cells with IL-4 and GM-CSF for 5 days. DCs were infected for 1 h at m.o.i. of 0.1, washed two times and cultured for 24 h. In some cultures, infected DCs were treated with IFN-α. After this time, infected DCs were co-cultured with homologous non-adherent cells (>95% of $CD3^+$) during 5 days. Some non-adherent cells were stained with CFSE in order to evaluate proliferation. Expression of cytokines mRNA (IL-10, and TGF-β) were analyzed by real time PCR, supernatants from the co-cultures were collected for cytokine analysis (IL-10 and IFN-γ), and the expression of $Foxp3^+CD25^+$ cells was analyzed by flow cytometry.

Our results showed that American type viruses reduced proliferation (1.8 fold difference, $p<0.05$); in two European type ones proliferation was 2.5 higher ($p<0.05$), and the other three European type viruses did not show significant differences ($p>0.05$). In presence of IFN-α, the proliferation with American type was not substantially increased ($p>0.05$), but it inhibited the ability of PRRSv to replicate. Heat-inactivated PRRSV did not modify proliferation ($p>0.05$). DCs infected with American type viruses induced an increased number of cells expressing $Foxp3^+CD25^+$ phenotype compared with the mock treated one (4.6 fold different, $p<0.05$); IFN-α treatment reduced $Foxp3^+CD25^+$ cells but this was not significantly different ($p>0.05$); heat inactivated virus was not different from the mock treated one ($p>0.05$). However, European type virus did not induce cells with $Foxp3^+CD25^+$ phenotype ($p>0.05$). The expression of mRNA IL-10 was 3 fold higher in DC infected with American type virus. ELISA analysis of American and three European type-infected DCs showed unsignificant differences, only one European type virus was able to increase the production of IL-10 ($p>0.05$). Finally, American type viruses increased the expression of mRNA TGF-β 5 fold.

In conclusion, these results suggest that American, but not European type PRRSV, induce the expression of Tregs cells ($Foxp3^+CD25^+$), and this induction could be dependent of TGF-β.

EFFECTIVENESS OF A PRRS MODIFIED-LIVE VIRUS VACCINE PREPARED IN A NOVEL PORCINE ALVEOLAR MACROPHAGE CELL LINE

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An innovative porcine alveolar macrophage cell line, designated ZMAC-1, was generated and its utility to manufacture an effective PRRS modified live virus (MLV) vaccine was examined. This cell line was found to be 100% susceptible to infection by PRRSV, as evidenced by the successful immunofluorescence staining for viral proteins at 20 hr after infection. To compare the efficacy of stocks of the MLV vaccine Prime Pac PRRS (Schering-Plough Animal Health) prepared in either ZMAC-1 or the simian cell line MARC-145, a standard immunization-challenge study was conducted. Six 8 week-old pigs were initially vaccinated intramuscularly with an equivalent dose (10^4 $TCID_{50}$) of the Prime Pac vaccine grown in either ZMAC-1 or MARC-145 cells, while two additional groups of three animals were not immunized. All of these animals, as well as one of the two groups of unvaccinated controls, were challenged 4 weeks later with 10^4 $TCID_{50}$ of an "atypical PRRS abortion storm" virus isolate (NADC-20). While the unvaccinated animals experienced an average body weight (BW) loss of –5±4 lb by 7 days after the virulent virus challenge, the PRRS virus-naïve controls had gained on average 19.7±6 lb during this time interval. In contrast, at 7 days post-challenge, the animals vaccinated with the MLV virus grown in either ZMAC-1 or MARC-145 cells exhibit average BW gains of 8.2±5.2 and 9.3±3.6, respectively. Thus, statistically the Prime Pac MLV vaccine grown in either cell line was equally effective at reducing the negative effect of the exposure of pigs to a highly virulent PRRS virus on their growth. Remarkably, analyses of the virus load in serum and lung lavage samples from PRRS virus-immunized and challenged animals revealed that the vaccine virus grown in ZMAC-1 cells was significantly ($P=0.015$) more effective at reducing the extent of viremia at 7 days post-challenge and also at eliminating virulent virus from their lungs by 10 days post-challenge. These observations suggest that the effectiveness of a PRRS MLV virus vaccine is not only, as it is commonly believed, determined by its genetic similarity to the challenge virus, but is also influenced by how it is produced.

PRESENCE OF IL-12 IN SEMINAL PLASMA FROM PRRSV-INFECTED AND NON-INFECTED BOARS

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Variability in the duration and quantity of PRRSV shedding in semen has been observed, but it is unknown what local immunological factors might influence the duration and viral load in this mucosal compartment. Cytokines act as immunomodulators that can influence viral infectivity and have been measured in seminal plasma from other species however, there is no information on cytokine levels in boar seminal plasma. Therefore, the objective of this study was to measure cytokine levels in PRRSV-infected (n=8 boars; 23 seminal plasma samples) and non-infected boars (n=5 boars, 9 seminal plasma samples). PRRSV infected boars were inoculated with 4 x 10^4 $TCID_{50}$/ml of a low virulent wild-type PRRSV strain MN 30-100 and semen was collected through 15 days post inoculation. Semen was centrifuged at 600xg to remove sperm and supernatants were stored at -80 C until analysis. A sandwich ELISA was performed (R & D Systems Inc., Mpls., MN) using swine specific monoclonal antibodies to detect innate, proinflammatory (IL-1β, IL-8, IL-6, TNF α), regulatory (IL-10) and T helper 1 (IL-12) cytokines. Due to the observance of inhibitors when ELISA standards were spiked into seminal plasma, a 100 kD Amicon filter (Millipore, Corp., Billerica, MA) was used to remove high molecular weight proteins, glycoproteins and lipids from the sample prior to performing the immunoassay. Both PRRSV infected and non-infected boars had levels of IL-12 ranging from 140-1024 pg/ml with a mean of 696 ± 233 pg/ml. Other cytokines, at this point in time, appear to have levels which were negligible or below the limit of detection of the assays. The significance of IL-12 in boar seminal plasma is unknown. However, IL-12, in general, promotes inflammatory and cytotoxic T lymphocyte responses which may stimulate the known uterine inflammatory response documented in sows after insemination and may protect against transmission of viral infection *in utero*.

FARROWING RESULTS FOLLOWING A PRRSV CHALLENGE IN PREGNANT SOWS IMMUNIZED WITH A LIVE VIRUS AND SUBSEQUENT MULTIVALENT PRRSV SUBUNIT PROTEIN VACCINE: A PRELIMINARY STUDY.

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The purpose of this study was to investigate and evaluate how anti-PRRSV envelope proteins (EP) antibodies play roles in the ability of protection against a heterologous PRRSV in pregnant sows. The sows had been exposed with a farm-specific PRRSV during their acclimatization. Then each sow was inoculated at least twice with multivalent PRRSV subunit protein vaccines. The last vaccine was given at 34 days of gestation. Six pregnant sows under the same parity were obtained and divided into 3 groups depending on the antibody quantity (high, medium and low by Western immunoblot analysis) to EP of the vaccines. All sows were inoculated 30 days before the due date with serum that was collected during acute PRRS outbreak in a different farm. Two sows each with high, medium and low EP antibody farrowed 20, 16 and 9 live-born pigs, respectively. Two sows each with high, medium and low EP antibody farrowed 5, 8 and 18 born-dead pigs, respectively. At birth, PRRSV was not detected in 11 of 11 piglet pools of the 4 sows with high and medium EP antibody but PRRSV PCR was positive for 2 of 3 pools of the 2 sows with low EP antibody. Results of PRRSV detection from the piglets at 3 and 14 days of age were similar to those at birth. These results indicate that transplacental infection did not occur in the sows with medium to high EP antibody at the time of challenge. The results also suggest that EP antibody levels measured by Western immunoblot may be a useful indicator for the protection. Repeated experiments with more sows are required to conclude these results.

PRRSV REPLICATION AND SUBSEQUENT IMMUNE RESPONSES IN SWINE OF VARIOUS AGES

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This investigation was performed to determine if the age of the host at the time of PRRSV infection influenced the *in vivo* growth characteristics of North American PRRSV and the resulting host immune response. The study design utilized a total of ninety pigs from the same PRRSV-free genetic source, comprising 30 pigs in each of three different age groups: three-week-old weaned piglets, growing pigs at 16-20 weeks of age, and mature, non-bred sows at 3 $\pm$ 1 parity. On day 0, 10 pigs from each age group received one of the following treatments: JA142 virulent PRRSV (parental isolate of vaccine), avirulent PRRSV (Ingelvac® PRRS ATP), or placebo. Viremia and the humoral response in the PRRSV-exposed animals were monitored for 63 days. Data collected illustrated distinct trends among the age groups. The $TCID_{50}$ evaluation of viremia following PRRSV exposure revealed that the three-week-old piglets generated the highest virus titers and maintained the live virus in the bloodstream for the greatest length of time. A comparison of titer results for the pigs at 16-20 weeks of age and the sows demonstrated modest statistical differences. The PRRSV quantitative RT-PCR assay further confirmed this age effect, demonstrating higher viral genome titers and longer persistence in the blood of weaned piglets. The IDEXX Herdcheck® 2XR ELISA also confirmed age-related variability in response following PRRSV exposure. The older animals, both sows and 16-20 weeks of age pigs, seroconverted sooner and achieved higher group average S/P ratios than the three-week-old piglets. This study confirmed previous research which stated that PRRSV isolates of varying virulence levels exhibit distinct trends *in vivo*, and also revealed that the pigs' age at the time of PRRSV exposure results in a noticeable difference in viremic and immunological outcomes.

USE OF REVERSE GENETICS TO DEVELOP A LIVE ATTTENUATED PRRSV DIVA VACCINE: PROOF-OF- CONCEPT AND PURSUIT OF AN OPTIMAL MARKER

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Availability of a DIVA (Differentiating Infected from Vaccinated Animals) vaccine is central for the control and eradication of PRRS. Epidemiological as well as regulatory considerations dictate that a PRRSV DIVA vaccine be designed in base of a negative marker (i.e., a marker absent from the vaccine strain but consistently present in wt strains). While technically straightforward in the case of DsDNA viruses (i.e., pseudorabies virus) deleting antigen-coding sequences from the genome of a live-attenuated RNA virus vaccine is a more difficult task. Previous studies in our laboratory identified numerous B-cell linear epitopes consistently recognized by convalescent serum of pigs infected with PRRSV. Based on their immuno-dominance, amino acid (aa) conservation and their degree of reactivity with field and reference antisera, we have focused on two epitopes which we consider serological marker candidates: one in NSP2 and the other in the M protein. Using site-directed mutagenesis we deleted these 15 aa epitopes in our PRRSV cDNA infectious clone (FL-12). In the case of the NSP2 protein (predictably, the viral protein most likely to tolerate large deletions) we were able to successfully rescue a mutant that fulfilled all the requirements for a DIVA marker vaccine: 1) efficient growth in vitro and in vivo, 2) active sero-conversion by regular ELISA with absence of a marker-specific ELISA response in 100 % (n=15) of the vaccinated animals and 3) rapid sero-conversion to peptide marker specific ELISA when animals were super-infected with an unrelated wt PRRSV strain at 110+ days PV. Deletion of the entire peptide marker candidate from the M gene was lethal for virus recovery. Alternatively, by substituting 5 aa at a time within the M peptide marker, we could recover a viable mutant virus, although it still resulted in a “marker positive” virus Efforts center now on preparing a viable M-marker mutant deprived of marker’s antigenicity.

In summary, our results provide proof-of-concept that DIVA PRRS virus vaccines can be developed. Efforts should now focus on identifying the optimal marker, capable of reliably inducing a detectable serologic response in the diverse universe of wt PRRSV strains.

REPLICATION OF PRRSV IN PORCINE PLASMACYTOID DENDRITIC CELLS IS NOT REQUIRED FOR INHIBITION OF THEIR INTERFERON-α GENERATIVE FUNCTION

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Infection of pigs with PRRSV results in a temporary loss in the ability of the host's plasmacytoid dendritic cells (PDC) to produce interferon (IFN)-α when exposed to external stimulants. A similar phenomenon is observed *in vitro*, although recovery can not be verified due to the cells' limited viability when cultured. This phenomenon does not appear to be dependent on virus replication since: 1) UV light inactivation of PRRSV infectivity did not alter the virus's inhibitory phenotype when assessed by using a peripheral blood mononuclear cell population (PBMC), containing PDC, and either transmissible gastroenteritis virus or deoxyoligonucleotides as a stimulant; 2) prior treatment of either PBMC or purified PDC with chloroquine, a lysosomotropic agent that blocks the pH-dependent step of PRRS virus entry into cells, had no noticeable impact on virus-mediated repression of the cells' IFN-α generative ability; and 3) PDC did not seem to support PRRS virus replication as indicated by a similar distribution of virus N protein in cells infected with either live or inactivated virus and a failure to detect PRRS virus ORF 7 transcription or release of virus from infected PDC. Since this pathogen probably does not replicate in PDC, the mechanism for suppression of induced IFN-α synthesis likely involves host cell interaction(s) with proteins associated with incoming, PRRS virus particles.

CERTAIN PRRSV PROTEINS INHIBIT IFN-B PROMOTER ACTIVATION.

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To antagonize the antiviral effects of interferons, viruses have developed specific antagonists as counter majors. The viral proteins that target type I interferon system either inhibit the production of IFNs or interfere with the downstream signaling pathway elicited by the IFNs. PRRSV is known to be a poor inducer of IFN-α both *in vitro* (MARC-145 and swine pulmonary alveolar macrophages) and *in vivo*. It has long been speculated that some PRRSV protein(s) is/are involved in mediating this type I interferon antagonism. To investigate this matter we cloned the viral structural and non structural genes into a CMV immediate early promoter-driven expression vector. The possible repression of IFN-β promoter activity by these genes was investigated with chloramphenicol acetyl transferase (CAT) assay. To this end Hela cells were cotransfected with an IFN-β CAT reporter plasmid, a plasmid expressing interferon regulatory factor-3 (IRF-3) for activating the IFN-β promoter and the individual viral gene expression constructs. The non structural protein1, 2 and 11 (nsp1, nsp2, nsp11) were found to be strong repressors (5-20 fold downregulation) of the IFN-β promoter. In addition non structural protein 4 (nsp4) and the nucleocapsid (N) protein exhibited moderate repression effect (1.5 to 2.5 fold downregulation) on IFN-β promoter. This implicates that nsp1, nsp2, nsp4, nsp11 and N protein may be involved in viral evasion of innate immune response.

GENETIC MARKER DEVELOPMENT IN THE NSP2 REGION OF A US TYPE I PRRSV.

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Porcine reproductive and respiratory syndrome virus (PRRSV) continues to be a major problem in the pork industry worldwide. The limitations offered by current PRRSV vaccines require the development of a new generation of vaccines. One of the key steps in future vaccine development is to include markers for diagnostic differentiation of vaccinated animals from those naturally infected with wild-type virus. Using a cDNA infectious clone of type 1 PRRSV, we have constructed several negative marker viruses, which contain deletions of immunodominant B-cell epitopes in the non-structural protein 2 (nsp2) region. Growth characterization in MARC-145 cells showed that all of the mutant viruses had a one-log reduction in titer compared to that of original cloned viruses, except the ΔES3 mutant replicated to a level comparable to that of the parental virus. To compliment the marker identification, we developed individual B-cell epitope-based ELISAs. Pigs immunized with the deletion mutants completely lacked antibodies directed against the corresponding deleted epitope, while pigs immunized with the parental virus generated high levels of antibodies to the nsp2 epitopes. The current research efforts are to determine whether a specific nsp2 mutant could confer the protection against a heterologous strain challenge. These marked-PRRSV recombinants, in conjunction with a diagnostic test, enable serological differentiation of vaccinated animals from infected animals and would be useful tools in PRRSV eradication programs. The identification of a deletion-permissive region on the nsp2 gene allows a rational approach to the insertion of protective epitopes and may be relevant for the design of cross-protective marker vaccines.

ANTIBODY RESPONSE OF NON-STRUCTURAL PROTEINS: IMPLICATION FOR DIAGNOSTIC DETECTION AND DIFFERENTIATION OF TYPE 1 AND TYPE 2 PRRSV

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In an effort to further characterize the humoral immune response of pigs to PRRSV, the kinetics of appearance of antibody response directed to the PRRSV non-structural proteins (nsp) was determined in pigs experimentally exposed to the virus using direct enzyme-linked immunosorbent assays (ELISA). The nsp1, nsp2 and nsp7 induced higher antibody response than the other nsps, which can be detected as early as 14 dpi, and last more than 202 dpi. Antibodies to nsp8 can be detected at 21 dpi, but the titer remains low. The nsp7 based ELISA performed better than the other nsps-based ELISA in regard to antigen preparation and diagnostic test application. Using nsp7 recombinant protein as antigen, we further validated a dual enzyme-linked immunosorbent assay (nsp7 Dua-ELISA) for the simultaneous detection and differentiation of serum antibodies directed against type1 and type2 PRRSV. Receiver operating characteristic analysis based on 1061 known positive and 1164 known negative samples showed good specificity (96.8% to type1 and 97.3% to type2) and sensitivity (99.0% to type1 and 99.8 % to type2) of the nsp7 dua-ELISA. To differentiate Type1 and Type2 PRRSV, a total of 912 sera originating from experimentally challenged pigs were used. Positive sera were correctly differentiated in 346 of 349 samples, indicating a high differentiation capability of this dual ELISA. The capability of the nsp7 dua-ELISA for detecting serum antibody response from pigs infected with various genetically different field strains was determined. 668 samples submitted to the SDSU diagnostic laboratory were tested. The nsp7 dua-ELISA possessed 97.2% agreement with the IDEXX ELISA. Taken together, the Dua-ELISA is the first differentiatial ELISA for PRRSV serology based on nsp. It is convenient with respect to antigen production, and it is reliable, economical, and highly sensitive and specific. Thus, it is considered to be a potential tool for routine diagnostics, epidemiological surveys, and outbreak investigations.

TESTICLE AND UMBILICAL CORD JUICE ARE SUITABLE SAMPLES FOR SEROLOGICAL CONTROL OF PRRS

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Bleeding breeding pigs is expensive, laborious and sometimes detrimental to the animal. Alternatives are meat-juice, colostrum or oral fluids (e.g. Salmonella, EP, PRRSV). This paper focuses on the use of testicle- (TJ) and umbilical cord juice (UCJ) for the serological diagnosis of PRRSV (ELISA; Idexx, Ludwigsburg). Sample dilution differed from manufacturer's instructions: TJ and UCJ were diluted 1/10 and 1/2, respectively. For all samples a cut-off of 30% was used. Testicles had to be cut before the freeze-thaw-cycle to get sufficient sample fluid. For analysis of TJ and UCJ blood was collected at the time of castration (3-7 days after farrowing) from the sow and from piglets. Umbilical cords were collected by the farmer immediately at farrowing (4-5 cm). Mean and maximum (brackets) reactivity of piglets within a litter were compared to the reactivity of the sow.

Results: a. TJ: 25 farms, 153 sera from sows and 483 testicle fluids were analysed. 86 (83) and 54 (55) were negative and positive for TJ and sow serum, respectively. Only 6 (4) TJ samples scored negative, although the sows gave positive or equivocal results. On the opposite 8 (11) TJ samples were positive although sows were scored negative. On herd level 12 (13) and 12 (12) herds were classified negative and positive by both materials, respectively. Only one herd was negative by TJ. b. UCJ: In 5 herds 144 umbilical cords from 50 litters were analysed. 12 (17) UCJ were positive from 3 (3) herds. In one of these herds an acute clinical PRRSV-outbreak was observed. In a second herd abortions are still under investigation.

Conclusions: TJ is an easily accessible alternative sample for testing individual sows just to avoid bleeding of sows. In individual herds it seemed to be slightly more sensitive than serum from sows. This might be explained by concentration of IgG in colostrum. Preliminary data on UCJ are promising however not yet conclusive. Currently, samples are analysed for viral genome sequences.

COMPARATIVE INFECTION EFFICIENCY OF PRRSV FIELD ISOLATES ON MA-104 CELLS AND PORCINE ALVEOLAR MACROPHAGES

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The purpose of this study was to determine if PPAM is a more sensitive tool to isolate field strains PRRSV than MA104. To compare the sensitivity of both cells, PPAM and MA 104 were inoculated with fifty sera samples from pig farms chronically affected with PRRSV. PRRSV-VR2332 strain was used as a control positive. We found that out of 50 sera samples tested 20 (40%) PRRSV field strains were isolated by one passage on PPAM; after three blind passes only one (2%) field strain grew in MA104. VR2332 grew better in MA104 reaching a titer of 107 TCDI50/ml in 72 h, when PPAM were infected with VR 2332 (m.o.i. 2), the highest titer obtain was 102 TCDI50/ml. Additionally, when PRRSV VR-2332 was used as a control positive, it did not replicate on PPAM. To evaluate PRRSV infectivity on cells, the cyto-pathogenic effect, immunofluorescence and RT-PCR were used. In this study we found than PPAM are more sensitive to isolate PRRSV field strain than MA 104 cells and that VR 2332 PRRSV strain grew better in continuous cell line, in this case MA104.

COMPARISON OF THE PRODUCTION RESULTS OF FOUR FARMS IN DIFFERENT PRRS CONTROL SCENARIOS.

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This study compared the main productive parameters of 4 swine farms of central Mexico of 1,000 animals each. All four farms have different PRRS control scenarios, the first is negative to the presence of the virus, the second is positive due to autogenous inoculation of the farm for PRRS control; the third farm is positive to the virus due to use of specific avian immunoglobulins for PRRSV control and the fourth is a farm that uses an attenuated commercial vaccine to control PRRSV infection. The results of the trial show that many productive parameters of the reproductive area are better in some farms that are PRRSV positive than in the negative farm, nevertheless the growing area there is a big difference in the productive parameters of positive farms vs negative farm. There are also great differences amongst positive farms productive parameters depending on the control scenario.

STIMULATION OF INTERFERON-GAMMA SECRETION AND LYMPHOCYTE PROLIFERATION BY PRRS GP5 PEPTIDE 6 FROM LYMPH NODE LYMPHOID CELLS FROM PRRSV INFECTED, BUT NOT FROM UNINFECTED, PIGS

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This study was designed to determine the optimum concentration of peptide P6 that would stimulate IFN-gamma secretion and proliferation by lymphocytes taken from pigs that had been infected with PRRS virus for a minimum of 6 months. Isolate VR-2332 was inoculated intranasally and intramuscularly into nursery pigs, and the pigs were maintained for 6-7 months before use. Uninfected age-matched pigs were held as controls. Sets of one infected and one control pig were euthanized, and several doses of chemically synthesized P6 were used to stimulate mesenteric lymph node cells in an ELISPOT assay for IFN-gamma secretion and to stimulate proliferation of PKH-67 labeled cells and detect that proliferation by flow cytometry. IFN-gamma was secreted from infected pig lymphocytes, but not from control pig lymphocytes, in response to P6. Similarly, lymphocyte proliferation was detected with infected pig lymphocytes, but not with control pig lymphocytes. The optimum P6 concentration for both assays was 5 ug/ml, the same dose that had previously been used to generate preliminary data that had suggested that P6 was a stimulatory peptide. These data suggest that P6 from GP5 can stimulate a potentially protective immune response by pig T lymphocytes.

INHIBITION OF PRRSV REPLICATION BY QUINOLONE-CONTAINING ANTIBIOTICS

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Antiviral agents for PRRSV remain an area that is largely unexplored. In contrast, antibiotics for non-virus microbial infections are widely used in the swine industry. This work was based on the observation that the incorporation of certain bacterial antibiotics into cell culture medium inhibits PRRSV replication in MARC-145 cells and porcine macrophages. For example the incorporation of the quinolone-containing compound Plasmocin, as well as the quinolones nalidixic acid and ciprofloxacin, resulted in potent anti-PRRSV activity in vitro. Anti-PRRSV activity was dose dependent in infected MARC-145 cells and cultures of primary alveolar porcine macrophages (PAMs). When used at suboptimal concentrations, nalidixic acid synergized with antiviral cytokines to inhibit PRRSV replication in MARC-145 and PAM cells. The antiviral activity of quinolones correlated with reduced actin expression, which points to a possible mechanism for antiviral activity. Plasmocin also inhibited replication of the closely related arterivirus, lactate dehydrogenase elevating virus (LDV). These results are significant to the development of antiviral strategies with potentially reduced toxicity, and provide a model system to better understand arterivirus replication.

SECTION 4: PRRS-RELATED DISEASES

PRRS-RELATED DISEASE: PORCINE CIRCOVIRUS-ASSOCIATED DISEASES (PCVAD)

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Type 2 porcine circovirus (PCV2)-associated diseases (PCVAD) including postweaning multisystemic wasting syndrome is an important global swine disease that poses serious economic threat to the swine industry worldwide. Numerous studies indicated that porcine reproductive and respiratory syndrome virus (PRRSV) potentiated PCV2 replication in pigs, and co-infection of pigs with PRRSV and PCV2 increases the severity of PRRSV-induced interstitial pneumonia in pigs. Currently PCV2 infection in pigs is widespread, and co-infection of pigs with both PRRSV and PCV2 occurs naturally in the field. Therefore, prevention of PCV2 infection will not only prevent PCVAD but will also have important implications in PRRSV management. To prevent and control PCVAD, we recently developed a genetically engineered vaccine, designated chimera PCV1-2, in which the capsid gene of the non-pathogenic PCV1 is replaced by the immunogenic capsid gene of PCV2 in the genomic backbone of PCV1. Subsequent immunogenicity, pathogenicty and challenge studies in pigs revealed that the chimeric PCV1-2 virus is attenuated in pigs but induces strong protective immunity against PCV2 infection and PCVAD. A vaccine based on the chimeric PCV1-2 virus, Suvaxyn® PCV2 One Dose™, has received full licensure from the USDA, and is currently on the market (Fort Dodge Animal Health Inc). The vaccine effectively prevents the development of PCV2 viremia and control PCV2-associated lymphoid depletion. By using the chimeric virus strategy, we also identified five different but overlapping conformational epitopes within residues 47 to 63, 165 to 200 and the last four amino acids at the C-terminus of the PCV2 capsid protein. These antigenic epitopes identified on the PCV2 capsid protein should provide valuable information for further structural analyses of PCV2 capsid protein and for the development of second-generation vaccines against PCV2 infection and PCVAD.

POTENTIAL OF PRRSV AS A DUAL VACCINE VECTOR FOR PRRS AND PCV2

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The recent development of infectious clones for PRRSV allows us to manipulate the viral genome and generate altered PRRSVs. In the present study, PRRSV-mediated foreign gene expression was explored. An expression cassette made to couple GFP with a PRRSV-specific transcription regulatory sequence was inserted into the viral genome immediately downstream of the replicase gene ORF1b. GFP was expressed in infected cells and was useful for monitoring virus infection in real time. The successful expression of GFP in PRRSV allowed us to further develop PRRSV as a vaccine vector. Since porcine circovirus type 2 (PCV2) is an etiologic agent for post-weaning multisystemic wasting syndrome in pigs, the PCV2 capsid protein gene was cloned and inserted in PRRSV to generate PRRSV-PCV2. Expression of the PCV2 capsid protein was confirmed by RT-PCR and immunofluorescence in virus-infected cells. To assess the immunogenicity of recombinant PRRSVs, three groups of five pigs were immunized twice intramuscularly with wild-type PRRSV, PRRSV-PCV2, or PRRSV-GFP, and blood samples were collected weekly for five weeks. GFP antibody began to rise at seven days post-infection and continued to rise for at least five weeks as determined by ELISA. PCV2 antibody increased until three weeks post-infection and then maintained a constant level thereafter. Our results demonstrate that a PRRSV-based vector is able to induce antibody responses in pigs to the products of inserted genes, and show a potential for PRRSV as a multivalent swine vaccine.

PRRS MLV VACCINATION DECREASES PCV2 VIREMIA

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Objective: Literature suggests that controlling co-factors in PCVAD is essential for the management of the disease. The objective of the study was to investigate describes the effect of vaccination against PRRSV on quantitative PCV2 PCR results in serum samples.

Methods: The qPCR PCV2 protocol utilized a TaqMan-based, real-time PCR using a standard curve created from serial dilutions of a plasmid encoding the open reading frame 2 (ORF2) of PCV2 to reveal the viral load in serum samples. Diagnostic data from 23 farms with PCVAD were available and included in the statistical analysis. The effects of PRRS vaccination status (yes/no) on qPCR PCV2 were analysed by means of the Wilcoxon-rank-sum test. Results were considered statistically significant if $p \leq 0.05$.

Results: Vaccination of pigs against PRRS had no effect on qPCR for PCV2 in 4 – 12 week old pigs ($p > 0.05$). However, vaccination against PRRS resulted in significantly lower qPCR results in animals 13 weeks or older compared to non-vaccinated animals. Interestingly, PRRS vaccinates had significantly lower viral loads when peak wasting disease was observed in the herds.

Conclusions: Vaccination against PRRS appears to decrease the magnitude of viremia of PCV2 as measured by qPCR in PRRS positive and PCVAD affected herds.

MOLECULAR CHARACTERIZATION OF A HIGHLY PATHOGENIC STRAIN OF PRRSV ASSOCIATED WITH PORCINE HIGH FEVER SYNDROME IN CHINA

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A devastating infectious disease, designated locally as "porcine high fever syndrome (PHFS)", spread almost all over China in 2006 resulting in culling up to 20 millions of pigs. Due to the complexity of the swine industry and the lack of an effective animal disease prevention and control régime, the causative agent(s) remained elusive. We conducted epidemiological investigation of PHFS, and our results showed that the major etiologic agent of the PHFS was a highly virulent form of PRRSV. Based on clinical observation and nucleotide sequencing analysis of ORF5, we further characterized a representative strain, designated as PRRSV JX143. The full-length genomic sequence of JX143 was determined by RT-PCR and nucleotide sequencing, which is 15320 nucleotides in-length excluding poly (A) tail. Phylogenetic analysis showed that JX143 is distinctly related to the prototype of Type II PRRSV VR-2332, with modest nucleic acid identity of 89%, while the highest genetic homology (96%) was detected with a Chinese strain, PRRSV HB-1. ORF5-based PCR-RFLP analysis showed that JX143, along with another 20 PRRSV isolates, belong to 1-4-3 pattern, which had not been detected in China. Surprisingly, all PHFS related PRRSV isolates share the same discontinuous deletions of one and 29 amino acids in nsp2-coding region, which are different from those deletions seen in MN184 strain. It is of great interest to investigate if the nsp2 deletion correlates with the phenotypic changes, mainly increased pathogenicity. To this end, we developed an infectious cDNA clone of PRRSV JX143, and the rescued virus displayed the same virological and clinical properties with those of the parental virus, which further confirmed that PRRSV JX143 was the causative agent for PHFS. We concluded that a highly pathogenic variant of PRRSV, represented by JX143, played major role in the PHFS outbreak in China.

DETECTION OF PCV2 IN RETROSPECTIVE CASES ASSOCIATED WITH PRRS

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The objective of this study was to determine the prevalence of porcine circovirus type 2 (PCV2) in retrospective cases of pigs naturally infected with PRRSV. One hundred ninety six pigs representing 140 pig herds that were diagnosed PRRSV infection between February 2004 and November 2005 were used in the study. A total of 34.2% of pigs appeared positive for PCV2 for organ homogenate pools of lung, liver, spleen, kidney, and inguinal lymph node. For serum, however, only 25.5% was found to be positive for PCV2 by polymerase chain reaction (PCR). The positive rate for PCV2 was much higher in weaned pigs than in suckling piglets as determined by PCR for both serum and organ homogenate pools. This result indicates that many pigs in the examined areas were co-infected with PCV2 and PRRSV, and the prevalence of PCV2 infection varied between suckling pigs and weaned pigs.

DEVELOPMENT OF A MULTIPLEX REAL-TIME QUANTITATIVE-PCR ASSAY FOR DETECTION AND DIFFERENTIATION OF PCV 2A AND 2B GENOTYPES AND ITS USE IN AN EPIDEMIOLOGICAL SURVEY

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In 2005, the Québec swine industry has experienced a significant increase of PMWS, which was associated with a new PCV-2 genotype named PCV-2b (Gagnon et al., 2007). Furthermore, characteristic PMWS histopathological lesions are likely to be more severe in pigs infected with PCV-2b virus compared to PCV-2a virus (Carman et al., 2007). Consequently, it became essential to differentiate PCV-2 viruses found in clinical samples submitted to our diagnostic laboratory. Following genomic analyses, it was found that the most variable PCV-2 gene between both genotypes was ORF2. Then, a hypervariable region was identified between position 222 nt and 279 nt of ORF2. Thus, this region was targeted to develop a multiplex real-time quantitative-PCR assay (MRTQ-PCR). It was determined that the MRTQ-PCR was at least 1000 times more sensitive than a previously described standard multiplex PCR (Ouardani et al, 1999). Additionally, the MRTQ-PCR can efficiently detect and differentiate both PCV-2 genotypes. Following a retrospective epidemiologic study where 40.2% of the submitted cases had severe to moderate PMWS and 39.3% had no PMWS, it was found that 91.8%, 4.1% and 4.1% of those cases were positive for PCV-2b, 2a or both genotypes, respectively. Following logistic regression statistical analysis, PMWS presence correlates significantly with the presence of PRRSV ($p < 0.0001$). Furthermore, the development of PMWS correlate significantly with the viral titer of PCV-2 ($p < 0.0001$), but not with the viral titer of PRRSV ($p = 0.73$) in lung and lymph nodes. Interestingly, the presence of *S. suis* epidemic serotypes (1/2, 1, 2, 3, 4 or 7) was significantly associated with PMWS ($p=0.0223$). No statistical association was identified between the PMWS and other pathogens like SIV and *M. hyopneumoniae*.

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PREVALENCE OF PCV2 AND PRRSV+PCV2 CO-INFECTION IN SWINE POPULATIONS IN MEXICO

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In this work we evaluated the prevalence of porcine circovirus type 2 (PCV2) and co-infections between PRRSV and PCV2 in swine populations of Mexico.

By IMPA assay, a retrospective serological survey in serum samples (n=659) collected between 1972 and 2000 revealed that serum antibodies to PCV2 were detected since 1973, with increasing proportions across the time. The analysis of serum samples from pigs from 11 states of Mexico (n=997) as well as from backyard pigs from the rural area of Mexico City (n=695) showed that 97% and 92% of those pig populations, respectively, had antibodies to PCV2. These results show evidence of enzootic PCV2 infection in Mexico at least since 1973, much before the first description of postweaning multisystemic wasting syndrome (PMWS) in the country (2001), and provide the earliest PCV2 infection evidence in the American continents. In addition, those results show that PCV2 antibodies are present in pigs produced under intensive and extensive farms.

Semen and serum from boars from three different farms (A, n=15; B, n=9; C, n=8) were analyzed to quantify PRRSv and PCV2 by real time PCR. Positive samples were sequenced (ORF5 and a fragment of ORF2 of PRRSV and PCV2, respectively). Antibodies to PRRSV and PCV2 were evaluated by ELISA and IPMA in serum samples, respectively. In farm A, 7 out of 15 boars were PRRSv PCR positive in semen and serum, and 3 out of 7 positive boars were infected with two different strains of the virus (<95% of homology). In this farm, 14 out of 15 boars were PCR positive to PCV2 in semen and serum and sequence analysis revealed the presence of two genotypes of PCV2 (<95% of homology). Serological studies showed that all boars from farm A were seropositive to PRRSV and PCV2. In farm B, 9 out of 9 boars were PCR positive to PRRSV in serum, and only 3 in semen. All boars were PCR positive to PCV2 on semen. In farm C, all boars (n=8) were PCR negative to PRRSV and PCV2 in semen and serum. Serologically, all boars from farms B and C had antibodies to PCV2. The present results showed that boars can be infected by two different strains of PRRSV, and that all boars analyzed had been previously infected with PCV2; PCV2 shedding in semen occurred only in those concomitantly infected with PRRSV.

PRRS AND RESEARCH ON PRRS IN VIETNAM.

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Porcine reproductive and respiratory syndrome virus (PRRSV) is a pathogen causing an important disease in pigs in most swine raising areas. The antibody against the PRRSV was detected for the first time in Vietnam in 1997 in 10 of 51 pigs imported from the States. Although all these pigs were slaughtered at a short time after the detection of virus-specific antibody, the PRRSV infected other pigs in the surrounding and the results of serological surveys at pig breeding farms in later years showed that 1,3% to 68,3% pigs had anti-PRRSV antibody in tested serum. However, there were no PRRSV-related outbreaks reported from 1997 to early 2006.

In March 2007, PRRSV-related outbreaks in pigs occurred in HaiDuong province in the North of Vietnam and rapidly spread to the other 6 provinces of the Red River Delta namely HungYen, QuangNinh, ThaiBinh, BacNinh, BacGiang and HaiPhong with more than 31,000 pigs affected. With comprehensive control measures, the epidemic was controlled after about one month. In June 2007, severe PRRSV-related outbreaks occurred in pigs in QuangNam province in the Center of Vietnam and uncontrollably spread to the surrounding provinces namely ThuaThienHue, DaNang and QuangNgai with more than 33,000 pigs affected with high mortality (23%). In July 2007, PRRS outbreaks occurred in LongAn province and BaRia – VungTau province in the South of Vietnam with 131 pigs affected. The PRRSVs were isolated from the diseased pigs using the MARC-145 cells. The challenge experiment was carried out using the isolated PRRSV from diseased pig of BacGiang province with 5 conventional pigs. The autopsies were performed on 2 pigs at 10 days post-challenge. As a result, multiple lesion sites in viscera were found: (1) pneumonia; (2) spleen haemorrhagic spots; (3) foci of the yellow-white necrosis in liver; (4) ulcers in the small intestine and (5) slight hemorrhages in the tonsil. Ten samples from different diseased pigs of several provinces were sent to the National Veterinary Service Laboratories at Plum Island and Ames in the US, under the collaboration with the USDA and FAO, for further identifying and characterizing the agents. Furthermore, with the assistance of the Chinese experts, the PRRSVs recently isolated in diseased pigs in Vietnam were classified as highly pathogenic PRRSV by using the RT-PCR detection kit to evidence target fragment of 400bp from the RNA of the PRRSV (Nsp2 1594-1680 Mutant).

DECREASED MORTALITY AND INCREASED PERFORMANCE FOLLOWING VACCINATION AGAINST PCV2

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In the past year, there have been several new developments in the control of porcine circovirus associated disease (PCVAD), including the release of commercial vaccines against porcine circovirus type 2 (PCV2). The purpose of this study was to conduct a blinded, randomized, well controlled clinical field trial to evaluate the efficacy of a commercial vaccine. The study utilized 485 commercial, mixed breed pigs located on a PRRSV free, farrow-to-finish farm. The farm had a known history of PCVAD, including the identification of PCV2b. Morbidity, mortality and performance data were recorded. Blood samples were collected from a subgroup of 52 pigs and sera analyzed for PCV2 antibody by IFA and for virus load using a real time SYBR Green PCR technique. For vaccinated pigs, overall mortality was reduced by 50% and finisher growth rate increased by 9.3%. PCV2 vaccinated pigs were 8.8 kg heavier in the same number of days to market compared to unvaccinated control pigs. Vaccinated pigs seroconverted and maintained anti-PCV2 titer levels ranging between 640 and 20,000. PCR showed an approximate 10 fold reduction in mean virus load in the serum of vaccinated pigs. These data show that vaccination in the control of PCVAD results in significant decreases in morbidity, mortality, and virus load, while improving performance.

COLLAGENOUS LECTIN GENE POLYMORPHISMS ASSOCIATED WITH THE SEVERITY OF PNEUMONIA IN PIGS INFECTED WITH PRRS VIRUS

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Immune responses to the PRRSV and other pathogens are influenced by genetic background. Collagenous lectins such as mannan binding lectins (MBLs), ficolins (FCNs) and surfactant protein-A (SP-A) have various antiviral and antibacterial functions, and pig FCN-alpha can bind and neutralize PRRSV. Various single nucleotide polymorphisms (SNP) are associated with susceptibility to various infections in humans. In pigs, a SNP at position –1081 in the promoter region of the pig *MBL2* gene is associated with low expression of MBL-C and with the occurrence of various infectious diseases diagnosed at necropsy. The objective of this study was to see if collagenous lectin SNPs are associated with the severity of pneumonia in pigs diagnosed at post-mortem with PRRSV. The severity of pneumonia in sections was graded by various histological criteria, including peribronchial lymphocytic infiltrates, alveolar neutrophils, alveolar macrophages and immunoreactive PRRSV antigen. DNA was extracted from fresh spleen sections and archived formalin-fixed, paraffin embedded blocks, and specific SNPs in lectins and various other innate immune genes were determined by Sequenom® MassARRAY analysis. Multiple linear regression was used to assess the association between severity of pneumonic lesions in PRRSV-infected pigs and SNPs in collagenous lectin genes. These studies indicate that SNPs within the *MBL1* and *MBL2* genes were most significantly associated with variation in the severity of lung lesions in PRRSV-infected pigs. The risk allele with the highest association with increasing severity of interstitial pneumonia was the SNP at position –1081 in the promoter region of the pig *MBL2* gene. A SNP in the coding region of FCN-alpha was associated with increased risk for PRRSV, porcine circovirus type II (PCV-2) and swine influenza virus (SIV) infections, but the same mutation was associated with lower lung scores where the severity of pneumonia in PRRSV-infected pigs was graded. These preliminary studies suggest that polymorphic genes within the innate immune system may be associated with disease severity in pigs infected with PRRSV.

EVIDENCE FOR RECOMBINATION BETWEEN PCV2A AND PCV2B IN THE FIELD

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Genomic sequence analysis demonstrates that porcine circovirus type 2 (PCV2) isolates are divided into distinct genotypes. Historically, swine herds in the U.S. have been infected with the PCV2a genotype. In 2005, PCV2b was identified in the U.S. and coincided with outbreaks of with of porcine circovirus disease (PCVD). A differential PCR technique incorporating PCV2 genotype-specific primers was used to identify the presence of PCV2a and PCV2b in tissues and sera from diagnostic field cases. The results of 97 diagnostic submissions showed that both PCV2a and PCV2b were present in 25% of samples. The construction of phylogenetic trees using whole genome sequences from diagnostic submissions showed that one isolate, 0737A, was only loosely associated with other PCV2b sequence. Analysis of the variable sites between representative PCV2a and PCV2b DNA sequences and the 0737A sequence, showed that 0737A was a chimera, with the ORF1 region from PCV2a and ORF2 from PCV2b. This study demonstrates that pigs can be naturally infected with multiple PCV2 genotypes and that PCV2a/PCV2b recombination events occur in the field.

STUDY ON THE FARM TRIAL OF PORCINE RECOMBINANT IFN-ALPHA TO TREAT HIGHLY PATHOGENIC PRRSV

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So-called ‘‘high fever’’ ravaged more than 10 provinces in southern China in the summer of 2006. Interferon(IFN) as a biological agent is a broad anti-virus drug. The clinical veterinary application of porcine WBC-IFN has been reported in controlling kinds of animal viral infection diseases, but the use of porcine recombinant IFN has not been reported for prevention and treatment of swine infected by highly pathogenic PRRSV. This study evaluated the farm trial of the porcine recombinant IFN-alpha made from the Secretive Expression of Pichia Pastoris for prevention and treatment of the highly pathogenic PRRSV.

1. In December of 2006, Huadou pig farmers in Guangzhou raised 38 grown pigs, 16 of them were confirmed to be the sick pigs infected by the high pathogenic PRRSV. After clinical treatment, the pig body temperature started to drop, they ate some fodders from the second day, stopped to snivel from the third day, the body temperature returned to the normal 38.6C, and they resumed the ordinary fodders after treatment. As result, the rest of them has been cured other than 2 fatal pigs and 89% curing rate was achieved.

2. On the basis of the invasion of 1000 pigs farm in Lechang of Shaoguan, most of them were confirmed to show the sick pigs infected by the high pathogenic PRRSV in December of 2006. 28 severe infected pigs and 8 initial infected pigs were selected to conduct the comparative trial in the random groups. After clinical treatment, the body temperature started to drop and they ate some fodders from the second day, the body temperature returned to the normal 39.2C and they recovered the ordinary fodders after trial. The rest of them recovered except for 6 fatal pigs and the curative ratio was 70%.

3. In May of 2007, the epidemic of the highly pathogenic PRRSV infection occurred in Xinhui pig farmers in Jiangmen and most of the young pigs and part of the grown pigs were confirmed to be severely infected. Six initial infected pigs were used in the control group, 31 severely infected pigs were divided into the random groups: 5 pigs/per group for the comparative trial. After clinical treatment, the body temperature started to drop and they ate some fodders from the second day, stopped to snivel from the third day, the body temperature returned to the normal 39.3C and they recovered the ordinary fodders after trial. The rest of them had been recovered except for 6 fatal pigs and the cure rate was 82%.

DISCUSSION The above results showed significant difference between the treatment groups and control groups against highly pathogenic PRRSV and over 81%curing rate was achieved. Although porcine recombinant IFN-alpha is a broad anti-virus biological drug, the time of administration is very important. To our farm trial, the porcine recombinant IFN-alpha is effective in the treatment of initial infected pigs, but it is not effective for severely infected pigs close to death. Therefore, it is recommended for the treatment of initial infected pigs.

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