

**The NC-229 (PRRS) Committee
presents the**

**2005
International PRRS
Symposium**

**Research into Methods for the Integrated
Control, Prevention, and Elimination of PRRS**

**Westport Conference Center, St. Louis, Missouri
December 2-3, 2005**

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**International PRRS Symposium
Westport Conference Center, St. Louis, Missouri
December 2-3, 2005**

NC-229 Planning Committee

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PROGRAM and SCHEDULE OF ACTIVITIES

FRIDAY DECEMBER 2, 2005.

15:00 Registrants check in (Pre-registration required. No walk-ins accepted)
Conference Room B - available for poster set-up and viewing
18:00 Conference Room A - Keynote Presentation: *Engineering porcine coronavirus genomes for vaccine development - the PRRS virus case*. Luis Enjuanes. Centro Nacional de Biotecnología, Campus Universidad Autónoma, Cantoblanco, Madrid
18:45 Reception and cash bar

SATURDAY DECEMBER 3, 2005

VIRAL GENOME

Moderators: Dongwan Yoo, Fernando Osorio

8:00 Keynote Presentation: *Neutralization and virulence determinants of equine arteritis virus: Implications for PRRS virus pathogenesis and immunity*. Udeni B.R. Balasuriya. Department of Veterinary Science, University of Kentucky
8:45 The virus, PRRSV. D. Yoo*
9:15 PRRS virus small envelope protein as a potential viroporin required for uncoating and replication (#18). C. Lee*, W.J.B. Hanna, G.A. Woolley, D. Yoo
9:30 Significance of structural genes of PRRS virus for virulence and attenuation (#15). B.J. Kwon*, I.H. Ansari, A.K. Pattnaik, F.A. Osorio

2005 International PRRS Symposium

- 9:45 Influence of N-linked glycosylation of PRRS virus GP5 on virus infectivity, antigenicity, and ability to induce neutralizing antibodies (#2). I.H. Ansari*, B.J. Kwon, F.A. Osorio, A.K. Pattnaik
- 10:00 Nonstructural protein 2 mutations of PRRS virus strain VR-2332 infectious clone based on deletions seen in RFLP184 isolates are viable (#8). J. Han, K.S. Faaberg*, Y. Wang, H. Liu
- 10:15 Break

DIAGNOSTICS

Moderators: Jane Christopher-Hennings, Steve Kleiboeker

- 10:30 Using a PRRS risk assessment tool to evaluate herds and nucleotide sequencing for epidemiology. P. Yeske
- 10:50 A practitioner/researcher's perspective on PRRS virus diagnostics: past, present and future. R.B. Baker
- 11:05 An improved method for PRRS virus surveillance and monitoring (#31). R. Simer*, J. Prickett, E.-M. Zhou, J. Zimmerman
- 11:20 Detection of isotype-specific anti-PRRS virus antibodies in swine oral fluid samples (#32). E.-M. Zhou, R. Simer, J. Zimmerman
- 11:35 Evaluation of a new field test to detect PRRS virus antibodies in swine sera (#28). E. Mende, J.P. Cano, H.S. Joo
- 11:50 Detection of PRRS using an in-solution and fiber-immobilized FRET immunosensor (#26). B. Heits, D. Lichlyter, S. Kleiboeker, S.A. Grant*
- Noon Luncheon Buffet served in the Terrace Restaurant

IMMUNE RESPONSE

Moderators: Federico Zuckermann, Joan Lunney

- 13:00 Keynote Presentation: *Immunity and vaccines to animal coronaviruses: Lessons for PRRS virus*. Linda Saif. Food Animal Health Research Program, The Ohio State University
- 13:45 PRRS virus modulates the innate immune function of porcine plasmacytoid dendritic cells (#33). G. Calzada-Nova*, R.J. Husmann, W.M. Schnitzlein, F.A. Zuckermann
- 14:00 Effect of PRRS virus on monocyte-derived dendritic cells (#39). J. Hernández*, F. Osorio
- 14:15 B-cell responses to PRRS virus infection (#46). P. Mulupuri*, G.N. Hirsch, S.A. Dee, J. Zimmerman, M.P. Murtaugh.
- 14:30 Genetic alteration of PRRS virus in the capsid (N) protein nuclear localization signal attenuates virus replication (#16). J.G. Calvert*, C. Lee, D.G. Hodgins, S.K. Welch, R. Jolie, D. Yoo
- 14:45 Differential immunity in pigs with high and low responses to PRRS virus (#44). J.K. Lunney*, D. Petry, P. Boyd, D. Kuhar, E. Blankenship, R. Johnson
- 15:00 Break

ECOLOGY, EPIDEMIOLOGY, AND PRRS VIRUS ELIMINATION

Moderators: Scott Dee, Tony Goldberg

- 15:15 Experiences with the national PRRS eradication program in Chile. M. Rojas, H. Rojas Olivarria
- 15:35 Environmental stability of PRRS virus. J.R. Hermann*, A. Burkhardt, M. Roof, K.-J. Yoon, K.M. Bryden, S.J. Hoff, R. Evans, J. Zimmerman
- 15:55 PRRS virus infection patterns in nursery pigs (#59). C. Dewey*, O. Melnichouk, R. Friendship, D. Hayden
- 16:15 Modeling the dynamics of PRRS virus infection within a herd: Early disease detection and evaluation of herd immunity (#68). C.A. Muñoz-Zanzi*, A. Rovira
- 16:35 Evolutionary biology of PRRS virus (#76). K.-J. Yoon*, S.-H. Cha, W.-I. Kim, C.-C. Chang, J. Zimmerman, P.M. Dixon
- 16:55 PRRS virus control in large-scale commercial settings: incorporating scientific information into herd-level management decisions (#65). J.F. Lowe,* T.L. Goldberg, F.A. Zuckermann, L.D. Firkins
- 17:15 Adjourn. Please pick up your posters in Conference Room B after the meeting.

ABSTRACTS

Poster No.

SECTION 1: VIRAL GENOME

The complete nucleotide sequence analysis of a Thai PRRS virus isolate.....	1
A. Amonsin ¹ , S. Puranaveja ² , N. Pariyotorn ¹ , P. Wongyanin ² , S. Suradhat ³ , R. Thanawongnuwech ^{2*}	
¹ Veterinary Public Health, ² Veterinary Diagnostic Laboratory, ³ Veterinary Microbiology, Chulalongkorn University, Bangkok, Thailand	
Influence of N-linked glycosylation of PRRS virus GP5 on virus infectivity, antigenicity, and ability to induce neutralizing antibodies	2
I.H. Ansari, B.J. Kwon, F.A. Osorio, A.K. Pattnaik. Nebraska Center for Virology and Depart of Veterinary & Biomedical Sciences, University of Nebraska-Lincoln, Lincoln, NE	
Characterization of PRRS virus mutation after persistent infection in pigs.....	3
C.-C. Chang, ¹ J.J. Zimmerman, ² K.-J. Yoon. ² ¹ Dept. of Veterinary Medicine, College of Agriculture, Chiayi University, Taiwan, ² Dept. of Vet. Diagnostic and Production Animal Medicine, Iowa State University, Ames, IA	
Mapping PRRS virus genetic determinants of macrophage host range & immune modulation	4
G. Delhon ¹ , F. Zuckermann ¹ , D. Rock ¹ , I. Guðmundsdóttir ² , G. Risatti ^{2*} ¹ Department of Pathobiology, University of Illinois at Urbana-Champaign, Urbana, IL 61802. ² Department of Pathobiology, University of Connecticut, Storrs, CT	
The PRRS virus nucleotide sequence database	5
K.S. Faaberg ^{1*} , T. Wennblom ^{1,3} , C. Mahlum-Wees ² , E.F. Retzel ³ , J.E. Collins ² . ¹ Department of Veterinary and Biomedical Sciences and ² Minnesota Veterinary Diagnostic Laboratory, University of Minnesota, Saint Paul, MN; ³ Center for Computational Genomics and Bioinformatics, University of Minnesota, Minneapolis, MN	
<i>In-vitro</i> characterization of a European-like Type 1 PRRS virus full-length cDNA infectious clone.....	6
Y. Fang*, B. Neiger, J. Mann, N. Benson, E.A. Nelson. Dept. of Veterinary Science, South Dakota State University, Brookings, SD	
Changes of microelement content in MARC-145 cells before and after infection with virulent and attenuated strains of PRRS virus	7
T.V. Grebennikova ¹ , A.V. Syroeshkin ² , M.I. Musienko ¹ , A.D. Zaberezhny ^{1*} , T.I. Aliper. ¹ ¹ NARVAC R&D, D. I. Ivanovski Virology Institute, Moscow, Russia, ² Research Oceanography Institute, Moscow, Russia	
Nonstructural protein 2 mutations of PRRS virus strain VR-2332 infectious clone based on deletions seen in RFLP184 isolates are viable	8
J. Han, K. S. Faaberg*, Y. Wang, H. Liu. Department of Veterinary and Biomedical Sciences, University of Minnesota, Saint Paul, MN	
Interference of PRRS virus replication on Marc-145 cells using DNA-based short interfering RNAs	9
Y.-X. He, R.-H. Hua, Y.-J. Zhou, H.-J. Qiu, G.-Z. Tong.* National Key Laboratory of Veterinary Biotechnology, Harbin Veterinary Research Institute, Chinese Academy of Agricultural Sciences, Harbin 150001, P.R. China	

Genetic analysis of PRRS virus in Mexico.....	10
J. Hernández ^{1*} , G. Yepiz-Plascencia ² , F. Osorio ³ . ¹ Laboratory of Immunology, ² Laboratory of Marine Biotechnology, Research Center for Food and Development (CIAD, A.C.), Hermosillo, Sonora, Mexico. ³ Department of Veterinary and Biomedical Sciences, University of Nebraska-Lincoln, Lincoln, NE	
Physical characterization of the PRRS virus virion.....	11
C.R. Johnson ¹ , B.J. Saxton ¹ , M.C. Fuentes ¹ , L.B. Anderson ² , T.P. Krick ² , M.P. Murtaugh ^{1*} . ¹ Department of Veterinary & Biomedical Sciences, ² Department. of Biochemistry, Molecular Biology and Biophysics, University of Minnesota, St. Paul, MN	
Defining the cellular targets of PRRS virus blocking MoAb (7G10)	12
J.-K. Kim, S. Kapil.* Department of Diagnostic Medicine-Pathology, Kansas State University, Manhattan, KS	
Subcellular localization of the non-structural proteins of PRRS virus.....	13
D.-Y. Kim ^{1*} , M. Kerrigan ¹ , P. Schneider ¹ , R. Rowland. ¹ ¹ Dept. of Diagnostic Medicine and Pathobiology, College of Veterinary Medicine, Kansas State University, Manhattan, KS	
Generation of a PRRS virus infectious clone from an attenuated vaccine strain	14
B.J. Kwon, I.H. Ansari, F.A. Osorio, A.K. Pattnaik. Nebraska Center for Virology and Department of Veterinary & Biomedical Sciences, University of Nebraska-Lincoln	
Significance of structural genes of PRRS virus for virulence and attenuation.....	15
B.J. Kwon, I.H. Ansari, A.K. Pattnaik, F.A. Osorio. Nebraska Center for Virology and Department of Veterinary & Biomedical Sciences, University of Nebraska-Lincoln	
Genetic alteration of PRRS virus in the capsid (N) protein nuclear localization signal attenuates virus replication	16
C. Lee ¹ , D.G. Hodgins ¹ , J.G. Calvert ^{2*} , S.K. Welch ² , R. Jolie ² , D. Yoo ¹ , ¹ Dept. of Pathobiology, Ontario Veterinary College, University of Guelph, Guelph, Ontario, ² Pfizer Animal Health, Kalamazoo, MI	
PRRS virus structural protein mutation studies.....	17
H. Liu, K.S. Faaberg*, Y. Wang, J. Han. Department of Veterinary and Biomedical Sciences, University of Minnesota, Saint Paul, MN	
PRRS virus small envelope (E) protein as a potential viroporin required for uncoating and replication	18
C. Lee ^{1*} , W.J.B. Hanna ² , G.A. Woolley ³ , D. Yoo ¹ . ¹ Dept. of Pathobiology, ² Dept. of Biomedical Sciences, University of Guelph, Guelph, ³ Dept. of Chemistry, University of Toronto, Toronto, Ontario	
The PRRS virus N protein possesses a non-classical nuclear export signal sequence.....	19
J.M. Rowland,* R.R.R. Rowland. Department of Diagnostic. Medicine./Pathobiology. Kansas State University, Manhattan, KS	
Use of a PRRS virus infectious clone to evaluate in vitro quasispecies evolution.....	20
S.K. Schommer,* S.B. Kleiboeker. Department of Veterinary Pathobiology, College of Veterinary Medicine, University of Missouri-Columbia	
Potential of PRRS virus as a vaccine vector for foreign gene expression.....	21
C. Song ^{1*} , J.G. Calvert ² , S.K. Welch ² , D. Yoo ¹ . ¹ Department. of Pathobiology, Ontario Veterinary College, University of Guelph, Guelph, Ontario, ² Pfizer Animal Health, Kalamazoo, MI	

Identification and characterization of a cellular gene essential for PRRS virus infection	22
S.-K.W. Welch*, D.E. Slade, S.L. Shields, R. Jolie, R.M. Mannan, R.G. Ankenbauer, J.G. Calvert. Pfizer Animal Health, Kalamazoo, MI	
The virus, PRRS virus	23
D. Yoo. Department of Pathobiology, University of Guelph, Guelph, Ontario, Canada	
Identification and characterization of a putative cellular receptor for PRRS virus	24
E.-M. Zhou, Q.-S. Qin. Department of Veterinary Diagnostic and Production Animal Medicine, Iowa State University, Ames, IA	
 SECTION 2: DIAGNOSTICS	
A novel DNA Micro analyzer using PCR process	25
S. Bhattacharya ^{1*} , S. Grant ¹ , S.B. Klieboeker ² , K. Gangopadhyay ³ , S. Gangopadhyay ³ . ¹ Department of Biological Engineering Department, ² Veterinary Diagnostic Laboratory, ³ Department of Electrical Engineering, University of Missouri, Columbia	
Detection of PRRS using an in-solution and fiber-immobilized FRET immunosensor	26
B. Heits ¹ , D. Lichlyter ¹ , S. Kleiboeker ² , S.A. Grant ¹ . ¹ Department of Biological Engineering, University of Missouri-Columbia. ² Department of Veterinary Pathobiology, University of Missouri-Columbia	
The effect of genotypic and biotypic differences among PRRS viruses on the immune response and serologic assessment of pigs to PRRS virus infection.	27
W.-I. Kim ^{1*} , W. Johnson ² , K.-J. Yoon ¹ . Veterinary Diagnostic and Production Animal Medicine, Iowa State University, Ames, IA; ² Boehringer-Ingelheim Vetmedica, Inc.	
Evaluation of a new field test to detect PRRS virus antibodies in swine sera.....	28
E. Mende, J.P. Cano, H.S. Joo*. College of Veterinary Medicine, University of Minnesota, St. Paul, MN	
Sampling of adult boars for early detection of PRRSV by PCR using a new technique.	29
D.L. Reicks ^{1*} , C. Muñoz-Zanzi ² , K. Rossow ² . ¹ Swine Vet Center, St. Peter, MN. ² University of Minnesota, College of Veterinary Medicine, St. Paul, MN	
Detection of PRRS seroconversion and persisting antibody titers in swine following controlled infection during a long-term study	30
A. Rice ^{1*} , V. Leathers ¹ , L. Plourde ¹ , R. Rowland ² , R. Molina ³ , J. Hermann ³ , J. Zimmerman ³ . ¹ Infectious Diseases R&D, Production Animal Services, IDEXX Laboratories, Westbrook, ME. ² Dept. of Diagnostic Medicine and Pathobiology, Kansas State University, Manhattan, KS ³ Dept. of Vet Diagnostic & Production Animal Medicine, Iowa State University, Ames, IA	
An improved method for PRRS virus surveillance and monitoring.....	31
R. Simer ² , J. Prickett ¹ , E.-M. Zhou ¹ , J. Zimmerman ¹ . ¹ Department of Veterinary Diagnostic and Production Animal Medicine, Iowa State University, Ames, IA. ² Perryton, TX	
Detection of isotype-specific anti-PRRS virus antibodies in swine oral fluid samples	32
E.-M. Zhou ¹ , R. Simer ² , J. Zimmerman ¹ . ¹ Veterinary Diagnostic Laboratory, Iowa State University, Ames, IA. ² Perryton, TX	

SECTION 3: IMMUNE RESPONSE

PRRS virus modulates the innate immune function of porcine plasmacytoid dendritic cells.....	33
G. Calzada-Nova*, R.J. Husmann, W.M. Schnitzlein, F.A. Zuckermann. Department of Pathobiology, University of Illinois, Urbana, Illinois	
<i>Ex-vivo</i> assessment of the PRRS virus cross protective immune response over time.....	34
S.-H. Cha ¹ , W.-H. Wu ² , R. Molina ² , J. Zimmerman ² , K.-J. Yoon ^{1,2} . ¹ Veterinary Microbiology and Preventive Medicine and ² Veterinary Diagnostic and Production Animal Medicine, College of Veterinary Medicine, Iowa State University, Ames, IA	
Effects of PRRS virus virulence and antigen-presenting cells on T cell activation and antiviral cytokine production.....	35
W. Charemtantanakul*, R. Platt, J.A. Roth. Department of Veterinary Microbiology and Preventive Medicine, Iowa State University, Ames, IA	
Mapping of B-cell linear epitopes on Nsp2 and structural proteins of a North American strain of PRRS virus	36
M. de Lima ^{1,2} , A.K. Pattnaik ¹ , E.F. Flores ² , F.A. Osorio ¹ . ¹ Nebraska Center for Virology and Dept of Veterinary and Biomedical Sciences, University of Nebraska-Lincoln. ² Dept of Microbiology and Parasitology, Federal University of Santa Maria, Santa Maria, RS, Brazil	
Immunization of pigs with viral replicon particles expressing PRRS virus GP5 & M proteins ...	37
M.M. Erdman ^{1*} , K.I. Kamrud ² , D.L. Harris ^{1,3} . ¹ Dept of Animal Science, Iowa State University, ² AlphaVax Inc, Research Triangle Park, NC, ³ Dept of Veterinary Diagnostic and Production Animal Medicine, Iowa State University, Ames, IA	
Permissiveness of porcine alveolar macrophages for PRRS virus replication in culture is dependent on culture conditions	38
N. Gaudreault*, C. Wyatt, R.R.R. Rowland. Diagnostic Medicine/Pathobiology, Kansas State University, Manhattan, KS	
Effect of PRRS virus on monocyte-derived dendritic cells.....	39
J. Hernández ^{1*} , F. Osorio ² . ¹ Laboratory of Immunology, Research Center for Food and Development (CIAD, A.C.), Hermosillo, Sonora, Mexico. ² Department of Veterinary and Biomedical Sciences, University of Nebraska-Lincoln, Lincoln, NE	
Impact of PRRS virus on classical swine fever vaccine.....	40
S. Kesdangakonwut ¹ , W. Sada ³ , S. Lacharoje ¹ , S. Suradhat ² , R. Thanawongnuwech ^{1*} . ¹ Veterinary Pathology, ² Veterinary Microbiology, Chulalongkorn University, ³ Clinic for Swine, Mahanakorn University of Technology, Bangkok, Thailand	
Influence of genetic difference on cross protection among PRRS viruses.....	41
W.-I. Kim ¹ , S.-H. Cha ¹ , K.-J. Yoon ^{1,2} . Departments of ¹ Veterinary Microbiology and Preventive Medicine and ² Veterinary Diagnostic and Production Animal Medicine, College of Veterinary Medicine, Iowa State University, Ames, IA	
Experimental infection of pigs with European-like (Type 1) PRRS virus isolates of U.S. origin.....	42
S. Lawson ¹ , Y. Fang ¹ , R.R.R. Rowland ² , J. Christopher-Hennings ¹ and E.A. Nelson ^{1*} . ¹ Dept. of Veterinary Science, CIDRV, South Dakota State University, Brookings, SD and ² Dept. of Diagnostic Medicine/Pathobiology, Kansas State University, Manhattan, KS	

PRRS virus activates NF- κ B via reactive oxygen species (ROS) production and increases matrix metalloproteinase (MMP) expression in NF- κ B dependent manner.....	43
S.-M. Lee*, S.B. Kleiboeker. Department of Veterinary Pathobiology, College of Veterinary Medicine, University of Missouri-Columbia	
Differential immunity in pigs with high and low responses to PRRS virus.....	44
J.K. Lunney ¹ *, D. Petry ² , P. Boyd ¹ , D. Kuhar ¹ , E. Blankenship ² , R. Johnson ² ¹ APDL, BARC, USDA, Beltsville, MD and ² University of Nebraska, Lincoln, NE	
Type I interferon responses to PRRS virus infection	45
L.C. Miller*, C.G. Chitko-McKown, W.W. Laegreid. Animal Health Research Unit, USDA, ARS, Roman L. Hruska U.S. Meat Animal Research Center, Clay Center, NE	
B-cell responses to PRRS virus infection.....	46
P. Mulupuri ¹ *, G.N. Hirsch ¹ , S.A. Dee ² , J. Zimmerman ³ , M.P. Murtaugh ¹ . ¹ Department of Veterinary and Biomedical Sciences, ² Veterinary Population Medicine, University of Minnesota, St Paul, MN. ³ Department of Veterinary Diagnostic and Production Animal Medicine, Iowa State University, Ames, IA	
Antibody responses of PRRS virus-exposed sows following killed virus vaccination pre-farrowing.....	47
D. Nilubol ¹ *, B. Thacker ² , E. Thacker ² . ¹ Chulalongkorn University, Thailand, ² Iowa State University, Ames, IA	
PRRS virus fails to activate the unfolded protein response.....	48
J.M. Rowland*, R.R.R. Rowland. Department of Diagnostic Medicine/Pathobiology, Kansas State University, Manhattan, KS	
Identification and characterization of a novel cellular protein interacting with the PRRS virus nucleocapsid (N) protein.....	49
C. Song*, D. Yoo. Department of Pathobiology, Ontario Veterinary College, University of Guelph, Guelph, Ontario, Canada	
Immunization of sows with a recombinant pseudorabies virus expressing the GP5 of PRRS virus confer protection against PRRS.....	50
Z.-J. Tian, H.-J. Qiu, G.-Z. Tong*. National Key Laboratory of Veterinary Biotechnology, Harbin Veterinary Research Institute, Chinese Academy of Agricultural Sciences, Harbin 150001, P. R. China	
Microsatellite markers developed in the entire SLA region and their availability in various porcine breeds.....	51
H. Uenishi ¹ *, M. Tanaka ² , A. Ando ³ , C. Renard ⁴ , P. Chardon ⁴ , M. Domukai ² , N. Okumura ² , T. Awata ¹ . ¹ Natl. Inst. Agrobiological Sci., Tsukuba, Ibaraki, Japan. ² STAFF-Institute, Tsukuba, Ibaraki, Japan. ³ Dept. Mol. Life Sci., Div. Basic Mol. Sci. & Mol. Med., Tokai Univ. Sch. Med., Isehara, Kanagawa, Japan. ⁴ Laboratoire Mixte de Radiobiologie et d'Etude du Génome, INRA-CEA, Domaine de Vilvert, Jouy-en-Josas, France	
Mapping PRRS virus T cell epitopes.....	52
C. Wyatt* ¹ , R.R.R. Rowland ¹ , D. Smith ² . ¹ Diagnostic Medicine/Pathobiology, Kansas State University, Manhattan, KS 66506. ² Transplant Immunology Laboratory, Baylor University Medical Center, Dallas, TX	
Construction and characterization of recombinant PRV expressing PRRS virus gp5 and M proteins	53
Z. Liu ¹ *, A.K. Pattnaik ² , F. Osorio ² , S.I. Chowdhury ¹ . ¹ College of Veterinary Medicine, Kansas State University, Manhattan, KS. ² Veterinary Biomedical Sciences, University of Nebraska, Lincoln, NE	

Epitope mapping of structural proteins of PRRS virus	54
Y.-J. Zhou, T.-Q. An, J.-X. Liu, H.-J. Qiu, G.-Z Tong*. National Key Laboratory of Veterinary Biotechnology, Harbin Veterinary Research Institute, Chinese Academy of Agricultural Sciences, Harbin, 150001, The People's Republic of China	
SECTION 4: ECOLOGY, EPIDEMIOLOGY, ELIMINATION	
Transmission of PRRS virus to pigs via virus-contaminated pork: A risk assessment	55
L. Alban ¹ , T. Drew ² , P. Have ³ , M.-F. Le Potier ⁴ , M.S. Murtaugh ⁵ , H. Nauwynck ⁶ , G. Wellenberg ⁷ , J.M. Sánchez Vizcaino ⁸ , M. Wierup ⁹ , J. Zimmerman ¹⁰ . ¹ Danish Bacon and Meat Council, Copenhagen, Denmark; ² Veterinary Laboratories Agency, Addlestone, United Kingdom; ³ Danish Institute for Food and Veterinary Research, Copenhagen, Denmark; ⁴ Agence Française de Sécurité Sanitaire des Aliments, Ploufragan, France; ⁵ University of Minnesota, St. Paul, Minnesota; ⁶ Ghent University, Merelbeke, Belgium; ⁷ Animal Health Service Institute, Deventer, The Netherlands; ⁸ Universidad Complutense de Madrid, Madrid, Spain; ⁹ Swedish University of Agricultural Sciences, Uppsala, Sweden, ¹⁰ Iowa State University, Ames, Iowa	
Assessment of vertical transmission from parity one sows infected with low dose and mild virulent PRRSV isolate	56
J.P. Cano, R. Morrison, S. Dee. College of Veterinary Medicine, University of Minnesota, St Paul, MN	
An evaluation of isolate pathogenicity in the shedding and transmission of PRRS virus by aerosols	57
J.G. Cho ¹ , S.A. Dee* ¹ , J. Deen ¹ , K. Faaberg ² , H.S. Joo ¹ . ¹ Swine Disease Eradication Center, ² Veterinary Diagnostic Laboratory, University of Minnesota, College of Veterinary Medicine, St. Paul, MN	
PRRS research at the University of Minnesota.	58
S.A. Dee ^{1,2} , P. Davies ^{1,2} , C. Pijoan ^{1,2} , H.S. Joo ^{1,2} , B. Morrison ^{1,2} , T. Molitor ^{1,2} , C. Muñoz Zanzi ^{1,2} , K. Faaberg ³ , K. Rossow ¹ , M. Rutherford ³ , M.P. Murtaugh ^{2,3*} . Departments of ¹ Veterinary Population Medicine and ³ Veterinary & Biomedical Sciences, and ² Swine Disease Eradication Center, University of Minnesota, St. Paul, MN	
PRRS virus infection patterns in nursery pigs	59
C. Dewey*, O. Melnichouk, R. Friendship, D. Hayden. Population Medicine, University of Guelph, Guelph, Ontario Canada	
PRRS virus: Emergence of novel strains in Britain	60
J.P. Frossard*, D. Westcott, B. Naidu, G. Sharp, C. Russell, NGA Woodger, T. Drew. Veterinary Laboratories Agency (Weybridge), New Haw, Addlestone, Surrey KT15 3NB, U.K.	
Stability of PRRS virus in aerosols	61
J.R. Hermann ¹ , A. Burkhardt, M. Roof ⁴ , K.-J. Yoon, K.M. Bryden ³ , S.J. Hoff ⁴ , J. Zimmerman ¹ . ¹ College of Veterinary Medicine, ³ College of Engineering, ⁴ College of Agriculture Iowa State University, Ames, IA. ⁴ Boehringer Ingelheim, Ames, IA	
Sampling pig respiratory exhalations for aerosolized pathogens	62
J.R. Hermann ¹ , K.J. Yoon ¹ , R.B. Evans ¹ , S.J. Hoff ² , J. Zimmerman ¹ . ¹ College of Veterinary Medicine, ² College of Agriculture, Iowa State University, Ames, IA	
Origin of PRRS virus: Towards a better understanding beyond the epidemiology	63
T.Y.Lam, F.C.C.Leung*. Department of Zoology, The University of Hong Kong, Hong Kong Special Administrative Region (HKSAR), China	

Molecular epidemiology of PRRSV in Hong Kong	64
V.Y.Y. Li*, F.C.C. Leung. Department of Zoology, The University of Hong Kong, Hong Kong Special Administrative Region (HKSAR), China	
PRRS virus control in large-scale commercial settings: incorporating scientific information into herd-level management decisions	65
J.F. Lowe ^{1*} , T.L. Goldberg ² , F.A. Zuckermann ² , L.D. Firkins ² . ¹ The Maschhoffs, Inc., Carlyle, Illinois, ² College of Veterinary Medicine, University of Illinois	
Management of PRRS persistence: Identification of persistently infected swine	66
R.M. Molina ^{1*} , J. Hermann ¹ , R.R.R. Rowland ² , J. Christopher-Hennings ³ , E. Nelson ³ , J. Lunney ⁴ , K-J Yoon ¹ , J. Zimmerman ¹ . ¹ Iowa State University, Ames, IA; ² Kansas State University, Manhattan, KS; ³ South Dakota State University, Brookings SD; ⁴ USDA, ARS, BARC, Beltsville, MD	
Experimental quantification of effect of PRRS vaccination on PRRSV transmission	67
E. Mondaca-Fernandez*, C. Muñoz-Zanzi, R. Morrison. Dept of Clinical and Population Sciences, College of Veterinary Medicine, University of Minnesota, St. Paul, MN	
Modeling the dynamics of PRRS virus infection within a herd: Early disease detection and evaluation of herd immunity	68
C.A. Muñoz-Zanzi*, A. Rovira. Department of Veterinary Population Medicine, College of Veterinary Medicine, University of Minnesota. St. Paul, MN	
Suppression of PRRS virus infection by boar seminal plasma	69
T. Okinaga*, D.E. Reeves, D.J. Hurley. Department of Population Health, Food Animal Health and Management Program, College of Veterinary Medicine, University of Georgia, Athens, GA	
Management of PRRS persistence: Studies at the population level	70
R.R.R. Rowland ^{2*} , R. Molina ¹ , J. Hermann ¹ , J. Christopher-Hennings ³ , E. Nelson ³ , J. Lunney ⁴ , V. Leathers ⁵ , J. Zimmerman ¹ . ¹ Iowa State University, ² Kansas State University, ³ South Dakota State University, ⁴ BARC, ⁵ IDEXX Laboratories, Inc.	
The effect of PRRS virus infection on growth performance	71
R.R.R. Rowland ^{2*} , J. Nietfeld ² , R. Molina ¹ , J. Hermann ¹ , J. Zimmerman ¹ . ¹ Iowa State University, Ames, IA; ² Kansas State University, Manhattan, KS	
Full-length genome analysis of European-like PRRSV in the U.S.: Identification of non-structural protein regions as potential epidemiological tools	72
P. Schneider ¹ , R.R.R. Rowland ¹ , J. Mann ² , B. Neiger ² , N. Benson ² , P. Steen ² , J. Christopher-Hennings ² , E.A. Nelson ² , Y. Fang ^{2*} . ¹ Dept. of Diagnostic Medicine/Pathobiology, Kansas State University, Manhattan, KS and ² Dept. of Veterinary Science, South Dakota State University, Brookings, SD	
Relationships among immunity, reproductive performance, and viral genetic variation in swine infected with PRRS virus on farms	73
K. Vashisht, J.E. Lowe, R. Husmann, L.D. Firkins, F.A. Zuckermann, T.L. Goldberg. Department of Veterinary Pathobiology, College of Veterinary Medicine, University of Illinois, Urbana, IL	
Protection against heterologous PRRSV challenge in pregnant sows immunized with multivalent PRRSV vaccines	74
M. Wagner ^{1*} , B.D. Roggow ¹ , H.S. Joo ² . ¹ Faimount Veterinary Clinic LLP, Fairmont, MN, ² University of Minnesota, St. Paul, MN	

2005 International PRRS Symposium

Genetic divergence of ORF 5 during acute and persistent infection75
D. Waldner^{1*}, D. Zeman¹, A. Kasuske¹, S. Ropp¹, K. Fairbanks², E. Nelson¹, D. Benfield³. ¹Dept of Veterinary Science, Vet Science, S. Dakota State Univ., Brookings, SD., ²Pfizer Animal Health, Lincoln, NE and ³Food Animal Health Research Program, OARDC/Ohio State University, Wooster, OH

Evolutionary biology of PRRS virus76
K.-J. Yoon^{1*}, S.-H. Cha¹, W.-I. Kim¹, C.-C. Chang³, J. Zimmerman¹, P.M. Dixon². ¹Department of Veterinary Diagnostic and Production Animal Medicine, ²Department of Statistics, Iowa State University, Ames IA. ³ Department of Veterinary Medicine, College of Agriculture, Chiayi University, Taiwan

Suppression of PRRS virus by morpholino antisense oligomers77
Y. Zhang^{1*}, S. Fan¹, A. Kroeker², K. Wang¹, D. Stein², P. Iversen², X.-J. Meng³, D. Matson¹. ¹Center for Pediatric Research, Eastern Virginia Medical School, Norfolk, VA. ²AVI BioPharma, Corvallis, OR. ³Center for Molecular Medicine and Infectious Diseases, Virginia Polytechnic Institute and State University, Blacksburg, VA

SECTION 1: VIRAL GENOME

THE COMPLETE NUCLEOTIDE SEQUENCE ANALYSIS OF A THAI PRRS VIRUS ISOLATE

A. Amonsin¹, S. Puranaveja², N. Pariyotorn¹, P. Wongyanin², S. Suradhat³, R. Thanawongnuwech^{2*} ¹Veterinary Public Health, ²Veterinary Diagnostic Laboratory, ³Veterinary Microbiology, Chulalongkorn University, Bangkok, Thailand

Porcine reproductive and respiratory syndrome virus (PRRSV) is a causative agent of an important disease in pigs in most swine raising areas. In this study, the complete nucleotide sequence of a Thai PRRSV (01NP1) was determined. The 01NP1 contains 15,412 nucleotides with 2 untranslated regions (5' UTR and 3' UTR) and 8 open reading frames (ORFs) designated as ORF1a, ORF1b, and ORF2-7. In order to determine the genetic variation and genetic relatedness among PRRSV isolates, the complete nucleotide sequences of 01NP1 was compared with that of 7 US isolates and 1 EU isolate. Our results showed that the 01NP1 genome shares approximately 99.8% nucleotide identity with the live vaccine strain (Ingelvac PRRS MLV) and 99.7% nucleotide identity with 2 other US isolates, VR-2332 and BJ-4. Phylogenetic analysis also showed that the 01NP1 was closely related to BJ-4, MLV, and VR-2332. These finding suggested that the Thai PRRSV (01NP1) might have originated and evolved from the vaccine virus or its derivatives. The 5' UTR and 3'UTR and all 8 ORFs were very well conserved, however, amino acid differences were mostly observed in ORF1a (nonstructural regions). This report is the first report of complete nucleotide sequences of PRRSV in Thailand.

INFLUENCE OF N-LINKED GLYCOSYLATION OF PRRS VIRUS GP5 ON VIRUS
INFECTIVITY, ANTIGENICITY, AND ABILITY TO INDUCE NEUTRALIZING
ANTIBODIES

I.H. Ansari, B.J. Kwon, F.A. Osorio, A.K. Pattnaik. Nebraska Center for Virology and
Department of Veterinary & Biomedical Sciences, University of Nebraska-Lincoln, Lincoln, NE.

The porcine reproductive and respiratory syndrome virus (PRRSV) glycoprotein 5 (GP5) is the most abundant envelope glycoprotein and a major inducer of neutralizing antibodies *in vivo*. Three putative N-linked glycosylation sites (N34, N44, and N51) are located on the GP5 ectodomain, where a major neutralization epitope also exists. To determine which of these putative glycosylation sites are used in PRRSV life cycle and the role of the glycan moieties in induction of neutralizing antibodies, we generated a panel of GP5 mutants containing single and multiple amino acid substitutions at these sites. Transient expression of the wild-type (wt) as well as the mutant proteins and subsequent biochemical studies revealed that the mature GP5 contains high-mannose type sugar moieties at all three sites. These mutations were subsequently incorporated into a full-length cDNA clone to recover infectious PRRSV. Our results demonstrate that mutations involving N44 residue did not result in infectious progeny production, indicating that N44 is the most critical amino acid residue for viral infectivity. Viruses carrying mutations at N34, N51, and N34/51 grew to lower titers than the wt PRRSV and exhibited reduced cytopathic effect in MARC 145 cells. In serum neutralization assays, the mutant viruses exhibited enhanced sensitivity to neutralization by wt PRRSV-specific antibodies. Furthermore, inoculation of pigs with the mutant viruses induced significantly higher levels of neutralizing antibodies against the mutant as well as the wt PRRSV, thus suggesting that the loss of glycan residues in the ectodomain of GP5 enhances both the sensitivity of these viruses to *in vitro* neutralization as well as the immunogenicity of the nearby neutralization epitope. These results should have great significance for development of PRRSV vaccines of enhanced protective efficacy.

CHARACTERIZATION OF PRRS VIRUS MUTATION AFTER PERSISTENT INFECTION IN PIGS

C.-C. Chang¹, J.J. Zimmerman², K.-J. Yoon². ¹ Department of Veterinary Medicine, College of Agriculture, Chiayi University, Taiwan, ² Department of Veterinary Diagnostic and Production Animal Medicine, Iowa State University, Ames, Iowa

Objective The exact mechanisms that drive PRRSV persistency are unknown, but may involve the inability of pigs to efficiently mount a protective immunity against the virus and/or be attributed to the extensive genetic and antigenic variability known to exist among PRRSV isolates (Murtaugh et al., 1995). In an earlier study, we confirmed the existence of persistent PRRSV infection in pigs and provided an estimate of the rate of genetic change (Chang et al., 2002). Virus replication was continuously maintained in pigs by a series of pig-to-pig passages (n=13) using tissue homogenates collected at 60 days after inoculation from preceded passage for subsequent inoculation. It was observed that different PRRSV genes changed at different rates. Interestingly, the mutation rate of open-reading frame (ORF) 5 was lower than expected in comparison to the degree of variability observed in the field. This outcome raised the question of whether immune pressure plays a role in driving PRRSV evolution. The objective of this study was to address this question.

Methods A highly homologous PRRSV inoculum (CC-01) was derived from VR-2332, the North American prototype, through three rounds of plaque-cloning on MARC cells. Three pigs were inoculated with CC-01 and each pig was individually housed for 120 days. Sera were collected from all pigs at 7 days post inoculation (PI). After 120 days PI, tissues were collected from each principal pig and made into a homogenate. Each homogenate was inoculated to a 2-week-old pig (bioassay). Serum was collected from each bioassay pig at day 7 PI. Attempts were made to recover up to 30 plaque-cloned isolates from each of sera collected at day 7 PI from principal and bioassay pigs as previously described (Chang et al., 2002). Genotypic and phenotypic characteristics of each virus clone were compared to those of CC-01 by serum-virus neutralization, monoclonal antibody (MAb) analysis and sequencing for ORFs 1b (replicase), 5 (major envelope protein), and 7 (nucleocapsid).

Results and discussion One of 3 bioassay pigs became infected after exposure to tissue homogenates, suggesting that one of 3 principal pigs was persistently infected with PRRSV at 120 days PI. Eleven and 30 viral clones were recovered from day 7 sera collected from the principal and bioassay pig, respectively. Nucleotide variants (NV) and amino acid variants (AV) recovered from the principle and bioassay pigs and compared to CC-01 demonstrated that most mutations occurred in ORF5 (Table 1). A higher mutation rate in ORF5 may have been attributed to the selective immune pressure as ORF 5 product is located on the surface of virion.

Table 1. Nucleotide and amino acid variants

	ORF1b		ORF5		ORF7	
	NV	AV	NV	AV	NV	AV
Principal pig	1	1	5	3	0	0
Bioassay pig	1	0	4	3	1	0

The mutation rate of ORF5 was estimated at 5.33×10^{-3} at deduced amino acid level, which was not substantially different from that observed in our previous study (Chang et al., 2003). Consistent with previous observations, mutations were randomly distributed and mostly transitions. Change of amino acid at 151 of ORF 5 from glycine to arginine after in vivo replication was also observed, suggesting that glycine might be a marker for cell culture adaptation.

Even though variants appeared, no phenotypic changes were detected by MAb analysis or serum-virus neutralization assays, suggesting that no escape mutant emerged during this study. Hence the role of virus mutation in PRRSV persistence is in question.

MAPPING PRRS VIRUS GENETIC DETERMINANTS OF MACROPHAGE HOST RANGE AND IMMUNE MODULATION

G. Delhon¹, F. Zuckermann¹, D. Rock¹, I. Guðmundsdóttir², G. Risatti^{2*}. ¹ Department of Pathobiology, University of Illinois at Urbana-Champaign, Urbana, IL. ² Department of Pathobiology, University of Connecticut, Storrs, CT

Porcine reproductive and respiratory syndrome (PRRS), caused by the PRRS virus (PRRSV), is considered the most economically significant endemic infectious disease problem facing the US swine industry today. Although PRRS vaccines are available, issues of safety and efficacy have limited their effectiveness for disease control. Clearly, improved live attenuated virus vaccines that induce protective immunity while limiting aspects of viral spread and pathogenesis are important for effective control of PRRS in the future. Development of such vaccines requires an improved understanding of the genetic basis of viral virulence and host range, notably the PRRSV genetic determinants affecting cell and tissue tropism, including the ability to replicate in the primary target cell, the macrophage, and interaction with the host immune system. Here we will use genetic approaches involving a PRRSV infectious clone to 1) identify and characterize PRRSV genes/genetic determinants associated with macrophage host range, and 2) identify and characterize PRRSV genes/genetic determinants associated with suppression of a type I interferon response and modulation of pro- and anti-inflammatory cytokine expression *in vitro*. Work will contribute a fundamental understanding of mechanisms influencing pathobiologically significant PRRSV-macrophage interactions and will provide information necessary and critical for development of rationally designed live attenuated PRRS vaccines of increased safety, efficacy, and utility.

THE PRRS VIRUS NUCLEOTIDE SEQUENCE DATABASE

K.S. Faaberg^{1*}, T. Wennblom^{1,3}, C. Mahlum-Wees², E.F. Retzel³, J.E. Collins².

¹Department of Veterinary and Biomedical Sciences and ²Minnesota Veterinary Diagnostic Laboratory, University of Minnesota, Saint Paul, MN; ³Center for Computational Genomics and Bioinformatics, University of Minnesota, Minneapolis, MN

A PRRSV Sequence Database (PRRSV DB; <http://prrsv.ahc.umn.edu>) was developed in collaboration with the Minnesota Veterinary Diagnostic Laboratory (MVDL) and the University of Minnesota Center for Computational Genomics and Bioinformatics. Funded by the National Pork Board, the database now contains over 4400 ORF5 nucleotide sequences obtained predominantly from field isolate submissions to the MVDL from 1989-2005.

This web-based database is available to all individuals. The isolates were assigned a unique identifier and catalogued by year and place of isolation. The isolate identifier is hyperlinked to detailed sequence data concerning the specific nucleotide and amino acid sequence. At the top of the page are search options specific to the PRRSV DB, allowing refinement of parameters when desired. The database will compare a nucleotide sequence from the database or an uploaded sequence file to all sequences in the PRRSV database. Once the output is obtained, one may select from 2 to 25 sequences for further analysis. Available tools enable seamless progressive alignment, phylogeny, viewing and archiving. In addition, downloading of the sequence files is readily completed. Other diagnostic laboratories have indicated that their ORF5 nucleotide sequence archives will be added, as well as anticipated database expansion to include all sequences available at the National Center for Biotechnology Information (GenBank). The PRRSV DB will then house the world's most complete warehouse of PRRSV nucleotide sequence information.

IN-VITRO CHARACTERIZATION OF A EUROPEAN-LIKE TYPE 1 PRRS VIRUS
FULL-LENGTH CDNA INFECTIOUS CLONE

Y. Fang*, B. Neiger, J. Mann, N. Benson, E.A. Nelson. Dept. of Veterinary Science,
South Dakota State University, Brookings, SD

European-like PRRSV isolates (Type 1) have been identified in U.S. swine herds. In order to further characterize this group of U.S. Type 1 PRRSV and provide an essential tool for the future construction of a new generation of genetically engineered PRRSV vaccines for both Type 1 and Type 2 PRRSV, we constructed a full-length cDNA infectious clone of a U.S. Type 1 PRRSV, pSD 01-08. This infectious clone represents a recent member of this genotype, differentiating itself from the Lelystad virus infectious clone derived from a 15-year-old strain of PRRSV. In MARC-145 cell culture, this cloned virus was shown to be genetically stable and exhibited similar growth kinetics and plaque morphology as the parental virus. The parental virus, SD 01-08 was isolated from a group of 8-week-old pigs showing no clinical signs. Pathogenesis studies in our laboratory further confirmed that SD 01-08 possesses low virulence properties, but induces early and robust neutralizing antibody responses. We further attenuated this virus by passaging on MARC-145 cells 34 times (SD 01-08 P34), and SD 01-08 P34 was used to construct the full-length cDNA infectious clone. Virus titration on porcine alveolar macrophage cells showed that cloned virus and parental virus SD 01-08 P34 have a 3 log reduction in infectivity compared to the original isolate SD 01-08 P1. These results suggest that SD 01-08 may represent a good vaccine candidate strain. The full-length cDNA infectious clone derived from SD 01-08 P34 could be an ideal viral backbone for future recombinant PRRSV vaccine construction.

CHANGES OF MICROELEMENT CONTENT IN MARC-145 CELLS BEFORE AND AFTER INFECTION WITH VIRULENT AND ATTENUATED STRAINS OF PRRS VIRUS

T.V. Grebennikova¹, A.V. Syroeshkin², M.I. Musienko¹, A.D. Zaberezhny^{1*}, T.I. Aliper¹.
¹NARVAC R&D, D. I. Ivanovski Virology Institute, Moscow, Russia, ²Research Oceanography Institute, Moscow, Russia

Porcine reproductive and respiratory syndrome virus (PRRSV) is characterized by high genetic variability allowing fast reversions from attenuated to more virulent phenotype. In previous studies, 3 passages in cell culture of virulent North American PRRSV strain NADC-8 were characterized by virulence and entire genomic sequencing: the virus was virulent after passage 2, highly attenuated after passage 251, and partially reverted to wild type after one more passage in a pig (252p). The strains were provided by Dr. W. Mengeling. Only 4 amino acid changes were found between strains 251 and 252p. At the same time a reverse genetics system was created for the strain 251 consisting of a plasmid library of full-length genomic cDNA copies, with proven ability to generate infectious viral genomes and recombinant progeny viruses obtained from the genomes after transfection of cells. This provides an opportunity to introduce changes in the viral genomes and to study the consequent changes in viral phenotype. In addition to virulence in pigs and growth characteristics in cell culture, more phenotypic features of PRRSV could be accessed. Virus-infected cells could be tested for concentration of the following trace elements: Al, Cr, Mn, Ni, Fe, Cu, and Zn using atomic-absorption spectrophotometry. The accumulation of trace elements in the infected cells is determined as ratio between concentrations in the cells and in the growth media. Similar technology previously revealed correlations between pathogenicity and microelement profiles in studies of other viruses (Vanderheijden et al., 2003; Hornyak et al., 2004). In the present study the influence of virulent and attenuated strains of PRRSV with defined genomic differences on microelement profile of MARC-145 cells was investigated in order to learn more of the process of infection *in vitro*. The concentrations of seven microelements (Al, Cr, Mn, Ni, Fe, Cu, and Zn) in infected and non-infected cells were determined. Although viral replication was similar in all cases, there were distinct differences in the microelement profiles (changes of concentrations of Ni, Mn, and Fe) in infected cells. In particular, cells infected with the attenuated strain of PRRSV (NADC8-251) contained very low level of Ni, compared with its reverted counterpart NADC8-252P which led to very high level. The infection with European type PRRSV (strain Lelystad) also led to moderately high level of this microelement. The conclusion was made that certain mutations in PRRSV genome could be associated with characteristic changes in element profiles and at the same time with virulence.

NONSTRUCTURAL PROTEIN 2 MUTATIONS OF PRRS VIRUS STRAIN VR-2332
INFECTIOUS CLONE BASED ON DELETIONS SEEN IN RFLP184 ISOLATES ARE
VIABLE

J. Han, K.S. Faaberg*, Y. Wang, H. Liu. Department of Veterinary and Biomedical
Sciences, University of Minnesota, Saint Paul, MN

Isolates of a seemingly new PRRSV North American (NA) genotype appeared in Minnesota in 2002. Nucleotide sequence analysis on two of these RFLP184 field isolates determined that they were only 85 % similar to prototype strain VR-2332. The isolates were only 15,019 bases in length, compared to most NA strains, such as VR-2332, which are 15,411 bases long. The length difference was localized to the putative nonstructural protein 2 (Nsp2) region of ORF1a.

Using an infectious clone of prototype NA strain VR-2332 (pVR-FLV7), deletions of 333 nucleotides (nt) and 603 nt were made in the Nsp2 region. In frame insertions of the green fluorescent protein (GFP) were then completed in place of these deletions. The deletion clones, as well as the GFP insertion clones, were *in vitro* transcribed and then examined for infectivity when transfected into MA-104 cells. All mutants were viable. Mutant viruses obtained were then analyzed by one-step growth curves, plaque size, and expression of GFP. The viral mutants were also examined for several passages in MA-104 cells to determine the stability of the deletions and insertions. We found that the 333 nt deletion mutant, representing a 111 amino acid reduction in the size of putative Nsp2 replicated with almost the same kinetics as the parental infectious clone. Further deletion, as well as insertion of GFP, resulted in severely delayed replication kinetics. The insertion of GFP was apparently unstable, as the mutant virus showed the presence of two populations by passage 3 in MA-104 cells, one representing the full-length GFP mutant and one representing the length of the mutant without the GFP insertion.

INTERFERENCE OF PRRS VIRUS REPLICATION ON MARC-145 CELLS USING
DNA-BASED SHORT INTERFERING RNAS

Y.-X. He, R.-H. Hua, Y.-J. Zhou, H.-J. Qiu, G.-Z. Tong.* National Key Laboratory of
Veterinary Biotechnology, Harbin Veterinary Research Institute, Chinese Academy of
Agricultural Sciences, Harbin 150001, P.R. China

Porcine reproductive and respiratory syndrome (PRRS) is ubiquitous in swine producing areas of the world and is considered to be an economically important global disease. Since PRRS virus (PRRSV) naturally infects monocytic lineage, current immunological strategies against PRRS virus infection have not been so effective. RNA interference (RNAi) is an evolutionarily conserved phenomenon among diverse eukaryotes and has been considered as “the genome’s immune system.” Vector-based RNAi technology raises the possibility of using this phenomenon as a therapeutic tool against viral infection. In the present study, four siRNAs sequences (N95, N179, N218, and N294) specific for the relatively conserved regions of nucleocapsid protein gene (ORF7) of PRRSV were designed. Four short hairpin RNA (shRNA) expressing vectors were constructed by using a PCR-based strategy. Marc-145 cells were pre-transfected with shRNA expression vectors, and then infected with PRRSV, the interference effect of virus replication was measured by CPE, virus titer, western blot, immunofluorescence, and real-time PCR. The results indicated that one out of four shRNA expression vectors (N179) could effectively inhibit the replication of PRRSV effectively on Marc-145 cells.

GENETIC ANALYSIS OF PRRS VIRUS IN MEXICO

J. Hernández^{1*}, G. Yepiz-Plascencia², F. Osorio³. ¹Laboratory of Immunology, ²Laboratory of Marine Biotechnology, Research Center for Food and Development (CIAD, A.C.), Hermosillo, Sonora, Mexico. ³Department of Veterinary and Biomedical Sciences, University of Nebraska-Lincoln, Lincoln, NE.

This work describes the genetic analysis of ORF5 gene of 16 PRRS virus isolates collected from farms located in the state of Sonora, Mexico. Viral RNA was isolated from serum samples using a commercial kit. The ORF5 was amplified by a nested RT-PCR and the products were sequenced. The nucleotide or deduced amino acid sequences were analyzed with different programs including: CLUSTAL W, BIOEDIT, Treeview, TMAP, and EXPASY. The ORF5 displayed a range of genetic identities from 83 to 99% among the isolates. The similarity to American-type virus was below to 90%, while to European-type virus the similarity ranged between 60 to 63%. An evolutionary tree showed four main groups, two groups included only American type virus, the other one included three Mexican isolates together with other American-type virus and the last grouped only the last 13 Mexican isolates. The analysis of predicted protein sequences show that the Mexican virus presented differences in their N-glycosylation sites and in the predicted transmembrane segments. These results evidence the presence of a high genetic and biologic diversity of PRRSV isolates in Mexico.

PHYSICAL CHARACTERIZATION OF THE PRRS VIRUS VIRION

C.R. Johnson¹, B.J. Saxton¹, M.C. Fuentes¹, L.B. Anderson², T.P. Krick², M.P. Murtaugh^{1*}.
¹Department of Veterinary & Biomedical Sciences, ²Department of Biochemistry, Molecular Biology and Biophysics, University of Minnesota, St. Paul MN

The physical characteristics of a viral particle determine the surface features that are essential for initiation of the infection process and for neutralization of infectivity. Structural information about the PRRSV virion is based primarily on comparative analogies to other arteriviruses and interpretations of immunological investigations. We have analyzed PRRSV virion structure by subjecting purified virion preparations to mass spectrometry. Virions were dissolved and separated into constituent parts by electrophoresis or cation-exchange chromatography, followed by tryptic digestion. Peptide fragments were identified by mass spectrometry and database searching using whole genome assemblies of sequenced viruses. Crude virion preparations obtained by sedimentation of freeze-thaw lysates through a sucrose cushion contain essentially all known expressed proteins, presumably due to co-sedimentation of cellular replication complexes. Virions purified by rate zonal gradient centrifugation in sucrose also contained nonstructural as well as structural proteins. Equivalent results were obtained using strains VR-2332 and JA142, indicating that the protein composition of the PRRSV virion may be more complex than previously known.

DEFINING THE CELLULAR TARGETS OF PRRS VIRUS BLOCKING MOAB (7G10)

J.-K. Kim, S. Kapil.* Department of Diagnostic Medicine-Pathology, Kansas State University, Manhattan, KS

We have produced a monoclonal antibody (MAb) (7G10) that has high blocking activity against porcine reproductive and respiratory syndrome virus (PRRSV). In this study, we identified the components of 7G10 MAb-bound complex as the members of cytoskeletal filaments: vimentin, cytokeratin 8, cytokeratin 18, actin, and hair type II basic keratin. Vimentin bound to PRRSV nucleocapsid protein, and polyclonal anti-vimentin antibody showed PRRSV blocking activity. Vimentin was expressed on the surface of MARC-145, a PRRSV susceptible cell line. Simian vimentin rendered BHK-21, a non-susceptible cell line, susceptible to PRRSV infection. These results suggest that vimentin is part of PRRSV receptor complex and plays an important role in PRRSV binding with the other cytoskeletal filaments that mediate transportation of the virus in the cytosol.

SUBCELLULAR LOCALIZATION OF THE NON-STRUCTURAL PROTEINS OF PRRS
VIRUS

D.-Y. Kim^{1*}, M. Kerrigan¹, P. Schneider¹, R. Rowland¹. ¹Dept. of Diagnostic Medicine and Pathobiology, College of Veterinary Medicine, Kansas State University, Manhattan, KS

The structural protein, N, and the nonstructural protein, nsp1, of equine arteritis virus (EAV) localize to the nucleus. The purpose of this study was to characterize the intracellular localization properties of the non-structural proteins of porcine reproductive and respiratory syndrome virus (PRRSV) another member of the *Arteriviridae*. cDNAs for PRRSV nsp 1(PCP), 2(CP), 3(HD), 4(SP), 7(HD), 9(RdRp), 10(HEL) and 11 (coronavirus-like domain), 12(HD) were cloned from ORF1ab region of SD-23983, a North American type 2 isolate. The cloning of each nsp was based on the proteolytic cleavage sites found in EAV. cDNAs were cloned into the pEGFP-C3 expression vector and transfected into Vero cells. Proteins were tagged on the N-terminal end with enhanced green fluorescent protein (EGFP). Western blots of transfected cells probed with anti-GFP antibody showed products of the predicted size for each clone. Confocal microscopy of EGFP-labeled fusion proteins identified nsp 2, 3, 9, 12 in the perinuclear region of the cell, consistent with results reported for EAV. Similar to EAV, nsp1 localized to the nucleus. Interestingly, significant accumulation in the nucleus was observed for nsp 3, 4, 7, 10, 11 EGFP fusion proteins. Current studies are directed at determining the localization of non-structural proteins during infection and their co-localization with cellular components.

GENERATION OF A PRRS VIRUS INFECTIOUS CLONE FROM AN ATTENUATED
VACCINE STRAIN

B.J. Kwon, I.H. Ansari, F.A. Osorio, A.K. Pattnaik. Nebraska Center for Virology and
Department of Veterinary & Biomedical Sciences, University of Nebraska-Lincoln

Regardless of the fact that several avirulent strains of PRRSV are known, the molecular basis for the attenuation of PRRSV virulence remains to be determined. An attenuated vaccine strain of PRRSV (Prime Pac), generated through conventional serial passages in a susceptible cell line, has been available for some time. This virus has a number of mutations in all non-structural and structural open reading frames (ORFs) and seems to be a bona fide candidate to facilitate the study of the molecular basis of attenuation of virulence of PRRSV. As a complement to the experiments involving a virulent infectious clone (previously reported by our laboratory), we generated an infectious clone (PP-18) from this Prime Pac attenuated vaccine strain. The complete nucleotide sequence was determined and compared with parental vaccine virus. The viral genome is 15,520 nucleotides long excluding poly (A) tail, which is the same length as the parental virus. A number of nucleotide sequence changes were noted. A full-length cDNA clone flanked by an upstream T7 RNA polymerase promoter element and a unique *PacI* restriction enzyme site was assembled in pBR322. When capped RNAs generated by *in vitro* transcription of *PacI*-linearized plasmid were transfected into MARC-145 cells, infectious virus was recovered. The rescued virus had similar growth kinetics as the parental vaccine virus in both MARC-145 cells and porcine alveolar macrophages and could be differentiated from the other American type viruses by indirect fluorescent staining with specific Mabs (SDOW17 and SR30). The rescued virus showed the same fluorescence reactivity pattern as the parental virus. The phenotypic characteristics of the rescued and parental viruses will be determined and compared with the virulent strain using the pregnant sow model. The derivation of this infectious clone from the attenuated PRRSV vaccine strain should significantly facilitate ongoing molecular attenuation studies by providing an avirulent phenotypic background on which to evaluate the contribution that single wt PRRSV genes may have on virulence.

SIGNIFICANCE OF STRUCTURAL GENES OF PRRS VIRUS FOR VIRULENCE AND ATTENUATION

B.J. Kwon, I.H. Ansari, A.K. Pattnaik, F.A. Osorio. Nebraska Center for Virology and Department of Veterinary & Biomedical Sciences, University of Nebraska-Lincoln

Using reverse genetics, we generated a series of chimeric viruses containing specific genomic sequences of an attenuated PRRSV vaccine strain (Prime Pac) within the genomic context of a highly virulent infectious clone (FL-12). Eight viable chimeric viruses, encompassing the entire genome of PRRSV (Prime Pac), have been obtained. Five of these chimeras include all the non-structural open reading frames (ORFs): (1) 5' UTR and NSP1 and part of NSP2, (2) part of NSP2 and part of NSP3, (3) part of NSP3 to NSP8, (4) part of NSP9, and (5) part of NSP9 to NSP12 genes; while the remaining 3 chimeric viruses include all the structural ORFs: (6) part of NSP12, ORF2 and part of ORF3, (7) ORF3 to 7 and 3'UTR, and (8) the entire region spanning all the structural genes and the 3'UTR. By studying in vitro growth kinetics of these chimeras in both MARC 145 cells and porcine alveolar macrophages (PAM), we could approximately ascertain their virulence phenotype. However, clear-cut characterization of their virulence phenotype was obtained in vivo, upon inoculation of pregnant sows at day 90 of gestation. Most virulence determinants clustered in the structural genes of PRRSV. Some non-structural regions of the PRRSV genome (NSP3-8) exhibited a marked role in virulence. Meanwhile, other non-structural regions (NSP1-3, NSP10-12) showed an intermediate attenuation phenotype, while other non-structural (NSP9) or structural (ORF2) regions of the PRRSV genome could be ruled out as important determinants of virulence. We further dissected the structural regions for a finer mapping of individual ORFs of the PRRSV genome and generated 5 more chimeric viruses representing the majority of each individual ORF, 3 through 7. The in vitro growth kinetics in both MARC 145 cells and PAM and in vivo characterization in pregnant sows are currently in process. This approach should allow us to narrow down the relative contribution of individual ORFs on attenuation of virulence of PRRSV, thus opening the avenue for precise mapping of the critical regions and residues within the individual gene products that are important for attenuation.

GENETIC ALTERATION OF PRRS VIRUS IN THE CAPSID (N) PROTEIN NUCLEAR LOCALIZATION SIGNAL ATTENUATES VIRUS REPLICATION

C. Lee¹, D.G. Hodgins¹, J.G. Calvert^{2*}, S.K. Welch², R. Jolie², D. Yoo¹. ¹Dept. of Pathobiology, Ontario Veterinary College, University of Guelph, Guelph, Ontario, ²Pfizer Animal Health, Kalamazoo, MI

PRRS virus replicates in the cytoplasm of infected cell, but the viral nucleocapsid (N) protein is specifically localized in the nucleus and nucleolus. The biological function of the N protein nuclear localization is unknown. Using an infectious cDNA clone, the N protein nuclear localization signal (NLS) was mutated to knock out the nuclear function of the N protein. By transfection of cells with the mutated genomic clone, NLS-null mutant PRRS virus was generated. The NLS-null PRRS virus was fully infectious, but the titer was 100-fold lower than that of wild-type virus. Three groups of piglets were intranasally inoculated with wild-type, placebo, or NLS-null virus, and maintained for 4 weeks for clinical observation, viremia, and antibody responses. The NLS-null virus infected pigs had a lower mean duration of viremia and a low peak mean virus titer than in wild-type infected pigs, but developed higher neutralizing antibodies and higher ELISA antibody titers. Mutations were observed from the persisting virus in the tonsil of pigs inoculated with the NLS-null virus. These mutations were limited to the NLS locus, indicating strong selection pressure for reversion at the NLS locus. Our study indicates that the N protein nuclear localization is non-essential for virus replication in vitro but plays an important role in PRRSV pathogenesis in vivo.

PRRS VIRUS STRUCTURAL PROTEIN MUTATION STUDIES

H. Liu, K.S. Faaberg*, Y. Wang, J. Han. Department of Veterinary and Biomedical Sciences, University of Minnesota, Saint Paul, MN

As part of a collaboration with Northern Michigan University (NMU), South Dakota State University (SDSU) and University of Nebraska, the role that N-glycosylation of may play in viral neutralization is being examined by mutational analysis of PRRSV ORF5. An infectious clone of prototype strain VR-2332 (pVR-FLV7) was utilized for the studies in our laboratory. ORF5 of strain VR-2332 contains four potential N-glycosylation sites. Mutation of the asparagine residues to serine was completed for each possible combination of residues, which totaled 16 mutant subclones of pVR-FLV7. Each mutant was placed back into the cloned genome and verified for mutation retention. Full length *in vitro* RNA transcripts were produced for 6 of the mutants with 2-3 N-glycosylation sites removed. These transcripts were transfected into MA-104 cells and then cultured for 3 passages. Five of the mutants showed evidence of replication through passage 2; one survived to passage 3. After MA-104 growth trials with the remaining infectious clone mutants, analyses of simultaneous growth curves, plaque size determinations and retention/reversion of the induced mutations will be completed. Viruses obtained from the successful infectious clone constructs will be forwarded to NMU and SDSU for immunological studies.

The functional significance and the role of N-glycosylation in the minor glycoproteins are also being studied in collaboration with SDSU, Guelph University, and NMU. ORFs 2a, 2b, 3, and 4 of three significantly different North American strains have been subcloned and are in the process of structural evaluation. Production of additional protein specific antibodies and delineation of the minor structural proteins roles in PRRSV infection is the aim of this newly begun research.

PRRS VIRUS SMALL ENVELOPE (E) PROTEIN AS A POTENTIAL VIROPORIN
REQUIRED FOR UNCOATING AND REPLICATION

C. Lee^{1*}, W.J.B. Hanna², G.A. Woolley³, D. Yoo¹. ¹Dept. of Pathobiology, ²Dept. of Biomedical Sciences, University of Guelph, Guelph, ³Dept. of Chemistry, University of Toronto, Toronto, Ontario

PRRSV contains a small envelope (E) protein of 73 amino acids encoded by ORF2b residing within ORF2a. Using a full-length infectious cDNA clone, the translation initiation codon for the E gene was mutated, so that the mutant genomic clone (P129-ΔE) was unable to express E protein. Transfection of cells with P129-ΔE showed the absence of infectivity, indicating that the E protein is essential for PRRS virus replication. The P129-ΔE transfected cells, however, produced virus particles in the supernatant, and these particles contained viral genomic RNA, demonstrating that the E protein is not a requirement for virion assembly. Electron microscopy revealed that the particles assembled in the absence of E protein were similar in appearance to wild-type virions. Strand-specific RT-PCR indicated that the E-protein negative, non-infectious PRRS virus particles were able to enter cells but unable to proceed to further steps of replication, implicating interruption of the uncoating process. The E protein did not form a cysteine-linked multimer, but cross-linking studies show that E exists as non-covalently linked oligomers in cells. Our study suggests that the PRRS virus E protein may form pores in the viral envelope, facilitating uncoating of virus and therefore release of the viral genome in the cytoplasm.

THE PRRS VIRUS N PROTEIN POSSESSES A NON-CLASSICAL NUCLEAR EXPORT
SIGNAL SEQUENCE

J.M. Rowland,* R.R.R. Rowland. Department of Diagnostic Medicine./Pathobiology,
Kansas State University, Manhattan, KS

The 123 amino acid nucleocapsid (N) protein of porcine reproductive and respiratory syndrome virus (PRRSV) localizes to the nucleus and nucleolus of infected cells and when expressed as a single protein tagged with GFP. The role of N in the nucleolus appears to be related to the regulation of rRNA processing. Accumulation of N in the nucleus means that less N would be available for the assembly of nucleocapsids in the cytoplasm. The purpose of this study was to determine if N possessed a mechanism for export from the nucleus. The addition of leptomycin B (LMB), an inhibitor of the nuclear export shuttle protein, CRM1, and actinomycin D, an inhibitor of RNA polymerase I and II, blocked nuclear export. The C-terminal 34 amino acid polypeptide covering amino acids 90-123, when tagged with EGFP, was retained in the cytoplasm and could substitute for the nuclear export signal (NES) sequence of equine infectious anemia virus Rev (ERev) protein. However, the replacement of two hydrophobic residues within an LXL-like motif failed to prevent nuclear export. These data show that the C-terminal region of the PRRSV N possess a CRM1-dependent mechanism for N protein export from the nucleus; however, the NES deviates from the classical NES sequences found in HIV Rev and other viral proteins. Furthermore, inhibition of export by high concentrations of actinomycin D suggests that N protein export is dependent on de novo nucleolar rRNA synthesis.

USE OF A PRRS VIRUS INFECTIOUS CLONE TO EVALUATE IN VITRO
QUASISPECIES EVOLUTION

S.K. Schommer,* S.B. Kleiboeker. Department of Veterinary Pathobiology, College of
Veterinary Medicine, University of Missouri-Columbia

Genetic diversity is an important mechanism of viral persistence and pathogenesis for many RNA viruses and has been well established for PRRSV. Closely related virus populations in vivo, referred to as quasispecies, are comprised of a heterogeneous mix of related genetic variants, which are randomly generated as a result of errors by the viral RNA-dependent RNA polymerases. The establishment of full-length infectious cDNA clones for PRRSV allows manipulation of viral genomes; however, the rescued virus must be passaged. It is known that genetic changes occur during cell culture passage, but no studies of quasispecies evolution of PRRSV in vitro have been reported. In this study, the VR-2332 full-length infectious clone (Nielsen et al. 2003) was in vitro transcribed and used to transfect BHK21 cells. Infectious virus was recovered and serially passaged in MARC-145 cells. The RNA obtained from the in vitro transcription reaction as well as passages 1 and 3 from 2 independently transfected wells (A and B) were used to study quasispecies evolution in vitro. Four regions of the genome were selected for analysis, Nsp2, ORF3, ORF5, and ORF6. The master sequence for each sample derived from the infectious clone was the same as the original plasmid for all genetic regions. Analysis of the in vitro transcribed RNA showed that Nsp2, ORF3, and ORF6 all had low levels of genetic variation even though it was prepared directly from a single bacterial colony plasmid preparation. Increased passage number generally correlated with a decrease in the percentage of the master sequence. New quasispecies appear and disappear with passage, with no cases of a variant present in passage 1 of a well being present in passage 3, although the variants appeared at such low levels that they may not be detected with our sample size. In total, our data suggests that even with the use of an infectious clone it will be difficult, if not impossible, to create point mutations without accumulating other changes in the genome, especially if multiple in vitro passages are required to obtain sufficient viral titers.

POTENTIAL OF PRRS VIRUS AS A VACCINE VECTOR FOR FOREIGN GENE
EXPRESSION

C. Song^{1*}, J.G. Calvert², S.K. Welch², D. Yoo¹. ¹Department of Pathobiology, Ontario
Veterinary College, University of Guelph, Guelph, Ontario, ²Pfizer Animal Health,
Kalamazoo, MI

The potential of PRRS virus as a foreign gene expression vector was explored using an infectious cDNA clone of the North American genotype P129 strain. The green fluorescence protein (GFP) gene was engineered to contain the PRRSV-specific transcriptional regulatory sequence immediately upstream of the translation initiation of GFP, and the entire cassette was inserted between the ORF1b non-structural and ORF2a structural protein genes. When cells were transfected with the engineered full-length clone, cytopathic effect developed and infectious virus was produced. The reconstituted virus was fully viable and retained the GFP gene in the viral genome. The recombinant PRRS virus expressed GFP at high levels in infected cells, shown by fluorescent microscopy and radio-immunoprecipitation. The green virus was able to synthesize an extra subgenomic RNA species in addition to the six standard subgenomic RNAs, and GFP was translated from the extra subgenomic RNA. The green virus was named 'Kermit'. Kermit was genetically and phenotypically stable for at least up to 37 passages in cell culture and grew to a titer approaching wild-type virus. Our study demonstrates the potential of PRRS virus as a foreign gene expression vector and implies the usefulness of PRRS virus as a dual vaccine vector for swine disease.

IDENTIFICATION AND CHARACTERIZATION OF A CELLULAR GENE
ESSENTIAL FOR PRRS VIRUS INFECTION

S.-K.W. Welch*, D.E. Slade, S.L. Shields, R. Jolie, R.M. Mannan, R.G. Ankenbauer, J.G. Calvert. Pfizer Animal Health, Kalamazoo, MI

Porcine Reproductive and Respiratory Syndrome (PRRS) virus is a single-stranded positive-sense RNA virus classified in the Genus *Arterivirus*, Family *Arteriviridae*, Order *Nidovirales*. PRRS virus causes respiratory diseases in young pigs and reproductive failures in gilts and sows. The emergence and spread of PRRS virus has had a significant negative impact on the swine industries of many nations. In the host animal, PRRS virus primarily infects terminally differentiated porcine alveolar macrophages (PAM). Certain simian kidney cell lines (MA-104 and derivatives such as MARC-145) are the only immortalized cell lines known to support sustained, high-level PRRS virus infection. Several attachment and/or internalization factors have been identified, but none have demonstrated ability to convert a non-permissive cell to a PRRSV-permissive phenotype. We have identified a cellular gene from PAM cells, MARC-145 cells, and other sources that appears to be essential for PRRS virus infectivity. Stable expression of this gene is necessary and sufficient to render a number of common cell lines permissive to PRRSV infection. The sequence and characterization of this cellular gene will be presented.

THE VIRUS, PRRS VIRUS

D. Yoo. Department of Pathobiology, University of Guelph, Guelph, Ontario, Canada

PRRSV contains a genome capacity of encoding 7 structural proteins and 13 non-structural proteins. Despite the relatively slow progress in understanding their role during infection, recent studies have revealed some of the important protein functions, while some other functions may be extrapolated from their homologs of coronaviruses and other arteriviruses. The nucleocapsid protein is specifically localized in the nucleus and nucleolus of infected cell, implicating an important role for host cell function modification by PRRSV during infection. A small envelope protein is a newly identified protein for arteriviruses, and recent studies suggest that this protein may form pores in the viral envelope implicating possible ion-channel function. GP4 is a type I membrane protein with two hydrophobic regions at both termini. GP4 appears to be anchored in the membrane via glycosylphosphatidylinositol modification suggesting a role for signal transduction and 'lipid rafting'. Infectious cDNA clones have been developed for various PRRSV strains, and a functional reverse genetics technology has become available. This technology allows us to manipulate the PRRSV genome at specific sites and produce genetically-modified mutant viruses. The specific mutants may be used to examine phenotypic consequences upon infection. The reverse genetics technology is the most powerful tool in PRRSV research, and a rapid research progress is anticipated to understand the virus, PRRSV.

IDENTIFICATION AND CHARACTERIZATION OF A PUTATIVE CELLULAR
RECEPTOR FOR PRRS VIRUS

E.-M. Zhou, Q.-S. Qin. Department of Veterinary Diagnostic and Production Animal
Medicine, Iowa State University, Ames, Iowa

Porcine reproductive and respiratory syndrome virus (PRRSV) is an enveloped, positive single-stranded RNA virus in the family *Arteriviridae*, order *Nidovirales*. The virus has restricted tropism for cell binding and/or entry via cellular receptor(s). Among the six structural proteins, GP5, encoded by ORF5, is a major envelope protein and responsible for virus binding or entry into permissive cells. We have produced an anti-idiotypic monoclonal antibody (Mab2-3H) directly against the monoclonal antibody (Mab25C) specific for GP5 antigen and determined that Mab2-3H structurally and/or functionally mimicked GP5 and identified a putative PRRSV receptor on Marc-145 cells and PAM. Mab2-3H specifically precipitated a soluble protein with a MW of approximately 250KDa. Western blot immunoassay demonstrated the specific binding of Mab2-3H to 250KDa protein prepared from Marc-145 as well as from PAM. Studies are in progress to purify the protein for protein sequencing and elucidate its biological functions.

SECTION 2: DIAGNOSTICS

A NOVEL DNA MICRO ANALYZER USING PCR PROCESS

S. Bhattacharya^{1*}, S. Grant¹, S.B. Klieboeker², K. Gangopadhyay³, S. Gangopadhyay³.
¹Department of Biological Engineering Department, ²Veterinary Diagnostic Laboratory,
³Department of Electrical Engineering, University of Missouri, Columbia

A micro-fluidic assay to quickly analyze microscopic samples of DNA is being developed for field applications. The device is intended to provide quick identification of PRRS virus RNA. Prevention of PRRS in a naive herd depends on the ability to prevent introduction of infected animals; thus, the quick identification of the viral strain becomes critical to the pork industry. The microanalyzer consists of a micro-PCR chamber, micropumps, and microheaters. Additional components include gel electrophoresis micro-channels and solid core waveguide fluorescence collectors. The intended analyzer is a micro-fluidic platform that is principally based on the three-step polymerase chain reaction mechanism. It is fabricated using standardized soft lithography and replica molding techniques on inexpensive and easily available materials like soda lime glass and poly (dimethyl) siloxane (PDMS), a bio-friendly and visco-elastic polymer. The device is realized by a unique on chip fluid handling system controlled by an off chip mechanism comprising of a compressed nitrogen supply, several solenoid actuated valves and a computer run by labview code.

The micropumps and microheaters for the PCR chamber have been successfully designed and fabricated. This includes a successfully developed fabrication methodology, a variety of physical processes like mixing, pumping, and valving of fluids at the microscopic length scale. For fabrication of the device, a unique characterization of bond strength in terms of surface hydrophilicity using contact angle has been synthesized for an advanced prediction of good wafer level bonding. This study gives us a basis of estimation of bond strength with change in different plasma exposure parameters. The formulated generalized approach to estimation of good wafer level bonding is essential to fabrication of MEMS devices and is an important processing requirement for the MEMS industry. We have also mathematically modeled the involved heat transfer equations for optimization of the on-chip heater design for the PCR chamber. The detection starts with injecting the PCR mix in the PCR chamber. The microheaters fabricated by platinum sputtering on silicon is monitored using another film acting as an RTD (resistance temperature detector) film and controlled by a flexible labview code. The post PCR mix is transported into the gel channel using peristaltic pump. A set of sputtered platinum films is used as electrodes across the gel channel, which helps in the transport of the amplified sample across fixed distances in the horizontal length scale. An array of waveguides spatially located on the basis of the standardized distance traversed by the target base pairs are next used to pick up the fluorescence response and transport it to an off-chip spectrometer, confirming the presence of the target. The successful development of our lab-on-a-chip device will have several advantages over conventional bench top systems, which primarily include an overall reduction in size, reduced use of reagents, decreased power requirements, increased speed and accuracy of analysis, and increased portability for field use. We envision this assay as a highly sensitive analyzer tool with a capability to pick-up trace samples with high accuracy.

DETECTION OF PRRS USING AN IN-SOLUTION AND FIBER-IMMOBILIZED FRET IMMUNOSENSOR

B. Heits¹, D. Lichlyter¹, S. Kleiboeker², S.A. Grant¹. ¹Department of Biological Engineering, University of Missouri-Columbia. ²Department of Veterinary Pathobiology, University of Missouri-Columbia

Porcine Reproductive and Respiratory Syndrome Virus (PRRSV) is a devastating and highly contagious disease that causes dramatic losses in swine production. Some common symptoms of infection can include anorexia, fever, reproduction failure, difficult breathing, and multiple infections. These problems accompanied with some swine showing no signs of infection have created a desperate need for serologic tests in PRRS detection.

A transduction method being utilized to detect the PRRS virus is Fluorescence Resonance Energy Transfer (FRET). FRET utilizes two fluorophores, termed the donor and acceptor. Energy transfer occurs between the donor and acceptor when the two are in close proximity. When the antibody/protein A complex binds to the specific antigen, a conformational change occurs and the distance between the donor and acceptor shortens. This results in transfer of energy to the acceptor causing it to produce a characteristic wavelength of emission light.

In our experiments, SDOW-17 antibody was labeled with the donor fluorophore, Alexa Fluor 546, while protein A was labeled to the acceptor fluorophore, Alexa Fluor 594. Protein A preferentially binds to the antibody, thus creating FRET bioprobes. PRRS virus ($\sim 10^{4.2}$ particles per milliliter) was exposed to the bioprobes which were immobilized onto optical fibers or just in solution. A spectrofluorometer recorded changes in fluorescence.

The initial in-solution experiments demonstrated an average change in fluorescence of approximately 5% whereas the fiber immobilization experiments showed up to 20% change in fluorescence. The bioprobes immobilized to optical fibers may be a feasible, simple, and fast technique to detect the presence of PRRS virus.

THE EFFECT OF GENOTYPIC AND BIOTYPIC DIFFERENCES AMONG PRRS
VIRUSES ON THE IMMUNE RESPONSE AND SEROLOGIC ASSESSMENT OF PIGS
TO PRRS VIRUS INFECTION

W.-I. Kim^{1*}, W. Johnson², K.-J. Yoon¹. Veterinary Diagnostic and Production Animal
Medicine, Iowa State University, Ames, IA; ²Boehringer-Ingelheim Vetmedica, Inc.

A comparative serologic study was conducted to determine if serologic responses to PRRS virus infection can be influenced by biotypic and/or genotypic differences of the viruses. Five field and 2 cell-attenuated viruses were used to challenge one of 7 groups of 10 pigs each at a titer of 10^3 TCID₅₀ via nasal route. The viruses varied in their virulence to pigs and their genetic make-up. All of the inoculated animals became viremic during the study period. While some of the animals inoculated with the attenuated viruses remained seronegative until the end of the study (42 days PI), all of the animals inoculated with the field viruses developed ELISA and IFA antibodies regardless of the virus strains in the test. Thus, the IFA test may be used as a confirmatory test when a false-positive ELISA result is suspected or vice-a-versa. In contrast, there was a great degree of variation in the induction of SVN antibody by individual pig and by each virus. SVN antibody was highly specific for homologous viruses. Cross neutralization titers were correlated with homology of ORFs 4 and 5 among the viruses. In conclusion, the biotypic difference among PRRS viruses may influence the degree of humoral immune response in infected pigs to the virus. Cross neutralization of PRRS viruses can be adversely affected by antigenic variability.

EVALUATION OF A NEW FIELD TEST TO DETECT PRRS VIRUS ANTIBODIES IN SWINE SERA

E. Mende, J.P. Cano, H.S. Joo*. College of Veterinary Medicine, University of Minnesota, St. Paul, MN

A field-based test was developed for the detection PRRSV antibodies in swine sera. Viral antigen used was a detergent-extracted PRRSV infected cells. The test plates were prepared by coating the antigen on the bottom of petri dishes and overlaying agar gel. For the test, a filter paper disc is adsorbed with each test serum and placed on the agar of the test plate. This was incubated at 25 C for 2-3 hours, and then the agar was peeled off. After washing the plate, antigen-antibody reaction was visualized by reacting with peroxidase conjugate and a substrate. The results were read after 5-10 minutes and only positive samples showed color reaction. Various serum samples with known ELISA S/P ratios or history of PRRSV infection were tested, and the sensitivity and specificity of the test were evaluated. With the sera optimal incubation period of 3 hours, sensitivity was 100% (248/248) and specificity using the PRRSV-naive samples as the true negative was 99.7% (299/300). Using Kappa statistics, agreement of the test result with corresponding ELISA S/P values was 96.9% ($p < 0.001$). Antibody detection using whole blood was also possible with a longer incubation period. For these reasons, the gel-EIA can be validated as equivalent to that of ELISA test to detect PRRS virus antibodies.

SAMPLING OF ADULT BOARS FOR EARLY DETECTION OF PRRSV BY PCR
USING A NEW TECHNIQUE (BLOOD SWAB METHOD)

D.L. Reicks^{1*}, C. Muñoz-Zanzi², K. Rossow². ¹Swine Vet Center, St. Peter, MN.
²University of Minnesota, College of Veterinary Medicine, St. Paul, MN

Previous studies have shown that PRRS virus can shed in semen and this is a well-accepted fact. Serum PCR is more sensitive than semen PCR (Reicks 2005), and will pick up virus by PCR testing within 24-48 hours after an animal is infected. Thus to prevent infecting sow farms (a boar stud can supply as many as 50-100 sow farms), boars should be sampled in the serum (blood) rather than semen. This study evaluated the feasibility of using a new approach for blood collection in boars called the blood swab method. The blood swab method involves puncturing a vein (normally in the ear) with a needle and swabbing the blood with a polyester swab. The swab is then put into saline solution and finally tested by PCR. The procedure is done while the boar is on the collection dummy ejaculating.

Twenty-one boars were inoculated with a field isolate on day 0. Serum, blood swabs, mouth swabs, and semen samples were collected from each of two groups of 10 boars on a rotating basis, one group every 24 hours for 6 days. Rectal temperatures were recorded for all boars every 24 hours. The Results of the study showed that 60/61 boars were detected positive using the blood swab method compared with 61/61 with traditional serum collection methods. Both serum and blood swabs detected boars earlier and with higher frequency than semen (27/60 positive) or mouth swab samples (19/61 positive). There was no statistical difference between likelihood of detecting a positive boar with blood swab method compared to serum. There was less quantity of virus detected by the blood swab method when compared with serum, which can be explained by the dilution effect of the saline and using whole blood rather than serum. The procedure can be implemented as part of the routine monitoring program to detect PRRSV infection in boar studs. The blood swab method is much more convenient than collecting serum by the traditional snaring method and will detect virus much sooner and with greater sensitivity than semen PCR.

DETECTION OF PRRS SEROCONVERSION AND PERSISTING ANTIBODY TITERS
IN SWINE FOLLOWING CONTROLLED INFECTION DURING A LONG-TERM
STUDY

A. Rice^{1*}, V. Leathers¹, L. Plourde¹, R. Rowland², R. Molina³, J. Hermann³, J. Zimmerman³. ¹Infectious Diseases R&D, Production Animal Services, IDEXX Laboratories, Westbrook, ME. ²Dept. of Diagnostic Medicine and Pathobiology, Kansas State University, Manhattan, KS ³Dept. of Vet Diagnostic & Production Animal Medicine, Iowa State University, Ames, IA

The porcine reproductive and respiratory syndrome (PRRS) virus affects swine herd productivity and generates large financial losses. Detection of exposure through serum antibodies in sows is imperative to herd management; however, temporal changes in antibody response to the virus can affect the quality and sensitivity of diagnosis. To assess the long term persistence of anti-PRRS virus antibodies in swine, 109 naïve pigs were infected with VR-2332, a US strain of PRRS virus, and analyzed for seroreactivity using the IDEXX HerdChek* PRRS 2XR Antibody Test Kit. Sera collected weekly and/or biweekly for 202 days post-infection, tested positive within 14 days, consistent with the onset of peak phase antibody production. Only 7% of the pigs tested positive at day 7. All control animals tested antibody negative with s/p ratios < 0.4. At PID 112, 81 out of 83 animals (97.6%) continued to exhibit PRRS immunoreactivity. The seropositive response persisted through day 182 in 26 out of 28 animals (92.9%). These results indicate that serum antibodies against PRRS virus can be detected well into late phases of infection under the conditions of this study.

*HerdChek® is a trademark or registered trademark of IDEXX Laboratories, Inc. in the United States and/or other countries.

AN IMPROVED METHOD FOR PRRS VIRUS SURVEILLANCE AND MONITORING

R. Simer², J. Prickett¹, E.-M. Zhou¹, J. Zimmerman¹. ¹Department of Veterinary Diagnostic and Production Animal Medicine, Iowa State University, Ames, Iowa.
²Perryton, Texas

The long-term goal of this project is to evaluate testing pen-based oral fluid samples as a method of PRRSV surveillance or monitoring in nursery, grower, and finisher pigs. Isolation of PRRSV from individual pig oral fluid samples was first reported in 1997 (Wills et al. 1997). The project reported here focused on detection of PRRSV in pen-based oral fluid samples using PCR.

Methods: A field study was done to determine whether observations made under experimental conditions applied to production settings. Twelve pens containing approximately 25 finisher pigs each were sampled in a nursery unit in which PRRSV was believed to be circulating. Serum samples were collected from two pigs selected at random from each pen. At the same time, two pen-based oral fluid samples were collected from each pen. Serum and oral fluids were submitted for PCR testing.

Results: 20 of 24 (83%) pen-based oral fluid samples and 17 of 24 (71%) individual pig serum samples were PCR positive.

Conclusions: PCR results on pen-based oral fluid samples collected in the field corroborated earlier experimental data. Compared to individual pig sampling, pen-based oral fluid samples could offer significant advantages: 1) less labor for sample collection, 2) reduced sampling/testing costs, and 3) more comprehensive herd sampling. Longitudinal experimental and field studies are under way to expand upon these results.

DETECTION OF ISOTYPE-SPECIFIC ANTI-PRRS VIRUS ANTIBODIES IN SWINE
ORAL FLUID SAMPLES

E.-M. Zhou¹, R. Simer², J. Zimmerman¹. ¹Veterinary Diagnostic Laboratory, Iowa State University, Ames, Iowa. ²Perryton, Texas

PRRSV causes \$560 million in direct losses each year to the U.S. swine industry (Neumann et al., 2005). Control and/or elimination of PRRSV require improved surveillance and monitoring procedures. In this study, we report the detection of anti-PRRSV antibodies in oral fluid samples.

Methods: Pigs were infected with PRRSV VR-2332 and housed under experimental conditions. Oral fluids were collected by allowing pigs to chew on absorbent material suspended in the pen. Pen-based oral fluid samples were collected on DPI 35, 42, 48, 54, and 82 and assayed for anti-PRRSV antibodies using (1) the HerdChek® PRRS ELISA (IDEXX Laboratories, Inc.) and (2) an ELISA using a recombinant N antigen and peroxidase-conjugated goat anti-swine IgG or IgA.

Results: (1) HerdChek® PRRS ELISA: oral fluid samples collected DPI 35, 42, 48, 54, and 82 were negative at a 1:40 dilution. At a 1:2 dilution, the 35 and 42 DPI samples had S/P ratios of 0.661 and 0.628, respectively. (2) N antigen ELISA: oral fluid samples diluted 1:2 were positive for IgG anti-N antibodies on DPI 35 and 42. Oral fluid samples were positive for IgA anti-N antibodies on DPI 42, 48, 54, and 82 at dilutions of 1:2, 1:4, and 1:8. No IgM anti-N antibodies were detected in the samples we tested. Negative control samples were negative in all tests.

Conclusions: These results suggested the prolonged presence of a high level of IgA anti-N antibodies in oral fluids from PRRSV-infected pigs. Pen-based oral fluid samples may offer an efficient method to surveil and monitor pig populations.

SECTION 3: IMMUNE RESPONSE

PRRS VIRUS MODULATES THE INNATE IMMUNE FUNCTION OF PORCINE PLASMACYTOID DENDRITIC CELLS

G. Calzada-Nova*, R.J. Husmann, W.M. Schnitzlein, F.A. Zuckermann. Department of Pathobiology, University of Illinois, Urbana, Illinois

Plasmacytoid dendritic cells (PDCs) are the most potent source of interferon- α (IFN- α) and thus are primarily responsible for the initial protective response elicited during an acute virus infection. However, the limited amounts of this cytokine detected in pigs infected with porcine reproductive and respiratory syndrome virus (PRRSV) indicates that this pathogen somehow circumvents this innate immune reaction. To clarify this issue we evaluated the behavior of porcine PDCs exposed to PRRSV both *in vitro* and *in vivo*. When freshly purified PDCs were incubated with wild type PRRSV, the resultant IFN- α response was meager and at least 100-fold less intense than that registered in the presence of transmissible gastroenteritis virus (TGEV). Moreover, while the PDCs underwent physical changes in the presence of TGEV, contact with PRRSV failed to cause a similar type of maturation morphogenesis. That PRRSV was actually affecting PDC function was established by the demonstrated ability of this entity to repress the otherwise vigorous IFN- α responsiveness to TGEV of an isolated porcine peripheral blood mononuclear cell (PBMC) population that was partially comprised of PDCs. A similar impact of PRRSV on the performance of PDCs was observed during the temporal monitoring of the TGEV response of PBMCs obtained from pigs either infected with wild type PRRSV or vaccinated with an attenuated strain. In this case, a significant reduction in the amount of IFN- α secreted by the PBMCs in the presence of TGEV was noted when the cells were obtained within seven days after the introduction of PRRSV into the animals. After an additional seven days, the inhibition began to ameliorate and was no longer detected a week later. This phenomenon could not be attributed to the disappearance of circulating PDCs since the proportion of these cells in the PBMC population was approximately ten-fold greater than that measured in the same pigs prior to PRRSV infection. This immuno-modulating ability of PRRSV is rather unique since a similar type of impairment was not noticed when swine were infected with Aujeszky's disease virus.

EX-VIVO ASSESSMENT OF THE PRRS VIRUS CROSS PROTECTIVE IMMUNE RESPONSE OVER TIME

S.-H. Cha¹, W.-H. Wu², R. Molina², J. Zimmerman², K.-J. Yoon^{1,2}. ¹Veterinary Microbiology and Preventive Medicine and ²Veterinary Diagnostic and Production Animal Medicine, College of Veterinary Medicine, Iowa State University, Ames, IA

Sequential pig-to-pig passages of a homologous plaque-cloned PRRS virus (CC-01) originated from VR-2332 for 2 years demonstrated significant genetic changes in various genes of progeny viruses. Furthermore, genetic changes in structural genes resulted in antigenic changes allowing some of the progeny viruses to escape from neutralizing activity of antisera raised against CC-01. In the following study, the affect of genetic and antigenic variability on cross protection among PRRS viruses by cell-mediated immunity (CMI) was determined by assessing IFN- γ response of infected pigs to CC-01 and its progeny viruses collected at passages 7 and 13 which showed continuous antigenic drift from CC-01. A total of 9 pigs were experimentally inoculated intranasally with CC-01-P4 that was prepared by passing CC-01 twice in pigs. Blood samples were periodically collected from all pigs over 200 days post inoculation (PI) for ELISPOT assays to assess the kinetic of virus-specific IFN- γ producing T cells. On average, IFN- γ response to the homologous virus (CC-01-P4) was peak at 28 days PI and decreased over time, demonstrating a short-lived virus-specific CMI. At all sampling points, IFN- γ response was significantly higher to the homologous virus than to others. The response to viruses from the passage 13 was the least, indicating that optimal CMI may be restricted to the homologous virus. In conclusion, our study suggests that antigenic variability among PRRS viruses can adversely affect anti-viral activity of PRRS virus-specific CMI.

EFFECTS OF PRRS VIRUS VIRULENCE AND ANTIGEN-PRESENTING CELLS ON T CELL ACTIVATION AND ANTIVIRAL CYTOKINE PRODUCTION

W. Charentantanakul*, R. Platt, J.A. Roth. Department of Veterinary Microbiology and Preventive Medicine, Iowa State University, Ames, IA

Effects of porcine reproductive and respiratory syndrome virus (PRRSV) virulence and porcine antigen-presenting cells (APC) on T cell activation and antiviral cytokine production were studied using 3 virulent PRRSV isolates (VR-2385, SDSU-73, VR-2332) and a modified-live virus vaccine strain. The up-regulation of CD25 (alpha chain of IL-2 receptor) in response to concanavalin A (conA) and the expression of interferon gamma (IFN γ) and tumor-necrosis factor alpha (TNF α) in response to phorbol 12-myristate 13-acetate plus ionomycin (PMA/I) on naïve porcine T cells were observed. Naïve porcine T cells from PRRSV seronegative pigs were cultured with autologous lymphocytes, monocytes, monocyte-derived macrophages (M Φ), or monocyte-derived dendritic cells (DC) that were inoculated with each PRRSV isolate for 2 days. ConA or PMA/I were subsequently added to the cultures which were allowed to incubate for 2 more days in conA stimulation or for 12 hours in PMA/I stimulation, respectively. T cells co-cultured with monocytes infected with all 3 virulent PRRSV demonstrated significant reduction in %CD25⁺, %IFN γ ⁺, and %TNF α ⁺ cells as determined by flow cytometry when compared to those from uninoculated controls. In addition, the significantly reduced %TNF α ⁺ was observed in T cells co-cultured with monocytes infected with vaccine strain. The significant reduction in T-cell responses was not detected in T cells co-cultured with lymphocyte, M Φ , and DC inoculated with any PRRSV isolates. Heat-inactivated PRRSV did not induce significantly reduced T-cell responses in any cultures. Anti-swine IL-10 monoclonal antibodies when added at the beginning of the culture period inhibited the reduced T cell responses. The results of this study suggest that (1) PRRSV virulence plays an important role in T-cell suppression (2) monocytes play a major role among porcine APC in T-cell suppression during PRRSV infection and (3) T-cell suppression may be due to PRRSV-induced IL-10 expression.

MAPPING OF B-CELL LINEAR EPITOPES ON NSP2 AND STRUCTURAL PROTEINS
OF A NORTH AMERICAN STRAIN OF PRRS VIRUS

M. de Lima^{1,2}, A.K. Pattnaik¹, E.F. Flores², F.A. Osorio¹. ¹Nebraska Center for Virology and Dept of Veterinary and Biomedical Sciences, University of Nebraska-Lincoln. ²Dept of Microbiology and Parasitology, Federal University of Santa Maria, Santa Maria, RS, Brazil.

This study is aimed at identifying PRRSV B-cell linear epitopes that would be consistently recognized by the humoral immune response of naturally infected animals. To this end, 213 overlapping 15-mer synthetic peptides covering the whole amino acid sequence of a non-structural protein (nsp2) and all the structural proteins of a North American strain of PRRSV (NVSL97-7895) were used in a peptide-based enzyme-linked immunosorbent assay. Preliminary results from the analysis of 97 peptides derived from nsp2 have indicated the presence of ten immunodominant epitopes scattered along the sequence of the protein. These epitopes were consistently recognized by the majority of 15 animals experimentally infected with PRRSV FL12 (PRRSV derived from a full-length cDNA clone prepared from the NVSL97-7895 strain). Several other epitopes were reactive with about 50% of the sera tested. In addition, seroconversion kinetics has revealed that the antibodies recognizing these epitopes appear regularly between days 7 and 15 pi, remaining detectable until at least day 90 pi. Further analysis of the structural proteins and the serological reactivity of selected epitopes with field sera will provide us with insight about specific immunogenic regions that could serve as marker candidates, allowing serological differentiation between vaccinated and naturally infected pigs.

IMMUNIZATION OF PIGS WITH VIRAL REPLICON PARTICLES EXPRESSING
PRRS VIRUS GP5 AND M PROTEINS

M.M. Erdman^{1*}, K.I. Kamrud², D.L. Harris^{1,3}. ¹Dept of Animal Science, Iowa State University, ²AlphaVax Inc, Research Triangle Park, NC, ³Dept of Veterinary Diagnostic and Production Animal Medicine, Iowa State University

There is a clear and immediate need for improved PRRSV vaccines. The use of viral replicon particles (VRPs) merge subunit and live attenuated technologies providing a safe system of inducing protective immunity that mimics natural infection. Antibodies to PRRSV GP5-M heterodimer epitopes other than to just GP5 may be important for *in vivo* protection based on *in vitro* studies. The importance of a heterodimer similar to PRRSV GP5-M has already been established for equine arteritis virus (EAV), another member of the *Arteriviridae* family. Although bacterial vectors of PRRSV proteins have been investigated, the proposed importance of accurate protein (heterodimer) formation and glycosylation patterns favors the use of viral vectors. When considering the studies using viral recombinants or plasmid DNA, there are currently no reports expressing GP5 and M proteins in heterodimer formation. We thus propose the expression of PRRSV GP5-M heterodimer in VRPs and evaluation as a vaccine to protect pigs from homologous and heterologous PRRSV challenge.

PERMISSIVENESS OF PORCINE ALVEOLAR MACROPHAGES FOR PRRS VIRUS REPLICATION IN CULTURE IS DEPENDENT ON CULTURE CONDITIONS

N. Gaudreault*, C. Wyatt, R.R.R. Rowland. Diagnostic Medicine/Pathobiology, Kansas State University, Manhattan, KS

Porcine reproductive and respiratory syndrome virus (PRRSV) targets a subpopulation of macrophages in the porcine lung. The overall goal of this project is to understand the molecular bases for the non-permissive vs. permissive state of porcine alveolar macrophages (PAMs) for PRRSV. The approach was to place PAMs in culture in medium consisting of RPMI with 10% FBS. After two hr the cells were washed and 15 hr later fixed and stained with FITC-SDOW-17. The percentage of PRRSV-permissive cells was determined by comparing the number of fluorescent cells with total number of cells counted in several microscopic fields. Results showed that PAMs placed in culture and infected one hr later were non-permissive. Permissiveness appeared between 8 and 24 hr after culture and peaked at around 48 hr. PAMs retained permissiveness for at least 96 hr. Even though the percentage of permissive macrophages differed between pigs, the pattern was the same. PAMs obtained from acutely infected pigs were also permissive for PRRSV when placed in culture. IL-10, LPS, and TNF-alpha, added at the initiation of culture, had a minimal effect. IL-4 had a positive effect, increasing both the percentage of permissive cells and virus yield in a dose-dependent manner; however, the effect of IL-4 appeared to be pig-specific. As expected, IFN-gamma blocked infection. Actinomycin D, an inhibitor of mRNA synthesis, when added at 0, but not at 48 hr, also prevented infection. We also followed the phenotypic properties of cells using flow cytometry and eight cell surface markers. A consistent up-regulation of CD14 was observed in PRRSV-infected cultures. These data indicate that *in vivo* PRRSV infection does not remove the population of cells that later become permissive for virus in culture. The permissiveness of PAMs is dependent on new mRNA synthesis and can be regulated by cytokines. Up-regulation of CD14 is consistent with *in vivo* observations of increased CD14 in lungs of infected pigs.

EFFECT OF PRRS VIRUS ON MONOCYTE-DERIVED DENDRITIC CELLS

J. Hernández^{1*}, F. Osorio². ¹Laboratory of Immunology, Research Center for Food and Development (CIAD, A.C.), Hermosillo, Sonora, Mexico. ²Department of Veterinary and Biomedical Sciences, University of Nebraska-Lincoln, Lincoln, NE

After capture and process antigens, mature dendritic cells (mDC) present the peptides to naïve cells, thereby inducing a cellular immune response that involves both CD4 T helper and cytolytic CD8 T cells. This work evaluated the effect of PRRSV on monocyte-derived DC. PBMC were obtained from 5 weeks old healthy pigs using Ficoll gradients. In order to get adherent cells, 5×10^6 PBMC were culture over night in DMEM with 10 % FCS. Adherent cells were stimulated in presence of IL-4 (20 μ g/ml) and GM-CSF (20 μ g/ml) during 8 days. At day 6 cells immature DC (iDC) were stimulated with LPS (2 μ g/ml) or PRRS virus (10^3 DICC₅₀). The effect of PRRS virus was evaluated in iDC or mDC through the expression of CD14, CD80/86, MHC-II, and SWC3. The viral antigen was analyzed by RT-PCR, FACS and titer in Marc-145 cells. Our results show that PRRSV virus indeed infects and replicates in DC, the outcome of which being with a meager expression of co-stimulatory molecules in DC. These results may provide evidence that interaction of PRRSV with DC may be one of the factors responsible for the delay in acquired immunity that is the hallmark for the pathogenesis of PRRS.

IMPACT OF PRRS VIRUS ON CLASSICAL SWINE FEVER VACCINE

S. Kedsangakonwut¹, W. Sada³, S. Lacharoje¹, S. Suradhat², R. Thanawongnuwech^{1*}.
¹Veterinary Pathology, ²Veterinary Microbiology, Chulalongkorn University, ³Clinic for Swine, Mahanakorn University of Technology, Bangkok, Thailand.

To investigate the effect of PRRSV infection on CSF vaccine efficacy, twenty-eight, 17-day-old pigs were divided into 5 groups (5-7 pigs/group). Group 1 served as a virulent CSF virus (Bangkok 1950) challenge group. Group 2 was vaccinated with CSF vaccine and challenged with the virulent CSF virus 3 weeks later. Group 3 was infected with a Thai-isolated PRRSV (01NP1, US genotype) and challenged with the virulent CSF virus 4 weeks later. Group 4 was infected with 01NP1 a week before CSF vaccination and challenged with the virulent CSF virus 3 weeks later. The negative control groups (Group 5) received no PRRSV or CSF challenge or no CSF vaccination. The pigs in Groups 1 and 3 showed clinical signs consisted with CSF infection within 2 days post CSF challenge (dpc), and all pigs died between 9 and 11 dpc. CSF virus could be recovered from pooled sera and tissues from both groups while PRRSV was recovered only from Group 3 until 14 days post PRRSV infection. Interestingly, the pigs in Group 4 had PRRSV viremia until 35 days post PRRSV infection and those pigs died after CSF challenge as early as 6 dpc. The pigs in Group 2 and Group 5 looked normal throughout the experiment. The results demonstrated that CSF vaccination during PRRSV infection could prolong PRRSV viremia and PRRSV infection significantly reduced the CSF vaccine efficacy leading to CSF vaccination failure.

INFLUENCE OF GENETIC DIFFERENCE ON CROSS PROTECTION AMONG PRRS
VIRUSES

W.-I. Kim¹, S.-H. Cha¹, K.-J. Yoon^{1,2}. Departments of ¹Veterinary Microbiology and Preventive Medicine and ²Veterinary Diagnostic and Production Animal Medicine, College of Veterinary Medicine, Iowa State University, Ames, Iowa

The following study was conducted to determine genetic elements of the virus with immunological significance for cross neutralization among various PRRS viruses which, in turn, can be used for viral classification. Sixty-nine field isolates collected between 1996 and 2002 were classified into susceptible (S) and resistant (R) groups based on *in vitro* susceptibility to the antiviral activity of sera raised against the VR-2332 strain. The R group was further classified into R1, R2, and R3 according to the number of consistent amino acid changes in ORF5, compared to the S group. Ten viruses each were selected from R and S groups and inoculated into pigs with passively transferred IgG at a rate of 1:≥16 VN titer against VR-2332 or normal serum globulin by IP injection. All of the viruses from the R groups escaped from passive antiviral humoral immunization. Viruses of the S group were further classified into Sc (completely inhibited), Sp (partially inhibited) or Svt (susceptible only by *in vitro* test) group. Then structural gene sequences (ORFs 2 to 6) of selected viruses (n=33) were aligned and compared by the classified groups to determine amino acid changes which were correlated with altered susceptibility to neutralizing activity of anti-VR-2332 antibody. The most significant changes were observed in ORF5 and corresponded with classification of virus susceptibility to neutralizing antibody. Some additional consistent amino acid changes were also observed in ORFs 3 and 4. In conclusion, genetic changes in certain regions of PRRS viral genome may be directly related to the effectiveness of virus neutralization.

EXPERIMENTAL INFECTION OF PIGS WITH EUROPEAN-LIKE (TYPE 1) PRRS
VIRUS ISOLATES OF U.S. ORIGIN

S. Lawson¹, Y. Fang¹, R.R.R. Rowland², J. Christopher-Hennings¹ and E.A. Nelson^{1*}.

¹Dept. of Veterinary Science, CIDRV, South Dakota State University, Brookings, SD and

²Dept. of Diagnostic Medicine/Pathobiology, Kansas State University, Manhattan, KS

Porcine reproductive and respiratory syndrome virus (PRRSV) exists as two major genotypes, designated as Type 1 (European-like) and Type 2 (North American-like). Type 1 isolates have only recently appeared in the U.S. The purpose of this study was to evaluate the pathogenic and immunological properties of representative U.S. Type 1 isolates obtained between 2001 and 2003. Forty 4-5 week-old pigs were divided into 5 groups (n=8) and inoculated intra-nasally with one of four different U.S. Type 1 PRRSV isolates (SD01-07, SD01-08, SD02-11, or SD03-15) or left as mock-infected controls. Isolates were chosen based on their location in different branches of the current U.S. Type 1 phylogenetic tree, origin from different geographical regions and presentation of a range of clinical signs in the field. Pigs were monitored daily for clinical signs and blood samples were collected twice weekly for the first two weeks and weekly thereafter. Three pigs from each group were euthanized at 14 dpi to evaluate acute lesions and the remaining pigs were retained for 12 weeks to monitor antibody responses, viral loads, and persistence. Clinical signs and pathology were variable between groups but generally remained mild. Viremia was monitored using semi-quantitative real-time PCR. Challenged animals seroconverted, as detected by the IDEXX ELISA, by approximately 14 dpi. Neutralizing antibody responses against the homologous challenge isolates reached detectable levels as early as 21 dpi and were particularly robust, reaching titers as high as 1:128 by 56 dpi. However, sera did not neutralize selected North American (Type 2) PRRSV isolates and demonstrated intermediate levels of neutralization against other European-like isolates and the European prototypic strain, Lelystad virus. This study represents the first detailed analysis of the relative virulence, persistence potential and pathogenesis of diverse U.S. Type 1 PRRSV isolates. It also provides a resource of well-characterized serum samples to serve as control materials for other researchers and diagnostic laboratories.

PRRS VIRUS ACTIVATES NF- κ B VIA REACTIVE OXYGEN SPECIES (ROS) PRODUCTION AND INCREASES MATRIX METALLOPROTEINASE (MMP) EXPRESSION IN NF- κ B DEPENDENT MANNER

S.-M. Lee*, S.B. Kleiboeker. Department of Veterinary Pathobiology, College of Veterinary Medicine, University of Missouri-Columbia

Virus-host interactions lead to both activation and inhibition of complex cellular pathways, resulting in antiviral responses as well as enhanced viral replication and virulence. Despite years of research, little is known about intracellular signaling pathways that play key roles after PRRSV infection and the role of these pathways in PRRSV pathogenesis. NF- κ B is a critical regulator of innate and adaptive immune function as well as cell proliferation and survival. Therefore, many viruses have evolved strategies to modulate NF- κ B pathway to their advantage. The present study demonstrated for the first time that a virus belonging to the *Arteriviridae* family activates NF- κ B in host cells, characterized by translocation of NF- κ B from the cytoplasm to the nucleus, increased DNA binding activity and NF- κ B regulated gene expression. NF- κ B regulated gene expression was increased at later times post-infection in a viral dose- dependent manner and UV-inactivation of PRRSV significantly decreased NF- κ B activation. It was also shown that the elevated NF- κ B activity was the consequence of I κ B protein degradation, a cellular NF- κ B inhibitor, in PRRSV infected cells. Over expression of the dominant negative form of I κ B α significantly suppressed NF- κ B activation in PRRSV infected cells but did not affect viral replication and viral cytopathic effect, suggesting that NF- κ B may not be required for efficient PRRSV replication. PRRSV infection induced oxidative stress in cells by generating ROS and the involvement of ROS in NF- κ B activation by PRRSV was demonstrated by reduced NF- κ B binding activity in the presence of antioxidants (pyrrolidinedithiocarbamate and N-acetylcysteine), suggesting ROS as a mechanism by which NF- κ B was activated by PRRSV infection. Moreover, NF- κ B dependent expression of MMP-2 and MMP-9, which play a role in immune responses by promoting infiltration of inflammatory cells, was observed in PRRSV infected cells. This implies that NF- κ B activation is a biologically significant aspect of PRRSV pathogenesis. The results presented here provide a basis for understanding molecular pathways of pathology and immune evasion associated with disease caused by PRRSV.

DIFFERENTIAL IMMUNITY IN PIGS WITH HIGH AND LOW RESPONSES TO PRRS
VIRUS

J.K. Lunney^{1*}, D. Petry², P. Boyd¹, D. Kuhar¹, E. Blankenship², R. Johnson² ¹APDL,
BARC, USDA, Beltsville, MD and ²University of Nebraska, Lincoln, NE

Duroc/Hampshire (HD) crossbred pigs (100) and NE Index line (I) pigs (100) were infected with porcine reproductive and respiratory syndrome virus (PRRSV) and evaluated for resistance/susceptibility. Controls (100/line) were uninfected littermates to each infected pig. Viremia (V), weight change (WTΔ), and rectal temperature at 0, 4, 7, and 14 days post-infection (dpi) were recorded. Lung, bronchial lymph node (BLN), and blood were collected at necropsy (14 dpi). Line differences, and line by treatment interactions across days, indicated genetic variation in responses to PRRSV (Petry et al., 2005). Principal component analyses were used to rank pigs for phenotypic response to PRRSV. Pigs classed as low (L) responders had high WTΔ, low V, and few lung lesions; high (H) responders had low WTΔ, high V, and many lesions. I pigs had a quicker response to PRRSV than HD pigs as indicated by the differences in viremia in L class pigs. RNA from frozen lung and BLN tissue of the 7 highest and lowest responders per line, and their littermates, were extracted. cDNA expression of 12 specific innate and Th1 immune markers was evaluated in a 2*2*2 factorial design. HD pigs had a greater magnitude of difference in expression than I pigs. Significant under-expression of L pigs for certain immune genes, relative to controls, was detected in lung and BLN, particularly in I. Serum levels of the immune cytokines affirmed the lung differences. Low pre-infection serum levels of the innate cytokine, interleukin-8, were significantly associated with PRRSV resistant, L pigs. Following infection, low expression of interferon-gamma in cDNA and in serum was also correlated with resistance. These data are critical for genetic association studies to fine map candidate genes and to determine causative alleles.

TYPE I INTERFERON RESPONSES TO PRRS VIRUS INFECTION

L.C. Miller*, C.G. Chitko-McKown, W.W. Laegreid. Animal Health Research Unit, USDA, ARS, Roman L. Hruska U.S. Meat Animal Research Center, Clay Center, NE.

Porcine reproductive and respiratory syndrome virus (PRRSV) causes highly significant losses to the swine industry worldwide. Productive infection occurs almost exclusively in cells of the monocyte-macrophage lineage both *in vitro* and *in vivo*, predominantly in alveolar macrophages of the lung. Thus, innate immune responses of the alveolar macrophages comprise the initial defense against PRRSV. Previously we have demonstrated that PRRSV infection does not result in induction of type I interferons (IFN- α and - β) by MARC-145 cells as would be expected with most RNA viruses. The results are significant because both IFN- α and IFN- β are members of the innate immune system, which is typically viewed as the first responder of the immune system. Activation of this response signals other branches of the immune system to become activated and mount a protective response. The fact that PRRSV is capable of suppressing the activation of this response may explain the general delayed immune response to PRRSV infection. Further studies on the dynamics of this process, time and dose kinetics and analysis of other genes in the type I interferon pathway ongoing in primary porcine macrophages. Elucidation of the mechanism of PRRSV suppression of the type I interferon response may provide targets for novel vaccination approaches to control this important disease.

B-CELL RESPONSES TO PRRS VIRUS INFECTION

P. Mulupuri^{1*}, G.N. Hirsch¹, S.A. Dee², J. Zimmerman³, M.P. Murtaugh¹. ¹Department of Veterinary and Biomedical Sciences, ²Veterinary Population Medicine, University of Minnesota, St Paul, MN. ³Department of Veterinary Diagnostic and Production Animal Medicine, Iowa State University, Ames, IA

Humoral responses to PRRSV are focused primarily on serum antibody titers. However an understanding on the source of serum antibody titers i.e., induction and localization of antibody secreting cell (ASC) and memory B-cell responses is necessary to understand the induction and maintenance of anti-PRRSV immunity as well as to gain insights into mechanisms of viral persistence. Here, the ELISPOT assay was used to characterize total and antigen-specific ASC and memory B-cell responses to non-structural protein 2 (Nsp2), nucleocapsid and glycoprotein 5 (GP5) polypeptides of the PRRSV. PRRSV infection did not substantially affect global B-cell responses, but there was a significant B-cell response to a variety of PRRSV antigens. Nsp2 showed the strongest antigen-specific IgG secreting B-cell and memory B-cell responses, followed by nucleocapsid and GP5. IgG secreting B-cells are significantly localized to sternal lymph node (SLN) and spleen at 37 days of PRRSV infection, indicating that SLN and spleen are the major sites of antibody production. Antigen-specific actively secreting B-cell responses reached a peak by 37 days of PRRSV infection, and declined by 98 days of infection. However memory B-cell responses remained high even at 150 days of infection. In contrast to actively secreting B-cells, there was no significant difference in tissue distribution of memory B-cell responses. Both ASC and memory B-cell responses were extremely low in bone marrow, although it is regarded as the primary site of antibody production in vertebrates. Since no difference in IgG total B-cell responses was observed in a variety of lymphoid tissues in both infected and un-infected pigs in acute and persistent PRRSV infection, we also conclude that there was no polyclonal B-cell activation in PRRSV infection.

ANTIBODY RESPONSES OF PRRS VIRUS-EXPOSED SOWS FOLLOWING KILLED VIRUS VACCINATION PRE-FARROWING

D. Nilubol^{1*}, B. Thacker², E. Thacker². ¹Chulalongkorn University, Thailand, ²Iowa State University, Ames, IA

The serological response of PRRSV-exposed sows and the antibody levels of their offspring following the use of killed virus vaccination pre-farrowing were investigated. Forty multiparous sows from a PRRSV field virus infected herd with no history of KV use, past use of MLV were used in this study. No clinical signs of active PRRSV infection were present at the time of the study, although the herd had several previous PRRSV outbreaks. Twenty randomly selected sows were vaccinated with KV according to label directions at 60 and 75 days of gestation. The remaining sows served as non-vaccinated controls. Serum was obtained from all 40 sows prior to vaccination and at 3 days after farrowing. PRRSV serum antibodies were measured by ELISA and SN assays. SN assays were performed using the MLV and KV PRRSV parent strains, VR-2332 and VR-2402, respectively. From the 40 sows, 6 vaccinated and 6 non-vaccinated sows were selected for further study based on parity and serum neutralizing (SN) and ELISA antibody titers prior to KV vaccination. Colostrum was collected and assayed for SN antibodies and blood was collected from 4 piglets from each sow and assayed for antibodies using ELISA and SN assays. KV increased antibody levels compared to the non-vaccinated sows as measured by ELISA and SN. SN antibodies against VR-2402 were significantly increased while no increase in SN antibodies against VR-2332 was observed. KV significantly increased SN antibody levels in the colostrum and the offspring. Similar to the response observed in sows, the increased SN antibody levels in pigs were primarily against VR-2402. In contrast, the SN antibodies in colostrum were found with both VR-2402 and VR-2332. The results of the study demonstrated that the use of KV in PRRSV exposed sows pre-farrowing can booster the levels of SN antibodies in colostrum and the offspring. The increased SN antibodies were mainly against VR-2402, suggesting a heterologous booster effect. However, their protective effect against other viruses was not assessed in this trial.

PRRS VIRUS FAILS TO ACTIVATE THE UNFOLDED PROTEIN RESPONSE

J.M. Rowland*, R.R.R. Rowland. Department of Diagnostic Medicine/Pathobiology,
Kansas State University, Manhattan, KS

The activation of innate immune response pathways plays a critical role in the control of virus infections. The antiviral properties of the unfolded protein response (UPR) are related to the detection of perturbations in ER function, such as the accumulation of misfolded or aggregated proteins. One outcome of UPR activation is the altered splicing of XBP-1 mRNA, which codes for a transcription factor that is rapidly transported to the nucleus. Replication of porcine reproductive and respiratory syndrome virus (PRRSV) is primarily restricted to the ER-Golgi, culminating in the disintegration of the ER and formation of double membrane vesicles. In preliminary work, PRRSV replication did not induce apoptosis or influence normal cell-cycle progression, suggesting that cells fail to recognize the presence of PRRSV. In this study, MARC 145 cells were infected with low passage and cell-adapted PRRSV isolates SD23983 P4 and P136, respectively. Un-infected cultures served as negative controls and culture wells treated with the UPR activator, DTT, served as positive controls. At two days after infection and one hr after DTT treatment, total RNA was extracted and XBP-1 amplified by RT-PCR using gene-specific primers. Un-spliced XBP-1 was detected by the presence of a Pst-1 restriction site. Results showed that DTT treatment resulted in the accumulation of Pst-resistant XBP-1 cDNA; whereas PCR products from PRRSV-infected and control cultures retained sensitivity to Pst-1. These results provide further support for the ability of PRRSV to block the induction of antiviral responses during replication.

IDENTIFICATION AND CHARACTERIZATION OF A NOVEL CELLULAR PROTEIN
INTERACTING WITH THE PRRS VIRUS NUCLEOCAPSID (N) PROTEIN

C. Song*, D. Yoo. Department of Pathobiology, Ontario Veterinary College, University of Guelph, Guelph, Ontario, Canada

PRRS virus replicates in the cytoplasm of infected cell, but the nucleocapsid (N) protein is specifically localized in the nucleus and nucleolus. The N protein nuclear localization suggests its role in modulation of host cell function and biosynthesis during PRRS virus infection. Using N as a bait in the yeast two hybrid system, we screened more than 3 million yeast colonies and identified a novel cellular protein specifically interacting with the N protein. The specific interaction of N with this protein was confirmed in cells by mammalian two-hybrid assay and in vitro by co-immunoprecipitation and GST pull-down assays. Two isoforms were generated to yield p40 and p32, and they shared the C-terminal 246 amino acids. The C-terminal 82 amino acids displayed a sequence motif of I-mfa (inhibitor of MyoD family), and we named this protein PICP (primate I-mfa domain containing protein). The N-terminal extension of p40 contained a putative nuclear localization signal, and accordingly p40 was found to localize in the nuclear compartment while p32 was predominantly cytoplasmic. The C-terminus of PICP contains a cysteine-rich domain, resembling a zinc-finger-like structure, and we show that PICP is indeed a zinc-binding protein. Unexpectedly, the N protein itself was found to contain a transcription inhibitory activity as it down-regulated the retrovirus long terminal repeats (LTR) promoter activities. Our data implicate an important regulatory role of the N protein during PRRS virus infection.

IMMUNIZATION OF SOWS WITH A RECOMBINANT PSEUDORABIES VIRUS EXPRESSING THE GP5 OF PRRS VIRUS CONFER PROTECTION AGAINST PRRS

Z.-J. Tian, H.-J. Qiu, G.-Z. Tong*. National Key Laboratory of Veterinary Biotechnology, Harbin Veterinary Research Institute, Chinese Academy of Agricultural Sciences, Harbin 150001, P. R. China

Objectives

Porcine reproductive and respiratory syndrome (PRRS) virus causes reproductive failure in pregnant sows including premature farrowing, late-term abortion, and increased dead-born and weak-born pigs. The objective of this study was to investigate the efficacy of sows vaccinated with a recombinant pseudorabies virus expressing the GP5 of PRRSV (rPRV-GP5) (Qiu et al, 2005, Vet Immunol Immunopathol) following a challenge with a virulent PRRS virus.

Materials and Methods

Animal Experiment A: Nine PRV- and PRRSV-free multiparous sows were randomly divided into two groups. Group A (n=6), each sow was immunized with $2 \times 10^{7.0}$ PFU of rPRV-GP5 by IM routes before gestation; Group B (n=3) were no vaccinated as negative controls, respectively. Sows of groups A and B were challenged by IN inoculation with $10^{5.5}$ TCID50 of PRRSV strain CH-1a during 75-90 days of pregnancy.

Animal Experiment B: The trial was performed in a 500-sow farrow-to-finish pig farm, infected with PRRSV. 68 sows were immunized with rPRV-GP5, 68 sows were vaccinated with a commercial inactivated PRRSV vaccine twice at 3-4 weeks interval and a commercial live PRV vaccine.

Results

The results were shown that sows in lab or field had a significantly mortality reduction after vaccination with rPRV-GP5 (Tables 1, 2).

Table 1 Protection rate of immunized sows after challenge with virulent PRRSV

Groups	Number of sows	No. of newborn piglets	No. of stillbirth	No. of weak- born pigs	Protective rate (%)
rPRV-GP5	6	71	4	6	85.9%
Control	2	30	9	7	46.7%

Table 2 The efficacy of rPRV-GP5 in immunized pregnancy sows in farm

Groups	Number of sows	No. of newborn piglets	No. of stillbirth and mummy	Survived piglets ratio (%)
rPRV-GP5	68	696	40	94.3%
PRRSV+PRV vaccines	68	706	85	87.9%

MICROSATELLITE MARKERS DEVELOPED IN THE ENTIRE SLA REGION AND
THEIR AVAILABILITY IN VARIOUS PORCINE BREEDS

H. Uenishi^{1*}, M. Tanaka², A. Ando³, C. Renard⁴, P. Chardon⁴, M. Domukai², N. Okumura²,
T. Awata¹. ¹Natl. Inst. Agrobiological Sci., Tsukuba, Ibaraki, Japan. ²STAFF-Institute,
Tsukuba, Ibaraki, Japan. ³Dept. Mol. Life Sci., Div. Basic Mol. Sci. & Mol. Med., Tokai
Univ. Sch. Med., Isehara, Kanagawa, Japan. ⁴Laboratoire Mixte de Radiobiologie et d'Etude
du Génome, INRA-CEA, Domaine de Vilvert, Jouy-en-Josas, France

The genes encoded within the swine leukocyte antigen (SLA) region are closely related with immune functions. Studies of association between diseases and SLA require a precise method for genotyping the SLA alleles. The SLA region is highly polymorphic and many observations suggest that there is much duplication and deletion of the genetic loci in the region. The genotyping method based on polymorphic markers is a possible solution to the difficulty with the typing of genes per se. We developed 40 microsatellite markers in the entire swine leukocyte antigen (SLA) region with its flanking sequence, spanning over 2.35 Mb, which has been already determined. The average span between markers was 59 kb, and the largest interval between markers was 127 kb. We also evaluated polymorphisms of length for the markers using 97 pigs derived from 12 breeds, including representative commercial breeds. All of the markers were successfully amplified in genomic DNA and shown to be polymorphic. They will be valuable for association studies between SLA and immunological traits such as disease susceptibility.

MAPPING PRRS VIRUS T CELL EPITOPES

C. Wyatt*¹, R.R.R. Rowland¹, D. Smith². ¹Diagnostic Medicine/Pathobiology, Kansas State University, Manhattan, KS. ²Transplant Immunology Laboratory, Baylor University Medical Center, Dallas, TX

Knowledge of PRRSV T cell epitopes is required for understanding PRRSV immunity and for the design of new vaccines. The purpose of this project is to develop the techniques needed to identify T cell epitopes, and to begin an analysis of T cell epitopes in GP5 and M proteins, because these structural proteins are expressed at high levels in PRRSV infected cells. In order to minimize variation due to MHC polymorphism, we have selected 5 groups of SLA haplotype matched pigs by PCR-SSP typing of SLA class I antigens. Twenty pigs were selected from 40 SLA typed. Fifteen pigs are currently inoculated with PRRSV isolate VR-2332, and five pigs are being maintained as controls. At present, neutralizing and non-neutralizing antibody titers are being evaluated. RT-PCR is being used to follow viremia. When pigs are virus negative in serum for 2 consecutive weeks, blood and mesenteric nodes will be collected and PBMC and lymph node lymphoid cells will be labeled with PKH67 and cultured in the presence of several stimulators, including chemically synthesized individual peptides representing overlapping 30-mer peptides that represent the GP5 protein. Proliferation of CD4⁺ T cells will be assessed by dual fluorescence flow cytometry. Additional PBMC and lymph node lymphoid cells will be assessed by ELISPOT, and the number of cells secreting IFN- γ will be determined. Once developed, these assays will be used to evaluate additional PRRS virus proteins.

CONSTRUCTION AND CHARACTERIZATION OF RECOMBINANT PRV
EXPRESSING PRRS VIRUS GP5 AND M PROTEINS

Z. Liu^{1*}, A.K. Pattnaik², F. Osorio², S.I. Chowdhury¹. ¹College of Veterinary Medicine, Kansas State University, Manhattan, KS. ²Veterinary Biomedical Sciences, University of Nebraska, Lincoln, NE

Our lab is constructing PRV recombinant viruses expressing PRRSV gP5 and M proteins. PRVgE-deleted/PRRSV gP5 or M ORF expressing vectors have been constructed. Currently, transfection experiments and plaque purification of the recombinant PRV viruses are in progress. In the next several months, identification and characterization of the PRV recombinants expressing PRRSV gP5 and M proteins will be completed.

EPITOPE MAPPING OF STRUCTURAL PROTEINS OF PRRS VIRUS

Y.-J. Zhou, T.-Q. An, J.-X. Liu, H.-J. Qiu, G.-Z Tong*. National Key Laboratory of Veterinary Biotechnology, Harbin Veterinary Research Institute, Chinese Academy of Agricultural Sciences, Harbin, 150001, The People's Republic of China

In this study, six structural proteins (GP2-GP5, M, and N) of PRRSV CH-1a strain were expressed in *E. coli* and used to develop monoclonal antibodies (MAbs). A total of 16 anti-N MAbs, 15 anti-GP5 MAbs, 5 anti-GP3 MAbs, one anti-M MAb, one anti-GP4 MAb and one anti-GP2 MAb were obtained. The structural proteins were divided into two through four overlapped fragments and expressed in *E. coli* respectively. The reactivity of different fragments with MAbs was probed in indirect ELISA. After broad epitope mapping with overlapping protein fragments, the fragments that could be recognized by MAbs were serially truncated from both ends until the smallest functional unit that was recognized by each MAb was identified. An immunodominant epitope cluster composed of H⁵⁴FPLA⁵⁸, K⁵²PHFPLA⁵⁸ and E⁵¹KPHFP⁵⁶ was localized in the middle of N protein, which was well conserved among PRRSV isolates (including both NA-type and European-type). An immunodominant epitope E¹¹⁷SAAGFHPIAAND¹²⁹ was found in M protein for the first time. The minimal antigenic epitopes recognized by GP5 MAbs were R¹⁵¹LYRWR¹⁵⁶, E¹⁶⁹GHLIDLKRV¹⁷⁸ and Q¹⁹⁶WGRL²⁰⁰, and substitution analysis showed that 5 MAbs could only recognize the peptide with L²⁰⁰ and 4 MAbs could recognize both L²⁰⁰ and P²⁰⁰. Antigenic epitopes of GP3 were W⁷⁴CRIGHDRCGED⁸⁵ and Y⁶⁷EPGRSLW⁷⁴, respectively. Western blot analysis with sera from BJ-4 and CH-1a infected pigs found that peptides within GP3 (50-65aa, 58-72aa, 66-81aa, 73-87aa, 80-95aa, 88-101aa and 94-109aa) were all immunodominant epitopes. In addition, antigenic epitopes of GP2 and GP4 were located in region 38-148aa and 61-178aa, respectively.

Taken together, this study of epitope mapping may be helpful in understanding the molecular properties of structural proteins of PRRSV, and the relationship between immunogenicity or pathogenesis and gene variations of the virus, as well as may also be useful for diagnosis of PRRSV infection based on antigenic epitope or developing a new strategy for vaccine design.

SECTION 4: ECOLOGY, EPIDEMIOLOGY, ELIMINATION

TRANSMISSION OF PRRS VIRUS TO PIGS VIA VIRUS-CONTAMINATED PORK: A RISK ASSESSMENT

L. Alban¹, T. Drew², P. Have³, M.-F. Le Potier⁴, M.S. Murtaugh⁵, H. Nauwynck⁶, G. Wellenberg⁷, J.M. Sánchez Vizcaino⁸, M. Wierup⁹, J. Zimmerman¹⁰. ¹Danish Bacon and Meat Council, Copenhagen, Denmark; ²Veterinary Laboratories Agency, Addlestone, United Kingdom; ³Danish Institute for Food and Veterinary Research, Copenhagen, Denmark; ⁴Agence Française de Sécurité Sanitaire des Aliments, Ploufragan, France; ⁵University of Minnesota, St. Paul, Minnesota; ⁶Ghent University, Merelbeke, Belgium; ⁷Animal Health Service Institute, Deventer, The Netherlands; ⁸Universidad Complutense de Madrid, Madrid, Spain; ⁹Swedish University of Agricultural Sciences, Uppsala, Sweden, ¹⁰Iowa State University, Ames, Iowa

To prevent introduction of PRRSV, some countries have placed restrictions on trade in pork. To evaluate the likelihood of transmission of PRRSV via pork from infected pigs, the European Food Safety Authority conducted a risk assessment of 1) the probability of infectious PRRSV in pork at the time of slaughter, and 2) the amount of infectious virus in pork after bleeding, maturation, storage, transport, chilling, and thawing. Finally, a simulation was performed based on a farmer feeding 16-hours old swill containing 500g of raw pork to 10 pigs. The assumptions of the model were based on data from published papers and simulations were performed with the software program @Risk. According to the model, 1.9% of carcasses might harbor low amounts of PRRSV at slaughter ($<10^3$ TCID₅₀/g). Infectious virus declines by about 1 log both during maturation and thawing of frozen pork. No virus inactivation occurs during freezing, but inactivation occurs at $>4^\circ\text{C}$. According to the model, 32% of 50g swill-servings would contain PRRSV in low amounts ($<10^1$ TCID₅₀). In a worst-case scenario, it was assumed that all scraps originated from a carcass harboring PRRSV at slaughter. This would result in a mean of $10^{1.53}$ TCID₅₀ in a 50g-serving (90% C.I.: 10^0 - 10^2 TCID₅₀). The question is whether such low levels of PRRSV are capable of infecting naïve pigs via the oral route.

ASSESSMENT OF VERTICAL TRANSMISSION FROM PARITY ONE SOWS
INFECTED WITH LOW DOSE AND MILD VIRULENT PRRSV ISOLATE

J.P. Cano, R. Morrison, S. Dee. College of Veterinary Medicine, University of Minnesota,
St Paul, MN

Introduction PRRSV costs \$560.32 million to US swine producers every year¹. The first and most important step to control and eradicate PRRSV from infected populations is to “stabilize” herd immunity via different approaches like herd closure, intentional mass exposure or gilt acclimatization^{2,3,4,5}. Afterwards, the challenge for swine practitioners is to evaluate the virus “activity” in these chronically infected herds. The most commonly used strategy to perform this evaluation might be testing sera from piglets for PRRSV PCR. Questions about number of litters to be sampled, age and clinical signs of the piglets or the effect of pooling on PCR results do not have clear answers today. The aim of this study is to propose a protocol to sample piglets at birth to evaluate PRRSV chronically infected herds.

Materials and Methods 12 PRRSV naïve pregnant sows were individually housed in isolation rooms at the University of Minnesota. Sows were identified and then assigned to three different groups (4 sows per group). Sows in group A were intramuscularly injected with 3 ml of sterile MEM, group B with 10¹ TCID₅₀ total dose of PRRSV isolate MN 30-100 and sows in group C were also intramuscularly inoculated with 10² TCID₅₀ total dose of PRRSV MN 30-100. All sows were injected at 90 days of gestation. All individuals were PRRSV PCR and ELISA negative the day of injection, all sows in groups B and C resulted PRRSV PCR positive five days post inoculation (DPI) and again all exposed sows were PRRSV ELISA and PCR positive 14 DPI.

Four specific objectives were included in the study: a) To determine the proportion of viremic piglets at birth, 96 hours and at weaning, PRRSV real time PCR was performed in individual serum samples and proportions among groups and age were compared using Fisher’s Exact Test. b) To identify clinical signs (fuzziness, light weight, dome head pigs, dyspnea, diarrhea) associated with viremic piglets individual information was recorded from 1 to 18 days of age. c) Sensitivity and specificity of blood swab technique for PRRSV PCR were compared to serum collection technique by paired sample analysis. d) Effect of pooling serum or swab samples for PRRSV PCR was studied by obtaining the *maximum dilution still positive* from the relationship between the quantitative PCR result and the threshold. The experiment is being performed at this time and final results will be obtained at the end of September, 2005.

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AN EVALUATION OF ISOLATE PATHOGENICITY IN THE SHEDDING AND TRANSMISSION OF PRRS VIRUS BY AEROSOLS

J.G. Cho¹, S.A. Dee*¹, J. Deen¹, K. Faaberg², H.S. Joo¹ ¹Swine Disease Eradication Center, ²Veterinary Diagnostic Laboratory, University of Minnesota, College of Veterinary Medicine, St. Paul, MN

The objective of this study was to evaluate the role of animal age, bacterial co-infection and porcine reproductive and respiratory syndrome virus (PRRSV) isolate pathogenicity on the shedding and transmission of PRRSV by aerosols. To evaluate patterns of PRRSV shedding in aerosols, animals were grouped according to age (2 months vs. 6 months) and infected with either a mildly virulent PRRSV isolate (MN-30100) or a virulent isolate (MN-184). Selected animals in each group were also co-infected with *Mycoplasma hyopneumoniae* (*M hyo*). Pigs were anesthetized and aerosol samples collected on alternating days from days 1-21 post-PRRSV infection using a specialized mask. Concentrations of PRRSV RNA in aerosol samples were determined by quantitative TaqMan real-time polymerase chain reaction (RT-PCR) and expressed in units of TCID₅₀/ml. The role of isolate pathogenicity in the airborne transmission of PRRSV was assessed by exposing naïve recipient pigs to aerosols from pigs infected with either a mildly or highly virulent isolate. Recipient pigs were evaluated over a 14-day period post-exposure and tested on days 7 and 14 PI by qualitative TaqMan RT-PCR and ELISA. Animal age, *M hyo* co-infection and PRRSV isolate pathogenicity did not significantly influence PRRSV aerosol concentrations. However, infection with the MN-184 isolate did impact the likelihood of aerosol shedding (p=0.00005, OR=3.22) as well as aerosol transmission (p=0.04) while *M hyo* co-infection demonstrated tendencies toward positively influencing shedding frequency (p=0.053). Results indicate that the shedding and transmission of PRRSV through aerosols may be isolate dependent.

PRRS RESEARCH AT THE UNIVERSITY OF MINNESOTA.

S.A. Dee^{1,2}, P. Davies^{1,2}, C. Pijoan^{1,2}, H.S. Joo^{1,2}, B. Morrison^{1,2}, T. Molitor^{1,2}, C. Muñoz Zanzi^{1,2}, K. Faaberg³, K. Rossow¹, M. Rutherford³, M.P. Murtaugh^{2,3*}. Departments of ¹Veterinary Population Medicine and ³Veterinary & Biomedical Sciences, and ²Swine Disease Eradication Center, University of Minnesota, St. Paul, MN

Control of porcine reproductive and respiratory syndrome (PRRS) requires a better understanding of epidemiological and ecological factors that contribute to disease spread and persistence, both within animals and in the environment. A variety of PRRS research projects at the University of Minnesota are being conducted in laboratory settings, in controlled field settings and in collaboration with swine veterinarians and producers to identify risk factors associated with herd infection, maintenance of endemic infection in sow herds, and transmission of disease from sow herds to nurseries. Studies also are being performed to improve methods for disease diagnosis, elucidate genetic determinants of pathogenesis and immunogenicity, and explore basic mechanisms of viral interaction with the host cell. The research progress and accomplishments are reported as part of the USDA NC-229 multi-state project on PRRS.

PRRS VIRUS INFECTION PATTERNS IN NURSERY PIGS

C. Dewey*, O. Melnichouk, R. Friendship, D. Hayden. Population Medicine, University of Guelph, Guelph, Ontario Canada

The purpose of the study was to describe the spread of naturally occurring PRRS virus in nursery pigs from commercial farms. Eight herds with ongoing respiratory disease were selected by practicing veterinarians. On each farm, the 21 sows with the youngest pigs at the time of the visit were bled. Five healthy pigs nursing these sows were also bled, ear tagged and then re-bled every other week until they were 10 – 12 weeks old. Pigs were randomly assigned to be bled on the odd or even weeks. The serum samples were evaluated using the original IDEXX ELISA test. It was assumed that pigs became infected 10 days prior to a rise in the S/P ratio. The proportion of pigs that were seropositive due to recent infection was modeled using a catalytic infection model. Constant and linearly increasing infection models were evaluated using the coefficient of determination.

Depending on the herd of origin, 40% to 85% of pigs had maternal immunity in the first week of life. There were two distinct patterns of spread of the PRRS virus through nursery barns. There were 6 farms with a rapid spread of the virus where 50% of the pigs were infected by 5 to 7 weeks of age. In these herds, 90% of pigs were infected by 8.5 weeks of age. In the 2 farms with slow spread, only 20% to 40% of the pigs were infected by the end of the nursery phase. The linear infection model best explained the spread of infection which suggests that the virus is spread by pig to pig contact.

PRRS VIRUS: EMERGENCE OF NOVEL STRAINS IN BRITAIN

J.P. Frossard*, D. Westcott, B. Naidu, G. Sharp, C. Russell, N.G.A. Woodger, T. Drew.
Veterinary Laboratories Agency (Weybridge), New Haw, Addlestone, Surrey KT15 3NB,
U.K.

This research aims to identify novel strains of PRRSV associated with disease in pigs in Britain, with special emphasis placed on those associated with severe respiratory disease and/or high numbers of stillbirths and neonatal deaths. Recent reports of anomalies in serological testing had suggested increasing diversity and/or evolution of the virus.

The diversity of strains was assessed by analyzing the genetic sequence of ORF7 and ORF5 from PRRS viral RNA from tissue samples obtained from clinical cases of the disease. These were then compared with previously obtained data from strains isolated in Britain between 1991 and 1995.

Phylogenetic analysis of the data reveals a much greater diversity among recent isolates than had been seen with early isolates from Britain. For the recent isolates, two major branches were identified, one very different to historical British isolates, and a second group, some of which are rather more closely related to historical isolates, but nevertheless containing a very diverse member within that lineage. This alignment also indicated that diagnostic primers recently described would be unlikely to provide efficient amplification of the more recently isolated strains of PRRSV.

These findings demonstrate that PRRSV strains circulating in British herds have significantly increased in diversity between 1991 and 2005. Whether this is a result of rapid evolution or the introduction of new lineages remains to be determined. We present these findings, along with some preliminary data on the evolutionary relationship among these strains, and other European isolates.

STABILITY OF PRRS VIRUS IN AEROSOLS

J.R. Hermann¹, A. Burkhardt¹, M. Roof⁴, K-J Yoon¹, K.M. Bryden³, S.J. Hoff⁴, J. Zimmerman¹. ¹College of Veterinary Medicine, ³College of Engineering, ⁴College of Agriculture Iowa State University, Ames, IA. ⁴Boehringer Ingelheim, Ames, IA.

The long-term goal of our research is to develop accurate models of aerosol transmission of respiratory pathogens. Modeling aerosol transmission requires estimates of: 1) viral shedding in aerosols, 2) stability (half-life) of infectious virus in aerosols, and 3) estimates of the probability that a given dose is sufficient to infect a susceptible animal. The objective of the research reported here was to estimate the stability of aerosolized virus at various temperatures and relative humidities.

A 24-jet Collison nebulizer was used to aerosolize PRRSV into a dynamic aerosol toroid (DAT) (Goldberg et al. 1958) housed in an atmospheric chamber capable of maintaining temperatures between 4°C to 40°C and relative humidities from 10% to 90%. Air samples were taken over time from the DAT using an AGI-4 all-glass impinger. Rhodamine B aerosolized with the PRRSV served as a tracer to measure physical loss of the virus.

Infectious PRRSV and PRRSV RNA were quantified at each sampling point using TCID₅₀ and PCR assays, respectively. Half-life and associated confidence intervals (Bryan et al., 1990) were estimated for infectious virus and viral RNA at each combination of temperature x relative humidity.

SAMPLING PIG RESPIRATORY EXHALATIONS FOR AEROSOLIZED PATHOGENS

J.R. Hermann¹, K.J. Yoon¹, R.B. Evans¹, S.J. Hoff², J. Zimmerman¹. ¹College of Veterinary Medicine, ²College of Agriculture, Iowa State University, Ames, IA

The long-term goal of our research is to model aerosol transmission of respiratory pathogens. Modeling aerosol transmission requires estimates of: 1) viral shedding in aerosols, 2) stability (half-life) of infectious virus in aerosols, and 3) the probability that exposure to a specific dose will produce infection in a susceptible animal. The objective of the research reported here was to quantify pathogens exhaled by acutely infected individual pigs.

Analytical sensitivity of the air sampling system: To be able to interpret the results of animal sampling, it was necessary to determine the analytical sensitivity of the air sampling system. Ten-fold dilutions of PRRSV were aerosolized using a 24-jet Collison nebulizer. The aerosol cloud was sampled for 5 minutes using each of three impingers (AGI-30, AGI-4, and SKC BioSampler®). The quantity of PRRSV aerosolized by the nebulizer and the quantity collected by the impingers were determined by quantitative RT-PCR and a regression line plotted. The threshold of detection of the AGI-30, AGI-4, and SKC BioSampler® impingers was $1 \times 10^{1.05}$, $1 \times 10^{1.21}$, and $1 \times 10^{0.97}$ TCID₅₀, respectively.

Sampling respiratory exhalations from acutely infected pigs: Respiratory exhalations were captured directly from pigs inoculated with one or more of the following: PRRSV (ATCC VR-2332, MN-184, VR-2385), porcine circovirus type 2 (PCV-2), and *Mycoplasma hyopneumoniae*. Most pigs were sampled on post inoculation days 0, 1, 4, 6, 8, 11, 13, and 15. In addition, buccal or nasal swabs were collected to document the presence of the target pathogen in the upper respiratory tract. Likewise, samples of ambient air were collected and assayed for the presence of airborne target pathogens. PRRSV, PCV-2, and *M. hyopneumoniae* were detected in buccal or nasal swabs. *M. hyopneumoniae* was detected in air samples. If present in respiratory exhalations, PRRSV, PCV-2, and *M. hyopneumoniae* are excreted at levels below the detection threshold of this system.

ORIGIN OF PRRS VIRUS: TOWARDS A BETTER UNDERSTANDING BEYOND THE
EPIDEMIOLOGY

T.Y. Lam, F.C.C. Leung*. Department of Zoology, The University of Hong Kong, Hong Kong Special Administrative Region (HKSAR), China.

Porcine reproductive and respiratory syndrome virus (PRRSV) is categorized into two genotypes: North American-type (NA) and European-type (EU), which correspond to two simultaneous outbreaks in North America (1987) and Europe (1990), respectively. Although the two PRRS outbreaks started almost simultaneously, the sequence similarity of the two genotypes were found to be around 60-70% suggesting that PRRS ancestry virus separated and evolved independently in two continents a long time before the outbreaks. In this study, the time of PRRS ancestor diverged into NA-type and EU-type, and the most recent common ancestors (MRCA) of both genotypes were dated from worldwide ORF2-7 nucleotide sequences (download from Genbank) using Bayesian and traditional inference approaches. The molecular datings were done under the non-clock and clock conditions. The clock-evolving sites of the PRRSV nucleotide sequences were recovered using Site-Stripping Clock Detection (SSCD) procedure, and the SSCD method was compared with the selective pressure analysis as to evaluate different inference properties of different ORF sequences. Based on these data, an origin of all PRRSV has been identified to be in existence since A.D. 1900s. The MRCA of NA-type and EU-type have been dated both at around 1970s, suggesting that the PRRSV entered the domestic pig population at that time. Present results also demonstrate PRRS precursor virus took around 70 years (inferred) to evolve and reach a status leading to outbreaks in domestic pig farms in North America and Europe. Since there were no seropositive detection in domestic pigs before mid-1980s, the hypothesis that intermediate hosts such as wild boars preserving the virus for more than half century before cross-transmitting the PRRSV to domestic population around 15 years before the outbreaks is strengthened by this result.

MOLECULAR EPIDEMIOLOGY OF PRRSV IN HONG KONG

V.Y.Y. Li*, F.C.C. Leung. Department of Zoology, The University of Hong Kong, Hong Kong Special Administrative Region (HKSAR), China

The epidemic history of the European (EU) and North American (NA) genotype of porcine reproductive and respiratory syndrome virus in Hong Kong was studied using phylogenetic analysis and Bayesian approach. The enveloped glycoprotein and nucleocapsid genes were sequenced from 163 new isolates of PRRSV identified and collected in Hong Kong pig farms between 2001 and 2005 for the phylogenetic analysis. Multiple distinct clusters of both genotypes have been identified. Through coalescent-based analysis, we showed that the great variety arose through separate introductions starting in the early to late 1990s. The most possible reason is due to the multiple introductions of infected swine during replacement of gilts and purchasing PRRSV infected frozen semen. Moreover, 17 pig samples in 15 farms were found to be coexisted with both genotypes. Although no new variant was found, it is important to keep close monitoring on the newly evolved variant of both genotypes and pig farms are advised to reinforce bio-security measures to prevent further introduction and generation of new variants in Hong Kong.

PRRS VIRUS CONTROL IN LARGE-SCALE COMMERCIAL SETTINGS:
INCORPORATING SCIENTIFIC INFORMATION INTO HERD-LEVEL
MANAGEMENT DECISIONS.

J.F. Lowe,^{1*} T.L. Goldberg,² F.A. Zuckermann,² L.D. Firkins² ¹The Maschhoffs, Inc.,
Carlyle, Illinois. ²College of Veterinary Medicine, University of Illinois

Over the last decade, practicing veterinarians in their desire to solve clinical issues have used methods for PRRSV control that have not been universally successful and, in some cases, have not been based on principles tested in a controlled scientific manner. In many cases, this has resulted in acceptance of clinical PRRS within production systems and pressure on veterinarians to manage status quo instead of leading control or elimination strategies that would increase economic returns to the industry. The challenges for the practicing veterinarian are to incorporate the available data into their practices, but more importantly, for the scientific community to provide answers to critical questions that continue to challenge the industry.

Current scientific information suggests that there are challenges with control strategies that rely on managing chronically PRRSV infected populations. Current commercial management strategies typically involve exposure to live PRRSV, either MLV or field virus. In chronically infected farms, there are quasi-species of PRRSV circulating both in the herd and within individual animals in the herd (Goldberg, Lowe et al. 2003). In addition, immunity to PRRSV is imperfect; resulting in the ability of pigs to be infected while having an immune response (Allende et al. 2000; Osorio et al. 2002; Lowe et al. 2005). Data from experiments on controlled exposure to PRRSV suggests there is a significant reproductive cost. When gilts were exposed to field PRRSV prior to puberty they had a lower number of pigs born alive per gilt in the breeding cohort compared to those gilts exposed to MLV vaccine at the same age (5.5 pigs/ gilt vs 7.9 pigs per gilt). When comparing these data gilts from the same source cohort that were never exposed to PRRSV there are differences of 3.3 and 0.9 pigs born alive in the first litter per gilt introduced. Together, these data suggest that the cost of managing PRRSV infected populations may be higher than the industry assumes and that management of infection should only be an intermediate step prior to elimination of PRRSV from the herd.

Dee demonstrated that PRRSV can be eliminated from populations with a combination of herd closure followed by internal segregation of infected and naïve populations within the herd (Dee and Joo 1997). We have successfully implemented this technique in more than 12 herds to date, with dramatically improved performance following elimination. As an industry we still are lacking the tools to control infection in endemically infected areas to allow long term elimination of the virus. Even though we have made large strides in understanding how the virus is transmitted between populations, there is still much to learn so that we can develop tools to prevent infection of naïve herds. Maybe more importantly are additional tools to control infection and viral shedding in infected populations prior to elimination strategies. History (i.e. PRV and CSF) tells us that until we have effective methods of building immunity we will have little chance of eliminating the disease from areas with a high density of swine.

MANAGEMENT OF PRRS PERSISTENCE: IDENTIFICATION OF PERSISTENTLY
INFECTED SWINE

R.M. Molina^{1*}, J. Hermann¹, R.R.R. Rowland², J. Christopher-Hennings³, E. Nelson³, J. Lunney⁴, K-J Yoon¹, J. Zimmerman.¹ ¹Iowa State University, Ames, IA. ²Kansas State University, Manhattan, KS. ³South Dakota State University, Brookings SD. ⁴USDA:ARS: BARC, Beltsville, MD

The objectives of this experiment were to (1) establish a better estimate of the proportion of PRRSV carriers over time and (2) identify virological or immunological correlates of persistent infection or immunity for diagnostic use.

109 2-week-old pigs were inoculated with PRRSV ATCC VR-2332; 56 age-matched animals served as uninoculated controls. Infected animals were housed in a single room (648 ft²) until PID 40, then split between two rooms. Serum and whole blood were collected at 2 week intervals through post inoculation day (PID) 202. On approximately the same sampling schedule, subsets of pigs were euthanized and 18 tissues collected for analysis, with the final necropsy on PID 203. Immediately following collections, samples were shipped first priority overnight to collaborating laboratories. PCR results on tonsil samples showed the following pattern of persistent infection:

DPI	7	28	42	56	84	98	112	119	133	147	161	175	189
PCR+	$\frac{3}{3}$	$\frac{3}{3}$	$\frac{3}{3}$	$\frac{3}{3}$	$\frac{2}{2}$	$\frac{3}{3}$	$\frac{1}{3}$	$\frac{5}{10}$	$\frac{6}{10}$	$\frac{3}{10}$	$\frac{3}{10}$	$\frac{2}{10}$	$\frac{1}{10}$
No.	3	3	3	3	2	3	3	10	10	10	10	10	10

Additional results are forthcoming, but persistent infection in a low percentage of the population even beyond 200 DPI appears to be a certainty.

EXPERIMENTAL QUANTIFICATION OF EFFECT OF PRRS VACCINATION ON
PRRSV TRANSMISSION

E. Mondaca-Fernandez*, C. Muñoz-Zanzi, R. Morrison. Dept of Clinical and Population Sciences, College of Veterinary Medicine, University of Minnesota, St. Paul, MN

To know if vaccination can be an option when designing regional PRRSV control or eradication programs, we conducted an experiment where the goal was to test if transmission of a field strain of PRRSV among vaccinated pigs is significantly affected compared to that among non-vaccinated pigs. The rate of transmission of an infectious agent can be expressed as the basic reproduction rate R_0 : the number of secondary cases caused by one infectious animal. The experimental design was a 2x2 factorial design with the factors being vaccinated vs. non-vaccinated recipients and vaccinated vs. non-vaccinated challenged (seeder) pigs. A PRRSV vaccine (Ingelvac[®] PRRS MLV, Boehringer Ingelheim, USA) was administered intramuscularly to fifty, 3-week-old piglets. A field isolate (MN-30100) of PRRSV was used as the challenge virus in this experiment at 10^4 TCID₅₀/ml. The groups of vaccinated recipients had approximately the same number of infected pigs (1/8 and 2/8) compared to the non-vaccinated recipients (0/8 and 1/8). The two rates of transmission were not different (0.598 vs. 0.227; $p > 0.05$). The strain used in the trial - considered a low virulence strain - appeared to have low transmissibility in pig populations, independent of the vaccination status of the pigs ($R_0 < 1$). The low rate of transmission associated with the low virulence raises an important question: is virulence of a particular strain associated with its transmissibility? To answer this question we are conducting a study comparing rates of transmission between strains with diverse levels of virulence. Findings will be presented.

MODELING THE DYNAMICS OF PRRS VIRUS INFECTION WITHIN A HERD:
EARLY DISEASE DETECTION AND EVALUATION OF HERD IMMUNITY

C.A. Muñoz-Zanzi*, A. Rovira. Department of Veterinary Population Medicine, College of Veterinary Medicine, University of Minnesota, St. Paul, MN.

Understanding the dynamics of PRRSV infection within a herd is a critical step in developing measures for control and prevention of PRRS virus infection. The objective of this study was to develop a simulation model of the dynamics of PRRS virus infection within a herd. Specific objectives included the investigation of the relationships between herd immunity and virus transmission and between virus transmission and disease detection. We used mathematical modeling to describe the natural spread of PRRS virus from pig to pig over time (host-level modeling) and the changes in the levels of viremia and PRRS virus antibodies within the infected pigs over time (within-host modeling). Information obtained included: number of pigs becoming infected at any given time, the temporal distribution of viremic pigs and shedding level, and the temporal distribution of pigs with specific levels of immune response after infection. Subsequently, the model was used to identify the conditions under which a herd became immune (transmission was stopped) and to evaluate various diagnostic testing strategies for the cost-effective monitoring of herd disease status.

SUPPRESSION OF PRRS VIRUS INFECTION BY BOAR SEMINAL PLASMA

T. Okinaga*, D.E. Reeves, D.J. Hurley. Department of Population Health, Food Animal Health and Management Program, College of Veterinary Medicine, University of Georgia, Athens, GA

PRRS virus shed in the boar seminal plasma is suspected to be one of the transmission routes for sows. Virus can be transmitted venereally or by artificial insemination (AI). We hypothesized that seminal plasma has an ability to suppress virus infection. Thus, dilution of seminal plasma for AI with semen extender increases the chance of virus infection. The objective of this research is to determine the role of seminal plasma in suppression of PRRS virus infection. PRRS virus was incubated with MARC-145 cells in the presence of boar seminal plasma. PRRS virus production after 4 days of incubation with virus, and PRRS virus infection with 2 hr incubation with virus followed by 16 hr incubation in medium were assessed by detection of virus with SR30 antibodies. Seminal plasma at 1/80 of dilution in culture medium completely suppressed virus production and infection. At 1/320 of dilution, the infection was still suppressed by 40%. Incubation of virus suspension for 1 hr with seminal plasma (1:1 v/v) prior to addition to the cells also decreased the virus infection. Viability of the cells incubated with seminal plasma was confirmed to be same as the cells incubated with medium. We conclude that boar seminal plasma suppresses the infection of PRRS virus to MARC-145 cells. We will further evidence about how the boar seminal plasma suppress the infection. (Support provided by University of Georgia New Faculty Grant and the Food Animal Health and Management Program.)

MANAGEMENT OF PRRS PERSISTENCE: STUDIES AT THE POPULATION LEVEL

R.R.R. Rowland*², R. Molina¹, J. Hermann¹, J. Christopher-Hennings³, E. Nelson³, J. Lunney⁴, V. Leathers⁵, J. Zimmerman¹. ¹Iowa State University. ²Kansas State University. ³South Dakota State University. ⁴USDA:ARS:BARC, ⁵IDEXX Laboratories, Inc.

Studies of PRRSV infection in individual animals do not reflect the outcomes observed in swine production systems. The purpose of this study was to perform an extensive analysis of virus replication and immunity in a population of 109 pigs (and 60 control pigs) over a period of up to 203 days. The objectives were to (1) create a sample resource for the PRRS research community, (2) identify virological/immunological correlates of persistence and clearance, (3) develop a model for persistence at the population level. The “Big Pig” project incorporated a multi-disciplinary, multi-institutional approach including significant support from industry. The study began with the infection of pigs with VR-2332 and ended with the necropsy of the last pigs on August 25, 2005. During the course of the study, over 20,000 samples, including serum and tissues were distributed to five institutions. Analyses performed in-parallel included QT-PCR of serum and tonsil, cytokine gene expression in lymphoid tissues, serology, neutralizing activity, histopathology, immunohistochemistry, and immune cell phenotyping. Several “satellite” projects were also supported. This study illustrates both the spirit and power of collaboration within the NC-229/CAP framework. Current findings and conclusions are presented in a variety of related abstracts.

THE EFFECT OF PRRS VIRUS INFECTION ON GROWTH PERFORMANCE

R.R.R. Rowland*², J. Nietfeld², R. Molina¹, J. Hermann¹, J. Zimmerman¹. ¹Iowa State University, Ames, IA. ²Kansas State University, Manhattan, KS

PRRSV infections broadly impact the swine production system, contributing to a variety of syndromes, such as PRDC and PMWS. One outcome is the appearance of pigs that grow relatively slowly, so-called “poor-doers.” The objective of this study was to evaluate the effect of PRRSV infection in a relatively large population of experimentally infected pigs over a period of 200 days. The study was initiated by infecting 109 2-week-old pigs with VR-2332; 56 age-matched animals served as un-inoculated controls. Pigs were randomly assigned to either infected or control groups. At 2 week intervals, a subset of pigs was randomly removed, weighed, and necropsied. Serology and RT-PCR were used to monitor infection. Serology was performed on sera from control pigs. Results showed that all PRRS pigs were productively infected and that the control groups remained PRRS free throughout the study period. Within about two weeks after infection, a distinct subpopulation of pigs emerged within the infected group. Approximately 15% of pigs appeared to be smaller and had acquired a “rough” appearance. Weights at the time of necropsy showed a high degree of variability in the PRRSV group, but not in controls. For example, at day 133 PID, the range in weights was 242-271 lbs (mean=258, n=10) for the control group versus 150-251 (mean 217, n=5) for the PRRSV group. Microhistology of lymphoid and non-lymphoid tissues, serology, and PCR showed that all pigs were negative for circovirus infection. These results describe a subpopulation of PRRSV-infected pigs that show defects related to growth. This effect was not observed in control pigs and could not be attributed to a secondary infection.

FULL-LENGTH GENOME ANALYSIS OF EUROPEAN-LIKE PRRSV IN THE U.S.:
IDENTIFICATION OF NON-STRUCTURAL PROTEIN REGIONS AS POTENTIAL
EPIDEMIOLOGICAL TOOLS

P. Schneider¹, R.R.R. Rowland¹, J. Mann², B. Neiger², N. Benson², P. Steen², J. Christopher-Hennings², E.A. Nelson², Y. Fang^{2*}. ¹Dept. of Diagnostic Medicine/Pathobiology, Kansas State University, Manhattan, KS and ²Dept. of Veterinary Science, South Dakota State University, Brookings, SD

European-like (Type 1) porcine reproductive and respiratory syndrome virus (PRRSV) isolates have been found circulating in U.S. swine herds since 1999. Phylogenetic analysis using ORF5 nucleotide sequences from 16 U.S. Type 1 isolates from geographically separated swine herds showed that 15 of 16 isolates formed a well-supported monomorphic clade. This clade is further branched into four subgroups. To more fully understand the extent of genetic diversity, full-length sequences of four European-like PRRSV isolates, SD01-07, SD01-08, MO-8257, and SD03-15, representing each of these four subgroups were determined. Comparative analysis with the genomic sequences of European prototypic strain, Lelystad virus (LV) and North American prototypic strain, VR-2332 revealed that SD 01-07 shared 89.6%-97.7% nucleotide identity with that of LV, but only 44.8% -72.8% identity with VR-2332, while SD01-08 shared 90.9%-100% identity with that of LV and 43.9%-72.8% with VR-2332. MO-8257 shared 92.1%-99.3% identity with that of LV and 44.9%-70.8% identity with VR-2332, and SD03-15 shared 90.1%-97.9% identity with that of LV and 44.8%-75.0% identity with VR-2332. Nsp 1 β , nsp2, nsp6, and nsp12 were identified as the most variable non-structural protein regions. In comparison to the phylogenetic analysis of ORF5, which has been used most frequently for PRRSV genetic diversity and evolution studies previously, nsp2 analysis showed similar topology with that of ORF 5 phylogenetic trees. This study developed the largest Type 1 PRRSV full-length genome sequence database currently available and establishes a genetic basis for future studies.

RELATIONSHIPS AMONG IMMUNITY, REPRODUCTIVE PERFORMANCE, AND
VIRAL GENETIC VARIATION IN SWINE INFECTED WITH PRRS VIRUS ON
FARMS

K. Vashisht, J.E. Lowe, R. Husmann, L.D. Firkins, F.A. Zuckermann, T.L. Goldberg.
Department of Veterinary Pathobiology, College of Veterinary Medicine, University of
Illinois, Urbana, IL

This study was a longitudinal investigation of a cohort of pigs on a commercial swine farm in the Midwestern United States and was designed to investigate the relationships between viral genetic changes, host immunity, and reproductive disease. Thirty PRRSV-naïve replacement gilts were exposed to PRRSV (either wild-type virus or modified live vaccine) by intramuscular injection upon their introduction to the farm. Serial clinical samples (blood, serum, and/or tonsil biopsies) were collected until 85 days of the first gestation. Tonsil biopsies were used for RT-PCR testing for viral RNA and genetic characterization of PRRSV (ORF 5 sequences). Ten weeks post-infection, two weeks after pigs had been intermingled with resident non-study pigs, viral RNA was recovered from 6 study pigs and 8 non-study pigs. Two genetic clusters of ORF5 sequences were identified at this time: one cluster closely related to the exposure strain, and one genetically divergent cluster. ORF5 nucleotide sequences in the first cluster (nine pigs; three study animals and six non-study animals) differed from the exposure strain by an average of 10.77% (range: 10.5 – 11.1 %). ORF5 nucleotide sequences in the second cluster (five pigs; three study animals and two non-study animals) differed from the exposure strain by an average of 2.54% (range: 1.0-5.1%). Observed changes in ORF5 were randomly distributed throughout the gene. These results indicate that, of the study animals, three pigs had become re-infected with a co-circulating, genetically divergent viral variant at the time of sampling. Cellular and humoral immune responses were examined in all pigs using ELISPOT and FFN tests, respectively. The mean ELISPOT response of pigs from which no PRRSV was recovered was 205 ± 114 PRRSV-reactive T-lymphocytes/million PBMC. The mean ELISPOT response of the 3 pigs infected with viral variants closely related to the exposure strain was 213 ± 27 . The mean ELISPOT response of the 3 pigs that had become re-infected with genetically distinct viruses was 136 ± 53 . Mean FFN values for the three groups of pigs were 8, 25.4, and 3.18 respectively. These results indicate that animals exposed to one PRRSV strain can become re-exposed to another genetically divergent strain, and that poor immunity to the virus may facilitate re-infection. Finally, regardless of the type of virus used for conditioning, there was a positive correlation ($r = 0.63$) between the number of pigs born alive and the intensity of the virus-specific Inf-gamma response, indicating that cellular immunity provides some protection from clinical disease even for pigs housed in an environment characterized by multiple, co-circulating viral strains.

PROTECTION AGAINST HETEROLOGOUS PRRSV CHALLENGE IN PREGNANT
SOWS IMMUNIZED WITH MULTIVALENT PRRSV VACCINES

M. Wagner^{1*}, B.D. Roggow¹, H.S. Joo². ¹Faimount Veterinary Clinic LLP, Fairmont,
MN, ²University of Minnesota, St. Paul, MN

The purpose of this study was to investigate ability of protection against highly pathogenic PRRSV in 100-day pregnant sows. The sows had been infected with a live PRRSV during their acclimatization and subsequently immunized with multivalent PRRSV protein vaccine. The challenge PRRS virus A and B were isolated from two different farms during acute PRRS outbreaks. In site 1, 4 vaccinated and 4 naïve pregnant sows were inoculated intranasally with challenge virus A. In site 2, 3 vaccinated and 3 naïve pregnant sows were inoculated similarly with challenge virus B. Following the challenge, clinical signs were observed daily and blood samples were collected from sows and piglets. In site 1, 2 vaccinated and 4 naïve sows showed off-feed, and 1 vaccinated and 4 naïve sows aborted. In site 2, 3 vaccinated sows farrowed 29 healthy pigs, while all 3 naïve sows aborted 10-12 days post-inoculation. Mortality of the naïve sows was observed in both sites. These findings indicated that the protection was partial in site 1 but was complete in site 2. The present results suggest that the use of multivalent PRRS virus protein vaccine could be beneficial in reducing clinical losses during PRRS outbreak in commercial swine farms.

GENETIC DIVERGENCE OF ORF 5 DURING ACUTE AND PERSISTENT INFECTION

D. Waldner^{1*}, D. Zeman¹, A. Kasuske¹, S. Ropp¹, K. Fairbanks², E. Nelson¹, D. Benfield³.
¹Department of Veterinary Science, South Dakota State University, Brookings, SD., ²Pfizer
Animal Health, Lincoln, NE and ³Food Animal Health Research Program, OARDC/Ohio
State University, Wooster, OH.

Porcine reproductive and respiratory syndrome virus (PRRSV) is a single-stranded RNA virus with a high degree of genetic diversity. The objective of this study was to investigate the evolution of ORF5 in pigs from 0 to 126 dpi. Two-week old pigs were either mock-inoculated or inoculated with a plaque-purified virus, strain SDSU93-23983 and direct PCR products from serum, lung, palatine tonsil and inguinal lymph node were cloned and sequenced from pigs euthanized at intervals from 6 hpi to 126 dpi. Of 622 clones, the most common change was at aa 33 (21/45 Gly to Ser; 2/45 Gly to Asn). Only two aa changes V²⁹ to A and D³⁰ to N were seen in immunoepitope A and 3 aa changes in epitope B two L⁴¹ and the other at N⁴⁴. N-glycosylation sites were eliminated in three sequences at aa 34, one at aa 44 and 5 new glycosylation sites were added. Results of this study suggest that ORF5 gene continues to evolve from 0 to 126 dpi in infected pigs but the significance of these changes as related to persistence could not be established.

EVOLUTIONARY BIOLOGY OF PRRS VIRUS

K.-J. Yoon^{1*}, S.-H. Cha¹, W.-I. Kim¹, C.-C. Chang³, J. Zimmerman¹, P.M. Dixon².
¹Department of Veterinary Diagnostic and Production Animal Medicine, ²Department of
Statistics, Iowa State University, Ames IA. ³ Department of Veterinary Medicine, College
of Agriculture, Chiayi University, Taiwan

[Abstract not available at time of printing]

SUPPRESSION OF PRRS VIRUS BY MORPHOLINO ANTISENSE OLIGOMERS

Y. Zhang^{1*}, S. Fan¹, A. Kroeker², K. Wang¹, D. Stein², P. Iversen², X.-J. Meng³, D. Matson¹. ¹Center for Pediatric Research, Eastern Virginia Medical School, Norfolk, VA. ²AVI BioPharma, Corvallis, OR. ³Center for Molecular Medicine and Infectious Diseases, Virginia Polytechnic Institute and State University, Blacksburg, VA

Porcine reproductive and respiratory syndrome virus (PRRSV) is a single-stranded RNA virus containing a 15-Kb genome and the causative agent of a contagious disease that has been causing heavy economic losses in the swine industry. During PRRSV replication, a nested set of subgenomic RNAs with 5'- and 3'-coterminal ends are generated in infected cells. In PRRSV genomic sequence, there are high homologous regions among different strains in the same genotype although the PRRSV sequences are variable across strains as are other RNA viruses. In this study, the potential of antisense phosphorodiamidate morpholino oligomers (PMOs) to suppress PRRSV replication in cell culture was assessed. PMOs are analogs of short DNA oligomers with a modified backbone, resulting in highly specific binding and complete resistance to nucleases. Six PMOs with different target regions in the PRRSV genome were synthesized and tested. One PMO compound (PMO-1) was found to be effective in blocking PRRSV replication in cells treated in a dose-dependent manner in comparison with a control PMO. PMO-1 treatment of the cells with PRRSV infection led to reduction of viral yield by up to 4.5 logs. Immunofluorescence assay with anti-PRRSV monoclonal antibodies SDOW17 confirmed the observation. Cell viability assay showed no cytotoxicity of PMO-1, indicating that inhibition of PRRSV replication was due to an anti-viral PMO effect. To demonstrate the sequence specificity of the PMO, the PMO-1 target sequence with up to four point mutations were cloned into a luciferase reporter plasmid. Cell-free translation assay showed that PMO-1 had reduced ability to inhibit the translation of target sequence with several point mutations, while a control PMO had no effect on the target translation, indicating the sequence specificity of the PMO-1 binding. These results suggest potential for PMO-1 to be an effective antiviral for control of PRRSV infection.

**The NC-229 (PRRS) Committee thanks CRWAD
for its support of the International PRRS Symposium**

The Conference of Research Workers in Animal Diseases (CRWAD) was established in 1920. CRWAD is a non-profit organization and has been so since its origin. The sole purpose of CRWAD is to discuss and disseminate the most current research advances in animal diseases. The annual meeting is held each November or December where research scientists from around the world present their recent research in oral or poster presentation formats. The meeting averages 600 in attendance and 325 presentations.

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