RESEARCH REPORT
SWINE HEALTH

Title: Development of Real-time, multiplex PCR/RT-PCR assays for improved PRDC pathogen detection - **NPB #03-114**

Investigator: Steven B. Kleiboeker

Institution: University of Missouri

Date Received: June 28, 2004

Abstract: Porcine respiratory disease complex (PRDC) continues to be a significant economic problem for swine producers. Porcine reproductive and respiratory syndrome virus (PRRSV), Swine influenza virus (SIV), Porcine circovirus-2 (PCV-2), *Mycoplasma hyopneumoniae*, *Actinobacillus pleuropneumoniae*, and *Pasturella multocida* are among the most important pathogens contributing to PRDC. Recently, a new generation of PCR/RT-PCR equipment has been developed. These machines use sophisticated, sensitive detection techniques which allow quantification of the amplification reaction as it occurs. This methodology has been termed “real-time” and/or “quantitative” PCR (for DNA pathogen detection) or RT-PCR (for RNA pathogen detection). Although not yet widely used in veterinary diagnostic laboratories, Real-time PCR/RT-PCR offers several advantages over traditional molecular diagnostic techniques. In this project multiplex real-time PCR or RT-PCR assays were developed and validated for the detection of: 1) PRRSV (both NA and European strains) and SIV; 2) PCV-2 and *M. hyopneumoniae*; and 3) *P. multocida* and *A. pleuropneumoniae*. Comparison of the analytical and diagnostic parameters of these assays to traditional culture and PCR/RT-PCR based approaches demonstrated that the real-time assays were consistently equal or superior. Approaches were investigated to improve the efficiency of sample preparation and assay performance. In addition, an analysis of laboratory-associated costs for real-time PCR/RT-PCR was performed.

Introduction: Porcine respiratory disease complex (PRDC) continues to be a significant economic problem for swine producers, resulting in increased death loss and morbidity, decreased feed efficiency and disruption of pig flow.[2, 4] While a number of pathogens contribute to PRDC,[10, 11, 12] the viral pathogens Porcine reproductive and respiratory syndrome virus (PRRSV), Swine influenza (SIV), and Porcine circovirus 2 (PCV2), as well as the bacterial pathogens *Mycoplasma hyopneumoniae*, *Actinobacillus pleuropneumoniae*, and *Pasturella multocida,* are typically considered among the more important pathogens of this disease complex. In the past, the diagnosis of PRDC pathogens is accomplished by a combination of serology, traditional virus isolation and bacterial culture or individual PCR/RT-PCR assays. An important drawback to traditional culture methods is that they are typically slow, especially with pathogens such as PRRSV, PCV2 or *M. hyopneumoniae*. In addition, culture for some pathogens is very difficult which results in low diagnostic sensitivity. While the use of a molecular approach (PCR/RT-PCR) reduces the turnaround time and may improve sensitivity, the costs involved with running individual assays for a number of PRDC pathogens can be prohibitive. Additionally, to achieve adequate analytical sensitivity many PCR/RT-PCR assays use a "nested" (two-round) amplification strategy which increases the turnaround time and provides the opportunity for cross-contamination between samples and potential false-positive results.

Recently, a new generation of PCR/RT-PCR equipment has been developed. These machines use sophisticated, sensitive detection techniques which allow quantification of the amplification reaction as it occurs. This methodology has been termed "real-time" and/or "quantitative" PCR. Although not yet widely used in veterinary diagnostic laboratories, Real-time PCR/RT-PCR offers several advantages over traditional molecular diagnostic techniques. Advantages include: 1) The analytical sensitivity of Real-time PCR is significantly increased as compared to traditional PCR/RT-PCR; 2) The ability to use probes with different "colors" allows multiplex detection of pathogens without the need to amplify products that are distinctly different in size; 3) Quantitative detection allows accurate estimation of pathogen load which can be developed as prognostic indicators of disease severity and/or progression; 4) Post-amplification analysis of results is automated which reduces the potential for human error in interpretation of results; and 5) Post-amplification sample handling is avoided thus reducing the risk of contaminating the laboratory with the amplification product (which can result in false-positive results).

Objectives: The objectives for this project were three-fold: Specific Objective 1 was to develop and validate multiplex Real-time RT-PCR assays for the detection of North American PRRSV, European PRRSV and SIV; Specific Objective 2 was to develop and validate multiplex Real-time PCR assays for the detection of PCV2, *M. hyopneumoniae*, *P. multocida*, and *A. pleuropneumoniae*; and Specific Objective 3 was to improve the efficiency of Real-time, multiplex detection of PRDC pathogens.

Materials and Methods: The oligonucleotide primers and dual-labeled "Taqman" probes shown in Table 1 were developed and validated in this project. The primers and probes shown for *A. pleuropneumoniae* were previously reported in the literature. All other primer and probe sets are novel and were developed specifically for this project. The dual-labeled probes for North American PRRSV, PCV-2 and *P. multocida* detection were labeled with 6-FAM and BHQ1, the probes for European PRRSV, *M. hyopneumoniae* and *A. pleuropneumoniae* were labeled with HEX and BHQ2, and the probe for SIV was labeled with Cy5 and Iowa Black.

Quantitative (TaqMan) RT-PCR and PCR. Amplification of RNA or DNA from the respective pathogens was performed using the Qiagen QuantiTect Probe RT-PCR kit or the Qiagen QuantiTect Probe PCR kit (Qiagen, Inc., Valencia, CA) with thermocycling and detection performed in a Stratagene Mx4000 (Stratagene, Inc., La Jolla, CA). Samples were analyzed in duplicate or triplicate. For RT-PCR thermocycling conditions were: 50ºC (30 min), 95ºC (15 min), followed by 40 cycles of denaturation (94ºC, 15 s) and annealing/extension (60ºC, 60 s) with fluorescence data collected at the end of the annealing/extension step. For PCR thermocycling conditions were: 95ºC (15 min), followed by 40 cycles of denaturation (94ºC, 15 s) and annealing/extension (60ºC, 60 s) with fluorescence data collected at the end of the annealing/extension step. For each assay the forward and reverse oligonucleotide primers were used at a final concentration of 0.3 µM (each) and the dual-labeled probe was used at a final concentration of 0.2 µM (each). All oligonucleotide primers and dual-labeled probes were synthesized by Integrated DNA Technologies, Inc. (Coralville, IA). Negative control reactions, in which RNA or DNA extracted from normal (unaffected) swine tissues or serum was added as template to the RT-PCR reaction mixture, did not produce a signal for any of the quantitative assays. For each assay, the following samples were tested and found to be negative (except when the specific target pathogen was present): RNA or DNA purified from lung and lymphoid tissue of a PRRSV-seronegative pig were amplified in the multiplex qRT-PCR or qPCR assay and found to be negative. Additionally, RNA and DNA purified from MARC-145 cells, cultured porcine alveolar macrophage cells, PK-15 cells, and characterized stocks of North American and European PRRSV, Swine influenza A virus (H1N1, H1N2, and H3N2 strains), Porcine circovirus type 1, Porcine circovirus type 2, *M. hyopneumoniae*, *P. multocida, A. pleuropneumoniae,* Porcine respiratory coronavirus, Transmissible gastroenteritis virus, Vesicular stomatitis virus (Indiana and New Jersey strains), Porcine parvovirus, Porcine enterovirus, Porcine encephalomyocarditis virus, Bovine viral diarrhea virus types 1 and 2, or *Mycoplasma spp.M. hyopneumoniae*

Table 1. Oligonucleotide primers and probes used for real-time detection of PRRSV, SIV, PCV-2, *M. hyopneumonia, A. pleuropneumoniae, and P. multocida*

Target	5′→3′ Primer sequence (position)	5′→3′ Probe sequence (position)	GenBank Acc #
North American PRRSV	for: ATGATGRGCTGGCATTCT (15,257-15,274) rev: ACACGGTCGCCCTAATTG (15,353-15,370)	TGTGGTGAATGGCACTGATTGACA (15,307-15,330)	U87392
European PRRSV	for: GCACCACCTCACCCAGAC (14,792-14,809) rev: CAGTTCCTGCGCCTTGAT (14,851-14,868)	CCTCTGCTTGCAATCGATCCAGAC (14,819-14,842)	M96262
Swine influenza virus	for: TTGTGTTCACGCTCACCG (210-227) rev: CCATTRAGGGCATTYTGG (265-282)	AGGACTGCAGCGTAGACGCTTTGT (241-264)	AF251406
PCV-2	for: ATTACCAGCGCACTTCGG (768-785) rev: GGGTCCGCTTCTTCCATT (836-853)	AGCAGCAACATGCCCAGCAAGAA G (812-835)	AY288134
*A. pleuro-pneumoniae**	for: AGTGCTTACCGCATGTAGTGGC (202-223) rev: TTGGTGCGGACATATCAACCTTA (224-244)	CGATGAACCCGATGAGCCGCC (224-244)	L06318
M. hyo	for: TGCCGGTGAATGTTTCTG (893-910) rev: CCGGCGAGAAACTGGATA (966-983)	CCGGAATTGATAGCTCAAATTACG AA (928-953)	X67286
P. multocida	for: CCGCAAACATTTCTCGCT (1113-1130) rev: GGCCATTCAGGGGACTCT (1194-1211)	CGTCAGGAAGGAGAAACGTTTACA GG (1138-1163)	X52478

* This assay was previously reported by Angen et al., 2001

VI. Results:

<u>Specific Objective 1</u> – Develop and validate multiplex Real-time RT-PCR assays for the detection of North American PRRSV, European PRRSV and SIV

Novel real-time RT-PCR assays have been developed for NA PRRSV, EU PRRSV and SIV and fully validated. Heterologous competitor RNA has been synthesized for each of these three assays, and this RNA is used both as a positive control and for generation of standard curves. As a result of collaborations in the PI's laboratory, the North American PRRSV assay is also currently being independently evaluated by two additional laboratories, which have both reported excellent assay performance on a variety of research and clinical samples. All three assays have been combined into a multiplex assay. Most importantly, all three pathogens can be detected simultaneously and the sensitivity of detection and quantification of viral RNA is not significantly reduced in the multiplex format.

Quantitative RT-PCR (qRT-PCR) assay sensitivity and specificity. Oligonucleotide primers and probes were selected from conserved regions of the PRRSV (NA and EU) and the SIV genomes based on nucleotide alignments, generated by MegAlign (version 5.01; DNAStar Inc., Madison, WI) of all available sequences in the public domain databases (data not shown). For each oligonucleotide primer and dual-labeled probe, a maximum of a single base mismatch in any individual sequence was identified by nucleotide alignment. None of these mismatches were within 3 bases of the 3′ end of the oligonucleotide primers. The NA PRRSV forward primer contained a degenerate base (a mixture of A and G) since both nucleotides were commonly present in available sequence data. For NA PRRSV, the region amplified is from the 3′ end of the nucleocapsid gene (ORF 7) and the 3′ untranslated region of the genome. For EU PRRSV, the region amplified is from the nucleocapsid gene (ORF 7). For SIV, the region amplified is from the M protein gene.

To assess the sensitivity of the qRT-PCR assays, serial ten-fold dilutions of virus stocks of NA PRRSV, EU PRRSV and SIV were analyzed in parallel by virus isolation, qRT-PCR and nested RT-PCR (nRT-PCR) (Table 2). Nested RT-PCR was only performed for NA PRRSV isolates. For this analysis qRT-PCR was performed in a multiplex format, with NA and EU PRRSV plus the SIV oligonucleotide primer sets and dual-labeled probes present in each reaction mixture. Virus isolation detected virus at concentrations of 1 and 10 $TCID_{50}$/0.1 ml for the NA and EU PRRSV viral stocks, respectively. For both strains of PRRSV, qRT-PCR reproducibly detected viral RNA at 100-fold lower concentrations than virus isolation, and for SIV qRT-PCR reproducibly detected viral RNA at 10-fold lower concentrations than virus isolation. Nested RT-PCR for NA PRRSV detected viral RNA to the same level as qRT-PCR.

Field isolates of PRRSV, collected between 1992 and 2003, were used to assess the ability of the multiplex qRT-PCR assay to detect a broad range of PRRSV strains. For this analysis qRT-PCR was performed in a multiplex format. For the NA PRRSV assay, a total of 74 field strains that were detected by virus isolation were also positive by qRT-PCR assay. North American PRRSV isolates tested included the prototype strain VR-2332, as well as its attenuated vaccine derivative IngelVac MLV, and the IngelVac ATP vaccine strain. For the EU PRRSV assay, a total of nine field strains that were detected by virus isolation were positive by the quantitative RT-PCR assay. European PRRSV strains tested included the prototype Lelystad isolate plus eight U.S. field isolates of European PRRSV. For both NA and EU PRRSV isolates, all field and vaccine strains identified by virus isolation in MARC-145 or PAM culture were also

found to be positive by either the NA or European PRRSV quantitative assay, giving both assays an analytical sensitivity of 100% compared to virus isolation. Field isolates of SIV, collected between 1988 and 2003, were used to assess the ability of the multiplex qRT-PCR assay to detect a broad range of SIV strains. A total of 22 SIV strains (Table 3) were tested using the multiplex qRT-PCR assay and all were found to be positive. It is important to note that H1N1, H3N2 and H1N2 were all detected by this assay.

To compare the diagnostic performance of the NA PRRSV qRT-PCR assay to traditional nRT-PCR, 149 RNA samples purified from a combination of virus isolates, swine serum, or swine tissue samples were tested in parallel by both qRT-PCR (containing oligonucleotide primers and probes for NA and EU PRRSV plus SIV) and nRT-PCR for NA PRRSV. The diagnostic sensitivity of the qRT-PCR was found to be 100% and the specificity was found to be 97.5% compared to nRT-PCR. For the two samples that were positive by qRT-PCR yet negative by nRT-PCR, a second independent RNA extraction was performed and found to be positive when retested by qRT-PCR for NA PRRSV but not by nRT-PCR, indicating that these results may be true positives rather than false positives and thus that qRT-PCR may be somewhat more sensitive than nRT-PCR. All 149 RNA samples tested for this data set were found to be negative for the EU PRRSV qRT-PCR assay.

Diagnostic performance of the NA PRRSV qRT-PCR assay was also compared to nRT-PCR and virus isolation using samples collected following experimental inoculation of 14 day-old piglets seronegative for PRRSV with one of two virulent strains of NA PRRSV (Table 4). Serum collected from all control (sham-inoculated) piglets at days 1, 2 and 3 post-inoculation (p.i.) were negative by virus isolation, qRT-PCR (containing oligonucleotide primers and probes for both NA and European PRRSV) and nRT-PCR for NA PRRSV. For inoculated piglets, viral RNA was detected in serum at 1, 2 and 3 days p.i. Virus isolation for PRRSV was also positive as early as 1 day p.i. The diagnostic

Table 2. Diagnostic sensitivity comparison of virus isolation, real-time RT-PCR and nested RT-PCR

North American PRRSV				European PRRSV			Swine influenza virus		
$TCID_{50}$/ 0.1 ml	Virus isol.	Real-time RT-PCR (Ct)	Nested RT-PCR	$TCID_{50}$/ 0.1 ml	Virus isol.	Real-time RT-PCR (Ct)	$TCID_{50}$/ 0.1 ml	Virus isol.	Real-time RT-PCR (Ct)
1000	Pos	17.7	Pos	1000	Pos	25.4	1000	Pos	23.1
100	Pos	20.9	Pos	100	Pos	29.1	100	Pos	26.7
10	Pos	24.7	Pos	10	Pos	32.6	10	Pos	30.3
1	Pos	28.8	Pos	1	Neg	35.9	1	Pos	34.9
0.1	Neg	31.3	Pos	0.1	Neg	38.9	0.1	Neg	39.7
0.01	Neg	35.2	Pos	0.01	Neg	Neg	0.01	Neg	Neg
0.001	Neg	Neg	Neg	0.001	Neg	Neg	0.001	Neg	Neg

Table 3. Swine Influenza A virus isolates tested by multiplex, real-time RT-PCR

SIV strain[a]	Type	C_T for multiplex qRT-PCR assay
A/Swine/Indiana/1726/88	H1N1	27.05
A/Swine/Wisconsin/125/97	H1N1	25.50
A/Swine/Wisconsin/235/97	H1N1	24.22
A/Swine/Wisconsin/238/97	H1N1	23.62
A/Swine/Wisconsin/458/98	H1N1	23.09
A/Swine/Wisconsin/R5E/01	H3N2	24.99
A/Swine/Wisconsin/R5I/01	H3N2	25.09
A/Swine/Wisconsin/R6E/01	H3N2	19.16
A/Swine/Wisconsin/R6H/01	H3N2	25.40
A/Swine/Wisconsin/R7C/01	H3N2	25.10
A/Swine/Wisconsin/RP5G/01	H3N2	23.39
A/Swine/Wisconsin/R30G/01	H1N2	22.34
A/Swine/Wisconsin/R33F/01	H1N2	26.47
A/Swine/Wisconsin/R36C/01	H1N2	23.08
A/Swine/Wisconsin/R46F/01	H1N2	36.14
A/Swine/Wisconsin/H02NJ56371/02	H1N1	24.04
A/Swine/Wisconsin/H02PW7/02	H1N1	22.81
A/Swine/Wisconsin/H02AS8/02	H3N2	23.81
A/Swine/Wisconsin/H03HB4/03	H3N2	24.04
A/Swine/Wisconsin/H03G1/03	H1N1	21.15
A/Swine/Wisconsin/H03LS4/03	H1N1	22.45
A/Swine/Wisconsin/H03HS5/03	H1N1	25.94

[a]Supplied as a gift from Dr. Chris Olsen, Univ. of Wisconsin

Table 4. Serum virus titration, quantitative RT-PCR and nested RT-PCR results following inoculation of piglets with North American PRRSV strains

			dpi 1			dpi 2			dpi 3		
group	treatment	Animal no.	titration result[a]	qRT-PCR[b]	nRT-PCR	Titration result	qRT-PCR	nRT-PCR	Titration result	qRT-PCR	nRT-PCR
1	control (uninoc.)	1	0	neg	neg	0	0	neg	0	0	neg
		2	0	neg	neg	0	0	neg	0	0	neg
		3	0	neg	neg	0	0	neg	0	0	neg
		4	0	neg	neg	0	0	neg	0	0	neg
		5	0	neg	neg	0	0	neg	0	0	neg
		6	0	neg	neg	0	0	neg	0	0	neg
		7	0	neg	neg	0	0	neg	0	0	neg
		8	0	neg	neg	0	0	neg	0	0	neg
2	inoc (isol 1)	9	0	neg	neg	2.5	4.76	POS	3.5	5.27	POS
		10	0	neg	neg	2.75	5.21	POS	2.5	4.53	POS
		11	0	neg	neg	2.5	ND	ND	2.25	4.19	POS
		12	0	neg	neg	2.75	4.05	POS	2.5	4.73	POS
		13	0[d]	2.5	POS	3.5	5.54	POS	2.25	4.86	POS
		14	0[d]	2.17	POS	3	6.05	POS	3.5	6.44	POS
3	inoc (isol 2)	15	3.75	4.7	POS	4	5.78	POS	4.25	ND[c]	ND
		16	3	4.15	POS	3	5.19	POS	3.5	ND	ND
		17	1.75	3.27	POS	3.25	5.27	POS	3.75	5.78	POS
		18	0	neg	neg	3.25	5.23	POS	4.25	ND	ND
		19	3	4.41	POS	3	5.78	POS	4	6.12	POS
		20	2.75	4	POS	4.5	6.07	POS	4.25	ND	ND
		21	0[d]	3.63	POS	3.25	5.73	POS	3.5	5.57	POS

[a]Expressed as Log_{10} $TCID_{50}$/ml with titrations performed on MARC-145 cells
[b]Expressed as Log_{10} viral RNA copies/ml
[c]Not determined
[d]Virus isolation positive upon secondary passage on fresh MARC-145 cells

sensitivity of qRT-PCR was found to be 100% and the specificity was found to be 96.6% compared to virus isolation (Table 3). For the single sample that was positive by qRT-PCR yet negative by virus isolation, a second independent RNA extraction was performed and found to be positive when retested by both qRT-PCR and nRT-PCR, indicating that these results may be true positives rather than false positives. When comparing NA PRRSV qRT-PCR and nRT-PCR for these samples, there was 100% agreement of results.

Simultaneous detection of RNA from NA PRRSV, EU PRRSV, and SIV

The ability to detect NA or EU PRRSV RNA in a multiplex assay format was assessed by comparing amplification curves of reactions performed with either a single set of oligonucleotide primers and probe (i.e. single reaction) or two sets of oligonucleotide primers and dual-labeled probes (i.e. multiplex reaction). RNA purified from serial ten-fold dilutions of viral stocks was amplified for NA PRRSV, EU PRRSV, and SIV (FIG. 1A, 1B, and 1C respectively). Representative experiments are shown for samples run in triplicate. Replicate experiments gave equivalent results. In general overlapping amplification curves were produced and C_T values did not change. However, for the final two dilutions of European PRRSV, C_T values for the multiplex reaction were increased by a value of ≤1 cycle compared to the C_T values for the single reaction and the final three dilutions of SIV had C_T decreases of approximately 1-2.

The ability to simultaneously detect two viral RNA targets was assessed by performing multiplex reactions with relatively high concentrations of one target viral RNA in reaction mixtures containing approximately 5, 50, 500, or 5000-fold lower concentrations of the second viral RNA target (data not shown). In the reaction mixtures containing high levels of NA PRRSV RNA and decreasing concentrations of EU PRRSV RNA, the C_T values generated with simultaneous detection of two viral RNA targets were increased ≤1 cycle and the amplitude of the amplification curves was slightly reduced compared to reactions which did not contain an excess of NA PRRSV. For the reaction mixtures containing high levels of EU PRRSV RNA and decreasing concentrations of NA PRRSV RNA, the C_T values generated for simultaneous detection were only increased at the final dilution of NA PRRSV viral RNA although slight decreases in the amplitude of the curves were observed for each dilution.

<u>Specific Objective 2</u> – Develop and validate multiplex Real-time PCR assays for the detection of PCV2, *M. hyopneumoniae*, *P. multocida*, and *A. pleuropneumoniae*.

The individual published real-time assay for *A. pleuropneumoniae* (as referenced in the original proposal) has been established in this laboratory and found to be acceptable. A compatible novel assay was developed for *P. multocida.* Novel assays were also developed for PCV-2 and *M. hyopneumoniae.* These assays are in the final stages of validation. The diagnostic performance of the PCV-2 and *M. hyopneumoniae* multiplex qPCR assay and the *P. multocida* and *A. pleuropneumoniae* multiplex qPCR assay was assessed by comparing the results of the qPCR assays to those of traditional single PCR assays. Known (sequenced) positives from previous diagnostic laboratory submissions for each of these pathogens (detected by either traditional PCR or culture methods) were first re-tested by the multiplex qPCR assays. The number of DNA samples tested were 14 for PCV-2, 11 for *M. hyopneumoniae*, 22 for *P. multocida* and 9 for *A. pleuropneumoniae*. All were found to be positive for the respective pathogen by the qPCR multiplex assay. Next, 7 serial ten-fold dilutions were performed for 2 isolates for each pathogen and tested by both traditional PCR and the multiplex

qPCR assay. For each isolate tested the multiplex qPCR had a positive result (defined as $C_T \leq 35$) at every dilution that was positive with the traditional PCR assay. Additionally, in 3 out of 8 of the serial dilutions, the qPCR assay was positive at a one additional dilution of the isolate, indicating greater

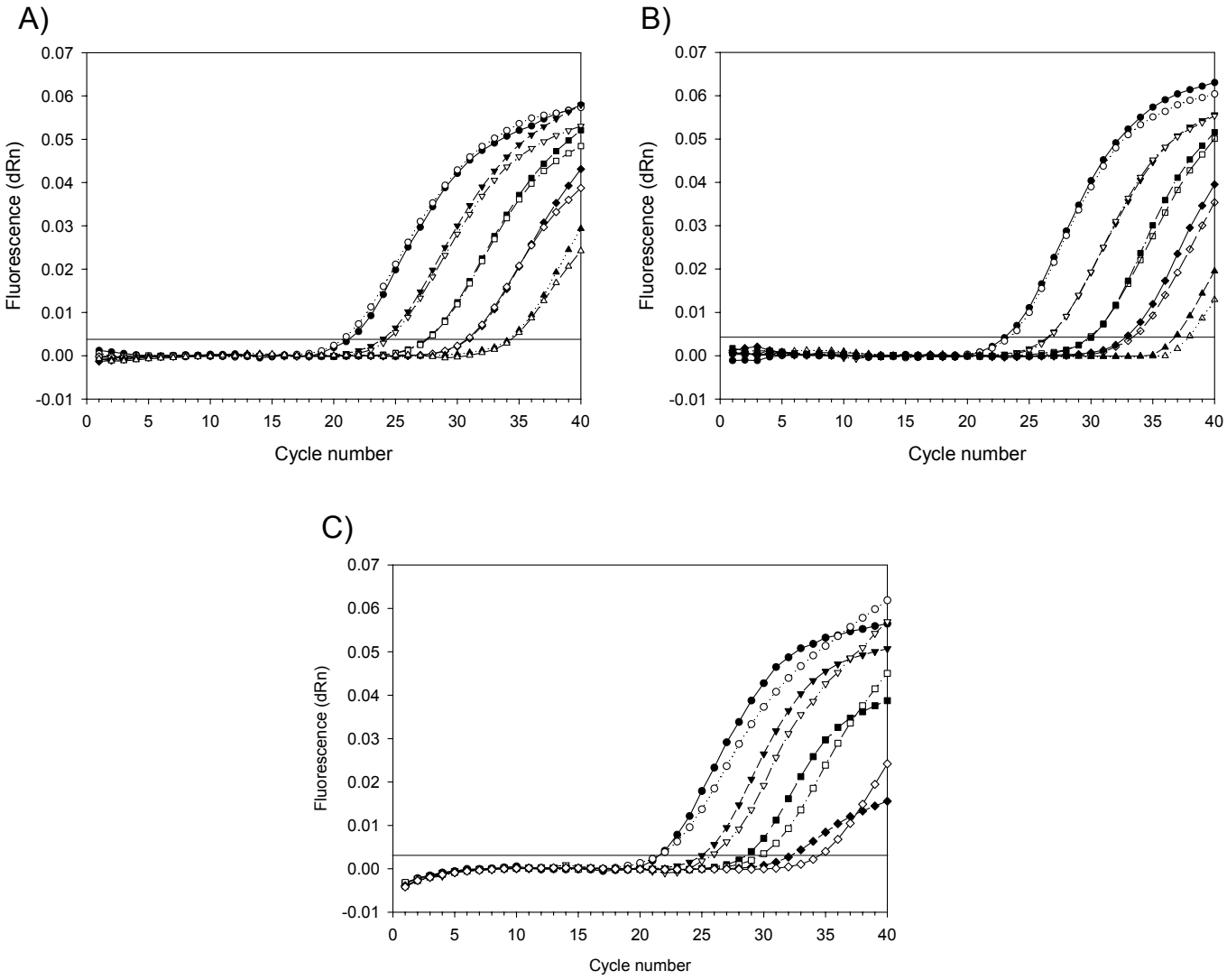

FIG. 1. Individual and multiplex quantitative (TaqMan) RT-PCR amplification of North American or European PRRSV. Serial ten-fold dilutions of viral stocks derived from field isolates were amplified either as individual reactions (closed symbols), containing a single oligonucleotide primer set and dual-labeled probe, or as multiplex reactions (open symbols), containing three sets of oligonucleotide primers and dual-labeled probes for detection of NA PRRSV, European PRRSV and SIV RNA. Amplification plots of RNA purified from undiluted (circles), 1:10 (inverted triangles), 1:100 (squares), 1:1,000 (diamonds) or 1:10,000 (triangles) dilutions of a North American PRRSV isolate (A) or a European PRRSV isolate (B) or an SIV isolate (C) are shown. Symbols represent means of triplicate values. The horizontal line at approximately 0.004 fluorescence units (dRn) indicates the threshold for a positive reaction. dRn, baseline-corrected normalized fluorescence.

analytical sensitivity for the qPCR assay compared to the traditional PCR assay. For the PCV2 and *M. hyopneumoniae* multiplex and the *P. multocida*, and *A. pleuropneumoniae* multiplex, field isolates have not yet been tested.

Simultaneous detection of PCV-2 and *M. hyopneumoniae* in a multiplex format was assessed by comparing amplification curves of reactions performed with either a single set of oligonucleotide primers and probe (i.e. single reaction) or two sets of oligonucleotide primers and dual-labeled probes (i.e. multiplex reaction). In general overlapping amplification curves were produced and C_T values did not change. However, for the final two dilutions of PCV-2, C_T values for the multiplex reaction were increased by a value of ~1 cycle compared to the C_T values for the single reaction.

<u>Specific Objective 3</u> – Improve the efficiency of Real-time, multiplex detection of PRDC pathogens

For this objective, the use of two standard equipment items has been evaluated and found to improve the efficiency of pathogen detection. First, the use of a vacuum manifold for routine sample processing has reduced the time required for extraction of 24 samples from approximately 1 hour to 25 minutes. In addition to reducing the time required, use of a vacuum manifold greatly reduces the need to transfer tubes from racks to a centrifuge rotor and consequently makes the task of sample extraction less tedious. The use of 96-well extraction plates was also evaluated and found to greatly improve the speed of sample extraction. However, serum or fluid samples were the only sample types that were acceptable for the 96-well plate format. Other samples, such as tissue or semen, routinely blocked the flow of liquid through the silica matrix filter which in turn caused excessive delays in sample processing in addition to increasing the cost due to the need for re-extraction of individual samples. In addition to improvement of sample extraction techniques, the use of repeating electronic pipettes has improved the efficiency of pathogen detection. Specifically, electronic pipettes have been most useful in aliquoting the final reaction mix into the PCR tubes. It is important to note that incorporation of a vacuum manifold, 96-well extraction plates, and an electronic pipette can be accomplished for a one-time cost of approximately $1000 (depending on supplier of equipment).

A cost analysis (not including equipment purchases) has been performed. We have demonstrated no decrease in analytical sensitivity (or any other adverse effects) by using a final reaction volume of 20 µl (slightly less than a “half-reaction”) as opposed to the 50 µl reaction recommended in the manufacturer’s instructions (data not shown). Using a 20 µl final volume reduces the “per replicate” cost from $2.27 to $0.90 for real-time RT-PCR and from $1.79 to $0.71 for real-time PCR (not including reactions in each group of samples for negative and positive controls). Given that the “per reaction” costs of oligonucleotide primers and dual-labeled fluorescent probes is less than $0.20 per amplification target, multiplex detection of pathogens does not contribute significantly to the cost of analysis. The costs for RNA sample extraction are $3.30 and for DNA extraction are $1.90, including the cost of extraction reagents and labor costs of $15/h. Thus, the reagent and labor costs for an RNA sample extracted a single time and run in duplicate is approximately $5.50 and for a DNA sample extracted a single time and run in duplicate is approximately $2.61. Duplicate extractions followed by real-time amplification of each extraction increases the cost to $8.80/sample for RNA samples and $5.25/sample for DNA samples.

Discussion: Simultaneous, sensitive detection of a broad range of pathogens that have been implicated in PRDC will provide an important management tool for swine

producers. While the diagnostic performance of these assays is very important, considerations such as turnaround time and cost to the producers must also be optimized to provide maximum benefit to the industry. Real-time PCR/RT-PCR offers many advantages in terms of sensitivity and improved diagnostic accuracy (mainly through decreased false positive results) when compared to traditional PCR/RT-PCR. In addition, it is anticipated that use of Real-time PCR/RT-PCR will improve the efficiency of diagnostic efforts and thus decrease the cost of these assays to producers. As the new technology of Real-time PCR is adopted by veterinary diagnostic laboratories, it is important to maximize the benefits of this approach through a sound, scientific approach to assay development. Accurate, affordable, and rapid detection of PRDC pathogens will greatly assist swine veterinarians and producers in their efforts to control this economically important disease complex.

Lay interpretation: A number of pathogens (both viruses and bacteria) cause respiratory disease in swine. Often the clinical signs of disease are similar for infected pigs. Therefore to accurately diagnose the cause, multiple tests must be performed on each sample. The ability to simultaneously detect the most important PRDC pathogens in three assays improves the efficiency and reduces the cost of diagnostic testing. In this project, three "multiplex" assays were developed for the detection of 1) PRRSV (both NA and EU strains) and SIV; 2) PCV-2 and *M. hyopneumoniae*; and 3) *P. multocida* and *A. pleuropneumoniae*. These assays were based on state-of-the-art "real-time" PCR methods that allow detection of the pathogen at the same time that quantification of pathogen loads is made. It was demonstrated that these assays had diagnostic sensitivity equal to or exceeding standard PCR methods and culture methods. Use of vacuum "manifolds" for sample extraction and electronic repeating pipettors was shown to improve the efficiency of sample preparation and analysis without effecting diagnostic sensitivity and accuracy. The actual laboratory-associated costs of performing an individual assay (with a single sample preparation and real-time RT-PCR performed in duplicate) was calculated to be $5.50 for RT-PCR and $2.61 for PCR, not including assessment of positive and negative control reactions.

References:

1. Angen O, J Jensen, and D Lavritsen. 2001. *J Clin Microbiol.* 39:260-265.
2. Dee, SA. 1996. *Swine Health and Prod.* 4:147-149.
3. Egli, C, B Thur, L Liu, and MA Hofmann. 2001. *J Virol Methods.* 98:63-75.
4. Halbur PG. 1996. Proc Natl Pork Prod Council Pork Summit, Des Moines, IA.
5. Kamp EM, Bokken GC, Vermeulen TM, de Jong MF, Buys HE, Reek FH, Smits MA. 1996. *J Vet Diagn Invest.* 8:304-309.
6. Lichtensteiger CA, Steenbergen SM, Lee RM, Polson DD, Vimr ER. *J Clin Microbiol.* 1996 34:3035-3039.
7. Mattsson JG, Bergstrom K, Wallgren P, Johansson KE. 1995. *J Clin Microbiol.* 33(4):893-7.
8. Pijoan C. and Molitor, T. 1992. Proceedings of International Pig Veterinary Society.
9. Rovira A, M Balasch, J Segales, et al. 2002. *J Virol.* 76:3232-3239.
10. Thacker EL. 2001. *Vet Clin North Am Food An Pract.* 17:551-565.
11. Thacker EL, Thacker BJ, and Janke BH. 2001. *J Clin Microbiol.* 39:2525-2530.
12. Thacker EL, Halbur PG, Ross RF, Thanawongnuwech R, Thacker BJ. 1999. *J Clin Microbiol.* 37:620-627.
13. van Elden, LJR, M Nijhuis, P Schipper, et al. 2001. *J Clin Microbiol*. 39:196-200.