

Figure 1 Diagram of the Open Reading Frame 5 (ORF5) region of the PRRSV genome, the location of the oligonucleotide primers and the length of the amplified products. F, forward primer, NR, non-nested reverse primer, SR, semi-nested reverse primer.

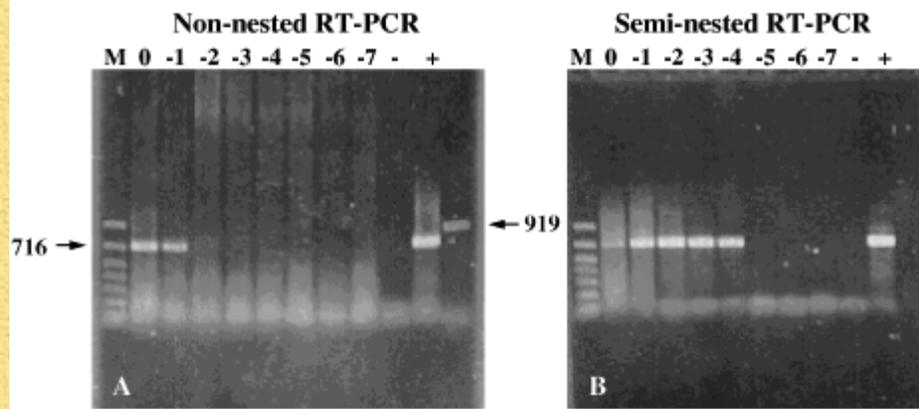


Figure 2. Sensitivity of A) non-nested RT-PCR, and B) semi-nested RT-PCR amplification of total RNA isolated from PRRSV infected Marc-145 cells serially diluted into noninfected cells (See Table 1 for details of dilutions) M, marker; - no RNA control; +, PRRSV genomic RNA control; Panel A, lane 12, product of semi-nested RT-PCR first round amplification.

Table 1
Sensitivity of non-nested vs semi-nested RT-PCR

	Undiluted	10 ⁻¹	10 ⁻²	10 ⁻³	10 ⁻⁴	10 ⁻⁵
Non-nested	+	+	+/-	-	-	-
Semi-nested	+	+	+	+	+	-
Total Cells	120,000	120,000	120,000	120,000	120,000	120,000
Infected Cells	60,000	6,000	600	60	6	0.6
Infectious Units	2,800,000	280,000	28,000	2,800	280	28

The undiluted Marc-145 cell population was determined to be 50% infected with PRRSV by assaying a replicate culture with an Indirect Fluorescence Antibody (IFA) test. The undiluted cells were serially diluted 1:10 into 300,000 uninfected cells. Total RNA was isolated from 300,000 Marc-145 cells for each dilution. 1µg RNA was used for each RT-PCR reaction, equivalent to ~40% of the total RNA isolated, or 120,000 cells.

1 CCID₅₀/ml = 0.7 infectious units

Table 2
Comparison of methods for detecting PRRSV in
alveolar macrophages from lung lavage fluid.

Non-nested RT-PCR	Semi-nested RT-PCR	Virus Isolation
3/8	7/8	8/8
nd	10/12	12/12

A total of 20 samples were tested. Eight of these were tested by all three methods. The remaining 12 were tested by semi-nested RT-PCR and virus isolation only. Fractions represent the number of positive results over the total number of samples tested by the indicated procedure. nd = not done.

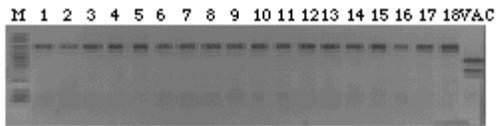


Figure 3. Differentiation between samples from pigs infected with wild type or vaccine virus. *M*HuI restriction enzyme digest of final 716 bp RT-PCR product. *M*, marker; #1-18 wildtype virus; vac, vaccine virus.

Detection of Porcine Reproductive and Respiratory Syndrome Virus (PRRSV) in Alveolar Macrophage of Infected Pigs by Semi-Nested Set RT-PCR

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Abstract:

A semi-nested set RT-PCR was developed to increase the sensitivity of a current RT-PCR method used in RFLP for the differentiation of porcine reproductive and respiratory syndrome virus (PRRSV) isolates (Wesley et al., Proc. AASP, 1996). An additional primer was designed outside of one of the original primers which amplify ORF 5. The sensitivity of the semi-nested set PCR was determined using infected MARC-145 cells serially diluted 1:10 into uninfected cells prior to RNA purification. Six infected cells out of 120,000 total cells was sufficient for detecting PRRSV by this method. This is equivalent to 280 infectious units. This is 1000-fold more sensitive than the non-nested RT-PCR method. The semi-nested set RT-PCR method was able to directly detect PRRSV infected alveolar macrophages isolated from both experimentally and naturally infected pigs, thereby eliminating the need for cell culture replication prior to detection. Several of the infected alveolar macrophage samples detected by semi-nested RT-PCR were PRRSV negative by the non-nested RT-PCR method. With few exceptions the presence of PRRSV was confirmed by virus isolation from lavage fluid.

Introduction:

Porcine Respiratory and Reproductive Syndrome Virus (PRRSV) is an enveloped, single-stranded, positive sense RNA virus tentatively classified in the arteriviridae family. Typical PRRSV infections are characterized by reproductive failure and respiratory distress in pigs of all ages, which is most severe in neo-natal and nursery-age pigs.

A RT-PCR method coupled with a RFLP assay has been developed for the differentiation of PRRSV strains (Wesley et al., Proc. AASP, 1996). This technique is useful in studying the epidemiology of PRRS in swine herds. It is also being used to differentiate between a current modified-live-virus vaccine, RespPRRS/Repro (distributed by NOBL Laboratoris, Inc), and wild-type virus in the field.

Previous attempts to circumvent the time consuming cell culture amplification of PRRSV by directly assaying alveolar macrophages from infected pigs have been unsuccessful. To improve the sensitivity we have developed a semi-nested RT-PCR method. The data presented here is a comparison of the sensitivity of non-nested and semi-nested RT-PCR using serially diluted PRRSV infected cells and alveolar macrophages from PRRSV infected pigs.

Materials and Methods:

RNA EXTRACTION

Total cellular RNA was extracted from PRRSV-infected MARC-145 cells and alveolar macrophages with a modified version of the single-step RNA isolation method (Chomczynski and Sacchi, *Anal. Biochem.* 162, 156,1987) using Trizol Reagent(Registered Trademark) (GIBCO BRL Life Technologies, Inc.), and resuspended in 25 ul TE (10 mM Tris-HCl [pH 8], 1 mM EDTA, made with nuclease-free water (AMRESCO)).

NON-NESTED RT-PCR

1 ug of RNA was mixed with a prepared RT-PCR reaction buffer containing 1x PCR buffer (10 mM Tris-HCl [pH 8.3], 50 mM KCl, 1.5 mM MgCl); 0.2 mM (each) dGTP, dATP, dCTP, and dTTP; 50 pmol each of the primers F and NR; 1.25 U of *Taq* DNA polymerase; 10 U Rnasin; and 2.5 U Reverse Transcriptase. The tubes were incubated at 56C for 1 hr, then 95C for 5 min, then 25 amplification cycles, each cycle consisting of 10 sec at 94C, 30 sec at 55C, and 30 sec at 72C. The amplification was followed by a final elongation step of 10 min at 72C.

SEMI-NESTED RT-PCR

First amplification round:

The first amplification was identical to the non-nested RT-PCR with the exception of the primers and cycling temperatures: Primers F and SR; 25 amplification cycles consisting of 10 sec at 94C, 30 sec at 50C, and 30 sec at 72C.

Dilutions:

After amplification the PCR products were diluted 1:100 into TE and 10 ul of each dilution was used for the second amplification round.

Second amplification round:

10 ul of each diluted PCR product from the first amplification was mixed with a prepared PCR reaction buffer containing 1x PCR buffer (10 mM Tris-HCl [pH 8.3], 50 mM KCl, 1.5 mM MgCl), 0.2 mM (each) dGTP, dATP, dCTP, and dTTP, 50 pmol each of the primers F and NR, and 1.25 U of *Taq*. Semi-nested RT-PCR is approximately 1000-fold more sensitive than non-nested RT-PCR when compared using PRRSV infected Marc-145 cells serially diluted into a constant number of non-infected Marc-145 cells. Semi-nested RT-PCR is also more sensitive than non-nested RT-PCR when alveolar macrophages from pig lung lavage fluid are assayed directly.

The increased sensitivity provided by semi-nested RT-PCR enables us to circumvent the time consuming steps of in vitro virus amplification prior to analysis by allowing us to directly assay alveolar macrophages from PRRSV infected pigs.

DNA polymerase. The tubes were amplified for 35 cycles, each cycle consisting of 10 sec at 94C, 30 sec at 55C, and 30 sec at 72C. The amplification was followed by a final elongation step of 10 min at 72C.

Discussion and Conclusions:

Semi-nested RT-PCR is approximately 1000-fold more sensitive than non-nested RT-PCR when compared using PRRSV infected Marc-145 cells serially diluted into a constant number of non-infected Marc-145 cells. Semi-nested RT-PCR is also more sensitive than non-nested RT-PCR when alveolar macrophages from pig lung lavage fluid are assayed directly.

The increased sensitivity provided by semi-nested RT-PCR enables us to circumvent the time consuming steps of in vitro virus amplification prior to analysis by allowing us to directly assay alveolar macrophages from PRRSV infected pigs.

Questions and Answers

Q:What are the sequences of your RT-PCR primers?

A: The primer sequences have not been released. They will be released after final patent approval and commercial licensing is obtained.

Q: Is your semi-nested RT-PCR technique sensitive enough to detect virus in nasal swabs from infected pigs?

A: I have not yet tried to use the semi-nested RT-PCR technique to detect PRRSV in nasal swabs. In general, people have found that nasal swabs are not as reliable as serum samples or lung lavage fluid in detecting PRRSV by virus isolation. It would be interesting to see how these samples compare by the semi-nested RT-PCR method.