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Development of Real-Time PCR Methods for the Detection of CD163 and Porcine Reproductive and Respiratory Syndrome Virus *N* Genes in Marc-145 Cell

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Abstract: The objective of this study was to develop an RNA-dependent real-time reverse-transcriptase PCR (real-time RT-PCR) Method for the detection of relative levels of CD163 and Porcine Reproductive and Respiratory Syndrome Virus (PRRSV) *N* genes in Marc-145 cells. Primers were designed based on the sequence of highly conservative region of *PRRSV N*, *CD163* and *β-actin* gene as the reference gene. The specificity and sensitivity of real-time RT-PCR Method were determined with the amplification signals Tm peaks in positive controls and the standard curve generated from *PRRSV N* gene positive plasmid and total RNA dilution end-point standard curve from Marc-145, respectively. The minimum detection levels for *PRRSV N* gene, *CD163* and *β-actin* gene were 10 copies, 0.25 and 0.025 ng total RNA per reaction mixture, respectively. The R^2 and efficiency of standard curves were 0.983 and 102.166% for CD163, 1 and 101.453% for PRRSV N and 0.996 and 90.969% for *β-actin* genes. The developed real time RT-PCR Method described in this report is more rapid, specific and sensitive than the conventional RT-PCR for the detection of relative levels of *PRRSV N* and *CD163* gene in Marc-145 cell line.

Key words: PRRSV, CD163, SYBR green I, quantitative real-time PCR, China

INTRODUCTION

Porcine Reproductive and Respiratory Syndrome (PRRS) was first reported in the United States in 1987 with the characteristics of reproductive failure in sow and respiratory problems in young pigs (Collins *et al*., 1992). The causative agent of PRRS is PRRS virus (PRRSV), a member of the family Arteriviridae in the order Nidovirales (Cavanagh, 1997). PRRSV genome is positive stranded RNA with about 15 kb in length and encodes at least nine Open Reading Frames (ORFs) (Conzelmann *et al*., 1993).

PRRSV infection of permissive cells depends on the interaction with the receptors or mediators on the cells CD163, a macrophage-specific protein in the Scavenger Receptor Cysteine-Rich (SRCR) superfamily, functions as one of the cellular receptors PRRSV (Calvert *et al*., 2007). Experimental results have showed that transfection with CD163 cDNA is necessary and sufficient to render a variety of non-permissive cell lines fully permissive to PRRSV with production of progeny virus (Calvert *et al*., 2007). CD163 transcripts can be readily isolated by RT-PCR from cells originating from dog (DH82), mouse (peritoneal macrophages), monkey (Marc-145 and Vero) and human (U937) and can serve as PRRSV entry receptors when transfected into non-susceptible hamster kidney, porcine kidney and feline kidney cell lines. However, not all the original CD163-expressing donor cells (with the exception of Marc-145) are themselves PRRSV permissive. The reasons may be the level of CD163 in these cells is low since that constitutive high-level expression of a CD163 protein in the recombinant cells increases the chance of PRRSV uptake (Welch and Calvert, 2010) and possible requirement of other cellular receptors or mediators along with CD163 to complete the virus attachment and internalization.

The SYBR Green I-based real time reverse transcription PCR (real-time RT-PCR) Method is widely used for quantifying mRNA levels (Bustin, 2002). This technique is sensitivity and less time consuming and has a high degree of potential automation compared to the

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conventional quantification methods such as Northern blot analysis or RNase protection assay. Although, no single standard operating protocol exists (Bustin, 2002), it would be reasonable to presume that the real-time RT-PCR Method can be developed and standardized. Therefore, to obtain a reliable quantification method, each real-time RT-PCR Method has to be optimized taking into account the different successive steps in this multistep procedure such as lysis cells, RNA extraction, DNase treatment, cDNA synthesis and finally real-time PCR.

In the current study, researchers developed a rapid and sensitive two-step SYBR green real-time RT-PCR Method to detect the relative levels of *PRRSV N* and *CD163* genes for the determination of the relationship between PRRSV infection and CD163 expression in Marc-145 cells.

MATERIALS AND METHODS

Cells and virus: Marc-145 cells, derived from African green monkey kidney cells MA-104 were cultured in DMEM (GIBCO) supplemented with 10% Fetal Bovine Serum (FBS) and 1% penicillin/streptomycin/glutamate. Cell cultures were maintained in a humidified atmosphere of 5% CO_2 at 37°C. The American prototype highly pathogenic PRRSV (HP-PRRSV) strain, SD-16 (Wang *et al*., 2012) was isolated by plaque purification and cultivated on Marc-145 cells. Virus stocks were prepared and tittered in Marc-145 cells and stored in aliquots at -80°C until use.

Total RNA extraction and cDNA synthesis: Total RNA of Marc-145 and PRRSV infected Marc-145 cells were extracted using High Pure Isolation kit (Roche). RNA concentrations were quantified using optical density determination at 260 nm (OD260) by microplate reader (BioTek). cDNA was generated with oligo (dT) from 1 μg of RNA using Transcriptor High Fidelity cDNA Synthesis kit (Roche) according to the manufacturer's instructions.

Primers design and synthesize: Real time RT-PCR detection primers for *CD163*, *PRRSV N* and *β-Actin* genes were designed base on the published *Chlorocebus aethiops* mRNA for CD163 (GeneBank ID: JF753553), HPRRSV SD-16 mRNA for N (GenBank ID: JX087437) and *Cercopithecus aethiops* mRNA for β-Actin (GenBank ID: AB004047) using primer express 3.0 Software (Applied Biosystems) and synthesize by Invitrogen, China. The specificity of the primer sequences was verified by comparison to the GenBank nr database using BLASTn searches configured for short and nearly exact matches. The three gene specific primer sequences are shown in Table 1.

Table 1: Primer pairs for the quantitative real-time RT-PCR

Primers	Orientation	Sequence	Product length (bp)
CD163	Sense	TTCACTGCACTGGGACTGAG	144
	Antisense	AGGACAGTGTTTGGGACTGG	
PRRSV-ORF7	Sense	AGATCATCGCCCAACAAAAC	144
	Antisense	GACACAATTGCCGCTCACTA	
β-Actin	Sense	TCCCTGGAGAAGAGCTACGA	194
	Antisense	AGCACTGTGTTGGCGTACAG	

Plasmids: Fragments of *CD163*, *PRRSV N* and *β-actin* genes were ligated in pMD18-T (TAKARA) and designated pMD-CD163, pMD-N and pMD-actin, respectively.

Conventional RT-PCR: The conventional RT-PCR assay was performed on a 2720 thermal cycler (Applied Biosystems) using the primers shown in Table 1. cDNA were synthesized from total RNA of Marc-145 and *PRRSV N* gene plasmid pMD-N and performed in 10 fold dilutions in ultrapure water. The PCR reaction mixture contained 0.5 μL of forward and reverse primers (12 μM each), 0.5 μL dNTPs (10 mM), 0.5 μL cDNA or pMD-N, 10 μL 2×Phusion HF PCR Master Mix (NEB) and 8 μL ultrapure water. The PCR conditions were as follows: PCR activation for 30 sec at 98°C, 30 cycles of amplification (30 sec at 98°C, 30 sec at 60°C and 15 sec at 72°C) and a final extension step at 72°C for 5 min. The resulting PCR products were analyzed by electrophoresis on an ethidium bromide stained 1.0% agarose gel.

Real-time RT-PCR: Real-time PCR was optimized by hot-start condition using FastStart Universal SYBR Green Master (ROX) kit (Roche). SYBR Green I and ROX were used as the reporter and passive reference dyes, respectively. Reactions were performed using a Step One Plus Real-Time PCR System (Applied Biosystems). The reaction mixture contained 10 μL of 2xFastStar Universal SYBR Green Master Mix, 5 μL cDNA or PRRSV N plasmid, 0.5 μL each of forward and reverse gene-specific primers (12 μM each) (Table 1) and 4 μL ultrapure water. The following thermal profile was used for all real-time PCRs: 95°C 10 min; 95°C 15 sec and 60°C 60 sec for 40 cycles; 95°C 30 sec; 60°C, 20 sec and 95°C, 30 sec for 1 cycle. Relative levels were performed using β-actin mRNA as an endogenous control.

Analytical specificity: The specificity of the primers was tested using cDNA synthesized from total RNA of PRRSV infected Marc-145 cells, the pMD-CD163, pMD-N and pMD-actin plasmids as the positive control. Tm value of amplification product was analyzed by melt curve assay to determine the amplification specificity.

Analytical sensitivity: Serial 10 fold dilutions of cDNA synthesized from 250 ng total RNA of Marc-145 cell were performed in ultrapure water to determine the detection limit. Same dilutions were performed on pMD-N plasmid (2.08×10^6 copies uL^{-1}) to ascertain the detection limit of *PRRSV N* gene copy number. The lowest levels of *N*, *CD163* and *β-actin* genes from total RNA of Marc-145 cells were set as the detection limits.

Detection of samples: Marc-145 cells was infected with HP-PRRSV SD16 strain at 0.01 MOI at 4°C for 1 h after washing out unattached viruses with PBS, the cells were cultured at 37°C for 0, 1, 2, 6, 9, 12 and 24 h. The relative levels of *PRRSV N* and *CD163* genes in Marc-145 cell at each time point were detected by quantitative Real-Time RT-PCR. Uninfected Marc-145 was used as the normal control.

RESULTS AND DISCUSSION

Validation of the real time RT-PCR Method: Gene fragments shown in Table 1 were chosen as the targets for the detection of *PRRSV N*, *CD163* and *β-actin* genes by real-time PCR. The specificity was assessed with cDNA synthesized from total RNA of PRRSV infected Marc-145 cells and constructed plasmids pMD-CD163, pMD-N and pMD-actin were used as the positive controls. The results showed that the target genes were amplified correctly (Fig. 1). The Tm analysis showed peaks of cDNA detection were coincident with the plasmid positive control, CD163 at ~83.34°C (Fig. 1a), PRRSV-N at ~79.12°C (Fig. 1b) and β-actin at ~81.1°C (Fig. 1c) while negative controls did not show any Tm.

For the detection limit of *CD163* and *β-actin* genes in total RAN quantitation, a series of 10 fold dilutions of cDNA synthesized from total RNA of Marc-145 cells were measured. The results showed that the minimum level of *CD163* gene was 10^{-6} dilution and the total RNA detection quantitation was 0.25 ng (Fig. 2a) and β-actin was 0.025 ng (Fig. 2c). The detection limit of HP-PRRSV *SD-16 N* gene measured with pMD-N plasmid was up to 10^{-7} dilution (Fig. 2b) and the copies number was upto 10 copies.

Dilution end-point standard curves of *CD163* and *β-actin* genes were created between Ct value and template quantitation using serial dilutions of cDNA in ultrapure water. The equation of the total RNA quantitation versus the threshold Cycle (Ct) values for CD163 was $y = -3.271x + 29.998$ with a R^2 of 0.983, the reaction efficiency was 102.166% (Fig. 2d) and β-actin was $y = -3.559x + 24.864$ with a R^2 of 0.996, the reaction efficiency was 90.969% (Fig. 2f). Standard curve of

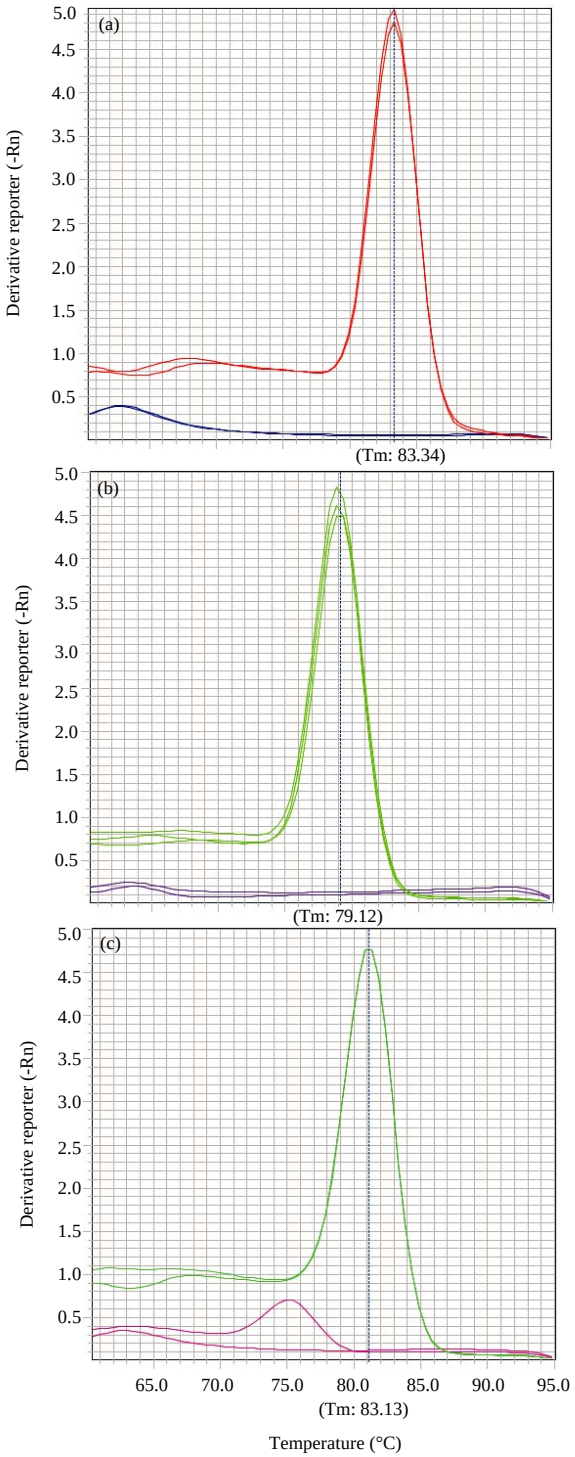

Fig. 1: Melting curve analysis of the amplification products of CD163; a) PRRSV N; b) β-Actin and c) specific gene sequence in specificity test

PRRSV N gene was created between Ct value and template copy number using serial dilutions of pMD-N

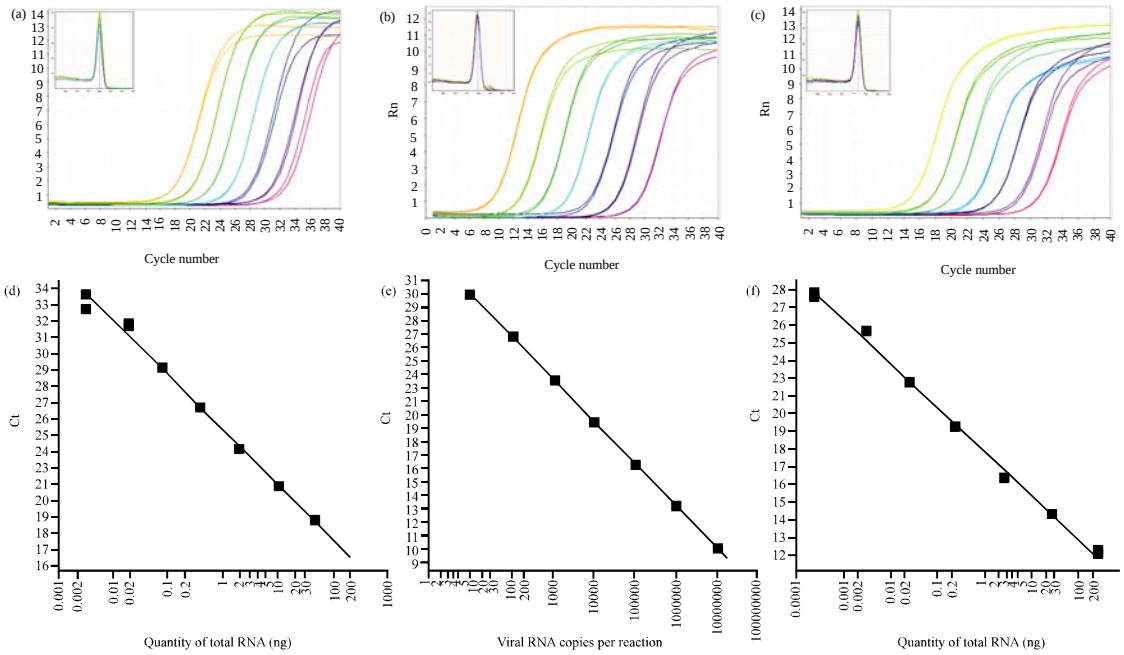

Fig. 2: Standard curves generated by real-time RT-PCR amplification of serial dilutions of total RNA (CD163 and β-Actin) and pMD-N (*PRRSV N* gene). Colony forming units of a) CD163; b) PRRSV N and c) β-Actin of amplification curves (from left to right over the baseline) were 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6} and 10^{-7}. The straight lines were linear regression; d) CD163 was y = -3.271x + 29.998 with a R^2 of 0.983, the reaction efficiency was 102.166%; e) *PRRSV N* gene was y = -3.288x + 33.348 with a R^2 of 1, the reaction efficiency was 101.453%; f) and β-actin was y = -3.559x + 24.864 with a R^2 of 0.996, the reaction efficiency was 90.969%

plasmid in ultrapure water. The equation of the plasmid copy numbers versus the threshold Cycle (Ct) values for *PRRSV N* gene was y = -3.288x + 33.348 with a R^2 of 1, the reaction efficiency was 101.453% (Fig. 2e). Base on these results, the R^2 and E value were suitable to analyze the relative levels of *PRRSV N* and *CD163* genes.

Comparison of real-time RT-PCR and conventional RT-PCR: The detection limit of the real-time RT-PCR assay and the conventional RT-PCR assay were compared. In real-time RT-PCR assay, *CD163* gene was detected at a 10^{-6} dilution with a corresponding Ct value of 31.84±0.18 (Fig. 2a), *β-actin* gene at 10^{-7} dilution with a corresponding Ct value of 27.66±0.09 (Fig. 2c) and *PRRSV N* gene at 10^{-7} dilution with a corresponding Ct value of 29.99±0.08 (Fig. 2b). In parallel, the analytical sensitivity of the conventional RT-PCR for CD163 was 10^{-4} dilution (Fig. 3a), β-actin was 10^{-6} dilution (Fig. 3c) and PRRSV N was 10^{-5} dilution (Fig. 3b). These results indicated that the sensitivities of the real-time RT-PCR assay for the detection of CD163 (Fig. 2a and 2d) and PRRSV N (Fig. 2b and e) genes were two log unit increases and one log unit increase for *β-actin* gene (Fig. 2c and f) than that of the conventional RT-PCR assay.

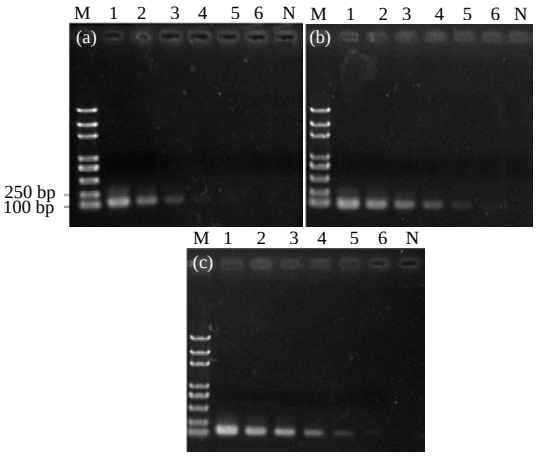

Fig. 3: The sensitivity of conventional RT-PCR for detection of the a) *CD163*; b) *PRRSV-N* and c) *β-Actin* gene. Lanes 1-6: amplification product of 10 fold dilution series of sample cDNA synthesized from total RNA of *Marc1-145* and *PRRSV N* gene plasmid pMD-N ranging from 10^{-1} to 10^{-6} dilutions; Lane N: negative control having no template; Lane M: DL2000 Plus DNA Maker (Transgene)

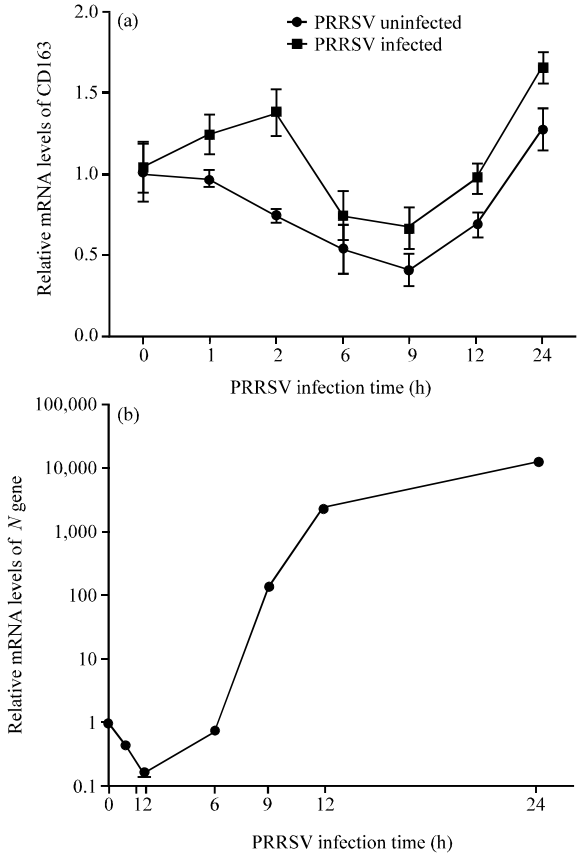

Fig. 4: a) The relative CD163 mRNA and b) relative PRRSV N mRNA levels at different time post PRRSV infection of Marc-145 cells

Detection of *PRRSV N* and *CD163* genes in PRRSV infected Marc-145 cells: The levels of *CD163* and *PRRSV N* genes in PRRSV infected Marc-145 cells were detected using the established SYBR I real-time RT-PCR Method. The result showed that the levels of CD163 mRNA was increased from 1-24 h post PRRSV infection compared with uninfected Marc-145 cells (Fig. 4a) while that of PRRSV N mRNA was decreased after PRRSV attachment 2 h later then increased (Fig. 4b).

CONCLUSION

The real-time RT-PCR Method developed in this study is sensitive, specific and rapid method for detection and quantitation of *CD163* gene and PRRSV infection levels in Marc-145 cell.

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