Detection of genotype 1 Porcine Reproductive and Respiratory Syndrome virus in swine, using one-step Real-Time PCR for the ORF7 gene

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Abstract

Porcine Reproductive and Respiratory Syndrome (PRRS) is the most devastating and economically challenging disease to the swine industry worldwide due to reproductive failure. The main objective of the current study was to evaluate the sensitivity and accuracy of Real-Time RT-PCR method in the detection of PRRS virus and also estimation of the pathogen load in samples with clinical signs. The primers used for the detection of PRRS virus were represented by primers with a specific sequence for the ORF7 gene of the PRRS virus. More important, the primers attachment process was influenced by punctual mutations of the viral strand belonging to the ORF7 gene. 114 samples were tested to identify the presence of PRRS virus, genotype I and 14 of them were found to be positive, using OneStep PCR. Those samples were used to test the specificity of the TaqMan probe and robustness of Real-Time RT-PCR reaction. According to the results, only the samples which presented some specific punctual mutations (4 in total, all from one particular region of Romania) at the genome level of ORF7, were positive, due to primer sequence specificity and complementarity. The Real-Time RT-PCR method has been increasingly adopted by swine producers and veterinarian laboratories as one of the most trustful techniques, combining rapidity, specificity and efficiency for detecting and monitoring the spread of PRRS virus.

Keywords: Porcine Reproductive and Respiratory Syndrome, real-time PCR, virus variants.

1. Introduction

Porcine Reproductive and Respiratory Syndrome is the most devastating and economically challenging disease to the swine industry worldwide due to reproductive failure including late abortions, high mortality in weaned pigs and respiratory disorders associated with secondary bacterial infections [1]. The etiological agent is the PRRS virus (PRRSV), a spherical, lipid-enveloped single-stranded positive RNA virus, classified in the order Nidovirales, family *Arteriviridae* [2,3]. The length of PRRSV is approximately 15 kilobases (kb) with nine overlapping open reading frames [4,5]. PRRSV is classified in two different genotypes, North American and European being officially recognized as Type 1 (EU genotype) and Type 2 (NA genotype) [6]. In Romania only Type 1 of PRRSV is present and there are minimal economic losses caused by the disease [7].

In the last seven years technical advances have improved PRRSV detection systems and made these technologies affordable for swine researchers and veterinarians. There are several methods for diagnosis, most of them being accomplished by serological methods. For detection of PRRSV there are also used: virus isolation techniques, reverse transcriptase-

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polymerase chain reaction, nested PCR and real time RT-PCR [8,9]. There are some important drawbacks to cell culture based viral detection because it is time-consuming and laborious, often requiring 7–14 days for completion. In addition, virus isolation for some field strains and diagnostic samples is difficult, and these results in low diagnostic sensitivity [10]. Proper use of the assays can provide critical data for PRRSV control, but an "over-interpretation" can generate poor decisions resulting in failure of control efforts [11].

Real-time RT-PCR approach allows rapid quantification of target RNA or DNA in a single tube, having enhanced assay specificity and allowing simultaneous detection of multiple targets without the need to have amplification products of different sizes. Moreover, post-amplification analysis, that reduces sample manipulation is automated and real-time assays are a ''closed-tube system,'' reducing the risk of contamination, which is an important cause of false-positive results [8]. The values derived from Real-time RT-PCR assay allow accurate estimation of pathogen load in cases with clinical signs of PRRS. Real time methods using SYBR Green and TaqMan® chemistries have been previous reported for the detection of both NA and EU genotypes [8,9,11-14]. However TaqMan® methods require a perfect match between the probes and the region targeted, otherwise a significant decrease in the sensitivity of the system occur [15].

Because PRRSV is one of the most rapidly evolving RNA virus the purpose of the study was to make a local population screening and strain identification on Romanian farms, using a sensitive and accurate real-time RT-PCR method.

2. Materials and methods

The biological material consisted of 114 samples from pig farms from different regions of Romania. The samples were represented by organs homogenates (liver, lungs, and kidney) collected from dead pigs and since sampling were stored at -80 ^{O} C until further analysis.

Viral genomic RNA was isolated using an RNeasy Mini Kit (Qiagen), as instructed by the manufacturer. First, 200 µl tissue homogenates was disrupted in 350 µl Buffer RLT and then homogenized. After an equal volume of ethanol was added (550 µl), the samples were moved in the RNeasy Mini spin column (total RNA binds to the membrane) and subsequently washed with 700 µl Buffer RW1 and twice with 500 µl Buffer RPE to remove the contaminants. The RNA was eluted in 30 µl RNase-free water. Each step (wash and elution) was followed by centrifugation.

Before using the RNA molecules for different molecular procedures, it is necessary to verify their purity and integrity. The integrity and the purity of the RNA solution were evaluated using BioSpec-nano Micro-volume UV-Vis Spectrophotometer.

One step PCR involves carrying out both reverse-transcription (cDNA synthesis) and PCR amplification in the same tube, without being necessary the addition of other components, once the reaction began, reducing this way the risk of contamination.

The One Step PCR (Qiagen One Step RT PCR) mix contained: 14.8 µl nuclease free water, 5.1 µl OneStep RT-PCR Buffer, 1.1 µl dNTP, 0.4 µl forward primer (ORF 7B-F 5'-GCCCCTGCCCAICACG-3'), 0.4 µl reverse primer (ORF 7C-R 5'-TCGCCCTAATTGAATGGTGA-3') and 1.1 µl OneStep RT-PCR Enzyme Mix. The final volume was of 24.4 µl (22.9 µl One Step PCR mix and 1.5 µl RNA extract).

The reactions were carried out on a Corbett thermocycler with the following programme: 30 min - 50° (1 cycle); 15 min - 95° (1 cycle); 45 s - 95° (45 cycles), 45 s - 55° (45 cycles) and 60 s - 72° (45 cycles). The PCR products were analyzed by electrophoresis in 2% agarose gels stained with ethidium bromide.

The Real Time PCR mix (Qiagen One Step RT PCR) contained: 3.9 µl OneStep RT-PCR Buffer, 0.7 µl dNTP mix, 0.8 µl OneStep RT-PCR Enzyme Mix, 0.2 µl forward primer (F: 5´-CGCAATCAGGCGCA CWGTATG- 3'), 0.2 µl Reverse primer (R: 5´-AGAAAAGTACAGCTC CGATGG-3'), 0.4 µl probe (5'-6FAM/3'-BHQ1) and 7.8 µl nuclease free water. The final volume of 20 µl (14 µl Real-Time-PCR mix and 6 µl RNA extract) was filled in the LightCycler glass capillaries.

Second mix included: 3.9 µl OneStep RT-PCR Buffer, 0.7 µl dNTP mix , 0.8 µl OneStep RT-PCR Enzyme Mix, 0.2 µl forward primer (F: 5´-CGCAATCAGG CGCACWGTATG-3´), 0.2 µl reverse primer (R: 5´-AGAAAAGTACAGCTCCGATGG-3´), 0.4 µl probe (5'-6FAM/3'-BHQ1) and 9.8 µl

nuclease free water. In this case, the reaction was set up in a final volume of 20 µl containing 16 µl RT-PCR mix and 4 µl RNA.

For each assay the forward and reverse primers were used at a final concentration of 20 µM (each). The primers were used to confirm the presence of PRRS European genotype strain. Primers sequences were complementary with the ORF7 gene.

The reactions were carried out on a LightCyler 2.0 (Roche) thermocycler with the following run program: RT (reverse transcription) – 15 min. at 45^{O} C, 1 cycle; RT inactivation – 10 min. at 95^{O}, 1 cycle; PCR (quantification and amplification) – 10s at 95^{O} C, 30s at 60^{O} C (single), 30s at 72^{O} C, 45 cycles and 30s at 40^{O} C (cooling step), 1 cycle.

The DNA sequencing was performed for both forward and reverse strands using BigDye Terminator Kit v3.1 (Applied Biosystems) and conducted on a 3130 Genetic Analyzer (Applied Biosystems). The obtained sequences were proofread manually, truncated to the real dimensions of ORF7 gene (387 bp) using BioEdit version 7.1.3.0 and aligned using CLUSTAL W application.

3. Results and discussion

A classic PCR was carried out with the same set of primers and the resulted amplicons were subject of gel electrophoresis "Figure 1". In this reaction all the tested samples were positive. Samples were sequenced and the sequences obtained were aligned with existing data in GenBank "Figure 4", yielding specific region of the genome of PRRSV ORF7. The failure of the Real Time PCR reaction is due to some punctual mutations in the probe binding site, shown in "Figure 4".

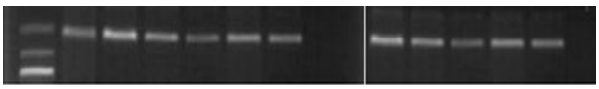

Figure 1. Gel electrophoresis of 10 tested samples. The left lane is represented by a molecular ruler of 100 bp, the samples from lanes 1-6 are correspondent for samples from Braila (Rom4), Braila (Rom22), Braila (Rom23), Cluj (Rom 24), Sample from Arad, positive control *Porcilis vaccine* and a negative control, represented by nuclease free water; the samples from the lanes 7-11 are correspondant to sample from Arad, two samples from Iasi, sample from Satu Mare and sample from Braila.

These samples were analyzed by Real Time PCR technique. Results from the study are shown in "Figure 2" and "Figure 3" and were statistically analyzed by comparing the differences in threshold cycle (Ct) values between the samples and the controls (positive and negative controls).

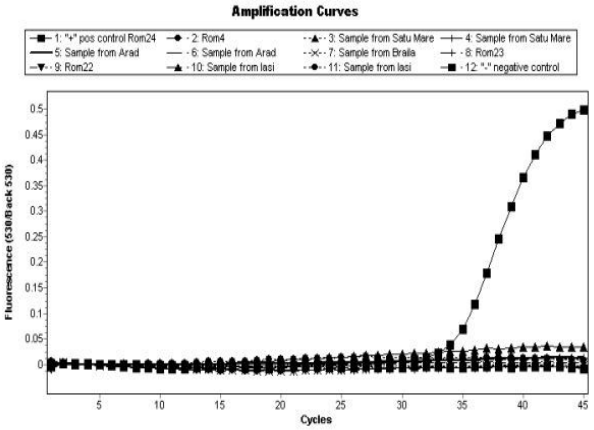

Figure 2. Amplification plots for the first 10 samples obtained after extraction. The vertical line at approximately 0.005 fluorescence units is indicating the threshold for a positive reaction.

By analyzing the Ct values, the viral concentration of the samples can be evaluated easily because the values are directly proportional to the amount of viral RNA. Real–Time PCR reaction is one of the most sensitive method because it can detect the smallest viral concentration. In the first case, were tested 10 samples from different important Romanian pig farms (Satu Mare, Arad, Braila and Iasi). We used the reference sample Rom24 (from Cluj area) as positive control. All the samples were negative, less the positive control.

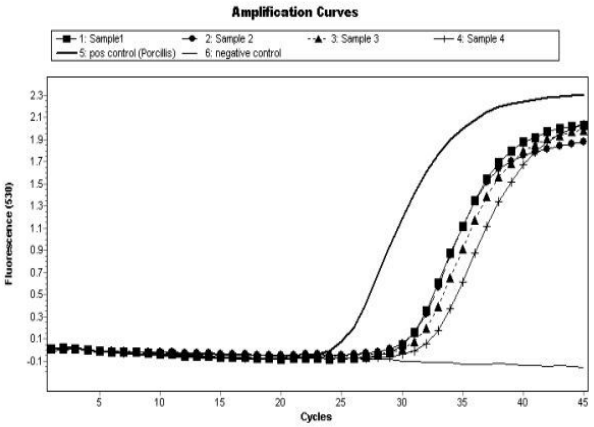

Figure 3. The results from the second Real-Time PCR reaction in which all the samples were positive from the same area (Cluj). The vertical line at approximately 0.005 fluorescence units is indicating the threshold for a positive reaction.

In the second case, were we tested 4 samples only from Cluj area. Results of Real-Time PCR reaction showed that all 4 samples were positive. PRRS Porcilis vaccine (based on the European strain DV of PRRS virus) was used as positive control in this reaction. For the tested samples on Real Time PCR, the Ct values for the PRRSV isolates and control samples were 29.36 (Sample 1), 29.62 (Sample 2), 30.25 (Sample 3), 31.27 (Sample 4) and 24.14 (Porcilis) respectively, the Ct value for Porcilis vaccine was significantly higher than the values that characterized the other samples.

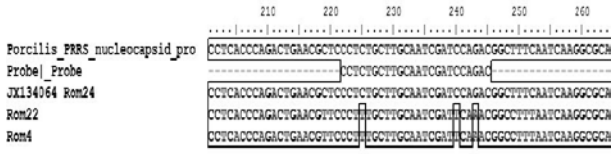

Figure 4. Sequences shown the tree point mutations from the sample Rom22 and Rom4. All the samples, except Rom14, had same mutations in the probe binding region

Negative control which was added as template to the Real-Time PCR reaction mixture, did not produce a signal for any of the quantitative assays. The comparison between the amplification curves obtained for the two controls and the samples revealed the accuracy of this molecular technology.

The current study's main objective was to evaluate the sensitivity and accuracy of Real-Time RT-PCR method in detection and also to estimate the pathogen load in the cases with clinical signs of PRRS virus. The analyzed samples consisted in pig tissue and organ homogenates collected randomly from different pig farms on the Romanian territory.

In order to avoid mismatches in the primer and probe binding sites, the sequences should be periodically subjected to control and sequence analysis. The use of an internal positive control gave the opportunity to find out whether the reaction failed or to identify false-negative results.

The purpose of this study was to evaluate and verify a Real-Time RT PCR method that is sensitive, fast and – unlike other methods that use probes (TaqMan) – is not able to tolerate mismatches in the probe region. Because PRRSV is one of the most variable and rapidly evolving RNA viruses the probe hybridization is the key point in this assay. In TaqMan assay when a mismatch is present in the probe binding site, the probe is displaced rather than hydrolyzed and the quencher (Q) molecule remains in the close proximity of the reporter (R). This can lead to signal loss, reduced sensitivity and possible false negative results which happened in the first Real Time PCR assay. The samples that were negative in the first assay presented specific punctual mutations in the probe binding region. Only the tested samples from Cluj didn't have these punctual mutations.

4. Conclusions

The principal objective of the present study was to evaluate the sensitivity and accuracy of Real-Time RT-PCR method in detection of PRRS virus and also estimation of the pathogen load in samples with clinical signs. 114 samples were tested to identify the presence of PRRS virus, genotype I, 14 of them were found to be positive on ONE Step PCR. Evaluating the specificity of Real Time RT assay for the positive samples, we found out that only 4 samples, all from one particular region of Cluj, were positive using this method. The failure of the Real Time PCR reaction is due to some specific punctual mutations in the probe binding site. More important, the primers attachment process was influenced by punctual mutations of the viral strand belonging to the ORF7 gene, leading to the conclusion that a routine analysis for detection can provide some false positive results. Moreover it is strongly suggested to share and determine ORF7 sequences of new emerging strains from all over the world, in order to provide a source of continuous information for the molecular diagnostic methods.

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