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The impact of porcine reproductive and respiratory syndrome virus genetic heterogeneity on molecular assay performances

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A B S T R A C T

The remarkable economic losses due to porcine reproductive and respiratory syndrome (PRRS) have stated the control and eradication of this disease is one of the main issues of swine modern farming. The limited cross-protection of vaccine-induced immunity compelled the adoption of strict biosecurity measures that must be associated with the prompt diagnosis of infection. In our study four RT-PCR methods, a RT-PCR, a SYBR Green I and two hydrolysis probes, were compared to evaluate their respective benefits and disadvantages. One hundred and seventy samples originating from 50 farms located in northern Italy were tested with all assays and performances were evaluated using a Bayesian approach to deal with the absence of a Gold Standard. Sequencing the complete of ORF7, the segment targeted by all methods, allowed a gain of insight into the genetic variability of Italian strains and to investigate the role of mismatches on assay sensitivity. Our study evidenced that methods based only on primers-genome interaction better tolerate PRRSV genetic variability, demonstrating a greater sensitivity (Se): SYBR Green I (Se = 98.4%) and RT-PCR (Se = 99%) outperform both in-house (Se = 71.4%) and commercial (Se = 91.7%) probe-based methods. On the other hand, probe-based assays allowed an easier genotyping of PRRSV strains and implementation of the internal control system (IC). Phylogenetic analysis allowed demonstration of a presence of two clades circulating continuously in northern Italy since 1996, when their probable ancestors were collected.

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1. Introduction

Porcine reproductive and respiratory syndrome virus (PRRSV) emerged as a disease of swine in the late 1980s. Although more than 20 years have elapsed since its discovery, PRRS is still the most prevalent swine disease, with a huge economic impact (Lunney et al., 2010). Great genetic heterogeneity has been demonstrated among different strains. Two main genotypes (type I and II) have been identified sharing only 50–70% nucleotides and 50–80% similarity of amino acids (Forsberg, 2005). In addition, mean nucleotide diversity within the genotype has been estimated to be about 12.5–15%, while a maximum genetic distance of 21–30% has been reported within genotype I and II, respectively (Cho and Dee, 2006; Murtaugh et al., 2010; Pesch et al., 2005; Shi et al., 2010a, 2010b, 2013). The persistent nature of infection, coupled with the limited efficacy of vaccines, has made the control of PRRS particularly problematic (Chand et al., 2012). Avoiding the introduction and minimizing vertical and horizontal spread of the virus within the farm, play a major role in control and eradication of PRRS (Rowland and Morrison, 2012). In order for this strategy to be effective, there needs to be accurate diagnostic tools for identification of infected herds or animals (Corzo et al., 2010; Thanawongnuwech and Suradhat, 2010). Several methods have been validated to detect PRRSV infection. Although serology represents a popular choice, this method is disadvantageous because immune response is highly variable and, even if such antibody titer may fall rapidly after infection, it is not possible to easily discriminate between present vs. past infection or active vs. passive immunity. Moreover, negative or low positive ELISA results do not rule out persistent infection (Batista et al., 2004). Virus isolation is time-consuming and requires a certain expertise. Immunohistochemistry or immunofluorescence assays are used for antigen detection but their sensitivity is still a concern (Martínez et al., 2008). PCR and real time PCR have been used widely for viral infection diagnosis and genotype

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identification (Belák, 2007). This success is due to their usual enhanced sensitivity, larger dynamic range and reduced risk of cross contamination. The use of a specific probe facilitates an increased specificity compared to conventional agarose gel-based PCR assays. Besides the development of multi-color real-time PCR cyclers, it has also been made possible to combine several assays within a single tube. This allows the simultaneous detection and discrimination between different pathogens as well as genotypes of the same microorganism, while providing an easy implementation of internal control (IC) systems (Gunson et al., 2008; Hoffmann et al., 2006; Wu et al., 2008; Zheng et al., 2013). Several commercial diagnostic tests are also available, making it possible to avoid the complex phase of in-house method validation. A major challenge in RT–PCR and real time RT-PCR is represented by problems in designing specific primers/probes able to deal with the PRRSV genetic variability (Hoffmann et al., 2009). This issue is relevant particularly for commercial kits sold on a large scale. However, national and international animal trade is exposed constantly to new strains, imposing on local laboratories to update continuously. Therefore, it is not surprising that the lack of sensitivity in diagnosis of PRRSV infection of both in-house and commercial kits has been reported by previous studies and laboratory experiences (Toplak et al., 2012; Wernike et al., 2012).

The aim of this study is to compare performances, advantages and disadvantages of different in-house and commercial RT-PCR and real time RT-PCR methods in detecting PRRSV from samples collected from a large area in northern Italy. The sequence of field samples has been performed to gain insight into the genetic heterogeneity of Italian viruses and to explore its relation with assay sensitivity.

2. Material and methods

2.1. Field samples

A total of 170 aliquots originating from as many pigs (80 lungs and 90 sera) were selected on the basis of diagnostic activity results obtained using the RT-PCR method (see below) from stored samples delivered during the 2010–2012 time period to Istituto Zooprofilattico delle Venezie (IZSVe). One hundred and fifty-one positive and 19 negative samples have been included in the study to challenge the assays with a broader spectrum of PRRSV strains. The samples originated from 50 farms located in three regions of northern Italy, with unknown previous sanitary status.

Two hundred microliters of serum samples were extracted using High Pure Viral RNA Kit (Roche Diagnostics, Monza, Italy). Lung samples were homogenized after an addition of 10 ml of PBS for each gram of tissue. Two hundred microliters of homogenate were extracted using High Pure RNA Tissue Kit (Roche Diagnostics, Monza, Italy). Before extraction, each aliquot was added with 2 μl of solution containing 2×10^5copies/μl of RNA Internal Control (IC) (Hoffmann et al., 2006) immediately following an addition of lysis buffer. The same extract was subdivided in four aliquots for successive testing. All samples and RNA aliquots were stored at −80 °C until processing.

2.2. Test samples with RT-PCR and real time RT-PCR

Each aliquot was tested with each of four RT-PCR-based methods. Two in-house real time RT-PCR assays (here defined as Probe and SYBR) described by Drigo et al. (2014) were used with minor modification. Briefly, for TaqMan-based One-Step qRT-PCR 2 μl of extracted RNA were added to a standard reaction mix containing 0.2 μl of SuperScript® III RT/Platinum®Taq Mix (Life Technologies™, Monza, Italy), 0.3 μM of primer PRRSVf1 and PRRSVr2, 0.4 μM of primer EGBP-1F and EGBP-2R, 0.2 μM of PRRSV specific probes (EU-1, EU-2, US) and 0.4 μM of EGBPp. Sterile nanopure water was added to bring the final volume to 10 μl. Cycling parameters were 50 °C for 15 min, 95 °C for 2 min, 40 cycle of 95 °C for 10 s and 60 °C for 30 s. The fluorescence signal was acquired for each cycle at the end of the extension phase. Similarly, for SYBR Green One-Step qRT-PCR 2 μl of extracted RNA were added to a standard reaction mix containing 0.2 μl of SuperScript® III RT/Platinum® Taq Mix (Life Technologies™, Monza, Italy), 0.4 μM of primer PRRSVf1 and PRRSVr2, 0.1 μM of primer EGBP-1F and EGBP-2R. Sterile nanopure water was added to bring the final volume to 10 μl. Cycling parameters were 50 °C for 15 min, 95 °C for 5 min, 40 cycles of 95 °C for 10 s and 60 °C for 30 s. The fluorescence signal was acquired for each cycle at the end of the extension phase. After incubation for 1 min at 40 °C, melting curves were performed by gradually raising temperatures from 64 °C to 95 °C with a continuous collection of fluorescence data.

A commercial real time RT-PCR kit (ADIAVET™), was included in the study in order to determine its diagnostic sensitivity with regard to the highly variable “Italian cluster” (Shi et al., 2010a).

In-house and commercial real time RT-PCRs were performed on a LightCycler®480 system (Roche, Monza, Italy) and 7900HT Fast Real Time PCR System (Life Technologies™, Monza, Italy), respectively.

All samples were tested again with RT-PCR (Persia et al., 2001) to avoid a bias in sensitivity and specificity estimate during assay comparisons due to time-dependent RNA degradation. Also in this case, genotyping was possible due to the use of specific primers resulting in different amplicon lengths. Specific primer and probes are summarized in Table 1.

2.3. Sequences and phylogenetic analysis

The complete ORF7 of all positive samples to at least one of the methods defined previously was amplified as described by Oleksiewicz et al. (1998). Both strands of each amplicon were sequenced using the Byg Dye terminator v3.1 sequencing kit.

Table 1
List of primers and probes used for in-house developed assays.

Primer/Probe	Oligonucleotides	Assay	Reference
RT	5′-TCGCCCTAAT-3′	RT-PCR	Persia et al. (2001)
US/EU-F	5′-ATGGCCAGCCAGTCAATC-3′	RT-PCR	Persia et al. (2001)
EU-R	5′-GATTGCAAGCAGAGGGAGCGTTC-3′	RT-PCR	Persia et al. (2001)
US-R	5′-GGCGCACAGTATGATGCGTAG-3′	RT-PCR	Persia et al. (2001)
PRRSVf1	5′-GGGGAATGGCCAGYCAGTCAA-3′	qRT-PCR	Lurchachaiwong et al. (2008)
PRRSVr2	5′-GCCAGRGGAAAATGKGGCTTCTC-3′	qRT-PCR	Lurchachaiwong et al. (2008)
US	5′-HEX-CTGGGTAAGATCATCGCCCAGGA-3′-IABkFQ	qRT-PCR	Lurchachaiwong et al. (2008)
EU-1	5′-FAM-TTGGCCTGTCCTCCCCTAGGTTG-3′-IABKFQ	qRT-PCR	Lurchachaiwong et al. (2008)
EU-2	5′-FAM-ATGATGAAATCCCAGCGCCAGCGGT-3′-IABkFQ	qRT-PCR	Present study
EGFP-1-F	5′-GACCACTACCAGCAGAACAC-3′	IC System	Hoffmann et al. (2006)
EGFP-2-R	5′-GAACTCCAGCAGGACCATG-3′	IC System	Hoffmann et al. (2006)
EGFP-Cy5	5′-Cy5-AGCACCCAGTCCGCCCTGAGCA-IBRQ-3′	IC System	Hoffmann et al. (2006)

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