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Direct detection of the porcine reproductive and respiratory syndrome (PRRS) virus by reverse polymerase chain reaction (RT- PCR)

P. Suárez[1], R. Zardoya[2], C. Prieto[1], A. Solana[1], E. Tabarés[3], J. M. Bautista[2], and J. M. Castro[1]

[1]Departamento de Patología Animal I and [2]Departamento de Bioquímica y Biología Molecular IV, Universidad Complutense de Madrid, Facultad de Veterinaria, Madrid, and [3]Departamento de Microbiología, Universidad Autonoma de Madrid, Facultad de Medicina, Madrid, Spain

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Summary. A method for direct detection of the porcine reproductive and respiratory syndrome (PRRS) virus was developed, based on reverse transcription of the viral RNA coupled to DNA amplification by polymerase chain reaction. A set of primers was designed from Lelystad virus sequence within ORF 7 encoding nucleocapsid protein. From seven Spanish field isolated strains the 312 bp amplified fragment was cloned and sequenced. Alignment with Lelystad virus sequence revealed a 96–97% homology. A maximum sensitivity of 6.7 $TCID_{50}$ was achieved with the reported procedure in experimentally infected swine alveolar macrophages cultures. The sensitivity obtained in crude clinical samples from experimentally infected 3-weeks old pigs was approximately 10^2 $TCID_{50}$. High specificity for the PRRS virus was demonstrated for the method, as none of the seven common swine virus assayed rendered DNA amplification product.

Introduction

What was initially referred to as "mystery swine disease", which causes reproductive failures in sows and respiratory disorders in piglets [9], was first reported in North America in 1987 [8]. The etiological agent was first isolated in 1990 in Europe [16] and designated Lelystad virus (LV), and then in 1992 in the U.S.A. [4], being both virus antigenically distinct but structurally related [17]. This disease is also known by other different names, such as "swine infertility and respiratory syndrome" (SIRS) and "porcine reproductive and respiratory syndrome" (PRRS).

It has been reported that the causative viral agent of PRRS preferentially replicates in vitro in primary cultures of swine alveolar lung macrophages (SAM) [2]. Electron microscopic studies have shown that the PRRS virus is

50-65 nm in size, has spherical shape and is enveloped with a nucleocapsid of 30–35 nm [2]. The polyadenylated RNA genome of 15.1 kb from the PRRS virus has been recently sequenced and the production of a 3′ coterminal nested set of subgenomic RNAs in infected cells has been demonstrated. A total of eight open reading frames (ORFs) has been identified encoding virus-specific proteins [11]. The nucleotide sequence and the organization of the genome indicate that PRRS virus is a member of a new recently proposed family of viruses, the *Arteriviridae*, comprising lactate dehydrogenase-elevating virus (LDV), equine arteritis virus (EAV), and simian hemorrhagic fever virus (SHFV) [13]. Subclinical infections and persistent viremia with concurrent antibody formation are characteristic properties of these viruses, as well as clinical and immunological differences among isolated strains. Concurring with field evidence indicating differences in virulence of PRRS virus isolates, antigenic variability between European and American isolates has been demonstrated [17].

Since no vaccine or therapeutic measures are available for the treatment or control of PRRS, the diagnosis of infection is very important in the implementation of control based on identification and elimination of persistently infected pigs. These animals shed PRRS virus with normal antibody titers against the virus [19].

Current methods for detecting PRRS virus in clinical samples rely on serial passages in SAM and subsequent detection of PRRS antigens in the infected cells by immunofluorescence or immunoperoxidase staining [16, 18]. Both are labour-intensive and time-consuming and do not provide rapid diagnostic results nor direct detection of the viral agent.

Since no reports have been published on methods for direct detection of the PRRS virus, the purpose of this study was the development and optimization of a RT-PCR system for the amplification of a cDNA fragment from the viral RNA genome, setting up sensitivity and specificity of the technique, as well as its application in clinical samples. The variability found in the sequences analyzed from seven spanish field isolates is also reported.

Materials and methods

Virus strains

Reported experiments were performed with the Spanish PRRS virus strains 5710, 5711, 2228, 2910, 3211, 4606 and 5999 field isolated in the north of Spain (Navarra and Catalonia) between December 1991 and November 1992 and kindly supplied by Dr. E. Espuña from Hipra Laboratory (Girona, Spain). Additionally, for the specificity test, the following viruses were assayed: hog cholera virus (HC), Alfort/187 strain, obtained from the Central Laboratory of Spanish Ministry of Agriculture at Algete; swine influenza virus, A/Belgium/1/83 (H1N1) strain, and A/Ghent/1/84 (H3N2) strain, supplied by Dr. Pensaert, Dept. Virology, Veterinary Faculty, Ghent, Belgium; transmissible gastroenteritis virus (TGE), Purdue strain obtained from Dr. Enjuanes, Centro Nacional de Biotecnología, Madrid, Spain; porcine parvovirus (PPV), NADL-8 strain given by Dr. Mengeling, National Animal Disease Center, Iowa, U.S.A., bovine virus diarrhea (BVD), Singer strain provided by Dr. McClurkin, National Animal Disease Center, Iowa, U.S.A., and Aujezsky Disease Virus (ADV) NIA-3 strain, obtained from Intervet S.A. Laboratories, Salamanca, Spain.

Cell culture and virus isolation

PRRS virus was propagated on SAM cultures. Growth was carried out in 75 cm^2 bottles containing 25 ml of Dulbecco's Minimal Eagle's Medium (DMEM; Gibco/BRL Uxbridge, U.K.), suplemented with antibiotic and 10% fetal calf serum. The initial cell concentration was 2.6×10^6 cells/ml. After 8–16 h of incubation at 37 °C, medium was discarded and 2 ml of a virus suspension with a titer of 10^5 $TCID_{50}$ (50% tissue culture infectious doses)/ml were inoculated. After 2 h, 25 ml of fresh medium were added. The virus-containing culture medium was harvested when most of the cells showed citophatic effect. After three freeze-thawed cycles, the supernatant containing virus was frozen and stored at −70 °C. Virus were recovered by a centrifugation at 140 000 × **g** for 2 h at 4 °C.

Virus titration

For virus titration, growth medium was employed as diluent, and serial ten-fold dilutions of samples were made in 96-wells microtitre plates. Titres were calculated according to the method of Reed and Muench [14], and expressed as log $TCID_{50}$/ml or log $TCID_{50}$/g depending on sample nature.

Experimental inoculation in pigs

Four 3-weeks old pigs were intranasally exposed with 1 ml of virus (strain 5710) per nostril with 5×10^6 $TCID_{50}$/ml. The experiment included a non-infected control pig. Pigs were sacrificd at 5 and 10 days post-infection, and samples were taken from lung, spleen, liver, kidney, tonsils and submaxillar and inguinal (superficial) lympho-nodules. 1 g of each sample was homogenized in potter with 9 ml of DMEM and subsequently centrifugated at 3 000 × **g** for 15 min. The supernatant was titrated as described above. For RT-PCR reaction 10 ml of each specimen (excepting plasma, 6 ml and buffy-coat, 1 ml) were centrifugated at 14 000 × **g** for 2 h at 4 °C and the pellet was employed for RNA extraction. Samples from blood (serum, plasma and buffy-coat) and lung macrophages were also collected at slaughter and directly titrated for PRRS virus. The 3 000 × **g** supernatant from viscera samples and the crude blood and lung macrophages samples were centrifugated at 14 000 × **g** and the pellet was employed for viral RNA extraction.

RNA extraction

Viral RNA was isolated according to Chomcyinski and Sacchi [3]. Essentially, viral pellets were subjected to a denaturing solution 4M guanidym thyocianate (Fluka Chemie, Buchs, Switzerland), 25 mM Sodium citrate pH 7, 0.5% Sarcosyl, 0.1 M ß-mercaptoethanol), and throughly mixed with 200 mM sodium acetate pH 4, one volume of phenol and 0.2 volumes of a chloroform:isoamylic alcohol (49:1) solution. The 15 min ice incubation was spinned at 10 000 × **g** for 20 min at 4 °C. Upper layer was recovered and mixed with 1 volume of isopropanol. After 1 h at −20 °C, samples were centrifuged and pellets resuspended in 150 µl of denaturing solution. One volume of isopropanol was added and after an hour of incubation at −20 °C the samples were again centrifuged. The resulting RNA pellet was washed with 70% ethanol and resuspended in Diethyl pyrocarbonate (DEPC) treated water at 65 °C for 10 min.

Reverse transcriptase-polymerase chain reaction (RT-PCR)

Two primers corresponding to ORF 7 were designed from the Lelystad virus sequence: forward 5′ GGGAATGGCCAGCCAGTCAATCAACTGT 3′ and reverse 5′ TGTAGAAG-TCACGCGAATCAGGCGCACT 3′. Viral RNA (25 µl) was heated at 65 °C for 5 min in the presence of 1 × RT buffer (50 mM Tris-HCl pH 8.3, 50 mM KCl, 10 mM $MgCl_2$,

0.5 mM spermidine, 10 mM DTT, 1 mM of each dNTP) and 5 μg of the reverse PCR primer in a final volume of 50 μl. Once cool to room temperature, 40 U of placental RNasin (Promega Corp., Madison, WI., U.S.A.) and 24 U of avian myeloblastosis virus reverse transcriptase (Promega) were added and the mixture incubated at 42 °C for 1 h. A PCR reaction was set up with 3 μl of the reverse transcription product in 50 μl of PCR buffer containing 50 mM KCl, 10 mM Tris-HCl pH 8.3, 1.5 mM $MgCl_2$, 0.001% gelatin, 0.05% Nonidet P40, 0.05% Tween 20, 300 μM of each dNTP and 300 ng of the corresponding primers. After a brief heating of 2 min at 94 °C ("Hotstart"), 1 unit of thermostable DNA polymerase Dynazyme (Fynnnzyme, Riihitontuntie, Espoo, Finland) and a thin layer of mineral oil (Sigma, St. Louis, MI, U.S.A.) were added and the PCR reaction started with the following programme: 1 min at 94 °C, 1 min at 60 °C and 1 min at 72 °C. After 35 cycles, the reaction was held at 72 °C for 10 min in order to elongate any uncomplete product. All PCR reactions were performed in The Minicycler (MJ Research Inc., Watertown, MA, U.S.A.) automated thermal cycler.

Cloning and sequencing

An aliquot of the PCR product (5 μl) was analyzed by electrophoresis through a 1.5% agarose gel. Once the expected 312 bp amplified fragment was observed, 1 μl of the remainder PCR product was mixed with 1 μl of the pGEM-T vector (Promega) and 3 units of T4 DNA ligase (Promega) in 1 × ligase buffer (30 mM Tris-HCl pH 7.8, 10 mM $MgCl_2$, 10 mM DTT, 1 mM ATP) and incubated 3 h at 15 °C, and 16 h at 4 °C. Transformation of JM109 competent cells was performed as described by Hanahan [7]. Positive clones from duplicate amplifications of each strain were selected and both strands were manually and automatically sequenced. Sequence data were analyzed by use of CGC program [5].

Results

Suitability of primers for amplification of the PRRS virus genome

Variability analysis of the most studied RNA virus has shown that RNA polymerase and nucleocapsid proteins are usually more conserved than the rest of viral proteins [6], and therefore ORF 7 (nucleocapsid) from the reported Lelystad virus sequence was chosen for the design of a pair of oligonucleotide primers for reverse transcription and subsequent PCR amplification. The forward primer is a 28-mer oligonucleotide, whose 5′ end is located at base 14 639 according to LV sequence. The reverse primer is also a 28-mer oligonucleotide whose 5′ end is located at base 14 950. After PCR amplification conditions were optimized, it was possible to consistently amplify a 312 bp fragment from all seven field isolates. The single product from each strain was cloned prior to sequencing. The resulting sequences were aligned with the corresponding LV sequence inside ORF 7. From this comparison, a homology between 96.0 and 97.6 was found (Fig. 1). It is remarkable that once translated the obtained sequence into protein all mutations except three were conserved, and even two of the detected amino acid changes (isolate 5710, $A_{14733} \rightarrow G$, Lys → Arg; and isolate 2910, $A_{14885} \rightarrow G$, Ser → Gly) will not produce a net charge change in the resulting capside protein in contrast with the change observed in isolate 2228 where a His is mutated to Asp ($C_{14783} \rightarrow G$).

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            14667
LV          GCCAGTTGCT  GGGTGCAATG  ATAAAGTCCC  AGCGCCAGCA  ACCTAGGGGA
PRRS 5710   **********  **********  **********  **********  **********
PRRS 5999   **********  **********  **********  **********  **********
PRRS 4606   **********  **********  **********  **********  **********
PRRS 2228   **********  **********  **********  **********  **********
PRRS 3211   **********  **********  **********  *******A**  **********
PRRS 5711   **********  **********  **********  **********  **********
PRRS 2910   **********  **********  **********  **********  **********
            14717
LV          GGACAGGCCA  AAAAGAAAAA  GCCTGAGAAG  CCACATTTTC  CCCTGGCTGC
PRRS 5710   ********A*  ****A*G***  **********  **********  ****A*****
PRRS 5999   **********  *******G**  **********  **********  ****A*****
PRRS 4606   **********  **********  **********  **********  ****A*****
PRRS 2228   **********  **********  **********  **********  ****A*****
PRRS 3211   **********  **********  **********  **********  **T*A*****
PRRS 5711   **********  *******G**  **********  **********  ****A*****
PRRS 2910   **********  **********  **********  **********  **********
            14767
LV          TGAAGATGAC  ATCCGGCACC  ACCTCACCCA  GACTGAACGC  TCCCTCTGCT
PRRS 5710   **********  **T*******  **********  ***C******T  **********
PRRS 5999   **********  **T*******  *T********  ***C******T  **********
PRRS 4606   **********  **T*******  **********  ***C******T  **********
PRRS 2228   **********  **T***G***  **********  ***C******T  **********
PRRS 3211   **********  **T*******  **********  ***C******T  *****T****
PRRS 5711   **********  **T*******  **********  ***C******T  **********
PRRS 2910   **********  **********  **********  ***C******T  **********
            14817
LV          TGCAATCGAT  CCAGACGGCT  TTCAATCAAG  GCGCAGGAAC  TGCGTCGCTT
PRRS 5710   **********  **********  **T*******  **********  **********
PRRS 5999   **********  **********  **T*******  **********  **********
PRRS 4606   **********  **********  **T*******  **********  **********
PRRS 2228   **********  **********  **T*******  **********  **********
PRRS 3211   **********  *********C  **T*******  **********  **********
PRRS 5711   **********  **********  **T*******  **********  **********
PRRS 2910   **********  **********  **********  ****T*****  **********
            14867
LV          TCATCCAGCG  GGAAGGTCAG  TTTTCAGGTT  GAGTTTATGC  TGCCGGTTGC
PRRS 5710   **********  **********  **********  *****C****  **********
PRRS 5999   **********  **********  *********C  *****C****  **********
PRRS 4606   **********  **********  **********  *****C****  **********
PRRS 2228   **********  **********  **********  *****C****  **********
PRRS 3211   **********  **********  **********  *****C****  **********
PRRS 5711   **********  **********  **********  *****C****  **********
PRRS 2910   **********  *******TG*  ***C******  **********  **********
            14917
LV          TCATAC
PRRS 5710   ******
PRRS 5999   ******
PRRS 4606   ******
PRRS 2228   ******
PRRS 3211   ******
PRRS 5711   ******
PRRS 2910   ******
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Fig. 1. Sequence alignment of the fragments amplified by RT-PCR within ORF-7 from the field isolated PRRS virus strains. Sequences are aligned to the Lelystad virus (LV) sequence from bp 14667 to 14922. Bases matching are indicated by asterisks in the sequence of the Spanish isolates

Determination of sensitivity and specificity

Sensitivity was determined directly from viral RNA and further from the cloned cDNA fragment. In the first case, RNA extracted from 1.9 ml of the virus stock was subjected to a 10-fold dilution protocol. Dilutions cover a 6.7×10^6 to 6.7×10^{-2} $TCID_{50}$/ml range. After RT-PCR was performed positive products were sound detected in lanes corresponding to dilutions ranging from 6.7×10^6 to 6.7 $TCDI_{50}$/ml as clearly shown in Fig. 2. No signal was observed when RNA extracted from an uninfected SAM culture was assayed.

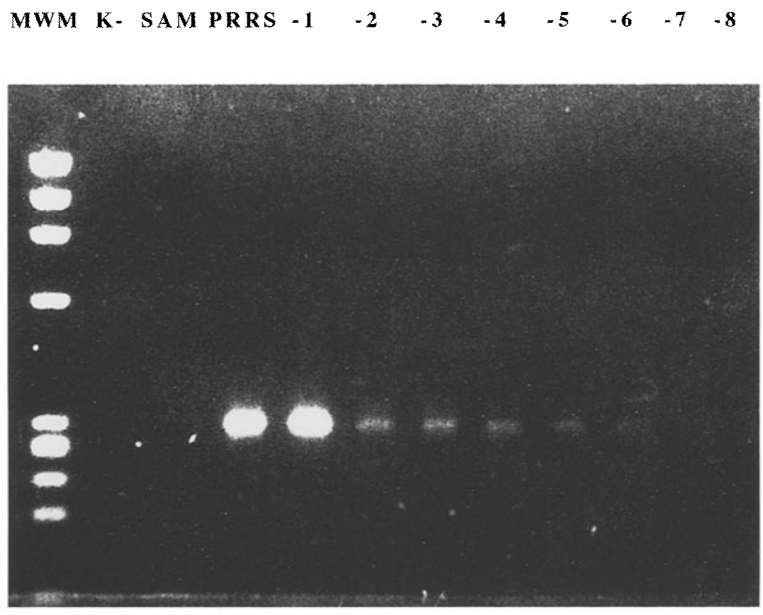

Fig. 2. RT-PCR sensitivity. Ten fold dilutions of the extracted PRRS virus RNA (6.7×10^6 $TCID_{50}$) from 1 to 10-8 (*PRRS, 1* to *8*) were subjected to a RT-PCR reaction. Product was clearly detected up to a 10^{-6} dilution which corresponds to 6.7 $TCDI_{50}$. RNA from swine alveolar lung macrophages (*SAM*) and a negative control (*K-*) without RNA were also subjected to the RT- PCR reaction rendering no product. *MWM* indicates the ∅X174 Hae III molecular weight markers employed in the 1.5% agarose gel showed

Specificity was also established for the RT-PCR reaction with the SRRS virus against other swine virus mentioned under Materials and methods. When all assayed viruses were adjusted to a 10^5 $TCDI_{50}$/ml titer and the RT-PCR was carried out, not only the DNA viruses (parvovirus and Aujeszky virus) rendered no amplification products but also RNA viruses (transmissible gastroenteritis virus and swine influenza virus) and the closest RNA *Togaviridae* viruses (hog cholera virus and bovine viral diarrhoea) yielded no amplification bands as shown in Fig 3, where only PRRS virus produced the expected size DNA fragment.

Fig. 4. Early detection of PRRS virus by RT-PCR in crude clinical samples from a experimental inoculated pig, 5-days post-infection. PRRS virus, strain 5710, was inoculated to 3-weeks old pigs and biological samples were treated as described under Materials and Methods. The 1.5% agarose gel shows the 312 bp amplification product obtained in the RT-PCR assay from the following samples: *1* PCR negative control; *2* positive control of PRRS virus from 1 ml of 5×10^6 $TCID_{50}$ SAM culture supernatant; *3* alveolar lung macrophages; *4* lung; *5* liver; *6* spleen; *7* kidney; *8* serum; *9* buffy-coat; *10* plasma; *11* submaxillar lymphonodule; *12* inguinal lympho-nodule; *13* tonsils. *MWM* indicates the ∅X174 Hae III molecular weight markers employed

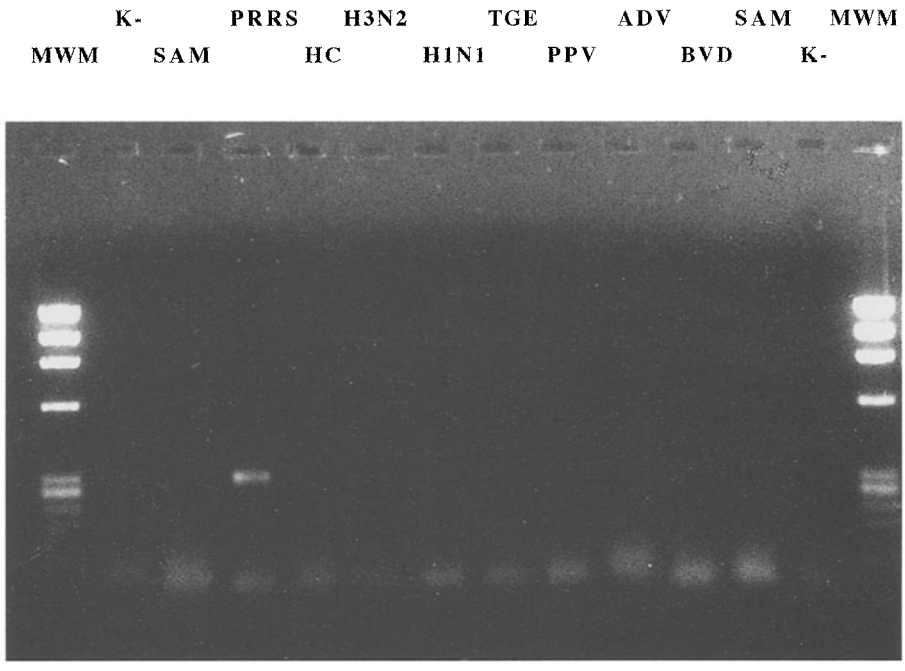

Fig. 3. RT-PCR specificity. Primers specificity was tested running a RT-PCR reaction with several swine viruses: Hog cholera virus, Alfort/187 strain (*HC*); swine influenza virus, A/Ghent/1/84 strain (*H3N2*) and A/Belgium/1/83 strain (*H1N1*); transmissible gastroenteritis virus, Purdue strain (*TGE*); porcine parvovirus, NADL-l8 strain (*PPV*); Aujezsky disease virus, NIA-3 strain (*ADV*); and bovine virus diarrhea, Singer strain (*BVD*). PRRS virus as positive control (*PRRS*), swine alveolar lung macrophages (*SAM*) and a negative control (*K-*) without virus were also subjected to the test. All virus were adjusted to 10^5 $TCID_{50}$ titer. *MWM* indicates the ∅X174 Hae III molecular weight markers employed in the 1.5% agarose gel

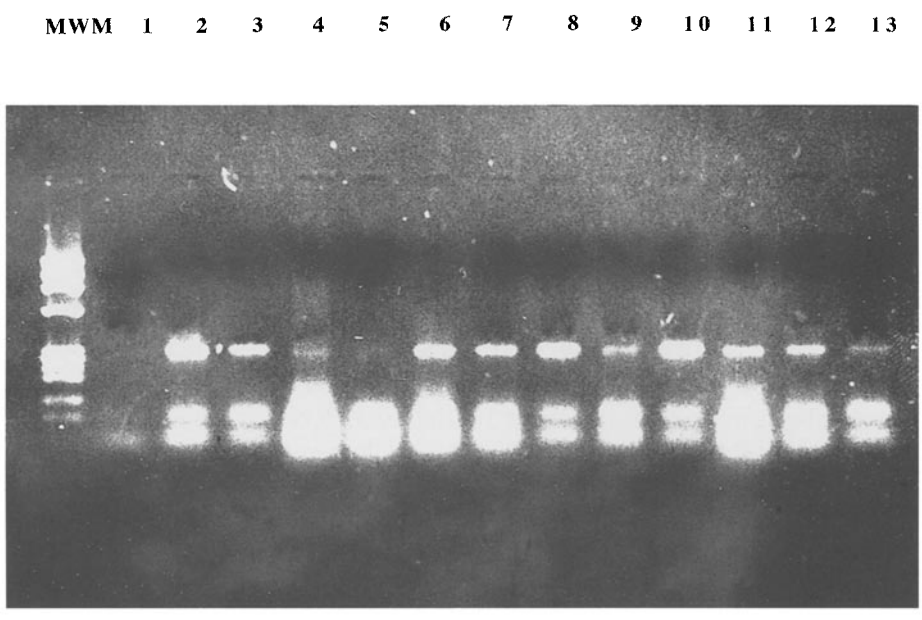

Table 1. Comparison of the direct detection of PRRS virus by RT-PCR and the level of detection by titration of the cytopathic effect on SAM cultures

	5 day post-infection		10 day post-infection	
	RT-PCR	titre	RT-PCR	titre
Macrophages	+	10^5	+	$10^{5.39}$
Lung	+	10^3	n	$10^{2.49}$
Liver	+	10^2	n	ud
Kidney	+	$10^{2.5}$	n	$10^{2.24}$
Spleen	+	10^2	n	$10^{1.75}$
Submaxillar ganglion	+	10^3	n	10^2
Plasma	+	$10^{3.5}$	+	$10^{2.74}$
Buffy-coat	+	$10^{3.5}$	n	ud
Inguinal Ln	+	10^2	n	$10^{1.75}$
Tonsil	+	10^3	n	$10^{1.75}$
Serum	+	10^4	+	10^3

All assays were conducted in duplicate from the same clinical samples treated as described under Materials and methods. Titres are given in $TCID_{50}$, undetectable titres are indicated by *ud.* RT-PCR positives or negatives are indicated by + or *n* respectively

PRRS virus detection in crude clinical samples

The experimentally infected pigs showed no clinical signs and only a mild transient fever (39.8–39.9 °C) appeared 5–6 days post-infection. As shown in Fig. 4, in pigs sacrified 5 days post- infection, PRRS virus was detected by RT-PCR in all analyzed samples by the presence of a single product of 312 bp. The amplified band from liver and lung was weaker than in the rest of tissues. After 10 days post-infection the virus was only detected in macrophages, plasma and serum (the latter showing a very weak band). These results are in agreement with the titration experiments on SAM cultures (Table 1) where it is possible to correlate titers above or around 10^2 $TCID_{50}$ and the presence of positive bands by RT-PCR.

Discussion

The results reported here demostrated the feasibility to amplify a 312 bp specific DNA fragment corresponding to PRRS virus RNA sequence encoding the nucleocapsid protein by two subsequent reactions. By priming with a specific antisense oligonucleotide annealed to the viral RNA it is generated a cDNA template for the 5′ (sense) primer, which let PCR amplification to occur, allowing a rapid method to directly detect the PRRS virus in cultures and in crude clinical samples. Additionally, no extra bands of amplification are obtained in the samples containing virus due to the high stringency employed during the PCR reaction and the specific sequence of the primer used for the cDNA synthesis,

making easy the interpretation of the results. On the other hand, even in cycles where annealing temperature of 37 °C was employed (preliminary assays not shown) no additional bands (not specific amplification) were obtained.

The variability found in the ORF 7 sequences from our isolates compared with the original report of the sequence (LV) is not very high, at least in the amplified region (6 to 10 bp changes out of 256). Furthermore, as capsid protein structure is not highly perturbated by the detected point mutations, as expected for this essential assembly protein, we are optimistic that our designed primers could be applied to detect a wide range of PRRS virus from several strains, at least in the European continent (as none of the American strains were available to us for analysis due to patent pending). This also suggests that our target primer-sequences lie within a relative conserved area of the PRRS virus genome, and this is a desirable feature for this kind of methodology.

From the specificity test carried out with other seven swine viruses, five of them carrying RNA genome, we can conclude that is not very probable to obtain false positives from samples submitted for routine PRRS virus detection co-infected with any of the assayed viruses which are the most common and prevalent in swine herds.

The results obtained by the RT-PCR assay over crude clinical samples have limits of detection around 10^2 $TCID_{50}$ as observed from the correlation with the titration experiments on SAM cultures of the equivalent biological samples. However, sensitivity of the RT-PCR technique is greatly improved by first inoculating the biological samples on SAM cultures reaching a limit of sensitivity about 6 $TCID_{50}$. In view of the results here reported, it is advisable either to use alveolar macrophages, serum or plasma as source of viral RNA for early detection of PRRS by direct RT-PCR (as in these samples clear positives were obtained), or either run a first (and unique) step of inoculation in SAM cultures of the suspected pathological samples to enhance the subsequent RT-PCR assay. These data suggest an increase in sensitivity of at least 10^5 times in relation with hybridization techniques for some DNA virus [1, 12], in addition to the well known lower levels of sensitivity of Northern compared with Southern hybridization techniques [10]. High sensitivity will be very appreciated for diagnostic if, like other togavirus, few PRRS viral particles are shed in body secretions by infected animals [15].

Up to date, detection of PRRS is based on the determination of the virus antigens in infected cell from specific cell cultures. This procedure has several limitations as at least three blind passages of the clinical sample on primary alveolar lung macrophages are required to improve the minimal titer to be detected by immunofluorescence or inmunoperoxidase staining [16, 18]. Moreover, in our hands the reliability of this latter diagnostic method has shown to depend on the macrophages batch, as we detected quite different levels of sensitivity even in macrophages from individuals of the same herd (results not shown). Based on the proven efficiency and reliability of the RT-PCR method as described here, to detect PRRS causative virus, as well as its low cost and fast routine make it the choice technique to screen a high number of samples.

As the pathogeny of this disease is not very well known, we are currently working out new improved methods of sample treatment from different pathological sources to detect the virus in order to advance our understanding of the pathogenic mechanism and ways of dissemination of the PRRS.

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References

1. Andino RH, Torres HN, Polacino PS, Shudel A, Palma EL (1987) Detection of bovine herpesvirus 1 nucleic acid sequences using a dot blot hybridization procedure. Am J Vet Res 48: 984–987
2. Benfield DA, Nelson E, Collins JE, Harris L, Goyal SM, Robison D, Christianson WT, Morrison RB, Gorcyca D, Chladek D (1992) Characterization of swine infertility and reproductive syndrome (SIRS) virus (isolate ATCC-VR2332). J Vet Diagn Invest 4: 127–133
3. Chomczynski PY, Sacchi N (1987) Single-step method of RNA isolation by acid guanidium thiocyanate-phenol-chloroform extraction. Anal Biochem 162: 156–159
4. Collins JE, Benfield DA, Christianson WT, Harris L, Hennings JC, Shaw DP, Goyal SM, McCullough S, Morrison RB, Joo HS, Gorcyca D, Chladek D (1992) Isolation of swine infertility and respiratory syndrome virus (isolated ATCC VR- 2332) in North America and experimental reproduction of the disease in gnotibiotic pigs. J Vet Diagn Invest 4: 117–126
5. Deveraux J, Haeberli P, Smithhies O (1984) A comprehensive set of sequence analysis programs for the VAX. Nucleic Acids Res 12: 387–395
6. Domingo E, Holland JJ, Ahlquist P (1988) RNA genetics, vol 3. Variability of RNA genomes. CRC Press, Boca Raton
7. Hanahan D (1983) Studies on transformation of *Escherichia coli* with plasmids. J Mol Biol 166: 557–561
8. Hill, H (1990) Overview and history of mystery swine disease (swine infertility respiratory syndrome). In: Proc Mystery Swine Disease Committee Meeting, Denver, October 6, 1990. Livestock Conservation Institute, Madison, pp 29–31
9. Loula T (1990) Clinical presentation of mystery pig disease in the breeding herd and suckling piglets. In: Proc Mystery Swine Disease Committee Meeting, Denver, October 6, 1990. Livestock Conservation Institute, Madison, pp 37–40
10. Mason PJ, Willians, JG (1985) Title of paper In: Rickwood D, Hames BD (eds) Nucleic acid hybridisation. IRL Press, Oxford, pp 113–137
11. Meulenberg JJM, Hulst MM, de Meijer EJ, Moonen PLJM, den Besten A, de Kluyver EP, Wensvoort G, Moormann RJM (1993) Lelystad virus, the causative agent of porcine epidemic abortion and respiratory syndrome (PEARS), is related to LDV and EAV. Virology 192: 62–72
12. Pacciarini M, Agresti A, Desimone F, Poli G, Torretta E, Siccardi AG, Meneveri R, Ginelli E (1988) Detection of bovine herpesvirus I (BHVI) semen infections by a dot-blot hybridization assay. Br Vet J 141: 55–63

13. Plagemann PGW, Moenning V (1992) Lactate dehydrogenase-elevating virus, equine arteritis virus, and simian hemorrhagic fever virus: a new group of positive-strand RNA viruses. Adv Virus Res 41: 99–192
14. Reed JJ, Muench RH (1938) A simple method to estimating fifty percent end points. Am J Hyg 27: 493–497
15. Ward P, Misra V (1991) Detection of bovine viral diarrhea virus, using degenerate oligonucleotide primers and the polymerase chain reaction. Am J Vet Res 52: 1231–1236
16. Weensvoort G, Terpstra C, Pol JMA, ter Laak EA, Bloemraad M, de Kluyver EP, Kragten C, Van Buiten L, Den Besten A, Wagenaar F, Broekhuijsen JM, Moonen PLJM, Zetstra T, de Boer EA, Tibben HJ, De Jong MF, Van't Veld P, Groenland GJR, Van Gennep JA, Voets M Th, Verheijden JHM, Braamskamp J (1991) Mystery swine disease in the Netherlands: the isolation of Lelystad virus. Vet Q 13: 121–130
17. Weensvoort G, de Kluijver EP, Luijtze EA, den Bestenl A, Harris L, Collins JE, Christianson WT, Chladek C (1992) Antigenic comparison of Lelystad virus and swine infertility and respiratory syndrome virus. J Vet Diagn Invest 4: 134–138
18. Yoon IJ, Joo HS, Christianson WT, Kim HS, Collins JE, Morrison RB, Dial GD (1992) An indirect fluorescent antibody test for the detection of antibody to swine infertility and respiratory syndrome virus in swine sera. J Vet Diagn Invest 4: 144–147
19. Zimmerman J, Sanderson T, Eernisse KA, Hill HT, Frey ML (1992) Transmission of SIRS virus from convalescent animals to commingled penmates under experimental conditions. In: International Symposium on Swine Infertility and Respiratory Syndrome, St Paul, 1992

Authors' address: Dr. J. M. Castro, Departamento de Patología Animal I, Universidad Complutense de Madrid, Facultad de Veterinaria, 28040 Madrid, Spain.

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