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Development of a real-time RT-PCR method for detection of porcine rubulavirus (PoRV-LPMV)

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A B S T R A C T

In order to provide a rapid and sensitive method for detection of the *Porcine rubulavirus* La Piedad-Michoacan-Mexico Virus (PoRV-LPMV), we have developed a specific real-time reverse transcriptase polymerase chain reaction assay. The detection of PoRV-LPMV, represents a diagnostic challenge due to the viral RNA being present in very small amounts in tissue samples. In this study, a TaqMan® real-time PCR assay was designed based on the phosphoprotein gene of PoRV-LPMV, to allow specific amplification and detection of viral RNA in clinical samples. Assay conditions for the primers and probe were optimized using infected PK15 cells and ten-fold serial dilutions of a plasmid containing the whole P-gene. The sensitivity of the developed TaqMan® assay was approximately 10 plasmid copies per reaction, and was shown to be 1000 fold better than a conventional nested RT-PCR. The performance of this real-time RT-PCR method enables studies of various aspects of PoRV-LPMV infection. Finally, the assay detects all current known variants of the virus.

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1. Introduction

La Piedad-Michoacan-Mexico virus (PoRV-LPMV) is the causative agent of the blue eye syndrome of pigs (Moreno-Lopez et al., 1986; Stephano et al., 1988). The clinical signs vary considerably and depend mainly of the age of the pig. Very young pigs are generally more affected by encephalitis, pneumonia and corneal opacity (Stephano et al., 1988). However, recent observations suggest that the virus also affects older pigs, with severe signs including high rates of neurological symptoms in both fattening and adult pigs (Sánchez-Betancourt et al., 2008), although this has not been experimentally demonstrated. The virus have been characterized and studied at the molecular level (Berg et al., 1991, 1992, 1997; Linné et al., 1992; Reyes-Leyva et al., 1999; Svenda et al., 1997; Sundqvist et al., 1990, 1992), the cellular level (Hjertner et al., 1997, 1998; Reyes-Leyva et al., 1997; Wiman et al., 1998) and through experimental infections (Allan et al., 1996; Stephano et al., 1988; Stephano, 2002; Hernandez-Jauregui et al., 2004; Hernandez-Jauregui et al., 2001).

The genome consists of approximately 15 kb of negative single stranded RNA. Early phylogenetic analyses indicated that the virus is most related to mumps virus and simian virus 5 (Berg et al., 1991, 1992, 1997; Sundqvist et al., 1992; Svenda et al., 1997, 2002), although the identity to these two viruses at the amino acids level was only about 40%. More recent genetic studies have shown that PoRV-LPMV is more closely related to a virus isolated from bats, the Mapuera virus (Wang et al., 2007). PoRV-LPMV has been classified in the order *Mononegavirales*, family *Paramyxoviridae*, subfamily *Paramyxovirinae*, genus *Rubulavirus* and species *Porcine rubulavirus* (ICTV data 2005).

Only one full-length genome of PoRV-LPMV has been characterized fully, an isolate from 1984 (LPMV/84). There is therefore limited information about the genetic variation of this virus. The F-gene of PoRV-LPMV has been sequenced from LPMV/84 and from the “San Fandila-1988′′ isolate. The F-gene from these two isolates differed at 16 nucleotides, giving four amino acids changes (Berg et al., 1997). In addition to the LPMV/84, the HN-gene has also been sequenced from 10 other isolates originating from 1990 to 2003 (Sánchez-Betancourt et al., 2008). Over these years only about 71 nucleotide positions differ in the HN-gene, corresponding to about four percent of the nucleotides in the gene. Since this protein

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probably is the major immunogenic protein of the virus and is prone to antigenic drift, one can assume that the other internal genes are more conserved, although this has not been shown of PoRV.

Concerning possible diagnostic tests, at present there are only serological tests and classical virus isolation techniques available (McNeilly et al., 1997; Nordengrahn et al., 1999). In addition, classical PCR technology has been used for various research purposes (Berg et al., 1992; Wiman et al., 1998; Cuevas et al., 2009). However, the real-time PCR method is not yet available. This technique has many advantages over classical PCR, such as better sensitivity and specificity (Belák and Thorén, 2001). Also, it is possible to quantify the exact levels of viral RNA, if needed, and the procedure is much less prone to contamination problems.

There are many unanswered questions concerning the PoRV-LPMV persistence in domestic pigs, and regarding possible reservoirs in nature, and it would therefore be advantageous to use real-time RT-PCR to investigate these important questions. The purpose of this study was to develop a sensitive and specific real-time RT-PCR assay based upon the TaqMan® technology to detect PoRV-LPMV. The developed assay was tested on various clinical materials, to evaluate its suitability, including representatives of all known variants of the virus. In addition, the sensitivity of the new real-time RT-PCR assay was compared to a conventional nested RT-PCR (Wiman et al., 1998).

2. Materials and methods

2.1. Virus, cell line and tissue samples

For the initial set-up and sensitivity tests, a plasmid containing the whole P-gene was used (Berg et al., 1992). Porcine Rubulavirus infected PK15 cells were used for testing the procedure,along with the original reference strain of LPMV (Moreno-Lopez et al., 1986), called "PoRV-LPMV′′ (Lamb et al., 2005). The virus was propagated in monolayers of PK15 cell line cultures with a titer of 107 TCID50%/ml. After infection of the cells and observation of cytopathic effects (48 h post-infection), the supernatant fluid was collected and titered according to standard methods. One ml of supernatant fluid was aliquoted into sterile cryotubes and frozen at −70 °C until further use. Mock-infected PK15 cells were included as a negative control.

Clinical samples were collected from pigs infected naturally and experimentally through a research project at the National Microbiology Research Center, INIFAP, in Mexico City. The experiments undertaken were previously approved by the Ethical and Animal Welfare Committee. In brief: 11 tissue samples were collected from naturally infected pigs; eleven samples came from experimentally infected pigs; five samples were obtained from viral isolates in PK15 cell culture from clinical cases of naturally infected pigs, and six tissue samples were collected from non-infected pigs (Table 1). Two of the selected samples were obtained from lung tissue, but most of the specimens were taken from the central nervous system, including the mid brain and olfactory bulb. The samples were kept at −80 °C until further use. All of the sampled pigs were certified free of Aujesky's disease and porcine reproductive respiratory syndrome.

The suitability of the assay for use in a clinical setting was verified using tissue samples from PoRV-LPMV infected pigs, and viral isolates of PoRV collected in Mexico from 2008 to 2010 genetically related to CI (1991), CII (1991), CIII (1999), PAC-1; PAC-2 (1990); PAC-3 (1992), PAC-4 (1993) and PAC 9 (2003) (Sánchez-Betancourt et al., 2012). One of the clinical samples was from semen. All tissue samples were tested in duplicate, and each PCR contained 50 ng/μl of total RNA.

Table 1
Results of real-time PCR assay suitability on clinical material – 50 ng/μl of total RNA from each tissue sample and viral isolates was used in each PCR reaction.

Natural Infected Pigs	Sample type	TaqMan result (Ct) PoRV-LPMV
1	Mid brain	28.25
2	Mid brain	29.26
3	Mid brain	>40
4	Mid brain	34.34
5	Olfactory bulb	33.41
6	Olfactory bulb	>40
7	Olfactory bulb	>40
8	Mid brain	26.17
9	Mid brain	31.09
10	Mid brain	33.73
11	Lung	29.45
Experimental infected pigs		
1	Olfactory bulb	30.42
2	Olfactory bulb	29.20
3	Olfactory bulb	28.3
4	Olfactory bulb	27.98
5	Olfactory bulb	29.03
6	Olfactory bulb	30.12
7	Olfactory bulb	25.11
8	Olfactory bulb	30.42
9	Mid brain	33.39
10	Mid brain	27.45
11	Lung	21.5
Viral isolation	Tissue culture PK 15 supernatant from	
c-1	Semen	29.76
c-2	Mid brain	16.93
c-3	Mid brain	17.58
c-4	Mid brain	21.8
c-5	Mid brain	21.96
Antigenic variant of PoRV	Tissue culture PK 15 supernatant from	
1	PAC 1	20.31
2	PAC 2	15.33
3	PAC 3	14.40
4	PAC 4	14.53
Reference strain	LPMV	24.65
Non-infected pigs		
1	Mid brain	–
2	Mid brain	–
3	Mid brain	–
4	Mid brain	–
5	Mid brain	–
6	Olfactory bulb	–

2.2. RNA isolation

Total RNA was extracted from the PoRV-LPMV reference strain and the mock-infected PK-15 cells, as well as from the clinical viral isolates and tissue samples, using Trizol according to the manufacturer's instructions (Invitrogen Life Technologies, Carlsbad, CA, USA). Briefly, 250 μl of cell culture supernatant or 100 mg of tissue samples were homogenized with a sterile single-use pestle (Kontes Glass, Vineland, NJ, USA) in Trizol liquid. The rest of the protocol was followed strictly. The RNA concentration was quantified spectrophotometerically, (NanoDrop ND-100, NanoDrop Technologies, Montchamin, DE, USA) at OD 260 nm and the purity was assessed by determining the OD 260/OD 280 ratio, where a ratio greater than 1.8 is usually considered to be an acceptable indicator of good RNA (Fleige and Pfaffl, 2006). The extracted RNA was kept at −80 °C until further use.

2.3. Quality of RNA and internal control

The quality of the RNA for each sample was tested by evaluating the expression of the housekeeping gene cyclophilin. Primers

for cyclophilin were designed as described by Duvigneau et al. (2005): forward primer [5′-TGCTTTCACAGAATAATTCCAGGATTTA-3′], reverse primer [5′-GACTTGCCACCAGTGCCATTA-3′] and the probe [5′-FAM TGCCAGGGTGGTGACTTCACACGCC-BHQ1-3′]. The real-time TaqMan assay was carried out using the one-step EZ RT-PCR master mix reagents kit (Applied Biosystems). 50 or 100 ng/μl of total RNA were added directly to the real-time PCR reaction containing 1× EZ buffer, 0.6 μM dNTP, 2.5 μM Mn(OAC)2), 0.4 μM reverse primer, 0.4 μM forward primer, 0.4 μM TaqMan® probe, 2.5 U r*Tth* polymerase in a total volume of 25 μl. The thermal cycling protocol was: 42 °C for 5 min and 60 °C for 20 min for reverse transcription; followed by the PCR step including an initial denaturation at 95 °C for 2 min followed by 48 cycles at 95 °C for 15 s and 60 °C for 60 s.

2.4. PoRV-LPMV primers and probe

The P-gene is one of the most highly expressed, and so the system on this gene was constructed accordingly. The oligonucleotide primers were designed to match the assay conditions, such as amplicon size and melting temperature. A multiple nucleotide sequence alignment was used to design a set of PCR primers specific to the P-gene of PoRV-LPMV. This set of primers was: forward primer [5′-TGG TGC AAC CCA GCA TGT-3′], the reverse primer [5′-GCA CTT TGC GGG GCA GG-3′] and the TaqMan® probe [5′-FAM-CAA GAT ACTACC AAT GCA CCT GT-BHQ-3′] labeled with 6-carboxyfluorescein (FAM) and with blackhole-quencher (BHQ1). These correspond to the positions 681–698, 718–740 and 746–762 of the P-gene (accession number AF416650), respectively. The amplicon had a total length of 60 base pairs.

2.5. PoRV-LPMV TaqMan real-time assay

The real-time TaqMan assay was carried out using r*Tth* DNA polymerase & EZ buffer Pack (Gene-Amp® Applied Biosystems). In the first step the concentration of primers, probe and other reagents, as well as annealing temperature, were optimized individually and then combined using the P-gene plasmid as template. In the final protocol, 50 or 100 ng of total RNA was added to the real-time PCR containing 1× EZ buffer, 0.6 μM dNTP, 2.5 μM $Mn(OAC)_2$, 0.4 μM reverse PoRV primer, 0.4 μM forward PoRV primer, 0.4 μM PoRV-LPMV TaqMan® probe and 2.5 U r*Tth* polymerase in a total volume of 25 μl. Real-time detection of the PCR products was performed on a Rotor-Gene 3000 (Corbett Research, Sydney, Australia) by measuring the fluorescence signal emitted at the end of each annealing step. All samples and controls were run in duplicate. A no-template control of nuclease-free water was included in each run. The thermal cycling protocol was: 42 °C C for 5 min and 60 °C for 20 min – for reverse transcription, followed by the PCR step including an initial denaturation at 95 °C for 2 min and 50 cycles of 95 °C for 15 s and annealing/extension at 58 °C for 60 s. However, the r*Tth* protocol described above when testing clinical samples was shown to give very low fluorescence signals, and so we switched to the AgPath-ID One-step RTPCR kit (Applied Biosystems, Foster City, CA, USA), which has been reported to have a greater sensitivity in comparison with other commercially available kits (Stephens et al., 2010). The original concentrations of primers and probe were maintained unmodified and the buffer and enzymes were added according to the manufacturer's instructions. The thermal cycling protocol was: 48 °C C for 30 min – for reverse transcription; followed by the PCR step including an initial denaturation at 95 °C for 10 min and 50 cycles of 95 °C for 15 s and annealing/extension at 58 °C for 60 s. A number of the positive PCR reactions were separated on a 2% agarose gel, and purified by Wizard SV Gel and PCR Clean up system (Promega, Madison, USA), according to the manufacturer's protocol. The purified PCR products were subsequently sequenced using the ABI PRISM BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA), following the manufacturer's instructions.

2.6. Evaluation of the sensitivity of the PoRV-LPMV PCR assay

For determination of the analytical sensitivity of the assay, the copy numbers of the DNA P-gene plasmid were calculated and ten-fold serial dilutions were made. The copy number of the plasmid ranged between 10^7 to 1 copy per reaction, and each concentration was run in triplicate. Based on these PCR results, a standard curve checking the sensitivity was generated. RNA from uninfected and PK15 infected cells were used to test the sensitivity of the assay. Briefly, duplications of ten-fold serial dilutions of total RNA were run to determine the detection limit.

2.7. Specificity test

The specificity was tested using the real-time RT-PCR system on genetically different porcine rubulavirus isolates as mentioned earlier and other relevant RNA viruses, such as vesicular stomatitis virus, borna disease virus, feline corona virus and rabies virus.

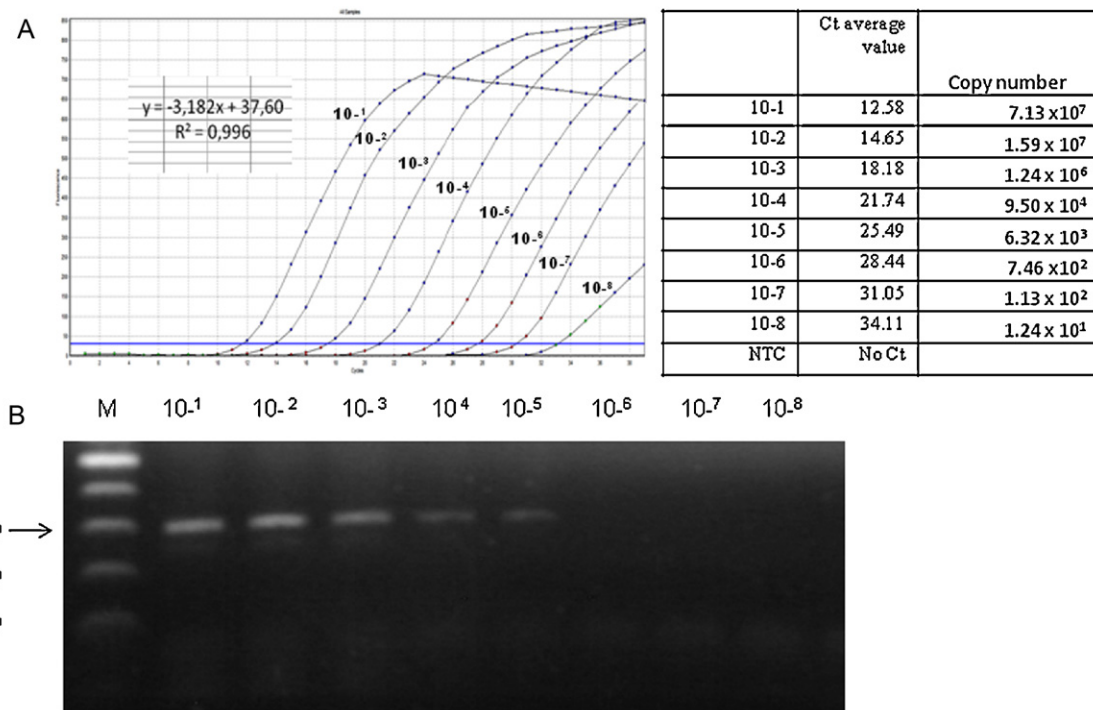
2.8. Sensitivity comparison between the real-time PCR assay and a nested PCR (nPCR)

The performance of the developed real-time RT-PCR assay was compared with the conventional nested PCR assay described by Wiman et al. (1998). Briefly, the sensitivity of the nPCR was evaluated and compared to that of the real-time PCR assay by running the PCR using the P-gene plasmid in copy numbers ranging between 10^8 and 10^1 copies per reaction. The external primers were [5′-CCAGTCCGAGGTTCATCATCCAC-3′] (sense) and [5′-TGCGGCCCTCGATTGCTTTC-3′] (antisense); and the internal primers were [5′-ATGAGGGCGATCTGATGGCG-3′] (sense) and [5′-ATCTCCGGCACATTGAGGGC-3′] (antisense). The first PCR was performed in a reaction mix containing 1× PCR buffer, 1.5 mM $MgCl_2$, 0.2 μM dNTP mix, 2.5 U Ampli Taq DNA polymerase (Perkin Elmer Cetus, Norwalk, CA, USA), 0.2 pmol of each external primer and 2 μl of P gene plasmid. The thermal cycling protocol was: five cycles of 94 °C for 1 min (denaturation), 62 °C for 1 min (annealing) and 72 °C for 2 min (extension). This was followed by 30 cycles of 94 °C for 1 min (denaturation), 58 °C for 1 min (annealing) and 72 °C for 2 min (extension), followed by 72 °C for 10 min. For the nested PCR, 5 μl of the first PCR reaction was transferred to a nPCR mix in a 50 μl solution as described above, but with the exception that the internal primers were used. The thermal cycling protocol was: 25 cycles of 94 °C for 1 min (denaturation), 58 °C for 1 min (annealing) and 72 °C for 2 min (extension), followed by 72 °C for 10 min. PCR products were analyzed by agarose gel electrophoresis (1.5% gel) and visualized by ethidium bromide staining. The final nested product was 298 bp.

3. Results

3.1. RNA quality control – cyclophilin gene

The quantity (OD: 260 nm) and purity (OD ratio: 260/280 nm absorption ratio > 1.80) were considered when selecting the samples. As an internal control of the RNA quality a cyclophilin gene PCR assay was performed on all of the RNA extracts. All samples included in this study were positive for the cyclophilin, indicating RNA of good quality.



	Ct average value	Copy number
10-1	12.58	7.13 x10⁷
10-2	14.65	1.59 x 10⁷
10-3	18.18	1.24 x 10⁶
10-4	21.74	9.50 x 10⁴
10-5	25.49	6.32 x 10³
10-6	28.44	7.46 x10²
10-7	31.05	1.13 x 10²
10-8	34.11	1.24 x 10¹
NTC	No Ct	

Fig. 1. Sensitivity comparison between the developed real-time PCR assay and a nested PCR using ten-fold serial dilutions of the P-gene plasmid (10^{-1}–10^{-8}). (A) The estimated detection limit achieved by new real-time PCR assay was approximately 10 copies per reaction (1.24×10^1); (B) the nested PCR amplification was able to detect at a dilution 10^5 (6.32×10^3 copies): M, 100 bp = marker.

3.2. Sensitivity test

The sensitivity of the assay was estimated by testing ten-fold dilutions of the plasmid ranging from 10^1 to 10^8 copies of plasmid per reaction. Quantitative analysis showed that the lower detection limit achieved was approximately 10 copies per reaction; and the Ct value at the detection limit point was 34.11 ± 0.94. The standard curve displayed a linear relationship between the Ct value and the copy number, with a reaction coefficient (R^2 value) of 0.996 and the reaction efficiency was approximately 100% ($Y = -3.186$). In comparison with the conventional nested PCR, the sensitivity of TaqMan assay was 1000 fold greater (Fig. 1A and B). The sensitivity of the assay was also tested using ten-fold serial dilutions of total RNA extracted from PK15 PoRV-LPMV infected cells, ranging from 1 to 0.0001 ng RNA/reaction, and showed a detection limit of 0.001 ng RNA/reaction.

3.3. Specificity test

A number of products from the PoRV-LPMV positive samples were sequenced in order to confirm the correct amplification. The sequence analysis showed that the product corresponded to the expected genomic region of PoRV-LPMV P-gene. Furthermore, the assay was found to be specific for PoRV-LPMV, as no amplification was seen when tested against other RNA viruses, such as vesicular stomatitis virus, borna disease virus, feline corona virus and rabies virus (data not shown).

3.4. Clinical samples

The suitability of the assay on clinical material was evaluated using thirty-three clinical samples. Twenty-two of these samples were recovered from naturally infected pigs and from experimentally infected pigs of PoRV-LPMV; and five samples were obtained from viral isolates in PK15 cell culture from clinical cases recovered from 2008 to 2010 in Mexico. Six tissue samples came from non-infected pigs. Most of the samples were obtained from brain tissue; however, two samples from lung tissue and one from semen were also included. A majority of the samples from the naturally and experimentally infected pigs as well as the viral isolates were positive for PoRV-LPMV, while the specimens from non-infected pigs remained negative (Table 1).

4. Discussion

PoRV-LPMV has been endemic in Mexico since the early 1980s, and it is still a problem for the pig-farming industry in Mexico (Moreno-Lopez et al., 1986; Stephano et al., 1988; Escobar-López et al., 2011). Where the virus came from and how it was introduced into pigs is unknown. But it was found that PoRV-LPMV is most closely related to Mapuera virus isolated from a fruit bat in Brazil in 1979 (Wang et al., 2007). In addition, in 2004 the presence of antibodies to PoRV from a non-hematophagous bat on the subtropical Pacific coast of Mexico was reported (Salas-Rojas et al., 2004). It is therefore possible that bats introduced the virus to pigs. However, other natural reservoirs are also possible. Until now it has been impossible to study this, since no suitable diagnostic method has been available. To be able to investigate the spread of PoRV-LPMV in nature and in domestic pigs, where the virus now is endemic, a new detection tool is necessary. A real-time RT-PCR assay was therefore developed for PoRV-LPMV, to be able to study these and other aspects of the viral disease.

Real-time RT PCR has many advantages over other diagnostic methods. For example, the assay has high sensitivity and specificity, and the procedure limits possible cross-contamination. For the assay presented here the P-gene was used since this gene is highly expressed (Hjertner et al., 1998), and because its sequence is available (Berg et al., 1992). As for many other negative stranded RNA viruses, the number of LPMV transcripts diminishes from the three-prime end of the genome. Therefore the genes closest to the

three prime end are the logical choice for a sensitive system as possible.

In this study material from different PoRV isolates was used, and the assay was shown to detect representatives of all known isolates from historical samples, from early collections to the present ones, independently of the antigenic and genetic divergence of PoRV isolates. This finding is highly relevant for diagnostic application, since it was recently suggested that there are genetically and antigenically different PoRV strains circulating in the swine population of the affected geographical area (Escobar-López et al., 2011; Sánchez-Betancourt et al., 2012).

The assay was optimized to be able to detect small numbers of viral RNA, and to avoid non-specific amplification. The viral RNA could be detected in 100% of tissue samples from experimentally infected pigs, and in all viral isolates from clinical cases recovered from infected pigs in the endemic area of the disease (Michoacán and Jalisco state of Mexico). Interestingly, one of the positive clinical samples was collected from semen. This finding could be an advantage that leads to a broader application of the real time PCR in the swine industry, since PoRV may be transmitted through semen (Solis et al., 2007). Viral excretion in the semen may thus play a major epidemiological role in the transmission of the disease.

Viral RNA could also be detected in the majority of the samples from naturally infected pigs, despite the specimens being taken from pigs that were killed one year after recovery from natural infection, thereby confirming the high sensitivity of this test. Thus, it is possible to detect the virus when the viral RNA is present in very small amounts in tissue samples, such as from pigs that have recovered from an acute infection. This observation is in agreement with those reported by Wiman et al. (1998). It also shows that this test can be used to study archived material. A small number of infected specimens produced a low fluorescent signal, high Ct value or gave negative results. This was, however, not surprising, as it has been demonstrated by Allan et al. (1996) that the distribution of the PoRV-LPMV in different tissues is very localized, and the localization of virus in the brain indicates an age dependent mode of spread.

Apart from PoRV-LPMV detection in the brain, two samples from the lung were also positive. Wiman et al. (1998) also found that, during acute infection in experimental infected pigs, the virus could be recovered and detected from respiratory tract tissue.

The use of a housekeeping gene (Duvigneau et al., 2005) as a quality control of the RNA extraction method was here adopted. It was important to confirm the absence of PCR inhibitors in each sample, thereby avoiding the possibility of false negative results. Using material from other relevant viruses, the real-time RT-PCR was shown to be specific for PoRV.

In summary, a sensitive and specific real-time RT-PCR assay for the detection of all known isolates of PoRV in clinical samples, with a detection limit of about ten copies per reaction and 0.001 ng RNA/reaction of total RNA, was developed. The assay can be used for further virological studies.

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