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Porcine rubulavirus LPMV RNA persists in the central nervous system of pigs after recovery from acute infection

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In order to study persistence of the porcine rubulavirus LPMV, we examined tissue samples collected from pigs 53 days after experimental infection. These pigs survived the initial infection and could clinically be considered to have recovered from the infection. Two of the pigs used in this study were chemically immunosuppressed during the last 4 days before necropsy. No infectious virus or viral antigen could be detected in any tissue using standard methods for virus isolation and detection. However, the presence of viral genomic RNA and mRNA could be demonstrated in the mid brain of the convalescent pig using an optimised RT-nested PCR. Mid brain, forebrain and lung were all shown to contain LPMV RNA in the immunosuppressed convalescent pigs. In addition we examined the P-gene editing in the recovered pigs and conclude that the viral genome is transcriptionally active in these pigs. The relevance of the persistence of LPMV for maintenance and spread within and/or between pig populations is discussed.

Keywords: paramyxoviridae; rubulavirus; persistent infection; P-gene editing

Introduction

Porcine rubulavirus LPMV (La Piedad Michoacan Mexico Virus), the causative agent of 'blue eye disease' in pigs, is a member of the paramyxoviridae family (Rima *et al*, 1995). Infection of pigs with LPMV causes encephalitis, pneumonia, and corneal opacity. In general the disease is more severe in young piglets (Stephano *et al*, 1988). LPMV has been analysed in great detail at the molecular level. The whole 15 kb genome has been sequenced and phylogenetic analysis has revealed the closest relatives to be mumps virus and simian virus 5 (Berg *et al*, 1991, 1992, 1997; Linné *et al*, 1992; Sundqvist *et al*, 1992; Svenda *et al*, 1997, 1998). Furthermore, the expression of the P-gene has been analysed. LPMV expresses the P-gene in a similar way to mumps virus, editing being necessary for expression of the P protein (Berg *et al*, 1992).

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A detailed sequential study of virus distribution in tissues of pigs experimentally infected with LPMV has recently been reported (Allan *et al*, 1996). Pigs inoculated at 3 days of age with LPMV suffered from severe clinical signs and by day 8 after infection they were either dead or moribund. In contrast, pigs inoculated with virus at 17 days of age showed only mild clinical signs. Virus could be demonstrated in tissue samples from the respiratory tract and in the central nervous system (CNS) in pigs inoculated both at 3 and 17 days of age. However, differences in virus distribution within the CNS were demonstrated. In the younger pigs, virus was widespread throughout the CNS. In contrast, virus was restricted to the olfactory bulb and mid brain in the older pigs and no virus could be detected in any organ at 14 days after infection i.e. the infection seemed to be cleared.

Since the first outbreak of LPMV, the disease has spread throughout Mexico with disease outbreaks recorded in many states. Stephano *et al* (1988)

suggested that subclinically infected pigs could serve as a reservoir for the virus. They noted that when susceptible pigs were introduced into a farm operating on a continuous flow basis with a prior history of LPMV infection the newly introduced group showed clinical signs of LPMV infection. However, when a sentinel pig was introduced into closed herd 6–12 months after resolution of an outbreak the pig remained asymptomatic (Stephano, 1990). It is possible that persistence of LPMV in convalescent pigs could serve as a reservoir for the virus and may reactivate in a situation of natural immunosuppression and spread to other susceptible pigs. Whether LPMV can persist in convalescent pigs in nature has not yet been demonstrated.

The porcine rubulavirus LPMV can establish persistent infections in porcine kidney cells (Hjertner *et al*, 1997, 1998). The persistent infection has been shown to be stable for at least one year in continuous culture. Virus was observed to be released from the cells but at lower infectious titers than those released from lytically infected cells. Several large subgenomic RNAs were associated with the persistently infected cell, a common phenomenon in many persistent infections (Re, 1991). Reduced expression of several viral proteins was also noted.

The aim of this study was to document the possible persistence of LPMV and viral RNA in pigs after recovery from clinical illness. The results show that no infectious virus particles could be detected in any tissue. Virus specific RNA, both genomic and mRNA, could however be detected in brain tissue 53 days after infection. Viral RNA could also be detected in lung tissue when the convalescent pig was treated with the immunosuppressive drug cyclophosphamide 4 days prior to necropsy. We also show that mRNA editing of the P-gene occurs in the convalescent pigs, indicative of active viral transcription in these pigs.

Results

The aim of this study was to investigate whether LPMV can persist *in vivo* and if persistent virus can be reactivated by immunosuppression (CPA treatment) of pigs after recovery from clinical illness. To do so in a controlled way we examined tissue materials from three convalescent pigs 53 days after infection. Tissue samples from these animals were subjected to histophatological examination, virus isolation, immunofluorescent staining of cryostat sections and analysis for the presence of viral RNA.

Clinical signs and histopathology

The three pigs inoculated at 17 days of age with LPMV-84 virus all showed mild clinical signs typical of porcine rubulavirus infection, including mild respiratory and nervous signs between 4 and 10 days after inoculation. All three pigs recovered and were clinically normal from 12 days after infection. During the immunosuppression (CPA treatment) no clinical signs were noted in pigs R220 and R221. Histopathological examination revealed no evidence of active inflammation in tissue sections from pig R219. Pig R220 and R221 however showed active inflammation, involving leptomeninges and brain tissue (meningoencephalitis). The brain areas affected included the olfactory system and adjacent frontal lobe and also mid brain and pons. The inflammatory response consisted of perivenous lymphocytic cuffing, neuronophagia and microglial nodule formation predominantly in the grey matter (Figure 1). This type of inflammatory response is characteristic of a viral infection in the CNS. The lesions are similar to those observed in the acutely infected pig. However, the distribution of the inflammatory response was different. In the acutely infected animal the inflammation was predominantly in the nasal submucosal tissue and adjacent olfactory areas. In the convalescent CPA treated pigs (R220 and R221) the inflammation is also present in brainstem structures.

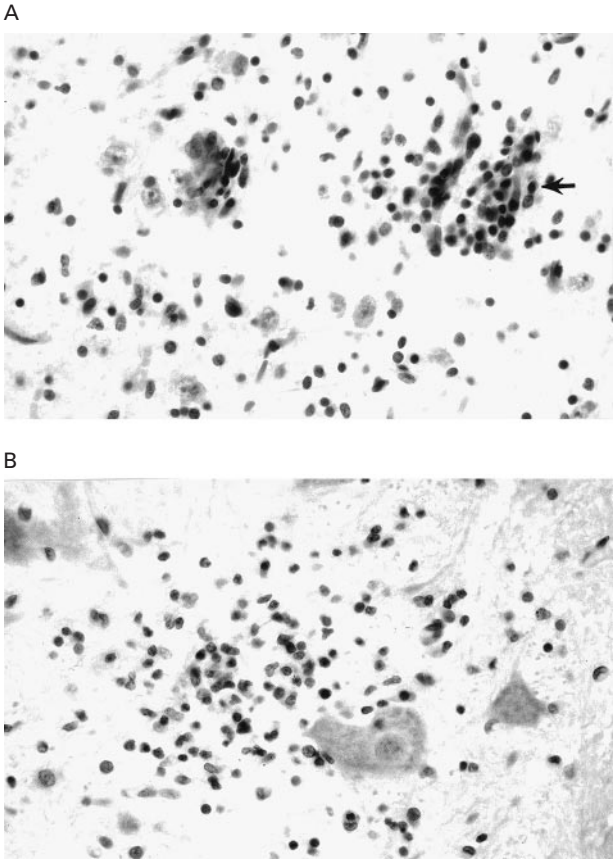

Figure 1 (**a**) Section through mid brain showing areas of active inflammation. The inflammatory infiltrate (arrow) is composed of lymphocytes located perivascularly ×2.5 magnification. (**b**) Brain stem nuclei with adjacent lymphocytic infiltrate ×2.5 magnification.

Absence of infectious virus and viral antigen in convalescent pigs

Virus isolation has previously been conducted on acutely infected pigs, infected at 17 days of age. Virus could be recovered from the CNS and this was restricted to the mid brain and olfactory bulb (Allan *et al*, 1996). However, in the convalescent pigs (R219, R220 and R221) tested in this study, no infectious virus could be detected in any tissue, including mid brain, forebrain and lung. The rectal and nasal swabs taken daily from day 49 after infection until necropsy also tested negative for infectious virus. In the acutely infected control pig (R233) virus was recovered from respiratory tract tissue and was also seen to be widespread in the CNS. No virus was detected in the uninfected control pig (R400). In addition immunofluorescent staining of cryostat sections was performed on the same tissues as were used for virus isolation, and no viral antigen could be detected in the recovered pigs or in the uninfected control pig. Viral antigen could be detected in the respiratory tract and in the CNS of the acutely infected control pig.

Presence of LPMV specific RNA in convalescent pigs

RT-nested PCR was performed using 1 μg of total RNA isolated from mid brain, forebrain and lung as template. Tissues which tested negative were retested with 10 times more (10 μg) of total RNA. In the P-gene RT-nested PCR analysis we included assays using appropriate primers for selection of LPMV genomic RNA (P genomic primer) or LPMV mRNA (oligo dT primer) in the RT reaction.

In the acutely infected control pig (R233), both NP- and P-gene specific LPMV RNA could be demonstrated in the organs tested (Figure 2 lanes 18 and 20, Figure 3b lanes 13–15, Figure 3c lane 6). In this pig nested PCR was not necessary and 1 ng RNA was enough to detect LPMV specific RNA (data not shown). No LPMV specific RNA could be detected in the uninfected control pig (R400) (Figure 2 lanes 11, 21, 22, Figure 3a lane 3, Figure 3b lanes 3–5 and Figure 3c lane 3). In the convalescent pig (R219), P gene specific LPMV mRNA could only be detected using 10 μg total RNA from mid brain (Figure 3a lanes 4,5) but not RNA from forebrain or lung (Figure 3b lane 6, Figure 3c lane 4). None of the tissues used in the assay tested positive for NP gene specific RNA (Figure 2 lanes 7, 8, 19). However, in convalescent pigs which had been immunosuppressed with CPA (R220 and R221) both NP and P gene specific LPMV RNA could be detected in mid brain and forebrain (Figure 2 lanes 6, 9, 10, Figure 3a lanes 7–9 and Figure 3b lanes 7–12). In one of the CPA treated convalescent pigs (R220) LPMV RNA was demonstrated in lung tissues as well, but the amount of RNA present was low (Figure 2 lane 23, Figure 3c lane 5). A summary of all these results is presented in Table 1.

The results presented here clearly demonstrate that both genomic RNA and mRNA are present in the brain of convalescent pigs. This indicates that LPMV can persist *in vivo*. Furthermore, the amount

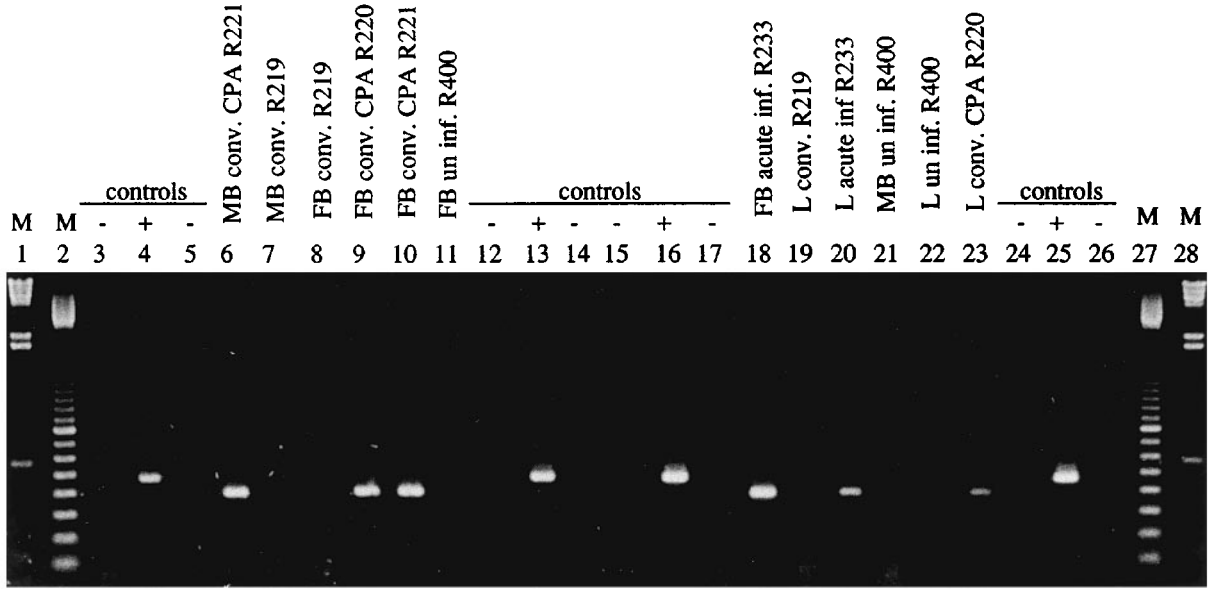

Figure 2 LPMV NP-gene specific RT-nested PCR products visualized by agarose gel electrophoresis. RT-nested PCR done with total RNA extracted from mid brain, forebrain and lung. Random hexamer primer are used in the RT reaction (lanes 6–11 and 18–23). M indicates 100 base pair (bp) ladder (lanes 2 and 27) and λ DNA *Hind*III digest (lanes 1 and 28). + indicates positive plasmid control (lanes 4, 13, 16 and 25). conv. indicates convalescent pig.

of detectable LPMV RNA increases in the brain of immunosuppressed pigs and is even detectable in the lungs of these animals.

Primer extension analysis demonstrates active transcription in convalescent pigs

Since some transcripts from the P-gene contain extra nontemplated nucleotides it is possible to assay for active transcription by identifying these mRNAs. The 298 base pair fragments shown in Figure 3a and b, were used in the primer extension assay. As expected, only the 28 nt product was seen to be present when the genome specific primer was used in the RT reaction (Figure 4a lanes 4–7). When oligo(dT) primer was used in the RT reaction, samples from the acutely infected control pig (R233) and the convalescent CPA treated pigs (R220 and R221) were shown to contain RNAs which gave both 28 and 30 nt products, as judged by comparison with the V and P plasmid controls (Figure 4a lanes 9–12). Surprisingly, the convalescent pig (R219) show only the extension product originating from a P specific transcript (Figure 4a lane 8). This is somewhat surprising, but may indicate that only a few molecules are being amplified in the RT-nested PCR, and that these organs contain quantities of LPMV RNA which are at the lower limit for detection by this method. Another explanation could be that the frequency of the P transcripts are much higher in this pig. To check this we tested mid brain from the convalescent pig (R219). This time we used an mRNA selection approach to increase the amount LPMV RNA/μg total RNA. We also tested mid brain from the convalescent CPA treated pig (R220). This time only the unedited transcript was detected in pig R219 (Figure 4b lanes 4, 5). However this mRNA selection approach may select the negative stranded genomic RNA since it can bind to the mRNA during the selection which will give a high background of genomic RNA.

This result endorses our view that the RNA amount is at the lower limit for detection. These observations further confirm that both genome RNA and mRNA of the P gene are present in the convalescent pigs, although in very small amounts. It also shows that mRNA editing of the P gene occurs, and that viral transcription is active.

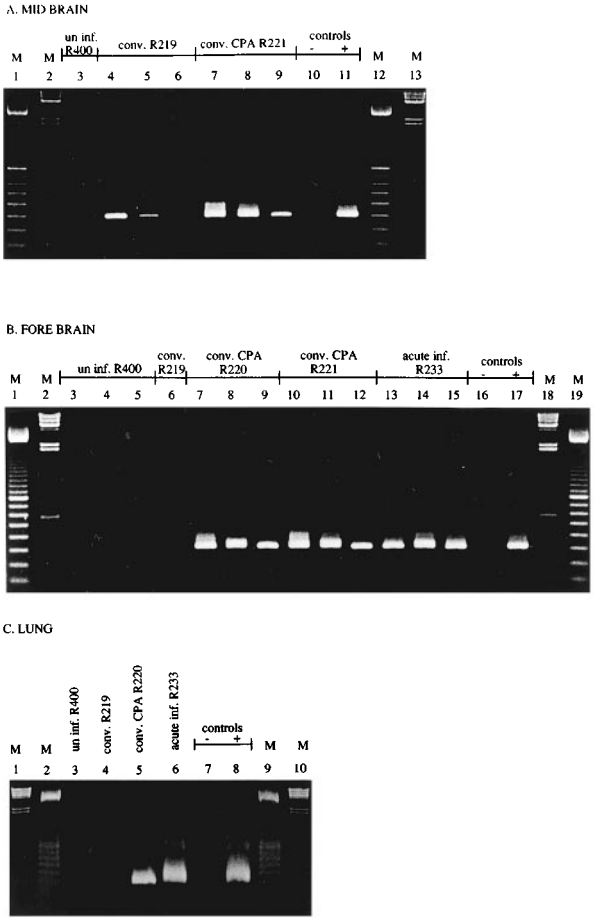

Figure 3 LPMV P-gene specific RT-nested PCR products visualized by agarose gel electrophoresis. conv. indicates convalescent pig. (**a**) RT-nested PCR done with total RNA extracted from mid brain. M indicates 100 base bair (bp) ladder (lanes 1 and 12) and λ DNA *Hind*III digest (lanes 2 and 13). Primers used in the RT reaction are; random hexamer (lanes 3, 4, 7, 10 and 11), oligo dT (lanes 5 and 8) and P347+ (lanes 6 and 9). (**b**) RT-nested PCR done with total RNA extracted from forebrain. M indicates 100 base pair (bp) ladder (lanes 1 and 19) and λ DNA *Hind*III digest (lanes 2 and 18). Primers used in the RT reaction are; random hexamer (lanes 3, 6, 7, 10, 13, 16 and 17), oligo dT (lanes 4, 8, 11 and 14) and P347+ (lanes 5, 9, 12 and 15). (**c**) RT-nested PCR done with total RNA extracted from lung. M indicates 100 base pair (bp) ladder (lanes 1 and 10) and λ DNA *Hind*III digest (lanes 2 and 9). Primer used in the RT reaction is random hexamer (lanes 3–8).

Discussion

New outbreaks of blue eye disease appear to emerge selectively in farms working with a continuous flow system suggesting that newly introduced pigs acquire the disease from pigs in the herd into which they are introduced (Stephano *et al*, 1988). This observation has led to the contention that a reservoir of LPMV is maintained in subclinically infected pigs. In view of a finding by Allan *et al* (1996) this seems unlikely. The study conducted by this group showed that no infectious virus could be isolated from day 14 post infection and onwards in LPMV infected pigs. An alternative explanation of the epidemiological data could be that persistently infected pigs which have recovered from the acute phase of infection act as a reservoir, shedding virus in situations of natural immunosuppression such as stress. The aim of this study was to elucidate whether the porcine rubulavirus LPMV could persist in pigs after full recovery from infection. In

three cases out of three, we have demonstrated that LPMV RNA persists in pigs for at least 53 days after infection. No infectious virus or viral antigen could be detected. Both genomic RNA and mRNA were detected at this time. In addition, RNA editing of the P gene was demonstrated, indicating that the viral genome was actively transcribed. According to these findings it seems likely that LPMV can persist at least in the form of viral genetic material, possibly without the production of infectious virus. This may be of importance for maintenance of the virus infection in the individual and could also have profound effects on the spread of virus within and/or between pig populations.

Results similar to these have been reported for Vesicular Stomatitis Virus, New Jersey strain (VSV-NJ) infections in hamster (Barrera and Letchworth, 1996) and in cattle (Letchworth *et al*, 1996). In hamsters, persistence of VSV RNA could be detected in the CNS 10–12 months after infection, but no infectious viral particles could be detected. In cattle, persistence of VSV RNA but not infectious viral particles could be detected in the tongue and in lymph nodes draining the tongue 5 months after infection. Our results agree well with these findings, indicating that persistence of viral RNA may be a fairly common feature after clearance of negative stranded RNA virus infections.

In a situation of immunosuppression, infectious virus could be produced and shed by persistently infected animals enabling infection of susceptible pigs. In our study, immunosuppression led to the detection of enhanced levels of LPMV RNA. Also, viral RNA could be detected in lung tissue. This indicates that a limited reactivation of the virus occurred. Immunosuppression of the pigs used in this study was carried out for a fairly short time, 4 days. Prolonged suppression might lead to further reactivation of the LPMV and the eventual production of infectious viral particles. Experiments utilising prolonged immunosuppression times will be of importance in elucidating whether recurring

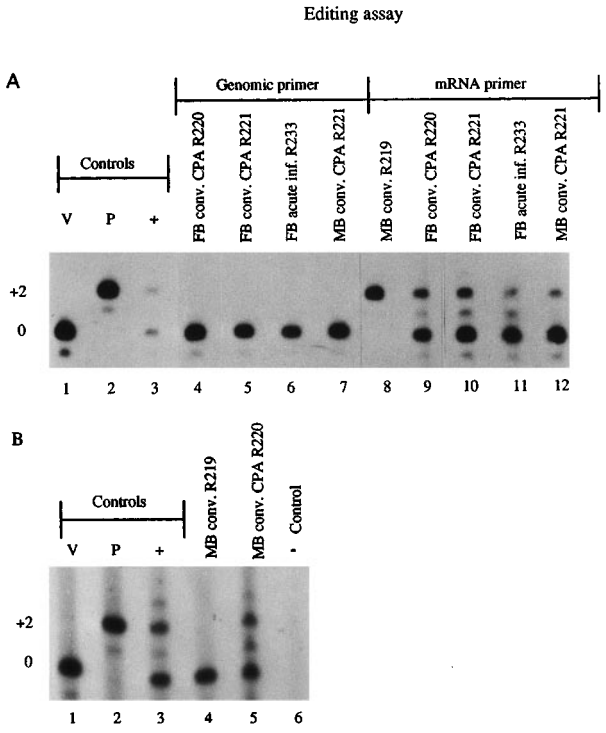

Figure 4 Primer extension analysis of RNA isolated from recovered and acute infected pigs. V indicates a plasmid control containing only V transcript cDNA (lane 1). P indicates a plasmid control containing only P transcript cDNA (lane 2). The V transcript is indicated with O. The P transcript is indicated with +2. conv. indicates convalescent pig. (**a**) Primers used in the RT reaction are; P-genomic (lanes 4–7) and oligo dT (lanes 8–12). (**b**) mRNA selected with oligo dT beads. RT reaction with random hexamer primer (lanes 3–6). Control indicates PCR product from the F-gene as a negative primer extension control.

Table 1. Summary of RT-nested PCR analysis of LPMV convalescent pigs

Animal	*Organ material*	*NP-gene PCR pd(N)⁶**	*P-gene PCR pd(N)⁶**	*P-gene PCR oligo(dT)**	*P-gene PCR genomic**
Convalescent CPA	Midbrain (1 μg RNA)	+	+	+	+
R221	Forebrain (1 μg RNA)	+	+	+	+
Convalescent CPA	Midbrain (1 μg RNA)	ND	+[a]	ND	ND
R220	Forebrain (1 μg RNA)	+	+	+	+
	Lung (10 μg RNA)	–	+	ND	ND
Convalescent	Midbrain (10 μg RNA)	–	+[b]	+	–
R219	Forebrain (10 μg RNA)	–	–	ND	ND
	Lung (10 μg RNA)	–	–	ND	ND
Uninfected	Midbrain (10 μg RNA)	–	–[b]	ND	ND
R400	Forebrain (10 μg RNA)	–	–	–	–
	Lung (10 μg RNA)	–	–	ND	ND
Acute infected					
R233 (no nested	Forebrain (1 μg RNA)	+	+	+	+
PCR needed)	Lung (1 μg RNA)	+	+	ND	ND

*Primers used in reverse transcription. ND indicates not done. [a] indicates that the organ is tested with mRNA selected material only. [b]indicates that the organ was tested with both mRNA and total RNA selected material.

outbreaks of blue eye disease in farms with a continuous flow system, originate from persistently infected pigs.

Several factors are of importance in promoting the establishment of persistent infection of a cell. The virus has to assume a non-lytic mode of replication preferably in a long-lived cell which is not subjected to immune surveillance and escape the immune response (Randall and Russell, 1991). Neurons lack constitutive expression of MHC class I, and are comparatively insensitive to the induction of de novo expression of these molecules. Furthermore, they are very long-lived and thus meet both requirements for establishment of persistent infection (Sedgwick and Dörries, 1991). LPMV has been shown to infect neuronal cells by immunofluorescent staining of cryostat sections (Kennedy, S personal communication).

It has been shown that the highest titers of virus in acutely infected pigs with LPMV can be found in the mid brain (McNeilly *et al*, 1997). Therefore, we decided to assay for RNA in brain tissue of convalescent pigs as an initial step in characterisation of the persistent state. Other organs however could be more important in terms of virus shedding and spread to other animals. We therefore extended our study to include lung tissue, and one of the immunosuppressed pigs was shown to harbour viral RNA in the lung.

In conclusion, both LPMV genome and mRNA can be recovered in pigs after recovery from the acute phase of the disease, indicating persistent infection of these pigs. Whether these pigs can shed infectious virus remains to be shown.

Materials and methods

Virus

A cell culture strain of the porcine rubulavirus, designated LPMV-84, was used throughout this study. A virus pool was prepared. The infectious titer of this working pool was calculated to be $10^{7.50}$ $TCID_{50}$/0.1 ml. This virus pool was examined for evidence of contamination with pseudorabies virus, classical swine fever virus, and hemagglutinating encephalitis virus using direct immunofluorescence (IF) staining of acetone-fixed cell culture preparations. No evidence of contamination of the LPMV preparation with these viruses was detected.

Pigs

All of the pigs used in this study were obtained from a closed, minimal disease Large White breeder/finisher unit. The animals were shown to be free of porcine rubulavirus, pseudorabies virus, porcine parvovirus and classical swine fever virus by indirect immunofluorescence using sera from the pigs and cell cultures infected with either of the three viruses.

Three 17-day-old pigs (R219, R220, R221) were experimentally infected by intranasal and eyedrop routes with $10^{7.00}$ $TCID_{50}$ of the pool of LPMV-84 virus described above. They were euthanized after infection by intravenous barbiturate injection at 53 days after inoculation. R220 and R221 were chemically immunosuppressed by inoculation with cyclophosphamide (CPA) by the intraperitoneal route (30 mg/kg body weight) at 49 days after infection and again at 51 days after infection. CPA has been used as an immunosuppressant drug in pigs (Mackie, 1981). Pig R219 was not chemically immunosuppressed. Rectal and nasal swabs were taken daily from all three pigs from 49 days after infection until necropsy. Pig R233 was incoculated with the same virus pool and dose at 3 days of age and sacrificed when clinical signs of virus infection were evident (Allan *et al*, 1996). Pig R400 served as an uninoculated control for the duration of the experiment.

At necropsy, tissue samples listed in Allan *et al* (1996), were taken for histopathological examination, virus isolation, immunofluorescent staining of cryostat sections and analysis for the presence of viral RNA.

Histopathology

Immediately following death the brain and spinal cord were extracted and fixed in 10% formalin. The nasal mucosa and olfactory projection to the nasal area were extracted in continuity with the rest of the brain. Following fixation in formalin the tissues were dissected according to a fixed protocol. The brain stem and cerebellum were dissected from the brain and then the cerebral hemispheres were divided into 10 coronal slices from anterior to posterior (frontal to occipital). The brain stem and cerebellum were then sectioned from mid brain to medulla in transverse slices. Sections were taken from the cervical, thoracic and lumbar spinal cord. Each of these sections was stained with Haematoxylin and Eosin (H&E).

Virus isolation and immunofluorescent staining of cryostat sections

Virus isolation has been described earlier (Allan *et al*, 1996). Briefly, tissue samples were suspended in Minimal Essential Medium, centrifuged, and the supernatant was inoculated into PK-15 cell cultures and incubated for 6 days at 37°C. The cell cultures were then freeze/thawed once and the cell lysates were inoculated into fresh PK-15 cell cultures, incubated and freeze/thawed again as described above. The resulting cell lysates were assayed for hemagglutination (HA) activity and by immunofluorescence (IF) using a hyperimmune antiserum to the LPMV-84 isolate prepared from a rabbit (Allan *et al*, 1996). Tissue samples for cryostat sectioning were processed and immunostained (McNeilly *et al*, 1991) using the same antiserum as above.

RT-nested PCR of LPMV RNA

Total RNA was purified using the RNaid® KIT (BIO 101), and polyA$^+$ RNA was purified using the Quick Prep®Micro mRNA Purification Kit (Pharmacia Biotech) according to the manufacturer's protocol. Either 1 or 10 μg RNA in a volume of 9 μl was heat denatured (70°C, 5 min), and reverse-transcribed in 20 μl containing 4 μl 5 × RT buffer (250 mM Tris-HCl pH 8.3, 375 mM KCl, 15 mM $MgCl_2$, 50 mM DTT), 4 μl 2.5 mM dNTP mix, 1 μl 33 u/μl RNA guard (Pharmacia Biotech), 1 μl 200 u/μl moloney murine leukeamia virus reverse transcriptase (Promega) and 1 μl 20 pmol/μl primer (random hexamers, oligo(dT) or LPMV genome (P-gene) specific; 5′-GGCGCACCCTACACTCCCCA-3′). The mixture was incubated at 20°C for 5 min and then 37°C for 90 min. The reverse transcriptase was subsequently inactivated at 98°C for 5 min. Ten μl of this viral cDNA was then amplified by nested PCR. Primers for the LPMV virus NP gene were designed from the NP gene sequence (Svenda *et al*, 1998) and had the following sequence: external primers, 5′-ATTCTC-CTTGCCTGCTGCTGCTAT-3′ (sense) and 5′-AGTG-CCAAGTATCGTGCCTGTTCA-3′ (antisense); internal primers, 5′-TCCCCCGATGCGATTATTGAG-3′ (sense) and 5′-CCCCCTTCGAGCTGGATTCTG-3′ (antisense). The final nested product was 375 bp (or 474 bp for the plasmid control). Primers for the LPMV virus P-gene extending over the editing site, were designed from the P-gene sequence (Berg *et al*, 1992) and had the following sequence: external primers, 5′-CCAGTCCGAGGTTCATCATCCAC-3′ (sense) and 5′-TGCGGCCCTCGATTGCTTTC-3′ (antisense); internal primers, 5′-ATGAGGGC-GATCTGATGGCG-3′ (sense) and 5′-ATCTCCGG-CACATTGAGGGC-3′ (antisense). The final nested product was 298 bp.

The first PCR was carried out in a 50 μl solution containing 5 μl 10 × PCR buffer (100 mM Tris-HCl, pH 8.3, 500 mM KCl, 0.01% gelatin), 3 μl 25 mM $MgCl_2$ 4 μl 2.5 mM dNTP mix, 0.2 μl 5 u/μl Ampli Taq® DNA Polymerase (Perkin Elmer), 1 μl 10 pmol/μl each of the external primers and 10 μl cDNA. The thermal cycling program consisted of: five cycles of 94°C for 1 min, 58°C (NP gene primer set) or 62°C (P gene primer set) for 1 min and 72°C for 2 min, followed by 30 cycles of 94°C for 1 min, 53°C (NP gene primer set) 58°C (P-gene primer set) for 1 min and 72°C for 2 min, followed by 72°C for 10 min. For the nested PCR, 5 μl of the first PCR reaction was transferred to a nested PCR mix in a 50 μl solution as above except that internal primers were used. The thermal cycling program consisted of: 25 cycles of 94°C for 1 min, 53°C (NP gene primer set) 58°C (P gene primer set) for 1 min and 72°C for 2 min, followed by 72°C for 10 min.

To be able to differentiate between the positive control and positive samples, we constructed a clone which gave a longer PCR product (474 bp) compared to the wild type (375 bp), and had a unique restriction site, enabling us to specifically digest the control PCR product. The polylinker in the pUC-19 plasmid was amplified by PCR using M13 universal and reversal primers (Pharmacia). The resulting PCR product was blunt and ligated into a filled in *Nsi*I site in a pUC19-NP-gene plasmid.

Total RNA extracted from lytically infected cells was included in each set of experiment as a positive control. In addition, we also included several negative dH_2O controls, to demonstrate that our routines were free from contamination. The final PCR product was analysed by agarose gel electrophoresis (1.5% gel), and visualized by ethidium bromide staining. Some of the PCR products were extracted from agarose gel slices using the QIAEX DNA Gel Extraction Kit (QIAGEN), according to the manufacturer's protocol, and subsequently used in primer extension analysis. To avoid contamination of the PCR reactions, careful routines for each step were set up, with separate rooms for: RNA extraction, RT-reaction, first PCR, nested PCR and PCR product analysis. UV-light and 10% chlorine solution were used after each step to destroy possible contaminants.

Quantification of the NP specific RT-nested PCR

A pcDNA3 plasmid construct containing the NP gene (pcDNA3-NP) was digested with *Xho*I. RNA was transcribed from the cleaved plasmid using T7 RNA Polymerase according to the protocol supplied by the manufacturer (Biolab). The reaction mixture was incubated at 37°C for 2 h, then treated with DNase I to remove the DNA template. After DNase I treatment the RNA was extracted with phenol-chloroform and then precipitated. The washed and dried RNA pellet was redissolved in diethyl pyrocarbonate treated water and extracted using the RNaid® KIT. The RNA was quantified spectrophotometrically at absorbance 260 nm, and a portion was analysed on an agarose gel to check the quality. To confirm that RNA and not original template DNA was amplified, the 'synthetic' RNA was assayed by nested PCR without reverse transcription. Extracted total RNA from mid brain from the negative control pig at a concentration of 0.1 μg/μl was used as carrier RNA to make 10-fold dilution series with the 'synthetic' RNA, which was subsequently used in the RT-nested PCR. Viral RNA could be detected at a dilution of 10^6 (1000 copies) but not 10^7 (100 copies). The sensitivity of the P gene RT-nested PCR was not tested, but our experiments indicate that it is at least as sensitive as the NP gene PCR or possibly slightly better.

Primer extension analysis

The primer extension analysis has been described previously (Berg *et al*, 1992). Briefly, the extracted PCR product was denatured with NaOH and then precipitated and annealed with a ^{32}P-5′ labelled

primer complementary to sequences immediately downstream of the editing site of the P-gene of LPMV. The extension was done with T7 DNA polymerase (Pharmacia Biotech) and a label mix consisting of dCTP, dGTP, dTTP and ddATP. The final products were either 28 nt (unedited transcript) or 30 nt (edited transcript) in length. They were separated by 15% PAGE-urea-gel electrophoresis and visualised by autoradiography.

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