Detection of porcine reproductive and respiratory syndrome virus, porcine circovirus type 2, swine influenza virus and Aujeszky’s disease virus in cases of porcine proliferative and necrotizing pneumonia (PNP) in Spain

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Abstract

Proliferative and necrotizing pneumonia (PNP) is a severe form of interstitial pneumonia characterised by hypertrophy and proliferation of pneumocytes type 2 and presence of necrotic cells within alveoli lumen. Many viral agents have been linked to PNP aetiology, with especial emphasis on porcine reproductive and respiratory syndrome virus (PRRSV). To gain knowledge on PNP causality, a retrospective study on 74 PNP cases from postweaning pigs from Spain was carried out. Coupled with histopathological examinations, the presence of porcine circovirus type 2 (PCV2) by in situ hybridization (ISH), and PRRSV, swine influenza virus (SIV) and Aujeszky’s disease virus (ADV) by immunohistochemical (IHC) methods, were investigated. PCV2 was the most prevalent viral agent in PNP cases (85.1%) followed by PRRSV (44.6%); 39.1% of PNP cases showed PCV2 as the solely detected agent, while only 4.1% had PRRSV as the unique pathogen. SIV and ADV were very sporadically detected in PNP cases, and always in co-infection with PCV2. Therefore, present data indicate that PCV2 is the most important aetiological agent in PNP cases from Spain and that PRRSV is not essential for the development of PNP. Taking into account the presented results and available literature, we suggest that PCV2 is possibly the main contributor to PNP cases in Europe while PRRSV could play a similar role in North America.

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Keywords: Swine; Proliferative and necrotizing pneumonia; Porcine reproductive and respiratory syndrome virus; Porcine circovirus type 2; Swine influenza virus; Aujeszky’s disease virus

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1. Introduction

Proliferative and necrotizing pneumonia (PNP) is a severe form of interstitial pneumonia described in pigs for the first time in 1990 in Quebec, Canada (Morin et al., 1990). Lungs with PNP are extensively affected, showing lack of pulmonary collapse, plum-red or grayish colour, moist and meaty aspect, and rubbery texture. Interlobular oedema is also common (Morin et al., 1990). Histologically, PNP is characterized by two main features: (a) lymphohistiocytic interstitial inflammation with hypertrophy and proliferation of type 2 pneumocytes, and (b) presence of clumps of necrotic inflammatory cells within alveolar spaces (Morin et al., 1990; Drolet et al., 2003). Moreover, other histopathologic lesions, such as hyaline membranes, intra-alveolar lymphoplasmacytic infiltrates, bronchial and bronchiolar necrosis or multinucleate giant cells, can also be present in some cases (Morin et al., 1990).

Initial studies attributed PNP causality to a novel strain of swine influenza virus (SIV) type A (Girard et al., 1992). However, subsequent investigations indicated the rare involvement of SIV, and porcine respiratory and reproductive syndrome virus (PRRSV) was proposed as the main causal agent of PNP (Larochelle et al., 1994). In the late 1990s, some authors suggested the possible involvement of porcine circovirus porcine type 2 (PCV2) in PNP cases (Ellis et al., 1999). Thereafter, a later study indicated that PNP was a result of co-infection of PRRSV and PCV2 (Pesch et al., 2000). However, participation of PCV2 has given contradictory results; Drolet et al. (2003) suggested that PRRSV was the main contributor to PNP and ruled out PCV2 as a significant one.

Therefore, to expand the current knowledge on PNP causality, the present work was aimed to determine the occurrence of PRRSV and PCV2 in cases of PNP from Spain. Moreover, the potential presence of SIV, which was initially suspected as the main cause of PNP (Girard et al., 1992), and Aujeszky’s disease virus (ADV), a viral agent also associated to respiratory disease in pigs in Spain (Gutierrez-Martin et al., 2000), were also investigated.

2. Materials and methods

2.1. Case selection

A retrospective study of 74 PNP cases from postweaning pigs received at the Pathology Diagnostic Service at the Veterinary School of Barcelona (Catalonia, Spain), between 2001 and 2005, was carried out. Selection of cases was based on the presence of the two microscopic hallmarks cited above (Morin et al., 1990).

2.2. Histopathological studies

Five consecutive 4 μm thick sections of each paraffin-embedded block including the PNP affected lung tissues were cut. One section was processed for histopathology, one for PCV2 nucleic acid detection by in situ hybridization (ISH), and the remaining were processed to detect PRRSV, SIV and ADV antigens by immunohistochemical methods, respectively.

Severity of the microscopic lesions was evaluated. The severity of hypertrophy and proliferation of type 2 pneumocytes and the presence of necrotic cells were semi-quantified following similar previously score systems (Opriessnig et al., 2004), including mild, moderate or severe lesional intensity, independently of multifocal or diffuse distribution. Concomitantly, the presence of other lesions, such as bronchial and bronchiolar epithelial necrosis, pulmonary necrosis, catarrhal-purulent bronchopneumonia and fibrous or fibrinous pleuritis were also noted.

2.3. In situ hybridization (ISH) to detect PCV2

A previously described ISH technique to detect PCV2 was performed (Rosell et al., 1999) using a 41 pb digoxigenin labeled DNA probe corresponding to ORF1 of PCV2 (DIG-CCT TCC TCA TTA CCC TCG CCA ACA ATA AAA TAA TCA AA). Briefly, lung tissue sections were placed on Probe On Plus glass microscope slides (Fisher Scientific). A work station (MicroProbe™ System, Fischer Scientific) was used to handle the slides, to control the temperature of the hybridization reactions and various incubations, and to minimize reagent consumption. Tissues were deparaffinized and rehydrated in diluted serial alcohols. They were then digested with 0.3%
pepsin and incubated with 100% formamide for 5 min at 105 °C. Sections were subsequently hybridized using the probe described above for 10 min at 105 °C and then for 30 min at 37 °C. High stringency washes were made with saline sodium citrate buffer to ensure a complete match between the target nucleic acid and DNA probe. After the washes, an antidigoxigenin antibody conjugated to alkaline phosphatase was applied to the sections. Colour was developed with nitroblue tetrazolium dye. Dye reduction to insoluble blue-black formazan indicated areas of probe hybridization. Tissue sections were counterstained with fast green, dehydrated, covered with coverslips, and examined. Controls included known negative-PCV2 and positive-PCV2 lung tissues. Presence of PCV2 was graded from low to high amounts (Quintana et al., 2001).

2.4. Immunohistochemistry (IHC) to detect PRRSV, ADV and SIV

Three avidin–biotin–peroxidase assays using the corresponding monoclonal antibodies to detect PRRSV, ADV and SIV were performed. Techniques were based on a previous published procedure (Halbur et al., 1994). Briefly, lung sections were cut and placed on silane–acetone-coated slides, deparaffinized and rehydrated in diluted serial alcohols. Antigen retrieval was performed using protease type XIV at 0.1% in Tris-buffered saline (TBS) during 8 min at 37 °C, followed by endogenous peroxidase blocking in 3% peroxide for 30 min. After washing, blocking of unspecific bindings was carried out for 1 h with cattle albumin–TBS solution (0.1%). Each primary monoclonal antibody was applied on the corresponding slide tissue, and was incubated overnight at room temperature. Monoclonals antibodies used to detect PRRSV, SIV and ADV were SDOW17 (Rural Technologies, USA), C65331 M (Tib Molbiol, Germany) and ICI (kindly donated by Dr. Hans Nauwynck, University of Ghent, Belgium), respectively, with corresponding dilutions of 1:500, 1:200 and 1:100 in TBS. After three TBS washes, the secondary antibody (biotinylated goat anti-mouse linking antibody) at 1:200 and peroxidase-conjugated avidine complex were applied on the slides during 60 min. After washing three times with TBS, sections were finally incubated in 3’-3-diaminobenzidine (DAB) and hydrogen peroxide for 10 min. Slides were subsequently counterstained with Harris’ haematoxylin, dehydrated, covered with a coverslip, and examined microscopically. Negative control procedures included omission of primary antiserum. Moreover, lung tissue sections from known PRRSV-, SIV- and ADV-negative, and PRRSV-, SIV- and ADV-positive pigs were included as negative and positive controls, respectively.

3. Results

Pathogen detection results are summarised in Table 1. PCV2 nucleic acid was detected in 63 out of 74 cases (85.1%), PRRSV antigen in 33 cases (44.6%), SIV antigen in 3 cases (4.1%) and, finally, ADV antigen in a single case (1.4%). Twenty-nine out of 74 cases (39.1%) had PCV2 as the only present viral pathogen. In contrast, only 3 cases (4.1%) had PRRSV as the only detected viral agent. Thirty PNP cases (40.5%) showed PCV2 and PRRSV co-infection. SIV and ADV were occasionally found, and always concurrently with PCV2. Eight PNP cases (10.8%) did not show the presence of any of the investigated viral agents.

<table>
<thead>
<tr>
<th>Pathogen</th>
<th>No. of positive</th>
<th>% Positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCV2 only</td>
<td>29</td>
<td>39.1</td>
</tr>
<tr>
<td>PRRSV only</td>
<td>3</td>
<td>4.1</td>
</tr>
<tr>
<td>SIV only</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>ADV only</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>PCV2 + PRRSV</td>
<td>30</td>
<td>40.5</td>
</tr>
<tr>
<td>PCV2 + SIV</td>
<td>3</td>
<td>4.1</td>
</tr>
<tr>
<td>PCV2 + ADV</td>
<td>1</td>
<td>1.4</td>
</tr>
<tr>
<td>At least one virus</td>
<td>66</td>
<td>89.2</td>
</tr>
<tr>
<td>None</td>
<td>8</td>
<td>10.8</td>
</tr>
</tbody>
</table>

Table 1
Summary of pathogen detection results in 74 lungs with PNP lesions

<p>| Amount of PCV2 nucleic acid detected by in situ hybridization in the 74 PNP studied cases |
|---------------------------------------------|---------------------------------|------------------|</p>
<table>
<thead>
<tr>
<th>Pathogen</th>
<th>No. of cases</th>
<th>% Cases</th>
</tr>
</thead>
<tbody>
<tr>
<td>High</td>
<td>37</td>
<td>50.0</td>
</tr>
<tr>
<td>Moderate</td>
<td>14</td>
<td>18.9</td>
</tr>
<tr>
<td>Low</td>
<td>12</td>
<td>16.2</td>
</tr>
<tr>
<td>None</td>
<td>11</td>
<td>14.9</td>
</tr>
<tr>
<td>Total</td>
<td>74</td>
<td>100</td>
</tr>
</tbody>
</table>
PCV2 nucleic acid labelling varied from low to high amounts (Table 2). PCV2 genome was mostly detected in clusters of necrotic cells (Fig. 1) and macrophages (intracytoplasmatic location, mainly). Viral nucleic acid was also found in bronchial epithelial cells (mainly in the nucleus) (Fig. 2), mucous glandular cells, blood monocytes within pulmonary vessels, smooth muscle cells (mainly in the nucleus), lymphocyte-like cells and/or plasmalike cells, endothelial cells, and fibroblast-like cells around peribronchial/olar areas. PCV2 nucleic acid was also present in the necrotic respiratory epithelium (Fig. 3). As a general rule, PCV2 genome distribution in lung tissue was diffuse when moderate to high amounts of virus were present, while it was...
peribronchial/olar when the amount of PCV2 labelling was relatively low.

PRRSV antigen was never found in an amount comparable to that of PCV2 nucleic acid, and it was mostly observed in the cytoplasm of macrophages; in one PNP case, viral antigen was also detected in cytoplasm of pneumocytes (Fig. 4). SIV antigen was mostly present within the nucleus of bronchial/olar epithelial cells. Finally, ADV antigen was associated with scattered areas of pulmonary necrosis.

Histopathological findings are summarised in Table 3. Forty-five out of 74 PNP cases (60.8%) had moderate to severe hypertrophy and proliferation of type 2 pneumocytes. Fifty-five out of 74 PNP cases (74.3%) had moderate to high amounts of necrotic cell clumps in alveoli. These percentages were fairly similar in PNP cases in which both PRRSV and PCV2 were detected and cases in which only PCV2 was present. Necrosis of bronchial/olar epithelium was detected in 57 out of 74 (77.0%) cases of PNP, showing similar percentages in PCV2-PRRSV co-infected animals as well as in animals where only one or no viral agents was detected. Thirty out of 74 PNP cases (40.5%) had concomitant catarrhal-purulent bronchopneumonia. Moreover, 13 out of 74 (17.7%) cases had fibrous or fibrinous pleuritis. Pulmonary necrosis was only noted in one PNP case (1.4%), in which ADV and PCV2 were concurrently detected.

### 4. Discussion

The present study further insights on the potential infectious agents involved in the occurrence of PNP in field cases of postweaning pigs. Globally, the results showed that all studied viruses were present in PNP cases, with fairly variable prevalence. PCV2 was the most frequently detected agent in the pig lung tissues analysed (85.1%), which is in agreement with results obtained in a German study (Pesch et al., 2000) using PCR as virus detection technique. Moreover, a fairly high number of PNP cases had PCV2 as the only detected agent (39.2%). As a whole, the present study indicates that PCV2 seems to be the main aetiological agent of PNP in postweaning pigs in Spain. However, our results are almost completely the opposite from those of Drolet et al. (2003). These authors ruled out PCV2 as a determining factor of PNP since none of the
cases from their study showed the presence of PCV2 alone.

Furthermore, Drolet et al. (2003) suggested that PRRSV was the main contributor to PNP. Our results showed the detection of PRRSV alone in only 4% of the total PNP cases and 40% of cases with PRRSV and PCV2 co-infection. Therefore, our results indicated that, although important when co-infection with PCV2 occurs (Pesch et al., 2000), PRRSV is not essential for the development of PNP. Considering the differences between the results of the Canadian study (Drolet et al., 2003) and the results from the present work together with the ones of the German study (Pesch et al., 2000), it seems that the aetiology of PNP presents important differences between North America and Europe. Potential differences between pathogenicity of different strains of PRRSV (Halbur et al., 1995, 1996) and/or PCV2 (Opriessnig et al., 2006), epidemiological context, host susceptibility and environmental or management factors could explain the situation.

One may argue the use of different detection techniques for PRRSV to explain differences between our study, IHC, and the Canadian one, ISH (Drolet et al., 2003). Although it has been demonstrated that the sensitivity of PRRSV detection by ISH is slightly higher than by IHC, the agreement of both techniques is very high, especially in lung tissues (Larochelle and Magar, 1997; Cheon and Chae, 2000). Therefore, the lesser sensitivity of IHC versus ISH cannot explain differences in PRRSV detection as marked as the ones observed between Canada and Spain.

The role of PCV2 seems to be more significant when we consider the amount of nucleic acid in the lung tissues. Although the aim of the present work was not to elucidate the mechanism for PNP development, which is yet poorly understood, the presence of moderate to high amount of PCV2 could directly explain the degree of lesions observed. Indeed, the amount of PCV2 nucleic acid showed a closed association with the severity of typical PNP lesions, especially with the presence of clusters of necrotic cells. On the other hand, as had been previous described (Drolet et al., 2003), the amount of PRRSV antigen was relatively low and it seems difficult to explain the degree of pneumonia observed as a direct effect of the virus.

Obtained data also confirm the rare involvement of SIV in PNP (Larochelle et al., 1994; Drolet et al., 2003) and indicate a similar situation for ADV, since both agents were detected in a few numbers of PNP cases and none of them were found alone (always together with PCV2). Therefore, those viral agents must be ruled out as significant etiological contributors to PNP.

In the present work, we only evaluated the presence of viral agents because, to date, no bacteria have been consistently related with PNP causality. While the presence of SIV was evaluated because it was initially suspected as the main cause of PNP (Girard et al., 1992), the presence of PRRSV and PCV2 were assessed because both has been repeatedly cited in the PNP causality hypotheses (Larochelle et al., 1994; Pesch et al., 2000; Drolet et al., 2003). Moreover, the presence of ADV was also investigated since this viral agent is also associated to respiratory disease in pigs in Spain (Gutierrez-Martín et al., 2000) and able to cause lung necrosis (Segalés et al., 1997). However, no viruses were demonstrated in about 11% of PNP cases. This rate is similar to that of previous retrospective studies (Larochelle et al., 1994; Drolet et al., 2003). At this time we cannot rule out the involvement of other non-studied or unknown viruses or even bacteria in the PNP causality and, therefore, further studies are needed to expand the possible involvement of other pathogens in the PNP aetiology.

Most of the PNP cases analysed showed the presence of more than one virus or one virus together with evidence of bacterial infection (catarrhal-purulent bronchopneumonia or fibrinous/fibrous pleuritis). This etiological mixture is a usual feature of porcine respiratory disease complex (PRDC), a serious health problem in postweaning pigs (Kim et al., 2003), and our data would support the idea that PCV2 plays a significant role in field cases of PRDC (Harms et al., 2002; Kim et al., 2003).

Harms et al. (2001) reported a higher degree of pneumonia in PRRSV–PCV2 experimentally co-infected cases than in cases infected only with one of them. Nevertheless, in spite there were an important number of viral co-infections in the studied PNP cases, severity of the lesions were not specially different when comparing viral co-infected PNP cases and those showing the presence of a single viral pathogen or even those with no agent involvement.

The present study analysed PNP cases from postweaning pigs exclusively. This is due to the fact that no cases of this pathological condition have been detected in lung tissues of lactating or adult pigs...
examined during the last 10 years in our laboratory (data not shown). However, PNP has been described in pigs as early as 15 days of life (Morin et al., 1990), and the mentioned study from Drolet et al. (2003) included PNP cases from lactating pigs.

In summary, factors involved in the development of PNP are yet poorly understood, but available results of our and previous works (Pesch et al., 2000; Drolet et al., 2003) indicate that both PCV2 and PRRSV can be present alone or concomitantly in PNP cases. Moreover, we may speculate that PCV2 is probably the most important aetiological agent in PNP cases in postweaning pigs in Europe while PRRSV may probably play the same role in North America.

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