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Detection of European Porcine Reproductive and Respiratory Syndrome Virus in Porcine Alveolar Macrophages by Two-colour Immunofluorescence and In-situ Hybridization-immunohistochemistry Double Labelling

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Summary

Two groups of five pigs aged 6 weeks were each infected oronasally with one of two different European isolates of porcine reproductive and respiratory syndrome virus (PRRSV). The animals were killed sequentially at 4, 7, 14 or 21 days post-inoculation for examination. The methods used consisted of histopathology, and mono- and double-labelling techniques based on in-situ hybridization, immunofluorescence and immunohistochemistry. Porcine alveolar macrophages (PAMs) contained large amounts of PRRSV antigen and PRRSV RNA, as shown by double labelling with (1) either PRRSV immunofluorescence or PRRSV-specific in-situ hybridization with digoxigenin-labelled riboprobes, and (2) immunolabelling with Mac 387 antibody for calprotectin. Expression of PRRSV-RNA was not detectable in cytokeratin-positive hypertrophic and proliferating pneumocytes or in cells of alveolar ducts or bronchiolar epithelium. The use of two-colour immunofluorescence with confocal laser scanning microscopy and double labelling with in-situ hybridization-immunohistochemistry showed that PAMs were the only pulmonary target cells. This contradicts earlier reports that epithelial pulmonary cells may also be infected by PRRSV.

Introduction

Since its first appearance in 1987 in the USA (Keffaber, 1989) and 1990 in Europe (Lindhaus and Lindhaus, 1991), porcine reproductive and respiratory syndrome (PRRS) has become endemic in many swine-producing countries. In addition to causing infertility and abortion in sows, PRRS virus (PRRSV) is, together with swine influenza virus and porcine respiratory coronavirus, an important cause of respiratory disease in juvenile pigs. Multisystemic wasting syndrome after co-infection with porcine circovirus also contributes to economic losses in the field (Rosell *et al.*, 1999). PRRSV has been isolated in the USA [e.g. strains VR-2332 (Benfield *et al.*, 1992), ISU79 and ISU1894 (Meng *et al.*, 1996)], Europe (Lelystad virus; Wensvoort *et al.*, 1991) and Japan (EDRD-1; Saito *et al.*, 1996). Isolates of PRRSV, now classified in the genus *Arterivirus* (family *Arteriviridae*, order *Nidovirales*), exhibit remarkable genetic and antigenic variation (Halbur *et al.*, 1995). Extensive experimental studies

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have shown that all European strains correspond to USA isolates of low virulence (Beyer *et al.*, 2000; van Reeth and Nauwynck, 2000). Susceptibility, clinical signs and lesions vary markedly, depending particularly on viral strain (Rossow, 1998) but also on infection route, age of pigs, their immune status and genetic predisposition, and on viral co-infection (Ellis *et al.*, 1999) or bacterial superinfection.

It is widely accepted and consistent with the known biological properties of arteriviruses that in infected pigs PRRSV replicates preferentially in alveolar macrophages (Rossow, 1998). ''Co-localization'' of isotopically labelled PRRSV-RNA within macrophages labelled by the monoclonal antibody (mAb) Mac 387 has been demonstrated in porcine lymph nodes but not in lungs (Lawson *et al.*, 1997). Studies on the tropism of PRRSV have been limited by the identification of infected pulmonary cells on the basis of morphological criteria only (Rossow *et al.*, 1996). The use of immunohistochemistry (IHC) or in-situ hybridization (ISH) on serial sections has suggested, however, that European, North American and Korean strains of PRRSV replicate in bronchiolar epithelial cells (Pol *et al.*, 1991), alveolar duct cells (Magar *et al.*, 1993), and pneumocytes (Pol *et al.*, 1991; Sur *et al.*, 1996; Cheon *et al.*, 1997). We describe here the results of a double labelling procedure on cryostat and paraffin-wax sections to investigate the tropism of PRRSV in the lungs of experimentally infected pigs. The methods used were (1) two-colour immunofluorescence analysed by confocal laser scanning microscopy, or (2) non-radioactive ISH-IHC double staining.

Materials and Methods

Animals and Procedure

The design of the infection study in general accorded with previously published procedures (Beyer *et al.*, 1998, 2000). Briefly, 10 conventionally raised, 6-week-old weaner pigs were infected oronasally with 10^5 tissue culture infectious doses 50% ($TCID_{50}$) of PRRSV. Five animals (nos 1–5) received the isolate ''Cobbelsdorf'' and five (nos 6–10) the isolate ''I10''; four animals (nos 11–14) were mock-inoculated, serving as controls. Initially, all pigs were serologically negative for PRRSV. On days 4, 7, 14 and 21 days post-inoculation (p.i.), pigs (one or two from each of the three groups) were killed and subjected to necropsy. The lungs of all pigs were immunohistochemically negative for porcine circovirus 2 (PCV-2; antibody kindly provided by J. Ellis, Saskatoon).

Tissue Samples

Pneumonic lung samples from three different sites were either snap-frozen in *n*-heptane and stored at −70°C until used for the preparation of cryostat sections or immediately fixed in 4% buffered paraformaldehyde and processed for paraffin-wax embedding. For histopathological examination, serial sections (3 μm) were dewaxed, placed on organosilane-coated slides and stained with haematoxylin and eosin (HE). For double labelling procedures, cryostat or paraffin-wax sections (3 μm) were used.

Immunofluorescence, Two-colour Immunofluorescence and Confocal Laser Scan Microscopy

Cryostat sections were fixed with acetone (−20°C) for 10 min and than treated at room temperature with bovine serum albumin (BSA) 5% in phosphate-buffered saline (PBS) for 20 min. Sections were incubated with PRRSV nucleocapsid protein (N)-specific mAbs; mAb P3/27 (IgG1) was applied for evaluation of PRRSV antigen distribution and quantification, as previously described (Beyer *et al.*, 2000), and mAb P14/a34-3 (IgG2a) was used for two-colour immunofluorescence. Both mAbs were diluted 1 in 2 in PBS containing BSA 2% (2% BSA-PBS). As secondary antibody for P14/a34-3, a fluorescein-isothiocyanate (FITC)-labelled goat anti-mouse IgG2a (γ) was used (Caltag Laboratories, Burlingame, CA, USA), diluted in 2% BSA-PBS and mixed at the ratio of 3:1 with 0.005% Evans blue solution to decrease autofluorescence of granulocytes. For immunohistochemical double labelling, the sections were incubated consecutively with mAb Mac 387 (IgG1; Linaris Biologische Produkte, Wertheim-Bettingen, Germany), which recognizes cytoplasmic antigen L1 (calprotectin) in macrophages (Brandtzaeg *et al.*, 1988) and, as secondary antibody, an indocarbocyanin (Cy5)-labelled goat anti-mouse IgG1 (Caltag Laboratories; 1 in 800 in PBS). Sections were sealed in glycerol buffer containing 1,4-diazobicyclo(2,2,2)-octane (DABCO) 25 mg/ml. For scanning and photography, an LSM510 (Zeiss, Göttingen, Germany) was used. FITC was irradiated at 488 nm and detected via a 505–530 nm band pass filter. Cy5 was irradiated at 633 nm and detected with a 650 nm long pass filter. The pinhole diameters of each detection channel were set at 100 μm. Two-channel frame-by-frame multitracking was used for detection to avoid ''crosstalk'' signals. The different frames were scanned separately, with appropriate installation of the optical path for excitation and

emission of each scan (according to the manufacturer's instructions).

In-situ Hybridization (ISH)

For ISH, a PRRSV strand-specific digoxigenin-labelled RNA probe was used. A 245 bp DNA fragment, located in the overlapping ORF 6 and 7 of PRRSV, was amplified by reverse transcriptase–polymerase chain reaction (RT-PCR) (5′ primer: 5′-GCC TTT AGC ATC ACA TAC ACA CC-3′, position 3962–3984 and 3′ primer: 5′-CAC AAT CTG CAT CTG GAA GTG AT-3′, position 4184–4206; position according to Conzelmann *et al.*, 1993), starting from RNA obtained from a lymph node of a piglet infected with PRRSV "Cobbelsdorf". The DNA was cloned into the pGEM-T-Easy plasmid vector and in-vitro transcription was performed with the RiboMAX™ system (Promega Corp., Madison, WI, USA) to yield digoxigenin-11-dUTP (Roche Molecular Biochemicals, Mannheim, Germany) sense (Sp6) and antisense (T7) riboprobes. ISH was performed essentially as described by Zurbriggen *et al.* (1998) and always preceded immunohistochemistry. Briefly, after dewaxing, slides were incubated for 20 min in 0.2 M HCl to remove basic proteins, followed by a 2-min wash in 2 × standard saline citrate (SSC). For proteolytic digestion, slides were incubated for 15 min (37°C) in proteinase K (Roche) 5 μg/ml. After postfixation for 5 min with 4% paraformaldehyde, two washes in 2 × SSC, and acetylation in 0.1 M triethanolamine buffer containing acetic anhydride 0.25% (v/v), the sections were prehybridized for 60 min (50°C) in 50% formamide, 4 × SSC, 2 × Denhard's solution and yeast-RNA 500 μg/ml. Hybridization was performed overnight under the same conditions with addition of 10% dextran sulphate (w/v) and labelled probes (5 ng/μl). To digest any unbound probe, the sections were treated with RNase T1 and DNase-free RNase (Roche). The sections were then given 10-min washes, twice in 2 × SSC (55°C) and twice in 0.2 × SSC (55°C). For immunological probe detection, the sections were incubated for 2 h with an anti-digoxigenin-alkaline phosphatase conjugate (1 in 500; Roche). Nitro-blue tetrazolium (NBT) and 5-bromo-4-chloro-4-indolylphosphate were used as substrates for colour reaction. Overnight development of the dark blue signal was stopped in Tris-EDTA (ethylenediaminetetraacetic acid) buffer (pH 8.0).

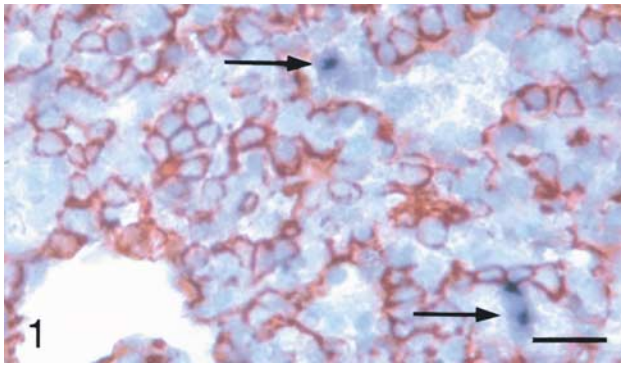
Immunohistochemistry (IHC)

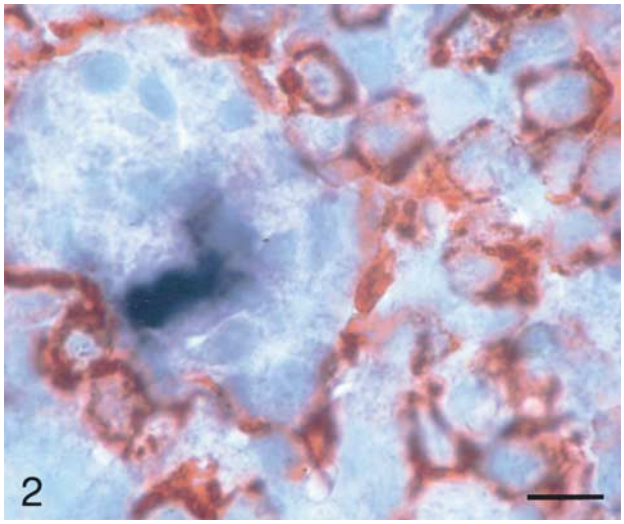
ISH was followed by IHC with the avidin–biotin–peroxidase complex (ABC) method, as described previously (Teifke *et al.*, 1998). For the detection of a broad spectrum of cytokeratins in epithelial pulmonary cells, sections were treated with a mixture of three murine mAbs (AE1, AE3 and Ks 13.1; Linaris Biologische Produkte) against different cytokeratins (1–8, 10, 13–16, 19). To detect porcine alveolar macrophages (PAMs), mAb Mac 387 was used as described above. For epitope recognition on paraffin-wax sections, antigen demasking procedures were necessary. The sections were microwave-irradiated for 10 min in a Histosafe-Enhancer (Linaris Biologische Produkte) for cytokeratin labelling or digested with 0.1% pronase (Linaris Biologische Produkte), according to the manufacturer's instructions, to detect calprotectin. By means of the ABC method and an immunoperoxidase kit (Vectastain Elite ABC Kit, Vector Laboratories, Burlingame, CA, USA), a bright red signal was produced from the substrate, 3-amino-9-ethylcarbazole (Dako AEC substrate-chromogen system; Dako, Carpinteria, CA, USA). The sections were counterstained with Mayer's haematoxylin, and mounted with aqueous medium (Aquatex; Merck, Darmstadt, Germany).

Morphometric analysis and quantification of Mac 387-positive cells (number/μm^2) or cytokeratin-positive tissue area (%) were performed with the KS300® image analysis system (Zeiss). Calibration and threshold determination were carried out once for each section, with a × 20 objective. On each tissue section, at least 20 randomly selected neighbouring fields were measured, starting within the lung area showing the most prominent pneumonic lesions. The median values of the parameters measured were calculated.

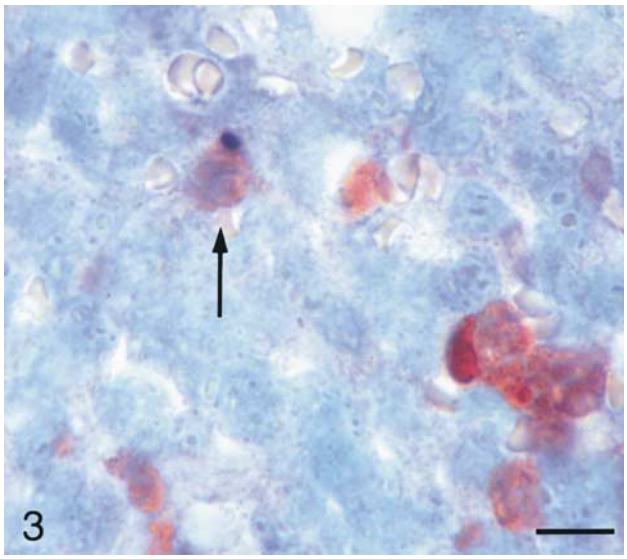
Results

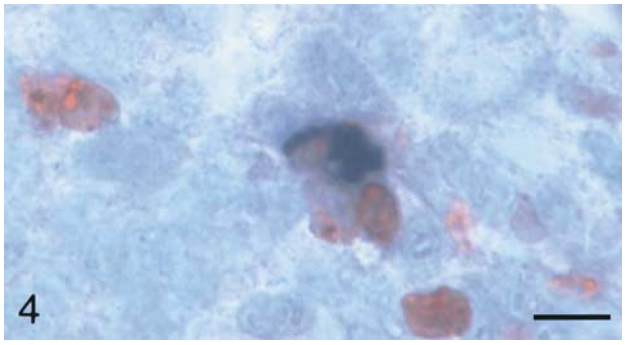
PRRSV-RNA was present in the cytoplasm of large, round to oval cells, which were scattered throughout the lung but concentrated in collapsed alveoli or within the intra-alveolar cellular debris of pneumonic areas (Fig. 1). Occasionally, the strong intensity of the hybridization signal overrode the cellular borders (Fig. 2). On paraffin-wax sections, immunohistochemical double labelling with Mac 387 identified PRRSV-infected cells as pulmonary macrophages (Figs 3 and 4). In addition, PRRSV-RNA-positive cells with strong hybridization signals that remained unlabelled by Mac 387 had the





Figs 1 and 2. Lung of pig no. 2, inoculated with PRRSV-isolate "Cobbelsdorf"; 7 days p.i. Double labelling with PRRSV-RNA in-situ hybridization and broad-spectrum cytokeratin immunohistochemistry. Haematoxylin counterstain. Paraffin wax-embedded tissue. (Fig. 1) Between large numbers of cytokeratin-expressing pneumocytes are solitary cytokeratin-negative cells with intense cytoplasmic PRRSV-RNA-specific hybridization signals (arrows). Co-localization of PRRSV-RNA within cytokeratin-positive cells is not observed. Bar, 50 μm. (Fig. 2) A nest of PRRSV-RNA-positive, cytokeratin-negative cells is surrounded by numerous hyperplastic cytokeratin-positive pneumocytes. Bar, 20 μm.





Figs 3 and 4. Lung of pig no. 8, inoculated with PRRSV-isolate "I10"; 14 days p.i. Double labelling with PRRSV-RNA in-situ hybridization and Mac 387 immunohistochemistry. Haematoxylin counterstain. Paraffin wax-embedded tissue. (Fig. 3) Mac 387 labelling is mainly confined to abundant cytoplasm of macrophages. Many macrophages lack a PRRSV-RNA-specific hybridization signal. In the cytoplasm of several Mac 387-positive cells only small spots (arrow) of hybridization signals for PRRSV-RNA are detectable. Bar, 20 μm. (Fig. 4) Co-localization of the red Mac 387 labelling with the black PRRSV-RNA hybridization signal to the abundant cytoplasm of larger oval cells This unequivocally identifies these cells as PRRSV-infected macrophages. Bar, 20 μm.

morphological features of pulmonary macrophages; however PRRSV-RNA was not detected in cytokeratin-positive cells. By means of immunolabelling, PRRSV-antigen was detectable as brilliant fluorescence with mAbs P3/27 and P14/a34-3. Nucleoprotein of PRRSV was detectable in cells lining the alveolar walls (Fig. 5A). The number of PRRSV antigen-positive cells peaked on day 7 p.i. and, except for day 4 p.i. was usually substantially higher than the number of cells with PRRSV-specific hybridization signals (Table 1). In advanced stages of pneumonia, the number of PRRSV antigen-positive cells had decreased strikingly. Two-colour immunofluorescence revealed unequivocally that cells positive for PRRSV antigen were also positive for the macrophage-specific antigen calprotectin (Figs 5B and 5C). The number of Mac 387-positive cells (mainly macrophages with a few granulocytes, as judged by their morphology)

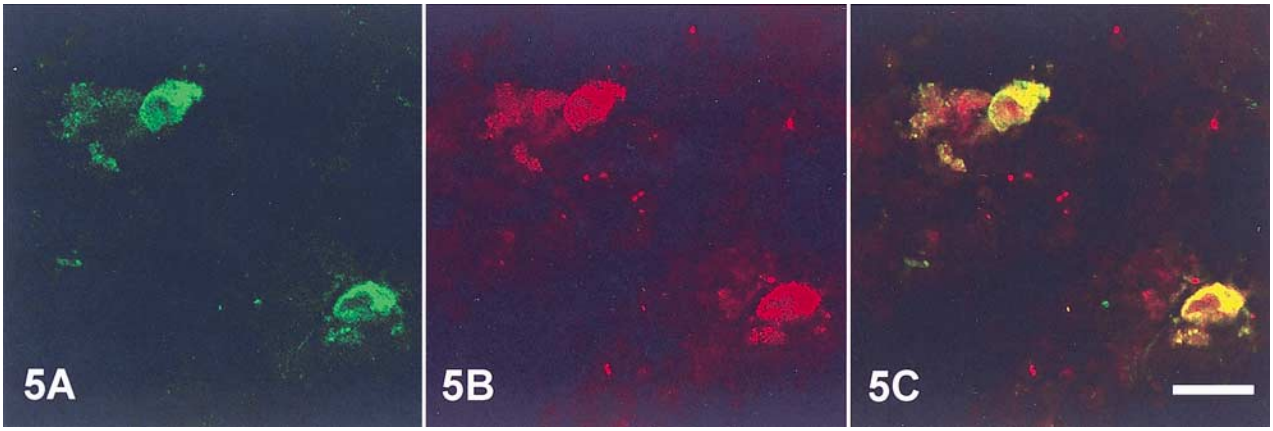


Fig. 5 A,B,C. Lung of pig no. 7, inoculated with PRRSV-isolate "I10"; 7 days p.i. Two-colour immunofluorescence, cryostat section. (A) Bound PRRSV-N-specific mAb P14/a34-3 corresponds to green FITC fluorescence. (B) Bound mAb Mac 387 corresponds to red Cy5 fluorescence. (C) In the overlay, yellow represents PRRSV-infected alveolar macrophages (PAMs). Two PRRSV-infected PAMs are located in the alveolar septa, together with cellular debris. Brilliant cytoplasmic FITC fluorescence for PRRSV-antigen, co-localized with Mac 387 specific Cy5-fluorescence. Bar, 12 µm.

increased rapidly after infection to values 10 times higher than in mock-inoculated controls. Only a small fraction of Mac 387-positive cells showed PRRSV-specific fluorescence, but all cells positive for PRRSV antigen displayed Mac 387-specific fluorescence. The cytokeratin-labelling pattern on paraffin-wax sections and morphometric quantification of cytokeratin-positive areas reflected the hypertrophy and greatly increased proliferative activity of type II pneumocytes early in PRRSV infection. It was notable that PRRSV-RNA could not be detected within cytokeratin-positive bronchiolar epithelium, alveolar ducts or densely packed pneumocytes.

Discussion

Previous reports have described the detection of PRRSV antigen in nasal turbinate epithelium (Rossow *et al.*, 1996), pulmonary epithelial cells (Pol *et al.*, 1991), type II pneumocytes (Halbur *et al.*, 1994), and vascular endothelium (Halbur *et al.*, 1995) by means of mono-labelling techniques on consecutive sections. We had been unable to confirm these observations in an earlier study (Beyer *et al.*, 2000). The present study was designed to investigate the pulmonary tropism of two European PRRSV isolates. The double labelling technique enabled a large number of lung samples to be examined. The striking increase in the number of macrophages and to a lesser extent the number of granulocytes seen shortly after inoculation was possibly due to the prolonged secretion of interleukin (IL)-1, one of the main stimuli for the production of monocyte-attracting chemokines (van Reeth and Nauwynck, 2000). Attraction of granulocytes may result from release of an alveolar macrophage chemotactic factor, AMCF II, which is selectively "upregulated" in PAMs shortly after infection (Beyer *et al.*, 1998). Only a small percentage of PAMs in bronchoalveolar lavage fluid from infected animals was found to be PRRSV antigen-positive (Duan *et al.*, 1997). This accords with our observation of a large number of Mac 387-positive but PRRSV antigen- and PRRSV-RNA-negative cells in lung tissue. The lower number of PRRSV-RNA-positive cells than PRRSV antigen-positive cells from day 7 p.i. onwards may have reflected differences in chemical as well as biological half-life, i.e. pronounced degradation of viral RNA during tissue processing or a shorter half-life of viral mRNA than that of accumulating viral protein. ISH reliably detects a small proportion of PRRSV-infected cells in formalin-fixed, paraffin wax-embedded tissues (Lawson *et al.*, 1997). Two-colour immunofluorescence with laser scan microscopy showed that nearly all cells per visual field positive for PRRSV antigen were also immunolabelled by Mac 387. ISH-IHC was less effective in demonstrating co-localization of viral RNA in Mac 387-positive cells. The strong black hybridization signal probably overrode the faint red Mac 387-specific signal, resulting in a larger number of PRRSV-RNA-positive cells than of cells with dual labelling. Mac 387 reacts with the leucocyte antigen L1, also known as calprotectin, a myelomonocytic protein

Table 1
Results of mono- and double-labelling based on in-situ hybridization, immunofluorescence and immunohistochemistry to detect PRRSV in the lungs of experimentally infected pigs

			Results obtained with*					
Pig no.	*PRRSV strain*	*DPI*	*ISH for PRRSV-RNA*	*IF for PRRSV antigen*	*IHC for Mac 387 reaction*	*IHC for cytokeratin [Positive area (%)]*	*ISH/IHC for PRRSV and Mac387*	*ISH/IHC for PRRSV and cytokeratin*
1	Cobbelsdorf	4	39	21	1870	27·5	12	0
2	Cobbelsdorf	7	82	281	1699	17·3	25	0
3	Cobbelsdorf	7	197	NT	924	18·7	21	0
4	Cobbelsdorf	14	0	0	1622	15·5	0	0
5	Cobbelsdorf	21	0	13	159	16·7	0	0
6	I10	4	130	111	1750	20·4	12	0
7	I10	7	237	496	1604	23·8	21	0
8	I10	14	40	147	1545	22·3	0	0
9	I10	14	84	NT	1824	14·3	3	0
10	I10	21	73	82	1146	17·5	7	0
11	None (controls)	4	0	0	156	22·6	0	0
12	None (controls)	7	0	0	363	19·8	0	0
13	None (controls)	14	0	0	225	17·2	0	0
14	None (controls)	21	0	0	184	23·9	0	0

* Positive cells/cm^2 (median values).
ISH, in-situ hybridization; IF, immunofluorescence; IHC, immunohistochemistry; DPI = days post-inoculation; NT = not tested.

abundantly expressed by peripheral neutrophils and monocytes. Possibly the intracellular calprotectin of Mac 387-negative, PRRSV-RNA-positive macrophages is reduced in response to chemotaxin-mediated release of calprotectin, as described for neutrophils (Hetland *et al.*, 1998).

In conclusion, the results clearly demonstrated that in 6-week-old pigs at 4–21 days p.i., PRRSV was present in Mac 387-positive pulmonary macrophages (probably PAMs) but not in cytokeratin-positive bronchiolar epithelium or pneumocytes. The apparent contradiction with results of other authors may be due to differences in experimental conditions. Thus, for example: Pol *et al.* (1991), Rossow *et al.* (1996) and Cheon *et al.* (1997) infected 1-week-old piglets; Halbur *et al.* (1994) used 3-week-old animals; Magar *et al.* (1993) used naturally infected pigs aged 6–7 weeks; and the duration of the study of Sur *et al.* (1996) was greater than that of the present experiments. However, double-labelling techniques have not previously been applied to PRRSV-infected lung tissue. Our findings support the general view, that macrophages are the principal target cells for PRRSV in the lung. The data suggest, moreover, that European PRRSV replicates not only primarily but also exclusively in alveolar macrophages during early pulmonary infection in weaner pigs.

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