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Abstract

Porcine Reproductive and Respiratory Syndrome virus (PRRSV) is one of the main component of the porcine respiratory disease complex (PRDC), which strongly impact the pig production. Although PRRSV is often considered as a primary infection that eases subsequent respiratory coinfections, the possibility that other PRDC components may facilitate PRRSV infection has been largely overlooked. The main cellular targets of PRRSV are respiratory macrophages among them alveolar macrophages (AM) and pulmonary intravascular macrophages (PIM). AM, contrarily to PIM, are directly exposed to the external respiratory environment, among them co-infectious agents. In order to explore the possibility of a co-infections impact on the capacity of respiratory macrophages to replicate PRRSV, we proceed to *in vitro* infection of AM and PIM sampled from animals presenting different sanitary status, and tested the presence in the respiratory tract of these animals of the most common porcine respiratory pathogens (PCV2, *Actinobacillus pleuropneumoniae*, *Mycoplasma hyopneumoniae, Mycoplasma hyorhinis, Mycoplasma floculare, Pasteurella multocida, Bordetella bronchiseptica*, *Streptoccocus suis)*. In this exploratory study with a limited number of animals, no statistic differences were observed between AM and PIM susceptibility to *in vitro* PRRSV infection, nor between AM coming from animals presenting very contrasting respiratory coinfection loads.

Introduction

Respiratory infections are one of the major cause of disease in pigs, leading to economic losses as well as to antibiotics overuse. Because of its sanitary importance, this condition has been given a name: porcine respiratory disease complex (PRDC). The major PRDC components described so far are swine influenza A virus (swIAV), porcine reproductive and respiratory syndrome virus (PRRSV), and porcine circovirus 2 (PCV2) as well as bacteria such as *Actinobacillus pleuropneumoniae*, *Mycoplasma hyopneumoniae*, *Pasteurella multocida, Bordetella bronchiseptica* and *Streptoccocus suis* (Opriessnig et al., 2011). Among them, PRRSV, because of the duration of infection (more than a month), its immunomodulatory properties as well as the emergence of highly pathogenic strains, is considering as one of the pillars of PRDC (Montaner-Tarbes et al., 2019).

The pathogens constituting the PRDC are thought to interact with each other as well as with the host tissues, in a complex manner (among others see (Ellis et al., 2004; Lévesque et al., 2014; Thacker et al., 1999) (Saade et al., 2020)). Indeed, whereas the adaptive immune response can largely be considered as pathogen-specific, the immediate inflammatory response and the countermeasures triggered by the pathogens to quench this response may impact the infectious capacities of a second pathogen. Moreover, some infections may trigger trained immunity (especially on monocytes and macrophages, the main targets of PRRSV), and the persistent character of the majority of these microbes may led to long term tissue remodelling.

In a previous study we observed that pulmonary intravascular macrophages (PIM) were equivalently susceptible as alveolar macrophages (AM) to PRRSV infection, and this *in vitro* as well as *in vivo* (Bordet et al., 2018). However, these preliminary data pointed on a lower variability between animals in PIM PRRSV susceptibility compared with AM PRRSV susceptibility. Since AM, seated in the airways, are more exposed to respiratory pathogens than PIM, which are protected from external environment by three tissue layers (epithelium, interstitium and endothelium), we postulated that the variability of AM susceptibility to PRRSV infection might be due to factors present in the airways such as coinfectious agents. In order to test our hypothesis, we set up an original approach consisting in monitoring ongoing *in vivo* recurrent respiratory infections and at the same time *in vitro* AM and PIM capacities to replicate PRRSV, in animals presenting different sanitary status. Since this influence between the pathogens and the components of the respiratory immune system might come from interactions lasting several weeks, and that persistent infections are naturally much more susceptible to be involved in coinfections situations, we focused on the following persistent PRDC components:

PCV2, *Actinobacillus pleuropneumoniae*, *Mycoplasma hyopneumoniae* and the related *Mycoplasma hyorhinis and Mycoplasma floculare*; *Pasteurella multocida, Bordetella bronchiseptica*, *Streptoccocus suis* and excluded *a priori* the short term swIAV infection that, moreover has been already shown to downregulate PRRSV infection *ex vivo* (Dobrescu et al., 2014).

Materials and Methods

Pig lung cells collection. Lung tissue samples were obtained from 5- to 7-month-old Large White conventionally bred sows coming from two origins: 6 animals from the Unité Experimentale de Physiologie Animal de l'Orfrasière (UEPAO, Tours, France) and 6 from PORCI MAUGES slaughterhouse (Beaupréau, France). Finally, 4 supplementary Large White specific pathogen free (SPF) 2 to 3-month-old sows from the highly controlled SPF facility of ANSES, Ploufragan (France) free of numerous respiratory pathogens such as PRRSV, swine Influenza virus, porcine circovirus type 2, *Mycoplasma hyopneumoniae*, *Actinobacillus pleuropneumoniae*, *Pasteurella multocida*, *Bordetella bronchiseptica* and *Haemophilus parasuis* were also tested. In order to reduce animal experiments, all the animals were euthanized in the course of the regular management process of the herds, hence the absence of trial number or experimental authorisation and the age difference according to the origin of the animals. Tracheo-bronchial tissue and lymph nodes were sampled and directly frozen in dry ice for subsequent pathogens detection (Zimmerman et al., 2012). A broncho-alveolar lavage (BAL) procedure was then performed twice on the isolated left lung with 250 mL of PBS supplemented with 2mM EDTA (PBS/EDTA), to collect AM. Next, a 1-cm slice of external lung parenchyma was dissected from the same lung. Tissues were minced and incubated in nonculture-treated Petri dishes, to avoid plastic adherence of macrophages, for 2 h at 37 °C in complete RPMI, consisting of RPMI 1640 supplemented with 100 IU/mL penicillin, 100 mg/mL streptomycin, 2 mM L-glutamine, and 10% inactivated foetal calf serum (FCS) (all from Invitrogen, Paisley, UK), containing 2 mg/mL collagenase D (Roche, Meylan, France), 1 mg/mL dispase (Invitrogen), and 0.1 mg/mL Dnase I (Roche). Cells were passed through 40 µm cell strainers and red blood cells lysed with erythrocytes lysis buffer (10 mM NaHCO3, 155 mM NH4Cl, and 10 mM EDTA). Next, cells were washed with PBS/EDTA, counted, and step-frozen in FCS/10% dimethyl sulfoxide (Sigma-Aldrich, St Louis, MO).

Cell infection and viral titration. Parenchymal and alveolar macrophages of one conventional and one controlled animal were systematically thawed and infected in parallel. Because of technical reasons, not enough PIM could be retrieved from animals 9, 11, 12 from Beaupréau slaughterhouse (Fig 1A), leading to only 3 conventional PIM infection tests. Thawed cells were first enriched in macrophages and depleted in dead cells by 1.065 density iodixanol gradient (Optiprep®, Nycomed Pharma, Oslo, Norway). These gradient-enriched mononuclear phagocyte cell preparations were further enriched in macrophages by 2 h plastic adherence, leading, from bronchoalveolar lavage to an AM purity of 86% +/-8%, and for parenchyma to a PIM purity of 51%+/-20%. Enriched macrophages were then cultured in complete RPMI for 24 h in flat-bottom 96-well plates at 3.10^5 cells/well and then infected at a multiplicity of infection (MOI) of 10^{-3} with PRRSV virus Lena strain in complete RPMI. At 24 hpi, plates were centrifuged and pelleted cells were lysed in 350 µL of RNA extraction buffer (RLT Buffer, QIAGEN). These MOI and incubation time have been set to measure the PRRSV titre during the linear phase of the infection, in order to observe weak infectivity differences between the different macrophages. Total RNA were extracted using RNeasy Plus Mini kit (QIAGEN) according to the manufacturer's instructions. RNA were reverse transcribed using oligo(dT) and random primers (BioRad iScript Reverse Transcription supermix). RNA samples were treated with DNAse I Amp Grade (Invitrogen) (1 U/µg of RNA). The absence of genomic DNA contamination was validated by the use of treated RNA as a template directly in PCR. Total RNA quantity and quality were assessed using Nanophotometer (Implen, Munich, Germany). cDNA was generated with a virus reverse transcriptase in the iScript Reverse Transcription Supermix for RT-qPCR (Bio-Rad, Hercules,

CA, USA) from 100-200 ng of RNA free of genomic DNA per reaction. Diluted cDNA (4X) was combined with primer/probe sets and IQ SYBR Green Supermix (Bio-Rad) according to the manufacturer's recommendations. Real-time assays were run on a CFX Connect Real-Time PCR Detection System (Bio-Rad). Samples were normalized internally by simultaneously using the average Cycle quantification (C*q*) of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) reference gene in each sample. Then, qPCR data were expressed as relative values after Genex macro analysis (Bio-Rad) (Vandesompele et al., 2002) using the *Cq* from the samples for the different transcripts. TTC AGT TCC GGT GA); GAPDH (F: CTT CAC GAC CAT GGA GAA GG, R: CCA AGC AGT TGG TGG TAC AG), TBP1 (F: AACAGTTCAGTAGTTATGAGCCAGA, R: AGATGTTCTCAAACGCTTCG), ActB (F: CACGCCATCCTGCGTCTGGA, R: AGCACCGTGTTGGCGTAGAG), HPRT1 (F: GGACTTGAATCATGTTTGTG, R: CAGATGTTTCCAAACTCAAC), IFN-α (generic see (Sang et al., 2011)) (F: GGC TCT GGT GCA TGA GAT GC, R: CAG CCA GGA TGG AGT CCT CC), IFN-ß (F: GTT GCC TGG GAC TCC TCA A, R: CCT CAG GGA CCT CAA AGT TCA T), Mx1 (F: AGT GTC GGC TGT TTA CCA AG, R: TTC ACA AAC CCT GGC AAC TC), Mx2 (F: CCG ACT TCA GTT CAG GAT GG, R : ACA GGA GAC GGT CCG TTTA C) and PKR (F: CAC ATC GGC TTC AGA GTC AG, R: GGG CGA GGT AAA TGT AGG TG). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a reference gene since it was endowed with one of the most stable expression in the whole porcine lung (Delgado-Ortega et al., 2011) as well as specifically in lung macrophages (Maisonnasse et al., 2016). Then, qPCR data were expressed as relative values after Genex macro analysis (Bio-Rad) (Vandesompele et al., 2002). In all experiments other reference genes (TBPI or ActB and HPRT1) were used in parallel with GAPDH and gave comparable results. All the AM/PIM were negative for PRRSV before *in vitro* infection.

PCR for pathogens detection. Tracheobronchial tissue and lymph node were thawed and mechanically (Lysing matrix E FastPrep24, MpBio) disrupted before DNA extraction using QIAamp cador Pathogen kit (INDICAL BIOSCIENCE). A proteinase K enzymatic lysis was then performed. A PCR was then run using previously described primers for PCV2 (F: TTT AGG GTT TAA GTG GGG GGT C, R: CCG GAT CCA TGA CGT ACC CAA GGA GGC G, 470 bp fragment expected) *Pasteurella multocida* (F: AAG GGA TGT TGT TAA ATA GAT AGC, R: GCT TCG GGC ACC AAG CAT AT, 410 bp fragment expected) *Haemophilus parasuis* (F: GTG ATG AGG AAG GGT GGT GT, R: GGC TTC GTC ACC CTC TGT, 820 bp fragment expected), *Streptococcus suis* (F: TTC TGC AGC GTA TTC TGT CAA ACG, R: TGT TCC CTG GAC AGA TAA AGA TGG, 700 bp fragment expected) (Cheong et al., 2017) or for *Mycoplasma* species (*M. hyopneumoniae* F: TTCAAAGGAGCCTTCAAGCTTC, 1,000 bp fragment expected; *M. floculare* F: GGGAAGAAAAAAATTAGGTAGGG, 754 bp fragment expected; *M. hyorhinis*, F: CGGGATGTAGCAATACATTCAG, 1129 bp fragment expected, and a common reverse primer R: AGAGGCATGATGATTTGACGTC) (Stakenborg et al., 2006). The PCR was performed in a 20 µL reaction mixture containing 4 µL of extracted DNA (containing a minimum of 25 ng of DNA), 0,5 µM of each primer, 1,5 to 2,5 mM of MgCl2, 0,1 mM of deoxynucleoside triphosphate and 0,02 U of GoTaq G2 Flexi DNA Polymerase (Promega). The PCR was carried out for 40 cycles consisting of denaturation for 20 seconds at 94°C, annealing for 30 seconds at Tm and extension for 30 seconds at 72°c using thermal cycle (BioRad). Then, qPCR data were expressed as relative values after Genex macro analysis (Bio-Rad) (Vandesompele et al., 2002) using the *Cq* from the samples for the different transcripts.

Statistical analysis. Data were analysed using Graph Pad Prism (version 6) and Mann-Whitney test was used to compare relative expressions. Non-parametric approaches were chosen due to the few number of samples available. The Mann-Whitney's test was used to compare unpaired samples based on ranking. The AM and PIM from the same animal were considered as paired samples. For paired samples, the Wilcoxon matched-pairs signed rank test was used. When scatter plots are used, the mean is depicted by a horizontal bar. Factor Analysis for Mixed Data (FAMD) was performed using FactoMineR package of RStudio (version 3.6.1) in order to investigate the relation between

isolated pathogens and gene expression.

Results and Discussion

AM and PIM were first collected from animals coming from 2 facilities presenting distinct health status: the controlled pig breeding facility from the UEPAO (INRA, Nouzilly, France) and the PORCI MAUGES slaughterhouse (Beaupréau, France) which processes animals from the conventional farms of the surrounding area, known from the regional veterinary services as a PRRSV-free area (Blanquefort and Benoit, 2000). The AM and PIM were infected *in vitro* at a multiplicity of infection (MOI) of 0.001. Twenty-four hours later the cellular viral load was measured using RT-qPCR. No significant differences were observed neither between AM from the two different origins nor between AM and PIM from the same animals (Fig 1A). Conversely, we could observe a correlation ($r=0.78$, $p=0.02$) of *in vitro* PRRSV infections between AM and PIM from the same animal (Fig 1B), in contradiction with the hypothesis that AM exposed to airways co-infections would be differentially susceptible to PRRSV than PIM. Finally, standard deviations of PRRSV titres presented no differences between AM and PIM from the two origins (Fig 1C).

Respiratory infections occurring *in vivo* at the time of AM and PIM collection were then detected by PCR on tracheo-bronchial lymph nodes and bronchial epithelium tissue (Figure 2), using previously described primers (Cheong et al., 2017; Stakenborg et al., 2006). Strikingly, none of the tested pathogens were detected in controlled animals whereas all the conventional animals were positive for at least one of the tested pathogens (Table 1), *P. multocida* (4 animals), *S. suis* (3 animals) and PCV2 (3 animals) being the most frequent infections. One animal was detected positive for 5 pathogens simultaneously (animal n°3, for PCV2, *H. parasuis*, *M. floculare*, *P. multocida* and *S. suis*). However, no link could be inferred between PRRSV *in vitro* titre upon AM infection, and the *in vivo* detection of one or more respiratory pathogens. To note, no animals presented *M. hyopneumoniae* and *M. hyorhinis* infections.

Type I IFNs responses were then evaluated in respiratory macrophages upon *in vitro* PRRSV infection by testing IFNα (generic), IFNβ and three interferon stimulating genes Mx1, Mx2 and PKR transcripts. No differences were observed between PRRSV mock or infected AM or between AM from the two different origins (Figure 3A), neither in raw transcriptomic expression, nor in induction (fold increase) upon PRRSV infection (data not shown). Once again, no consistent difference could be observed between AM and PIM from the same animals.

Finally, in order to test the possibility of a non-identified respiratory infection occurring in both controlled and conventional animals, we also tested AM from the highly controlled SPF facility of ANSES, Ploufragan (France). The same infections and measures than with controlled and conventional AM were performed. AM from SPF animals were as susceptible to PRRSV infection as AM from controlled and conventional animals (Figure 3B). We observed, as expected, a clear upregulation of IFNβ upon PRRSV infection in all conditions (Figure 3C). More globally, the only difference which can be observed for type I IFN and ISG responses (Figure 3D) was the lower IFNβ transcript level before PRRSV infection in AM from SPF animals, which might be due either to the SPF status of the animals or to their lower ages (3-month-old compared with 5 to 7-month-old for conventional and controlled animals).

In order to globally analyse our quantitative (PRRSV titre, IFN-I related genes fold change upon *in vitro* PRRSV infection) as well as categorical (presence or not of other pathogens in the respiratory tract) data, we proceeded to a Factor Analysis for Mixed Data (FAMD) (Fig 3E). According to the great variability as well as to the small size of the samples, no significant links between AM origins, type of respiratory infections and IFN-related genes induction could be observed. The analysis of the quantitative variables however showed an interesting segregation on the first and second axis

(encompassing respectively 26% and 25% of the total variability of the sample) of PRRSV-Lena, the three ISG transcripts measured (Mx1, Mx2, PKR) and IFNα, but not IFNβ transcriptomic induction. Thus ISG genes expressions correlation with PRRSV titre argues for the induction by PRRSV of type I IFN protein(s) expression.

In conclusion, although the difference of pathogens load from conventional and controlled animals was striking, no difference of respiratory macrophages PRRSV infection susceptibility could be observed, neither according to the pathogens' identities nor to the tissue location of the macrophages (AM and PIM), invalidating our hypothesis of a recurrent airways infection leading to the onset of AM more susceptible to PRRSV infection. One possibility would be that the observed AM susceptibility variation in our preliminary study was due to *M. hyorhinis* infections as reported by others (Lee et al., 2016; Thacker et al., 1999), a pathogen that was not detected in the animal tested in this study.

Despite this negative result, that might be confirmed by a larger scale campaign, our study allowed us to highlight a clear correlation between AM and PIM PRRSV susceptibility *in vitro*, in agreement with their high similarity (Bordet et al., 2018; Maisonnasse et al., 2016). Moreover, we could note that in the *Pays de la Loire* area (France), conventional, commercial pigs presented systematically one or several respiratory infections in perfect agreement with the concept of CRP, with 4 out of 6 (2/3) of the conventional pigs presenting at least two simultaneous infections, among them 3 out of 4 presented at least 3 pathogens.

This study is a first step showing the feasibility and interest of testing in parallel, on conventional animals, the *in vivo* presence of respiratory pathogens as well as the *in vitro* sensitivity, response and functions of AM in presence of different respiratory mimicking conditions such as PRRSV, PCV2 or Influenza infections as well as TLR-ligand stimuli or oxidative stress, for instance, allowing to explore the possible impact of long term respiratory infections on AM, one of the primary respiratory barrier.

Acknowledgments

We gratefully thank the slaughterhouse Porci Mauges, (Beaupréau, France), for giving us access to porcine lungs. Thanks for the UEPAO, (INRA, Nouzilly, France) and especially for Sylvain Bourgeais for their work and help in samples collection. We thank Olivier Bourry for providing alveolar macrophages from ANSES SPF facility (Ploufragan (France)).

Declarations of interest: None

Funding:

FM is supported by an establishment grant from the Région Pays de la Loire (RFI Food for tomorrow-Cap aliment).

GS PhD is funded by Foundation Philippe Jabre (Lebanon).

Figures Legends

Figure 1: AM and PIM from different animals' origins were not distinctly susceptible to PRRSV infection.

Gradient and plastic-adherence enriched alveolar macrophages (AM) and pulmonary intravascular macrophages (PIM) were infected by Lena strain PRRSV at an MOI of 10^{-3} for 24h. PRRSV titre was measured by RT-qPCR. Each symbol represents one animal. Macrophages from controlled animals (Ctred), macrophages from conventional animals (Conv). A) PRRSV titre using GAPDH as reference gene (Genex macro analysis (Bio-Rad)). The correspondence between animal identity numbers (for correspondence with Table 1) and symbols are indicated in the cartouche. B) Correlation plot of AM

and PIM PRRSV titre according to data depicted in A) using Spearman test. C) Standard deviations of data depicted in A).

Figure 2: Detection of respiratory pathogens.
Example of positive samples detected by PCR of PCV2 (PCV2 - 470 bp), *Streptococcus suis* (*S. suis* - 700 bp), *Haemophilus parasuis* (*H. parasuis* - 820 bp), *Pasteurella multocida* (*P. multocida* - 410 bp), and *Mycoplasma* species (*M. hyopneumoniae* - 1,000 bp, *M. floculare* - 754 bp, *M. hyorhinis* - 1,129 bp) from Tracheal epithelium and trachea-bronchial lymph node. The results for all pigs are shown in Table 1.

Figure 3: AM and PIM from different animals' origins did not distinctly respond to PRRSV infection.
A) Transcriptomic expression of type I IFNα and β genes and three representative interferon stimulated genes Mx1, Mx2 and PKR from *in vitro* infected AM and PIM from different origins. The samples are the same, with the same identification as in Figure 1. B) Comparison of *in vitro* PRRSV infection of AM from SPF animals with the previously depicted (Figure 1) PRRSV infected AM from controlled and conventional animals. C) Transcriptomic expression of type I IFNα and β genes and three representative interferon stimulated genes Mx1, Mx2 and PKR from *in vitro* infected AM from SPF animals. The samples are the same, with the same identification as in Figure 3B. D) Factor Analysis for Mixed Data (FAMD) plotting together data from Figure 1, Table 1 and Figure 3.

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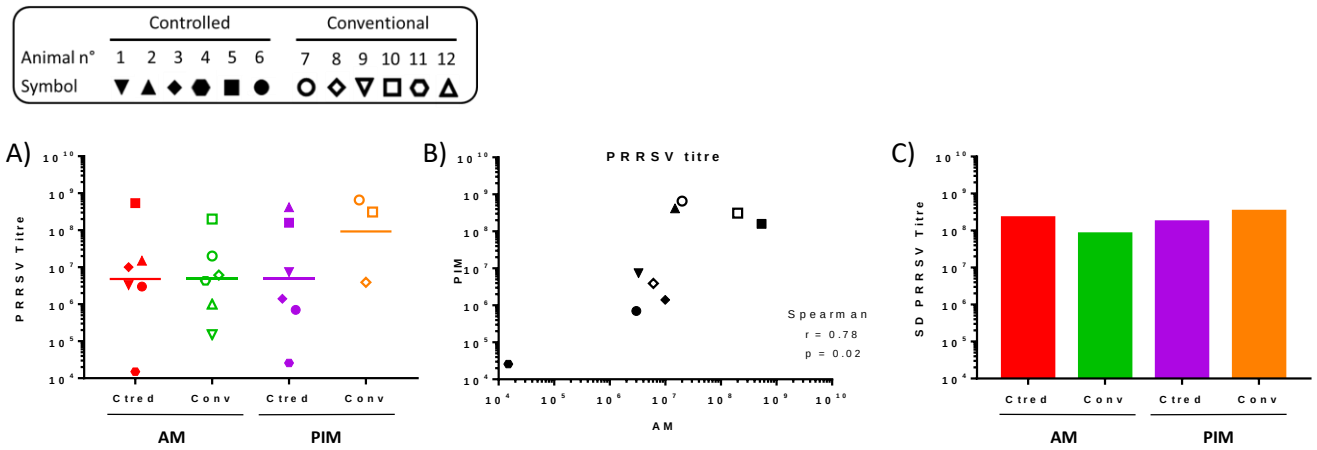

Figure 1: AM and PIM from different animals' origins were not distinctly susceptible to PRRSV infection

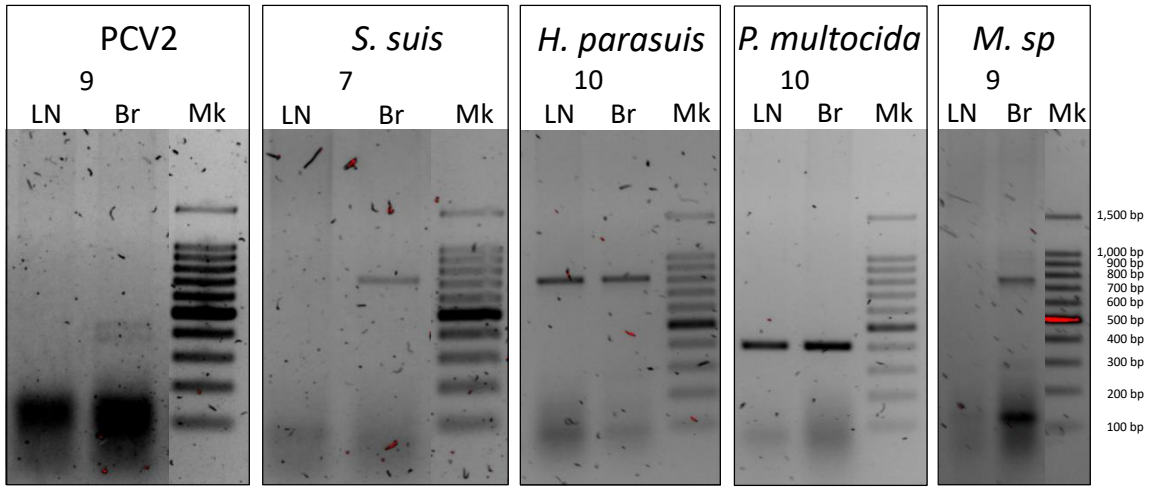

Figure 2: Detection of respiratory pathogens

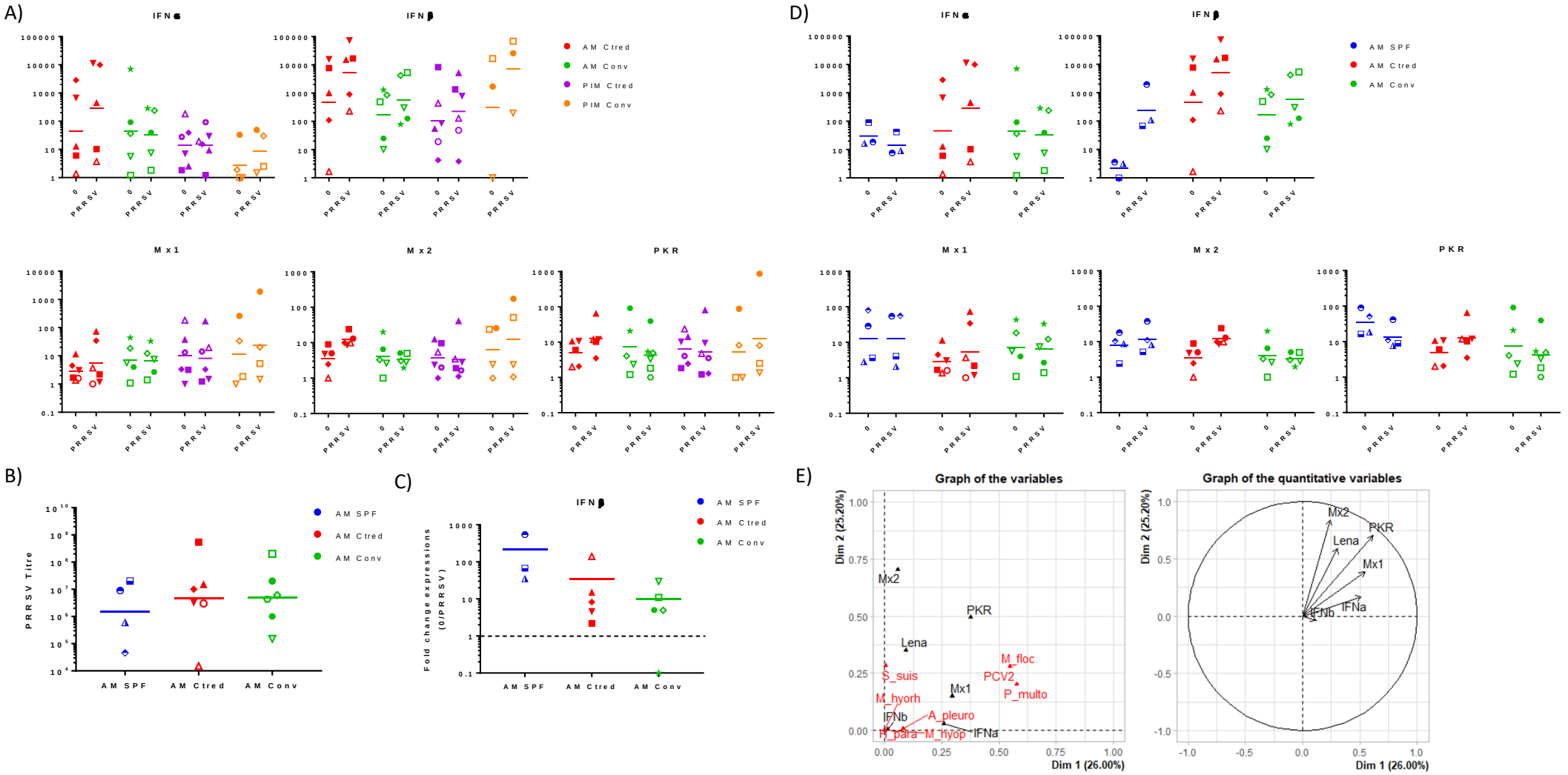

Figure 3: AM and PIM from different animals' origins did not distinctly respond to PRRSV infection.

		Controlled												Conventional											
		1		2		3		4		5		6		7		8		9		10		11		12	
		Br	LN	Br	LN	Br	LN	Br	LN	Br	LN	Br	LN	Br	LN	Br	LN	Br	LN	Br	LN	Br	LN	Br	LN
in vivo infection (whole tissues PCR detection)	PCV2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	+	-	-	+	-
	A. pleuro	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-
	H. para	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	-	-	-	-
	M. floc	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	+	-
	M. hyop	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	M. hyorh	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	P. multo	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+	+	+	+	+	-
	S. suis	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+	-	+	-	-	-	-	-	-	-
in vitro titre (AM)	PRRSV	3,3E+06		1,5E+07		1,E+07		1,5E+04		5,4E+08		3,E+06		2,0E+07		6,1E+06		1,5E+05		4,3E+06		2,0E+08		1,E+06	

Table 1: Conventional but not controlled animals presented viral and bacterial respiratory infections.

Bronchial epithelium (Br) and trachea-bronchial lymph node (LN) of animals from different sanitary status (Controlled, from UEPAO, INRA and Conventional, from commercial slaughterhouse) were tested by PCR for the presence of different respiratory pathogens as illustrated in Figure 2. The titre of *in vitro* PRRSV-infected AM (MOI 0,01, 24h post-infection, RT-qPCR-relative expression) from the same animals are depicted in the last row (see Figure 1). The second row depict the identification number of each animal as identified in figure 1).