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## Prospecting potential links between PRRSV infection susceptibility of alveolar macrophages and other respiratory infectious agents present in conventionally reared pigs

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1   Prospecting potential links between PRRSV infection susceptibility of alveolar macrophages and other  
2   respiratory infectious agents present in conventionally reared pigs.

3

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9

10

## 11 Abstract

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

27

## 28 Introduction

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

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

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

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

63

#### 64 Materials and Methods

65 **Pig lung cells collection.** Lung tissue samples were obtained from 5- to 7-month-old Large White  
66 conventionally bred sows coming from two origins: 6 animals from the Unité Experimentale de  
67 Physiologie Animal de l'Orfrasière (UEPAO, Tours, France) and 6 from PORCI MAUGES slaughterhouse  
68 (Beaupréau, France). Finally, 4 supplementary Large White specific pathogen free (SPF) 2 to 3-month-  
69 old sows from the highly controlled SPF facility of ANSES, Ploufragan (France) free of numerous  
70 respiratory pathogens such as PRRSV, swine Influenza virus, porcine circovirus type 2, *Mycoplasma*  
71 *hyopneumoniae*, *Actinobacillus pleuropneumoniae*, *Pasteurella multocida*, *Bordetella bronchiseptica*  
72 and *Haemophilus parasuis* were also tested. In order to reduce animal experiments, all the animals  
73 were euthanized in the course of the regular management process of the herds, hence the absence  
74 of trial number or experimental authorisation and the age difference according to the origin of the  
75 animals. Tracheo-bronchial tissue and lymph nodes were sampled and directly frozen in dry ice for  
76 subsequent pathogens detection (Zimmerman et al., 2012). A broncho-alveolar lavage (BAL)  
77 procedure was then performed twice on the isolated left lung with 250 mL of PBS supplemented with  
78 2mM EDTA (PBS/EDTA), to collect AM. Next, a 1-cm slice of external lung parenchyma was dissected  
79 from the same lung. Tissues were minced and incubated in nonculture-treated Petri dishes, to avoid  
80 plastic adherence of macrophages, for 2 h at 37 °C in complete RPMI, consisting of RPMI 1640  
81 supplemented with 100 IU/mL penicillin, 100 mg/mL streptomycin, 2 mM L-glutamine, and 10%  
82 inactivated foetal calf serum (FCS) (all from Invitrogen, Paisley, UK), containing 2 mg/mL collagenase  
83 D (Roche, Meylan, France), 1 mg/mL dispase (Invitrogen), and 0.1 mg/mL Dnase I (Roche). Cells were  
84 passed through 40 µm cell strainers and red blood cells lysed with erythrocytes lysis buffer (10 mM  
85 NaHCO<sub>3</sub>, 155 mM NH<sub>4</sub>Cl, and 10 mM EDTA). Next, cells were washed with PBS/EDTA, counted, and  
86 step-frozen in FCS/10% dimethyl sulfoxide (Sigma-Aldrich, St Louis, MO).

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

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

128 **PCR for pathogens detection.** Tracheobronchial tissue and lymph node were thawed and  
129 mechanically (Lysing matrix E FastPrep24, MpBio) disrupted before DNA extraction using QIAamp  
130 cador Pathogen kit (INDICAL BIOSCIENCE). A proteinase K enzymatic lysis was then performed. A  
131 PCR was then run using previously described primers for PCV2 (F: TTT AGG GTT TAA GTG GGG  
132 GGT C, R: CCG GAT CCA TGA CGT ACC CAA GGA GGC G, 470 bp fragment expected) *Pasteurella*  
133 *multocida* (F: AAG GGA TGT TGT TAA ATA GAT AGC, R: GCT TCG GGC ACC AAG CAT AT, 410  
134 bp fragment expected) *Haemophilus parasuis* (F: GTG ATG AGG AAG GGT GGT GT, R: GGC TTC GTC  
135 ACC CTC TGT, 820 bp fragment expected), *Streptococcus suis* (F: TTC TGC AGC GTA TTC TGT CAA  
136 ACG, R: TGT TCC CTG GAC AGA TAA AGA TGG, 700 bp fragment expected) (Cheong et al., 2017)  
137 or for *Mycoplasma* species (*M. hyopneumoniae* F: TTCAAAGGAGCCTTCAAGCTTC, 1,000 bp fragment  
138 expected; *M. flocculare* F: GGAAGAAAAAATTAGGTAGGG, 754 bp fragment expected; *M.*  
139 *hyorhinis*, F: CGGGATGTAGCAATACATTGAG, 1129 bp fragment expected, and a common reverse  
140 primer R: AGAGGCATGATGATTTGACGTC) (Stakenborg et al., 2006). The PCR was performed in a  
141 20  $\mu$ L reaction mixture containing 4  $\mu$ L of extracted DNA (containing a minimum of 25 ng of DNA),  
142 0,5  $\mu$ M of each primer, 1,5 to 2,5 mM of MgCl<sub>2</sub>, 0,1 mM of deoxynucleoside triphosphate and 0,02 U of  
143 GoTaq G2 Flexi DNA Polymerase (Promega). The PCR was carried out for 40 cycles consisting of  
144 denaturation for 20 seconds at 94°C, annealing for 30 seconds at T<sub>m</sub> and extension for 30 seconds at  
145 72°C using thermal cycle (BioRad). Then, qPCR data were expressed as relative values after Genex  
146 macro analysis (Bio-Rad) (Vandesompele et al., 2002) using the *C<sub>q</sub>* from the samples for the different  
147 transcripts.

148  
149 **Statistical analysis.** Data were analysed using Graph Pad Prism (version 6) and Mann-Whitney test  
150 was used to compare relative expressions. Non-parametric approaches were chosen due to the few  
151 number of samples available. The Mann-Whitney's test was used to compare unpaired samples  
152 based on ranking. The AM and PIM from the same animal were considered as paired samples. For  
153 paired samples, the Wilcoxon matched-pairs signed rank test was used. When scatter plots are used,  
154 the mean is depicted by a horizontal bar. Factor Analysis for Mixed Data (FAMD) was performed  
155 using FactoMineR package of RStudio (version 3.6.1) in order to investigate the relation between

156 isolated pathogens and gene expression.

157

158

## 159 Results and Discussion

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

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

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

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

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

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

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

216 Despite this negative result, that might be confirmed by a larger scale campaign, our study allowed  
217 us to highlight a clear correlation between AM and PIM PRRSV susceptibility *in vitro*, in agreement  
218 with their high similarity (Bordet et al., 2018; Maisonnasse et al., 2016). Moreover, we could note  
219 that in the *Pays de la Loire* area (France), conventional, commercial pigs presented systematically  
220 one or several respiratory infections in perfect agreement with the concept of CRP, with 4 out of 6  
221 (2/3) of the conventional pigs presenting at least two simultaneous infections, among them 3 out of  
222 4 presented at least 3 pathogens.

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

228

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234

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236

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241

242

#### 243 Figures Legends

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

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

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

254

255 Figure 2: Detection of respiratory pathogens.

256 Example of positive samples detected by PCR of PCV2 (PCV2 - 470 bp), *Streptococcus suis* (*S. suis* -  
257 700 bp), *Haemophilus parasuis* (*H. parasuis* - 820 bp), *Pasteurella multocida* (*P. multocida* - 410 bp),  
258 and *Mycoplasma* species (*M. hyopneumoniae* - 1,000 bp, *M. flocculare* - 754 bp, *M. hyorhinis* - 1,129  
259 bp) from Tracheal epithelium and trachea-bronchial lymph node. The results for all pigs are shown in  
260 Table 1.

261

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

263 A) Transcriptomic expression of type I IFN $\alpha$  and  $\beta$  genes and three representative interferon  
264 stimulated genes Mx1, Mx2 and PKR from *in vitro* infected AM and PIM from different origins. The  
265 samples are the same, with the same identification as in Figure 1. B) Comparison of *in vitro* PRRSV  
266 infection of AM from SPF animals with the previously depicted (Figure 1) PRRSV infected AM from  
267 controlled and conventional animals. C) Transcriptomic expression of type I IFN $\alpha$  and  $\beta$  genes and  
268 three representative interferon stimulated genes Mx1, Mx2 and PKR from *in vitro* infected AM from  
269 SPF animals. The samples are the same, with the same identification as in Figure 3B. D) Factor  
270 Analysis for Mixed Data (FAMD) plotting together data from Figure 1, Table 1 and Figure 3.

271

## 272 Citations

273 Blanquefort, P., Benoit, F., 2000. Eradication of Porcine Reproductive and Respiratory Syndrome virus  
274 in 125 herds in "Pays de la Loire" (France). *Vet. Res.* 31, 94–95.

275 <https://doi.org/10.1051/vetres:2000008>

276 Bordet, E., Maisonnasse, P., Renson, P., Bouguyon, E., Crisci, E., Tiret, M., Descamps, D., Bernelin-  
277 Cottet, C., Urien, C., Lefèvre, F., Jouneau, L., Bourry, O., Leplat, J.-J., Schwartz-Cornil, I., Bertho,  
278 N., 2018. Porcine alveolar macrophage-like cells are pro-inflammatory pulmonary intravascular  
279 macrophages that produce large titers of porcine reproductive and respiratory syndrome virus.  
280 *Sci. Rep.* 8. <https://doi.org/10.1038/s41598-018-28234-y>

281 Cheong, Y., Oh, C., Lee, K., Cho, K., 2017. Survey of porcine respiratory disease complex-associated  
282 pathogens among commercial pig farms in Korea via oral fluid method. *J. Vet. Sci.* 18, 283.

283 <https://doi.org/10.4142/jvs.2017.18.3.283>

284 Delgado-Ortega, M., Melo, S., Meurens, F., 2011. Expression of SOCS1-7 and CIS mRNA in porcine  
285 tissues. *Vet. Immunol. Immunopathol.* 144, 493–498.

286 <https://doi.org/10.1016/J.VETIMM.2011.08.002>

287 Dobrescu, I., Levast, B., Lai, K., Delgado-Ortega, M., Walker, S., Banman, S., Townsend, H., Simon, G.,  
288 Zhou, Y., Gerdts, V., Meurens, F., 2014. In vitro and ex vivo analyses of co-infections with swine  
289 influenza and porcine reproductive and respiratory syndrome viruses. *Vet. Microbiol.* 169, 18–  
290 32. <https://doi.org/10.1016/j.vetmic.2013.11.037>

291 Ellis, J., Clark, E., Haines, D., West, K., Krakowka, S., Kennedy, S., Allan, G.M., 2004. Porcine circovirus-  
292 2 and concurrent infections in the field, in: *Veterinary Microbiology*. Elsevier, pp. 159–163.

293 <https://doi.org/10.1016/j.vetmic.2003.10.008>

294 Lee, J.A., Oh, Y.R., Hwang, M.A., Lee, J.B., Park, S.Y., Song, C.S., Choi, I.S., Lee, S.W., 2016.

295 *Mycoplasma hyorhinis* is a potential pathogen of porcine respiratory disease complex that  
296 aggravates pneumonia caused by porcine reproductive and respiratory syndrome virus. *Vet.*

297 *Immunol. Immunopathol.* 177, 48–51. <https://doi.org/10.1016/j.vetimm.2016.06.008>



- 298 Lévesque, C., Provost, C., Labrie, J., Hernandez Reyes, Y., Burciaga Nava, J.A., Gagnon, C.A., Jacques,  
299 M., 2014. *Actinobacillus pleuropneumoniae* Possesses an Antiviral Activity against Porcine  
300 Reproductive and Respiratory Syndrome Virus. *PLoS One* 9, e98434.  
301 <https://doi.org/10.1371/journal.pone.0098434>
- 302 Maisonnasse, P., Bouguyon, E., Piton, G., Ezquerra, A., Urien, C., Deloizy, C., Bourge, M., Leplat, J.J.,  
303 Simon, G., Chevalier, C., Vincent-Naulleau, S., Crisci, E., Montoya, M., Schwartz-Cornil, I.,  
304 Bertho, N., 2016. The respiratory DC/macrophage network at steady-state and upon influenza  
305 infection in the swine biomedical model. *Mucosal Immunol.* 9, 835–849.  
306 <https://doi.org/10.1038/mi.2015.105>
- 307 Montaner-Tarbes, S., del Portillo, H.A., Montoya, M., Fraile, L., 2019. Key Gaps in the Knowledge of  
308 the Porcine Respiratory Reproductive Syndrome Virus (PRRSV). *Front. Vet. Sci.* 6, 38.  
309 <https://doi.org/10.3389/FVETS.2019.00038>
- 310 Opriessnig, T., Giménez-Lirola, L.G., Halbur, P.G., 2011. Polymicrobial respiratory disease in pigs.  
311 *Anim. Heal. Res. Rev.* 12, 133–148. <https://doi.org/10.1017/S1466252311000120>
- 312 Saade, G., Deblanc, C., Bougon, J., Marois-Créhan, C., Fablet, C., Auray, G., Belloc, C., Leblanc-  
313 Maridor, M., Gagnon, C.A., Zhu, J., Gottschalk, M., Summerfield, A., Simon, G., Bertho, N.,  
314 Meurens, F., 2020. Coinfections and their molecular consequences in the porcine respiratory  
315 tract. *Vet. Res.* 51, 80. <https://doi.org/10.1186/s13567-020-00807-8>
- 316 Sang, Y., Rowland, R.R.R., Blecha, F., 2011. Interaction between innate immunity and porcine  
317 reproductive and respiratory syndrome virus. *Anim. Heal. Res. Rev.* 12, 149–167.  
318 <https://doi.org/10.1017/S1466252311000144>
- 319 Stakenborg, T., Vicca, J., Butaye, P., Imberechts, H., Peeters, J., De Kruif, A., Haesebrouck, F., Maes,  
320 D., 2006. A Multiplex PCR to Identify Porcine Mycoplasmas Present in Broth Cultures. *Vet. Res.*  
321 *Commun.* 30, 239–247. <https://doi.org/10.1007/s11259-006-3226-3>
- 322 Thacker, E.L., Halbur, P.G., Ross, R.F., Thanawongnuwech, R., Thacker, B.J., 1999. *Mycoplasma*  
323 *hyopneumoniae* Potentiation of Porcine Reproductive and Respiratory Syndrome Virus-Induced  
324 Pneumonia. *J. Clin. Microbiol.* 37, 620–627.
- 325 Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, A., Speleman, F., 2002.  
326 Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of  
327 multiple internal control genes. *Genome Biol.* 3, research0034.1. [https://doi.org/10.1186/gb-](https://doi.org/10.1186/gb-2002-3-7-research0034)  
328 [2002-3-7-research0034](https://doi.org/10.1186/gb-2002-3-7-research0034)
- 329 Zimmerman, J.J., Karriker, L.A., Ramirez, A., Schwartz, K.J., Stevenson, G.W. (Eds.), 2012. Diseases of  
330 Swine. John Wiley & Sons, Inc., Ames, Iowa, USA, pp. 383–885.
- 331

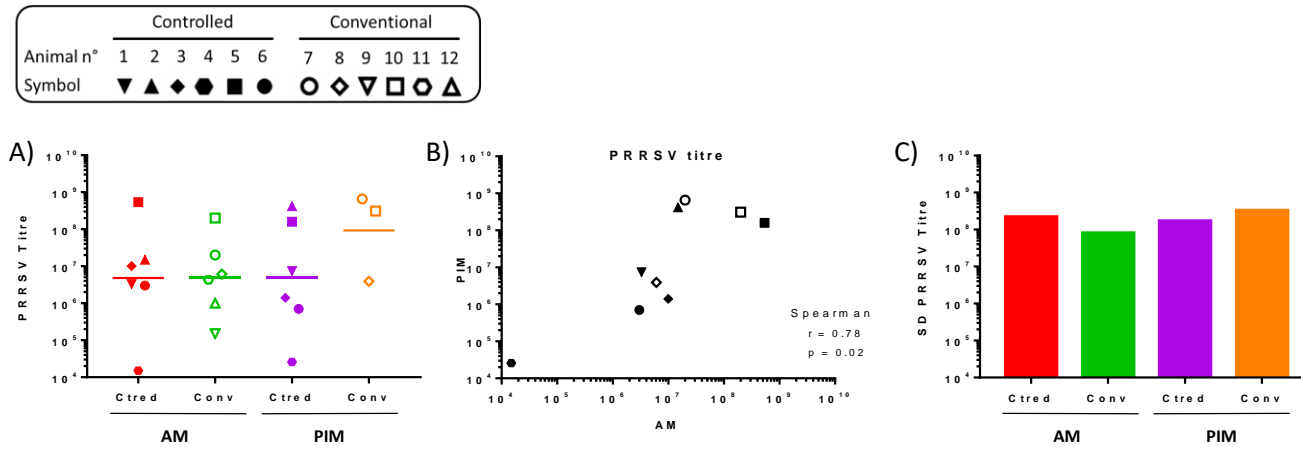


Figure 1: AM and PIM from different animals' origins were not distinctly susceptible to PRRSV infection

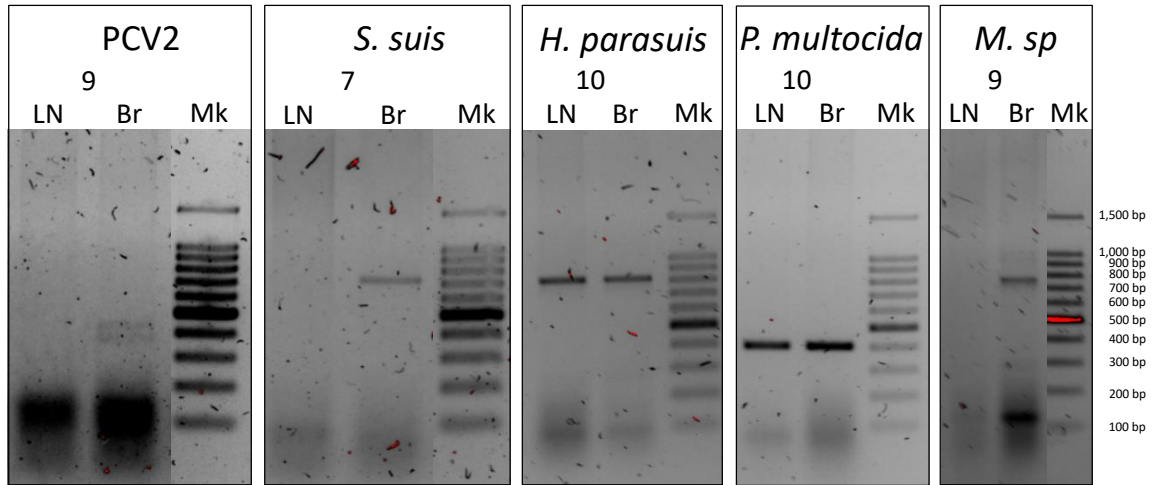


Figure 2: Detection of respiratory pathogens

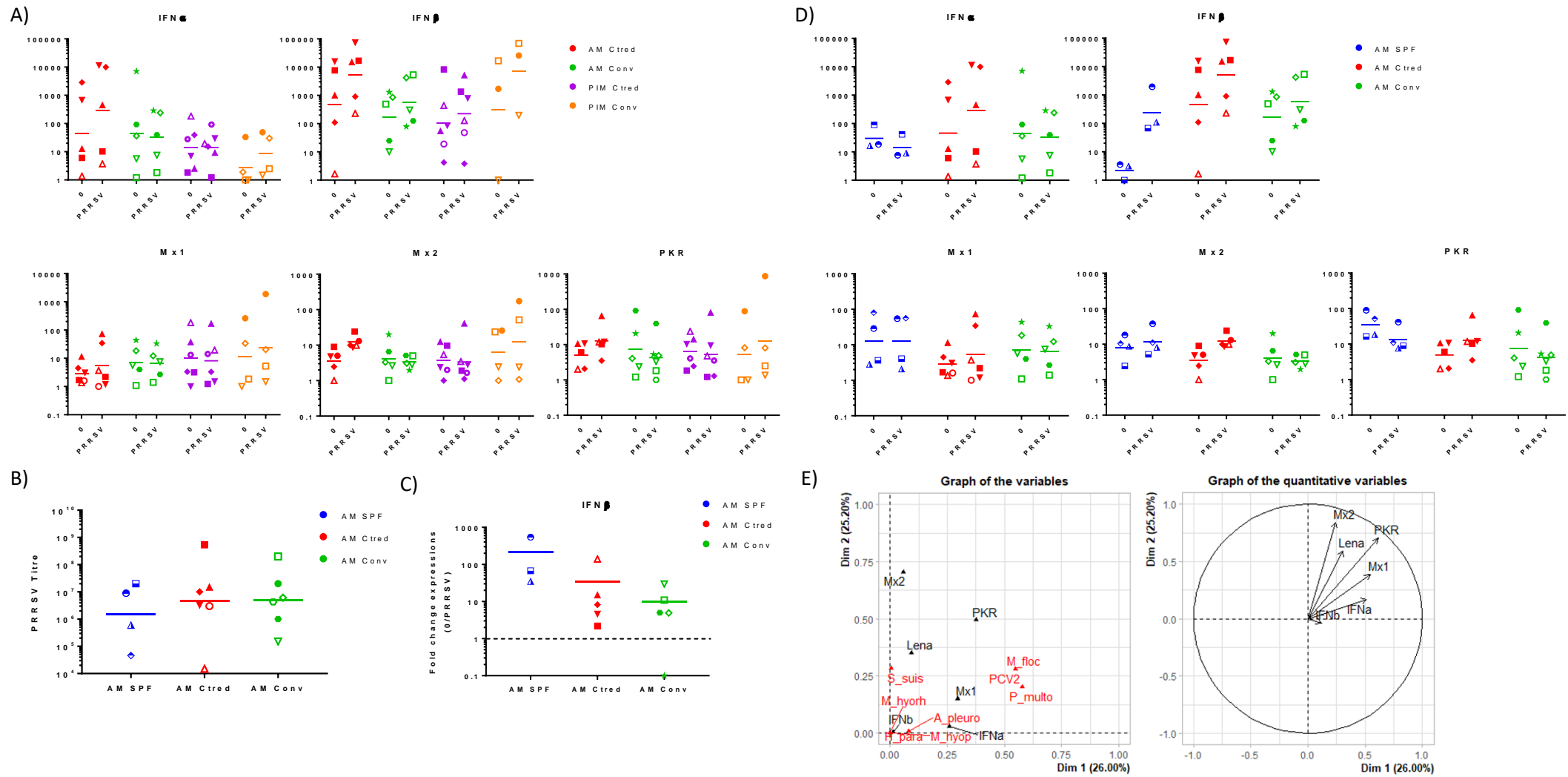


Figure 3: AM and PIM from different animals' origins did not distinctly respond to PRRSV infection.

		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
<i>in vivo</i> infection (whole tissues PCR detection)	PCV2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	+	-	-	+	-
	<i>A. pleuro</i>	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-
	<i>H. para</i>	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	-	-	-	-
	<i>M. floc</i>	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	+	-
	<i>M. hyop</i>	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	<i>M. hyorh</i>	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	<i>P. multo</i>	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+	+	+	+	+	-
<i>S. suis</i>	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+	-	+	-	-	-	-	-	-	-	
<i>in vitro</i> 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).