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1 **EXPLORATORY METAGENOMIC ANALYSES OF PERIWEANING**

2 **FAILURE-TO-THRIVE SYNDROME (PFTS) AFFECTED PIGS**

3

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19

20 **ABSTRACT**

21 Modern swine farming is characterized by the emergence of several syndromes whose
22 aetiology is unclear or has a multi-factorial origin, including periweaning failure-to-
23 thrive syndrome (PFTS). In fact, its specific aetiology remains elusive, although several
24 causes have been investigated over time. The present study aimed to investigate the
25 potential role of viral agents in PFTS-affected and heathy animals by evaluating the
26 virome composition of different organs using a metagenomics approach. This analysis
27 allowed demonstrating a higher abundance of *Porcine parvovirus 6* (PPV6) in healthy
28 subjects while *Ungulate bocaparvovirus 2* (BoPV2), *Ungulate protoparvovirus 1* (PPV)
29 and *Porcine circovirus 3* (PCV-3) were increased in pigs with PFTS. No differential
30 abundance of RNA viruses were found between PFTS-affected and control pigs.
31 Remarkably, this is the first molecular characterization of PPV6 and BoPV2 in Spain
32 and one of the few all around the world, supporting their apparent widespread
33 circulation. Interestingly, PCV-3 has been recently identified in several clinical-
34 pathological conditions as well as in healthy pigs, while BoPV2 pathogenic potential is
35 unknown. Although obtained results must be taken as preliminary, they open the door
36 for further studies on the potential role of these viruses or their combination as
37 predisposing factor/s for PFTS occurrence.

38

39 Key words: periweaning failure-to-thrive syndrome (PFTS); metagenomics, *Porcine*
40 *circovirus 3*; *Porcine parvovirus 6; Ungulate bocaparvovirus 2; Ungulate*
41 *protoparvovirus 1*; case-control

INTRODUCTION

Episodes of failure to thrive and growth retardation can be occasionally seen in the nursery phase. However, they were typically neglected because of the marginal economic significance.[1] In most of the cases, those situations were usually attributed to the lack of proper adaptation of the piglet from the liquid diet during the lactation period to the solid one at nursery.[2] This is expectable to a certain low percentage of piglets and modern pig production deals with this problem by means of introducing solid feed to piglets during lactation, use of fermented liquid feeds in nursery[2] and by creating a friendlier environment for the piglet at weaning.[3] By 2008, in North-America, several unrelated farms experienced a significantly increased number of piglets with anorexia, lethargy, progressive debilitation and death mainly within the first week after weaning.[4] Such scenario was clinically described with names such as postweaning catabolic syndrome, postweaning wasting-catabolic syndrome, failure to thrive syndrome, and postweaning fading pig-anorexia syndrome.[4]

The emergence of more frequent and relevant outbreaks of this syndrome in Canada prompted to develop a more precise case definition. By 2010, the condition was named periweaning failure-to-thrive syndrome (PFTS). Clinically, PFTS-affected pigs are characterized by progressive loss of weight and debilitation in absence of discernible and detrimental infectious, nutritional, management or environmental factors.[4] Although not constant, a number of diseased pigs show an unusual repetitive oral behaviour such as licking, chewing or chomping,[5] which makes the condition easier to suspect. Although several histopathological lesions have been evidenced in PFTS, the current definition includes the presence of superficial gastritis, thymic atrophy and small intestine villous atrophy.[6]

66 While clinical signs were initially suspected to be caused by an unknown
67 infectious agent, the role of most known parasitic, bacterial and viral agents were ruled
68 out; most of the studied pathogens were not found in PFTS affected piglets or they were
69 identified with comparable frequency in healthy and diseased pigs.[5] Additionally,
70 attempts to reproduce the disease by pig inoculation with tissue homogenates of
71 diseased animals were unsuccessful.[7] This evidence was not supportive of the
72 hypothesis that PFTS may have an infectious etiology.
73 Interestingly, some studies highlighted the potential genetic predisposition to the
74 disease,[8,9] which would be ascribable to variability in genes involved in brain
75 development and metabolism and part of signalling pathways associated to neurological
76 and depressive disorders in human beings.[10,11]
77 PFTS was diagnosed in Spain for the first time in 2012,[12] and although the
78 condition had a significant impact in some farms, the incidence of the condition has
79 apparently decreased. Nevertheless, the true aetio-pathogenesis of PFTS is still obscure
80 and unresolved, and the role of an unknown pathogen cannot be completely excluded,
81 especially considering that the current knowledge of the virosphere is still at its infancy
82 and more viral species are continuously being discovered.[13-16] Based on these premises,
83 an exploratory metagenomics analysis was performed for the first time to evaluate the
84 presence of previously unknown viral pathogens in PFTS pigs and in healthy
85 counterparts.
86
87 **MATERIALS AND METHODS**

88 **Farm and piglet selection**

89 After initial diagnosis of PFTS in Spain[12] an increasing number of clinical cases were
90 further characterized as PFTS based on established criteria.[4] The affected farm from

91 which PFTS affected pigs were selected was located in North-Eastern Spain. It
92 consisted of a 360-sow farm producing 18 kg pigs, with a historical nursery mortality of
93 1.78% before 2010. From October 2010 to April 2012, nursery mortality increased to an
94 average of 5.3% and most of the difference between periods accounted for piglets
95 undergoing wasting during the first two weeks post-weaning. The farm was
96 seronegative against Aujeszky's disease virus, *Porcine reproductive and respiratory*
97 *syndrome virus* (PRRSV) and *Mycoplasma hyopneumoniae*.

98 Three 6 week-old, cross-bred female piglets with clinical signs consistent with
99 PFTS were selected, euthanized with an intravenous overdose of pentobarbital and
100 necropsied. At necropsy, tissue samples (lung, intestine, kidney, liver and brain) were
101 collected and frozen in -80°C until processing for Next Generation Sequencing (NGS)
102 as described below. Also, a wider set of tissues (including also nasal turbinates, heart,
103 tonsil, inguinal superficial lymph node, spleen and stomach) were collected, fixed by
104 immersion in 10% buffered formalin, embedded in paraffin and sectioned at 4 μm for
105 histopathological analyses. All three animals displayed thymus atrophy, serous atrophy
106 of the fat, atrophy and fusion of villi and mild lympho-plasmacytic gastritis. *Porcine*
107 *circovirus 2* (PCV-2) and PRRSV were also ruled out by means of
108 immunohistochemical methods.

109 Three age-matched cross-bred healthy piglets (two males and one female) from
110 a 500-sow farm that never displayed clinical signs compatible with PFTS were selected
111 as negative controls. The herd was seronegative against Aujeszky's disease virus and
112 PRRSV, but seropositive to *M. hyopneumoniae*. These animals were subjected to the
113 same procedures as mentioned above for the affected pigs, and did not have any
114 noticeable gross or histological lesion.

115

116 **DNA and RNA extraction**

117 DNA and RNA were extracted from brain, intestine, kidney, liver and lung of three
118 healthy animals and three PFTS cases.

119 For total DNA extraction, 30 mg of each frozen tissue was homogenized in Tris-
120 buffer, followed by three freeze-thaw cycles. Samples were then centrifuged to pellet
121 nuclei and large cellular aggregates, and filtered through 0.45 µm filter. To remove
122 remaining host genomic DNA, the samples were treated with DNase. Finally, DNA was
123 extracted using NucleoSpin Blood (Macherey-Nagel) according to manufacturer's
124 instructions. At this point, pools were generated mixing equimolar amounts of DNA
125 treated as explained above, from brain (pool A), intestine (pool B), and kidney, liver and
126 lung (pool C) from PFTS affected and healthy piglets. The quality and quantity of DNA
127 pools were verified by Bioanalyzer and spectrophotometry (Nanodrop).

128 For total RNA extraction, 30 mg of each frozen tissue was treated with DNAse
129 and homogenized in Trizol reagent followed by RNA isolation using Ambion Ribo Pure
130 columns as recommended by the manufacturer (Life Technologies). Contaminating
131 DNA was removed from RNA preparations using TURBO DNA-free Kit (Ambion, Life
132 Technologies). The integrity of purified RNA was confirmed by Bioanalyzer and
133 concentration determined by spectrophotometry (Nanodrop). According to preliminary
134 NGS experiments, ribosomal RNA was present in the RNA samples in excessive
135 amounts. Therefore, a ribosomal RNA (rRNA) removal was tested and subsequently
136 incorporated in the protocol. RiboMinus Eukaryote System v2 (Ambion, Life
137 Technologies) was used to remove the rRNA from 5 µg of pooled total RNA according
138 to recommendations. Removal of rRNA was verified by Bioanalyzer. At this point,
139 pools were generated including total RNA from brain (pool A), intestine (pool B), and
140 kidney, liver and lung (pool C) from all tested animals

141

142 **Library preparation and Next Generation Sequencing.**

143 Sequencing libraries were prepared for each pool and were run as recommended by the
144 manufacturer (Ion Torrent, Life Sciences). Each library was run in a 318 chip and post-
145 sequencing processing was done based on quality, removing low quality reads and
146 polyclonal reads. Resulting files for each pool were used in the data analysis.

147

148 **NGS data analysis**

149 Quality of obtained FASTQ files was visually inspected using PRINTSEQ[17] and reads
150 shorter than 30 bp, with an average Phred quality score lower than 30 or with more than
151 one base with a quality lower that 15, were removed. Additionally, tag removal and
152 trimming of poor quality bases, poly-A/T and poly-N at 5' and 3' tails were performed
153 using the same software.

154 To remove the host genome contamination, obtained reads were aligned to the
155 *Sus scrofa* reference genome using bowtie2.[18] Unaligned reads were processed for viral
156 taxonomic classification using Kraken,[19] benefiting of the computational power offered
157 by the Galaxy platform.[20] Results were further confirmed using MGmapper.[21] In both
158 cases, the most updated version of the viral database, available on the respective web
159 server (https://usegalaxy.org/ and https://cge.cbs.dtu.dk/services/Mgmapper-2.4), was
160 selected as reference dataset for the analysis.

161 The classified reads count was normalized and the presence of statistically
162 significant differences in the abundance of viral reads between PFTS and healthy
163 animals in different tissues was assessed using the *metagenomeSeq*[22] library
164 implemented in R.

165 More particularly, normalization was performed using the *cumNorm* function.
166 The cumNormStatFast function was used to select the most appropriate percentile to
167 normalize the reads counts . Statistical testing of differences between diseased and
168 healthy animals was assessed on all tissues altogether using the Zero-inflated Log-
169 Normal mixture model, implemented in the *fitFeatureModel* function. Similarly, for the
170 comparison among different tissues, the Limma's topTable function for F-tests and
171 respective contrast functions, implemented in the same R library, were used.
172
173 **Reference based alignment**
174 The whole reference genomes of viruses detected to be differentially present between
175 healthy and diseased animals were downloaded from RefSeq and used for reference-
176 based alignment using Bowtie2.[18] Samtools[23] was used to convert, sort and remove
177 duplicates from the obtained SAM files. Finally, sample specific coverage and
178 consensus sequence were generated using QUASR.[24] The obtained consensus
179 sequences, originating from the three pooled individuals, have been made available in
180 GenBank (Accession Numbers: MH558676-MH558679).
181 To confirm the obtained viral classification, for each examined viral species, a
182 set of sequences was downloaded from the GenBank and aligned with the consensus
183 sequence obtained from experimental samples. A species-specific phylogenetic tree was
184 reconstructed to confirm and improve the viral classification using the Maximum
185 likelihood method implemented in PhyML,[25] selecting as substitution model the one
186 with the lowest AIC calculated using JmodelTest.[26] The robustness of the clade
187 reliability was evaluated using the fast non-parametric version of the aLRT
188 (Shimodaira–Hasegawa [SH]-aLRT), developed and implemented in PhyML 3.0.[27]
189

RESULTS

Next generation sequencing analyses.

Around 500 thousand NGS reads were obtained for each sample; most of those passed the quality evaluation step and were included in the final analysis. A more detailed summary of available reads is reported in Table 1.

Considered reads were assigned to several viral families using the Kraken analysis (Supplementary tables 1 and 2). However, only four DNA viruses' abundances differed significantly ($P < 0.001$) between healthy and diseased animals: Porcine parvovirus 6 (PPV6), *Ungulate bocaparvovirus 2* (BoPV2), *Ungulate protoparvovirus 1* (PPV) *and Porcine circovirus 3* (PCV-3). PPV6 was mostly represented in healthy animals while the other viruses (BoPV2, PPV and PCV-3) were more abundant in diseased ones (Table 2). No statistical difference was observed in viral tissue distribution with the only exception of BoPV2, which was detected only in the intestine PFTS animals (Table 2).

No differences were observed in RNA viruses' frequency, but a low number of reads was mapped to RNA viruses of potential veterinary interest with the exact match k-mers approach implemented in Kraken. Differently from the DNA analysis, where Kraken and MGmapper provided essentially concordant results, Kraken RNA results could not be confirmed with the MGmapper analyses, which identified no relevant RNA viruses.

Reference based alignment

The complete genome sequence was reconstructed for the four above mentioned viruses. The posterior sequence comparison through BLAST and phylogenetic analysis confirmed the taxonomic classification. More specifically, percentages of identity of

215 99%, 95%, 99% and 99% were proven compared to the most closely related sequences
216 published in the GenBank, for PPV6, BoPV2, PPV and PCV-3 respectively. Reference
217 based alignment of detected RNA viruses of potential interest for swine industry yielded
218 negative results, suggesting the identification of unspecific matches due to the small k-
219 mer size using Kraken and confirming MGmapper results.

220 No among-organ differences were observed in the consensus sequences of the
221 considered viruses.

222

223 DISCUSSION

224 The development of isolation- and sequence-independent metagenomics tools has
225 remarkably increased the detection of previously unknown viruses during overt clinical
226 disease episodes.[15,28,29] Nevertheless, the detection of a new virus species does not
227 imply a causal nexus and reliable proof of the pathogenic role are often hard to be
228 proven. In fact, the modern livestock farming is characterized by the identification of
229 several clinical syndromes whose aetiology is unclear or, more frequently, has a multi-
230 factorial origin. Accordingly, several infectious diseases are considered to be
231 "conditioned diseases", whose overt clinical manifestation occurs only in presence of
232 several co-factors and/or co-infections.[30]

233 The present study reports the comparison of healthy pigs' virome in different
234 tissues with the one of subjects experiencing PFTS. The NGS analysis performed on
235 pooled samples allowed achieving relevant sequence numbers for each tissue, which
236 were assigned to a number of specific taxonomic units. Interestingly, a low number of
237 RNA reads were classified as viral species. Since the total read number for each pool
238 was comparable between RNA and DNA pools (Table 1) and the same bioinformatics
239 approach was used, a less effective removal of host or other microorganism RNA during

240 sample processing was likely. Alternatively, the presence of a higher number of reads
241 originating from unidentified RNA viruses is possible. Although no differences were
242 detected in RNA virus abundance, four species belonging to DNA-virus groups
243 displayed a statistically significant different abundance between healthy and diseased
244 animals: PPV6, BoPV2, PPV and PCV-3. Among those, PPV6 (family *Parvoviridae*,
245 subfamily *Parvovirinae*, proposed genus *Copiparvovirus*)[31] was the only virus sequence
246 overrepresented in healthy animals and was detected in all considered tissue pools. The
247 current information about this species is scarce, however, its detection in USA,[32]
248 China,[31] Poland[33] and Spain (present study) allows speculating a widespread
249 distribution of this virus. Moreover, there is no clear association between PPV-6
250 infection and clinical outcome in pigs.[31,32]

251 On the contrary, the other two members of the family *Parvoviridae*, subfamily
252 *Parvovirinae* (BoPV2 and PPV) were more abundant in diseased animals. Particularly,
253 BoPV2, genus *Bocaparvovirus*, was only detected in the intestine of PFTS animals. The
254 herein described strain represents the first PBoV2 detection in Spain and one of the few
255 genetic characterizations all around the world. BoPV2 has been previously detected in
256 Sweden,[34] Germany[35] and China.[36] This genus include several viral species, and some
257 of them have been associated to enteric and respiratory disease in animals and human
258 beings.[37] Although no proof has been reported supporting the pathogenic role of
259 ungulate bocaparvoviruses in swine, a higher infection prevalence has been described in
260 pigs co-infected with other pathogens, providing support for a potential facilitating role
261 in other infections.[38,39] PPV (genus *Protoparvovirus)* is a well-established pathogen of
262 swine, with a noteworthy impact on animal health. However, its relevance is
263 substantially linked to reproductive disorders after foetal infection and no significant
264 effects on post-natal pigs have been reported except for a transient lymphopenia.[40]

265 Consequently, a clear role of these viruses in the observed clinical signs cannot be
266 claimed.

267 Remarkably, the present study demonstrates the presence of higher abundance of
268 PCV-3 sequences in all the considered tissues of PFTS pigs only. This recently
269 discovered virus has already been detected in samples from pigs suffering from several
270 clinical syndromes,[41-43] even though its pathological role has not been proven yet.[44-47]

271 The simultaneous detection of PCV-3 and different porcine parvoviruses in
272 PFTS affected pigs is at least of interest. Both experimental and field studies
273 demonstrated that co-infection with PPV increase the effect of PCV-2 in causing porcine
274 circovirus 2-systemic disease (PCV-2-SD).[48,49] Similarly, the first detection of porcine
275 boca-like virus occurred in the background of PCV-2-SD.[34] If a similar synergistic
276 effect between PCV-3 and detected parvoviruses is involved in PFTS emergence will
277 need further investigations.

278 Previous studies have revealed evidence against the infectious aetiology of
279 PFTS, including the failure to reproduce disease through the use of inocula from
280 diseased animal tissue homogenates.[7] While this evidence is undoubtedly suggestive of
281 a non-infectious cause, it cannot be considered definitive. In fact, the presence of the
282 etiological agent cannot be sufficient to elicit clinical signs' appearance in the
283 framework of a multi-factorial disease hypothesis. A genetic predisposition has also
284 been reported, linking the disease to a hereditary reduced stress resistance during the
285 weaning phase.[10,11] However, an interaction between infection susceptibility, disease
286 development and genetic background has been already demonstrated for a number of
287 infectious diseases in different animal species,[50-52] and it might be also the case of
288 PFTS.

289 It must be stressed that no statistical test could be performed to assess the
290 presence of a significant difference in viral abundance tissue by tissue between healthy
291 and diseased animals. However, the comparable read numbers in different tissue pools
292 and the remarkable difference (typically presence vs absence) between healthy and
293 diseased animals in the considered samples, support a differential viral abundance in all
294 tissues of the compared groups, being the BoPV2 the only relevant exception.

295 The present study was considered as exploratory in nature, and some limitations
296 must be highlighted. On one hand, the limited number of considered animals and
297 sample pooling may hide the inference of obtained conclusions on a broader scale. On
298 the other hand, an additional control group (healthy pigs from the same PFTS affected
299 farm) would have been desirable to completely rule out herd-level differences unrelated
300 to PFTS. Therefore, further studies with higher number of animals and the three group-
301 comparison design might provide more solid evidence of the potential infectious
302 causation of PFTS. Similarly, the absence of a no-template control during library
303 preparation and sequencing steps does not allow excluding the presence of some
304 “contaminant” reads in the final dataset. In fact, a low number of reads classified as
305 DNA viruses was detected in RNA-based libraries and vice versa. However, the count
306 of these exceptions was orders of magnitude lower than the gap in the abundance of
307 those viral species demonstrated to significantly differ between PFTS and healthy
308 animals, supporting the reliability of the results. In spite of these limitations, this study
309 opens the door for a new aetiological hypothesis on the PFTS pathogenesis and
310 stimulates dedicated and more extensive studies to provide a clearer aetio-pathogenic
311 picture for this disease.

312

313

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320 **Ethics approval** Data for this study came from previous diagnostic animal studies
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323

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481 Table 1. Summary of obtained and included reads for each animal group and tissue.

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Nucleic Acid	Animal Group	Tissue Pool	Reads number	Mean length	Reads included in the analysis
DNA	Pools from Healty animals	Brain (pool A)	4475711	272	4271248
		Intestine (pool B)	5263610	270	5029314
		Kidney/liver/lung (pool C)	3867029	240	3443772
	Pools from PFTS animals	Brain (pool A)	4596751	246	3640701
		Intestine (pool B)	5519346	269	5267485
		Kidney/liver/lung (pool C)	4418469	251	4115840
RNA	Pools from Healty animals	Brain (pool A)	5980195	148	3110813
		Intestine (pool B)	6404609	143	3559730
		Kidney/liver/lung (pool C)	6044144	132	3333853
	Pools from PFTS animals	Brain (pool A)	6002491	114	3467236
		Intestine (pool B)	6219160	117	3738157
		Kidney/liver/lung (pool C)	5971371	102	3063793

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486 Table 2. Summary of reads assigned to the considered viral species in different animal categories and tissues.
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	Brain (pool A)		Intestine (pool B)		Kidney/liver/lung (pool C)				
Virus	PFTS	Healthy	PFTS	Healthy	PFTS	Healthy	Average Count in PFTS animals	Average count in healthy animals	P-value
PPV6	0	12060	0	73749	0	28286	0	38031.67	$P < 0.001$
BoPV2	0	0	14144	0	0	0	4714.67	0	$P < 0.001$
PPV	1553	3	17646	0	460091	0	159763.33	1	$P < 0.001$
PCV-3	9845	0	2599	0	5877	0	6107	0	$P < 0.001$

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