Release of fragmented host, cell-free, genomic DNA into the circulation of pigs during infection by virulent African swine fever virus

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Abstract

African swine fever virus (ASFV) causes a severe hemorrhagic disease in domestic pigs resulting in high case fatality rates. The virus replicates in circulating cells of the monocyte-macrophage lineage and within lymphoid tissues, e.g. tonsils, spleen and lymph nodes. The infection results in high fever and a variety of clinical signs from about 3 days post infection. In this study, it was observed that one of many changes resulting from ASFV-infection within pigs was a large (>1000-fold) increase in the level of circulating cell-free DNA (cfDNA), including the beta-actin gene, derived from the nuclei of host cells, in the serum. This change occurred in parallel with the increase in circulating ASFV DNA. In addition, elevated levels (about 30-fold higher) of host mitochondrial DNA (mtDNA) were detected in serum from ASFV-infected pigs, but with a much higher baseline level of mtDNA in sera from uninfected pigs. The host derived cfDNA is derived from dead cells which may, or may not, have been infected. For comparison, the release of the cellular enzyme, lactate dehydrogenase (LDH), a commonly used marker of cellular damage, was also found to be elevated during the infection. The cfDNA is readily detected in serum and is a more sensitive host marker of ASFV infection than the release of mtDNA or LDH. In addition, sera from pigs infected by classical swine fever virus (CSFV), which causes a clinically similar disease as ASFV, were also tested but this infection did not result in the release of cfDNA, mtDNA or LDH.

Author summary

African swine fever virus causes a severe hemorrhagic disease in domestic pigs and wild boar, which often leads to death within a week. The infection results in a spectrum of different clinical signs and other changes within infected animals. In this study, we have shown, for the first time, that one consequence of infection by a highly virulent strain of this

virus is the release into the blood of host genomic DNA, in a highly fragmented form. We found an increase of >1000-fold in the level of this cell-free DNA within the serum of infected animals. Furthermore, we also showed that the level of the small circular DNA from the cell mitochondria is also elevated in serum from infected animals as is the cellular enzyme lactate dehydrogenase but these changes were less marked and occurred later. The increase in the level of the cell-free host DNA is coincident with the increase in level of the viral DNA within blood and may act as a marker for infection by a highly virulent form of the virus. Remarkably, pigs infected by classical swine fever virus, which produces similar clinical signs, did not have elevated levels of these markers in their serum.

Introduction

African swine fever (ASF) is a severe hemorrhagic disease of domestic pigs and other members of the family *Suidae*, including wild boar [1, 2]. The disease is caused by infection with African swine fever virus (ASFV) and can have a case fatality rate of up to 100% in both domestic pigs and wild boar. In consequence, the disease can cause major economic losses as well as serious animal welfare issues. The virus is classified within the genus *Asfivirus*; it is a large DNA virus belonging to the *Asfarviridae* family, indeed it is the only member of this family [3]. The viral genome is about 190 kbp in length and includes over 150 genes, many of which have unknown functions [4].

There are over 20 different genotypes of the virus (identified from the sequence of the gene encoding VP72), which have been identified in various locations across Africa [5]. Different ASFV strains can vary markedly in their virulence. A highly virulent, genotype II, virus has become important globally following its introduction into Georgia in 2007 and its subsequent spread into many countries in Europe and Asia [6]. Recently, the disease has also occurred in Haiti and The Dominican Republic [7]. It has been introduced into multiple EU

countries and in 2022 new outbreaks occurred in eight of them including Germany, Italy, Slovakia and Poland [8]. In 2018, the virus spread further into Asia and has caused massive losses within the pig production industry in China and nearby countries, e.g. Vietnam, Laos, Cambodia and South Korea [9]. The virus continues to cause outbreaks in these different regions.

Infection of domestic pigs results in high fever (often >41 °C) together with a range of rather non-specific clinical signs, including lethargy and anorexia, that occur within a few days of infection [10, 11]. The presence of skin hemorrhages is often observed [12, 13], while vomiting and bloody diarrhea are sometimes recorded [12, 14]. Death can occur with few external signs but, during post mortem examinations, enlargement of the spleen and lymph nodes is typically observed along with internal bleeding [12, 14, 15]. The virus replicates in macrophages and monocytes within the blood but is also present at high levels within the tonsils, other lymph nodes, and the spleens of infected animals [2, 16, 17]. A feature of ASFV-infection in pigs is the loss (through apoptosis) of B- and T-lymphocytes in lymphoid tissues linked to the presence of infected monocytes [18]. It has been suggested that the infected monocytes signal to uninfected lymphocytes to enter apoptosis [19]. However, cell death by necrosis may also occur [11]. Hence, pigs that are acutely infected with ASFV can display severe lymphopenia [10, 20].

The death of nucleated cells can result in the release of cellular, genomic, DNA into the circulation system. This cell-free DNA (cfDNA) can be used as a biomarker for cell damage during organ transplant rejection or as a marker for various cancers (see review [21]). The concentration of cfDNA in plasma is normally very low but can be higher in serum due to some lysis of cells during blood clotting. The process of cell death by apoptosis results in the release of cellular contents within a range of extracellular vesicles, these can contain a variety of different molecules, including DNA [22]. These vesicles are present within serum/plasma.

91 The presence of the fragmented cfDNA in serum/plasma can be readily detected using
92 sensitive real-time quantitative PCR (qPCR) assays that have a small target size.

93 In this study, the production of cfDNA, derived from the host genome, in the blood of
94 ASFV-infected pigs has been examined in parallel with other markers of cellular damage. It
95 appears that the production and release of cfDNA, derived from the host genome, into the
96 serum is closely linked to the replication of ASFV in the infected pigs. For comparison, sera
97 from pigs infected with low and high virulence strains of classical swine fever virus (CSFV, a
98 pestivirus), which can cause similar clinical signs of disease and severe lymphopenia (with
99 highly virulent strains) in pigs as with ASFV, were also assayed for the same markers.

100 Materials and Methods

101 Samples from ASFV-infected pigs

102 *Experiment A*

103 From an experiment performed in 2022 (here termed experiment A), the samples used
104 here were from 4 male pigs (Landrace x Large White), about 12 weeks of age (numbered 13,
105 14, 15 and 20), which had been inoculated, using the intranasal route, with 4 $\log_{10}$ $TCID_{50}$ of
106 the genotype II ASFV POL/2015/Podlaskie strain (as used previously [23]). EDTA-blood
107 samples were obtained from these pigs at 0 dpi, 3, 5, 6 and 7 (euthanasia) dpi, while serum
108 samples were obtained at 0 dpi and at euthanasia only. Some separate results from this
109 experiment, have been described previously but there is no overlap with the samples analysed
110 here [24].

111 *Experiment B*

112 In another experiment, from 2020 (here termed experiment B), 12 male pigs
113 (Landrace x Large White) were inoculated by the intranasal route with 4 $\log_{10}$ $TCID_{50}$ of the
114 ASFV/POL/2015/Podlaskie, as above. Results from analysis of certain samples (EDTA-

115 stabilized blood (EDTA-blood) and peripheral blood mononuclear cells (PBMCs)) from these
116 pigs have been described previously [23, 25] but no analysis of serum samples, which are
117 described here, has been reported previously. The serum samples were obtained from blood
118 samples collected prior to inoculation at 0 dpi and at 3, 5 and 6 dpi. The pigs were euthanized
119 at 6 dpi.

120 In both experiments, water and a commercial diet for weaned pigs were provided *ad*
121 *libitum*. EDTA-blood and unstabilized blood samples (for serum preparation) were collected
122 prior to inoculation on day 0 and at indicated days post inoculation (dpi). All samples were
123 stored at -80◦C until further analysis. Rectal temperatures were recorded and a total clinical
124 score was calculated on all sampling days, as described previously [23]. The pigs were
125 euthanized at the end of the study period by intravascular injection of Pentobarbital following
126 deep anesthesia.

127 Animal care and maintenance, experimental procedures and euthanasia were conducted
128 in accordance with EU legislation on animal experimentation (EU Directive 2010/63/EU). The
129 original animal experiments were approved by the Ethical and Animal Welfare Committee of
130 the Generalitat de Catalunya (Autonomous Government of Catalonia; permit number: CEA-
131 OH/11744/2) and no new animal experiments were performed for the analyses presented here.

132 **Samples from CSFV-infected pigs**

133 Serum samples had been collected from six pigs that had been inoculated with a high
134 virulence genotype 2.1 strain (CSF1047, Israel, 2009) or a low virulence genotype 2.2 strain
135 (CSF0906, Bergen); this study has been described previously [26]. Briefly, the pigs, which
136 originated from a standard Danish swine herd, were inoculated by the intranasal route with 5
137 $\log_{10}$ $TCID_{50}$ of the CSFV (three pigs for each virus strain). Serum used for the current study
138 was prepared from blood samples collected prior to inoculation at 0 days post infection (dpi),

139 and at 4, 7, 10, 11 or 22 dpi as indicated [26]. These serum samples were stored frozen at -20
140 ◦C until further analysis.

141 **Laboratory analyses**

142 *Viral genome detection*

143 Nucleic acids were purified from whole blood or serum using the MagNA Pure 96
144 system (Roche) with the DNA/Viral NA 2.0 kit and the Viral NA Plasma external lysis S.V.
145 3.1. protocol, as described previously [14]. The extracted samples were analyzed for the
146 presence of ASFV DNA by qPCR or CSFV RNA by RT-qPCR using the CFX Opus Real-
147 Time PCR System (Bio-Rad, Hercules, CA, USA), essentially as described [27, 28]. For both
148 assays, a positive result was defined as a threshold cycle value (Ct) at which FAM dye
149 emission appeared above background within 42 cycles.

150 *Genomic and mitochondrial genome detection*

151 For host DNA detection, the level of the *Sus scrofa* cytoskeletal β-actin gene in the
152 samples was determined using the ACTB-F and ACTB-R primers as described [27], while the
153 level of the *Sus scrofa* mitochondrial cytochrome b gene was determined using an assay
154 developed by Forth [29]. The qPCRs were performed using the CFX Opus Real-Time PCR
155 System (Bio-Rad). A positive result was defined as a FAM (mitochondrial cytochrome b gene)
156 or HEX dye emission signal (β-actin gene) appearing above background within 42 cycles
157 (reported as Ct-values).

158 *Genome copy number determination*

159 Absolute quantification was used to determine the number of genome copies per ml by
160 reference to standard curves based on an endpoint dilution series of the ASFV pVP72 plasmid
161 [23] or generated from endpoint dilutions of artificially synthesized double stranded cDNA
162 (dsDNA) (from gBlock, Integrated DNA Technologies, Coralville, IA, USA). The chemically

163 synthesized double stranded cDNA corresponded to the nt 631-763 of the *Sus scrofa* actin
164 mRNA (GenBank: KU672525.1) and the nt 563-856 of the *Sus scrofa* isolate CRB3254
165 cytochrome b (CYB) gene (GenBank: KY236028.1).
166
167 *Lactate dehydrogenase (LDH) assays*

168 LDH activity was quantified in the serum samples using a lactate dehydrogenase
169 activity assay kit (catalog number MAK066, Sigma-Aldrich, St. Louis, MO, USA). The
170 assays were performed according to the manufacturer's instructions using serum samples
171 diluted 1:10 in 1x Dulbecco's phosphate buffered saline (1× DPBS) (Gibco Thermo Fischer
172 Scientific, Waltham, MA, USA) in order to ensure that all measurements were within the
173 linear range of the assay. Measurements were made using a Sunrise™ absorbance microplate
174 reader (Tecan, Männedorf, Switzerland). Results were calculated according to the
175 manufacturer's instructions and presented as milliUnits (mU)/mL.

176 *DNA fragment size determination*

177 For DNA size fragment size determination, DNA was isolated from serum samples
178 manually using the TRIzol™ Reagent (Thermo Fischer Scientific) according to the
179 manufacturer's instructions with minor modifications. Phase separation was achieved using 1-
180 bromo-2-chloropropane (Thermo Fischer Scientific), and following ethanol precipitation, the
181 DNA was resuspended in TE buffer (Thermo Fischer Scientific). Following this DNA
182 purification, AMPure XP beads (Beckman Coulter Life Sciences, Indianapolis, IN, USA) were
183 used to increase DNA concentration and purity. The DNA was then analyzed using the
184 Genomic DNA ScreenTape (Agilent, Santa Clara, CA, USA) on a 4200 TapeStation system
185 (Agilent).

186 *Data presentation*

187 Data analysis and presentation were performed using GraphPad prism 9.0 (GraphPad
188 Software, Boston, MA, USA)

189

190 **Results**

191 ***Characterization of ASFV infections in pigs - Experiment A***

192 *Assessment of body temperature, clinical signs and virus replication*

193 A group of 4 pigs (numbered 13, 14, 15 and 20) had been inoculated, using the
194 intranasal route, with the highly virulent genotype II ASFV/POL/2015/Podlaskie, as
195 described in Materials and Methods. EDTA-blood samples were collected from each pig
196 prior to inoculation (day 0) and at 3, 5, 6 and 7 days post inoculation (dpi). These whole
197 blood samples, from each sampling day, were assayed for the presence of ASFV DNA by
198 qPCR; note that results are presented in the graphs as 42-Ct values (see Figure 1A). As
199 expected, no ASFV DNA was present at 0 dpi. Low levels of ASFV DNA were detected at 3
200 dpi in 3 of the 4 pigs (as observed previously using this virus isolate, [23]) and much higher
201 levels were observed at 5, 6 and 7 dpi. Most of the animals were euthanized at 7 dpi but pig
202 13 was euthanized on day 6 due to severe clinical disease and thus could not be sampled on
203 day 7.

204 The body temperature and a clinical score (determined as described in Materials and
205 Methods) for each pig were also recorded on each of the sampling days (see Figure 2). A
206 marked increase in body temperature (Figure 2A) was apparent in each pig at 5 dpi and
207 various clinical signs (e.g. lethargy and anorexia) also began to appear at this time (Figure
208 2B) and developed further to give the highest clinical scores at 6 and 7 dpi. Prior to
209 euthanasia, reddening of the skin and neurological signs (unsteady walk and sometimes
210 convulsions) were observed. As indicated above, pig 13 had to be euthanized at 6 dpi. As

211 may be expected, the increased body temperatures and elevated clinical scores coincided with
212 the large increase in the presence of ASFV DNA in the blood (Figure 1A) essentially from 5
213 dpi.

214 To ensure that the DNA extractions and qPCR assays were functional, the ASFV
215 DNA was assayed as part of a duplex assay including primers and probes that also detected
216 the gene encoding the cytoskeletal β-actin (part of the genomic DNA (gDNA)) [30]. As
217 expected, high levels of this β-actin gene were detected in the whole blood samples that
218 included both the nucleated white blood cells (e.g. PBMCs) and the enucleated erythrocytes
219 that were collected from each pig on each sampling day (Figure 1A). However, it was noticed
220 that markedly elevated levels of β-actin gDNA were detected in the blood from 2 of the 3 pig
221 samples taken at 7 dpi (i.e. from pigs 14 and 20) when very high levels of ASFV DNA were
222 also detected. The higher levels of β-actin gDNA in the blood were also detected in these two
223 pigs at 6 dpi (Figure 1A).

224 It seemed possible that the elevated signals for β-actin gDNA at 6 and 7 dpi (Figure
225 1A) resulted from the destruction of ASFV-infected blood cells that could result in the release
226 of cellular genomic DNA into the blood. To test for the release of cellular DNA into the
227 blood, serum samples (lacking all blood cells) that had been prepared, from the same group
228 of animals, using unstabilized blood samples, collected prior to infection and at euthanasia (at
229 6 or 7 dpi) were also assayed for the presence of β-actin gDNA. At 0 dpi, the levels of β-actin
230 gDNA in the serum were low (Ct values 32.1-34.8) although readily detectable, see Figure
231 1B, but at euthanasia, the level of gDNA was much higher (Ct values 21.1-25.2), i.e. a
232 difference of about 10 cycles (ca. 1000-fold increase, as 2^{10}=1024). Consistent with the
233 assays using whole blood, no ASFV DNA was detected in the sera at 0 dpi (no Ct value) but
234 very high levels of ASFV DNA were present in the sera at euthanasia (Ct values 19.3 – 23.3)
235 (Figure 1B).

236 The levels of mitochondrial DNA (mtDNA) were also assessed in the serum samples
237 (Figure 1C) using a qPCR that targeted the mitochondrial cytochrome b gene (see Materials
238 and Methods). Quite high levels of mtDNA were found in the serum of uninfected pigs (Ct
239 values of about 24), however there was a marked increase in the level of mtDNA (about 6 Ct
240 lower at around 18, which represents a change of about 2^6 = 64-fold) in the serum from pigs
241 14 and 20 at 7 dpi. These two pigs also had the highest level of ASFV DNA in their serum at
242 that time (Figure 1B).

243 ***ASFV-infection of pigs- Experiment B***

244 To confirm these results, serum samples from a similar but separate experiment,
245 termed here Experiment B, that was performed in 2020 [23, 25] were analyzed. In this
246 experiment, sera had been collected throughout the time course of infection, from 3 separate
247 groups of ASFV-infected pigs, and were tested here for the presence of ASFV DNA, β-actin
248 gDNA and mtDNA. The Ct values from this experiment are presented within the
249 Supplementary Figure S1. To enable easy comparison of the levels of ASFV DNA, mtDNA
250 and gDNA in the serum samples from pigs 1-12, the Ct values obtained (and presented as 42-
251 Ct values in Supplementary Figure S1) were converted, by reference to standard curves, to
252 genome copy numbers/ml and are shown in Figure 3A, B, C.

253 Prior to inoculation at 0 dpi, no ASFV DNA was present in the animals (Figure 3A,
254 B, C, Supplementary Figure S1), however at 3 dpi low levels of the ASFV genome were
255 present in four animals (mean value in these 4 animals (pigs 2, 4, 7 and 11) was ca. 1.5 x 10^4
256 ASFV genomes/ml). By 5 dpi, the level of ASFV DNA in serum had increased dramatically
257 to between $10^{6.5}$ to$10^{8.5}$ genomes/ml (for pigs 1, 2, 4 and 10-12, the mean value was 1.95 x
258 10^8 genomes/ml) and, when euthanized at 6 dpi, the level of viral DNA remained very high at

259 up to $10^{8.6}$ genome copies/ml (the mean value for pigs 1, 2, 4, 7-12 was 2.7 x 10^8 genome
260 copies/ml serum).

261 The level of the β-actin gene, as cfDNA, was very consistent, on 0 dpi, at about 10^5
262 genome copies/ml (mean = 1.33 x 10^5 copies/ml) in the serum of the 12 pigs (Figure 3A, B,
263 C). It was little changed at 3 dpi (mean = 1.43 x 10^5 copies/ml) but had markedly increased at
264 5 dpi in the ASFV-infected animals at up to about 10^8 genome copies /ml (mean for pigs 1, 2,
265 4, 7-12 = 2.4 x 10^8 copies/ml) and continued at this high level (mean for pigs 1, 2, 4, 7-12 =
266 4.4 x 10^8 genomes copies/ml) at 6 dpi when these infected pigs also had very high levels of
267 ASFV DNA in their blood. Thus, during the time course of ASFV infection, the mean level
268 of gDNA (as measured by the level of the β-actin gene) in the sera had increased by over
269 3000-fold. Furthermore, there is an apparent correspondance between the accumulation of
270 ASFV DNA in serum and the increased presence of cfDNA, containing the β-actin gDNA.

271 The level of mtDNA in serum (see Figure 3A, B, C) for these 12 pigs at 0 dpi was
272 about 10^6 genome copies/ml (mean value = 2.5 x 10^6 copies/ml), and was similar at 3 dpi
273 (mean value = 1.7 x 10^6 copies/ml). At 5 dpi, some of the pigs had markedly elevated levels
274 of mtDNA (i.e. pigs 2, 4, 10 and 12), with a level of well over 10^7 genomes /ml (mean value
275 for these 4 pigs = 7.5 x 10^7 copies/ml). It is noteworthy that these 4 pigs also had very high
276 levels of ASFV DNA and β-actin gDNA in their serum at this time (Figure 3A, B, C).
277 Finally, at 6 dpi, most of the pigs that were infected with ASFV also had markedly elevated
278 levels of mtDNA in their serum with nearly 10^8 genomes/ml (mean value for pigs 1, 2, 4, 7-
279 12 = 7.5 x 10^7 copies/ml). Thus, between 0 and 6 dpi, the level of mtDNA in the serum of
280 ASFV–infected pigs increased by about 30-fold. It is noteworthy that, in total, 9 of the pigs
281 had markedly increased levels of mtDNA at 6 dpi but, interestingly, only four of these pigs
282 (pigs 2, 4, 10 and 12) had markedly elevated mtDNA at 5 dpi (Figure 3A, B, C) although

283 some other sera, i.e. from pigs 1, 9 and 11, contained high levels of both ASFV DNA and β-
284 actin gDNA on each of these days (Figure 3A, B, C).

285 These results are similar, both qualitatively and quantitatively, to those observed in
286 the ASFV-infection experiment A (Figure 1 and Figure 3D).

287 It appears that the level of mtDNA in serum is increased by ASFV infection but the
288 extent of this change is less marked than the change in β-actin gDNA (cfDNA), due ,in part,
289 to the higher background level of mtDNA in serum from uninfected animals. Furthermore,
290 the change in the level of mtDNA occurs later than the change in the level of gDNA and
291 ASFV DNA.

292 *Release of LDH activity*

293 Lactate dehydrogenase (LDH) is a cytoplasmic enzyme that can be released by cells
294 when tissue damage occurs (e.g. following a heart attack). To assess whether the release of
295 genomic DNA into blood was accompanied by release of LDH, the serum samples from the
296 12 ASFV-infected pigs from Experiment B, as analyzed in Figure 3A, B, C, were assayed for
297 the presence of LDH. It was found (Figure 4) that an increase in the level of LDH activity
298 was apparent at 5 or 6 dpi in many (but not all) of the pigs. Thus LDH release appeared to be
299 a less sensitive marker of cell damage due to ASFV infection than the release of cfDNA or
300 mtDNA.

301 *Source of cfDNA in ASFV-infected sera*

302 To assess the nature of the cfDNA in the serum of the pigs, selected samples were
303 extracted manually and analyzed to determine the size of the DNA fragments. At 0 and 3 dpi,
304 no DNA fragments were detected in this assay. However, it was found that at 5 or 6 dpi a
305 smear of DNA fragments was present in the extracted samples (Figure 5), these fragments
306 were up to about 1000 bp in length. There was some evidence for specific bands, within the

307 smear, at about 200 bp and 400 bp but there was not a clear ladder of DNA fragments
308 separated by about 180 bp that is indicative of apoptosis.
309
310 ***CSFV infection studies***
311 CSFV infection of pigs can cause very similar clinical signs of disease and
312 lymphopenia as observed with ASFV. To determine whether CSFV infection resulted in
313 similar changes in the presence of β-actin gDNA, mtDNA and LDH in the serum of the
314 infected pigs, samples from previously described CSFV-infected pigs [26] were assayed for
315 these markers. Two different strains of CSFV, a low virulence strain, CSFV Bergen, and a
316 highly virulent strain, CSFV Israel, had each been used to infect 3 pigs. Pigs inoculated with
317 CSFV Bergen had viremia at 7 and 10 dpi but 2 of the 3 animals survived and cleared the
318 infection as determined by the loss of CSFV RNA in the serum (Figure 6A), consistent with
319 the earlier results [26]. Pigs inoculated with the CSFV Israel had detectable CSFV RNA in
320 their sera at 4 dpi (Figure 6C) and this increased through to 11 dpi when the animals were
321 euthanized, these results are again consistent with those reported previously [26]. In contrast,
322 the levels of gDNA and mtDNA in the sera remained relatively constant throughout the time
323 course of infection by both strains of CSFV (Figure 6A and 6C). Similarly, there was no
324 apparent change in the level of LDH within the sera of these infected animals throughout the
325 time course of infection (Figure 6B and 6D). Thus, in contrast to the changes in the levels of
326 cfDNA, mtDNA and LDH seen in ASFV-infected pigs (Figures 1-4), there were no marked
327 changes in the levels of these markers within CSFV-infected pigs. It should be noted that the
328 prolonged storage of the samples from the CSFV-infected pigs prior to analysis does not
329 seem to have adversely affected the results. The detection of the viral RNA was consistent
330 with earlier studies on whole blood [26] and the basal levels of LDH were similar to those

331 observed in the recent samples of pig sera obtained prior to inoculation with ASFV (Figure
332 4).
333
334 **Discussion**
335 During the course of infection of pigs with a highly virulent ASFV
336 (ASFV/POL/2015/Podlaskie) many changes occur, the animals develop fever and a range of
337 different clinical signs can be apparent. Generally, the animals die within a week of being
338 infected. We have described previously changes in the expression of over 1000 genes in the
339 PBMCs isolated from ASFV-infected pigs [31]. It is well established that major changes
340 occur within the spleen and lymph nodes of ASFV-infected animals [11, 17] and significant
341 loss of B- and T- lymphocytes can occur without these cells being infected themselves,
342 presumably in response to signals received from the infected monocytes [16, 20].
343 We are unaware of any previous studies that have detected the large (>1000-fold)
344 increase in the level of cfDNA within the sera of ASFV-infected pigs that has been observed
345 here. The parallel detection of ASFV DNA and cfDNA by qPCR may be a convenient way of
346 following the process of infection within the pigs without requiring detection of other virus-
347 specific biomarkers. It seems that the appearance of gDNA in the serum is a much clearer
348 marker of cell death resulting from the ASFV infection than the increased level of mtDNA.
349 The elevated levels of mtDNA in serum were generally detected later in infection,
350 furthermore the mtDNA has a higher baseline signal in the serum of uninfected pigs (see
351 Figures 1 and 3). It had seemed possible that mtDNA would be a better marker for cell death
352 since there are many (hundreds to thousands) mitochondria per cell but there are clearly
353 differences in the way in which the circular mtDNA (ca. 16 kbp) and gDNA (within the
354 nuclei) will be liberated from cells and perhaps the mechanisms for clearance from the
355 circulation will also be distinct. Previous studies [32] have shown that the mean size of

356 mtDNA fragments in plasma is less than 100 bp, this may be because mtDNA, in contrast to
357 gDNA, is not protected by histones and hence does not exist within nucleosomes. The
358 mtDNA normally exists within protein-DNA complexes called nucleoids [33], which contain
359 other DNA binding proteins. The mtDNA is more densely packaged in the nucleoids than the
360 gDNA within nuclei [33]. The reported [32] small size of the fragmented mtDNA may mean
361 that the qPCR assays, in which the targeted mtDNA sequence is 274 bp [29], may be less
362 than optimal. For comparison, the mean size of cfDNA fragments derived from the human
363 genome is ca. 170 bp [32], thus potentially allowing for more efficient detection by qPCR
364 since the targeted sequence is only 114 bp in the β-actin assay [27].

365 It is likely that nucleated blood cells are among the sources of the fragmented gDNA
366 (cfDNA) [21] but we have not actually demonstrated this due to the expected absence of
367 tissue-specific markers within the short DNA fragments. It is probable that a variety of
368 different cell types (from within the blood itself) and possibly from other tissues (e.g. lymph
369 nodes and spleen) could contribute. From studies in human patients, cfDNA is reported to
370 have only a short half- life in the blood (30 min – 2hrs, [34]). There must be efficient
371 mechanisms to remove the DNA from the blood since apoptosis is a highly regulated
372 mechanism of cell death within an organism with very many cells going through this process
373 on a daily basis [22].

374 As indicated above, the size of cfDNA is generally small (mean length <200 bp, [32])
375 and can give an indication of the process of gDNA release. Laddering of the DNA,
376 corresponding to breakage at intervals between nucleosomes (with fragments differing by ca.
377 200 bp), is consistent with apoptosis whereas necrosis can be expected to yield cfDNA that is
378 more heterogeneous in size [21]. In our studies, we have observed a smear of DNA fragments
379 in samples collected from 5 or 6 dpi (Figure 5) when the level of cfDNA had markedly
380 increased (Figures 1 and 3). There was some evidence for bands at about 200 bp and 400 bp

381 but the pattern did not appear to represent only apoptosis. It may be that the DNA fragments
382 detected are generated by both apoptosis and necrosis. Furthermore, some degradation of the
383 fragments may occur within the serum which could increase the heterogeneity of the
384 fragment sizes.

385 The release of LDH into the circulation is a convenient marker for cell damage and is
386 widely used for this purpose. The studies presented here do indicate an increase in LDH
387 release into the serum in ASFV-infected animals (Figure 4) and Karalyan et al., [35] have
388 also observed an increase in LDH in sera that resulted from a genotype II ASFV infection in
389 pigs. However, in our studies this change was less consistent than the large increase in the
390 level of cfDNA or the smaller, relative increase in mtDNA identified here. Thus, the
391 production of cfDNA, and to a lesser extent mtDNA, seem to be clear markers for the severe
392 infection within animals produced by the highly virulent genotype II ASFV that is currently
393 circulating in many pig producing countries. The mechanism by which this cfDNA is
394 produced in ASFV-infected pigs is not yet known, however, the absence of such changes in
395 CSFV-infected pigs may suggest a specific role for ASFV-encoded products. Furthermore,
396 the linkage between the virulence of ASFV strains and the release of cfDNA remains to be
397 determined. Potentially, the production of cfDNA may be a useful biomarker for the severity
398 of the infection.

399

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403 **Author contributions**

404 ASO and CMJ performed the laboratory analyses. GJB drafted the manuscript and prepared
405 the Figures. LL, TBR, AB and GJB supervised the work. All authors reviewed the
406 manuscript.

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557 **Figure legends.**

558

559 **Figure 1. Detection of ASFV genomes and cellular DNAs in whole blood and serum**
560 **from ASFV-inoculated pigs in Experiment A.** Pigs were inoculated by the intranasal route
561 on day 0 and blood samples were collected on the indicated days. Following extraction of
562 nucleic acids the samples were assayed by qPCR. Panel A. Nucleic acids from whole blood
563 samples were assayed in a duplex assay for the presence of the cellular β-actin gene (as a
564 marker for DNA extraction) and for ASFV DNA as indicated. N.D. indicates not determined
565 as pig 13 was euthanized at 6 dpi. Panel B. Nucleic acids extracted from serum samples
566 collected on the indicated days were assayed, as for panel A, for the cellular β-actin gene
567 within genomic DNA (gDNA) and ASFV DNA as indicated. Panel C. Nucleic acids from
568 serum, as used in panel B, were assayed for the presence of mtDNA (targeting the
569 mitochondrial cytochrome b gene). All results are presented as 42-Ct. When no Ct was
570 obtained after 42 cycles, the samples were given a value of 42.

571

572 **Figure 2. Rectal temperatures and clinical scores in pigs inoculated with ASFV**
573 **(Podlaskie) in Experiment A.** The indicated pigs were inoculated with ASFV Podlaskie, as
574 in Figure 1, the rectal temperatures (panel A) and clinical scores (panel B), were assessed as
575 described in Materials and Methods and the results, from the same days as blood sampling
576 occurred (as shown in Figure 1), are shown. N.D. indicates "not determined" as the pig had
577 been euthanized at 6 dpi.

578

579 **Figure 3. Absolute levels of ASFV DNA, β-actin gDNA and mtDNA in serum from**
580 **ASFV-inoculated pigs in Experiment B.** Nucleic acids isolated from serum samples

581 collected from three separate groups of ASFV-inoculated pigs from Experiment B (as
582 described by Olesen et al.[23]) were assayed for the presence of ASFV DNA, β-actin gDNA
583 and mtDNA and absolute copy numbers/ml were determined from standard curves (panels A,
584 B, C for pigs in Groups 1, 2 and 3 respectively). Note, pigs 3 and 5 did not show any sign of
585 infection by the virus (the temperatures remained normal and there were no clinical signs of
586 disease). The Ct values are shown in Supplementary Figure S1. Data from Experiment A (as
587 shown in Figure 1B, C) were also converted to absolute genome copy numbers and are shown
588 in panel D.

589

590 **Figure 4. Release of cellular lactate dehydrogenase (LDH) from cells into serum within**
591 **ASFV-infected pigs (Experiment B).** Serum samples, collected on the indicated days, from
592 12 pigs inoculated with ASFV, in Groups 1-3, as used in Figure 3, were assayed (as described
593 in Materials and Methods) for the presence of the LDH enzyme, a marker for tissue damage.

594

595 **Figure 5. Characterization of DNA fragments in serum of ASFV-infected pigs**
596 **(Experiment B).** Nucleic acids extracted from the serum samples from four different pigs
597 (numbered 9-12) collected on the indicated days post infection (dpi) were purified manually
598 and concentrated (as described in Materials and Methods) prior to analysis on a TapeStation
599 system. The sizes of relevant molecular weight markers are indicated.

600

601 **Figure 6. Detection of CSFV genomes, β-actin gDNA and mtDNA in serum from CSFV-**
602 **inoculated pigs.** Pigs were inoculated with the low virulence CSFV Bergen or the highly
603 virulent CSFV Israel (as described by Lohse et al., [26]). Serum samples were collected on
604 the indicated days and stored frozen. Nucleic acids were extracted from these samples and

605 assayed for the presence of CSFV RNA, β-actin gDNA and mtDNA (panels A and C) as
606 described in Materials and Methods. The serum samples were also assayed for the presence
607 of the LDH enzyme (panels B and D). N.D. indicates not determined.

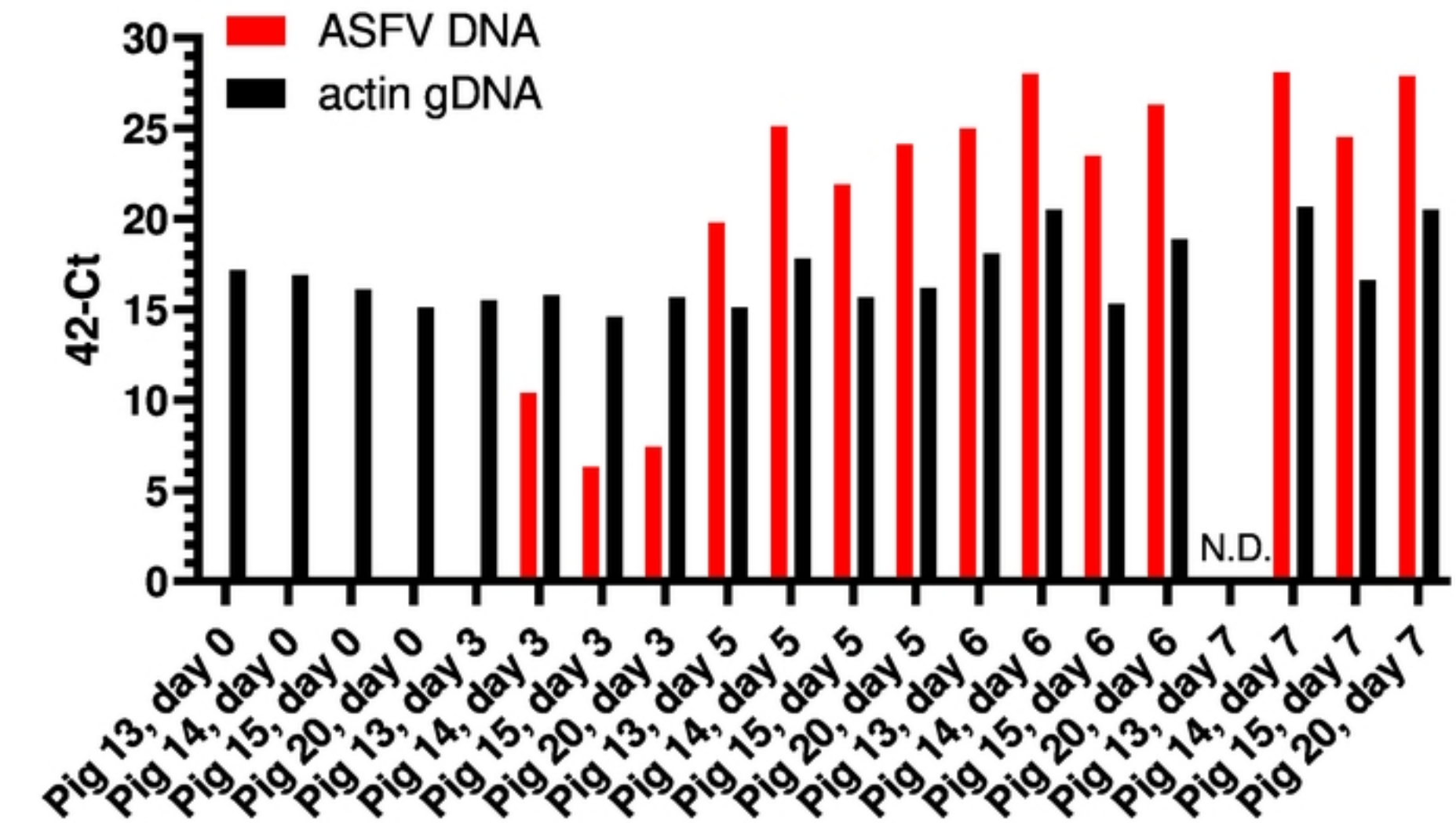
608

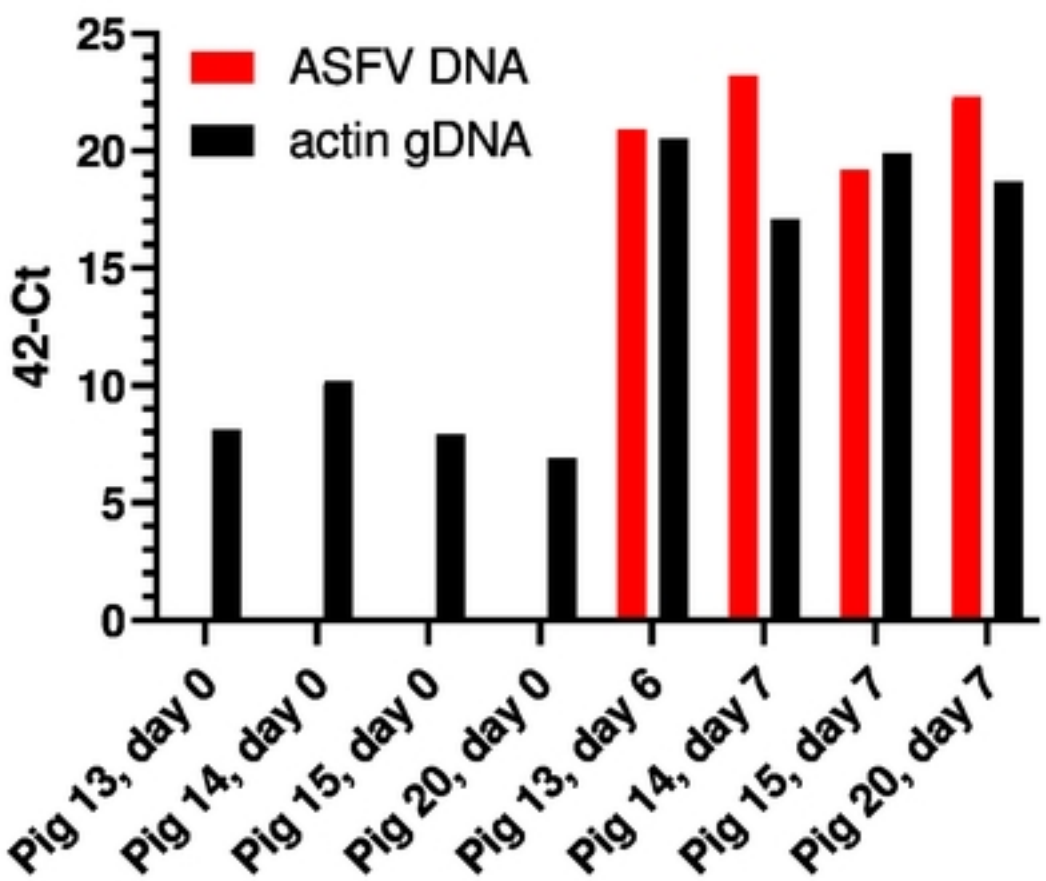
609

610 **Supplementary Figure S1.**

611 **Detection of ASFV DNA, β-actin gDNA and mtDNA in sera from ASFV-inoculated pigs**
612 **in Experiment B.** Nucleic acids were extracted from serum samples collected on the
613 indicated days from three groups of 4 pigs, (Groups 1 (panel A), Group 2 (panel B) and
614 Group 3 (panel C)) as described in Figure 3. The samples were assayed by qPCR for the
615 presence of ASFV DNA, the cellular β-actin gene (gDNA) and mtDNA as indicated. Results
616 are presented as 42-Ct values. Absolute genome copy numbers derived from these data, using
617 standard curves, are presented in Figure 3A, B and C. As indicated in Figure 3, pigs 3 and 5
618 showed no indications of becoming infected by ASFV following their inoculation with the
619 virus.

Figure 1





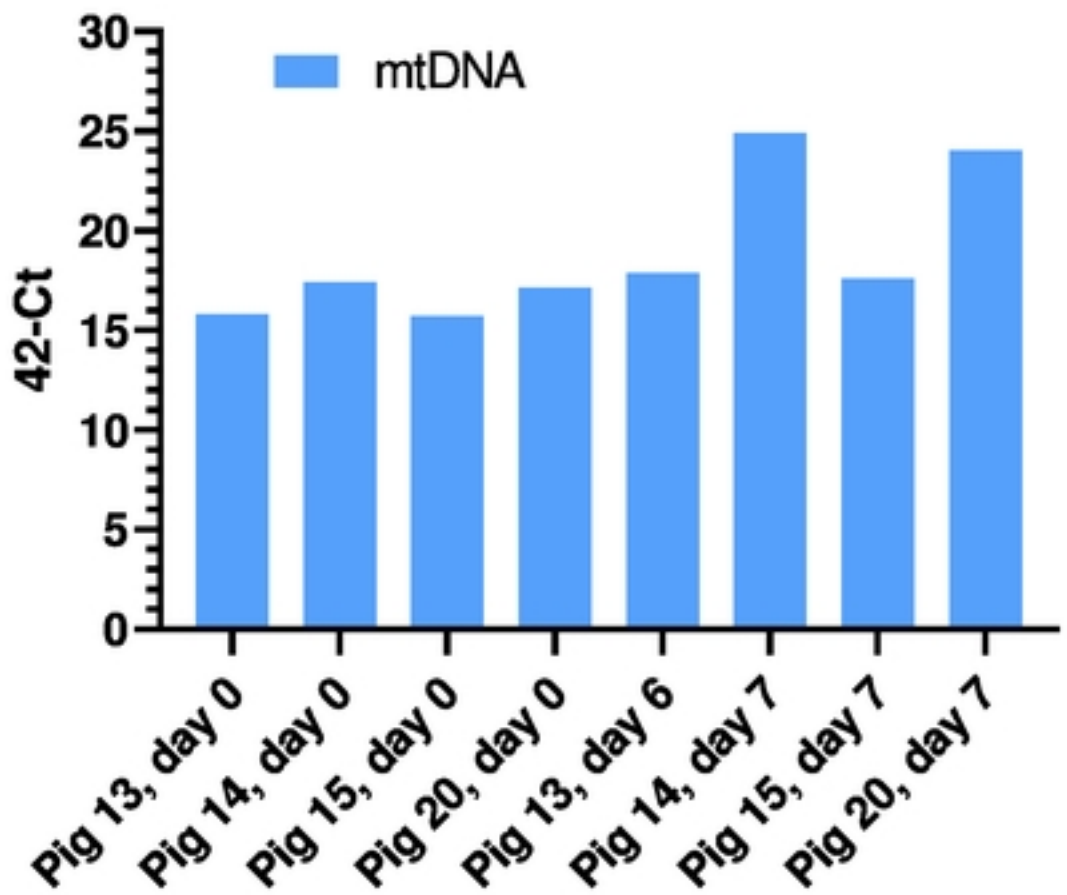


Figure 1

Figure 2

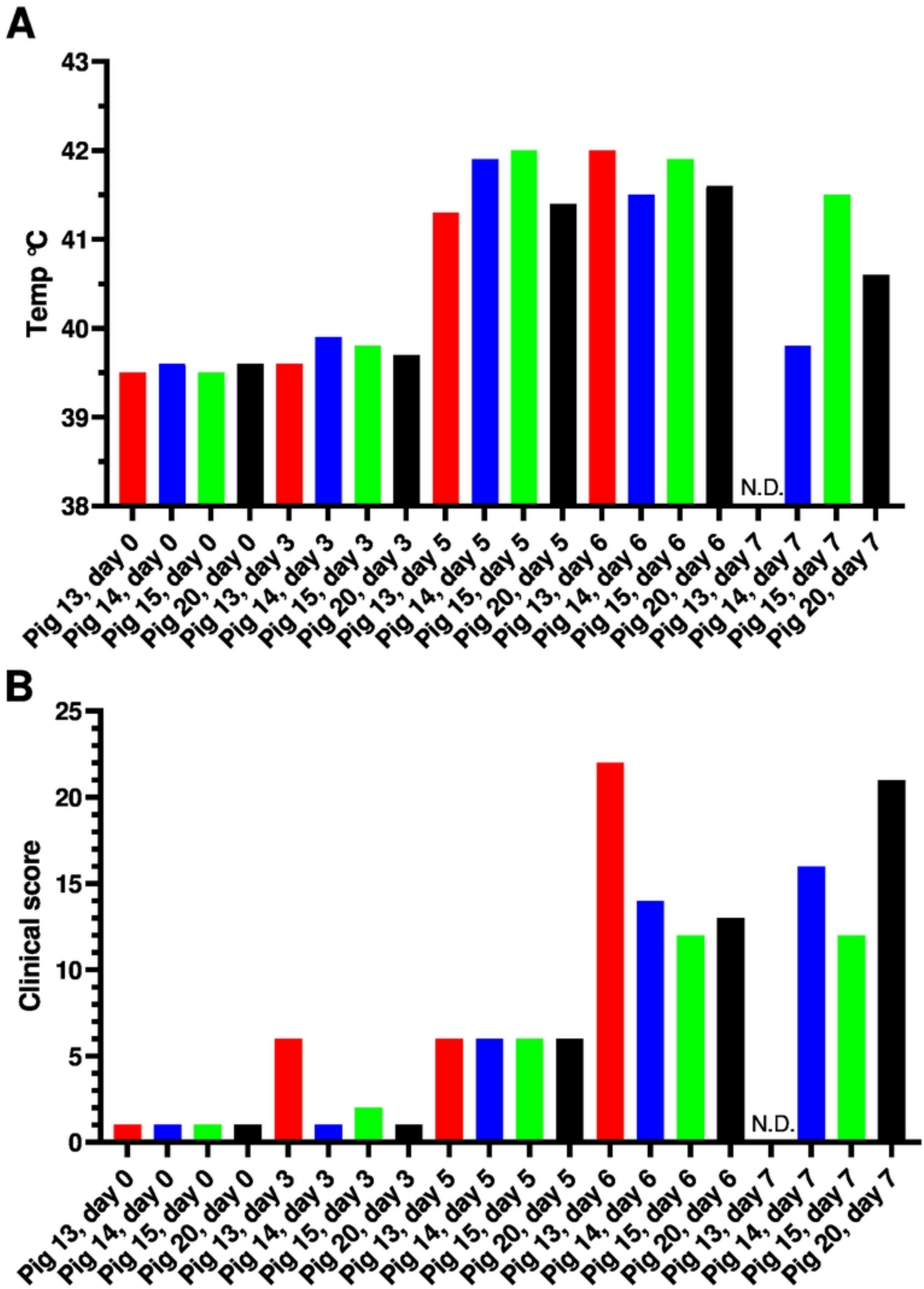


Figure 2

Figure 3

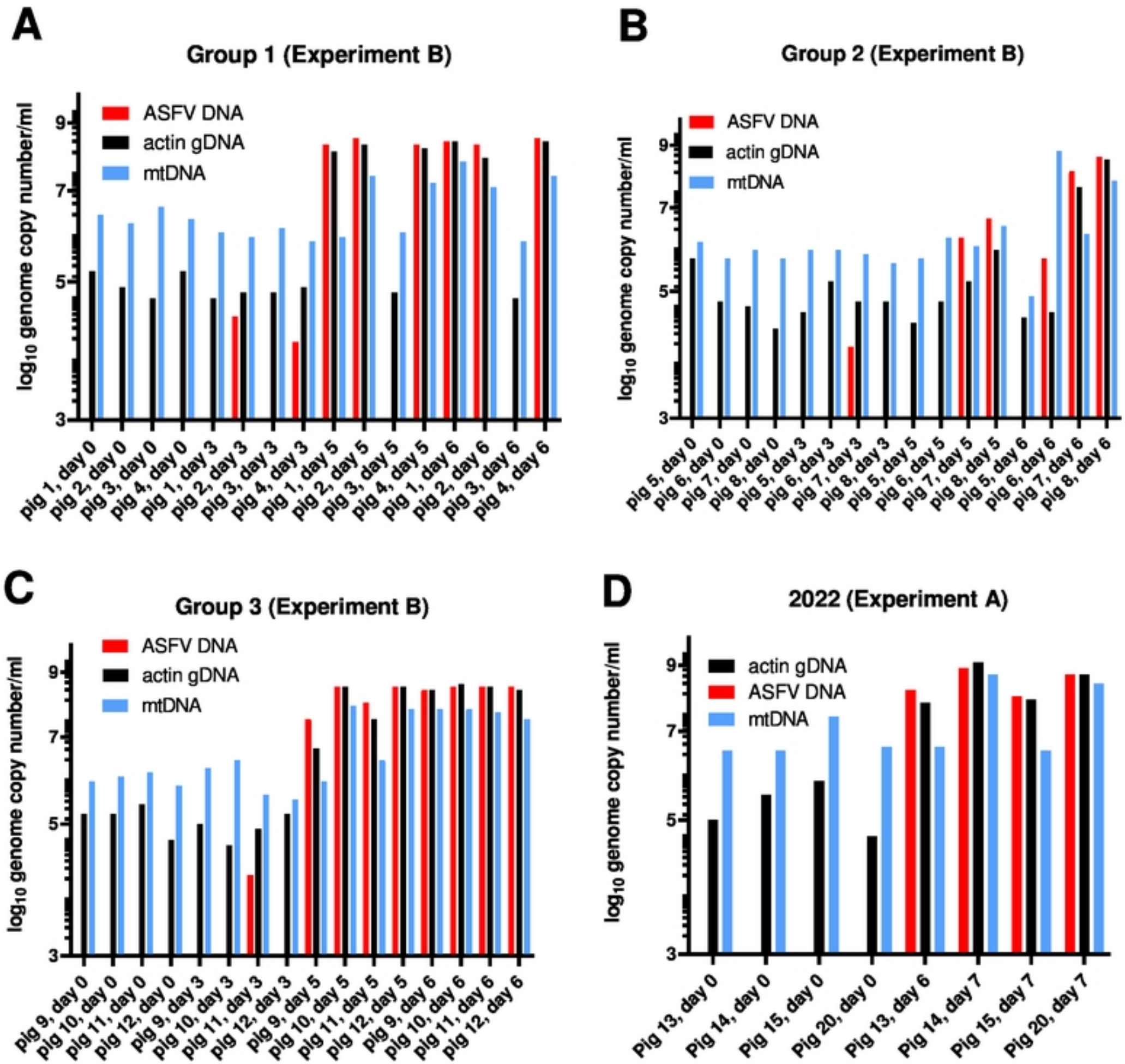


Figure 3

Figure 4

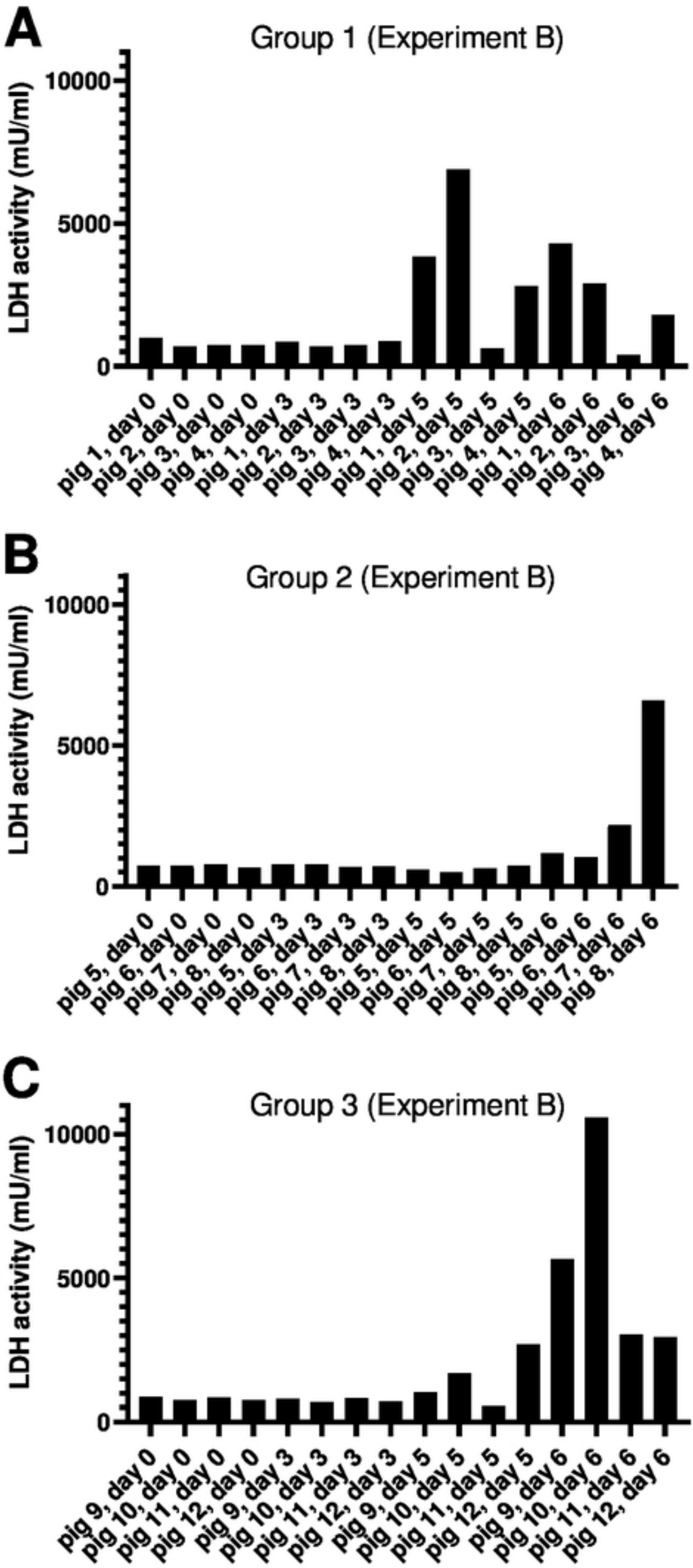


Figure 4

Figure 5

Pig 9	Pig 10	Pig 11	Pig 12	Pig 9	Pig 10	Pig 11	Pig 12	Pig 9	Pig 10	Pig 11	M	Pig 12	Pig 9	Pig 10	Pig 11	Pig 12
0 dpi	0 dpi	0 dpi	0 dpi	3 dpi	3 dpi	3 dpi	3 dpi	5 dpi	5 dpi	5 dpi		5 dpi	6 dpi	6 dpi	6 dpi	6 dpi

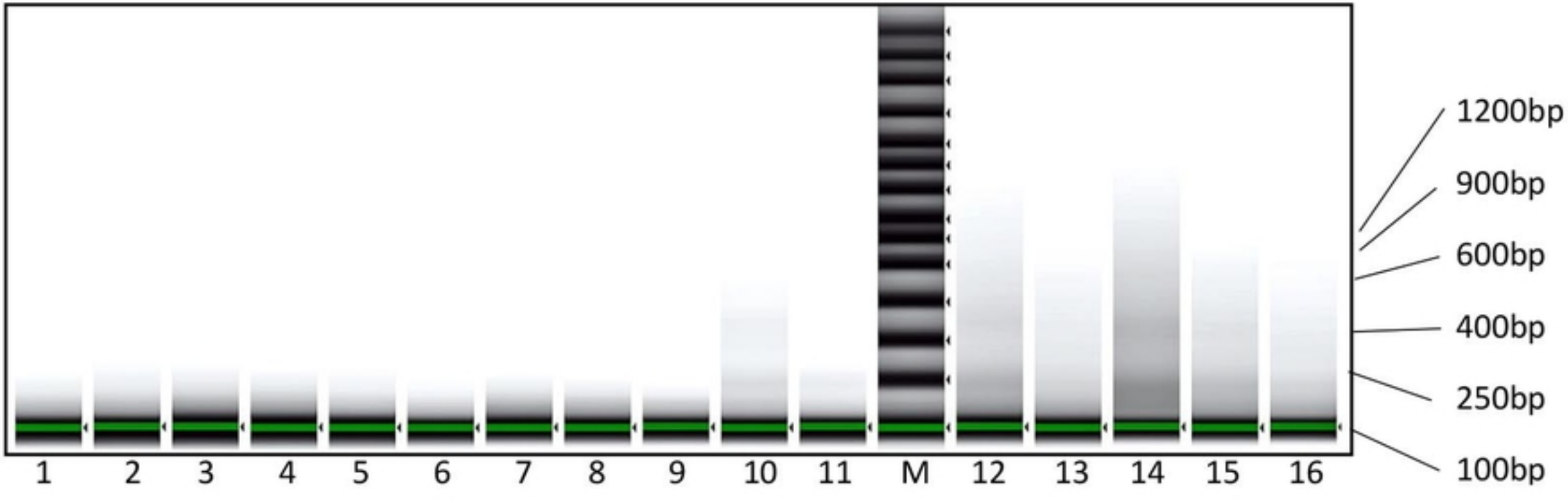


Figure 5

Figure 6

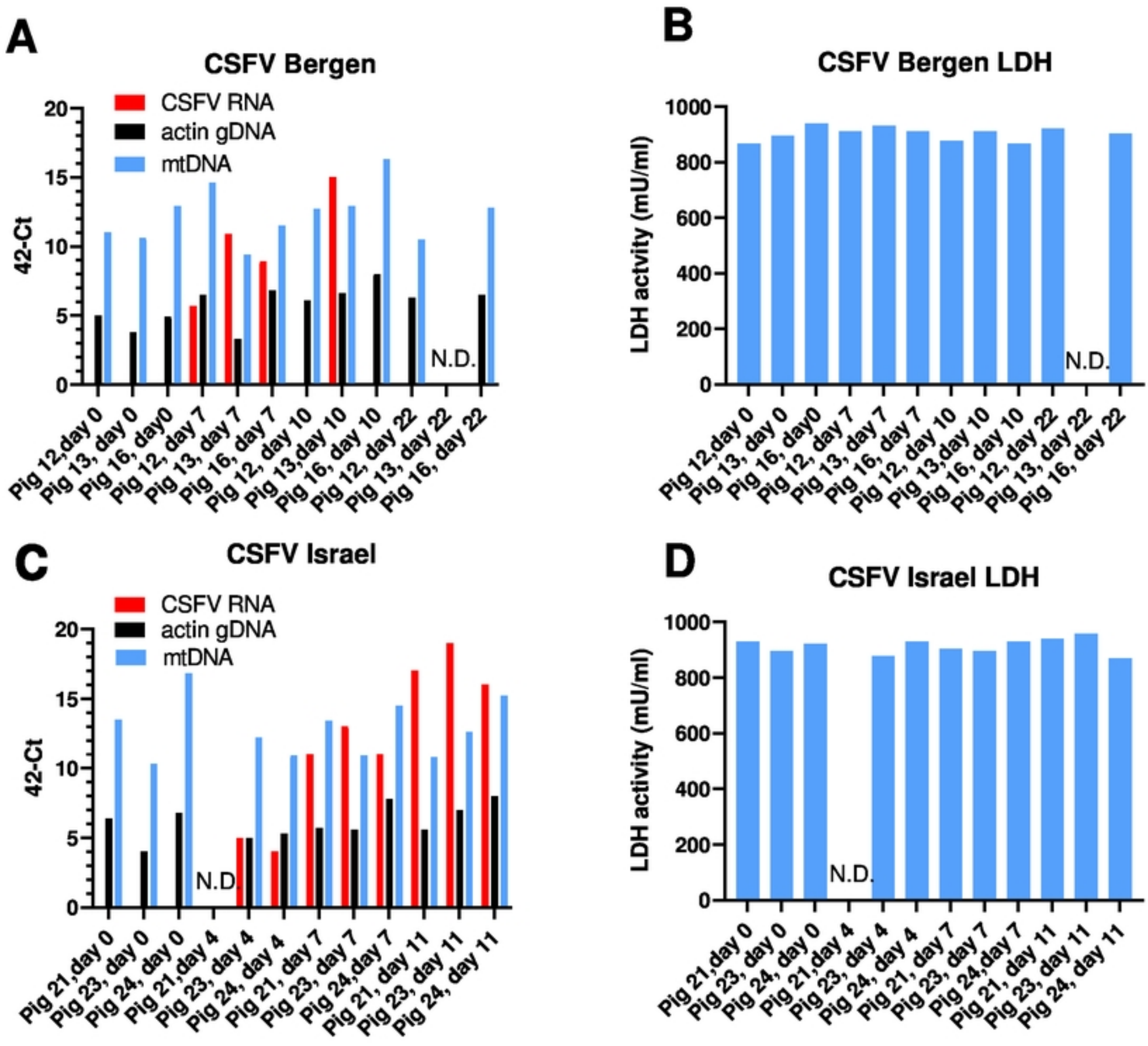


Figure 6