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1 **Evaluation of un-methylated DNA enrichment in sequencing of** 2 **African Swine Fever Virus complete genome.**

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12 **Abstract**

13 African swine fever is a febrile hemorrhagic fever disease that is caused by the African swine fever
14 virus (ASFV) and is lethal for domestic pigs and wild boar. ASFV also infects soft ticks of the genus
15 *Ornithodoros*, some species of which can act as a vector for ASFV. Whole genome sequencing of
16 ASFV is a challenge because, due to the size difference of the host genome versus the viral genome,
17 the higher proportion of host versus virus DNA fragments renders the virus sequencing poorly
18 efficient. A novel approach of DNA enrichment, based on the separation of methylated and un-
19 methylated DNA, has been reported but without an evaluation of its efficacy. In this study, the
20 efficiency of the un-methylated DNA enrichment protocol was evaluated for pig and tick samples
21 infected by ASFV. As expected, fewer reads corresponding to ASFV were found in the methylated
22 fraction compared to the un-methylated fraction. However, the sequencing coverage of the un-
23 methylated fraction was not improved compared to the untreated DNA. In our hands, the ASFV DNA
24 enrichment was inefficient for tick samples and very limited for pig samples. This enrichment process

25 represents extra work and cost without a significant improvement of ASFV genome coverage. The
26 efficiency of this enrichment approach and the cost/benefit ratio are discussed.

27

28 **Keywords:** African swine fever virus, NGS, enrichment, methylation

29 **1-Introduction**

30 African swine fever (ASF) is a highly contagious hemorrhagic fever in *Suidae* that is lethal for
31 domestic pigs and wild boar. The African swine fever virus (ASFV), a large enveloped DNA virus with a
32 genome size between 170 and 190 kb, is the etiologic agent of ASF and the only member of
33 *Asfarviridae* family. ASFV can infect two different hosts, *Suidae* and soft ticks of the genus
34 *Ornithodoros*, the last being a possible vector of ASFV (Boinas et al., 2011; Plowright et al., 1969).
35 Although ASFV is a large DNA virus, with a genome of 170 to 190 kb, this genome is far smaller than
36 the genomes of its two hosts. Pigs have a genome of 2.8 billion kb (Groenen et al., 2012) while ticks
37 have a genome estimated at 1 billion kb (Geraci et al., 2007). Standard DNA extraction methods, like
38 commercial kits using an affinity column or phenol chloroform, do not separate the host and viral
39 DNA genomes, leading to a heavily unbalanced output of sequences in favor of the host's genome,
40 even after cell culture amplification of the virus prior to sequencing. This is particularly critical for
41 samples that have low viral titers, such as tick samples, making the sequencing of the virus from
42 these samples highly challenging.

43 The first complete ASFV sequence, published in 1995, was of the non-virulent isolate BA71V,
44 a Vero cell culture adapted strain (Yáñez et al., 1995). This first full ASFV genome sequence, 170 kb
45 long, was obtained by Sanger sequencing after viral DNA fragmentation and cloning of the whole
46 genome into several plasmids. Despite the subsequent emergence and use of Next Generation
47 Sequencing (NGS), the sequencing of ASFV genomes has remained challenging, and ASFV sequences
48 have had to be completed or confirmed by PCR amplification of some DNA fragments followed by

49 Sanger sequencing (Chapman et al., 2011; de Villiers et al., 2010; Olesen et al., 2018; Zani et al.,
50 2018).

51 Different strategies for viral genome enrichment have been used, including
52 centrifugation/ultracentrifugation, filtration, and DNase and RNase treatments (Hall et al., 2014).
53 Genome capture, a more sophisticated technique based on a library of probes specifically designed
54 to selectively hybridize to a target genome, also has been used for the sequencing from clinical
55 samples of viruses that are difficult to sequence like Herpes Simplex Virus (Briese et al., 2015; Gaudin
56 and Desnues, 2018; Greninger et al., 2018). While very efficient, capture techniques are expensive,
57 and the preliminary knowledge of the viral sequences needed for the design of the probes also limits
58 their use for quasispecies studies.

59 In eukaryotes, DNA methylation is an epigenetic modification targeting CpG dinucleotides,
60 extremely stable DNA sequences frequently found in CpG islands that are involved in gene regulation
61 (Dor and Cedar, 2018). DNA methylation is also present in some large DNA viruses, such as
62 Herpesviruses and Iridoviruses, which replicate in the nucleus (Hoelzer et al., 2008). Interestingly,
63 contrary to most DNA viruses, the viral replication of ASFV occurs exclusively in the cytoplasm of
64 infected host cells (Dixon et al., 2013), whereas the DNA methylase is exclusively present in the
65 nucleus of the cell. The ASFV genome thus should avoid CpG methylation, as suggested by a recent
66 study demonstrating, on a small part of the genome, that ASFV DNA was un-methylated (Weber et
67 al., 2018). Recently, new complete genome sequences of African ASFV strains were obtained after an
68 enrichment step based on the separation of methylated and un-methylated DNA (Masembe et al.,
69 2018; Ndlovu et al., 2020). However, the efficiency of this approach on the different samples used
70 (high or low quantity of ASFV DNA) was not evaluated.

71 In this study, we investigated the efficiency of this un-methylated DNA enrichment strategy
72 for ASFV sequencing. Two types of samples infected with the Georgia2007/1 ASFV strain were
73 tested: 1) blood of viremic pigs, representing samples with a high quantity of ASFV DNA, and 2)

74 infected *Ornithodoros erraticus*, a European soft tick from Portugal, representing samples with a low
75 quantity of ASFV DNA. If efficient, sample enrichment based on methylated and un-methylated DNA
76 discrimination should separate host DNA from viral DNA. For comparison purposes, we sequenced
77 the untreated DNA (UD), the methylated fraction (MF) and the un-methylated fraction (UMF)
78 resulting from the enrichment process.

79 **2-Materials and methods**

80 **2.1-Infection of SPF pigs and soft tick *Ornithodoros erraticus***

81 The ASFV strain Georgia2007/1 was used for this experiment. This strain, member of the
82 ASFV genotype II group, was initially isolated from domestic pigs in Georgia in 2007 and was kindly
83 provided by Dr. Linda Dixon (OIE reference Laboratory, Pirbright Institute, UK). This strain has a
84 genome size of 189 344 bp coding for 269 ORFs (Forth et al., 2019). Five specific-pathogen-free (SPF)
85 pigs (889Geo, 936Geo, 941Geo, 6547Geo, 6518Geo) were inoculated intra-muscularly with 10^4 50%
86 hemadsorbing dose (HAD₅₀) of Georgia2007/1 ASFV strain. Heparin blood samples were collected on
87 viremia at day 3 post-inoculation. *Ornithodoros erraticus* (E geo F1-6, E geo M1-2) were infected by
88 feeding on Georgia2007/1 infected pigs and were frozen three months after the blood meal.

89 **2.2-DNA extraction**

90 DNA extractions were performed with the High Pure PCR Template Preparation Kit (Roche
91 Diagnostics, Meylan, France). For pigs, DNA extraction was realized from 400 µL of heparin blood
92 sample. Ticks, at 3 months post-infection, were washed 5 secs on sodium hypochlorite 2.6 % then 3
93 times in sterile Phosphate Buffered Saline (PBS). After washing, ticks were crushed in 200 µL of sterile
94 PBS with two steel beads of 3 mm and 4 mm at 25 Hz during 3 min with VWR Star-Beater (VWR
95 International bvba, Leuven, Belgium). Then 800 µL of sterile PBS were added and the whole solution
96 was clarified by centrifugation at 5000 g during 5 min. Supernatants were filtered on 0.22 µm Sterile
97 Millex filter (Merck Milipore, Carrigtwohill, Ireland) for E geo F1-2, E geo M1 and 0.45 µm filter for E

98 geo F3-6 and E geo M2. Filtered supernatants were centrifuged at 13000 g during 5 min and 400 μ L
99 of the supernatants were used for DNA extraction.

100 **2.3-Real-time PCR**

101 Each DNA extract was tested by quantitative real-time PCR for ASFV VP-72 and beta-actin
102 genes. The primers and probe used for ASFV VP-72 gene were previously described (Tignon et al.,
103 2011). The beta-actin primers and probes used in our study were also described for swine by Tignon
104 et al.. For the tick samples, the primers described by Duron et al. (2018) were used, however, instead
105 of SYBR Green, a Taqman probe was designed for our duplex real-time PCR Hex-5'-
106 CGAGAGGAAGTACTCCGTCTGG-3'-BHQ1.

107 **2.4-Quantification of DNA, sample enrichment, library preparation and sequencing**

108 DNA samples were assayed with Qubit Fluorometer (Invitrogen, Paisley, UK). For the pig
109 samples, DNA quantification was performed with 1 μ L of DNA; for the tick samples, DNA
110 quantification was performed with 10 μ L of DNA. The quantities of DNA obtained for each initial
111 sample are presented in Table 1.

112 After purification, all initial DNA samples were submitted to an enrichment step by
113 separating methylated DNA and non-methylated DNA with NEBNext Microbiome DNA Enrichment Kit
114 (NEW ENGLAND Biolabs, Evry, France) according to the manufacturer's protocol. More precisely, for
115 the pig samples, 100 ng of DNA were used for the NE library and the leftover (around 550 ng) for the
116 enrichment procedure. For the tick samples, 100 ng of DNA were also used for the NE library and the
117 leftover DNA, which varied between 260 ng and 450 ng, for the enrichment procedure. After the
118 enrichment procedure, each initial sample was then split into three fractions: 1) fraction 1, the initial
119 DNA sample, corresponding to an aliquot before the enrichment process (UD), 2) fraction 2, the
120 enriched methylated DNA sample (MF), and 3) fraction 3, the depleted methylated DNA sample
121 (UMF). DNA quantification of the MF and UMF fractions was performed after the enrichment

122 process. For library preparation, Ion Xpress Plus Fragment Library Kit (Thermo Fischer Scientific,
123 Frederick, Maryland, USA) was used and DNA fragments between 250 bp and 290 bp were size-
124 selected with Ion Xpress Barcode Adapters 1-96 kit (Thermo Fischer Scientific, Frederick, Maryland,
125 USA). For the DNA purification steps, magnetic beads from Agencourt AMPure XP Kit (Beckman
126 Coulter, Villepinte, France) were used.

127 All samples were sequenced with Proton Ion Torrent technology (Thermofischer Scientific,
128 Frederick, Maryland, USA). For the sequencing, the different libraries (UD, UMF, MF) were
129 multiplexed and simultaneously sequenced on a P1 chip for Proton sequencing. The P1 chip allows
130 the production of up to 100 M reads. The Phred Quality Score ≥ 20 was calculated for generated
131 bases and expressed as a percentage ($\% \geq Q20$). Raw data can be found in the GenBank SRA under
132 accession no. PRJNA643370.

133 **2.5-Bioinformatic analysis**

134 The reads were cleaned with Trimmomatic 0.36 software using the following parameters:
135 ILLUMINACLIP:oligos.fasta: 2:30:5:1: true; LEADING: 3; TRAILING: 3; MAXINFO: 40:0.2; MINLEN: 36
136 (Bolger et al., 2014). An alignment then was performed using Bowtie2 (version 2.2.5) (Langmead and
137 Salzberg, 2012) set on very fast (-D 5 -R 1 -N O -L 22 -i s,0,2.50) with cleaned down-sampled reads
138 on a local ncbi nucleotide database. This very fast alignment detected only the highly similar
139 sequences (>99% homology) and provided a snapshot picture of the data. The bam files were then
140 converted to a blast output readable by Krona taxonomic viewer (Ondov et al., 2011) to visualize the
141 gross organisms distribution in the different samples. For an accurate count of ASFV reads, the
142 sequence of strain Georgia2007/1, Genbank accession number FR682468.1 (Chapman et al., 2011),
143 was used as reference for a Bowtie2 alignment (command line option -al) versus all cleaned reads.

144 **2.7-Statistical analysis**

145 Statistical analyses were performed with RStudio software (version 1.1.463). For all data
146 analyses, three groups were considered: 1) the pool of UD fractions, 2) the pool of UMF fractions and
147 3) the pool of MF fractions. The Wilcoxon test was used for the statistical analysis of these three
148 groups. Our analysis focused on the impact that the enrichment process had on the NGS results, and
149 more specifically on ASFV genome enrichment and coverage between each group.

150 **3-Results**

151 **3.1-Detection and relative quantification of viral and host DNA in initial samples**

152 A duplex quantitative real-time PCR directed against ASFV and beta-actin was performed for
153 all samples and the ASFV/beta-actin ratios of the PCR Ct were calculated for the estimation of the
154 relative quantity of the genomes of ASFV and the hosts (Table 1). Assuming a similar efficiency of our
155 PCR reactions, a ratio of less than 1 indicates a higher level (by genome copy number) of ASFV
156 genomes than host genomes. Such a ratio (less than 1) was found for pig samples. For tick samples,
157 the ratio was superior to 1, excepting the E geo F1 and E geo M1 samples for which the ratio was
158 respectively 0.99 and 0.96.

159 **3.2-Quality analysis of generated data**

160 After the enrichment process, for pig samples, 158 ng to 205 ng of total DNA were obtained
161 for UMF and 208 ng to 325 ng for MF. For tick samples, 153 ng to 391 ng of total DNA were obtained
162 after enrichment for UMF and 9 ng to 24 ng for MF (Table 2).

163 The generated bases, reads and Phred quality scores are shown in Table 3. Briefly, for pig
164 samples, a mean number of 6 635 376 reads were generated for UD, 2 510 484 for UMF and
165 1 963 487 for MF. For tick samples, a mean number of 4 820 610 reads were generated for UD, 7 317
166 952 for UMF and 1 733 646 for MF.

167 The Phred quality scores were equivalent between all of the libraries, for both pig and tick
168 samples.

169 **3.3-Metagenomic analysis**

170 Due to a high variability in the number of reads obtained by samples, from 15 000 to 21×10^6
171 reads (Table 3), and for computing time efficiency, the metagenomic classification of the reads was
172 performed on subsets of the raw data ranging from 5 to 100% of the reads according to samples
173 (Table 4). The output of taxonomical assignment of the reads with the fast bowtie alignment varied
174 greatly according to the samples, with lower outputs for tick samples due to a poor representation of
175 this species in the databases. As far as possible, the order of magnitude in the number of reads
176 analyzed was kept in a range of hundreds of thousands of reads to be representative of the samples,
177 with the exception of the MF fraction of the 889Geo sample, which displays only 15 736 reads (Table
178 4).

179 The taxonomic classification of reads obtained from pig samples was divided into three main
180 groups: pig genome, ASFV genome and other sequences, which represent reads that are not assigned
181 to a specific taxon. For UD pig samples, 93% of detected reads belonged to the pig genome, 0.18% to
182 the ASFV genome and 6.82% to other sequences (Fig. 1A). For UMF pig samples, 83% of reads
183 belonged to the pig genome, 0.75% to the ASFV genome and 16.25% to other sequences (Fig. 1B).
184 For MF pig samples, 92.20% of reads belonged to the pig genome, 0.04% to the ASFV genome and
185 7.76% to other sequences (Fig. 1C). A statistical difference was found only for the proportion of ASFV
186 reads obtained in UD and UMF (p-value = 0.034).

187 The taxonomic classification of reads obtained from tick samples was divided into five
188 groups: tick, pig, ASFV, bacteria and other sequences. For UD tick samples, 38.63% were identified as
189 tick, 27.71% as pig, 0.03% as ASFV, 3.85% as bacteria and 32.78% as other sequences (Fig. 1D). For
190 UMF tick samples, 35.38% corresponded to tick, 25.71% to pig, 0.04% to ASFV, 4.48% to bacteria and
191 34.39% to other sequences (Fig. 1E). For MF samples, 36.63% corresponded to tick, 24.35% to pig, 0%

192 to ASFV, 1.34% to bacteria and 37.68% to other sequences (Fig. 1F). No statistical difference was
193 found between UD, UMF and MF samples.

194 **Fig. 1: Relative abundance of reads generated in the different samples classified according the**
195 **taxonomic origin.** Pig samples are presented in the left of the panel, and tick samples in the right. UD
196 samples are presented in **A** and **D**. UMF samples are presented in **B** and **E**. MF samples are presented
197 in **C** and **F**.

198

199 **3.4-Number and percentage of ASFV reads in all samples**

200 For all samples, the number of reads, the percentage and the mean coverage of reads aligned
201 on the ASFV reference are shown in Table 5.

202 For pig samples, the quantity of ASFV reads varied between 3000 and 71 286 for UD,
203 between 95 and 40 620 for UMF, and between 45 and 1220 for MF, representing 0.29%, 0.35% and
204 0.098% of total reads obtained for UD, UMF and MF of pig samples, respectively. The slight
205 difference in the number of ASFV reads between the UD and MF fractions was statistically significant
206 (p -value = 0.024).

207 For tick samples, the quantity of ASFV reads varied between 0 and 604 for UD, between 0
208 and 813 for UMF, and between 0 and 57 for MF. ASFV reads represented, for the best fraction,
209 0.002% of the total reads in tick samples (Table 5). No statistical difference was found between the
210 UD, UMF and MF samples.

211 ASFV genome sequencing coverages are also shown in Table 5. For pig samples, the mean
212 coverage was 15.11 for UD, 9.63 for UMF and 0.25 for MF fractions. For tick samples, the mean
213 coverage was 0.11 for UD, 0.12 for UMF and 0.005 for MF fractions.

214 **4-Discussion and conclusion**

215 In this study, we evaluated a new strategy of DNA enrichment for high-throughput
216 sequencing of the ASFV genome. The strategy, already used in two studies (Masembe et al., 2018;
217 Ndlovu et al., 2020) but without an evaluation of its efficacy, is based on a specificity of the ASFV
218 DNA genome which is not methylated, contrary to the host genome. Separation of methylated and
219 un-methylated DNA should result in an enrichment in ASFV reads in un-methylated samples. This
220 strategy was tested against samples corresponding to the different ASFV hosts, i.e., pig and tick
221 samples.

222 Before the enrichment process, ratios in genome copy number of ASFV and hosts were
223 roughly estimated (by PCR ratio) to be 1 in tick samples, and around 0.7 in pig samples. After
224 mapping the reads on the different genomes, the ASFV DNA indeed represented less than 0.4% and
225 0.01% of the reads obtained in pig and tick samples respectively.

226 For all pig samples, in the UMF fractions (depleted methylated DNA), the percentage of ASFV
227 genome reads increased from 0.18% to 0.75% and, conversely, the percentage of pig genome reads
228 decreased by 10% compared with the UD fractions. For tick samples, the proportion of reads
229 corresponding to the host genome decreased by 3% between UMF and UD, indicating a low
230 efficiency of the enrichment process. The decrease between the UD and UMF fractions was more
231 extensive for pig genome reads (10%) than the decrease for tick genome reads (3%). These
232 observations can be explained by the difference in DNA methylation between invertebrates and
233 vertebrates. Indeed, the DNA of invertebrates is less methylated than the DNA of vertebrates (Bird
234 and Taggart, 1980; Tweedie et al., 1997). However, the sequence of the whole *Ornithodoros erraticus*
235 genome is unknown, so the percentage of tick reads is probably underestimated. Moreover, the
236 proportion of ASFV reads in tick samples only increased in two samples out of eight and remained
237 lower than 0.01%, which in our case is insufficient to obtain the whole sequence of the ASFV
238 genome, with a mean coverage of less than 0.61.

239 In comparison with another enrichment approach for ASFV whole genome sequencing, DNA
240 extraction from erythrocytes of a viremic pig showed 0.84% of ASFV reads in their total reads with a
241 mean coverage of 103 reads per nucleotide (Olesen et al., 2018). This approach could be a good
242 strategy for fresh isolated erythrocytes but is not suitable for frozen whole blood.

243 In 2019, a workflow for efficient ASFV sequencing was proposed. This consisted of a target
244 enrichment approach by ASFV hybridization with probes fixed on magnetic beads followed by the use
245 of NGS technologies (either Illumina alone, or a combination of Illumina and Nanopore sequencing).
246 This target enrichment approach allows better coverage compared to the no enrichment approach,
247 and a smaller amount of data is generated during sequencing (Forth et al., 2019). However, this
248 approach has only been tested on the reference genome used for the design of the probes and a
249 strain close to this reference. Knowing that the core genome was constituted of 102 ortholog genes
250 on the 301 ortholog genes described in ASFV pan-genome (Wang et al., 2019), the efficiency of
251 enrichment by ASFV hybridization should be validated on other genotypes before being applied to
252 unknown strains.

253 Our study is the first to investigate the efficiency and the utility of the separation of
254 methylated and un-methylated DNA for the whole genome sequencing of the ASFV genome. Our
255 results suggest a low enrichment of ASFV DNA using this approach. The cost of the enrichment
256 process by separation of methylated and un-methylated DNA is around \$35 per sample. An increase
257 in the deepness of sequencing would most probably give similar results as EMD samples, with less
258 extra work on the samples and may be cheaper than the enrichment process. In comparison, the
259 target enrichment strategy, which costs approximately \$225 per sample, is most effective, resulting
260 in a significant reduction of data generated. However, the reduction of the sequencing costs does not
261 compensate for the extra cost of the enrichment process; the real advantage of this strategy is the
262 high quality of the sequence obtained.

263 In our hands, the enrichment of samples in un-methylated DNA for the sequencing of the
264 ASFV genome represented extra work and cost without a significant improvement in the final results
265 for very low ASFV load samples.

266 **Author contributions**

267 **Rémi Pereira De Oliveira:** Investigation, writing original draft, writing-review & editing.
268 **Pierrick Lucas:** Investigation. **Amélie Chastagner:** Writing-review & editing. **Claire De Boissesson:**
269 Investigation. **Laurence Vial:** Resources, writing-review & editing. **Marie-Frédérique Le Potier:**
270 Writing-review & editing. **Yannick Blanchard:** Conceptualization, writing-review & editing, validation
271 supervision.

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276 **Declaration of competing Interest**

277 The authors declare that they have no competing interests.

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388

389 **Table 1: Relative quantification of ASFV genome, host beta-actin and total DNA in pig and tick**
 390 **samples.**

Sample	Host	VP-72 ASFV PCR (Ct*)	Beta-actin PCR (Ct*)	DNA quantity (ng)	ASFV PCR/Beta- actin PCR ratio
889Geo	Pig	17.07	22.64	2610	0.74
936Geo		17.07	22.27	2180	0.77
941Geo		16.12	22.43	2130	0.72
6518Geo		15.88	22.11	2060	0.72
6547Geo		16.39	22.66	1540	0.72
E geo F1	Tick	27.2	27.1	554.4	0.99
E geo F2		33.95	29.33	392.4	1.16
E geo M1		27.13	28.35	594	0.96
E geo F3		35.51	28.5	374.2	1.24
E geo F4		38.77	28.85	357.5	1.34
E geo F5		33.79	28.39	330.8	1.19
E geo F6		31.29	28.26	410.8	1.10
E geo M2		31.11	28.66	407.3	1.08

391 *Ct = Cycle Threshold.

392 **Table 2: DNA quantity used for enrichment process and DNA quantity obtained after enrichment.**

Sample	Host	DNA for UD fraction (ng)	DNA for enrichment (ng)	DNA after enrichment for UMF fraction (ng)(% input)	DNA after enrichment for MF fraction (ng)(% input)	% DNA recovery (UMF+MF)
889Geo	Pig	99	550	178 (32)	282 (51)	84
936Geo		100	550	203 (37)	209 (38)	75
941Geo		98	550	162 (29)	275 (50)	79
6518Geo		99	550	205 (30)	290 (53)	90
6547Geo		98	550	158 (29)	326 (59)	88
E geo F1	Tick	100	454	368 (81)	24 (5)	86
E geo F2		100	292	210 (72)	9 (3)	75
E geo M1		100	494	347 (71)	23(4)	75
E geo F3		100	274	202 (74)	14(5)	79
E geo F4		100	257	172 (67)	9 (3)	70
E geo F5		100	230	153 (67)	9 (4)	70
E geo F6		100	310	392 (126)	13 (4)	131
E geo M2		100	307	246 (80)	10 (3)	83

393

394 **Table 3: Quantity of total bases generated, quality score of bases, quantity of total reads and mean**
 395 **read length per sample.** Quality of bases was analyzed by Phred Quality Scores ≥ 20 ($\% \geq Q20$) and
 396 expressed in percentage.

Fraction	Samples	Generated bases	$\% \geq Q20$	Quantity of total reads	Mean read length (bp)	
UD	pigs	889Geo	229 542 433	80.79	1 682 871	136
		936Geo	385 414 722	80.86	2 749 366	140
		941Geo	410 776 191	76.63	3 124 738	131
		6518Geo	452 263 244	81.64	3 792 151	119
		6547Geo	2 935 297 256	78.98	21 827 756	134
		mean for pigs	882 658 769	79.78	6 635 376	132
	ticks	E geo F1	1 213 297 206	82.27	9 285 185	131
		E geo F2	1 013 734 715	84.43	8 726 201	116
		E geo M1	855 764 828	87.38	7 243 049	118
		E geo F3	404 865 960	82.73	3 132 828	129
		E geo F4	468 405 245	83.33	3 618 451	129
		E geo F5	319 338 365	84.07	2 578 862	124
		E geo F6	177 777 191	84.00	1 437 348	124
		E geo M2	305 292 664	83.29	2 542 952	120
		mean for ticks	594 809 522	83.94	4 820 610	124
	global mean	705 520 771	82.34	5 518 597	127	
UMF	pigs	889Geo	285 321 178	80.59	2 246 476	127
		936Geo	222 820 790	80.98	1 751 451	127
		941Geo	17 393 456	82.28	150 956	115
		6518Geo	986 698 698	83.10	6 468 942	153
		6547Geo	248 717 004	80.28	1 934 593	129
		mean for pigs	352 190 225	81.45	2 510 484	130
	ticks	E geo F1	963 624 225	83.76	7 817 144	123
		E geo F2	940 491 921	83.99	8 008 483	117
		E geo M1	1 118 167 936	86.58	9 220 704	121
		E geo F3	152 407 597	82.92	1 203 993	127
		E geo F4	244 449 864	84.03	2 013 848	121
		E geo F5	324 824 629	84.41	2 728 169	119
		E geo F6	287 311 527	82.70	2 170 620	132
		E geo M2	148 976 803	84.68	1 380 652	108
		mean for ticks	522 531 813	84.13	4 317 952	121
	global mean	457 015 818	83.10	3 622 772	125	
MF	pigs	889Geo	2 151 395	80.31	15 736	137
		936Geo	56 192 863	76.39	414 846	135
		941Geo	104 536 025	79.83	754 918	138
		6518Geo	1 139 572 253	82.56	7 757 735	147
		6547Geo	126 458 347	80.45	874 201	145
		mean for pigs	285 782 177	79.91	1 963 487	140
	ticks	E geo F1	885 423 136	85.64	8 165 392	108
		E geo F2	254 288 416	84.29	2 169 858	117
		E geo M1	109 382 507	84.16	977 897	112
		E geo F3	53 307 894	85.23	445 062	120
		E geo F4	16 326 955	83.85	149 147	109
		E geo F5	17 295 136	82.75	135 737	127
		E geo F6	127 530 447	84.14	1 048 408	122
		E geo M2	80 531 098	84.62	777 670	104
		mean for ticks	193 010 699	84.34	1 733 646	115
	global mean	228 692 036	82.63	1 822 047	125	

397

398

Table 4: Number and percentage of the total reads used for metagenomics analysis.

Fraction	Samples		Number of reads used for metagenomics analysis	Percentage of reads used for metagenomics analysis
UD	pigs	889Geo	504 861	30%
		936Geo	824 810	30%
		941Geo	937 421	30%
		6518Geo	1 137 645	30%
		6547Geo	1 091 388	5%
		mean for pigs	899 225	25%
	ticks	E geo F1	464 256	5%
		E geo F2	436 310	5%
		E geo M1	724 305	10%
		E geo F3	939 848	30%
		E geo F4	1 085 535	30%
		E geo F5	773 659	30%
		E geo F6	431 204	30%
		E geo M2	762 886	30%
			mean for ticks	702 250
		global mean	778 010	23.12%
UMF	pigs	889Geo	673 943	30%
		936Geo	525 435	30%
		941Geo	150 956	100%
		6518Geo	646 894	10%
		6547Geo	580 378	30%
		mean for pigs	515 521	40%
	ticks	E geo F1	390 857	5%
		E geo F2	400 424	5%
		E geo M1	922 070	10%
		E geo F3	361 198	30%
		E geo F4	604 154	30%
		E geo F5	818 451	30%
		E geo F6	651 186	30%
		E geo M2	414 196	30%
			mean for ticks	551 199
		global mean	534 982	30.62%
MF	pigs	889Geo	15 736	100%
		936Geo	414 846	100%
		941Geo	452 951	60%
		6518Geo	775 774	10%
		6547Geo	524 521	60%
		mean for pigs	436 765	66%
	ticks	E geo F1	408 270	5%
		E geo F2	216 986	10%
		E geo M1	391 159	40%
		E geo F3	356 050	80%
		E geo F4	149 147	100%
		E geo F5	135 737	100%
		E geo F6	314 522	30%
		E geo M2	466 602	60%
			mean for ticks	304 809
		global mean	355 561	59.56%

402 **Table 5: Number and percentage of ASFV reads per sample and the sequencing coverage of the**
 403 **reference strain Georgia2007/1.**

Samples	UD			UMF			MF		
	ASFV reads	%	Cov.	ASFV reads	%	Cov.	ASFV reads	%	Cov.
889Geo	3000	0.18	2.20	5866	0.26	3.42	67	0.43	0.05
936Geo	5544	0.2	4.25	4594	0.26	3.25	45	0.01	0.03
941Geo	10760	0.34	7.68	95	0.06	0.06	72	0.01	0.06
6518Geo	14222	0.38	9.19	40620	0.63	33.96	1220	0.02	0.99
6547Geo	71286	0.33	52.21	10491	0.54	7.46	132	0.02	0.1
Mean	20962	0.29	15.11	12333	0.35	9.63	307	0.098	0.25
E geo F1	604	0.007	0.47	391	0.005	0.29	57	0.001	0.04
E geo F2	12	0	0.01	6	0	0	0	0	0
E geo M1	546	0.008	0.39	813	0.009	0.61	6	0.001	0
E geo F3	1	0	0	0	0	0	0	0	0
E geo F4	0	0	0	0	0	0	0	0	0
E geo F5	2	0	0	0	0	0	0	0	0
E geo F6	5	0	0	26	0.001	0.02	1	0	0
E geo M2	28	0.001	0.02	10	0.001	0.01	3	0	0
Mean	150	0.002	0.11	156	0.002	0.12	8	0.00025	0.005

404 Cov. = sequencing coverage in average number of reads per nucleotide position.

