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1 **Characterization of African swine fever virus IAP homologue expression in**
2 **porcine macrophages infected with different virulence isolates.**

3

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5

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14

15 **Abstract**

16 Genes modulating apoptosis are encoded by many viruses and have an important
17 role in viral evasion mechanisms. Our objective was to characterize the expression
18 of the IAP homologue gene of African swine fever virus (ASFV), *4-CL*, during *in*
19 *vitro* infection of porcine macrophages, the preferential target cell for viral
20 replication. Expression was compared along parallel infections by two naturally
21 occurring ASFV isolates of different virulence: highly virulent ASFV/L60 (L60) and
22 low virulent non-hemadsorbing ASFV/NH/P68 (NHV). Efficiency of macrophage
23 infection by both isolates was similar, as observed both by the percentage of
24 infected cells in culture and by virus progeny production. Our results showed that
25 transcription of *4-CL* initiates very early after infection with both isolates, since
26 specific mRNAs were observed and quantified at 1.5 h post-infection (p.i.).
27 However, the protein was produced later, from 4 h to 8 h p.i., around the same
28 time when viral DNA replication is reported to occur. *4-CL* protein was more

29 abundant in L60 than NHV infected cells, at both 8 h and 16 h p.i. The mRNA
30 levels, however, did not correlate with those of protein expression. Overall our
31 results suggest the existence of a post-transcriptional step in the regulation of 4-
32 CL gene expression.

33

34 **Keywords:** African swine fever virus; virulence; macrophage; inhibitor of
35 apoptosis; gene expression.

36

37 **1. Introduction**

38 African swine fever virus (ASFV) is a large double stranded DNA virus with 170-
39 190 kb, classified as sole member of *Asfarviridae* family (Dixon et al., 2005). It is
40 responsible for a highly contagious and fatal disease of domestic pigs,
41 representing a serious threat to swine industry worldwide.

42 In many viral infections, host cells trigger the apoptotic cell death program as a
43 defence mechanism, in order to abort viral replication and limit viral dissemination
44 in the organism. Thus, along evolution, especially large and complex animal
45 viruses with long replication cycles, acquired tools to prevent premature death of
46 the host cells, namely genes intervening in the apoptotic signalling cascades
47 (Benedict et al., 2002). Similarly, ASFV possesses in its genome at least three
48 genes implicated in apoptosis inhibition: a *bcl-2* homologue (Afonso et al., 1996;
49 Galindo et al., 2008), a lectin homologue (Hurtado et al., 2004), and a IAP
50 homologue (Nogal et al., 2001), the last being subject of this study. IAPs are key
51 inhibitors of apoptosis through their capacity to interfere with caspases, the
52 proteolytic enzymes acting at the heart of the apoptotic process, ultimately
53 responsible for cell demise (Shi, 2004). Current knowledge on the mode of action

54 of IAPs indicate that their inhibitory effect on caspases is exerted by direct
55 interaction with the processed active enzymes, by either blocking the catalytic part
56 of the enzymes via their BIR domains, or through E3 ubiquitin ligase activity from
57 RING domains, targeting caspases for rapid degradation via proteasome (Vaux
58 and Silke, 2005). The ASFV IAP, denominated 4-CL in the naturally occurring
59 virulent isolate Malawi Lil-20/1 (Neilan et al., 1997) and A224L in the non-virulent
60 Vero cell line adapted isolate Ba71V (Yanez et al., 1995), contains a single N-
61 terminal BIR-like motif, and instead of a C-terminal RING finger, possesses a
62 putative zinc finger motif of the C₄ type (Chacon et al., 1995). On a Vero cell line
63 adapted infection model, A224L deletion lead to higher caspase-3 activity and cell
64 death comparatively to infection with Ba71V parental virus (Nogal et al., 2001).
65 Also, over-expression of A224L inhibited caspase-3 activity in cells treated for
66 apoptosis induction, seemingly by a direct interaction, since the viral protein was
67 co-precipitated with active caspase-3, suggesting a mode of action similar to other
68 IAPs (Nogal et al., 2001). It was reported previously that A224L protein is
69 produced after the onset of viral replication, hence at a late phase of infection
70 (Chacon et al., 1995). However, viral DNA replication starts around 6 h post-
71 infection (p.i.), and morphogenesis and viral cycle completion takes several more
72 hours, being largely concluded at around 18 h p.i. (Brookes et al., 1996). Thus, it
73 would be important to asses more accurately when during the infection cycle the
74 viral IAP homologue is expressed.

75 ASFV isolates of different virulence circulate in nature and lead to different
76 outcomes of infection, ranging from acute and fatal to clinically unapparent forms
77 of disease. Relevant to viral pathogenesis, ASFV replicates preferentially in
78 porcine macrophages, known to play central roles for the development of immune

79 responses. Our studies have been focused on the characterization of viral-
80 macrophage interactions during infection with ASFV isolates of different virulence.
81 Such studies aim at identifying the still unknown viral or host factors subjacent to
82 the different immune responses and outcomes of infection. Given the important
83 role that genes modulating apoptosis may have for pathogenesis and viral evasion
84 of protective mechanisms, our objective was to further characterize the expression
85 of one of these genes, the IAP homologue denominated *4-CL*, during *in vitro*
86 infection of porcine macrophages by two naturally occurring ASFV isolates of
87 different virulence: highly virulent ASFV/L60 (L60) and low virulent non-
88 hemadsorbing ASFV/NH/P68 (NHV) (Leitao et al., 2001).

89

90 **2. Materials and methods**

91 2.1. Cells

92 Heparinized blood samples from crossbred Large White x Landrace pigs (six
93 month old) were obtained in aseptic conditions during bleeding at the abattoir.
94 Blood samples were incubated at 37 °C 15 minutes with 10 % v/v of a 5 % v/v
95 Dextran T500 solution in Hank's balanced saline solution. Supernatants were
96 collected, diluted 1:1 in culture medium (RPMI 1640 with 100 IU/ml penicillin, 100
97 µg/ml streptomycin, 20 mM HEPES), and seeded (100 ml/flask) in T175 tissue
98 culture (TC) flasks (Nunc 156502). Cultures were incubated at 37 °C for 48 h, and
99 non-adherent cells were removed by extensive washing in pre-warmed phosphate
100 buffered saline (PBS). Adherent macrophages were harvested by treatment with
101 cold 0.8 mM EDTA in PBS, washed in PBS, and viability of cells was determined
102 by Tripan blue dye exclusion. Macrophages were plated at 1.5×10^5 viable
103 cells/cm² for each assay, allowed to adhere for 3 h, and washed again to remove

104 non-adherent cells. Culture medium was RPMI 1640 with 100 IU/ml penicillin, 100
105 µg/ml streptomycin, 20 mM HEPES, supplemented with 10 % foetal calf serum.

106

107 2.2. Virus and viral infections

108 ASFV isolates used were the low virulent NHV and the highly virulent L60 (Leitao
109 et al., 2001). These were added to parallel macrophage cultures at MOI 3, unless
110 otherwise stated, and after 1 h adsorption cells were washed twice, new medium
111 was added, and incubation proceeded for the indicated times. Similarly treated
112 mock-infected cultures were kept in parallel as a control. At each independent
113 assay, infections/mock-infections were performed with cell cultures obtained from
114 the same pig donor.

115

116 2.3. RNA extraction

117 Total RNA was extracted from macrophage cultures on 6-well TC plates, at the
118 indicated times post-infection (p.i.), through TRIzol Reagent (Invitrogen) following
119 manufacturer instructions. Contaminating DNA was removed through “Turbo DNA-
120 free” system (Ambion).

121

122 2.4. Real time PCR

123 RNA (0.6 µg/reaction) was used for cDNA synthesis through Superscript II
124 Reverse Transcriptase (Invitrogen) and oligo pdT₁₂₋₁₈ for priming. 2 µl of 1:10
125 diluted cDNA was used in each amplification reaction. Primers for 4-CL sequence
126 were (5'-3') GCTCCATAGATGCACGAAATC and AAGCCGCAGTTTCTACTCCA,
127 amplifying a 193 bp sequence. Primers for house-keeping gene glyceraldehyde-3-
128 phosphate dehydrogenase (*GAPDH*) were designed according to the known

129 mRNA sequence from *Sus scrofa* (GenBank accession AF017079). Intron
130 locations were inferred from existing information for *Homo sapiens GAPDH*
131 (GenBank accession NM_002046). Chosen primers were (5'-3')
132 GTCGGAGTGAACGGATTTG (on the first exon-intron junction) and
133 CTTGACTGTGCCGTGGAA (on the third exon), amplifying a 168 bp sequence in
134 cDNA. Quantification of target sequences on cDNA was obtained through
135 standard curve construction (absolute quantification) (Whelan et al., 2003). The
136 above primers were used to obtain DNA of known concentration by PCR
137 amplification on cDNA, as previously described (Granfar et al., 2005). 0.6 μ M of
138 each primer, 1.5 mM MgCl₂, 200 μ M each dNTP and 59 °C annealing temperature
139 permitted to obtain specific amplification products, as confirmed in 2.5 % agarose
140 gel electrophoresis. Amplification products were purified through "High Pure PCR
141 Product Purification Kit" (Roche), quantified by spectrophotometry, and 1:5 serial
142 dilutions (200 000 – 64 molecules, 5-6 dilutions per assay) were used in real time
143 PCR. Commercial iTaq SYBR Green Supermix with ROX (Bio Rad) was used for
144 amplifications in "7300 System" apparatus (Applied Biosystems). Amplification
145 efficiency (E) was calculated from standard curve slopes (S) generated by the real
146 time PCR apparatus software, through the formula $E = 10^{-1/S} - 1$ (Revilla-Fernandez
147 et al., 2005). Efficiencies equal or superior to 95 % ($E \geq 0.95$) were considered
148 optimal, with regression coefficients for standard curves always >0.99, and cycle
149 thresholds (Ct) for first dilutions between cycles 14-18. Conditions that allowed
150 such efficiencies resulted similar for both primer pairs and were: 500 nM each
151 primer in reaction, 2 min and 45 sec at 95 °C initial denaturation, followed by 40
152 cycles of: 15 sec 95 °C denaturation, 15 sec 57 °C annealing, 45 sec 60 °C
153 extension. Optimal conditions for standard DNA were tested on cDNA. Serial

154 dilutions (1:2; 4-5 dilutions per assay) of a cDNA sample containing all
155 amplification targets were used in reaction with each primer pair. An arbitrary
156 number of molecules was attributed to first dilutions so that the software could
157 construct the standard curves (Cts for first dilutions were at 23-26 cycles). $E \geq 0.95$
158 were again obtained for all primer pairs, confirming optimal amplification also on
159 cDNA. Melting curves of amplification products confirmed the existence of a
160 unique melting temperature. Separation in 2.5 % agarose gel electrophoresis,
161 along with a suitable DNA ladder, confirmed fragment sizes. *4-CL* results were
162 further divided by *GAPDH* in the same cDNA sample as a normalization factor.

163

164 2.5. Evaluation of the percentage of infected macrophages in culture

165 Macrophages were plated on slides with culture chambers (Lab-Tek Chamber
166 Slide, Nunc), previously coated with Poly-L-Lysine solution (Sigma) to prevent cell
167 detachment. At the indicated times p.i., culture medium was removed and cells
168 were fixed with 4 % formaldehyde in PBS solution for 15 min, washed twice in
169 PBS and permeabilized with 0.1 % Triton X-100 in PBS for 20 min. Preparations of
170 cells infected for 8 h were incubated for 1 h with a rabbit serum anti-p32 early
171 ASFV protein (courtesy of Michael Parkhouse, IGC, Oeiras) diluted 1:200 in 0.05
172 % Tween 20 in PBS (PBS/T), washed three times in PBS/T and further incubated
173 for 1 h with a FITC-conjugated goat anti-rabbit IgG (10 $\mu\text{g}/\text{ml}$ in PBS/T).
174 Preparations of cells infected for 18 h were incubated with a polyclonal FITC-
175 conjugated anti-ASFV porcine serum (1:100 dilution in PBS) (courtesy of Benedita
176 Cruz, LNIV, Lisbon) during 1 h. After three washes in PBS/T, cells were incubated
177 with the DNA fluorochrome Hoechst 33258 (at 5 $\mu\text{g}/\text{ml}$ in PBS, 5 min), allowing the
178 observation of cell nuclei and assessment of total cell numbers. Further washes

179 were made as before and slides were mounted with PBS/glycerol (3/1) and
180 coverslips. Cells were visualized under a Leica DMR fluorescence microscope and
181 images were collected, at different culture fields, through a Leica DC200 camera
182 and Leica QFluoro Software.

183

184 2.6. Virus progeny titration

185 Parallel cultures on 48-well TC plates were infected with MOI 5 of each virus. After
186 18 h, virus in culture supernatants was titrated by observation of cytopathic effect
187 at end-point dilutions in macrophage cultures, as previously reported (Leitao et al.,
188 2001), and expressed as 50% tissue culture infectious dose (TCID₅₀).

189

190 2.7. Northern Blot

191 1 µg of total RNA was fractionated by electrophoresis on a specific hidrogel
192 (Clearose RNA Mini, Elchrom) after glyoxal denaturation, alongside with RNA
193 molecular weight markers (Promega). The gel was stained in ethidium bromide
194 solution and photographed on transilluminator. RNA was transferred to a nylon
195 membrane, fixed by UV radiation, and hybridized to a biotin-labelled anti-sense
196 RNA probe (Biotin RNA Labelling Mix, 10x conc., Roche) obtained by *in vitro*
197 transcription from a pGEM-T vector (Promega) with 4-CL sequence cloned. Probe
198 was complementary from nucleotides 100 to 950 downstream the ATG codon of 4-
199 CL ORF. Hybridization, washing and detection of probe on membranes were
200 through North2South Chemiluminescent Hybridization and Detection Kit (Pierce),
201 according to manufacturer instructions with some modifications: hybridization/pre-
202 hybridization solution was supplemented with 30 µg/ml yeast RNA (Roche) and 1
203 % SDS; pre-hybridization (30 minutes) and hybridization (overnight) were

204 performed at 70 °C; membranes were washed in 0.5 x SSC, 0.2 % SDS solution at
205 70 °C.

206

207 2.8. Western blot

208 Total cellular protein extracts were obtained from ethanol-phenol fractions kept
209 during RNA extraction through TRIzol Reagent, processed according to
210 manufacturer instructions (Invitrogen). Protein was quantified through Bradford
211 reagent (Sigma), and 25 µg were separated by SDS-PAGE electrophoresis
212 (through a 4 % concentrating gel and a 12 % resolution gel). An aliquot of 0.5 µg
213 purified A224L protein (courtesy of Y. Revilla, CBMSO, Madrid) was separated
214 together with protein samples. After transfer to nitrocellulose membranes, these
215 were incubated with blocking solution (5 % milk powder, 0.1 % Tween 20 in PBS)
216 for 2 h at room temperature, followed by overnight incubation at 4 °C with anti-
217 A224L rabbit serum, (courtesy of Y. Revilla, CBMSO, Madrid), diluted 1:750 in
218 blocking solution. After washings in 0.1 % Tween 20 in PBS, membranes were
219 incubated for 2 h at room temperature with goat anti-rabbit IgG HRP-conjugated (P
220 0448, Dako), diluted 1:50 000 in blocking solution. Washings were made as before
221 and membranes were incubated with quimioluminescent substrate (West Femto,
222 Pierce) for bound antibody detection. Band density was assessed on digital scans
223 of autoradiography films through ImageMaster TotalLab software (Amersham
224 Pharmacia Biotech).

225

226 2.9. Statistical analysis

227 Paired t-test was used to compare results between infections. Data were
228 transformed to their logarithms ($Y = \text{Log}_{10}$) for a maximal approximation to Normal

229 distribution, and differences where $p < 0.05$ were considered statistically significant.
230 The InStat GraphPad v.3 software was used.

231

232 **3. Results and discussion**

233 *4-CL* mRNA expression was quantified by real time PCR at different time-points of
234 1.5 h, 4 h, 8 h, 12 h, and 16 h, along L60 and NHV infections on porcine
235 macrophages. Interestingly, mRNA expression was obtained very early in both
236 infections, at 1.5 h p.i. (Fig.1). This finding diverges from a previous report
237 describing this gene as a late expression gene in Vero cell line infection model
238 (Chacon et al., 1995). Furthermore, this transcription was appreciable, since the
239 reference house-keeping gene used in our studies, *GAPDH*, is of abundant
240 expression in porcine macrophages (Foss et al., 1998), and both genes were
241 expressed to a similar order of magnitude. *4-CL* mRNA was more abundant in L60
242 than NHV infection at this very early time-point. At later times expression showed
243 a clear increase without further significant differences between infections, although
244 at 12 h to 16 h p.i. a tendency was observed for higher expression in NHV (Fig. 1).
245 In order to compare infection efficiencies by each viral isolate, we determined the
246 percentage of infected cells on macrophage cultures cultivated on slides at an
247 intermediate time of 8 h, and at a late time of 18 h p.i. After fixation and
248 permeabilization, cells were incubated with anti-ASFV antibodies and Hoescht
249 33258 DNA stain, and visualized under fluorescence microscopy. L60 and NHV
250 infected a similar percentage of the cells in culture at 8 h p.i. (on average 31%)
251 and 18 h p.i. (on average 68%) (Fig. 2a). The increase in infected cells along time
252 seemingly reflected a second round of infection, since budding of new virus can be
253 observed from 10 h p.i. (Brookes et al., 1996). Production of similar progeny titres

254 after 18 h infection were also observed (Fig. 2b), suggesting identical capacities of
255 L60 and NHV for in vitro infection of porcine macrophages during an 18 h time
256 period. Therefore, comparative results on this infection model do not seem to be
257 affected by different infection efficiencies.

258 In order to confirm early transcription of *4-CL* in infected macrophages, total RNA
259 extracted at 1.5 h, 8 h and 16 h from infected cultures was analysed by Northern
260 blot. RNA from 16 h mock-infected cultures was also analysed as a control of
261 specific hybridization. Production of *4-CL* transcripts at very early infection of 1.5 h
262 was confirmed, along with an increase in expression levels along time (Fig. 3a),
263 which at 8 h and 16 h p.i. was stronger in NHV infection when compared to L60.
264 Transcripts of approximately 1000 and 700 bases were similarly produced in both
265 infections, the larger one present at all times p.i., and the smaller at 8 h and 16 h.
266 A third and longer transcript, of about 1500 bases, was present only along NHV
267 infection, denoting a different pattern of expression in both infections. Since this
268 gene sequence was known for L60 (GenBank accession U91736) but not for NHV,
269 we sequenced it in this isolate (GenBank accession FJ394332). *4-CL* coding
270 sequence from NHV resulted similar to L60's, showing that the different
271 expression pattern observed does not result from differences at the level of the
272 gene coding sequence. This sequence was also similar in Ba71V (A224L)
273 (GenBank accession NC_001659), previously used by other authors for
274 confirmation of the anti-apoptotic function of this viral IAP homologue in
275 mammalian cells (Nogal et al., 2001).

276 Chacon et al. (1995) referred the detection in Northern blot of RNA species with
277 more than 1400 bases in immediate early and early RNAs, obtained from Vero
278 infected cells treated with inhibitors of protein and DNA synthesis. However, these

279 were assumed to result from non-specific probe hybridization, based solely in the
280 failure to detect primer extension products indicative of transcript initiation sites in
281 these RNAs. Transcription of ASFV genes usually starts at a short distance from
282 the translation initiation codon of the ORF (Almazan et al., 1992; Chacon et al.,
283 1995; Rodriguez et al., 1996), and ends at motifs of seven or more thymidylate
284 residues present in intergenic regions of the viral genome, identified as signals for
285 transcription termination (Almazan et al., 1992; Almazan et al., 1993). The different
286 mRNA sizes found in our infection model match closely with transcription initiation
287 21 to 24 nucleotides upstream the ORF translation initiation codon, as previously
288 identified for *A224L* transcripts in Vero cell infection model (Chacon et al., 1995),
289 and termination at one of three motifs of seven or eight thymidylate residues,
290 found 38, 407 and 845 bp downstream the termination codon, resulting in
291 transcripts with approximately 730, 1100 and 1550 bases (Fig. 3b). It is highly
292 unlikely that these transcripts correspond to “readthrough” sequences from an
293 upstream ORF, as reported for other ASFV consecutive genes (Almazan et al.,
294 1992; Galindo et al., 2000a; Galindo et al., 2000b). The ORF immediately
295 upstream *4-CL*, *A240L*, is a thymidylate kinase homologue expressed as a single
296 very early transcript of about 1000 bases in infected Vero cells (Yanez et al.,
297 1993). However, should transcription from *A240L* span *4-CL*, longer transcripts
298 with a minimum of 2100 bases would be visible in Northern blot, the distance
299 between *A240L* starting codon and the first thymidylate residues motif downstream
300 *4-CL* termination codon (sequence information according to GenBank accession
301 NC_001659). Also, no thymidylate residues motif is present within *4-CL* coding
302 sequence, making it improbable for transcripts arising from *A240L* to terminate
303 inside it. Moreover, the distance between *A240L* and *4-CL* translation initiation

304 codons is already 1405 bp, and the probe used in our Northern blot studies targets
305 4-CL mRNA from nucleotides 100 to 950 downstream its initiation codon.

306 In order to determine if transcription was accompanied by protein production in our
307 model of infection, cellular protein extracts obtained at 1.5 h, 4 h, 8 h and 16 h p.i.
308 were analysed by Western blot using a 4-CL specific serum. The viral 27 kDa
309 protein was clearly detected at 8 h and 16 h p.i. in both infections (Fig. 4a),
310 indicating that it is produced in macrophage host cells between 4 h and 8 h.
311 Therefore, although transcription is initiated at very early infection, translation
312 occurs later, probably after the onset of viral DNA replication, indicating a
313 regulation of this viral gene at the post-transcriptional level. Quantification of 4-CL
314 by band densitometry showed at both 8 h and 16 h higher abundance of this
315 protein in L60 infection (Fig. 4b), even though RNA quantification at the later time
316 pointed to higher transcription levels in NHV infection. The fact that mRNA and
317 protein expression levels run opposite, with 4-CL mRNA not leading directly to
318 protein production, supports the existence of post-transcriptional regulation of this
319 viral gene. Detection of the smaller transcript of 700 bases in both infections, at 8
320 h p.i., coincided with protein detection. It is possible that only this transcript is
321 translated into protein, the larger transcripts serving other yet unknown roles in
322 infected cells.

323 Independently of when the gene is transcribed, 4-CL protein is clearly produced at
324 phases of infection when viral DNA is actively replicating, and apoptosis inhibition
325 in the host cell is important for viral cycle completion. Also, this viral protein is
326 incorporated into viral particles (Chacon et al., 1995), and may therefore have also
327 a role in apoptosis inhibition upon viral entry into host cells. In fact, virus uncoating

328 in the cytoplasm is the signal triggering apoptosis (Carrascosa et al., 2002), which
329 takes place at a very early step of infection.

330 In conclusion, our results showed that although transcription of *4-CL* starts at very
331 early infection on porcine macrophages, the viral protein is produced later, from 4
332 h to 8 h p.i. The purpose of the very early transcripts of this gene, if any exists, is
333 presently unknown. However, given the appreciable levels to which they are
334 produced, it is tempting to speculate that they may have a regulatory function at
335 some level of the early infectious cycle. Furthermore, expression of *4-CL* at protein
336 level was higher during L60 infection when compared to NHV, not correlating with
337 mRNA expression levels, which supports a post-transcriptional regulation of the
338 ASFV IAP homologue gene expression.

339

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346

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- 426

427 **Figure legends**

428 **Fig. 1.** Quantification of *4-CL* mRNA expression along L60 and NHV infection on
429 macrophages. At the indicated hours post-infection (p.i.) total RNA was extracted, cDNA
430 synthesized, and sequences for *4-CL* and *GAPDH* house-keeping gene were quantified in
431 the same cDNA sample by real time PCR. Data represent the mean and standard error of
432 5 independent experiments, with results expressed as the ratio between *4-CL* and
433 *GAPDH* expression, for normalization purposes. L60 and NHV infections were run in
434 parallel on cell cultures obtained from the same pig donor at each experiment. *Significant
435 difference between L60 and NHV infections ($p=0.015$).

436

437 **Fig. 2.** Infected cells and progeny production on macrophage cultures infected by L60 and
438 NHV. (a) Percentage of infected cells at 8 h and 18 h post-infection (p.i.) as determined by
439 immunofluorescence assays (b) Titres of virus in culture supernatants at 18 h p.i. Results
440 are expressed as the \log_{10} of 50% tissue culture infectious dose (TCID₅₀). Data on graphs
441 represent the mean and standard error of 4 independent experiments. At each
442 experiment, L60 and NHV infections were run in parallel on cultures obtained from the
443 same pig donor.

444

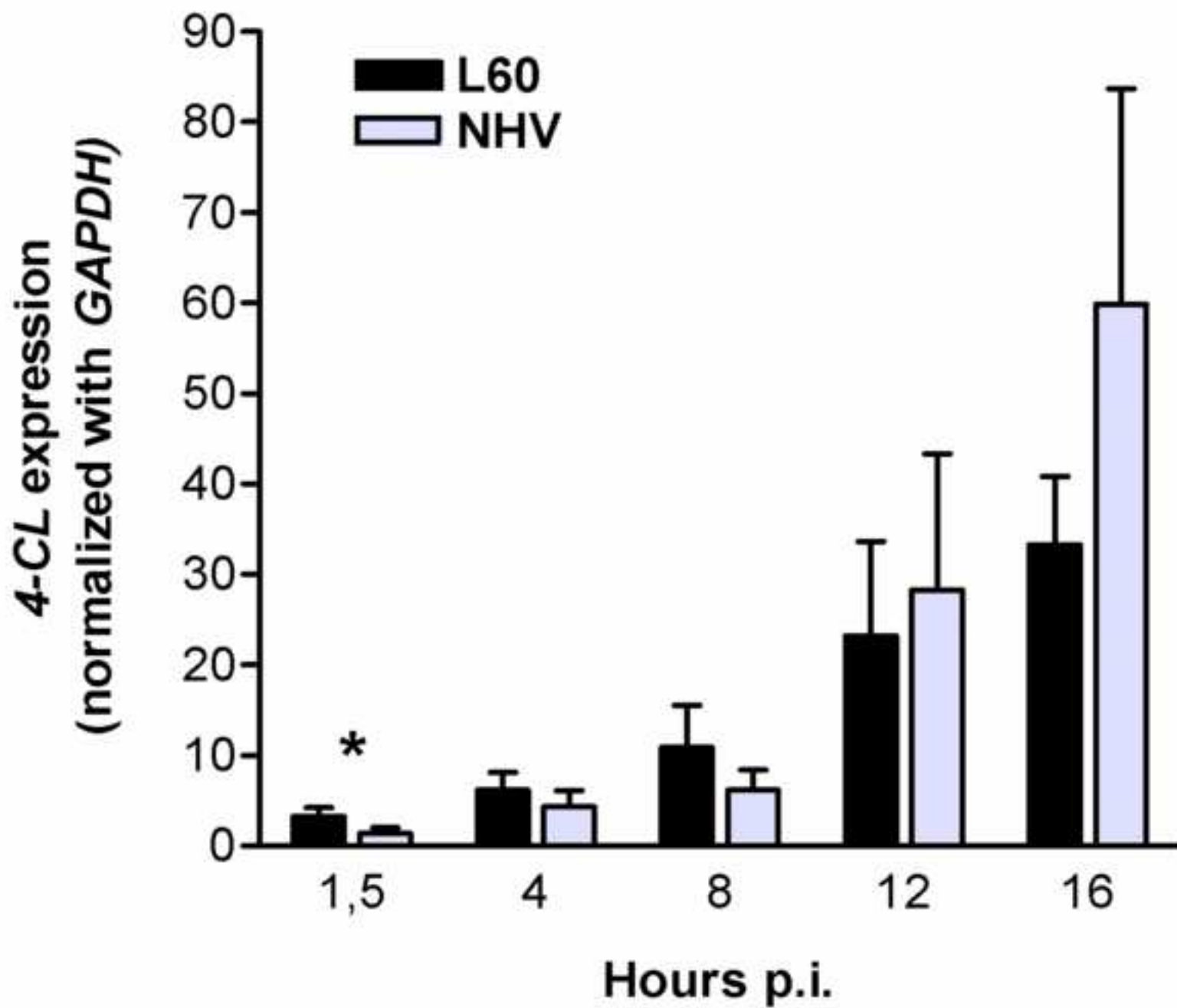
445 **Fig. 3.** *4-CL* transcripts produced during L60 (L) and NHV (N) infection on macrophages.
446 (a) Total RNA obtained at the indicated hours post-infection (p.i) or from a parallel mock-
447 infected 16 h culture (Mk), was separated in electrophoresis and hybridized to a specific
448 anti-sense RNA probe in Northern blot. On the left are indicated the sizes (in nucleotides)
449 of the RNA molecular weight marker used, and on the right the approximate sizes of the
450 transcripts detected. (b) Schematic representation of transcription initiation and
451 termination for this ORF. Underlined nucleotides represent initiation sites previously
452 described in Vero cell infection with adapted isolate Ba71V (Chacon et al., 1995), where
453 the ORF is named *A224L*. Possible termination sites, represented by stretches of 7-8 Ts
454 downstream the termination codon of the ORF, were depicted according to sequence
455 information for Ba71V deposited on GenBank (accession NC_001659). Distances are
456 represented in base pairs (bp).

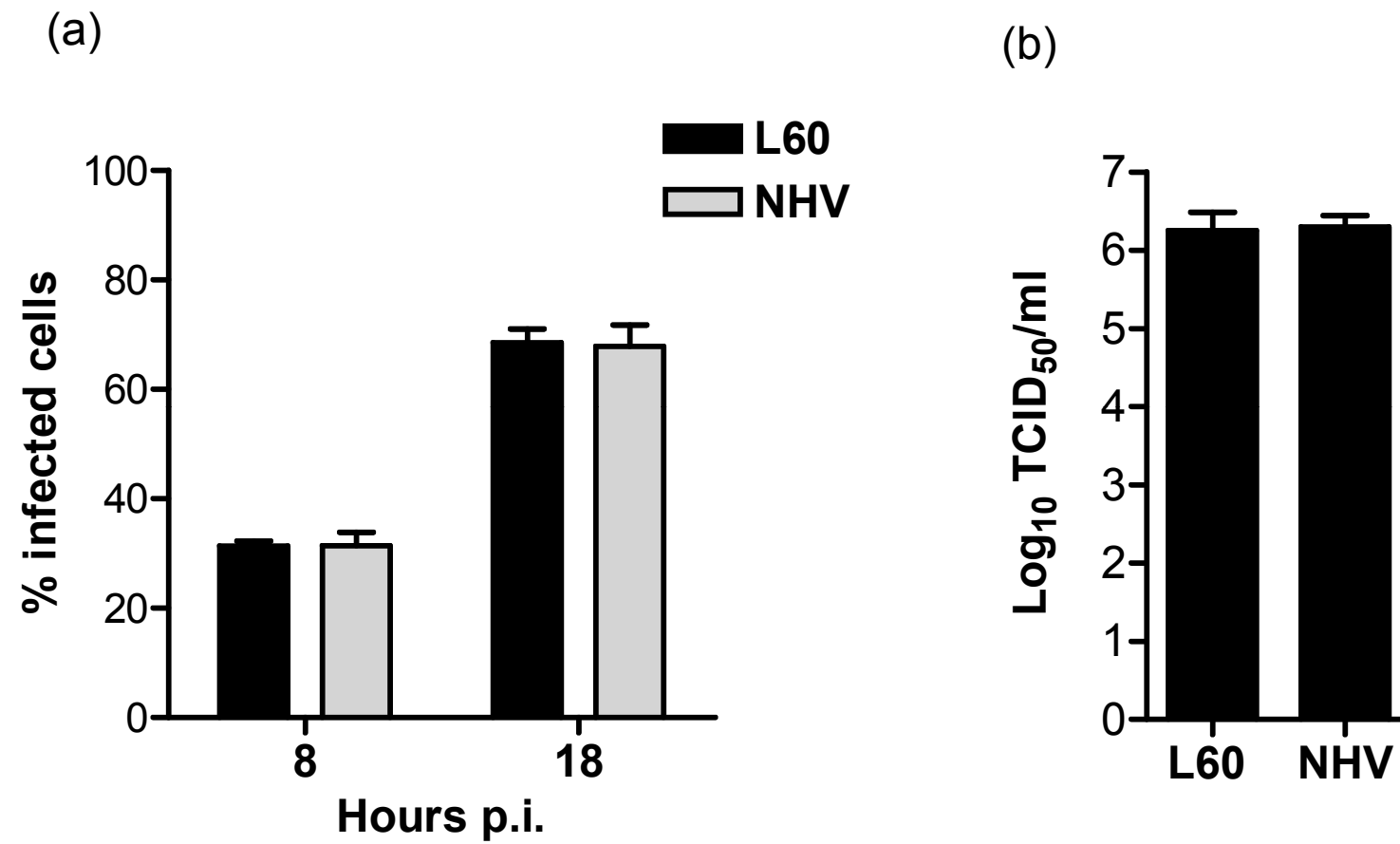
457

458 **Fig. 4.** Detection of *4-CL* protein along L60 (L) and NHV (N) infection on macrophages by
459 Western blot. (a) Protein extracts obtained at the indicated hours post-infection (p.i.), were
460 subjected to SDS-PAGE and analysed for the presence of the 27 kDa protein. A specific
461 serum raised for the same protein from Vero cell line adapted isolate Ba71V (named
462 *A224L* in this system) was used. An aliquot of purified *A224L* protein, as well as protein
463 extracted from mock-infected cells (Mk), were analysed in parallel to confirm specificity of

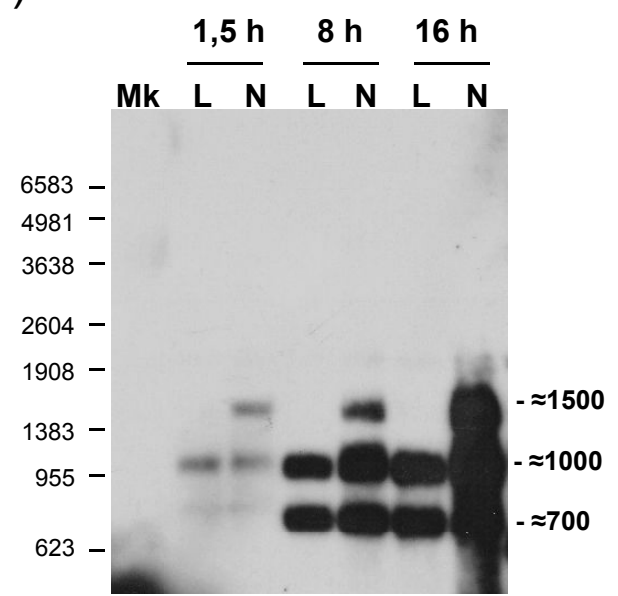
464 the serum. On the left side of the figure are represented the localization of bands from the
465 protein molecular weight markers used. (b) Ratio of 4-CL band densities between parallel
466 infections with L60 and NHV (L/N), in independent assays performed at the indicated
467 hours p.i. Average and standard errors are also presented (avg \pm se).
468

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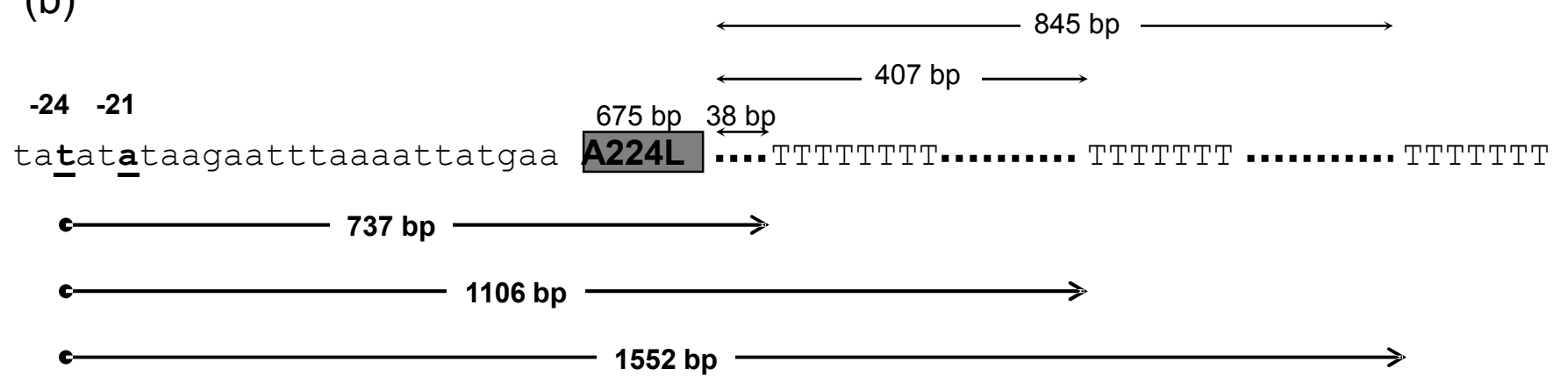




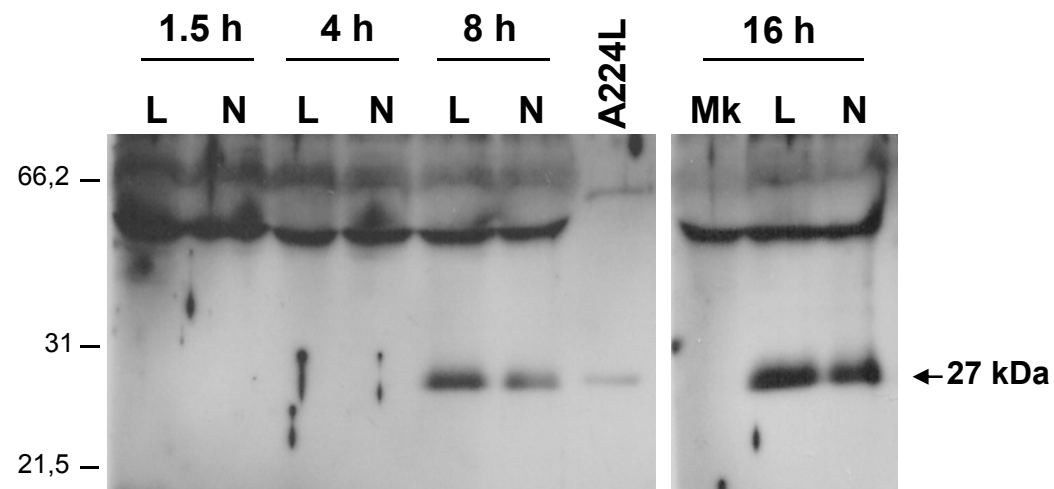
(a)



(b)



(a)



(b)

Ratio of 4-CL band density: L/N											
Assay	8 h p.i.					16 h p.i.					
	1	2	3	4	avg ± se	1	2	3	4	5	avg ± se
Ratio Value	3.4	1.9	1.6	1.2	2 ± 0.5	1.4	1.1	1.5	1.3	2.4	1.6 ± 0.2