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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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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 C<sub>4</sub> 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  $1.5 \times 10^5$  viable  
103 cells/cm<sup>2</sup> 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 pdT<sub>12-18</sub> 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  $\mu$ M of  
138 each primer, 1.5 mM MgCl<sub>2</sub>, 200 $\mu$ 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 (TCID<sub>50</sub>).

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 (TCID<sub>50</sub>). 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

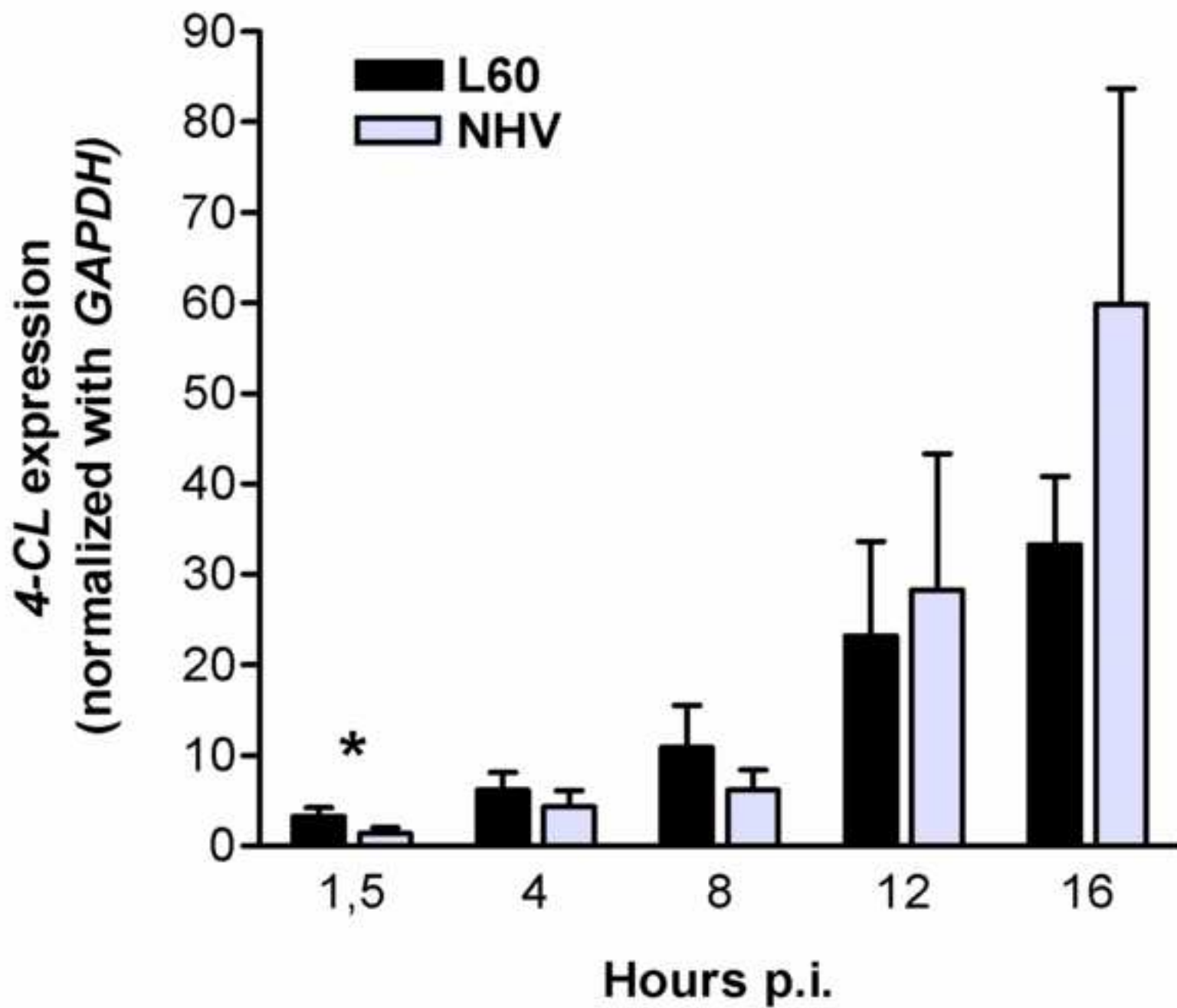
445 **Fig. 3.** *4-CL* transcripts produced during L60 (L) and NHV (N) infection on macrophages.  
446 (a) Total RNA obtained at the indicated hours post-infection (p.i) or from a parallel mock-  
447 infected 16 h culture (Mk), was separated in electrophoresis and hybridized to a specific  
448 anti-sense 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).

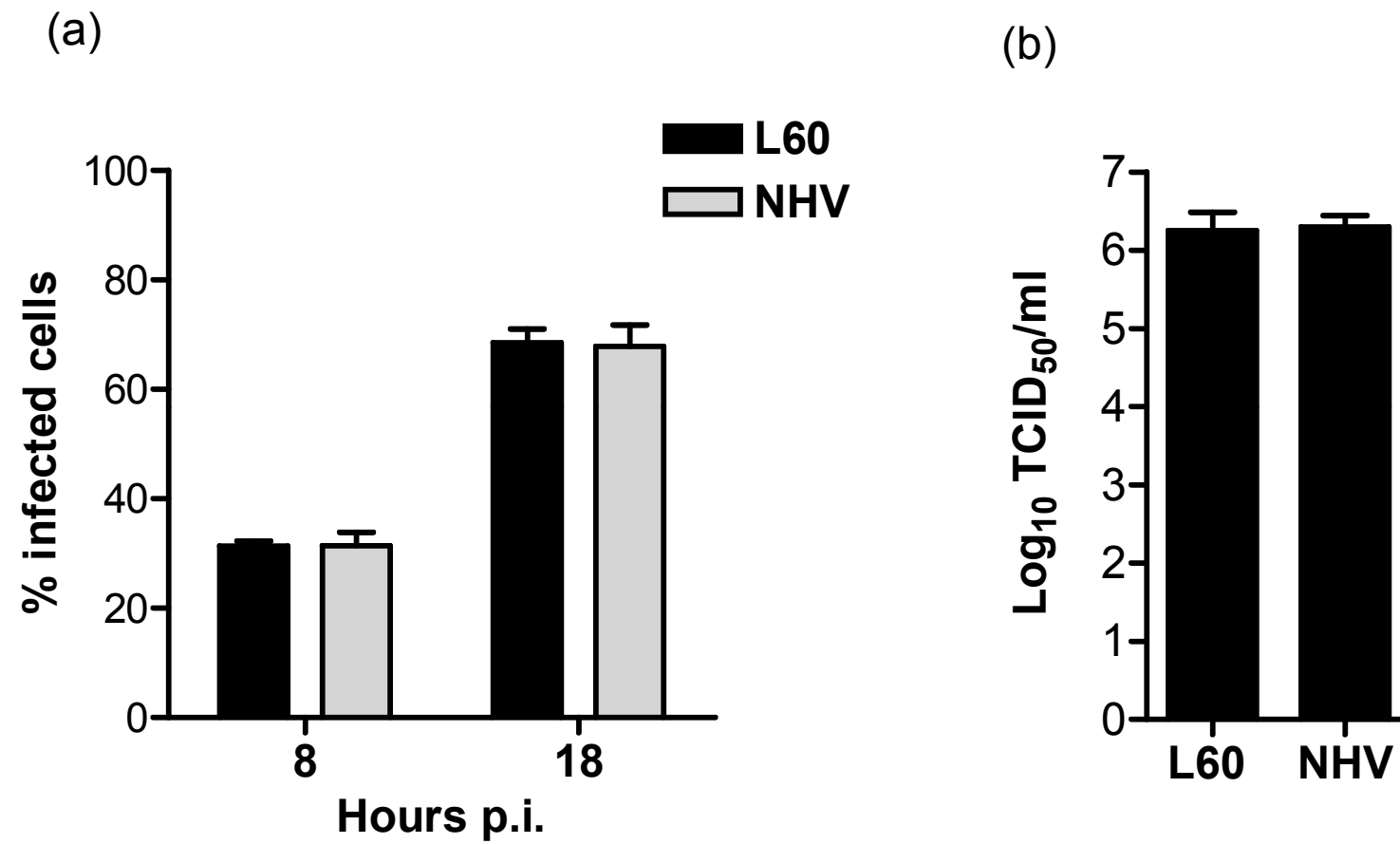
457

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

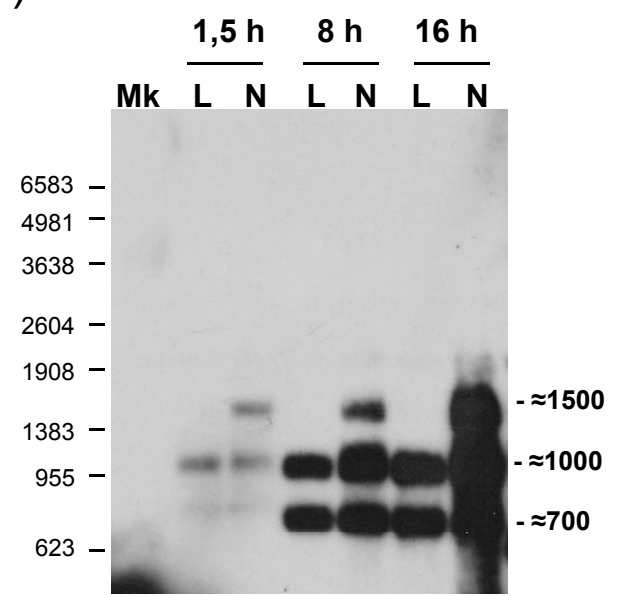
464 the serum. On the left side of the figure are represented the localization of bands from the  
465 protein molecular weight markers used. (b) Ratio of 4-CL band densities between parallel  
466 infections with L60 and NHV (L/N), in independent assays performed at the indicated  
467 hours p.i. Average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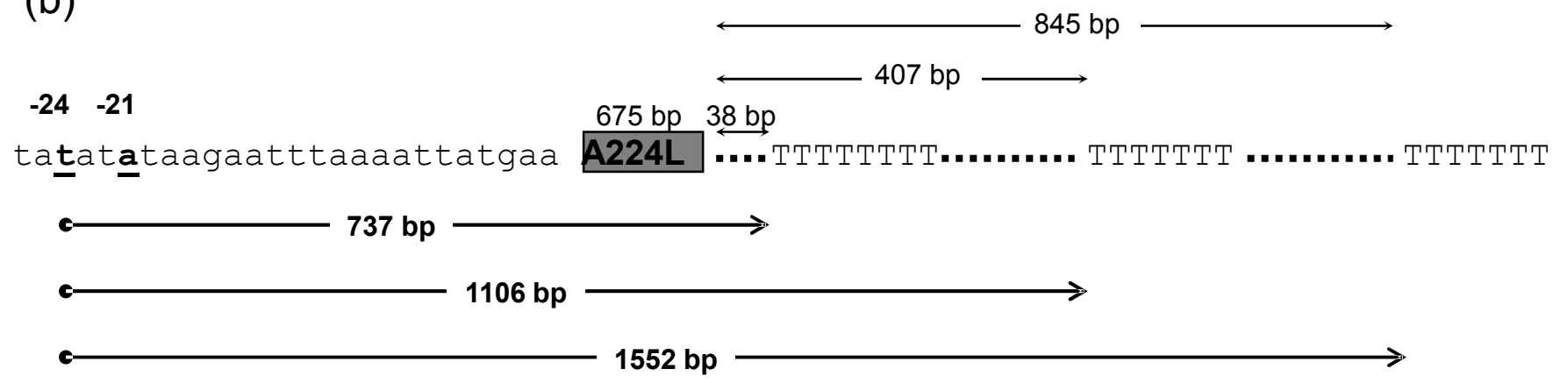




(a)

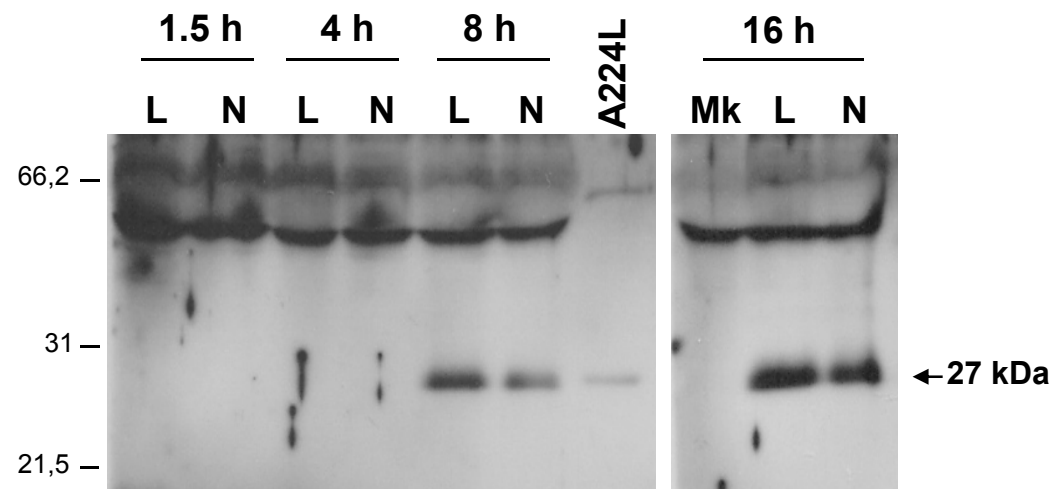


(b)





(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