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1

2 **Temperature sensitivity on growth and/or replication of H1N1,**
3 **H1N2 and H3N2 influenza A viruses isolated from pigs and birds**
4 **in mammalian cells**

5

6

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24 **Abstract 203 words**

25 Influenza A viruses have been isolated from a wide range of animal species,
26 aquatic birds being the reservoir for their genetic diversity. Avian influenza
27 viruses can be transmitted to humans, directly or indirectly through an
28 intermediate host like pig. This study aimed to define in vitro conditions that
29 could prove useful to evaluate the potential of influenza viruses to adapt to a
30 different host. Growth of H1N1, H1N2 and H3N2 influenza viruses belonging to
31 different lineages isolated from birds or pigs prior to 2005 was tested on MDCK
32 or NPTr cell lines in the presence or absence of exogenous trypsin. Virus
33 multiplication was compared at 33, 37 and 40°C, the infection site temperatures in
34 human, swine and avian hosts, respectively. Temperature-sensitivity of PB2-, NP-
35 and M-RNA replication was also tested by quantitative real-time PCR.
36 Multiplication of avian viruses was cold-sensitive, whatever cell type. By
37 contrast, temperature-sensitivity of swine viruses was found to depend on the
38 virus and the host cell: for an H1N1 swine isolate from 1982, multiplication was
39 cold-sensitive on NPTr cells and undetectable at 40°C. From genetic analyses, it
40 appears that temperature-sensitivity could involve other residues than PB2 residue
41 627 and could affect other steps of the replication cycle than replication.

42

43 **Keywords**

44 Avian influenza virus; Swine influenza virus; Temperature-sensitivity; NPTr
45 cells; Real-time quantitative PCR; PB2 627

46

47 **1. Introduction**

48 Influenza A viruses infect a wide range of animal species. All 16
49 hemagglutinins (HA) and 9 neuraminidases (NA) that determine influenza virus
50 subtypes have been isolated from wild aquatic birds. Thus, aquatic birds seem to
51 be the reservoir for the genetic diversity of influenza A viruses, from which they
52 can be transmitted to other species, like poultry, pig or human (Webster et al.,
53 1992). This phenomenon of crossing the species barrier was responsible for the
54 1918, 1957 and 1968 influenza pandemics and as seen more recently with the
55 emergence of the pandemic H1N1 2009 virus of swine origin is a major issue with
56 respect to human and animal public health.

57 In humans and pigs, influenza A viruses first replicate in the upper
58 respiratory tract at a temperature close to 33°C and 37°C, respectively, inducing a
59 respiratory syndrome (Alford et al., 1966). In aquatic birds, low pathogenic
60 influenza A viruses preferentially replicate in the intestinal tract at a temperature
61 close to 40°C, and the infection is usually asymptomatic (Webster et al., 1978). In
62 domestic poultry, some subtypes of influenza A viruses can cause devastating
63 epizootics leading to economic losses and sanitarian risks for humans and other
64 domestic animals. The HA is involved in host range and adaptation of influenza A
65 viruses through its binding specificity to the receptor i.e. sialic acids (SA). Indeed,
66 the HA of human and avian influenza A viruses preferentially binds to SA α 2,6-
67 and SA α 2,3-galactose, respectively (Connor et al., 1994; Suzuki et al., 2000),
68 which correlates well with their distribution at the site of virus multiplication: SA
69 α 2,6 on epithelial cells of the human upper respiratory tract and SA α 2,3 on

70 epithelial cells of the avian intestinal tract (Couceiro et al., 1993; Ito et al., 2000).
71 In the tracheal epithelium of pigs, both types of SA are present, which could
72 explain the susceptibility of pigs to both human and avian influenza A viruses
73 (Scholtissek et al., 1998). Consequently, pigs are considered as a "mixing vessel"
74 host, where reassortment among human, avian and swine influenza A viruses
75 could lead to new influenza virus lineages with zoonotic potential (Kuntz-Simon
76 and Madec, 2009; Webster et al., 1992). In addition to their possible transmission
77 to humans, swine influenza A viruses can also be transmitted to poultry (Suarez et
78 al., 2002) and wild ducks (Olsen et al., 2003).

79 Following receptor binding, entry involves the endocytosis of the virus
80 particle and the low pH-dependent fusion of the virus envelope with the
81 endosomal membrane. This process requires prior activation of the HA by
82 protease cleavage. The enzymes responsible are trypsin-like proteases secreted by
83 respiratory or intestinal tract cells that restrict virus multiplication to these tissues.
84 Therefore, in cell culture, the growth of influenza A viruses usually requires
85 exogenously added trypsin, since culture cells generally lack appropriate
86 endogenous proteases for HA cleavage (Klenk and Rott, 1988). However, some
87 H5 and H7 influenza A viruses possess a HA cleavable by ubiquitous and
88 intracellular proteases (Stieneke-Grober et al., 1992), allowing the virus to
89 replicate in other tissues and to spread systemically within the infected host.

90 Although the HA is a major determinant for host restriction it is not
91 necessarily sufficient. Indeed, some influenza A viruses of entire swine or avian
92 origin, thus not reassortants, have been directly transmitted to humans (Banks et

93 al., 1998; Claas et al., 1994; Kendal et al., 1977). These viruses had specific
94 characteristics that allowed them to replicate in humans, but were not able to
95 transmit from human to human and then spread in the human population. Other
96 determinants of host range or adaptation to a novel host were identified in the
97 virus internal proteins, especially in the polymerase complex subunits PB1, PB2
98 and PA. For the 1957 and 1968 pandemics, it was demonstrated that the emerging
99 viruses were reassortants between human and avian influenza viruses (Yasuda et
100 al., 1991). In both cases, reassortant viruses acquired not only NA and/or HA
101 genes but also the viral polymerase subunit PB1 gene from an avian influenza
102 virus, whereas other genes were acquired from a human influenza A virus
103 (Kawaoka et al., 1989; Scholtissek and Murphy, 1978). Experiments on the
104 compatibility of polymerase subunits showed that an avian origin of the PB1 gene
105 may have conferred a selective advantage to the 1957 and 1968 reassortant viruses
106 (Naffakh et al., 2000). In swine, reassortant viruses indicated that a PB1 gene of
107 human origin allows the virus to be infectious in pigs, whatever the swine or avian
108 origin of the two other subunits of the viral polymerase complex (Karasin et al.,
109 2006).

110 Reassortment experiments further showed that genomic segments NP, M,
111 NS and PB2 also seem to be involved in host restriction (Clements et al., 1992;
112 Geiss et al., 2002; Snyder et al., 1987; Tian et al., 1985). A well-characterised
113 host-range determinant is the residue 627 of PB2, a glutamic acid found in almost
114 all avian influenza A viruses versus a lysine in human influenza A viruses (Smith
115 et al., 2006; Subbarao et al., 1993). The nature of residue 627 of PB2 mostly

116 determines the cold-sensitivity at 33°C of the replication of avian viruses in
117 mammalian cells (Labadie et al., 2007; Massin et al., 2001) and has been involved
118 in the level of pathogenicity of the virus (Hatta et al., 2001; Munster et al., 2007)
119 and in its ability to replicate in the upper respiratory tract (Hatta et al., 2007).
120 Altogether, published data suggest that host restriction is a complex mechanism,
121 driven by specific gene constellations depending on the origin of the virus.

122 In an attempt to define in vitro conditions that could prove useful to
123 evaluate the potential of viruses to adapt to a different host, a comparative
124 analysis of multiplication capabilities of influenza A viruses isolated from pigs or
125 birds was performed at three temperatures (33, 37 and 40°C), in three cell types
126 (Madin-Darby Canine Kidney cell line or MDCK, Newborn Pig Tracheal cell line
127 or NPTr, chicken fibroblast DF1 cell line), and in the presence or absence of
128 exogenous trypsin. Three subtypes of influenza A viruses (H1N1, H1N2 and
129 H3N2) were selected based on the fact that these subtypes have also been
130 circulating in humans and are currently co-circulating in pigs and birds. The
131 replication capabilities of two H1N1 viruses belonging to the same avian-like
132 swine lineage and respectively isolated from turkey and swine were also
133 investigated by real-time PCRs targeting the PB2-, NP- and M- genomic vRNAs.

134

135 **2. Materials and Methods**

136 ***2.1. Cells and viruses***

137 Newborn Pig Trachea (NPTr, (Ferrari et al., 2003)) and Madin Darby
138 Canine Kidney (MDCK) cells were grown at 37°C with 5% CO₂, in MEM

139 supplemented with antibiotics and foetal calf serum (FCS, 10% and 5%
140 respectively). For MDCK cells, MEM was supplemented with 1% tricine.
141 Chicken fibroblast DF1 cells were grown at 39°C with 5% CO₂, in DMEM
142 supplemented with 10% FCS and antibiotics.

143 Regarding the viruses used, A/turkey/France/05016/05 (H1N1) (av-
144 H1N1/05), A/mallard/France/710/02 (H1N1) (av-H1N1/02) and
145 A/turkey/France/05045c/05 (H1N2) (av-H1N2/05) were isolated by the National
146 Reference Laboratory for Avian Influenza and Newcastle disease at the AFSSA-
147 LERAPP, Ploufragan, France. A/duck/Ontario/05/00 (H3N2) (av-H3N2/00) was
148 kindly provided by J. Pasick, Canadian Food Inspection Agency, National Centre
149 for Foreign Animal Disease, Winnipeg, Manitoba, Canada (OIE Reference
150 Laboratory for Avian Influenza). A/swine/Finistère/2899/82 (H1N1) (sw-
151 H1N1/82), A/swine/Scotland/410440/94 (H1N2) (sw-H1N2/94) and
152 A/swine/Gent/1/84 (H3N2) (sw-H3N2/84) were kindly provided by K. van Reeth,
153 Ghent University, Belgium. Influenza viruses were amplified at 36-37°C in 9-day-
154 old SPF embryonated chicken eggs. Allantoic fluids were collected at a maximum
155 of 4 days after inoculation and then subjected to centrifugal clarification before
156 use as viral stocks.

157

158 **2.2. Phylogenetic analyses**

159 For avian influenza A viruses, viral RNA was extracted from infectious
160 allantoic fluid according to a standardised procedure (Alexander, 2005). Viral
161 RNA was reverse transcribed using hexanucleotides as primers. cDNA was

162 subjected to PCR using segment-specific primers (primer sequences available
163 upon request). All 8 PCR products were purified from agarose gel by using the
164 Qiaquick gel extraction kit (Qiagen). The DNA sequences were determined by
165 sequencing both DNA strands by the dye terminator method (ABI Prism
166 DyeTerminator Cycle Sequencing Ready Reaction Kit, Applied Biosystems) on
167 an automatic DNA sequencer ABI 373XL (Applied Biosystems). Sequences are
168 available under the following accession numbers: FN395357 to FN395364 for av-
169 H1N1/05, AJ697878, AM157388, and FJ985246 to FJ985251 for av-H1N1/02,
170 FJ985238 to FJ985245 for H1N2-05045c, and AJ697864, AJ697881, and
171 GQ249055 to GQ249060 for av-H3N2/00.

172 Phylogenetic analyses for each of the 8 segments of each virus genome
173 were performed with representative avian, swine and human influenza A virus
174 counterpart sequences (depending on the segment, 111 to 270 sequences,
175 available on the National Center for Biotechnology Information website
176 (<http://www.ncbi.nlm.nih.gov/>)). Multiple sequence alignments were generated
177 using the CLUSTALW algorithm (Thompson et al., 1994). Phylogenetic analyses
178 were conducted using the MEGA 3.1 software (Kumar et al., 2004) by the
179 Neighbor-joining method implemented with the Kimura-2 parameters model.
180 Topology of the trees was evaluated by 1000 bootstrap analyses. For a review of
181 swine influenza virus lineages see (Kuntz-Simon and Madec, 2009). Data are
182 presented in Table 1.

183

184 ***2.3. Virus stock titration by TCID₅₀ determination***

185 Virus stock titres were determined by titration on MDCK or NPTr cells
186 and expressed as a tissue culture infectious dose 50% per millilitre (TCID₅₀/ml).
187 MDCK or NPTr cells were seeded 24 h before titration in 96-well plates at a
188 density of 3×10^4 cells/well in medium supplemented with FCS (10% or 5%,
189 respectively). Serial 10-fold dilutions (10^{-1} to 10^{-10}) of the virus stock or the viral
190 sample were made in MEM without FCS and supplemented with 2 or 0.2 $\mu\text{g/ml}$ of
191 trypsin-TPCK (Whorthington Biochemical Corporation). Cells were washed twice
192 with MEM without serum and then infected with virus dilutions (8 to 10 replicas
193 per dilution). At 72 h post-infection, the cytopathic effect was observed using an
194 inverted microscope and virus titres were calculated using the Reed and Muench
195 method. Virus titration was performed twice at least and did not differ by more
196 than one \log_{10} for a given virus suspension.

197

198 **2.4. Virus growth kinetics in cell culture**

199 NPTr and MDCK cells were seeded in 6-well plates at a density of 1×10^6
200 cells/well in medium supplemented with FCS (10% and 5%, respectively). 24 h
201 later, cells were washed twice with MEM without serum and then infected at a
202 multiplicity of infection (MOI) of 10^{-3} TCID₅₀/cell with either avian or swine
203 influenza viruses, or mock infected. After adsorption for one hour at room
204 temperature, the inoculum was removed. The cells were washed once with MEM
205 without serum and then incubated at 33, 37 or 40°C with MEM without serum but
206 supplemented, or not, with 2 $\mu\text{g/ml}$ of trypsin-TPCK. At 0, 12, 24 and 48 h post-

207 infection, virus titres were determined in the supernatants, on MDCK cells only,
208 as described in 2.3.

209

210 **2.5. Virus replication kinetics in cell culture**

211 NPTr cells were infected with either avian- or swine-H1N1 influenza
212 viruses, or mock-infected as described in section 2.4. At 0, 12, 24 and 48 h post-
213 infection, cells were washed twice in PBS and total RNA was extracted using the
214 MagAttract RNA Tissue Mini M48 kit and the M48 automated platform (Qiagen).
215 Total RNA was eluted in 100 µl RNase-free water and quantified with the Quant-
216 iT RNA kit and Qubit fluorometer (Invitrogen). Each sample was then subjected
217 to real-time PCRs as described below, to determine copy numbers of NP-, M-, or
218 PB2-vRNAs.

219

220 **2.6. SYBR Green real-time PCR**

221 Two-steps real-time PCRs specific for NP-, M-, or PB2-vRNA were
222 developed. First, total RNA (60 ng) was denatured and annealed at 65°C for 5 min
223 with 10 pmol of Uni12 primer (Hoffmann et al., 2001) and 1 µl of dNTP (10 mM
224 each). RNA was reverse-transcribed at 42°C for 70 min in a total volume of 30 µl,
225 using Superscript II reverse transcriptase (Invitrogen) according to the
226 manufacturer's protocol. Real-time PCR reactions were next performed in a final
227 volume of 25 µl with 12.5 µl of SYBR Green PCR Master Mix (Applied
228 Biosystems), 10 µl of cDNA (diluted 1:10) and 200 nM of each forward and
229 reverse primer (except for M PCR: 400 nM) (sequences available upon request).

230 Amplifications were performed using the following conditions: 2 min at 50°C, 10
231 min at 95°C, followed by 45 amplification cycles (15 sec at 95°C and 1 min at
232 60°C for NP and M PCRs or 15 sec at 55°C and 1 min at 60°C for PB2 PCR). The
233 program ended with a dissociation stage. Amplifications were carried out in 96-
234 well plates with 3 replicates per sample, using Applied Biosystems 7000 and 7500
235 Real-Time PCR Systems and analysed with the Sequence Detection Software. For
236 optimisation and standard curves, full-length PCR products of each viral segment
237 were used. RNA was extracted from allantoic fluids infected with either avian or
238 swine influenza viruses, using the RNeasy kit (Qiagen). cDNA corresponding to
239 each virus was synthesized using the Uni12 primer and the Superscript II
240 (Invitrogen) as described above. For each cDNA, specific amplification of NP-,
241 M-, and PB2-genes were performed using primers derived from Hoffman et al.
242 (Hoffmann et al., 2001) and the Taq Platinum High Fidelity polymerase
243 (Invitrogen), according to the manufacturer's protocol. PCR products of the
244 expected size were purified from an agarose gel using the Qiaquick gel extraction
245 kit (Qiagen). Purified products were then quantified with the Quant-iT DNAs kit
246 and Qubit fluorometer (Invitrogen) to determine the copy number. Serial 10-fold
247 dilutions of purified PCR products were used to establish standard curves. For all
248 subsequent analyses, one full-length PCR product was chosen as a standard for
249 each PB2-, NP- and M-specific real-time PCRs: av-H1N1-PB2 and sw-H3N2-NP
250 and -M. Ten independent serial dilutions of each standard were analysed to
251 determine limits of detection and quantification as described in a French reference
252 norm (XPT 90-471 from April 2006).

253

254 **2.7. Statistical analysis**

255 For virus titration experiments, analyses of variance were performed to
256 test the effect of trypsin concentration and cell type on the virus titres, assessed by
257 the \log_{10} of titres (TCID₅₀/ml). Analyses of variances were computed with the
258 GLM procedure of the SAS software (SAS, 2004). In addition, the Newman and
259 Keuls multiple comparison procedure was used to compare the different trypsin
260 concentrations, with respect to the different cell types.

261 For virus replication kinetics, statistical analyses were performed to test
262 the effect of the virus (avian or swine H1N1 influenza virus), of the temperature
263 (33°C, 37°C or 40°C) and of their potential interaction on the kinetics of
264 replication of three different viral genes (PB2, NP and M), assessed by the \log_{10} of
265 the copy number (three replicates). Linear mixed models (Littell et al., 1998)
266 including repeated effects were used. The virus, the temperature and the virus by
267 temperature interaction were introduced in the model as fixed effects in
268 accordance with the design of the study. The time post-infection (0, 12, 24 and
269 48 h) was incorporated in the model as a repeated measurement factor in order to
270 take into account the within-time covariability, specifying a first-order
271 autoregressive structure for the covariance matrix. In the case of a significant
272 virus by temperature interaction, an alternative to multiple comparison
273 procedures, that are not available with linear mixed models, contrasts procedures
274 were used to compare the different levels of the temperature, for each virus.

275 Linear mixed models were computed with the MIXED procedure of the SAS
276 software (SAS, 2004).

277

278 **3. Results**

279 ***3.1. Phylogenetic analyses***

280 Phylogenetic analyses of genomic segments of each virus were performed
281 as described in Materials and methods and are summarised in Table 1. Swine
282 influenza viruses used in this study belonged to well-known swine lineages (for a
283 review see (Kuntz-Simon and Madec, 2009)). Briefly, the sw-H1N1/82 belonged
284 to the avian-like swine lineage. Its 8 genomic segments were of avian origin. The
285 sw-H1N2/94 and sw-H3N2/84 belonged to human-like reassortant swine lineages:
286 they possessed internal genes from the avian-like swine H1N1 lineage and HA
287 and NA from human lineages. The av-H1N1/05 and av-H1N2/05 were found
288 closely related to sw-H1N1/82 (avian-like swine lineage) and to sw-H1N2/94
289 (human-like reassortant swine lineage), respectively. The av-H1N1/02 possessed
290 8 genomic segments of avian origin. All genes of the av-H3N2/00 were closely
291 related to human influenza viruses isolated in the 1970's.

292

293 ***3.2. Comparison of virus stock titres obtained on MDCK or NPTr cells in the*** 294 ***presence of 2 or 0.2 µg/ml of trypsin***

295 In order to perform virus titrations on MDCK, NPTr and DF1 cells, we
296 first tested the ability of monolayers of each cell type to resist in a medium
297 containing trypsin (data not shown). Monolayers of MDCK and NPTr cells

298 remained viable in a medium containing up to 2 $\mu\text{g/ml}$ of trypsin. In contrast,
299 monolayers of DF1 cells were disrupted in such a medium. At 0.2 $\mu\text{g/ml}$ of
300 trypsin, DF1 cell monolayers did not reproducibly resist. Thus, virus stock
301 titrations could only be performed on MDCK and NPTr cells.

302 The titres of H1N1, H1N2 and H3N2 influenza viruses isolated from pigs
303 or birds (further referred to as avian (or av-) and swine (or sw-), respectively)
304 were determined on MDCK and NPTr cells in the presence of 2 or 0.2 $\mu\text{g/ml}$
305 trypsin, as described in Materials and Methods. Calculated titres are presented in
306 Fig. 1.

307 On MDCK cells (left panel), titres were about 2 \log_{10} higher with 2 $\mu\text{g/ml}$
308 of trypsin than with 0.2 $\mu\text{g/ml}$ of trypsin, whereas on NPTr cells in the same
309 conditions, differences in titres were only about 0.4 \log_{10} . In the presence of 2
310 $\mu\text{g/ml}$ of trypsin, titres for all viruses were slightly but significantly ($p < 0.0001$)
311 higher on MDCK cells (black bars, left panel) than on NPTr cells (black bars,
312 right panel). In contrast, with 0.2 $\mu\text{g/ml}$ of trypsin, titres were globally slightly but
313 significantly ($p < 0.0001$) higher on NPTr cells (grey bars, right panel) than on
314 MDCK cells (grey bars, left panel). Taken as a whole, titres were significantly
315 ($p < 0.0001$) higher with 2 $\mu\text{g/ml}$ of trypsin (black bars) than with 0.2 $\mu\text{g/ml}$ (grey
316 bars). These results indicated that multiplication of av- or sw-H1N1, H1N2 and
317 H3N2 influenza viruses was less dependent on the trypsin-TPCK concentration on
318 NPTr cells than on MDCK cells.

319

320 **3.3. Comparison of virus growth kinetics at different temperatures on MDCK**
321 **and NPTr cells in the absence or presence (2 µg/ml) of trypsin-TPCK**

322 As described in Materials and Methods, the growth capabilities of av- and
323 sw-H1N1, H1N2 and H3N2 viruses were compared on MDCK and NPTr cells
324 infected at a low multiplicity of infection ($\text{MOI} = 10^{-3}$ TCID₅₀/cell) and then
325 incubated at 33, 37 or 40°C. At different times post-infection, the production of
326 infectious viral particles in the supernatants was evaluated by titration. The
327 kinetics of virus multiplication are presented in Fig. 2.

328 For viruses isolated from birds, in the presence of trypsin-TPCK (Fig. 2A,
329 bold lines), the kinetics of multiplication gave very similar profiles whatever the
330 host cell type or the virus used, with a delay in the titre raise at 33°C as compared
331 to 37 and 40°C. For each temperature considered, av-H1N1/05, av-H1N2/05 and
332 av-H3N2/00 viruses reached similar titres on MDCK and NPTr cells at 48 h post-
333 infection (p.i.). Similar titres were also reached at 48 h p.i. on MDCK and NPTr
334 cells for av-H1N1/02 virus, but only at 37 and 40°C. At 33°C, for av-H1N1/02
335 virus the titre at 48 h p.i. was nearly three log₁₀ lower on NPTr cells than on
336 MDCK cells.

337 In the absence of trypsin-TPCK (Fig. 2A, thin lines), the main profile
338 observed for avian viruses at all three temperatures was a delay in growth, with
339 titres starting to raise later than in the presence of trypsin-TPCK. Noticeably, for
340 av-H1N1/05 virus, no virus growth could be detected on MDCK cells at either
341 three temperatures. Similarly, av-H3N2/00 did not grow on MDCK cells when
342 incubated at 40°C.

343 For the viruses isolated from pigs, in the presence of trypsin-TPCK (Fig.
344 2B, bold lines), there was no general delay at 33°C as compared to 37°C on either
345 MDCK or NPTr cells. However, for sw-H1N1/82 on NPTr cells, the titre reached
346 at 48 h p.i. was more than one \log_{10} lower at 33°C than at 37°C. This difference at
347 33°C was not as clear on MDCK cells. Similarly, for sw-H1N2/94, the maximum
348 titre was reduced by one to two \log_{10} at 33°C as compared to 37°C on both cell
349 types. Moreover, at 40°C, virus growth was undetectable for sw-H1N1/82 on
350 NPTr cells and significantly reduced on NPTr as compared to MDCK cells for
351 sw-H1N2/94. As for sw-H3N2/84, whatever the temperature, it grew with similar
352 capabilities on both MDCK and NPTr cells.

353 In the absence of trypsin-TPCK (Fig. 2B, thin, dotted lines) the swine
354 viruses behaved differently from the avian viruses, since essentially no delay but
355 only a slight reduction (one to two \log_{10}) in the final titre reached at 48 h p.i. was
356 observed. The only noticeable difference was observed for sw-H1N1/82 on
357 MDCK cells at 40°C, for which the titre obtained was nearly three \log_{10} lower in
358 the absence of exogenous trypsin.

359

360 ***3.4. Comparison of virus replication kinetics at different temperatures on NPTr*** 361 ***cells in the presence of 2 $\mu\text{g/ml}$ of trypsin***

362 In order to determine if the temperature sensitivity observed in virus
363 growth was due to a defect in replication, we analysed the viral RNA levels over
364 time in NPTr cells infected with sw-H1N1/82 and av-H1N1/05 viruses using real-
365 time quantitative PCRs.

366 First, three SYBR green real-time PCRs were designed to specifically
367 detect and quantify the PB2-, NP-, and M-viral RNA segments. In order to obtain
368 a good efficiency of the real-time PCRs whatever the avian or swine origin of the
369 viral genome, thermocycling conditions and primer concentrations were optimised
370 on PB2-, NP- and M-full-length PCR products derived from avian and swine
371 influenza A viruses used in this study (data not shown). No detection limit could
372 be precisely determined for the three real-time PCRs, as they were below the
373 lowest amounts tested. Quantification limits could be estimated to be
374 approximately 220, 60 and 600 copies for our PB2-, NP- and M-specific real-time
375 PCRs, respectively.

376 NPTr cells were infected with either av-H1N1/05 or sw-H1N1/82 at a low
377 multiplicity of infection ($\text{MOI} = 10^{-3}$ TCID₅₀/cell) and were incubated at 33, 37
378 or 40°C. As described in Materials and Methods, total RNAs were extracted and
379 analysed by the three newly developed SYBR green real-time PCRs. Results are
380 presented in Fig. 3. For av-H1N1/05, the kinetics of replication of each vRNA
381 were very similar. At 12 h p.i., a delay was observed at 33°C as compared to 37°C
382 and 40°C. The difference between 33°C and 37°C was statistically confirmed for
383 the PB2 gene ($p < 0.05$) and with a level of significance of 10% ($p = 0.068$) for the
384 M gene, but not for the NP gene ($p > 0.05$). At 33°C the maximum level of vRNA
385 seemed to be reached at 48 h p.i. whereas it was already reached at 24 h p.i. at 37
386 and 40°C. For sw-H1N1/82, the vRNA kinetics were also very similar for the
387 three segments. A statistically significant delay in replication was observed at
388 33°C as compared to 37°C ($p < 0.05$ for the three genes), and at 48 h p.i. the copy

389 number was nearly two \log_{10} lower than at 37°C. At 40°C, we failed to detect
390 replication of any of the three vRNAs ($p < 0.05$ for the three genes). Altogether, the
391 real-time PCR results were very consistent with the virus growth results.

392

393 **4. Discussion**

394 The three subtypes of influenza A viruses that were used in this study
395 (H1N1, H1N2 and H3N2) were selected because they have been circulating in the
396 avian, swine and human populations. Furthermore, virus isolates were chosen in
397 order to fall within major lineages based on the phylogenetic analyses which
398 provided some insight on the genetic relationships between the viruses and their
399 human counterparts.

400 Three cell lines were originally chosen to compare the avian and swine
401 viruses. The MDCK cell line was derived from an adult female cocker spaniel in
402 1958 and was described to support replication of several influenza A viruses from
403 human, swine, equine and avian origin, forming plaques with high efficiency by
404 the addition of trypsin in the culture medium (Tobita et al., 1975). They are
405 widely used for the isolation of influenza A viruses from respiratory specimens
406 from human patients (Tobita et al., 1975) and for the multiplication of human and
407 swine influenza A viruses. DF1 cells consist of an established chicken fibroblast
408 cell line that has been used in recently published influenza studies (Labadie et al.,
409 2007; Lee et al., 2008). The NPTr cell line was selected because of the tissue (pig
410 trachea) from which it was established by serial culture, and the fact that it
411 supports the multiplication of several influenza A viruses from human, swine and

412 avian origin with similar infectious titres to those obtained in chicken embryos
413 (Ferrari et al., 2003).

414 Unfortunately, the DF1 cells proved highly sensitive to the trypsin
415 concentration in the culture medium and the cell monolayers inconsistently
416 resisted a 0.2 $\mu\text{g/ml}$ concentration of trypsin. In those conditions, titres obtained
417 for avian virus stocks on DF1 cells were low (data not shown) and in the same
418 range as those obtained on MDCK cells, i.e. two to three \log_{10} lower than titres
419 obtained on MDCK cells with 2 $\mu\text{g/ml}$ of trypsin. This seems to contrast with the
420 results of Lee et al. (2008), who evaluated the use of DF1 or QT6 cells, another
421 avian cell line, for the study of avian influenza A viruses, instead of primary avian
422 cells (Chicken Embryo Fibroblast, CEF) and MDCK cells, and concluded that
423 DF1 cells supported the growth of avian influenza A viruses as well as MDCK
424 cells. However, the trypsin concentrations used were different for each cell line
425 (0.75 versus 0 $\mu\text{g/ml}$ for MDCK cells and 0.05 versus 0 $\mu\text{g/ml}$ for DF1 cells),
426 making comparison difficult. As expected, on MDCK cells, the titres were higher
427 in the presence of 2 $\mu\text{g/ml}$ of trypsin than with only 0.2 $\mu\text{g/ml}$. In accordance with
428 the results of Ferrari et al. (2003), NPTr cells supported the multiplication of the
429 avian and swine viruses used and appeared less dependent on the addition of
430 exogenous trypsin in the culture medium, as similar virus titres were obtained for
431 both trypsin concentrations. Because of their tracheal origin, it is likely that NPTr
432 cells produce trypsin-like proteases that are excreted in the culture medium.
433 However, in order to facilitate comparisons with other studies, we decided to use

434 MDCK cells in the presence of 2 µg/ml of trypsin-TPCK for all the subsequent
435 titrations, as this is considered as a reference method for human viruses.

436 Multiplication kinetics were thus performed on MDCK and NPTr cells, in
437 the absence or in the presence of 2 µg/ml of trypsin-TPCK. DF1 cells were judged
438 too sensitive to exogenous trypsin and were not included. Three incubation
439 temperatures were chosen with respect to the temperature of the primary infection
440 site in human, swine and avian hosts, respectively. Indeed, human and swine
441 influenza A viruses first replicate in the upper respiratory tract, at a temperature
442 close to 33 and 37°C, respectively. In contrast, low pathogenic avian influenza A
443 viruses preferentially replicate in the bird intestinal tract at a temperature close to
444 40°C. Exogenous trypsin is believed to cleave the HA proteins at the surface of
445 virus particles. This step is required for the virus to gain infectiousness, as
446 cleavage of the HA is necessary to allow its conformational change for the fusion
447 process (Skehel and Wiley, 2000). The dependence on exogenous trypsin was
448 expected on MDCK cells only and for all viruses. It was therefore surprising that
449 the swine viruses did not appear to be highly dependent on exogenous trypsin to
450 grow efficiently on both cell types. Indeed, considering that a one log₁₀ difference
451 in titres simply reflects the accuracy error of the titration method, most differences
452 observed in the absence or presence of trypsin-TPCK could be regarded as not
453 significant. At 37 and 40°C, the av-H1N1/05 virus appeared extremely sensitive
454 to the absence of trypsin-TPCK when infecting MDCK but not NPTr cells.
455 Interestingly, all avian viruses clearly showed a delay in titre raise in the absence
456 of trypsin-TPCK on NPTr cells. It is possible that the proteases secreted by NPTr

457 cells are not fully efficient on the HA of avian viruses. More interestingly, we
458 observed that av-H3N2/00 required trypsin-TPCK only at 40°C on MDCK cells.
459 A clear dependence on trypsin was also noted for sw-H1N1/82 on MDCK cells,
460 also only at 40°C. However, it is not clear at present why or how exogenous
461 trypsin would be required only at 40°C for these viruses. Globally, for swine
462 viruses, in the presence of trypsin-TPCK, there was little difference between the
463 virus growth kinetics on MDCK or NPTr cells. The only two definite differences
464 were for sw-H1N1/82 (that will be discussed below) and for sw-H1N2/94. The
465 latter was the only swine virus to show a clear growth reduction on NPTr cells at
466 all temperatures. This would suggest that this virus might be less adapted to the
467 swine host. Noticeably, av-H1N2/02, with H1 and N2 sequences closely related to
468 the H1 and N2 of swine and human viruses, did not display a similar profile
469 suggesting that other genes could be involved.

470 Regarding the influence of temperature in the presence of trypsin-TPCK,
471 the results showed a clear sensitivity of viruses isolated from birds to a low
472 temperature of incubation, with a delay in growth observed at 33°C when
473 compared to 37 and 40°C. This was consistent with the fact that low-pathogenic
474 avian viruses replicate in the intestinal tract of infected birds, at a temperature
475 around 40°C. No significant growth reduction at 33°C was observed for swine
476 viruses. For swine influenza A viruses, to our knowledge, this is the first time that
477 temperature sensitivity has been studied and reported. Previous studies have
478 shown that viral replication and polymerase activity of avian influenza A viruses
479 are reduced at low temperature in several mammalian cells including MDCK cells

480 (Labadie et al., 2007; Massin et al., 2001). More specifically, the PB2 residue 627
481 (lysine for human (627K) and glutamic acid for most avian influenza A
482 viruses(627E)) was described to be involved in this cold-sensitivity of the avian
483 virus polymerase complex at 33°C in mammalian cells (Labadie et al., 2007;
484 Massin et al., 2001). Another study also showed that a lysine at position 627 of
485 PB2 confers an advantage for efficient growth of avian highly-pathogenic H5N1
486 viruses in the upper and lower respiratory tracts of mammals (Hatta et al., 2007).
487 In the present study, all avian, except av-H3N2/00, and swine viruses were
488 characterised by a 627E at this position. Interestingly, a 627K was found for av-
489 H3N2/00. Although the nature of this residue could account for the cold-
490 sensitivity observed in the growth kinetics of the avian H1N1 and H1N2 viruses,
491 it cannot explain the apparent lack of cold-sensitivity of the swine viruses.
492 Moreover, it cannot account for the definite lower growth capabilities at 33°C
493 noticed for av-H3N2/00 on MDCK cells. In fact, av-H3N2/00 was found to be
494 more sensitive to a low temperature than human H3N2 viruses (data not shown),
495 despite their close genetic relationship. This suggests that other determinants
496 besides PB2 residue 627 are involved.

497 A striking observation was the total lack of growth of sw-H1N1/82 on
498 NPTr cells at 40°C, which was not observed on MDCK cells nor for the closely
499 related av-H1N1/05 virus. This prompted us to analyse the replication of these
500 two viruses on NPTr cells using real-time PCRs targeting the PB2, NP and
501 genomic vRNA segments. Very similar replication kinetics were obtained for the
502 three genes tested, but differences might exist at earlier times of infection. We

503 showed a good correlation with the growth kinetics determined by virus titres in
504 the supernatant even if a light shift in time was observed, consistent with the fact
505 that replication must take place before new viral particles can be produced. The
506 delay in replication at 33°C confirmed the results of growth kinetics and was
507 perfectly consistent with the previously published data regarding the role of
508 residue 627E for avian viruses. Since av-H1N1/05 and sw-H1N1/82 are both
509 related to the avian-like swine lineage, it seems likely that similar mechanisms
510 involving residue 627E may prevail to explain their sensitivity at 33°C. However,
511 this sensitivity of replication at 33°C seemed more pronounced for sw-H1N1/82,
512 suggesting that additional determinants might be involved. In addition, the
513 absence of replication at 40°C was observed only for the swine virus and it seems
514 unlikely that residue 627E of PB2 could account for the sensitivity observed for
515 sw-H1N1/82 but not for other swine viruses. Thus, as clearly shown for H1N1
516 viruses, it seems that the temperature sensitivity of avian and swine viruses can be
517 an intrinsic property of the virus and that, even if two viruses belong to the same
518 genetic lineage, they might exhibit some features specific for the host they were
519 isolated from.

520 Several studies have recently been published focusing on influenza A virus
521 sensitivity to the incubation temperature and on host range determinants, all of
522 them trying to better understand the mechanisms behind cross-species infection
523 and host adaptation. Thus, using only human viruses as model, Steel et al (2009)
524 showed that residue 627E of PB2 could be compensated by mutating residue 701
525 from aspartic acid (D) to asparagine (N), resulting in a wild-type temperature

526 sensitivity phenotype and restoring transmission to mammals (Steel et al., 2009).
527 This could not explain the slightly better growth capabilities observed at 33°C for
528 av-H1N1/02 and av-H1N2/05 when compared to av-H1N1/05, as av-H1N2/05
529 and av-H1N1/05 were both characterised by the presence of a 701N residue in
530 PB2, and it would not explain why av-H3N2/00 showed temperature sensitivity at
531 33°C on MDCK cells. Interestingly, Scull et al (2009) showed that PB2 residue
532 627 is not always sufficient to confer sensitivity to low temperature (Scull et al.,
533 2009). Using human viruses as genetic background and HA and NA from an
534 Italian H7N1 influenza virus isolated from chicken, they demonstrated that the
535 glycoproteins could also be involved in the cold-sensitivity at 32°C. Although av-
536 H3N2/00 contains HA and NA that were found closely related to human virus
537 counterparts, it would be interesting to test this hypothesis to find an explanation
538 to the sensitivity at 33°C observed for this virus on MDCK cells.

539 Other hypotheses could be tested to explain some of the temperature
540 sensitivities we described. For example, the instability of the polymerase
541 complex, that would prevent replication from occurring, was proposed to be
542 responsible for a temperature sensitivity observed at 41°C for three mammalian
543 influenza viruses (Dalton et al., 2006). Two recent studies also highlighted a
544 putative role for NP in temperature sensitivity and as a determinant of host range.
545 Thus, residue 239 of NP was shown to be involved in the sensitivity at 39°C of
546 the vRNA packaging, possibly by affecting the interaction between NP and M1
547 (Noton et al., 2009). Finally, Gabriel et al (2008) described two point mutations
548 (701 in PB2 and 319 in NP) involved in the adaptation of an avian influenza strain

549 to mouse, demonstrating that these two residues provides more efficient binding
550 of the cellular importin α 1 protein in mammalian cells, resulting in an increased
551 transport of the virus genome into the nucleus (Gabriel et al., 2008). All these
552 hypotheses would be interesting to test using reverse genetics systems based on
553 each virus background.

554 In conclusion, data reported in this study suggested that a virus closely
555 related to a given genetic lineage defined by the host origin can possess specific
556 phenotypic growth characteristics when isolated from another host. It seems that
557 minor changes in nucleotide or amino acid sequences could be sufficient to allow
558 the adaptation of a strain from one host lineage to another host. Temperature
559 sensitivity might represent some specific signature of a virus, depending on the
560 host from which it was isolated, and could be due to residues other than residue
561 627 of PB2 and could affect other steps of the virus life cycle besides RNA
562 replication. Our results confirmed that temperature sensitivity of influenza viruses
563 is variable for each virus and might depend on the cell type supporting the
564 replication. More studies on various strains, including human influenza viruses,
565 would be necessary to better understand the variability in growth and replication
566 capabilities, and to possibly define in vitro conditions that could be used to predict
567 the risk for a virus to cross the species barrier and efficiently infect a new host.

568

569

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- 742
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744 **Figures**

745

746 **FIG 01:** Comparison of virus titres for influenza A viruses isolated from birds and
747 pigs, on MDCK and NPTr cells in the presence of 2 or 0.2 µg/ml of trypsin-
748 TPCK. MDCK and NPTr cells were infected with the indicated viruses in the
749 presence of 2 µg/ml (black bars) or 0.2 µg/ml (grey bars) of trypsin-TPCK as
750 described in Materials and Methods. At 72 h p.i., viral titres (TCID₅₀/ml) were
751 calculated by the Reed and Muench method. Results are expressed as the mean
752 log₁₀ per millilitre ± SD of two independent experiments. ND: not done.

753

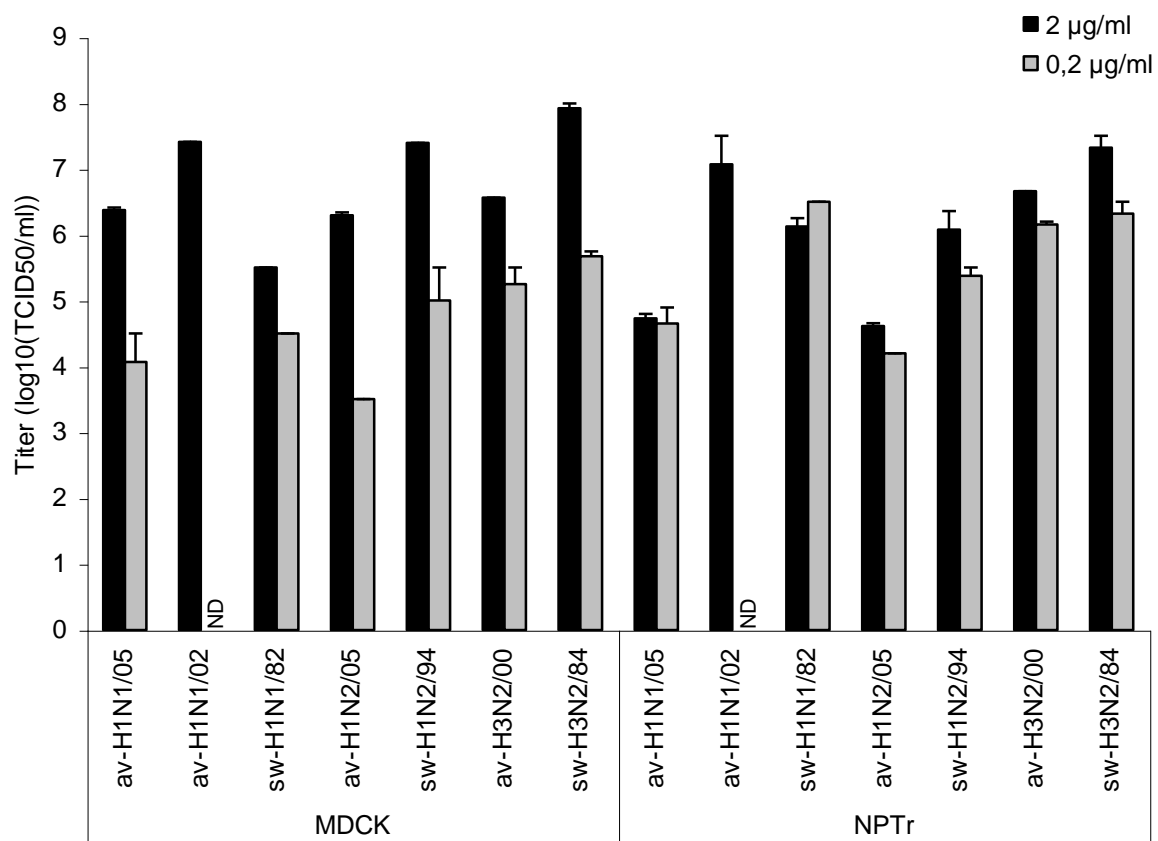
754 **FIG 02:** Kinetics of multiplication of influenza A viruses isolated from birds (A)
755 and pigs (B). MDCK and NPTr cells were infected at a MOI of 10⁻³ TCID₅₀/cell
756 with the indicated avian or swine viruses or mock infected. Infected cells were
757 incubated at 33, 37, or 40°C. Each growth curve was drawn using the virus titres
758 determined at 0, 12, 24, and 48 h post-infection, in the supernatant of infected
759 cells in the presence (bold lines) or absence (thin lines) of trypsin-TPCK. The
760 results are from one experiment representative of two independent experiments.

761

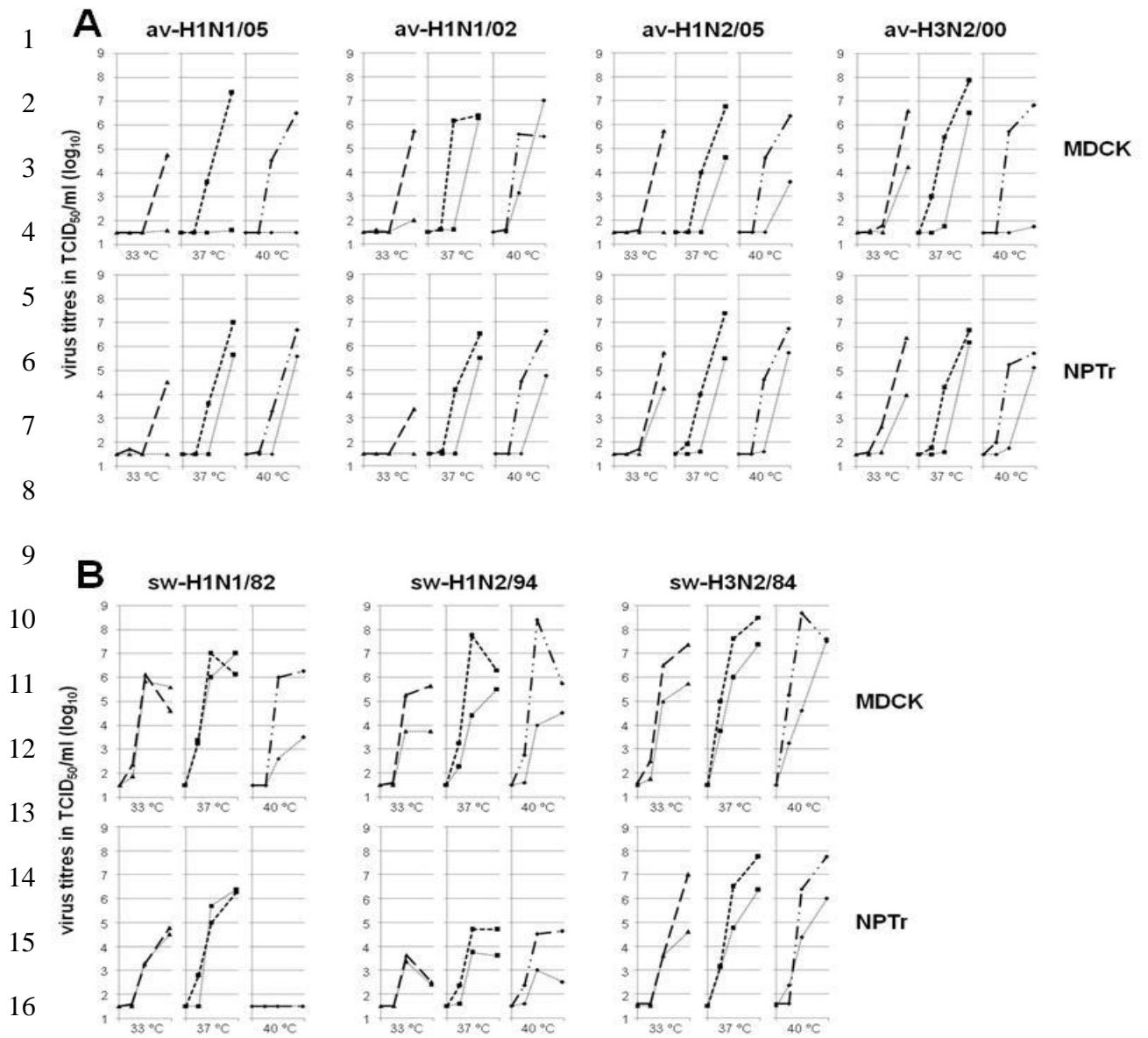
762 **FIG 03:** Kinetics of RNA replication for H1N1 influenza A viruses isolated from
763 birds and pigs, at 33, 37 and 40°C. NPTr cells were infected with (A) av-H1N1/05
764 or (B) sw-H1N1/82 viruses at a MOI of 0.001 TCID₅₀/cell or mock infected.
765 Infected cells were incubated at 40°C (solid symbols, solid lines), 37°C (solid
766 symbols, long dashed lines), or 33°C (open symbols, short dashed lines). At 0, 12,

767 24, and 48 h post-infection, the production of PB2-, NP- and M-vRNA in infected
768 cells was evaluated by quantitative real-time PCR. For each virus and each gene,
769 copy numbers were expressed as a percentage of the copy number obtained at
770 48 h post-infection, at 37°C (considered as 100%, values provided). The results
771 are from one experiment representative of two independent experiments.

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13 FIG 01



17 FIG 02

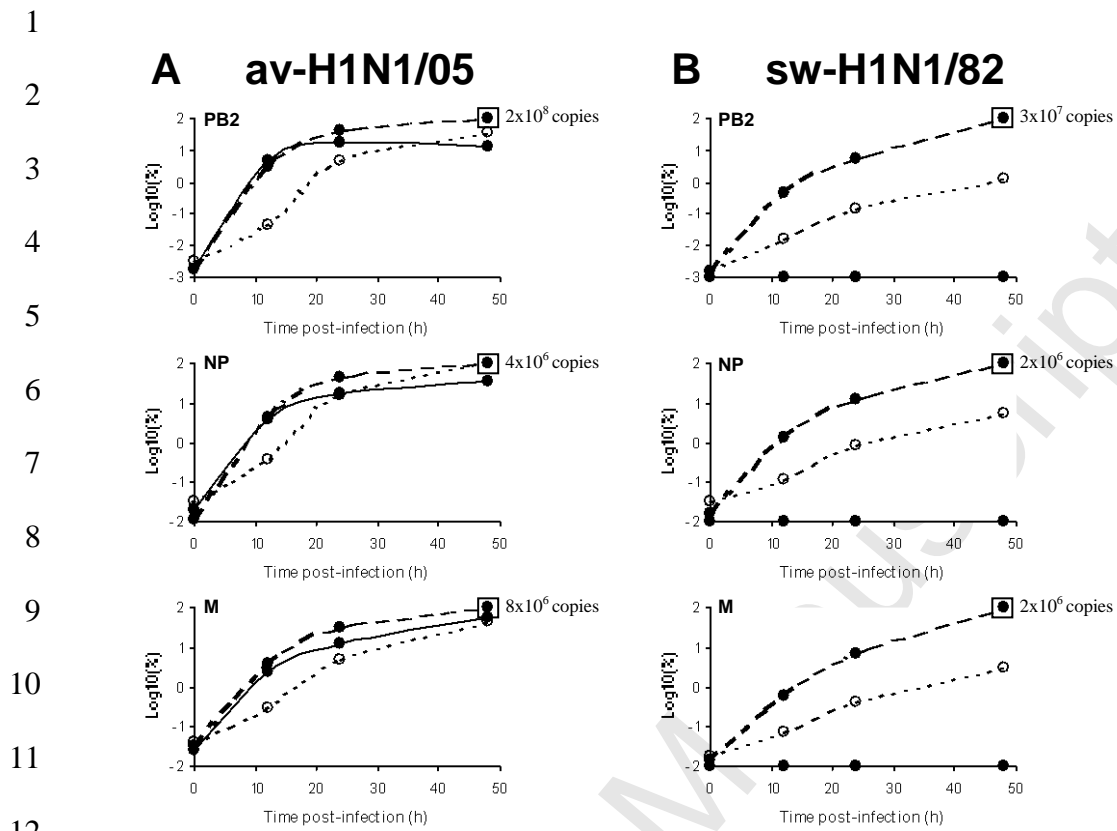


FIG 03

1 TABLE 1. Genetic characteristics of avian and swine influenza A viruses used

Virus strain	Lineage ^a	HA ^b	NA ^b	PB1 ^b	PB2 ^b	PB2-627 ^c	PA ^b	NP ^b	M ^b	NS ^b
Isolated from pigs										
A/swine/Finistère/2899/82 (H1N1)	avian-like swine	Sw ^{av}	Sw ^{av}	Sw ^{av}	Sw ^{av}	E	Sw ^{av}	Sw ^{av}	Sw ^{av}	Sw ^{av}
A/swine/Scotland/410440/94 (H1N2)	human-like reassortant swine	Sw ^{hu}	Sw ^{hu}	Sw ^{av}	Sw ^{av}	E	Sw ^{av}	Sw ^{av}	Sw ^{av}	Sw ^{av}
A/swine/Gent/1/84 (H3N2)	human-like reassortant swine	Sw ^{hu}	Sw ^{hu}	Sw ^{av}	Sw ^{av}	E	Sw ^{av}	Sw ^{av}	Sw ^{av}	Sw ^{av}
Isolated from birds										
A/turkey/France/05016/05 (H1N1)	-	Sw ^{av}	Sw ^{av}	Sw ^{av}	Sw ^{av}	E	Sw ^{av}	Sw ^{av}	Sw ^{av}	Sw ^{av}
A/mallard/France/710/02 (H1N1)	-	Av	Av	Av	Av	E	Av	Av	Av	Av
A/turkey/France/05045c/05 (H1N2)	-	Sw ^{hu}	Sw ^{hu}	Sw ^{av}	Sw ^{av}	E	Sw ^{av}	Sw ^{av}	Sw ^{av}	Sw ^{av}
A/duck/Ontario/05/00 (H3N2)	-	Hu	Hu	Hu	Hu	K	Hu	Hu	Hu	Hu

2 ^a For a review, see (Kuntz-Simon and Madec, 2009).

3 ^b Phylogenetic results were abbreviated as follows: Sw^{av}, genes closely related to
 4 avian-like swine influenza viruses; Sw^{hu}, genes closely related to human-like
 5 reassortant swine influenza viruses; Av, genes closely related to avian influenza
 6 viruses; Hu, genes closely related to human influenza viruses.

7 ^c E = glutamic acid; K = lysine