Veterinary Microbiology 142 (2010) 232–241

Contents lists available at ScienceDirect

Veterinary Microbiology

journal homepage: www.elsevier.com/locate/vetmic

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

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ARTICLE INFO

Article history:
Received 3 April 2009
Received in revised form 14 October 2009
Accepted 16 October 2009

Keywords:
Avian influenza virus
Swine influenza virus
Temperature sensitivity
NPTr cells
Real-time quantitative PCR
PB2 627

ABSTRACT

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

1. Introduction

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

In humans and pigs, influenza A viruses first replicate in the upper respiratory tract at a temperature close to 33 and 37 °C, respectively, inducing a respiratory syndrome (Alford et al., 1966). In aquatic birds, low pathogenic influenza A viruses preferentially replicate in the intestinal tract at a temperature close to 40 °C, and the infection is usually asymptomatic (Webster et al., 1978). In domestic poultry, some subtypes of influenza A viruses can cause devastating epizootics leading to economic losses and sanitarian risks for humans and other domestic animals. The HA is involved in host range and adaptation of influenza A viruses through its binding specificity to the

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doi:10.1016/j.vetmic.2009.10.012

receptor, i.e. sialic acids (SA). Indeed, the HA of human and avian influenza A viruses preferentially binds to SA α2,6- and SA α2,3-galactose, respectively (Connor et al., 1994; Suzuki et al., 2000), which correlates well with their distribution at the site of virus multiplication: SA α2,6 on epithelial cells of the human upper respiratory tract and SA α2,3 on epithelial cells of the avian intestinal tract (Couceiro et al., 1993; Ito et al., 2000). In the tracheal epithelium of pigs, both types of SA are present, which could explain the susceptibility of pigs to both human and avian influenza A viruses (Scholtissek et al., 1998). Consequently, pigs are considered as a "mixing vessel" host, where reassortment among human, avian and swine influenza A viruses could lead to new influenza virus lineages with zoonotic potential (Kuntz-Simon and Madec, 2009; Webster et al., 1992). In addition to their possible transmission to humans, swine influenza A viruses can also be transmitted to poultry (Suarez et al., 2002) and wild ducks (Olsen et al., 2003).

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

Although the HA is a major determinant for host restriction it is not necessarily sufficient. Indeed, some influenza A viruses of entire swine or avian origin, thus not reassortants, have been directly transmitted to humans (Banks et al., 1998; Claas et al., 1994; Kendal et al., 1977). These viruses had specific characteristics that allowed them to replicate in humans, but were not able to transmit from human to human and then spread in the human population. Other determinants of host range or adaptation to a novel host were identified in the virus internal proteins, especially in the polymerase complex subunits PB1, PB2 and PA. For the 1957 and 1968 pandemics, it was demonstrated that the emerging viruses were reassortants between human and avian influenza viruses (Yasuda et al., 1991). In both cases, reassortant viruses acquired not only NA and/or HA genes but also the viral polymerase subunit PB1 gene from an avian influenza virus, whereas other genes were acquired from a human influenza A virus (Kawaoka et al., 1989; Scholtissek and Murphy, 1978). Experiments on the compatibility of polymerase subunits showed that an avian origin of the PB1 gene may have conferred a selective advantage to the 1957 and 1968 reassortant viruses (Naffakh et al., 2000). In swine, reassortant viruses indicated that a PB1 gene of human origin allows the virus to be infectious in pigs, whatever the swine or avian origin of the two other subunits of the viral polymerase complex (Karasin et al., 2006).

Reassortment experiments further showed that genomic segments NP, M, NS and PB2 also seem to be involved in host restriction (Clements et al., 1992; Geiss et al., 2002; Snyder et al., 1987; Tian et al., 1985). A well-characterised host-range determinant is the residue 627 of PB2, a glutamic acid found in almost all avian influenza A viruses versus a lysine in human influenza A viruses (Smith et al., 2006; Subbarao et al., 1993). The nature of residue 627 of PB2 mostly determines the cold sensitivity at 33 °C of the replication of avian viruses in mammalian cells (Labadie et al., 2007; Massin et al., 2001) and has been involved in the level of pathogenicity of the virus (Hatta et al., 2001; Munster et al., 2007) and in its ability to replicate in the upper respiratory tract (Hatta et al., 2007). Altogether, published data suggest that host restriction is a complex mechanism, driven by specific gene constellations depending on the origin of the virus.

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

2. Materials and methods

2.1. Cells and viruses

Newborn Pig Trachea (NPTr, Ferrari et al., 2003) and Madin-Darby Canine Kidney (MDCK) cells were grown at 37 °C with 5% CO_2 in MEM supplemented with antibiotics and foetal calf serum (FCS, 10% and 5%, respectively). For MDCK cells, MEM was supplemented with 1% tricine. Chicken fibroblast DF1 cells were grown at 39 °C with 5% CO_2, in DMEM supplemented with 10% FCS and antibiotics.

Regarding the viruses used, A/turkey/France/05016/05 (H1N1) (av-H1N1/05), A/mallard/France/710/02 (H1N1) (av-H1N1/02) and A/turkey/France/05045c/05 (H1N2) (av-H1N2/05) were isolated by the National Reference Laboratory for Avian Influenza and Newcastle disease at the AFSSA-LERAPP, Ploufragan, France. A/duck/Ontario/05/00 (H3N2) (av-H3N2/00) was kindly provided by J. Pasick, Canadian Food Inspection Agency, National Centre for Foreign Animal Disease, Winnipeg, Manitoba, Canada (OIE Reference Laboratory for Avian Influenza). A/swine/Finistère/2899/82 (H1N1) (sw-H1N1/82), A/swine/Scotland/410440/94 (H1N2) (sw-H1N2/94) and A/swine/Gent/1/84 (H3N2) (sw-H3N2/84) were kindly provided by K. van Reeth, Ghent University, Belgium. Influenza viruses were amplified at 36–37 °C in 9-day-old SPF embryonated

chicken eggs. Allantoïc fluids were collected at a maximum of 4 days after inoculation and then subjected to centrifugal clarification before use as viral stocks.

2.2. Phylogenetic analyses

For avian influenza A viruses, viral RNA was extracted from infectious allantoïc fluid according to a standardised procedure (Alexander, 2005). Viral RNA was reverse transcribed using hexanucleotides as primers. cDNA was subjected to PCR using segment-specific primers (primer sequences available upon request). All eight PCR products were purified from agarose gel by using the Qiaquick gel extraction kit (Qiagen). The DNA sequences were determined by sequencing both DNA strands by the dye terminator method (ABI Prism DyeTerminator Cycle Sequencing Ready Reaction Kit, Applied Biosystems) on an automatic DNA sequencer ABI 373XL (Applied Biosystems). Sequences are available under the following accession numbers: FN395357–FN395364 for av-H1N1/05, AJ697878, AM157388, and FJ985246–FJ985251 for av-H1N1/02, FJ985238–FJ985245 for H1N2-05045c, and AJ697864, AJ697881, and GQ249055–GQ249060 for av-H3N2/00.

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

2.3. Virus stock titration by $TCID_{50}$ determination

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

2.4. Virus growth kinetics in cell culture

NPTr and MDCK cells were seeded in six-well plates at a density of 1×10^6 cells/well in medium supplemented with FCS (10% and 5%, respectively). 24 h later, cells were washed twice with MEM without serum and then infected at a multiplicity of infection (MOI) of 10^{-3} $TCID_{50}$/cell with either avian or swine influenza viruses, or mock infected. After adsorption for 1 h at room temperature, the inoculum was removed. The cells were washed once with MEM without serum and then incubated at 33, 37 or 40 °C with MEM without serum but supplemented, or not, with 2 μg/ml of trypsin-TPCK. At 0, 12, 24 and 48 h post-infection, virus titres were determined in the supernatants, on MDCK cells only, as described in Section 2.3.

2.5. Virus replication kinetics in cell culture

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

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

[a] For a review, see Kuntz-Simon and Madec (2009).
[b] Phylogenetic results were abbreviated as follows: Sw^{av}, genes closely related to avian-like swine influenza viruses; Sw^{hu}, genes closely related to human-like reassortant swine influenza viruses; Av, genes closely related to avian influenza viruses; Hu, genes closely related to human influenza viruses.
[c] E = glutamic acid; K = lysine.

2.6. SYBR Green real-time PCR

Two-steps real-time PCRs specific for NP-, M-, or PB2-vRNA were developed. First, total RNA (60 ng) was denatured and annealed at 65 °C for 5 min with 10 pmol of Uni12 primer (Hoffmann et al., 2001) and 1 μl of dNTP (10 mM each). RNA was reverse transcribed at 42 °C for 70 min in a total volume of 30 μl, using Superscript II reverse transcriptase (Invitrogen) according to the manufacturer's protocol. Real-time PCR reactions were next performed in a final volume of 25 μl with 12.5 μl of SYBR Green PCR Master Mix (Applied Biosystems), 10 μl of cDNA (diluted 1:10) and 200 nM of each forward and reverse primer (except for M PCR: 400 nM) (sequences available upon request). Amplifications were performed using the following conditions: 2 min at 50 °C, 10 min at 95 °C, followed by 45 amplification cycles (15 s at 95 °C and 1 min at 60 °C for NP and M PCRs or 15 s at 55 °C and 1 min at 60 °C for PB2 PCR). The program ended with a dissociation stage. Amplifications were carried out in 96-well plates with three replicates per sample, using Applied Biosystems 7000 and 7500 Real-Time PCR Systems and analysed with the Sequence Detection Software. For optimisation and standard curves, full-length PCR products of each viral segment were used. RNA was extracted from allantoïc fluids infected with either avian or swine influenza viruses, using the RNeasy kit (Qiagen). cDNA corresponding to each virus was synthesized using the Uni12 primer and the Superscript II (Invitrogen) as described above. For each cDNA, specific amplification of NP-, M-, and PB2-genes were performed using primers derived from Hoffmann et al. (2001) and the Taq Platinum High Fidelity polymerase (Invitrogen), according to the manufacturer's protocol. PCR products of the expected size were purified from an agarose gel using the Qiaquick gel extraction kit (Qiagen). Purified products were then quantified with the Quant-iT DNAds kit and Qubit fluorometer (Invitrogen) to determine the copy number. Serial 10-fold dilutions of purified PCR products were used to establish standard curves. For all subsequent analyses, one full-length PCR product was chosen as a standard for each PB2-, NP- and M-specific real-time PCRs: av-H1N1-PB2 and sw-H3N2-NP and -M. Ten independent serial dilutions of each standard were analysed to determine limits of detection and quantification as described in a French reference norm (XPT 90-471 from April 2006).

2.7. Statistical analysis

For virus titration experiments, analyses of variance were performed to test the effect of trypsin concentration and cell type on the virus titres, assessed by the $\log_{10}$ of titres ($TCID_{50}$/ml). Analyses of variances were computed with the GLM procedure of the SAS software (SAS, 2004). In addition, the Newman and Keuls multiple comparison procedure was used to compare the different trypsin concentrations, with respect to the different cell types.

For virus replication kinetics, statistical analyses were performed to test the effect of the virus (avian or swine H1N1 influenza virus), of the temperature (33, 37 or 40 °C) and of their potential interaction on the kinetics of replication of three different viral genes (PB2, NP and M), assessed by the $\log_{10}$ of the copy number (three replicates). Linear mixed models (Littell et al., 1998) including repeated effects were used. The virus, the temperature and the virus by temperature interaction were introduced in the model as fixed effects in accordance with the design of the study. The time post-infection (0, 12, 24 and 48 h) was incorporated in the model as a repeated measurement factor in order to take into account the within-time covariability, specifying a first-order autoregressive structure for the covariance matrix. In the case of a significant virus by temperature interaction, an alternative to multiple comparison procedures, that are not available with linear mixed models, contrasts procedures were used to compare the different levels of the temperature, for each virus. Linear mixed models were computed with the MIXED procedure of the SAS software (SAS, 2004).

3. Results

3.1. Phylogenetic analyses

Phylogenetic analyses of genomic segments of each virus were performed as described in Section 2 and are summarised in Table 1. Swine influenza viruses used in this study belonged to well-known swine lineages (for a review see Kuntz-Simon and Madec, 2009). Briefly, the sw-H1N1/82 belonged to the avian-like swine lineage. Its eight genomic segments were of avian origin. The sw-H1N2/94 and sw-H3N2/84 belonged to human-like reassortant swine lineages: they possessed internal genes from the avian-like swine H1N1 lineage and HA and NA from human lineages. The av-H1N1/05 and av-H1N2/05 were found closely related to sw-H1N1/82 (avian-like swine lineage) and to sw-H1N2/94 (human-like reassortant swine lineage), respectively. The av-H1N1/02 possessed eight genomic segments of avian origin. All genes of the av-H3N2/00 were closely related to human influenza viruses isolated in the 1970s.

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

In order to perform virus titrations on MDCK, NPTr and DF1 cells, we first tested the ability of monolayers of each cell type to resist in a medium containing trypsin (data not shown). Monolayers of MDCK and NPTr cells remained viable in a medium containing up to 2 μg/ml of trypsin. In contrast, monolayers of DF1 cells were disrupted in such a medium. At 0.2 μg/ml of trypsin, DF1 cell monolayers did not reproducibly resist. Thus, virus stock titrations could only be performed on MDCK and NPTr cells.

The titres of H1N1, H1N2 and H3N2 influenza viruses isolated from birds or pigs (further referred to as avian (or av-) and swine (or sw-), respectively) were determined on MDCK and NPTr cells in the presence of 2 or 0.2 μg/ml trypsin, as described in Section 2. Calculated titres are presented in Fig. 1.

On MDCK cells (left panel), titres were about 2 $\log_{10}$ higher with 2 μg/ml of trypsin than with 0.2 μg/ml of trypsin, whereas on NPTr cells in the same conditions,

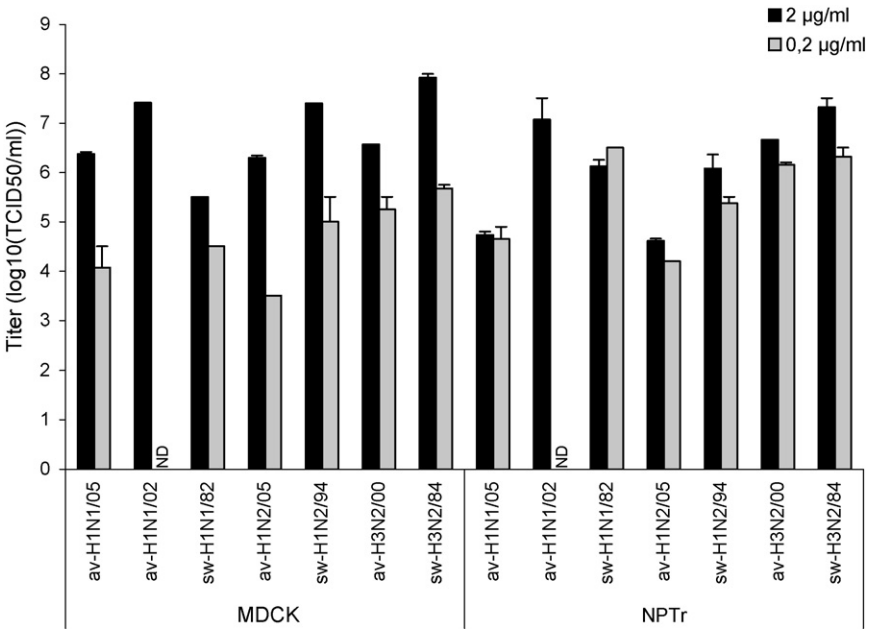

Fig. 1. Comparison of virus titres for influenza A viruses isolated from birds and pigs, on MDCK and NPTr cells in the presence of 2 or 0.2 μg/ml of trypsin-TPCK. MDCK and NPTr cells were infected with the indicated viruses in the presence of 2 μg/ml (black bars) or 0.2 μg/ml (grey bars) of trypsin-TPCK as described in Section 2. At 72 h p.i., viral titres ($TCID_{50}$/ml) were calculated by the Reed and Muench method. Results are expressed as the mean $\log_{10}$ per millilitre ± SD of two independent experiments. ND: not done.

differences in titres were only about 0.4 $\log_{10}$. In the presence of 2 μg/ml of trypsin, titres for all viruses were slightly but significantly ($p < 0.0001$) higher on MDCK cells (black bars, left panel) than on NPTr cells (black bars, right panel). In contrast, with 0.2 μg/ml of trypsin, titres were globally slightly but significantly ($p < 0.0001$) higher on NPTr cells (grey bars, right panel) than on MDCK cells (grey bars, left panel). Taken as a whole, titres were significantly ($p < 0.0001$) higher with 2 μg/ml of trypsin (black bars) than with 0.2 μg/ml (grey bars). These results indicated that multiplication of av- or sw-H1N1, H1N2 and H3N2 influenza viruses was less dependent on the trypsin-TPCK concentration on NPTr cells than on MDCK cells.

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

As described in Section 2, the growth capabilities of av- and sw-H1N1, H1N2 and H3N2 viruses were compared on MDCK and NPTr cells infected at a low multiplicity of infection (MOI = 10^{-3} $TCID_{50}$/cell) and then incubated at 33, 37 or 40 °C. At different times post-infection, the production of infectious viral particles in the supernatants was evaluated by titration. The kinetics of virus multiplication are presented in Fig. 2.

For viruses isolated from birds, in the presence of trypsin-TPCK (Fig. 2A, bold lines), the kinetics of multiplication gave very similar profiles whatever the host cell type or the virus used, with a delay in the titre raise at 33 °C as compared to 37 and 40 °C. For each temperature considered, av-H1N1/05, av-H1N2/05 and av-H3N2/00 viruses reached similar titres on MDCK and NPTr cells at 48 h post-infection (p.i.). Similar titres were also reached at 48 h p.i. on MDCK and NPTr cells for av-H1N1/02 virus, but only at 37 and 40 °C. At 33 °C, for av-H1N1/02 virus the titre at 48 h p.i. was nearly 3 $\log_{10}$ lower on NPTr cells than on MDCK cells.

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

For the viruses isolated from pigs, in the presence of trypsin-TPCK (Fig. 2B, bold lines), there was no general delay at 33 °C as compared to 37 °C on either MDCK or NPTr cells. However, for sw-H1N1/82 on NPTr cells, the titre reached at 48 h p.i. was more than 1 $\log_{10}$ lower at 33 °C than at 37 °C. This difference at 33 °C was not as clear on MDCK cells. Similarly, for sw-H1N2/94, the maximum titre was reduced by 1–2 $\log_{10}$ at 33 °C as compared to 37 °C on both cell types. Moreover, at 40 °C, virus growth was undetectable for sw-H1N1/82 on NPTr cells and significantly reduced on NPTr as compared to MDCK cells for sw-H1N2/94. As for sw-H3N2/84, whatever the temperature, it grew with similar capabilities on both MDCK and NPTr cells.

In the absence of trypsin-TPCK (Fig. 2B, thin, lines) the swine viruses behaved differently from the avian viruses, since essentially no delay but only a slight reduction (1–2 $\log_{10}$) in the final titre reached at 48 h p.i. was observed. The only noticeable difference was observed for sw-H1N1/82 on MDCK cells at 40 °C, for

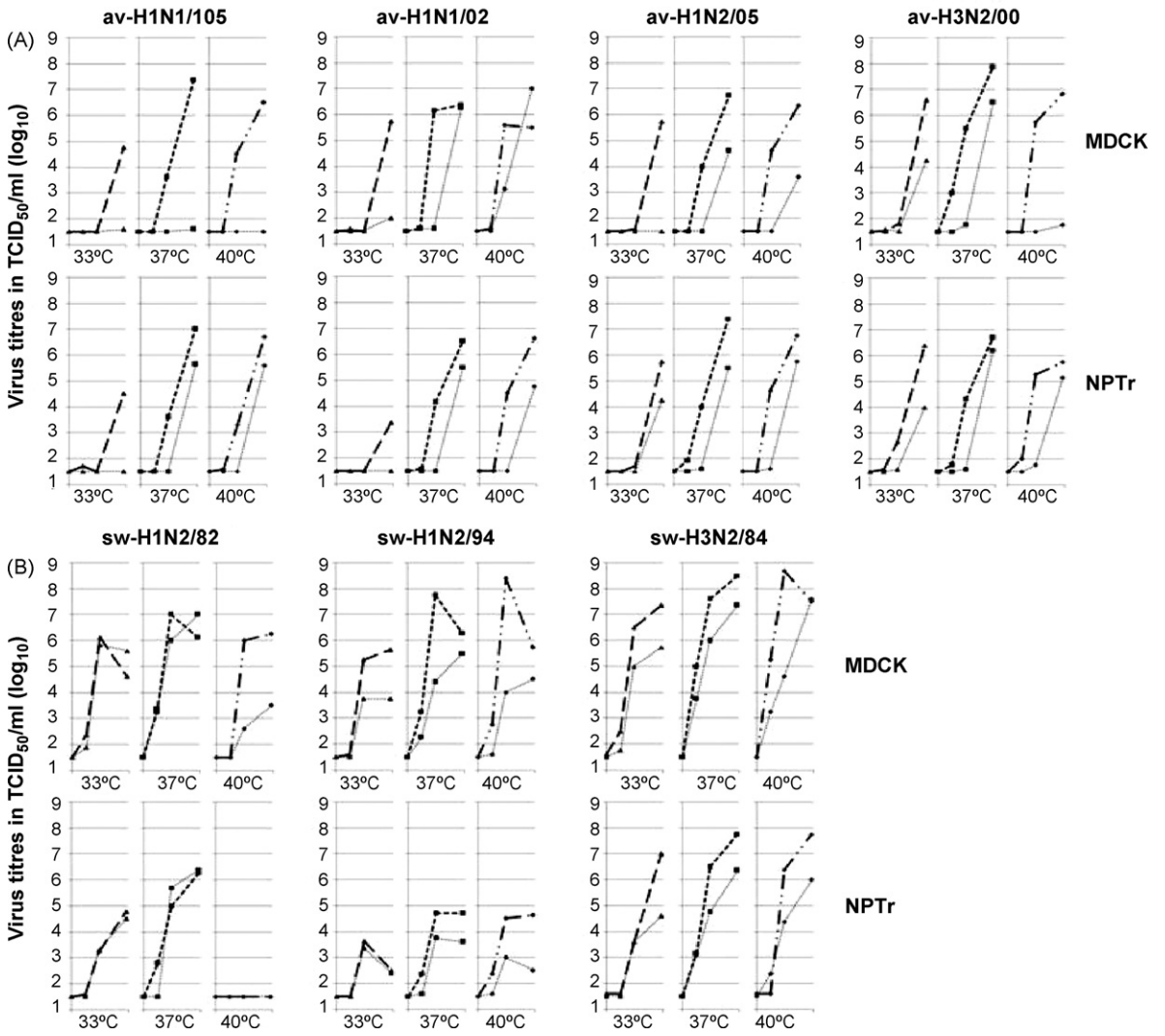

Fig. 2. Kinetics of multiplication of influenza A viruses isolated from birds (A) and pigs (B). MDCK and NPTr cells were infected at a MOI of 10^{-3} $TCID_{50}$/cell with the indicated avian or swine viruses or mock infected. Infected cells were incubated at 33, 37, or 40 °C. Each growth curve was drawn using the virus titres determined at 0, 12, 24, and 48 h post-infection, in the supernatant of infected cells in the presence (bold lines) or absence (thin lines) of trypsin-TPCK. The results are from one experiment representative of two independent experiments.

which the titre obtained was nearly 3 $\log_{10}$ lower in the absence of exogenous trypsin.

3.4. Comparison of virus replication kinetics at different temperatures on NPTr cells in the presence of 2 μg/ml of trypsin

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

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

NPTr cells were infected with either av-H1N1/05 or sw-H1N1/82 at a low multiplicity of infection (MOI = 10^{-3} $TCID_{50}$/cell) and were incubated at 33, 37 or 40 °C. As described in Section 2, total RNAs were extracted and analysed by the three newly developed SYBR green real-time PCRs. Results are presented in Fig. 3. For av-H1N1/05, the kinetics of replication of each vRNA were very similar. At 12 h p.i., a delay was observed at 33 °C as compared to

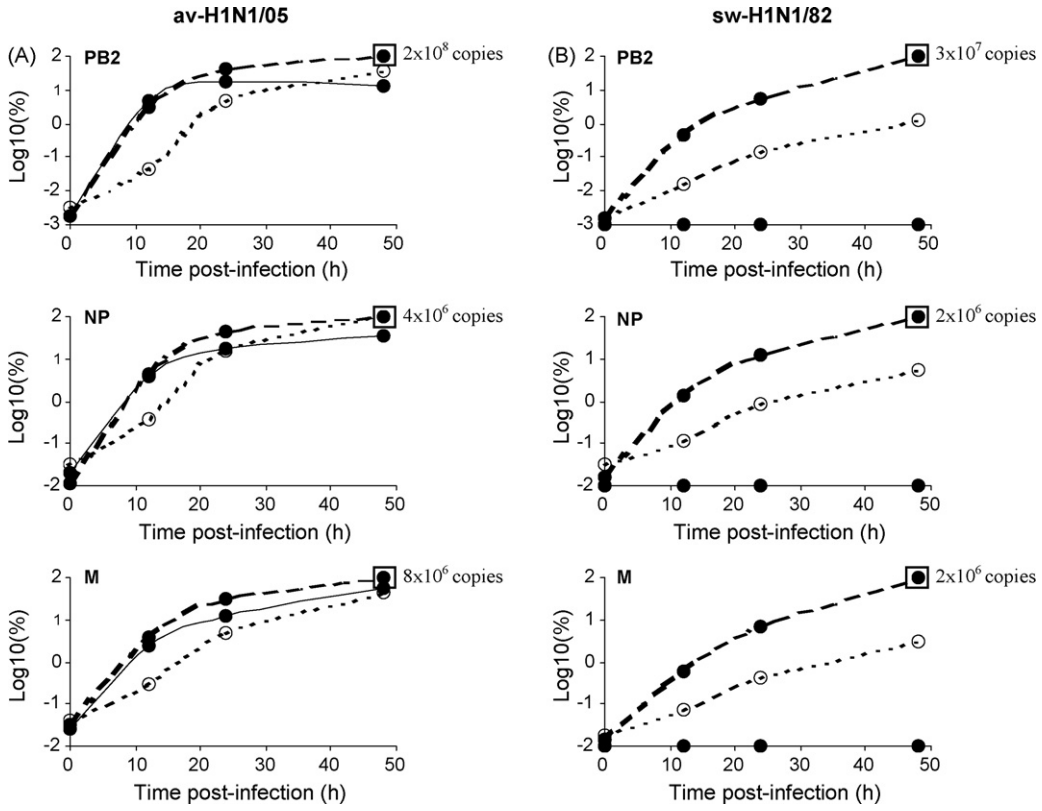

Fig. 3. Kinetics of RNA replication for H1N1 influenza A viruses isolated from birds and pigs, at 33, 37 and 40 °C. NPTr cells were infected with (A) av-H1N1/05 or (B) sw-H1N1/82 viruses at a MOI of 0.001 $TCID_{50}$/cell or mock infected. Infected cells were incubated at 40 °C (solid symbols, solid lines), 37 °C (solid symbols, long dashed lines), or 33 °C (open symbols, short dashed lines). At 0, 12, 24, and 48 h post-infection, the production of PB2-, NP- and M-vRNA in infected cells was evaluated by quantitative real-time PCR. For each virus and each gene, copy numbers were expressed as a percentage of the copy number obtained at 48 h post-infection, at 37 °C (considered as 100%, values provided). The results are from one experiment representative of two independent experiments.

37 and 40 °C. The difference between 33 and 37 °C was statistically confirmed for the PB2 gene ($p < 0.05$) and with a level of significance of 10% ($p = 0.068$) for the M gene, but not for the NP gene ($p > 0.05$). At 33 °C the maximum level of vRNA seemed to be reached at 48 h p.i. whereas it was already reached at 24 h p.i. at 37 and 40 °C. For sw-H1N1/82, the vRNA kinetics were also very similar for the three segments. A statistically significant delay in replication was observed at 33 °C as compared to 37 °C ($p < 0.05$ for the three genes), and at 48 h p.i. the copy number was nearly 2 $\log_{10}$ lower than at 37 °C. At 40 °C, we failed to detect replication of any of the three vRNAs ($p < 0.05$ for the three genes). Altogether, the real-time PCR results were very consistent with the virus growth results.

4. Discussion

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

Three cell lines were originally chosen to compare the avian and swine viruses. The MDCK cell line was derived from an adult female cocker spaniel in 1958 and was described to support replication of several influenza A viruses from human, swine, equine and avian origin, forming plaques with high efficiency by the addition of trypsin in the culture medium (Tobita et al., 1975). They are widely used for the isolation of influenza A viruses from respiratory specimens from human patients (Tobita et al., 1975) and for the multiplication of human and swine influenza A viruses. DF1 cells consist of an established chicken fibroblast cell line that has been used in recently published influenza studies (Labadie et al., 2007; Lee et al., 2008). The NPTr cell line was selected because of the tissue (pig trachea) from which it was established by serial culture, and the fact that it supports the multiplication of several influenza A viruses from human, swine and avian origin with similar infectious titres to those obtained in chicken embryos (Ferrari et al., 2003).

Unfortunately, the DF1 cells proved highly sensitive to the trypsin concentration in the culture medium and the cell monolayers inconsistently resisted a 0.2 μg/ml concentration of trypsin. In those conditions, titres obtained for avian virus stocks on DF1 cells were low (data not shown) and in the same range as those obtained on MDCK

cells, i.e. 2–3 $\log_{10}$ lower than titres obtained on MDCK cells with 2 μg/ml of trypsin. This seems to contrast with the results of Lee et al. (2008), who evaluated the use of DF1 or QT6 cells, another avian cell line, for the study of avian influenza A viruses, instead of primary avian cells (Chicken Embryo Fibroblast, CEF) and MDCK cells, and concluded that DF1 cells supported the growth of avian influenza A viruses as well as MDCK cells. However, the trypsin concentrations used were different for each cell line (0.75 μg/ml versus 0 μg/ml for MDCK cells and 0.05 μg/ml versus 0 μg/ml for DF1 cells), making comparison difficult. As expected, on MDCK cells, the titres were higher in the presence of 2 μg/ml of trypsin than with only 0.2 μg/ml. In accordance with the results of Ferrari et al. (2003), NPTr cells supported the multiplication of the avian and swine viruses used and appeared less dependent on the addition of exogenous trypsin in the culture medium, as similar virus titres were obtained for both trypsin concentrations. Because of their tracheal origin, it is likely that NPTr cells produce trypsin-like proteases that are excreted in the culture medium. However, in order to facilitate comparisons with other studies, we decided to use MDCK cells in the presence of 2 μg/ml of trypsin-TPCK for all the subsequent titrations, as this is considered as a reference method for human viruses.

Multiplication kinetics were thus performed on MDCK and NPTr cells, in the absence or in the presence of 2 μg/ml of trypsin-TPCK. DF1 cells were judged too sensitive to exogenous trypsin and were not included. Three incubation temperatures were chosen with respect to the temperature of the primary infection site in human, swine and avian hosts, respectively. Indeed, human and swine influenza A viruses first replicate in the upper respiratory tract, at a temperature close to 33 and 37 °C, respectively. In contrast, low pathogenic avian influenza A viruses preferentially replicate in the bird intestinal tract at a temperature close to 40 °C. Exogenous trypsin is believed to cleave the HA proteins at the surface of virus particles. This step is required for the virus to gain infectiousness, as cleavage of the HA is necessary to allow its conformational change for the fusion process (Skehel and Wiley, 2000). The dependence on exogenous trypsin was expected on MDCK cells only and for all viruses. It was therefore surprising that the swine viruses did not appear to be highly dependent on exogenous trypsin to grow efficiently on both cell types. Indeed, considering that a 1 $\log_{10}$ difference in titres simply reflects the accuracy error of the titration method, most differences observed in the absence or presence of trypsin-TPCK could be regarded as not significant. At 37 and 40 °C, the av-H1N1/05 virus appeared extremely sensitive to the absence of trypsin-TPCK when infecting MDCK but not NPTr cells. Interestingly, all avian viruses clearly showed a delay in titre raise in the absence of trypsin-TPCK on NPTr cells. It is possible that the proteases secreted by NPTr cells are not fully efficient on the HA of avian viruses. More interestingly, we observed that av-H3N2/00 required trypsin-TPCK only at 40 °C on MDCK cells. A clear dependence on trypsin was also noted for sw-H1N1/82 on MDCK cells, also only at 40 °C. However, it is not clear at present why or how exogenous trypsin would be required only at 40 °C for these viruses. Globally, for swine viruses, in the presence of trypsin-TPCK, there was little difference between the virus growth kinetics on MDCK or NPTr cells. The only two definite differences were for sw-H1N1/82 (that will be discussed below) and for sw-H1N2/94. The latter was the only swine virus to show a clear growth reduction on NPTr cells at all temperatures. This would suggest that this virus might be less adapted to the swine host. Noticeably, av-H1N2/02, with H1 and N2 sequences closely related to the H1 and N2 of swine and human viruses, did not display a similar profile suggesting that other genes could be involved.

Regarding the influence of temperature in the presence of trypsin-TPCK, the results showed a clear sensitivity of viruses isolated from birds to a low temperature of incubation, with a delay in growth observed at 33 °C when compared to 37 and 40 °C. This was consistent with the fact that low pathogenic avian viruses replicate in the intestinal tract of infected birds, at a temperature around 40 °C. No significant growth reduction at 33 °C was observed for swine viruses. For swine influenza A viruses, to our knowledge, this is the first time that temperature sensitivity has been studied and reported. Previous studies have shown that viral replication and polymerase activity of avian influenza A viruses are reduced at low temperature in several mammalian cells including MDCK cells (Labadie et al., 2007; Massin et al., 2001). More specifically, the PB2 residue 627 (lysine for human (627K) and glutamic acid for most avian influenza A viruses (627E)) was described to be involved in this cold sensitivity of the avian virus polymerase complex at 33 °C in mammalian cells (Labadie et al., 2007; Massin et al., 2001). Another study also showed that a lysine at position 627 of PB2 confers an advantage for efficient growth of avian highly pathogenic H5N1 viruses in the upper and lower respiratory tracts of mammals (Hatta et al., 2007). In the present study, all avian, except av-H3N2/00, and swine viruses were characterised by a 627E at this position. Interestingly, a 627K was found for av-H3N2/00. Although the nature of this residue could account for the cold sensitivity observed in the growth kinetics of the avian H1N1 and H1N2 viruses, it cannot explain the apparent lack of cold sensitivity of the swine viruses. Moreover, it cannot account for the definite lower growth capabilities at 33 °C noticed for av-H3N2/00 on MDCK cells. In fact, av-H3N2/00 was found to be more sensitive to a low temperature than human H3N2 viruses (data not shown), despite their close genetic relationship. This suggests that other determinants besides PB2 residue 627 are involved.

A striking observation was the total lack of growth of sw-H1N1/82 on NPTr cells at 40 °C, which was not observed on MDCK cells nor for the closely related av-H1N1/05 virus. This prompted us to analyse the replication of these two viruses on NPTr cells using real-time PCRs targeting the PB2, NP and genomic vRNA segments. Very similar replication kinetics were obtained for the three genes tested, but differences might exist at earlier times of infection. We showed a good correlation with the growth kinetics determined by virus titres in the supernatant even if a light shift in time was observed, consistent with the fact that replication must take place before new viral particles

can be produced. The delay in replication at 33 °C confirmed the results of growth kinetics and was perfectly consistent with the previously published data regarding the role of residue 627E for avian viruses. Since av-H1N1/05 and sw-H1N1/82 are both related to the avian-like swine lineage, it seems likely that similar mechanisms involving residue 627E may prevail to explain their sensitivity at 33 °C. However, this sensitivity of replication at 33 °C seemed more pronounced for sw-H1N1/82, suggesting that additional determinants might be involved. In addition, the absence of replication at 40 °C was observed only for the swine virus and it seems unlikely that residue 627E of PB2 could account for the sensitivity observed for sw-H1N1/82 but not for other swine viruses. Thus, as clearly shown for H1N1 viruses, it seems that the temperature sensitivity of avian and swine viruses can be an intrinsic property of the virus and that, even if two viruses belong to the same genetic lineage, they might exhibit some features specific for the host they were isolated from.

Several studies have recently been published focusing on influenza A virus sensitivity to the incubation temperature and on host-range determinants, all of them trying to better understand the mechanisms behind cross-species infection and host adaptation. Thus, using only human viruses as model, Steel et al. (2009) showed that residue 627E of PB2 could be compensated by mutating residue 701 from aspartic acid (D) to asparagine (N), resulting in a wild-type temperature sensitivity phenotype and restoring transmission to mammals. This could not explain the slightly better growth capabilities observed at 33 °C for av-H1N1/02 and av-H1N2/05 when compared to av-H1N1/05, as av-H1N2/05 and av-H1N1/05 were both characterised by the presence of a 701N residue in PB2, and it would not explain why av-H3N2/00 showed temperature sensitivity at 33 °C on MDCK cells. Interestingly, Scull et al. (2009) showed that PB2 residue 627 is not always sufficient to confer sensitivity to low temperature. Using human viruses as genetic background and HA and NA from an Italian H7N1 influenza virus isolated from chicken, they demonstrated that the glycoproteins could also be involved in the cold sensitivity at 32 °C. Although av-H3N2/00 contains HA and NA that were found closely related to human virus counterparts, it would be interesting to test this hypothesis to find an explanation to the sensitivity at 33 °C observed for this virus on MDCK cells.

Other hypotheses could be tested to explain some of the temperature sensitivities we described. For example, the instability of the polymerase complex, that would prevent replication from occurring, was proposed to be responsible for a temperature sensitivity observed at 41 °C for three mammalian influenza viruses (Dalton et al., 2006). Two recent studies also highlighted a putative role for NP in temperature sensitivity and as a determinant of host range. Thus, residue 239 of NP was shown to be involved in the sensitivity at 39 °C of the vRNA packaging, possibly by affecting the interaction between NP and M1 (Noton et al., 2009). Finally, Gabriel et al. (2008) described two point mutations (701 in PB2 and 319 in NP) involved in the adaptation of an avian influenza strain to mouse, demonstrating that these two residues provides more efficient binding of the cellular importin α1 protein in mammalian cells, resulting in an increased transport of the virus genome into the nucleus. All these hypotheses would be interesting to test using reverse genetics systems based on each virus background.

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

Acknowledgements

The authors would like to thank Carole Guillemoto, Stéphane Queguiner, Stéphane Gorin for technical assistance, and François-Xavier Briand for help in avian influenza virus phylogenetic analyses.

This work was supported by a "Programme transversal de recherche (PTR)" joined grant co-funded by the "Agence Française de Sécurité Sanitaire des Aliments" (AFSSA) and the Pasteur Institute.

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