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The E119D neuraminidase mutation identified in a multidrug-resistant influenza A(H1N1)pdm09 isolate severely alters viral fitness *in vitro* and in animal models

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ABSTRACT

We recently isolated an influenza A(H1N1)pdm09 E119D/H275Y neuraminidase (NA) variant from an immunocompromised patient who received oseltamivir and zanamivir therapies. This variant demonstrated cross resistance to zanamivir, oseltamivir, peramivir and laninamivir. In this study, the viral fitness of the recombinant wild-type (WT), E119D and E119D/H275Y A(H1N1)pdm09 viruses was evaluated *in vitro* and in experimentally-infected C57BL/6 mice and guinea pigs. In replication kinetics experiments, viral titers obtained with the E119D and E119D/H275Y recombinants were up to 2- and 4-log lower compared to the WT virus in MDCK and ST6GalI-MDCK cells, respectively. Enzymatic studies revealed that the E119D mutation significantly decreased the surface NA activity. In experimentally-infected mice, a 50% mortality rate was recorded in the group infected with the WT recombinant virus whereas no mortality was observed in the E119D and E119D/H275Y groups. Mean lung viral titers on day 5 post-inoculation for the WT ($1.2 \pm 0.57 \times 10^8$ PFU/ml) were significantly higher than those of the E119D ($9.75 \pm 0.41 \times 10^5$ PFU/ml, $P < 0.01$) and the E119D/H275Y ($1.47 \pm 0.61 \times 10^6$ PFU/ml, $P < 0.01$) groups. In guinea pigs, comparable seroconversion rates and viral titers in nasal washes (NW) were obtained for the WT and mutant index and contact groups. However, the D119E reversion was observed in most NW samples of the E119D and E119D/H275Y animals. In conclusion, the E119D NA mutation that could emerge in A(H1N1)pdm09 viruses during zanamivir therapy has a significant impact on viral fitness and such mutant is unlikely to be highly transmissible.

1. Introduction

Each year, influenza epidemics can be responsible for an average of 3–5 millions of severe cases and up to 500,000 deaths in the world (Dunning et al., 2014). The immunocompromised population is at particularly high risk of developing severe complications from influenza infections. For example, during an influenza season, 20–30% of stem cell transplant recipients with respiratory symptoms could test positive for influenza with a mortality rate of up to 28% (Choi et al., 2011). Since the 2009 pandemic, influenza A(H1N1)pdm09 strains have predominated in the United States and Europe during some influenza seasons such as in 2013–2014 (Epperson et al., 2014) and 2015–2016 (Chambers et al., 2016). Despite continuous genetic evolution, currently circulating influenza A(H1N1)pdm09 strains remain antigenically related to the prototype A/California/07/2009-like A(H1N1)pdm09 vaccine strain.

Immunisation programs are the main tool for the control and prevention of seasonal influenza infections (Hayden and Palese, 2002). However, the efficacy of influenza vaccines is lower in high-risk groups such as very young children, the elderly and severely immunocompromised patients. Consequently, antiviral agents are particularly needed for the management of influenza in such situations. In this regard, neuraminidase inhibitors (NAIs)

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constitute the antiviral class of choice (Moscona, 2005) as most seasonal A/H3N2 viruses isolated after 2005 and all A(H1N1) pdm09 viruses have been shown to be resistant to the adamantanes (amantadine and rimantadine) (Dong et al., 2015). NAIs target the active center of the influenza neuraminidase (NA) molecule, which is made of 8 functional (R-118, D-151, R-152, R-224, E-276, R-292, R-371, and Y-406; N2 numbering) and 11 framework (E-119, R-156, W-178, S-179, D-198, I-222, E-227, H-274, E-277, N-294, and E−425; N2 numbering) residues that are largely conserved among influenza A and B viruses (Colman et al., 1993). Two NAI compounds (i.e., oseltamivir and zanamivir) have been commercially available worldwide for more than a decade whereas peramivir (Birnkrant and Cox, 2009; Press release, 2014) and laninamivir (Kubo et al., 2010; Watanabe et al., 2010) are licensed in a limited number of countries. As for the adamantanes, the emergence of NAI-resistant viruses is a serious threat that may compromise the clinical utility of these agents.

We recently identified a double E119D/H275Y substitution in the NA gene of an influenza A(H1N1)pdm09 virus isolated from an immunocompromised patient who had received oseltamivir and zanamivir therapy (L'Huillier et al., 2015). In that study, we demonstrated that recombinant A(H1N1)pdm09 viruses containing the E119D mutation alone or in combination with the H275Y substitution demonstrated resistance to all tested NAIs (zanamivir, oseltamivir, peramivir and laninamivir). The aim of the present study was to evaluate the impact of the E119D NA substitution in the A(H1N1)pdm09 background on *in vitro* replicative capacity as well as virulence in mice and infectivity/transmissibility in guinea pigs.

2. Materials and methods

2.1. Cells

Madin-Darby canine kidney cells overexpressing the α2,6 sialic acid receptor (ST6-*Gal*I-MDCK) cells (kindly provided by Y. Kawaoka from the University of Wisconsin, Madison, WI) (Hatakeyama et al., 2005), human embryonic kidney 293 T and MDCK cells (ATCC) were maintained in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Carlsbad, CA), supplemented with 10% fetal bovine serum (Invitrogen) and antibiotics.

2.2. Generation of recombinant viruses

The pLLBA plasmid containing the A/Quebec/144147/2009 NA gene [A(H1N1)pdm09-like; GenBank No. FN434457−FN434464] was used for the introduction of single (E119D) or double (E119D/H275Y) substitutions (N1 numbering) using appropriate primers and the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, USA). Recombinant wild-type (WT), single (E119D) and double (E119D/H275Y) NA mutant A/Quebec/144147/2009 viruses were rescued by co-transfecting the 8 bidirectional pLLB plasmids into 293T cells by using the Lipofectamine 2000 reagent (Invitrogen) as previously described (Pizzorno et al., 2011). Supernatants were collected at 72 h post-transfection and used to inoculate MDCK or ST6-*Gal*I-MDCK cells in infection medium consisting on DMEM containing 1 μg/ml L-tosylamido-2-phenylmethyl chloromethyl ketone (TPCK)-treated trypsin (Sigma, Saint-Louis, Missouri, USA) and antibiotics. The resulting recombinant WT and mutant viruses were subsequently sequenced and titrated by plaque assays in their respective cell line. Briefly, confluent monolayers in 12-well plates were inoculated with viruses diluted in infection medium for 1 h at room temperature. After this adsorption step, the supernatant was removed and cells were washed with PBS and then overlaid with Eagle's minimum essential medium (MEM, Invitrogen) containing 0.8% agarose, 1% bovine serum albumin (BSA, Invitrogen) and 2 μg/ml of TPCK. After 3 days of incubation at 37 °C, the agar overlay was removed and plaques were visualized by staining with 0.1% crystal violet containing 10% formaldehyde.

2.3. In vitro replication kinetics experiments

Replicative capacities of the recombinant viruses were evaluated by infecting MDCK or ST6GalI-MDCK cells at a multiplicity of infection (MOI) of 0.0001 plaque-forming units (PFUs)/cell in 12-well tissue culture plates containing 5×10^5 cells/well. Supernatants were collected every 12 h until 72 h post-infection (PI) and titrated by plaque assays as described above in either cell line.

2.4. In vitro genetic stability

Recombinant viruses were submitted to four serial passages in MDCK cells. Confluent cells in 6-well plates were washed with phosphate-buffered saline (PBS) before viral inoculation at an MOI of 0.001 PFU/cell. After a 1 h adsorption at 37 °C, the supernatant was removed and fresh DMEM-TPCK medium was added. After 3 days, supernatants were used to infect freshly prepared confluent MDCK cells. The hemagglutinin (HA) and NA genes from supernatant samples were amplified by RT-PCR and sequenced by using an automated DNA sequencer (ABI Prism 377 DNA sequencer, Applied Biosystems, Foster City, CA).

2.5. In vitro analysis of neuraminidase surface expression and activity

The WT and mutant A/Quebec/144147/09 NA genes were inserted into an HDM plasmid (kindly provided by J. Bloom, California Institute of Technology, Passadena, California, USA) where the NA protein-coding sequence is directly fused to the V5 epitope tag, followed by an internal ribosome entry site (IRES) expressing the green fluorescent protein (GFP). Recombinant HDM plasmids (1 μg) were used to transfect 293 T cells in 12-well plates (4×10^5 cell/well). Twenty hours post-transfection, the cells were collected after a brief Trypsin-EDTA treatment, and resuspended in an isotonic buffer (Bloom et al., 2011). A fraction of these cells (approximately 20%) was tested for NA activity using the fluorogenic MUNANA substrate whereas the remaining amount of cells was stained with a 1/200 dilution of a mouse monoclonal anti-V5-Tag conjugated with a C3 fluorophore (Sigma, Saint-Louis, Missouri, USA) then the mean fluorescence intensity of quadruplicate samples was assessed by flow cytometry as previously described (Bloom et al., 2011).

2.6. Experimental infection of mice

Groups of twelve 18−22 g female C57BL/6 mice (Charles River, Lasalle, QC, Canada) were housed four per cage and kept under conditions which prevented cage-to-cage infections. Mice were anesthetized with isoflurane and received an intranasal inoculum of 10^5 plaque forming units (PFUs) of recombinant A(H1N1)pdm09 WT virus or its E119D and E119D/H275Y variants in 30 μl of PBS. One group of 6 uninfected mice was used as control. Animals were monitored daily for body weight loss and mortality over a 14-day period. Subgroups of four mice were sacrificed on day 5 PI, then their lungs were removed aseptically and homogenized in 1 ml of sterile PBS containing antibiotics. Lung homogenates were then centrifuged at 600*g* for 10 min and supernatants were used for determination of lung viral titers (LVT) by plaque assays in ST6GalI-MDCK cells.

2.7. Experimental infection of guinea pigs

Groups of four 6–8 week old female Hartley guinea pigs (Charles River, Saint-Constant, QC, Canada) housed in individual cages were anesthetized by isoflurane and received an intranasal inoculum of 4.5 log TCID50/ml in a total volume of 250 μL (125 μL per nostril) of the recombinant A(H1N1)pdm09 WT or its E119D or H275Y/E119D variants. Temperature of animals was measured daily by rectal thermometers until day 8 PI. Guinea pigs were weighed every day and nasal wash samples were also collected from animals on a daily basis during 10 days PI. Virus titers in nasal wash samples were determined by plaque assays using ST6GalI-MDCK cells. Serum samples were collected from each animal before intranasal infection and on day 14 PI to evaluate specific antibody levels against the A/Quebec/144147/2009 influenza strain using standard HA inhibition (HAI) assays. To evaluate direct contact-transmissibility, inoculated-contact animal pairs were established by placing a naive guinea pig into each cage 24 h after inoculation of the index animal. Contact animals were monitored for clinical signs and nasal wash/serum samples were collected as described for index guinea pigs.

All animal procedures were performed in a biocontainment level 2+ facility and approved by the Institutional Animal Care Committee of Armand Frappier Institute according to the guidelines of the Canadian Council on Animal Care.

3. Statistical analyses

Viral titers from *in vitro* replication kinetics experiments, surface NA expression/activity and viral titers from lungs of mice and from nasal washes of guinea pigs were compared by one-way ANOVA analysis of variance, with the Tukey's multiple comparison post-test.

4. Results

4.1. In vitro properties of recombinant A(H1N1)pdm09 NA mutants

In replication kinetics experiments using ST6GalI-MDCK cells, the peak viral titer obtained for the recombinant WT virus was observed at 48 h PI (mean titer of $1.27 \pm 0.41 \times 10^9$ PFU/ml) whereas the E119D and E119D/H275Y mutants peaked at 72 h PI with mean viral titers of $4.1 \pm 0.21 \times 10^7$ and $3.1 \pm 0.18 \times 10^6$ PFU/ml, respectively (Fig. 1A). At most time points, there was a significant (up to 4 log) reduction in viral titers for the E119D and E119D/H275Y mutants compared to the WT virus. A similar pattern was obtained when using MDCK cells where viral titers obtained with the E119D and E119D/H275Y recombinant mutants during the 24 h–60 h time points were up to 2.5 log lower than those obtained with the WT recombinant (Fig. 1B). In addition, contrasting with the recombinant WT virus, the E119D and E119D/H275Y NA mutants produced smaller and unclear viral plaques (Fig. S1). Sequence analysis of the NA gene from samples obtained at 72 h PI confirmed the expected genotypes for the WT as well as the E119D and E119D/H275Y variants.

After four serial passages in MDCK cells, a complete D119E reversion occurred in the recombinant E119D/H275Y mutant whereas a partial D119E reversion (≈70% D, 30% E) was detected in the single E119D mutant. No other NA changes were observed in any recombinant viruses. Sequence analysis of the HA gene revealed an I89L mutation that occurred at the fourth passage in the HA2 region of the E119D/H275Y mutant.

To assess the impact of the E119D substitution on NA surface expression and activity, we transfected 293T cells with plasmids encoding WT or mutant NA proteins. The E119D mutation alone

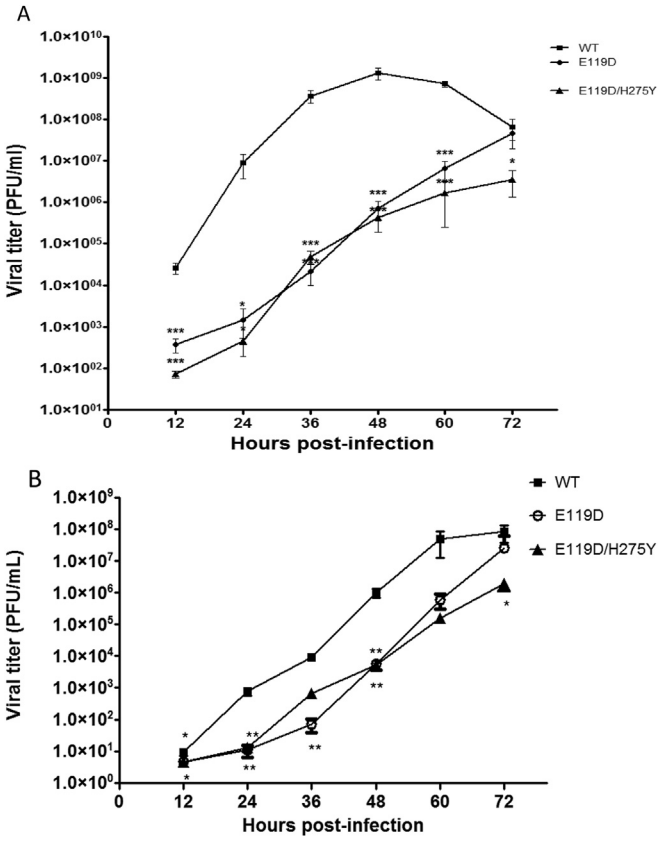

Fig. 1. Properties of recombinant A(H1N1)pdm09 viruses *in vitro*. Confluent ST6GalI-MDCK cells (A) or MDCK (B) were infected with recombinant viruses at a multiplicity of infection (MOI) of 0.0001 PFU/cell. Supernatants were harvested every 12 h until 72 h post-infection and titrated by standard plaque assays in the respective cell line. The mean values for three experiments with standard deviations are presented. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ as compared to the recombinant WT virus.

increased the surface expression of the NA protein by 18.2% compared to the WT NA ($P < 0.01$) while the H275Y substitution resulted in a significant decrease of NA expression (39.4% vs the WT, $P < 0.001$) (Fig. 2A). Cell surface expression of the double E119D/H275Y mutant was between those of the single mutants. On the other hand, NA activity assays showed that the single (E119D and H275Y) and double E119D/H275Y mutations were associated with a significant reduction of NA activity (by 67%, 14.2% and 95%, respectively, $P < 0.001$) as compared to the WT (Fig. 2B).

4.2. Properties of recombinant A(H1N1)pdm09 NA mutants in experimentally-infected mice

Intranasal inoculation of mice with 10^5 PFU of the recombinant viruses resulted in a mortality rate of 50% for the WT virus whereas all animals inoculated with the E119D or E119D/H275Y mutants survived. Accordingly, the WT group showed significantly greater mean body weight loss compared to the two mutant groups on days 4–14 PI (Fig. 3). The LVT determined on day 5 PI were also significantly higher for the WT group (mean of $1.2 \pm 0.57 \times 10^8$ PFU/ml) compared to those of the E119D (mean titer of $9.75 \pm 0.41 \times 10^5$ PFU/ml, $P < 0.01$) and the E119D/H275Y (mean titer of $1.47 \pm 0.61 \times 10^6$ PFU/ml, $P < 0.01$) mutant groups (Fig. 4). Sequence analysis of the NA gene from lung samples of mice collected on day 5 PI confirmed the presence of the desired genotype for the WT and E119D groups whereas a mixed population of GAC (≈55%) and GAA (≈45%) encoding for D and E, respectively,

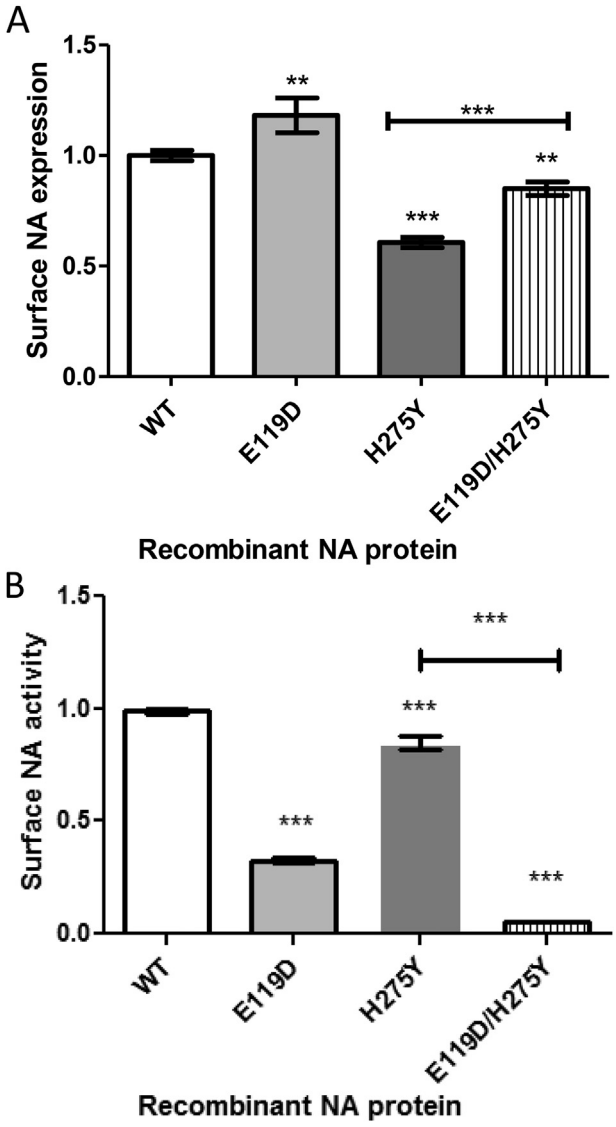

Fig. 2. Cell surface expression/activity of recombinant A(H1N1)pdm09 NAs. Cell surface expression of recombinant NAs expressed in 293 T cells was evaluated based on mean fluorescence intensity determined by flow cytometry analysis (A) whereas NA activity was determined by NA assays using the fluorogenic MUNANA substrate (B). $^{**}P < 0.01$; $^{***}P < 0.001$ for the mutants as compared to the WT or the H275Y group.

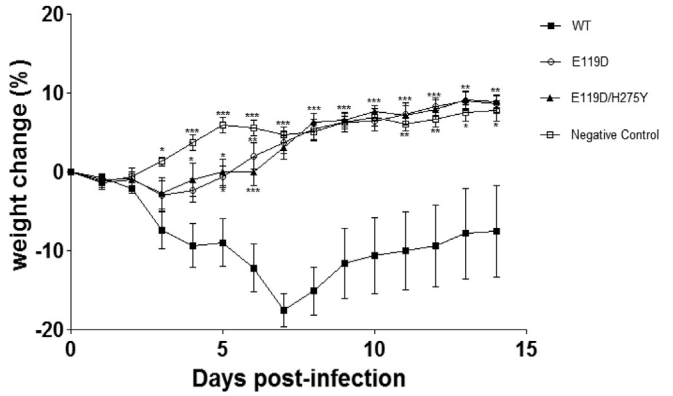

Fig. 3. Body weight losses induced by recombinant A(H1N1)pdm09 viruses in mice. Mean body weight loss ± standard error of the mean of mice inoculated intranasally with 10^5 PFUs of the recombinant A(H1N1)pdm09 WT virus or its E119D and E119D/H275Y variants. Percent body weight losses as compared to initial weights were recorded daily until day 14 post-inoculation. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ for the mutants as compared to the WT group.

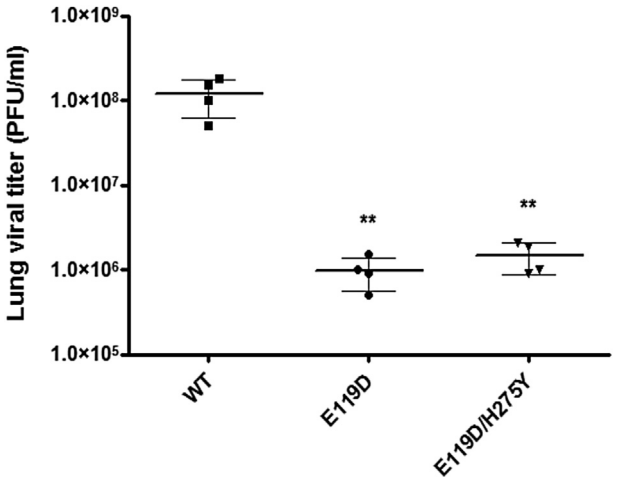

Fig. 4. Lung viral titers of recombinant A(H1N1)pdm09 viruses in mice. Mean lung viral titers ± standard error of mean for 4 mice infected intranasally with 10^5 PFUs of the recombinant A(H1N1)pdm09 WT virus or its E119D and E119D/H275Y variants. Titers were determined on day 5 post-inoculation by using plaque assays. $^{**}P < 0.01$ as compared to the WT group.

was observed in lung samples of the E119D/H275Y group.

4.3. Properties of recombinant A(H1N1)pdm09 NA mutants in experimentally-infected guinea pigs

Infectious viruses were recovered in nasal wash samples from all index guinea pigs except one from the WT group. Peak viral titers were reached on day 4 PI ($6.1 \pm 0.46 \times 10^6$, $1.41 \pm 0.91 \times 10^5$ and $1.9 \pm 0.25 \times 10^4$ PFU/ml for the WT, E119D and E119D/H275Y groups, respectively) (Fig. 5A). An increase in reciprocal HA inhibition titers against the A/Quebec/144147/2009 virus was observed on day 14 PI in 3 of 4 guinea pigs from the WT group (from <10 to 20–40) and in all 4 animals of the E119D (from <10 to 20–40) and E119D/H275Y (from <10 to 20–160) groups (Table S1). Of note, no virus could be recovered from the WT-infected guinea pig that did not seroconvert. Sequence analysis of the influenza A(H1N1)pdm09 NA gene from nasal wash samples obtained on day 4 PI revealed a D119E reversion in 50% of the samples from the E119D group and in all samples from the E119D/H275Y group. No other NA changes were observed in the three groups of guinea pigs.

In contact transmission guinea pigs, infectious viruses were recovered in nasal wash samples from 2, 1 and 2 animals in WT, E119D and E119D/H275Y groups, respectively, with peak viral titers being observed on day 8 PI ($2.15 \pm 0.16 \times 10^6$, 7.76×10^5 and $1.5 \pm 0.09 \times 10^4$ PFU/ml for the WT, E119D and E119D/H275Y groups, respectively) (Fig. 5B). An increase in reciprocal HAI titers against the A/Quebec/144147/2009 virus was observed in 2/4 guinea pigs from the WT group (from <10 to 20–40), in 1/4 guinea pigs of the E119D group (from <10 to 40) and in 2/4 animals of the E119D/H275Y group (from ≤10 to 80–640) (Table S1). Similarly, sequence analysis of the viral NA gene from nasal wash samples obtained on day 8 PI revealed a D119E reversion in all samples from the E119D and E119D/H275Y groups. No changes were observed at other codons in any of the three groups.

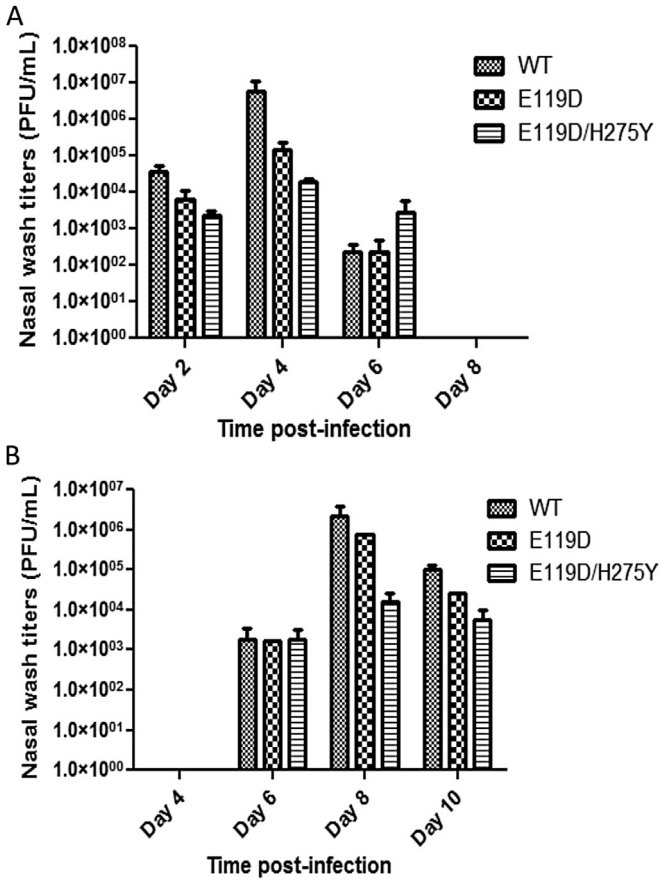

Fig. 5. Viral titers in nasal wash samples of index and contact guinea pigs. Mean viral titers ± standard error of the mean were determined in nasal washes by using standard plaque assays in groups of 4 index guinea pigs that received an intranasal inoculation of 250 μL containing 4.5 log TCID50/ml of recombinant A(H1N1)pdm09 wild-type (WT) virus as well as E119D and E119D/H275Y mutants (A) and in groups of 4 guinea pigs that were in direct contact with index guinea pigs (B). Note that there were undetectable levels of viruses on day 8 (panel A) and on day 4 (panel B).

5. Discussion

As for older human A(H1N1) viruses, resistance of the 2009 pandemic [A(H1N1)pdm09] viruses to oseltamivir, which is the most widely prescribed NAI, is mainly mediated by the H275Y NA mutation. Such variants are also resistant to peramivir but retain susceptibility to zanamivir and laninamivir (Pizzorno et al., 2011; Samson et al., 2014). Interestingly, our group and other investigators reported that A(H1N1)pdm09 H275Y variants conserved a good fitness and exhibited efficient contact transmission when assessed in ferrets and guinea pigs (Hamelin et al., 2010; Memoli et al., 2011; Seibert et al., 2010; Wong et al., 2012). Furthermore, we previously demonstrated that the A(H1N1)pdm09 H275Y mutant was at least as virulent as the WT virus in BALB/c (Hamelin et al., 2010) and C57BL/6 (Abed et al., 2014) mice. At this time, little information is available regarding the fitness and virulence of zanamivir-resistant A(H1N1)pdm09 viruses.

We previously reported that the introduction of E119A/G/V NA substitutions in the recombinant A/Quebec/144147/2009 pandemic strain resulted in reduced or highly reduced susceptibility to zanamivir and oseltamivir (Pizzorno et al., 2011). Moreover, *in vitro* passages of two A(H1N1)pdm09 strains (A/Quebec/144147/2009 and A/Auckland/3/2009) under laninamivir pressure induced a E119K change in the NA protein (Samson et al., 2014). These observations suggested that substitutions of the E119 residue are likely to be a dominant marker of zanamivir/laninamivir resistance in A(H1N1)pm09 viruses. Accordingly, influenza A(H1N1)pdm09 E119G/D variants were recovered from an 8-month old immunocompromised boy who had received zanamivir and oseltamivir therapy (Tamura et al., 2015). In addition, the E119D NA mutation emerged after zanamivir therapy in an immunocopromised patient infected with an A(H1N1)pdm09 virus (L'Huillier et al., 2015).

In our original clinical article, we reported that the E119D mutation, which occurred after eight days of zanamivir treatment, followed the emergence of the H275Y mutation which was detected after only one day of oseltamivir therapy (L'Huillier et al., 2015). The single E119D mutation increased IC50 values for zanamivir, oseltamivir, laninamivir and peramivir by 827-, 25-, 702- and 286-fold, respectively compared to the WT virus. The addition of the H275Y mutation further increased oseltamivir and peramivir IC_{50} values by 790- and >5000-fold, respectively, compared to the WT virus (L'Huillier et al., 2015). However, the virulence and transmissibility of these multi-drug resistant viruses were still uncharacterized.

In this study, we evaluated the impact of the E119D NA mutation on viral fitness as measured *in vitro* and in two animal models. We rescued recombinant WT, E119D and H275Y/E119D viruses and assessed their *in vitro* fitness using both MDCK and ST6GalI-MDCK cells. The fitness of the two mutants was attenuated in both cell lines which was in contrast with the findings of Baek and collaborators who recently reported that the A/California/04/2009 E119D and E119D/H275Y mutant viruses displayed efficient replication capacity in MDCK cells and were genetically stable (Baek et al., 2015). Notably, we previously reported that the D119E reversion occurred in the recombinant E119D/H275Y mutant after only two passages in ST6GalI-MDCK cells (L'Huillier et al., 2015) confirming the genetic instability of this resistance marker. In the present study, a complete D119E reversion also occurred in the recombinant E119D/H275Y mutant after four passages in MDCK cells whereas a partial D119E reversion was seen in the single E119D mutant. The E119D/H275Y mutant also acquired a I89L mutation in the HA2 subunit of the HA upon passages. This change is not part of the receptor binding site and seems unlikely to alter viral attachment for eventually compensating the NA default.

The detrimental impact of the E119D mutation on viral fitness was confirmed in experimentally-infected C57BL/6 mice where, contrasting to the WT virus, the E119D and E119D/H275Y mutants did not cause mortality or significant weight loss. Moreover, partial D119E reversion was observed in lungs of mice infected with the double E119D/H275Y recombinant hence reinforcing the genetic instability exhibited by this variant *in vitro* (L'Huillier et al., 2015). A rapid D119E reversion also occurred in the E119D/H275Y recombinant viruses recovered from all index guinea pigs as well as in 50% of animals infected with the single (E119D) recombinant mutant. The reversion was also observed in contact guinea pigs that were housed with the index animals. Noteworthy, the observed changes in NA genotypes are based on the Sanger sequencing method and it is possible that a more sensitive sequencing technique, such as deep sequencing, could have identified more reversion cases and at earlier times both *in vivo* and during *in vitro* passages. Of interest, a similar reversion to the WT genotype was reported by our group in nasal wash samples of ferrets infected with the recombinant A(H1N1)pdm09 virus harboring the E119G NA mutation (Pizzorno et al., 2013).

Our *in vivo* results are also discordant with those of Baek et al. who reported conserved virulence for the two mutants (E119D and E119D/H275Y) in BALB/c mice as well as retained infectivity and contact transmissibility in ferrets (Baek et al., 2015). The C57BL/6 mouse model used in our study appears to be more convenient to assess the impact of NA mutations on virulence as the A(H1N1)

pdm09 virus causes significant lethality in this mouse species without prior adaptation contrasting to the BALB/c species (Otte and Gabriel, 2011). The severely compromised fitness demonstrated in mice and *in vitro* for our double E119D/H275Y and single E119D mutants is also in agreement with a detrimental impact on NA velocity (Vmax), affinity (Km) (L'Huillier et al., 2015) and cell surface activity induced by these changes. Of interest, in a recent clinical report, the influenza A(H1N1)pdm09 E119D/H275Y variant that could be detected by pyrosequencing in the nasopharyngeal swab of an immunocompromised child under zanamivir therapy failed to grow in cell culture (Tamura et al., 2015).

Thus, the question remains as why the E119D and E119D/H275Y mutants demonstrate discordant *in vitro* and *in vivo* properties in the study reported by Baek (Baek et al., 2015) and in ours. It is difficult to make a direct comparison between these two studies due to some differences during *in vitro* experiments (different MOI and incubation time during the assessment of genetic stability) as well as different animal models (ferrets and BALB/c mice vs guinea pigs and C57Black6 mice) for *in vivo* evaluation of NA mutants. The difference in our results could also be related to the different viral backgrounds used in the two studies. Indeed, the A/California/04/2009 viral genome belongs to cluster I that appeared in early April 2009 and disappeared by the end of 2009 whereas the genome of A/Quebec/144147/09 strain belongs to cluster II that emerged later and has been circulating since (Christman et al., 2011). A few genetic differences obviously exist between the two strains as summarized in Table S2. In particular, the HA proteins differ by 3 amino acids (100S, 220 T and 338V in A/Quebec/144147/09 vs 100P, 220S and 338I in A/California/04/2009) and the NA proteins differ by 2 amino acids (106I, 248D in A/Quebec/144147/09 vs 106V, 248N in A/California/04/2009). As previously reported, the recombinant A/Quebec/144147/09 virus used in this study demonstrated a close genetic relatedness to the original clinical isolate (L'Huillier et al., 2015). Indeed, like A/Quebec/144147/09, the HA protein of the clinical isolate had S, T and V at positions 100, 220 and 338, respectively whereas there was a divergence at position 106 (I/V) of the NA protein.

The E119 residue is highly conserved in influenza A and B viruses (Colman et al., 1993). This codon plays a significant role in stabilizing the NA catalytic site, which is important for efficient cleavage of sialic acid residues (Yang et al., 2013). E119 is also involved in the interaction between the NA enzyme and either the guanidine group (present in zanamivir, laninamivir and peramivir) or the C4 hydroxyl group (present in oseltamivir) (L'Huillier et al., 2015). Replacement of glutamic acid by amino acids with a shorter side chain (such as aspartic acid) could significantly alter these interactions. In this study, enzymatic studies also revealed that the E119D NA mutation strongly reduced the surface NA activity.

In conclusion, the present investigation provides compelling evidence that, although some substitutions at residue E119 could occur in influenza A(H1N1)pdm09 viruses resulting in cross resistance to all licensed NAIs, these NA changes seem to significantly compromise viral fitness of most A(H1N1)pdm09 strains. This suggests that such variants are unlikely to have a significant clinical relevance outside the immunocompromised population. Nevertheless, the selective effect of NAI pressure and the eventual emergence of HA or other viral genetic changes with the potential to compensate for the E119D-associated NA default need to be considered. Such scenario reinforces the urgent need for developing alternate antiviral strategies against influenza A viruses.

Potential conflicts of interest

EB, MHJ, KD and CM are employees of the GSK group of companies.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.antiviral.2016.05.006

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