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The origin of the PB1 segment of swine influenza A virus subtype H1N2 determines viral pathogenicity in mice

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ABSTRACT

Swine appear to be a key species in the generation of novel human influenza pandemics. Previous pandemic viruses are postulated to have evolved in swine by reassortment of avian, human, and swine influenza viruses. The human pandemic influenza viruses that emerged in 1957 and 1968 as well as swine viruses circulating since 1998 encode PB1 segments derived from avian influenza viruses. Here we investigate the possible role in viral replication and virulence of the PB1 gene segments present in two swine H1N2 influenza A viruses, A/swine/Sweden/1021/2009(H1N2) (sw 1021) and A/swine/Sweden/9706/2010(H1N2) (sw 9706), where the sw 1021 virus has shown to be more pathogenic in mice. By using reverse genetics, we swapped the PB1 genes of these two viruses. Similar to the sw 9706 virus, chimeric sw 1021 virus carrying the sw 9706 PB1 gene was not virulent in mice. In contrast, replacement of the PB1 gene of the sw 9706 virus by that from sw 1021 virus resulted in increased pathogenicity. Our study demonstrated that differences in virulence of swine influenza virus subtype H1N2 are attributed at least in part to the PB1 segment.

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1. Introduction

Influenza A virus, a negative strand RNA virus in the family Orthomyxoviridae, contains eight RNA segments encoding up to twelve proteins. Influenza viruses can infect many animal species and some of them may give rise to pandemic strains in humans. One recent example is the case of the 2009 H1N1 pandemic (H1N1 pdm) virus. Most threatening is the possibility of another pandemic with virulence similar to the Spanish influenza of 1918, which cost more than 20 million lives worldwide (Hoehling, 1961; Johnson and Mueller, 2002; Palese, 2004). Influenza viruses have been isolated from avian species and mammals, including humans and pigs. Swine influenza viruses (SIVs) cause respiratory disease in pigs and have significant economic impact. Swine influenza was first

clinically recognized at the time of the 1918 Spanish influenza pandemic, and was later shown to be an influenza A virus of the H1N1 antigenic subtype called classical swine H1N1 (Shope, 1931). The swine influenza virus was derived from the same 1918 H1N1 virus that affected humans. Nowadays three main subtypes of SIVs are prevalent in pig populations worldwide: H1N1, H3N2 and H1N2. However, the origin and nature of SIVs differ on different continents (Karasin et al., 2002; Kuntz-Simon and Madec, 2009). Classical swine H1N1 viruses circulated in North America but were absent in Europe until 1976, when they were isolated from pigs imported to Italy from the United States (Nardelli et al., 1978).

In North America and Asia, classical swine H1N1 is more commonly isolated than avian-lineage H1N1; however, in Europe the most commonly isolated H1N1 SIV are of avian-lineage, known as “avian-like” swine H1N1 (Guan et al., 1996; Hinshaw et al., 1978). Since the introduction into the pig population of avian-like SIV from wild ducks in 1979, all eight genomic segments encoded by avian-like SIV are of avian virus origin (Pensaert et al., 1981). The “avian-like” swine H1N1 viruses are antigenically distinguishable from classical swine H1N1 influenza viruses. These “avian-like” viruses might have a selective advantage over classical swine H1N1

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viruses, as in Europe they have replaced the classical SIV (Brown, 2000). In addition, in the early 1970s, H3N2 viruses resembling the H3N2 strain responsible for the human 1968 Hong Kong influenza pandemic were introduced into European pigs (Harkness et al., 1972). In 1984 a reassortment event occurred, resulting in a new SIV strain encoding human-like H3N2 “Hong Kong influenza virus” surface glycoproteins (haemagglutinin-HA, neuraminidase-NA) and the internal genes of “avian-like” H1N1 (Campitelli et al., 1997). In the 1990s, these reassortant swine viruses replaced the original H3N2 human-like swine “1968 Hong Kong influenza” strains in pig populations in European countries (Kuntz-Simon and Madec, 2009).

As a result of further reassortment events, H1N2 viruses have originated in European swine populations twice. The first European SIV H1N2 subtype was reported in France in 1987 (Gourreau et al., 1994) and was the result of a reassortment event between the avian-like swine H1N1 virus and the European reassortant human-like swine H3N2 virus. This H1N2 virus encodes the HA segment of avian origin and the NA segment of human origin. This first European H1N2 SIV did not become widespread. In contrast, another distinct lineage of H1N2 virus became widespread in European pig populations, consisting of a reassortant variant of “human-like” H1N2 virus first identified in the UK in 1994, and encoding the HA and NA genes of human origin and the internal genes of avian origin. (Brown et al., 1995; Marozin et al., 2002; Van Reeth et al., 2000). Since 1998, these reassortant H1N2 SIVs have been isolated in Italy, Denmark and France (Balint et al., 2009; Kuntz-Simon and Madec, 2009; Marozin et al., 2002; Moreno et al., 2012). They are similar to the prototype H1N2 strains; the difference being that instead of a human origin HA they have the “avian-like” HA from H1N1 SIVs. In 2009 and 2010 the first isolations and demonstrations, respectively, of H1N2 viruses in pigs were reported in Sweden (Balint et al., 2009). The characterized Swedish isolates possessed avian-like SIV H1N2 HA and European H3N2 SIV-like NA (Metreveli et al., 2011). The Swedish isolates are similar to viruses described in Italy and Denmark (Moreno et al., 2012; Trebbien et al., 2013), but they are genetically distinct from other H1N2 viruses in Europe. There are several problematic areas regarding the study of the evolution of SIV, including a lack of surveillance worldwide and large gaps in the genetic and phenotypic information of SIV isolates. Continuous circulation of influenza A viruses in pigs can result in the production of new reassortant viruses of concern, as best exemplified by the appearance of the 2009 H1N1 pdm influenza virus. In addition to generating viruses with pandemic potential, reassortment and further mutation of genomic segments can alter the virulence of SIV. Studies by (Khiabani et al., 2009) have shown that together with the glycoprotein coding segments HA and NA, the PB1 segment appears frequently in reassortment events among swine influenza viruses, particularly in the 1957 (from avian species) and 1968 (from another avian virus) pandemic viruses as well as in swine viruses circulating since 1998 (Kawaoka et al., 1989; Richt et al., 2003; Zhou et al., 1999). However, the mechanism of the preferential reassortment of PB1 is still not fully understood. Since the novel Swedish lineage of avian-like reassortant H1N2 SIV appears to be gaining a stronger foothold among Swedish pig populations and causing more severe clinical disease, we have characterized whether the PB1 segment has a role in the differences in virulence in the mouse model between two independent isolates of H1N2 SIV in Sweden.

2. Materials and methods

2.1. Cells and viruses

293T and PK1 cells were maintained in Dulbecco's modified Eagle's medium with 10% fetal calf serum (FCS). MDCK cells were

grown in Eagle's minimal essential medium with 10% FCS. Viruses were grown in 10-day-old specific-pathogen-free chicken embryonated eggs at 37 °C (Charles River Laboratories, SPAFAS).

2.2. Rescue of recombinant influenza A viruses

The H1N2 SIV used in these studies included reverse genetics derived A/swine/Sweden/1021/2009 (r1021), A/swine/Sweden/9706/2010 (r9706), r1021 (9706 PB1) that encodes the PB1 gene segment from the r9706 virus strain, and r9706 (1021 PB1) that encodes the PB1 gene segment from the r1021 virus strain. Rescue plasmids used for the rescue of recombinant H1N2 SIV were constructed by methods described previously (Fodor et al., 1999; Gao et al., 2008; Neumann et al., 1999). Recombinant viruses were rescued as described previously (Gao et al., 2008). Briefly, 293T cells were transfected with eight ambisense pDZ vectors expressing viral genomic RNA and viral mRNAs, and incubated at 37 °C in a 5% CO₂ incubator for 24 h. After 24 h, the transected cells were harvested and inoculated into 10-day-old Specific Pathogen Free (SPF) eggs. The inoculated SPF eggs were incubated at 33 or 37 °C for 3 days. Allantoic fluids were harvested from the infected SPF eggs, and hemagglutination assay (HA) was performed to confirm the rescue of recombinant influenza viruses. The titers of the rescued viruses were determined by plaque assay in MDCK cells. The nucleotide sequence of the swapped PB1 segments was confirmed by sequence analysis.

2.3. Growth curves of recombinant and chimeric viruses

To analyze the replication phenotype of the recombinant viruses, PK1 cells were seeded at 10⁶ per well in 6 well plates (duplicates for each virus), were inoculated at a multiplicity of infection (MOI) of 0.01 PFU/cell, incubated for 1 h at 37 °C (plates were rocked every 10 min) for virus adsorption, and then incubated in serum-free MEM supplemented with trypsin-TPCK. At the indicated time points, 0.2 ml of supernatant was collected for virus titration by plaque assay using MDCK cells.

2.4. Infection of mice

Six to eight week-old female C57BL/6 mice (Jackson Laboratory, Bar Harbor, ME) were anesthetized with an intraperitoneal injection of a mixture of ketamine and xylazine (100 mg/kg and 10 mg/kg, respectively), and then 50 µl of infectious virus diluted in phosphate-buffered saline (PBS) was inoculated intranasally (i.n.). To determine virus pathogenicity, five animals per group were infected i.n. with different doses (1 × 10⁴, 1 × 10⁵, 1 × 10⁶ PFU), and were monitored daily for weight loss for up to 14 days post infection (p.i.). Mice showing more than 25% of body weight loss were considered to have reached predefined humane endpoints and were euthanized. To determine viral pulmonary replication kinetics and to examine pulmonary pathology by histology, groups of 12 mice were inoculated with the indicated virus (1 × 10⁵ PFU per mouse). Mice inoculated with PBS served as controls. At days 2 and day 4 p.i., 3 mice per experimental group per day were euthanized. For determination of virus titers, lungs were homogenized in 1 ml of PBS and viral titers determined by plaque assays with MDCK cells. On days 2 and 4 p.i. (D2, D4), three mice per group were sacrificed for histologic examination. All animal studies were performed according to institutional guidelines.

2.5. Histopathologic examination

For histopathological examination, the lungs from the sacrificed mice were removed and were infused with 10% neutral buffered formalin, and then paraffin-embedded for sectioning. Five micron

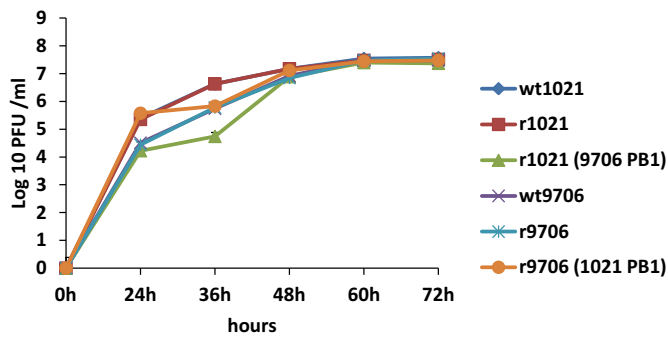


Fig. 1. Growth curves of recombinant and chimeric viruses. The replication kinetics of the recombinant viruses (sw1021 and sw9706) were compared to the replication of the PB1 exchanged recombinants r1021(9706 PB1) and r9706 (1021 PB1) in immortalized swine kidney cells (PK1 cells). Cells were inoculated at MOI of 0.01, and infection supernatants were collected at 24, 36, 48, 60 and 72 h. Bars show positive and negative standard deviations.

sections of each lung were cut and stained with Hematoxylin and Eosin for histopathology. Lung sections were examined using light microscopy and images were obtained using an Olympus DP21 digital camera system.

3. Results

3.1. Rescue of recombinant influenza A viruses

In order to determine the contribution of PB1 gene products of the two SIV to virus replication and pathogenicity, we rescued four recombinant swine H1N2 influenza viruses by reverse genetics. These recombinant viruses included the parental sw1021 and sw9706 strains, and the chimeric viruses r1021 (9706 PB1) that encodes the PB1 gene segment from the sw 9706 virus strain and r9706 (1021 PB1) that encodes the PB1 gene segment from the sw 1021 virus strain.

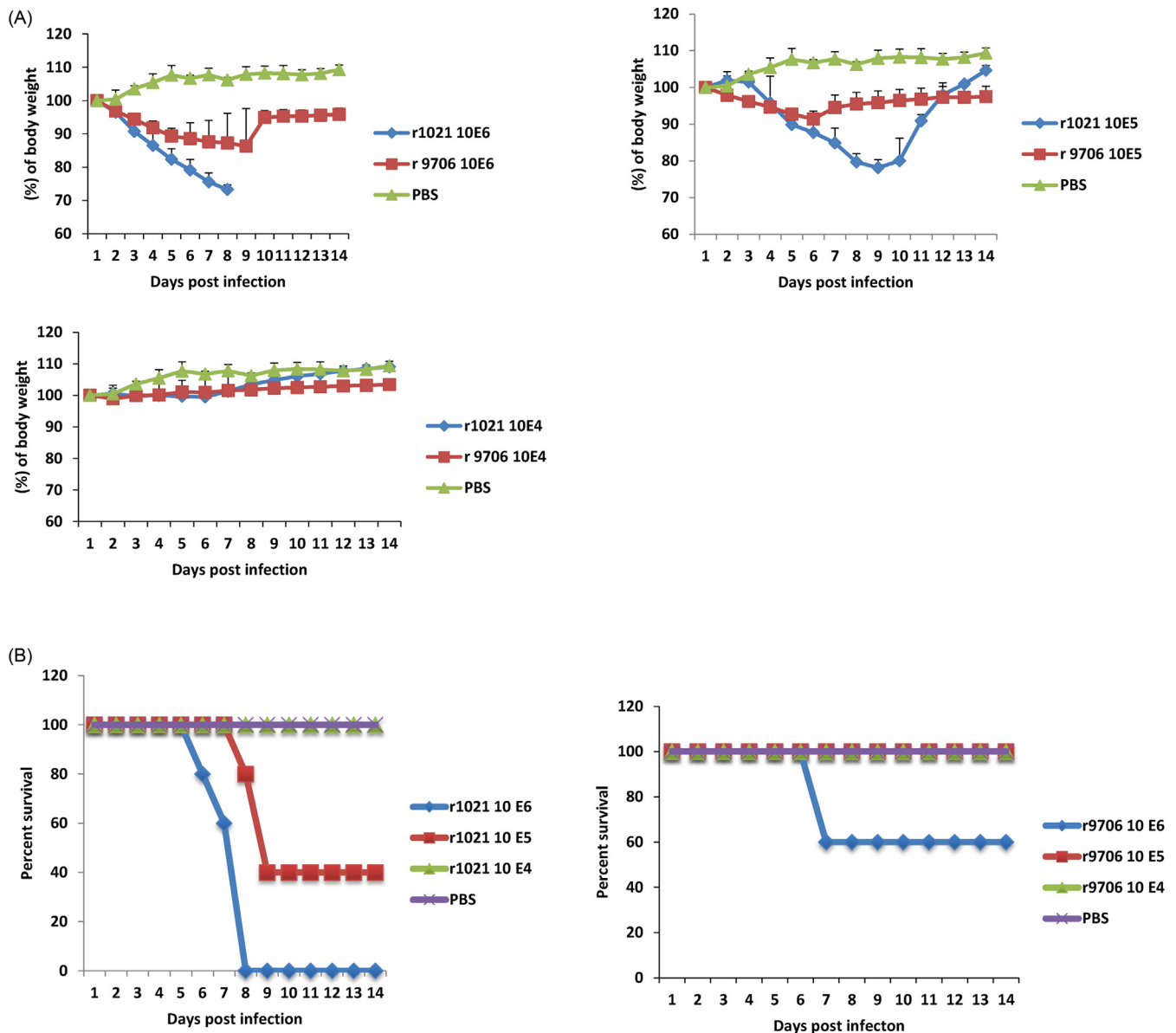


Fig. 2. Pathogenesis of recombinant viruses r1021 and r9706 in mice. (A) Five mice per virus were infected i.n. with different doses (1×10^4 , 1×10^5 , 1×10^6 PFU). Mice were weighed every day after infection for 14 days. Mice showing more than 25% of body weight loss were considered to have reached predefined humane endpoints and were euthanized according to the institutional guidelines. (B) Survival of mice infected with r1021 and r9706 viruses as described for panel A.

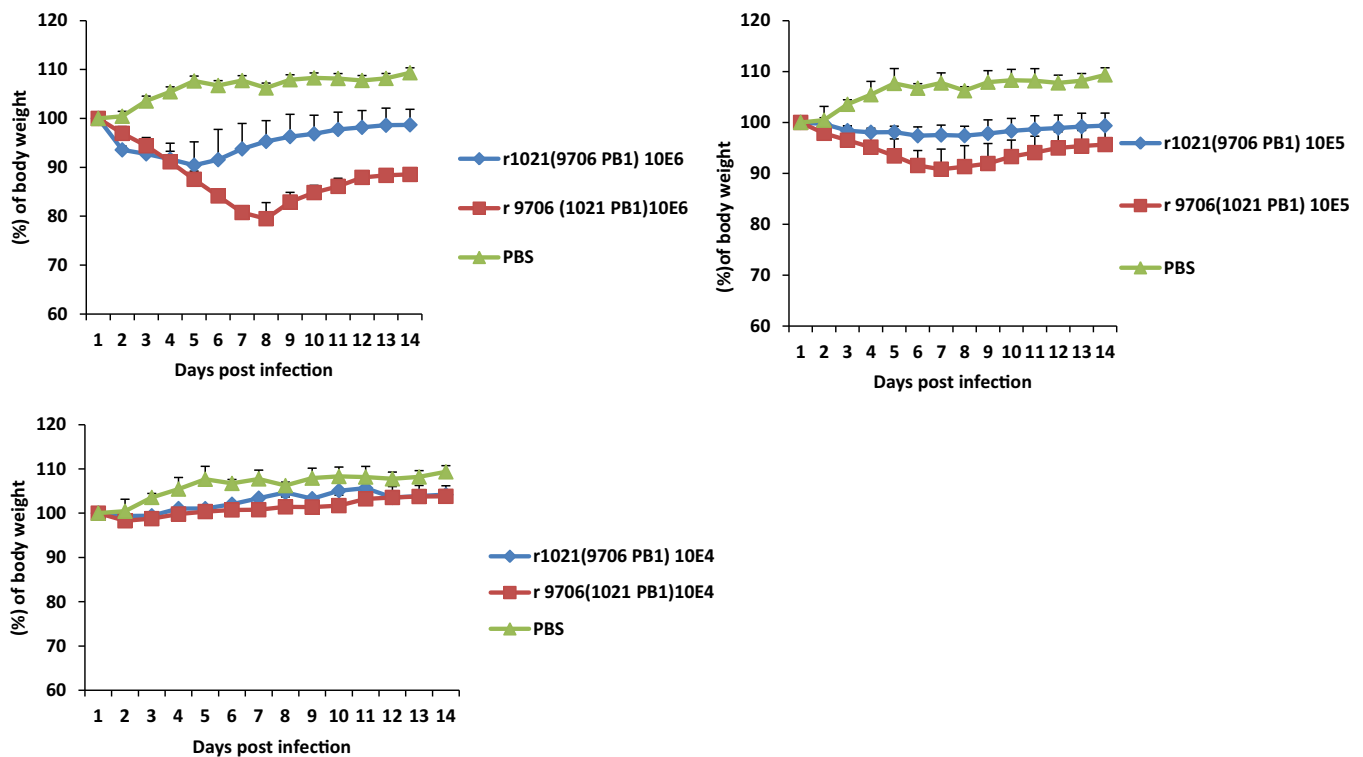


Fig. 3. Pathogenesis of chimeric r9706 (1021 PB1) and r1021 (9706 PB1) viruses in mice. Six to eight week-old female C57BL/6 were infected with increasing doses (1×10^4 , 1×10^5 , 1×10^6 PFU) of the chimeric r1021 (9706 PB1) and r9706 (1021 PB1) viruses and were monitored daily for weight loss for up to 14 days p.i. The chimeric virus r9706 (1021PB1), carrying PB1 gene from r1021, induced significantly higher weight loss than chimeric virus r1021 (9706 PB1).

3.2. Replication kinetics of recombinant swine influenza viruses in PK1 cells

The replication kinetics of the recombinant viruses (r1021 and r9706) were compared to the replication of the PB1 exchanged recombinants r1021 (9706 PB1) and r9706 (1021 PB1) in immortalized swine kidney cells (PK1 cells). Wild type sw 1021 and sw 9706 viruses were used as controls. PK1 cells were infected at an MOI of 0.01. Although there were slight differences in viral titers between 24 and 36 h p.i. of PK1 cells, all virus titers became equal by 48 hpi (Fig. 1). Plaque assays conducted in MDCK cells revealed no differences in plaque morphology (data not shown), suggesting that there were no differences in replication phenotypes among the different viruses.

3.3. PB1 gene segment contributes to virulence

To determine whether the parental viruses differ in pathogenesis, and whether the PB1 segment could contribute to any observed differences, the C57 BL/6 mouse model of influenza virus pathogenesis was utilized. Mice were infected with increasing doses (1×10^4 , 1×10^5 , 1×10^6 PFU) of the indicated viruses and were monitored daily for weight loss for up to 14 days p.i. At the highest inoculum tested, r1021 was more virulent than the r9706 virus as evidenced by significant weight loss resulting in mice achieving the predefined endpoint by day 8 post-infection (Fig. 2A and B). The calculated LD₅₀ was $1 \times 10^{4.9}$ PFU/ml and $1 \times 10^{6.1}$ PFU/ml for r1021 and r9706, respectively. In the case of chimeric viruses r1021 (9706 PB1) and r9706 (1021 PB1), mice infected with 1×10^6 PFU of r9706 (1021 PB1) showed a strong reduction in mouse weight loss (weight loss $\geq 21\%$) (Fig. 3). In contrast, only a mild reduction in weight loss (weight loss $\leq 10\%$) was observed for the mouse group inoculated with the r1021 (9706 PB1) virus. The switch in virulence resulting from the exchange of the PB1 segments showed that the PB1 gene

of the r1021 virus increased pathogenicity in the backbone of r9706 virus (Fig. 3). In order to understand the differences in the virulence caused by infection of these recombinant and chimeric viruses, we determined the kinetics of virus replication in the lungs of mice. Mice were infected i.n. with 1×10^5 PFU of these viruses. On days 2, and 4 p.i., three mice per group were sacrificed in order to determine viral titers in lung homogenates. The r1021 virus replicated with higher titer in mice lungs compared to r9706 at both days 2 and 4 (Fig. 4; $p < 0.001$). However, after the PB1 segment was swapped, the virus lung titers were changed accordingly, such that r9706 (1021 PB1) was detected at higher titers in the lung homogenates than r1021 (9706 PB1) (Fig. 4; $p < 0.001$). Comparison of the lung titers for the PB1 exchanged virus with the corresponding “wild-type” virus also revealed significant differences in virus titers such

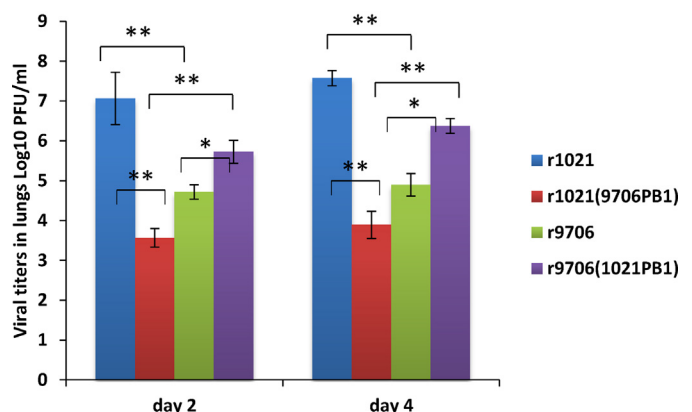


Fig. 4. Mouse lung virus titers. Six mice per virus group for r1021, r9706, r1021 (9706 PB1) and r9706 (1021 PB1) were infected with 1×10^5 PFU of each virus. Three mice from each group were sacrificed on days 2 and 4. Lung virus titers were evaluated by infection of MDCK cell. * $p < 0.01$; ** $p < 0.001$.

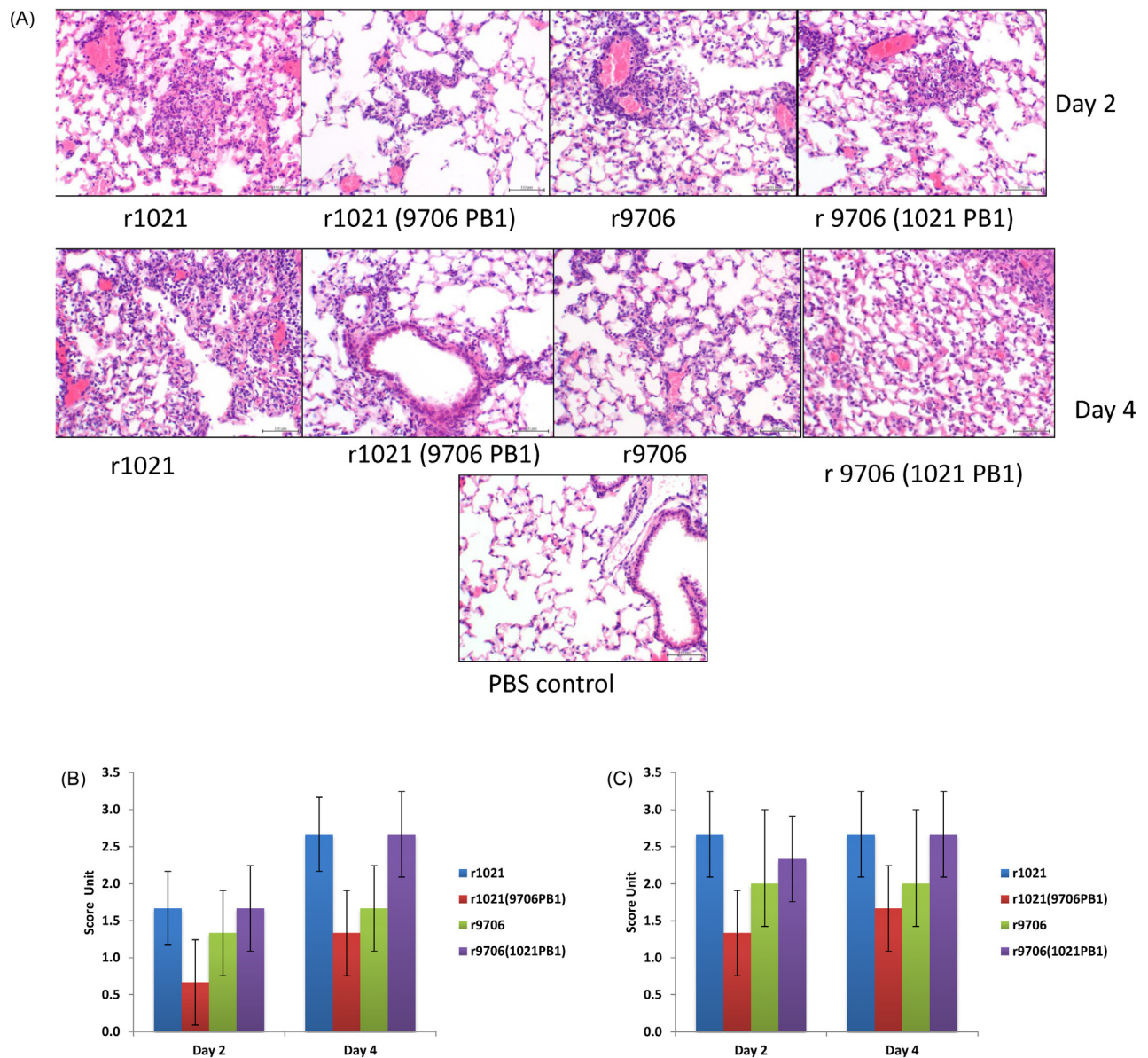


Fig. 5. Histopathologic examination. Six mice per group were infected intranasally with 1×10^5 PFU of r1021, r9706, r1021 (9706 PB1) or r 9706 (1021 PB1) viruses. On days 2 and 4 post-infection, three mice per group were euthanized. The lungs were extracted for histological examination. Histopathological analysis of infected mice revealed increased lesions associated with r1021 and r9706 (1021PB1) virus infection. The changes were characterized by infiltrates of leucocytes with varying intensity from a few cells to severe infiltrates, most pronounced on day 4 p.i. The inflammatory infiltrates at day 2 were predominantly focal but at day 4 tended to be diffuse (A), the bar represents 100 μ m and the picture was taken at magnification 20 $\times$. Bronchiolar epithelial (B) and vascular/perivascular (C) changes were scored as follows: "0" represents normal epithelium/endothelium without any inflammation; "1" represents mild changes with the focal presence of inflammatory cells and slight reactive atypia of the epithelium/endothelium; "2" represents moderate inflammation with the significant presence of inflammatory cells and marked reactive changes in the epithelium/endothelium and eventually the presence of a few necrotic cells; "3" represents the most pronounced inflammatory changes observed in these groups with eventually some epithelial/endothelial loss and multifocal/segmental necrosis.

(r9706 versus r9706 (1021 PB1) $p < 0.01$, and r1021 versus r1021 (9706PB1) $p < 0.001$). These results from the mouse pathogenesis studies suggest that the PB1 segment may contribute to the virulence of the Swedish H1N2 swine isolates. Macroscopically we observed that i.n. inoculation of mice with the r1021 virus resulted in gross pathology in lung appearance with prominent edema, at both day 2 and day 4. The chimeric virus r9706 (1021 PB1) also resulted in lesions detected in the lung of one infected mouse, but lung lesions were detected in all mouse infected lungs regardless of the virus by day 4 post-infection. Histological examinations for lung disease resulting from virus infection revealed that infection with r1021 or r9706 (1021 PB1) viruses resulted in various degrees of leukocyte infiltration, and focal to diffuse alveolar damage with

edema at both day 2 and day 4 p.i. (Fig. 5A). The histological findings were consistent with the increased bronchiolar epithelial (Fig. 5B) and vascular/perivascular (Fig. 5C) changes.

4. Discussion

SIV should be continuously monitored as a possible source of human influenza pandemic viruses. The introduction into swine of novel influenza virus genomic segments by reassortment of swine, avian and human influenza viruses could result in the generation of a human pandemic virus, as was the case for the swine origin 2009 H1N1 pdm virus. European avian-like SIV appears to be one of the ancestors of the 2009 H1N1 pdm virus. Here we characterized and

investigated the contribution of the PB1 gene segment to infection by swine influenza subtype H1N2 by using in vitro tissue culture and a mouse model. These H1N2 viruses are reassortant variants of SIV that have an avian-like SIV H1N1 HA and European H3N2 SIV-like NA (Metreveli et al., 2011). In this study, we have developed a reverse genetic system for these viruses and generated two recombinant SIV H1N2 viruses that recapitulated the same differences in virulence in mice, r1021 (more virulent) and r9706 (less virulent) as for the corresponding wild-type viruses, as well as recombinant viruses encoding swapped PB1 segments. Interestingly, our current findings show that the replacement of the PB1 segment of r9706 by that of r1021 increases the virulence and virus titers in the lungs of infected mice, while the opposite is true when PB1 r9706 is introduced into r1021. In our previous study (Metreveli et al., 2011) we showed the genotypic differences between the two swine influenza isolates in Sweden and the differences found in amino acid sequences of PB1 and PB1-F2 may explain their different phenotypes. Further studies will be needed to identify the amino acid changes responsible for these phenotypes. The reassortant H1N2 viruses may pose a threat for humans; since they have avian like H1 gene and therefore little pre-existing immunity is expected in humans. Our studies show that these H1N2 viruses differ in virulence due to the PB1 segment, which needs to be continuously monitored for these viruses during changes in virulence.

5. Conclusion

Overall, our findings show that the PB1 genomic segment of SIV H1N2 virus is a determinant of virulence and replication in mice. The selection of “virulent” highly replicative PB1s may be important when influenza viruses cross species barriers or when new viral strains are generated by reassortment. The successful interspecies transmission of influenza viruses depends on the viral gene constellation. Successful transmission between species can follow genetic reassortment with a progeny virus that contains a specific gene combination with the ability to replicate more efficiently in the new host. Continuous and efficient surveillance and further detailed phenotypic analysis can help to identify novel viruses with more potential to cross species and that pose health risks to humans.

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