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Pathogenicity and transmissibility of reassortant H9 influenza viruses with genes from pandemic H1N1 virus

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Both H9N2 avian influenza and 2009 pandemic H1N1 viruses (pH1N1) are able to infect humans and swine, which has raised concerns that novel reassortant H9 viruses with pH1N1 genes might be generated in these hosts by reassortment. Although previous studies have demonstrated that reassortant H9 viruses with pH1N1 genes show increased virulence in mice and transmissibility in ferrets, the virulence and transmissibility of reassortant H9 viruses in natural hosts such as chickens and swine remain unknown. This study generated two reassortant H9 viruses (H9N2/CA09 and H9N1/CA09) in the background of the pH1N1 A/California/04/2009 (CA09) virus by replacing either both the haemagglutinin (HA) and neuraminidase (NA) genes or only the HA gene with the respective genes from the A/quail/Hong Kong/G1/1997 (H9N2) virus and evaluated their replication, pathogenicity and transmission in chickens and pigs compared with the parental viruses. Chickens that were infected with the parental H9N2 and reassortant H9 viruses seroconverted. The parental H9N2 and reassortant H9N2/CA09 viruses were transmitted to sentinel chickens, but H9N1/CA09 virus was not. The parental H9N2 replicated poorly and was not transmitted in pigs, whereas both H9N2/CA09 and H9N1/CA09 viruses replicated and were transmitted efficiently in pigs, similar to the pH1N1 virus. These results demonstrated that reassortant H9 viruses with pH1N1 genes show enhanced replication and transmissibility in pigs compared with the parental H9N2 virus, indicating that they may pose a threat for humans if such reassortants arise in swine.

INTRODUCTION

H9N2 subtype avian influenza viruses are endemic in land-based poultry in Asia and have been documented to infect humans sporadically (Butt *et al.*, 2005; Lin *et al.*, 2000; Peiris *et al.*, 1999). Genetic characterization and phylogenetic analysis have revealed that there are three lineages of H9N2 viruses isolated from poultry in Asia: the H9N2/G1-like lineage represented by A/quail/Hong Kong/G1/1997 (H9N2/G1), H9N2/Y280-like lineage represented by A/duck/Hong Kong/Y280/1997 (H9N2/Y280) and an A/chicken/Korea/006/1996-like lineage (Guo *et al.*, 2000). In 1999, two human cases were reported to be infected with H9N2 subtype influenza A viruses causing mild respiratory symptoms (Peiris *et al.*, 1999), and these two H9N2 isolates are genetically and antigenically similar to the H9N2/G1 virus. In addition, it has been suggested that the H9N2/G1 strain is the internal gene donor for the 1997 H5N1 virus

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that caused several fatal human infections in Hong Kong (Guan *et al.*, 1999; Lin *et al.*, 2000). From 2002 to 2009, H9N2 viruses belonging to Y280-like and G1-like lineages were isolated occasionally from humans in Hong Kong and mainland China (Butt *et al.*, 2005; Cheng *et al.*, 2011). Infection of humans with avian H9N2 influenza viruses indicates that H9N2 viruses are able to cross the species barrier to infect humans directly from poultry without prior adaptation, although no human-to-human transmission has been found so far (Uyeki *et al.*, 2002).

H9N2 viruses have also been isolated from pigs in Asia. The first H9N2 swine isolate was identified from domestic pigs in Hong Kong in 1998 and belonged to the Y280-like lineage. Subsequently, different lineages of H9N2 viruses were isolated from swine in mainland China (Cong *et al.*, 2007; Peiris *et al.*, 2001; Shi *et al.*, 2008; Yu *et al.*, 2008, 2011). Furthermore, reassortment events in pigs have been detected between different H9N2 lineages or between the H9N2 virus and different subtypes (i.e. H5N1 and H6N2) of influenza A viruses (Cong *et al.*, 2007; Yu *et al.*, 2011). Sorrell *et al.* (2009) reported that H9N2 viruses containing internal genes from a human H3N2 virus can be transmitted efficiently via respiratory droplets in ferrets after adaptation by serial passages in ferret, raising the possibility that H9N2 viruses might acquire human-to-human transmissibility and initiate a human pandemic after reassorting with human or swine influenza viruses. Also of concern is that most of the current H9N2 isolates contain leucine at position 226 in the haemagglutinin (HA) receptor-binding site, which has been shown to mediate binding to mammalian α2,6-sialic acid-linked receptors and replication in human airway epithelial cells (Sorrell *et al.*, 2009; Wan & Perez, 2007).

The 2009 pandemic H1N1 viruses (pH1N1) caused the first flu pandemic of the 21st century. pH1N1 viruses not only infect humans but are also transmitted in other animal species including pigs (Liu *et al.*, 2012; Pasma & Joseph, 2010). Currently, pH1N1 viruses have been isolated from pigs worldwide (Howard *et al.*, 2011; Kim *et al.*, 2011; Moreno *et al.*, 2010; Sreta *et al.*, 2010; Starick *et al.*, 2011; Vijaykrishna *et al.*, 2010), and reassortments between pH1N1 and endemic swine influenza viruses in pigs have been documented (Howard *et al.*, 2011; Moreno *et al.*, 2011; Starick *et al.*, 2011; Vijaykrishna *et al.*, 2010). Such events may result in the generation of new potential pandemic strains with new HA genes for which humans lack pre-existing immunity. As both H9N2 and pH1N1 viruses are able to infect humans and swine, novel reassortant H9 strains might be generated via reassortment between H9N2 and pH1N1 viruses. Previous studies have shown that reassortant H9 viruses with pH1N1 genes exhibit higher virulence and transmissibility than the parental viruses in mice and ferrets (Kimble *et al.*, 2011; Sun *et al.*, 2011). However, so far, the replication capacity and transmissibility of reassortant H9 viruses with pH1N1 internal genes in influenza virus natural hosts – the chicken and pig – remain unknown. Here, we generated two reassortant H9 viruses (H9N1/CA09 and H9N2/CA09) in the genetic background of the pH1N1 strain A/California/04/2009 (CA09) by replacing either HA or both HA and neuraminidase (NA) genes with H9 and N2 genes from the influenza A/quail/Hong Kong/G1/1997 (H9N2) virus, and evaluated them for replication in different cell lines and for pathogenicity and transmissibility in chickens and pigs compared with the parental viruses.

RESULTS

Generation and characterization of parental and reassortant viruses

The parental (H9N2 and CA09) and two reassortant viruses generated by reverse genetics are shown in Fig. 1(a). Plaque assays showed that the H9N2, H9N2/CA09 and H9N1/CA09 viruses formed similar-sized plaques, which were larger than those formed by CA09. The CA09 virus formed tiny plaques in Madin–Darby canine kidney (MDCK) cells (Fig. 1b). Both parental and reassortant viruses replicated to similar titres in MDCK cells. However, in A549 cells, the reassortant H9N1/CA09 virus grew to significantly higher titres than the two parental viruses and the reassortant H9N2/CA09 virus throughout the course of infection at 24, 36 and 48 h p.i. (Fig. 1c).

Parental and reassortant H9N2 viruses transmitted in chickens

No clinical symptoms were observed in infected and contact chickens during the period of the study. The parental H9N2 and reassortant H9N2/CA09 viruses were detected in oropharyngeal swabs of >50 % of chickens (in five or eight of the nine chickens, respectively) on day 3 p.i. and mean virus titres ranged from 2.76 to 3.14 $\log_{10}$ ml^{-1} (Table 1). Only one chicken shed virus via the cloacal tract in the H9N2-infected (5 days p.i.) or H9N2/CA09-infected (3 days p.i.) group. On the other indicated days p.i., no virus was found in oropharyngeal or cloacal swabs collected from either challenged group (Table 1). This result indicated that the H9N2 and H9N2/CA09 viruses were shed through the respiratory tract in chicken, rather than from the intestinal tract. No virus was detected on the indicated days p.i. from either oropharyngeal and cloacal swabs collected from chickens infected with the H9N1/CA09 virus (Table 1). No macroscopic and microscopic lesions were observed in the lungs of any of the infected or contact chickens. Interestingly, no virus or viral antigen was detected by an immunohistochemistry (IHC) assay in tissues, including lungs, collected from all chickens infected with any of the three viruses on day 5 p.i. (data not shown). However, all infected chickens showed seroconversion at the end of the experiment on day 14 p.i., except for one chicken in the H9N2-infected group (Table 1).

No virus was found in oropharyngeal or cloacal swabs that were collected from contact chickens. However, seroconversion

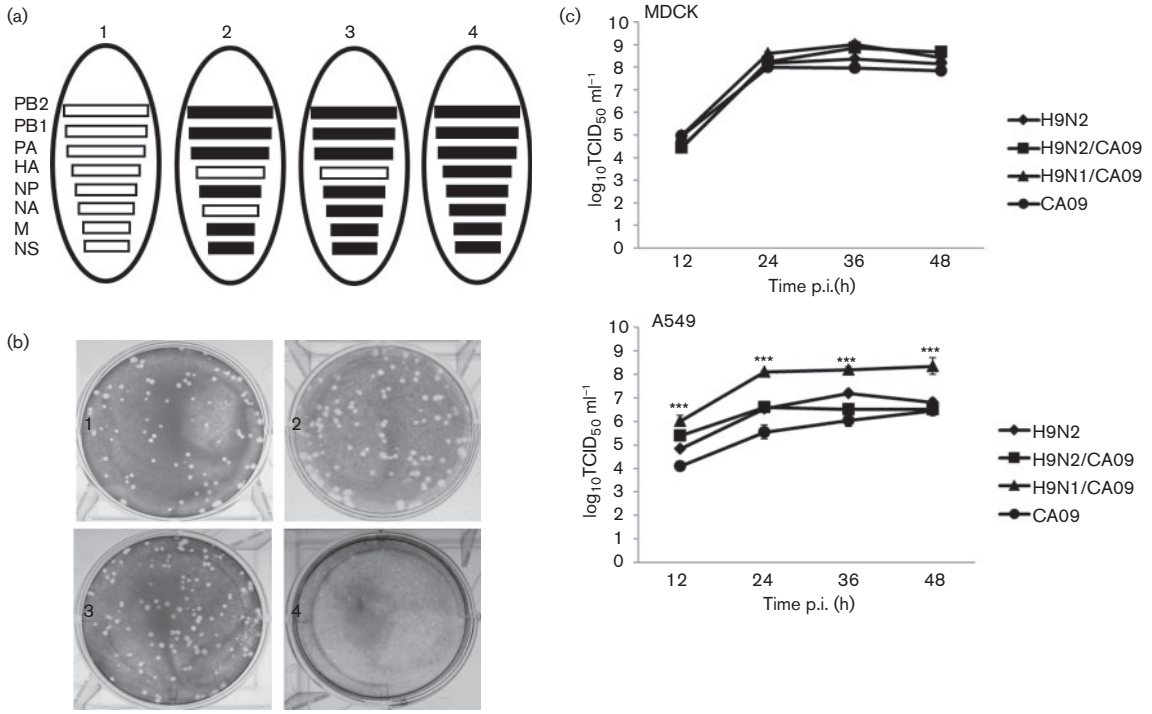

Fig. 1. Genotype, plaque morphology and growth kinetics of the influenza viruses in MDCK or A549 cells. (a) Genotype of the viruses. Open bars represent genes from A/quail/Hong Kong/G1/1997 (H9N2) and filled bars indicate genes derived from A/California/04/2009 (CA09). (b) Plaque sizes formed by the parental and reassortant viruses in MDCK cells at 2 days post-infection (p.i.). 1, H9N2; 2, H9N2/CA09; 3, H9N1/CA09; 4, CA09. (c) Growth dynamics of the parental and reassortant viruses in MDCK cells infected at an m.o.i. of 0.001 or in A549 cells infected at an m.o.i. of 0.1. Each data point on the curve indicates the mean ± SEM of three independent experiments. ***$P<0.001$.

was found in two (50 %) of four contact chickens in the parental H9N2 group, and in one (25 %) of four sentinel chickens in the H9N2/CA09 group at the end of the experiment. No contact chickens had seroconverted in the H9N1/CA09 group at the end of the experiment on day 13 post-contact (p.c.) (Table 1).

Reassortant H9 viruses show enhanced replication and transmissibility in pigs compared with the parental H9N2 virus

No obvious respiratory signs (e.g. coughing, sneezing) were observed in pigs infected with the parental or reassortant

Table 1. Virus shedding and antibody haemagglutination inhibition (HI) titres of chickens infected with wild-type and reassortant viruses on the indicated days p.i. or p.c.

Results are shown as the number of chickens shedding virus out of total numbers on days 3, 5, 7 and 9 p.i., or as the number of chickens that were seropositive out of total numbers on day 14 p.i. or day 13 p.c. NA, Not applicable (there were no contact chickens in the control group).

Virus	3 days p.i.*		5 days p.i.*		7 days p.i.		9 days p.i.		HI antibody 14 days p.i. (13 days p.c.)†	
	Trachea	Cloaca	Trachea	Cloaca	Trachea	Cloaca	Trachea	Cloaca	Infected	Contact
H9N2	5/9 (3.14 ± 0.19)	0/9	0/9	1/9 (3.5)	0/6	0/6	0/6	0/6	5/6 (139)	2/4 (160)
H9N2/CA09	8/9 (2.76 ± 0.11)	1/9 (4)	0/9	0/9	0/6	0/6	0/6	0/6	6/6 (126)	1/4 (80)
H9N1/CA09	0/9	0/9	0/9	0/9	0/6	0/6	0/6	0/6	6/6 (63)	0/4 (0)
Control	0/9	0/9	0/9	0/9	0/6	0/6	0/6	0/6	0/6 (0)	NA

*Numbers in parentheses indicate virus titres as $\log_{10}$ ml^{-1} (mean ± SEM).
†Numbers in parentheses indicate geometric mean HI titres for samples with a value ⩾20.

viruses. However, >80 % of pigs (ten of the 12) infected with the CA09 virus had a fever (≥39.9 °C) and ~70 % of animals (eight of the 12) had diarrhoea during the period of the experiment. In the other three challenged groups, there were limited numbers (one to three) of pigs with fever and diarrhoea. Macroscopic lung lesions were found in pigs in all inoculated groups, but no lesions were observed in the control pigs. CA09 virus caused significantly more severe macroscopic and histopathological lung lesions in all infected pigs than the other three viruses (Table 2). H9N2 only caused lung lesions in 25–50 % of the infected pigs on the indicated days p.i., whereas both reassortant H9N2/CA09 and H9N1/CA09 viruses induced lung lesions in 75–100 % of the pigs (Table 2). No significant difference was observed in lung lesion scores induced by these three viruses. H9N2 virus replicated poorly in pig lungs, and virus was isolated only from one of four bronchioaveolar lavage fluid (BALF) samples on days 3 and 5 p.i. (Table 3). In the other three infected groups, virus was detected in BALF from all of the infected pigs on days 3 and 5 p.i., except that virus was only found in one of four pigs of the H9N2/CA09-infected group at 3 days p.i. Virus was also found in the lungs of pigs infected with the parental CA09 or reassortant H9N1/CA09 virus on day 7 p.i. but not from those infected with parental H9N2 and reassortant H9N2/CA09 viruses (Table 3). In animals positive for virus isolation, no significant differences were observed in the virus titres of the four challenged groups.

The parental H9N2 virus was shed inefficiently, as only one out of 12 pigs shed virus on day 3 p.i. In contrast to the parental H9N2 virus, both reassortant viruses were shed more efficiently, similarly to the parental CA09 virus (Table 3). Nine (75 %) and six (50 %) of the 12 pigs on day 3 p.i. and six (75 %) and five (62.5 %) of the eight pigs on day 5 p.i. shed virus from the nasal cavity in the groups infected with the H9N2/CA09 and H9N1/CA09 reassortant viruses, respectively. On day 7 p.i. one (25 %) of four pigs was still shedding virus via the nasal cavity in the group infected with H9N1/CA09 virus, similar to the group infected with CA09 virus. No nasal shedding was found in pigs infected with H9N2 or H9N2/CA09 virus on day 7 p.i. (Table 3). All viruses were able to cause variable degrees of lung damage to infected pigs, i.e. mild to moderate bronchointerstitial pneumonia, atelectasis, acute to subacute bronchiolitis with epithelial necrosis, and variable lymphocytic cuffing of bronchioles. CA09 virus induced significant histopathological lung damage in pigs compared with the two reassortant and parental H9N2 viruses (Fig. 2), in agreement with the macroscopic lung scores observed. No significant differences were observed among the parental H9N2 and two reassortant viruses in microscopic lung lesions (Table 2). Viral antigens were detected in the lungs of pigs infected with the parental CA09 and two reassortant viruses on day 5 p.i. but not in the lungs of pigs infected with the parental H9N2 virus (Fig. 3).

No macroscopic lung lesions were observed in contact pigs of the parental H9N2 group. Sentinel pigs had significantly more severe lung lesions in the CA09 virus group when compared with the other two groups infected with the reassortant viruses. Only limited numbers of lung lesions were found in several contact pigs in the reassortant H9N2/CA09 and H9N1/CA09 groups (Table 2). No virus was found in the lungs of contact pigs in the H9N2 group on day 5 p.c. Virus was detected in the lungs of two and four of the four contact pigs in the H9N2/CA09 and H9N1/CA09 groups, respectively. CA09 virus was found in the lungs of all four contact pigs. Nasal virus shedding was found only in one contact pig in the H9N2 group on day 4 p.c., and none was detected on days 2 and 5 p.c. In both reassortant virus groups, virus was detected from nasal swabs collected from one to three pigs out of four animals on days 2, 4 and 5 p.c., which was similar to the results of the CA09 group (Table 3). Microscopic lung lesions were found in several contact pig lungs in the two reassortant and CA09 virus groups but not in those in the parental H9N2 virus group (Table 2). No significant difference was observed in lung lesions among the two reassortant and CA09 virus groups. Overall, these results indicated that both reassortant viruses, but not the parental H9N2 virus, were able to replicate efficiently in pigs and be transmitted to sentinels.

DISCUSSION

H9N2 subtype influenza A viruses are circulating widely in poultry in Asia. Since the 1990s, sporadic infection with H9N2 viruses in mammals including swine and humans has been reported (Bi *et al.*, 2010; Choi *et al.*, 2004; Dong *et al.*, 2011; Zhang *et al.*, 2009), and these events occur continuously (Yu *et al.*, 2011). Recent isolates of H9N2 possess leucine at position 226 in the receptor-binding site of the HA, which was found to contribute to human virus-like receptor specificity (Butt *et al.*, 2010; Wu *et al.*, 2010). A previous study showed that only minimal molecular changes were required for respiratory-droplet transmission of an avian H9N2 with human influenza internal genes and containing leucine at position 226 of the HA in a ferret model (Sorrell *et al.*, 2009), indicating that H9N2 subtype viruses might pose a threat for public health if they reassort with mammalian influenza viruses or adapt to mammals via mutation.

Both H9N2 and pH1N1 viruses are able to infect pigs. Recent studies have shown the high genetic compatibility of reassortants between H9N2 avian influenza virus and pH1N1 virus, and demonstrated that some reassortants have increased pathogenicity in mice (Sun *et al.*, 2011) and efficient respiratory-droplet transmission in a ferret model (Kimble *et al.*, 2011). In the present study, we tested reassortant viruses between an H9N2 and pH1N1 virus *in vitro* and in two influenza virus natural hosts, chicken and swine. *In vitro* studies showed that pH1N1 formed smaller-sized plaques in MDCK cells when compared with the parental and reassortant H9 viruses, but they exhibited similar growth kinetics in this cell line, suggesting that growth kinetics and virulence is not always consistent with plaque size formed by the virus, as is often discussed.

Table 2. Macroscopic and microscopic lung lesions in infected and contact pigs infected with parental and reassortant viruses on the indicated days p.i. or p.c.

Results are shown as the number of pigs with lung lesions out of the total number of pigs on the days indicated. Numbers in parentheses are the mean percentage of macroscopic or the mean score of microscopic lung lesions (±SEM). Significant differences ($P<0.05$) in the number of lung lesions between two groups are indicated by the same letter. NA, Not applicable (there were no contact pigs in the control group).

Virus	Infected pigs			Contact pigs
	Day 3 p.i.	Day 5 p.i.	Day 7 p.i.	Day 5 p.c.
Macroscopic lesions				
H9N2	2/4 (5.00±1.43)[a]	2/4 (2.50±1.79)[d]	1/4 (4.57)[g]	0/4 (0)
H9N2/CA09	3/4 (5.00±3.75)[b]	4/4 (10.07±4.20)[e]	3/4 (5.29±1.80)[h]	3/4 (1.38±0.33)
H9N1/CA09	4/4 (7.46±3.36)[c]	3/4 (8.10±2.85)[f]	3/4 (8.66±1.06)[i]	1/4 (2)
CA09	4/4 (35.00±8.93)[a,b,c]	4/4 (37.25±2.67)[d,e,f]	4/4 (28.07±4.02)[g,h,i]	4/4 (9.04±4.53)
Control	0/3 (0.00±0.00)	0/3 (0.00±0.00)	0/3 (0.00±0.00)	NA
Microscopic lesions				
H9N2	2/4 (0.75±0.25)[j]	2/4 (1.25±0.25)	2/4 (0.5±0.00)[m]	0/4 (0)
H9N2/CA09	3/4 (0.83±0.17)[k]	4/4 (1.13±0.24)	3/4 (0.83±0.17)[n]	3/4 (0.67±0.17)
H9N1/CA09	4/4 (1.38±0.38)	3/4 (0.83±0.17)[l]	3/4 (1.00±0.00)[o]	1/4 (0.50±0.00)
CA09	4/4 (2.50±0.20)[j,k]	4/4 (2.75±0.25)[l]	4/4 (2.25±0.32)[m,n,o]	3/4 (1.17±0.44)
Control	0/3 (0.00±0.00)	0/3 (0.00±0.00)	0/3 (0.00±0.00)	NA

Furthermore, *in vitro* results sometimes do not mirror *in vivo* viral pathogenicity data. This point was evidenced by our pig study, where the reassortant H9N1/CA09 virus displayed significantly less pathogenicity in pigs compared with the pH1N1 virus, despite forming larger plaques in MDCK cells and growing to higher titres in human A549 cells. Although the parental H9N2 and reassortant H9 viruses did not cause any clinical signs or lung lesions (macroscopic or microscopic) in infected chickens, seroconversion indicated that they were able to infect chickens. Interestingly, the reassortant H9N1/CA09 virus was not transmissible among chickens, whereas both the H9N2/CA09 and parental H9N2 viruses showed transmissibility in this host, suggesting that transmission of the reassortant among chickens is limited by the HA/NA combination. In contrast, both H9 reassortant viruses had increased infectivity and transmissibility in pigs compared with the parental H9N2 virus, and exhibited similar replication and transmissibility

Table 3. Virus titres of nasal swabs and lungs (BALF) of infected and contact pigs infected with parental and reassortant viruses on the indicated days p.i. or p.c.

Results are shown as the number of pigs positive for virus isolation out of the total number of pigs on the days indicated. Contact pigs were co-mingled with infected animals for 2, 4 and 5 days. Numbers in parentheses indicate the virus titres presented by $\log_{10}$ geometric mean ± SEM $TCID_{50}$ ml^{-1} among positive samples. No SEM is shown where only one pig shed virus. Significant differences ($P<0.05$) in virus titre between two groups are labelled with the same letter. NA, Not applicable (there were no nasal swabs or BALF samples on the indicated dates in the group).

Virus	Infected pigs			Contact pigs		
	3 days p.i.	5 days p.i.	7 days p.i.	2 days p.c.	4 days p.c.	5 days p.c.
Nasal swabs						
H9N2	1/12 (4.50)	0/8 (0)	0/4 (0)	0/4	1/4 (2.30)	0/4(0)
H9N2/CA09	9/12 (3.69±0.35)	6/8 (3.82±0.41)	0/4 (0)	1/4 (2.80)	2/4 (3.00±1.00)	2/4 (3.40±1.10)
H9N1/CA09	6/12 (3.33±0.47)	5/8 (2.94±0.31)	1/4 (2.50)	2/4 (2±0.00)	3/4 (2.67±0.67)	3/4 (3.33±0.71)
CA09	9/12 (3.74±0.39)	8/8 (3.36±0.20)	2/4 (2.75±0.25)	1/4 (3)	3/4 (5.57±0.45)	4/4(4.38±0.08)
Control	0/3 (0.00±0.00)	0/3 (0.00±0.00)	0/3 (0.00±0.00)	NA	NA	NA
BALF						
H9N2	1/4 (3.00)	1/4 (4.50)	0/4 (0)	NA	NA	0/4 (0)
H9N2/CA09	1/4 (4.80)	4/4 (4.53±0.16)	0/4 (0)	NA	NA	2/4 (3.00±0.30)[a|]
H9N1/CA09	4/4 (4.35±0.48)	4/4 (5.30±0.58)	1/4 (2.50)	NA	NA	3/4 (2.47±0.15)[b]
CA09	4/4 (4.95±0.86)	4/4 (5.60±0.43)	3/4 (3.33±0.17)	NA	NA	4/4 (6.47±0.17)[a,b]
Control	0/3 (0.00±0.00)	0/3 (0.00±0.00)	0/3 (0.00±0.00)	NA	NA	NA

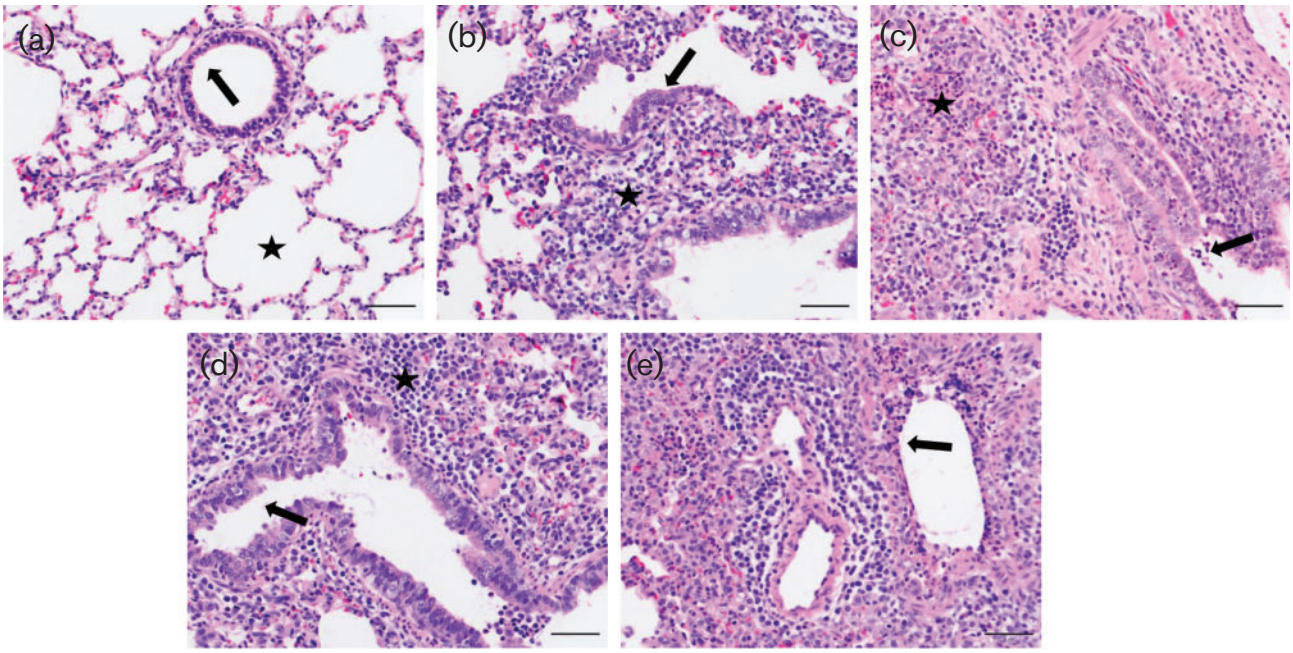

Fig. 2. Haematoxylin and eosin staining of microscopic lung sections from pigs infected with parental or reassortant viruses on day 5 p.i. (a) The bronchioles were lined by normal cuboidal epithelium (arrow) and the alveoli were clear (asterisk) in the control group. (b) In the parental H9N2 virus group, the alveolar interstitium and peribronchiolar areas contained small to moderate numbers of lymphocytes (asterisk). A mild bronchiolar epithelial hyperplasia (arrow) was noted. (c) In the reassortant H9N2/CA09 virus, the alveolar interstitium and peribronchiolar areas contained small to moderate numbers of lymphocytes and degenerate neutrophils (asterisk). Mild bronchiolar epithelial degeneration and necrosis (arrow) were observed. (d) In the reassortant H9N1/CA09 virus group, the peribronchiolar areas contained small to moderate numbers of lymphocytes and fewer degenerate neutrophils (asterisk). Mild bronchiolar epithelial hyperplasia and degeneration (arrow) were noted. (e) In the parental CA09 virus group, severe bronchiolar epithelial necrosis was seen (arrow). The peribronchiolar and interstitial areas contained moderate numbers of lymphocytes and fewer neutrophils. Bars, 50 μm.

in pigs to the pH1N1 virus. These results indicated that the H9N2 avian influenza viruses are able to gain the ability to replicate and be transmitted in pigs through reassortment with the pH1N1 virus. These reassortants have increased properties compared with the parental H9N2 virus to become established and maintained in this mammalian host. If this occurs in the future, H9 reassortant viruses might be candidates to cause the next human influenza pandemic.

The reassortant H9N1/CA09 virus was shed for longer, cleared later and transmitted more efficiently in pigs than the reassortant H9N2/CA09 virus. The only difference between these two reassortants was the NA gene (N1 or N2). A recent study showed that the balance of HA and NA confers respiratory-droplet transmissibility of pH1N1 in ferrets and that the NA of pH1N1 had significantly higher enzyme activity than that of swine influenza viruses (Yen *et al.*, 2011). The reassortant H9N2/CA09 virus might have matched avian-origin H9 and N2 genes for fitness in chicken, but an H9 and N1 gene combination might provide better fitness in swine.

A previous study demonstrated two-way transmission of the H9N2 virus between terrestrial and aquatic ducks, facilitating the generation of novel reassortant H9N2 viruses (Li *et al.*, 2003). Recent H9N2 isolates from domestic ducks in Vietnam in 2009 and 2010 belonged to the G1 sublineage and were shown to readily infect pigs (Nomura *et al.*, 2012). Reassortant H9 viruses have been found in diseased pigs in the field (Cong *et al.*, 2007). In this study, we demonstrated that H9 viruses were able to adapt to pigs by reassortment with pH1N1, resulting in increased pathogenicity and transmissibility in this host. It will be interesting to investigate whether reassortment of H9N2 viruses with other swine influenza viruses also leads to increased replication and transmission in the pig.

Taken together, previous results (Kimble *et al.*, 2011) and our studies demonstrate that H9N2 avian influenza viruses can reassort with pH1N1 virus, resulting in increased pathogenicity and transmissibility of H9 reassortant viruses in pigs and ferrets. This finding justifies and warrants continuous surveillance of influenza viruses in swine, especially in areas where avian influenza H9N2 viruses have been reported to infect pigs.

METHODS

Cell and viruses. Human embryonic kidney 293T cells were maintained in Opti-MEM (Invitrogen) supplemented with 10 %

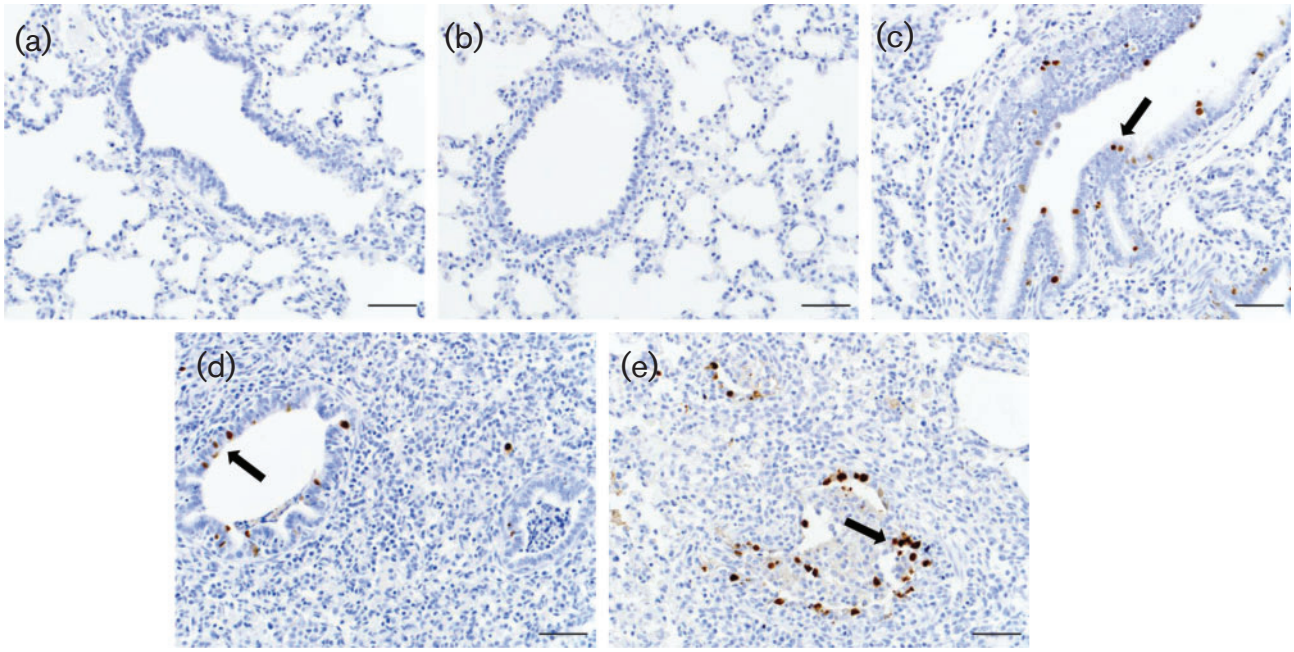

Fig. 3. IHC staining of microscopic lung sections from pigs infected with parental and reassortant viruses on 5 days p.i. (a, b) No positive immunohistochemical reaction was seen in controls (a) and in lung sections of pigs infected with the parental H9N2 virus (b). (c, d) A mild positive reaction (arrow) was seen in lung sections of pigs infected with the reassortant H9N2/CA09 virus (c) and with the reassortant H9N1/CA09 virus (d). (e) A moderate to strong positive reaction (arrow) was seen in lung sections of pigs infected with the CA09 virus. Anti-influenza A virus mAb against NP was used for IHC staining. Bars, 50 μm.

FBS (HyClone). MDCK cells were maintained in Eagle's minimal essential medium (MEM) with 5 % FBS, L-glutamine (Invitrogen), 1× MEM vitamins (Invitrogen) and 1 % antibiotics (Invitrogen). Human alveolar epithelial A549 cells were maintained in Dulbecco's MEM with 10 % FBS, L-glutamine, 1× MEM vitamins and 1 % antibiotics. Cells were inoculated with the respective viruses in MEM infecting medium containing 0.3 % BSA (Sigma), 1 μg tosyl phenylalanyl chloromethyl ketone-treated trypsin (Sigma) ml^{-1}, L-glutamine, 1× MEM vitamins and 1 % antibiotics.

Avian influenza virus A/quail/Hong Kong/G1/1997 (H9N2) and 2009 human pH1N1 A/California/04/2009 (CA09) viruses were generated by eight-plasmid reverse genetics as described previously (Mok *et al.*, 2011). Reassortant viruses were produced using reverse genetics in the background of the CA09 virus by replacing the CA09 HA or HA and NA genes with the respective H9N2 genes to produce H9N1/CA09 and H9N2/CA09 reassortant viruses, as depicted in Fig. 1(a). All viruses were plaque purified and passaged three times in MDCK cells and confirmed by sequencing.

Growth kinetics of parental and reassortant viruses. To evaluate the growth kinetics of the parental and reassortant viruses, monolayers of MDCK (m.o.i.=0.001) or A549 (m.o.i.=0.1) cells were cultured in six-well plates and infected with different viruses at the indicated m.o.i. Supernatants were collected at different time points (12, 24, 36 and 48 h p.i.) and titrated by infecting MDCK cells in 96-well plates. The $TCID_{50}$ ml^{-1} was calculated by the method of Reed & Muench (1938). Plaque assays were also conducted on MDCK cells to compare the size of plaques formed by different viruses.

Animal experiments

All animal experiments, including chicken and pig studies, were conducted in Biosafety Level 2+ facilities and approved by the Institutional Animal Care and Use Committee at Kansas State University.

Chickens. To determine the replication, pathogenicity and transmissibility of parental H9N2 and the two reassortant viruses, 4-week-old specific-pathogen-free (SPF) white leghorn chickens were used in this study. A total of 48 SPF chickens were allocated randomly to four groups (13 chickens in each infected group and nine chickens in the control group). Nine chickens from each group were infected with either 10^7 $TCID_{50}$ virus in 250 μl fresh MEM or 250 μl virus-free MEM (200 μl was applied to the nostrils and 50 μl was dripped into the eyes). At 2 days p.i., four naïve chickens were co-caged with the inoculated birds in each infected group, sharing food and drinking water. All birds were observed daily for clinical signs until the end of the experiment (14 days). Oropharyngeal and cloacal swabs were collected on days 0, 3, 5, 7 and 9 p.i. from the infected birds and on days 0, 2, 4, 6 and 8 days p.c. from the contact birds; swabs were used to infect MDCK cells to determine virus loads. Blood samples were collected on day 0 and on the last day of the experiment for HI assays. The HI assay was performed to test for antibodies against the inoculated H9 viruses, as described previously (Ma *et al.*, 2007). Three chickens from each group were euthanized on day 5 p.i. to investigate virus replication; the remaining birds were euthanized at the end of experiment. During necropsy, tissue samples of lung, heart, liver, spleen, brain, small intestine and large intestine were collected from each chicken for virus isolation and histopathological examination. IHC staining was performed using anti-influenza A virus mAb against NP (Thermo Scientific).

Pigs. Seventy-three 3–4-week-old swine influenza virus-negative and porcine reproductive and respiratory syndrome virus-negative pigs were allocated randomly to five groups (16 pigs in the four infected

groups and nine pigs in the control group). Twelve pigs in each infected group and the nine control pigs were inoculated intratracheally with 10^6 $TCID_{50}$ virus (H9N2, H9N2/CA, H9N1/CA and CA09) or virus-free MEM. Four contact pigs were co-mingled with each infected group on day 2 p.i. to investigate virus transmission. Four infected and three control pigs were necropsied on days 3, 5 and 7 p.i., and four contact pigs were euthanized on day 5 p.c. Nasal swabs were collected from infected pigs on days 0, 3, 5, 7 p.i. and from contact animals on days 0, 2, 4, 5 p.c. Blood samples were collected from all pigs before challenge and at the time of necropsy. During necropsy, the lungs were removed *in toto* from pigs. The percentage of gross lesions on each lung lobe was scored by a single experienced veterinarian. BALF was obtained by flushing each lung with 50 ml MEM, and viral titres of BALF and nasal swabs were determined in MDCK cells, as described previously (Ma *et al.*, 2007). The right cardiac lung lobe was collected and fixed in 10 % buffered formalin and stained with haematoxylin and eosin for histopathological examination. IHC staining was performed using anti-influenza A mAb against NP. Lung sections were examined by a veterinary pathologist in a blind fashion and given a score of 0–3 to reflect the severity of bronchial epithelial injury, as described previously (Richt *et al.*, 2003).

Statistical analysis. Macroscopic and microscopic lung scores and virus titres were analysed by analysis of variance in GraphPad Prism version 5.0 (GraphPad Software). A value of $P \leqslant 0.05$ was considered significant. Those response variables shown to have a significant effect by treatment group were subjected to comparisons for all pairs using a Tukey–Kramer test. Pair-wise mean comparisons between inoculated and control groups were made using Student's *t*-test.

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