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Replication and transmission of mammalian-adapted H9 subtype influenza virus in pigs and quail

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Influenza A virus is a major pathogen of birds, swine and humans. Strains can jump between species in a process often requiring mutations and reassortment, resulting in outbreaks and, potentially, pandemics. H9N2 avian influenza is predominant in poultry across Asia and occasionally infects humans and swine. Pandemic H1N1 (H1N1pdm) is endemic in humans and swine and has a history of reassortment in pigs. Previous studies have shown the compatibility of H9N2 and H1N1pdm for reassortment in ferrets, a model for human infection and transmission. Here, the effects of ferret adaptation of H9 surface gene segments on the infectivity and transmission in at-risk natural hosts, specifically swine and quail, were analysed. Reassortant H9N1 and H9N2 viruses, carrying seven or six gene segments from H1N1pdm, showed infectivity and transmissibility in swine, unlike the wholly avian H9N2 virus with ferret-adapted surface genes. In quail, only the reassortant H9N2 with the six internal gene segments from the H1N1pdm strain was able to infect and transmit, although less efficiently than the wholly avian H9N2 virus with ferret-adapted surface genes. These results highlight that ferret-adapted mutations on the haemagglutinin of H9 subtype virus do not restrict the ability of the virus to infect swine and quail, and that the ability to transmit in these species depends on the context of the whole virus. As such, this study emphasizes the threat that H9N2 reassortant viruses pose to humans and agricultural species and the importance of the genetic constellation of the virus to its ability to replicate and transmit in natural hosts of influenza.

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INTRODUCTION

Influenza A viruses (IAVs) are classified into subtypes based on the antigenic differences of the surface glycoproteins haemagglutinin (HA) and neuraminidase (NA) into 18 HA and 11 NA subtypes, with aquatic birds considered the natural hosts of all subtypes of except H17N10 and H18N11 viruses (Fouchier *et al.*, 2005; Gamblin & Skehel, 2010; Tong *et al.*, 2012, 2013; Webster *et al.*, 1992). IAVs of the H9N2 subtype are low-pathogenic viruses, and two geographically distinct lineages have been described – the North American and Eurasian lineages. Members of the Eurasian lineage jumped into poultry in Asia in the late 1980s causing outbreaks. These outbreaks have occurred in land-based poultry in many countries including China, Iran, Pakistan, Lebanon, Israel and Egypt (Barbour *et al.*, 2006; Davidson *et al.*, 2014; Naeem *et al.*, 1999; Nili & Asasi, 2003), and H9N2 viruses are currently endemic in poultry populations across much of Asia and the Middle East, mainly in chickens, quail and turkeys (Alexander, 2000; Guo *et al.*, 2000; Naeem *et al.*, 1999; Sun *et al.*, 2010).

The H9N2 IAV host range is not restricted to birds. Human and swine infections with H9N2 have been reported. Human infections with H9N2 viruses are relatively few and have been associated with direct contact with infected birds (Uyeki *et al.*, 2002). The first human isolates of H9N2 were recovered in 1999 in Hong Kong from two patients reporting mild respiratory symptoms (Peiris *et al.*, 1999). Studies showed that these isolates were of the Eurasian G1-like sublineage (Lin *et al.*, 2000). Subsequent isolates from sporadic human cases have been from the G1- and Y280-like lineages (Butt *et al.*, 2005, 2010; Cheng *et al.*, 2011). In 1998, the first swine H9N2 of the Y280-like sublineage was isolated in Hong Kong. Unlike human infections, swine infections appear

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to be more common and are associated with all sublineages (Cong *et al.*, 2007; Rui-Hua *et al.*, 2011; Xu *et al.*, 2008). Incidental and experimental infection of canines and felines with H9N2 have also been reported (Zhang *et al.*, 2013)

H9N2 viruses are of particular interest as they have been identified along with H7 and H5 as having pandemic potential (WHO, 2011). H9N2 viruses have also been identified as the donor, as well as recipient, of the internal genes to the highly pathogenic IAV H5N1 and the newly emerged H7N9 and H10N8 viruses that have infected humans in Asia (Chen *et al.*, 2014; Liu *et al.*, 2013; Wu *et al.*, 2013). Homosubtypic and heterosubtypic H9N2 reassortants have been isolated from both birds and swine in nature (Abolnik *et al.*, 2007; Cong *et al.*, 2007; Wang *et al.*, 2012; Wu *et al.*, 2015; Yu *et al.*, 2008, 2011). Under experimental conditions, H9N2 viruses also demonstrate compatibility for reassortment, especially with regard to human seasonal H3N2 and H1N1pdm viruses, with many reassortants showing increased infectivity, transmissibility and pathogenicity in mice and ferrets (Kimble *et al.*, 2011, 2014; Qiao *et al.*, 2012; Sorrell *et al.*, 2009; Sun *et al.*, 2011; Wan *et al.*, 2008)

We previously reported that a reassortant virus having surface genes from an avian H9N2 virus (A/guinea fowl/Hong Kong/WF10/1999) and internal genes from a human H3N2 virus (A/Memphis/14/1998) – herein referred to as 2WF10 : 6M98 – were transmitted only to direct-contact (DC) ferrets (Wan *et al.*, 2008). We further showed that following mammalian adaptation of the 2WF10 : 6M98 virus in ferrets, a ferret-adapted virus emerged, 2P10 : 6M98, in which only three amino acid changes in the surface genes (T189A in HA1, G192R in HA2 and I28V in the NA) were sufficient for airborne transmission to indirect-contact ferrets (Sorrell *et al.*, 2009). Transferring the HA gene segment of the 2P10 : 6M98 virus (with or without the NA gene segment) into the H1N1pdm backbone (1P10 : 7pdm or 2P10 : 6pdm) also allowed efficient airborne transmission in ferrets (Kimble *et al.*, 2011, 2014).

Whilst our previous results show that two mutations (T189A in HA1 and G192R in HA2) in the HA were essential for airborne transmission of H9 : H1N1pdm reassortants in ferrets, it is unknown whether these mutations would restrict the host range of the reassortant viruses. In this regard, it is important to note that the ferret is widely used as an animal model to study airborne transmission of influenza viruses; adaptive changes that lead to such a phenotype have been identified in the H9, H7 and H5 subtypes (Herfst *et al.*, 2012; Imai *et al.*, 2013; Kimble *et al.*, 2011; Ku *et al.*, 2014; Li *et al.*, 2014; Sorrell *et al.*, 2009; Sutton *et al.*, 2014; Wan *et al.*, 2008). However, further studies are lacking to demonstrate whether the ferret-adaptive mutations increase or restrict the host range of the virus. In this particular case, we aimed to investigate whether H9 HA ferret-adaptive mutations would affect the replication and transmission phenotype of these reassortants in pigs and quail. We chose the pig, an important agricultural species and intermediate host of influenza virus, and the quail, a land-based poultry species that has been shown to allow the replication of multiple influenza viruses (Makarova *et al.*, 2003). Our results showed that the replication and transmission of H9 : H1N1pdm reassortant viruses differed in the swine and quail hosts, and that molecular changes that confer efficient transmission can be virus and host specific.

RESULTS

Effects of ferret-adapted mutations on infectivity, transmissibility and pathogenicity of H9 subtype viruses in swine

To test the effect of mammalian-adapted (ferret-adapted) mutations of the H9 HA (Kimble *et al.*, 2011; Sorrell *et al.*, 2009) for replication and transmission in pigs and quail, five viruses were prepared (Table 1). Viruses containing the ferret-adapted H9 HA gene segment from the A/ferret/Maryland/P10_UMD/2008 (H9N2) virus (2P10 : 6WF10, 2P10 : 6pdm and 1P10 : 7pdm) were rescued in the context of the avian-origin A/guinea fowl/Hong Kong/WF10/1999 (H9N2) (WF10, G1 lineage) or the pandemic-origin A/Netherlands/602/2009 (H1N1) (H1N1pdm). Controls included reverse genetics versions of the wt WF10 and H1N1pdm viruses. Pigs were screened for prior influenza exposure and were negative by an ELISA prior to the study. IAV was not detected in nasal secretions of any pig at 0 days post-inoculation (p.i.). Negative-control pigs remained negative for virus isolation throughout the course of the experiment. Pigs, regardless of group,

Table 1. Genetic content and mutations in viruses used for each experimental group in pigs and quail

Virus group	HA subtype	NA subtype	Internal gene	Mutations (gene)
2P10 : 6WF10	H9 (ferret adapted)	N2 (ferret adapted)	Avian H9N2	T189A (HA1), G192R (HA2) I28V (NA)
2P10 : 6pdm	H9 (ferret adapted)	N2 (ferret adapted)	Pandemic H1N1	T189A (HA1), G192R (HA2) I28V (NA)
1P10 : 7pdm	H9 (ferret adapted)	N1(human adapted)	Pandemic H1N1	T189A (HA1), G192R (HA2)
WF10	H9 (avian)	N2 (avian)	Avian H9N2	NA
H1N1pdm	H1 (human adapted)	N1 (human adapted)	Pandemic H1N1	NA

NA, Not applicable.

showed no clinical signs of disease over the course of the study.

None of the 15 pigs directly inoculated (DI) with the 2P10 : 6WF10 (H9N2 virus with ferret-adaptive mutations in the background of a wt avian H9N2 virus) showed viral shedding at any sampling time points, nor did the DC pigs (data not shown). In contrast and as expected, all nasal swabs from the wt H1N1pdm DI group were positive from 2 to 5 days p.i. and had lower levels of viral shedding on day 6 (3/5 positive), and the pigs stopped shedding virus by 8 days p.i. (Fig. 1, and data not shown). All six H1N1pdm DC pigs were positive for virus by 2 days post-contact (p.c.) and continued shedding for up to 6 days p.c. before beginning to clear the virus, with low levels of virus shedding by 8 days p.c. (3/6 positive) and 10 days p.c. (1/6 positive). The 2P10 : 6pdm (H9N2 virus with ferret-adaptive mutations in the background of six internal gene segments from H1N1pdm) DI pigs shed virus from 2 to 6 days p.i., although the virus titre was, on average, $\sim$1.5 $\log_{10}$ lower than the group infected with the H1N1pdm virus. DC pigs in the 2P10 : 6pdm group became infected later when compared with the H1N1pdm group. Additionally, there was an apparent second round of transmission within the infected DC pigs, as two previously negative DC pigs began shedding at 10 days p.c., well after the DI pigs had cleared the virus. Overall, four of the six DC pigs became infected with 2P10 : 6pdm virus. Finally, all 1P10 : 7pdm (H9N1 virus with ferret-adaptive mutations in the background of seven gene segments from H1N1pdm) DI pigs shed virus from 2 to 6 days p.i. but were negative by 8 days p.i. (Fig. 1, and data not shown). The 1P10 : 7pdm virus was transmitted efficiently, as five of the six DC pigs began

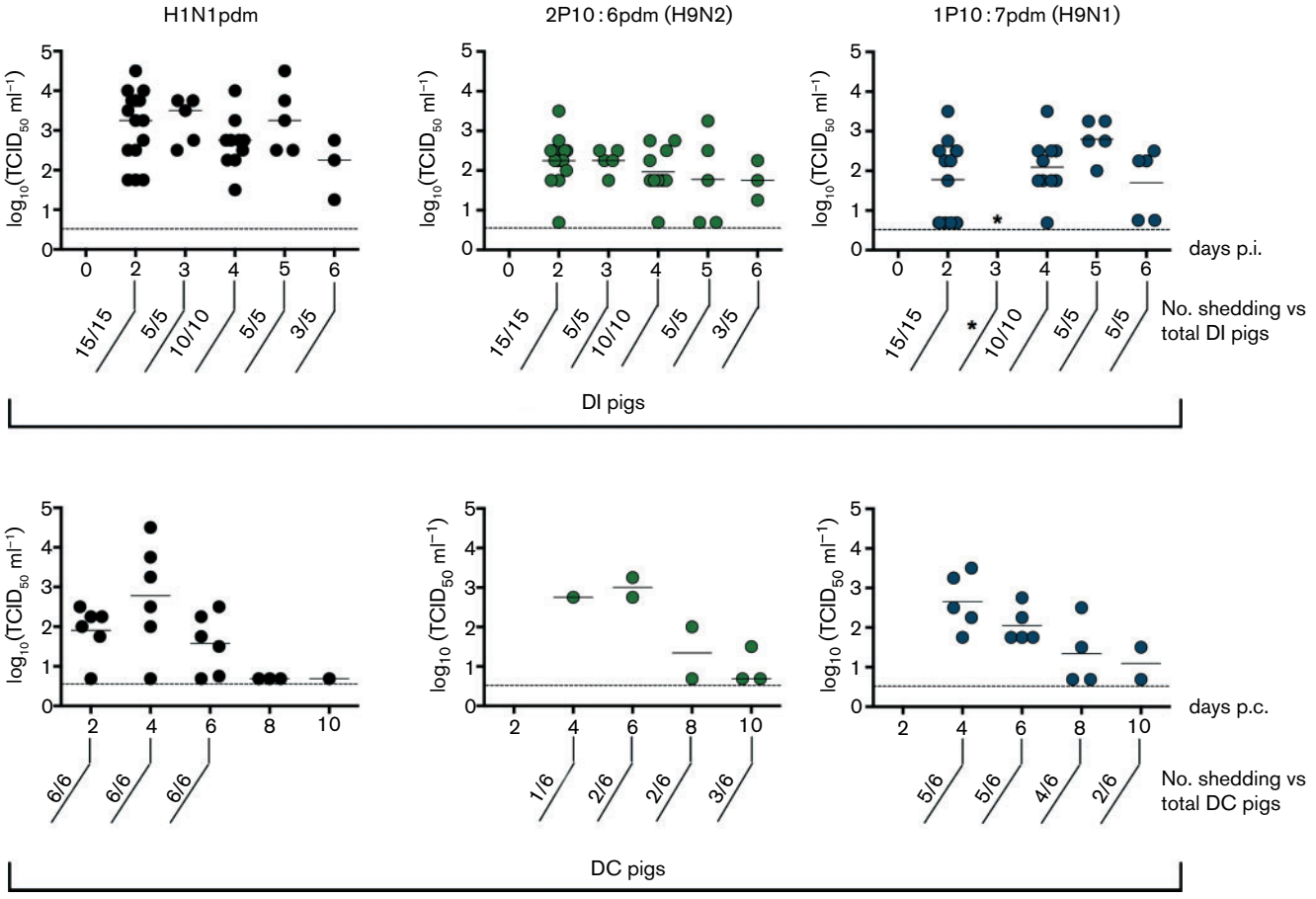

Fig. 1. Reassortant H9 viruses are transmitted in swine. Pigs (n=15 per group) were inoculated (DI) with the viruses indicated and nasal swabs were collected at various days p.i. The number of DI pigs positive for viral shedding in each group versus the total number of pigs is listed under each time point. At 3 and 5 days p.i., five DI pigs per group were sacrificed for virus titration in bronchoalveolar fluid. DC pigs (n=6 per group) were introduced in the same pen housing the DI pigs at 1 day p.i. Nasal swabs were collected from DC pigs and titrated as indicated above. The number of DC pigs positive for viral shedding in each group versus the total number of pigs is listed under each time point. Note the DC graphs were measured in days post-contact (days p.c.) not days p.i. The dashed line indicates the limit of detection and the asterisk denotes virus titers in samples just above limit of detection.

shedding by 4 days p.c. with clearance by 8–10 days p.c. There was also an apparent secondary transmission event in this group, as the one previously negative DC pig began shedding virus at 10 days p.c., making all six of the contact pigs positive for infection. Of note, nasal swabs collected from five DI pigs in the 1P10 : 7pdm virus group that were euthanized on day 3 p.i. were clearly positive for virus isolation but the titres were just above the limit of detection (indicated with an asterisk in Fig. 1). This observation was inconsistent with virus titres in nasal swabs collected from these and other DI pigs in this group at 2 and 4 days p.i., but was consistent with lower virus titres in the bronchoalveolar fluid (BALF) at 3 days p.i. in the same subset of five pigs (Fig. 2c). The significance of such findings remains to be discerned, but it does not change the observation that the 1P10 : 7pdm virus replicated in the respiratory tract of DI pigs.

Pathological analysis revealed that pigs inoculated with the 2P10 : 6WF10 virus had very few visible lung lesions at 3 days p.i. and were not different from the negative-control group at 5 days p.i. The H1N1pdm-infected pigs showed the most significant macroscopic lung lesions (Fig. 2a, b), with the mean percentage of lung affected (mean of five pigs per group) being 16.5 and 20.5 % at 3 and 5 days p.i., respectively. Pigs inoculated with the 2P10 : 6pdm and 1P10 : 7pdm viruses showed an intermediate level of gross pathology. The 2P10 : 6pdm group had means of 5.6 and 3.3 % at 3 and 5 days p.i., respectively. The 1P10 : 7pdm group had lesions covering 6.7 and 4.3 % of visible lung surface at 3 and 5 days p.i., respectively (Fig. 2a, b).

The 2P10 : 6WF10 group had no detectable virus in the BALF at either 3 or 5 days p.i., consistent with the nearly complete lack of pathology and lack of virus shedding at these time points (Fig. 2c). The H1N1pdm group had the highest mean titre of all five groups at both 3 and 5 days p.i. The 1P10 : 7pdm group had the lowest mean titre for both days; however, by 5 days p.i. the titre was nearly equivalent to the 2P10 : 6pdm virus group. Consistent with virus shedding in nasal swabs, BALF virus titres from the H1N1pdm-infected pigs were significantly higher than for the 1P10 : 7pdm group at both 3 and 5 days p.i.

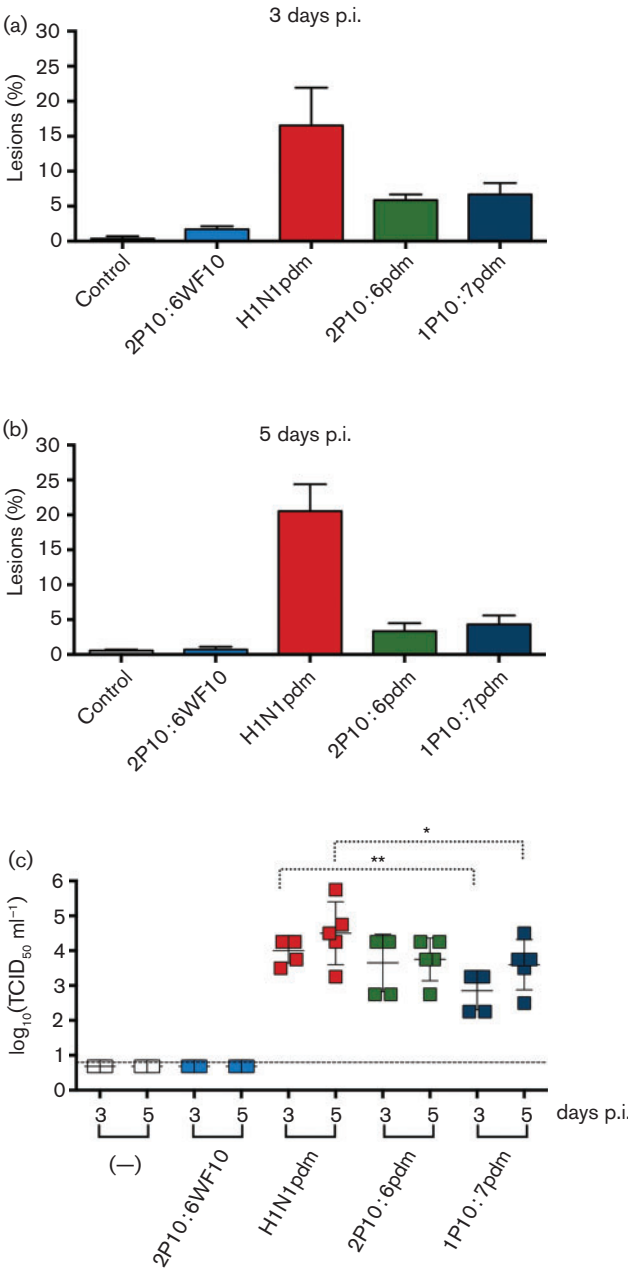

Fig. 2. H9 reassortants result in intermediate pathology and replication in swine lungs. (a, b) Five DI pigs per group were euthanized on day 3 (a) and day 5 p.i. (b). Lungs were scored for visible lesions and the total percentage of the lung surface affected was calculated as described previously (Halbur *et al.*, 1995). Results are shown as means ± SD. (c) BALF samples were collected at the time of necropsy and titrated for virus as $TCID_{50}$ in Madin–Darby canine kidney cells. Each square represents BALF titres per pig at different time points. A two-way ANOVA was used to determine significant differences with virus and time as factors. The dotted braces indicate statistically significant differences between groups (*$P<0.05$; **$P<0.01$). The dashed line indicates the limit of detection.

Effect of ferret-adapted mutations on infectivity, transmissibility and pathogenicity of H9 subtype viruses in quail

Virus replication in quail respiratory samples was evident in the 2P10 : 6pdm group (Fig. 3), in which DI quail shed virus in the trachea from 1 to 5 days p.i. and became negative by 7 days p.i. In contrast, DI quail in the 1P10 : 7pdm group were positive only at 1 day p.i., quickly clearing the virus and remaining negative for the remainder of the experiment. DI quail in the 2P10 : 6WF10 group were positive for virus from 1 to 5 days p.i. with the highest virus titres at 3 days p.i. This was similar to the wt WF10-infected quail, although

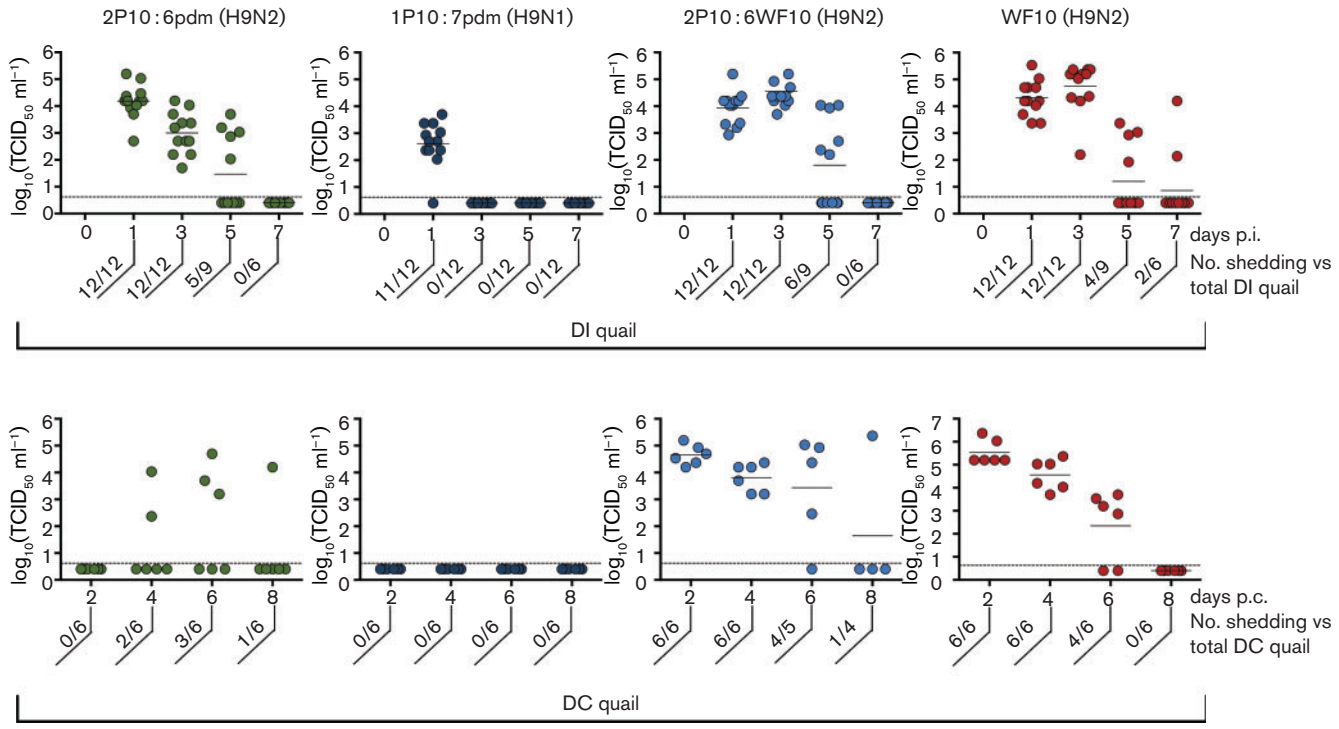

Fig. 3. H9 reassortant viruses replicate and transmit in quail. Quail (n=12 per group) were inoculated (DI) with the viruses indicated and tracheal and cloacal swabs collected at various days p.i. The number of DI quail positive for viral shedding in each group versus the total number of quail is listed under each time point. DC quail (n=6 per group) were introduced in the same pen housing the DI pigs at 1 day p.i. The number of DC quail positive for viral shedding in each group versus the total number of quail is listed under each time point. Note the DC graphs were measured in days p.c., not days p.i. The dotted lines indicate the limit of detection.

in this latter group, virus could be detected at 7 days p.i. None of the DI quail showed clinical signs of disease, consistent with previous observations (Perez *et al.*, 2003a). No virus was isolated from cloacal swabs in any of the groups except occasional shedding in the 2P10 : 6WF10 and wt WF10 groups. None of the PBS control quail shed virus (not shown).

In the DC groups, two of the six DC quail in the 2P10 : 6pdm group were positive for virus by 4 days p.i. and another began shedding on day 5, with these quail shedding for only 2–3 days. DC transmission did not occur in the 1P10 : 7pdm group, as none of the DC quail became positive during the course of the experiment, consistent with the limited shedding in the corresponding DI group. All DC quail in the 2P10 : 6WF10 and wt WF10 groups became positive by 2 days p.c., began to clear virus by 6 days p.c. and had stopped shedding by day 8, except for one DC quail in the 2P10 : 6WF10 group (Fig. 3). Two quail in the 2P10 : 6WF10 DC group were found dead on days 6 and 7 p.c.; however, the cause of death was ruled as idiopathic in nature. Virus titres in the lungs of inoculated quail collected at 4 and 6 days p.i. corresponded well with virus titres in tracheal swabs (Figs 3 and 4). Virus titres in the lungs of quail infected with viruses carrying the WF10 internal gene segments (2P10 : 6WF10 and wt WF10) were ~1.5 $\log_{10}$ higher compared with those having the pdm backbone (2P10 : 6pdm and 1P10 : 7pdm; Fig. 4).

SEROCONVERSION IN SWINE AND QUAIL IS CONSISTENT WITH H9 INFLUENZA VIRUS INFECTION

To measure seroconversion to the challenge virus, all surviving pigs were bled at 14 and 20 days p.i. and tested by ELISA for the nucleoprotein (NP) (Table 2). No PBS control pigs showed evidence of seroconversion (data not shown). From the 2P10 : 6WF10 group, only three of the remaining five DI pigs had seroconverted by 20 days p.i. and none of the DC pigs seroconverted, consistent with poor virus replication in this group. It should be noted that one DI pig in the 2P10 : 6pdm group and one DI pig in the H1N1pdm group were euthanized prior to serum collection due to unforeseen circumstances unrelated to the experiment. The DI H1N1pdm group showed seroconversion in three of the four remaining DI pigs at 14 days p.i. and in all four pigs at 20 days p.i. All six DC pigs in the H1N1pdm group seroconverted by 14 days p.i. The

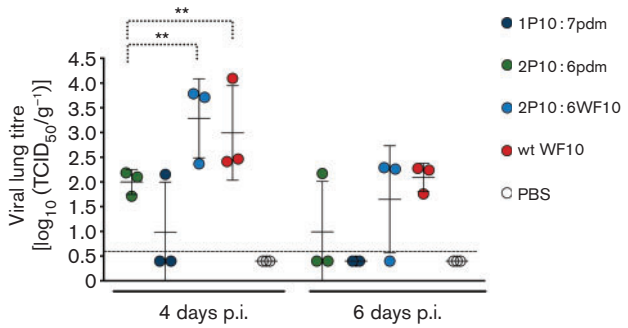

Fig. 4. Reassortant viruses replicate in quail lungs. On days 4 and 6 p.i., three DI quail per group were euthanized and lung samples were collected for virus titration. Each circle represents the lung titre per quail at different time points. A two-way ANOVA was used to determine significant differences with virus and time as factors. The dotted braces indicates statistically significant differences between groups (**$P<0.01$). The dotted line indicates the limit of detection.

DI pigs in the 2P10 : 6pdm group had two out of four seropositive at 14 days p.i. and all four at 20 days p.i.. The two DC pigs that were positive by virus isolation by 6 days p.c. seroconverted by 20 days p.i. and were the only DC pigs to do so. The five remaining DI pigs in the 1P10 : 7pdm virus group were seropositive by 20 days p.i., and four of the six DC pigs in this group seroconverted at 14 days p.i. but only three were positive by 20 days p.i.

Quail (six DI and six DC quail per group) serum samples were tested for seroconversion by haemagglutination inhibition (HI) assay (Table 2). None of the negative-control quail seroconverted. Of the six DI quail in the 1P10 : 7pdm group, only one did not seroconvert; however, HI titres were low (≤ 80), which is consistent with virus replication below the limit of detection. Also consistent with this observation was the lack of seroconversion in the DC quail in this group, indicating a lack of transmission. All DI quail in the other virus groups had significant HI titres (2P10 : 6pdm ≥ 80; 2P10 : 6WF10 and wt WF10 ≥ 320) against the respective homologous viruses, consistent with active virus replication. Likewise all DC quail in the 2P10 : 6WF10 and wt WF10 groups had HI titres of ≥ 320 consistent with efficient virus transmission. The DC quail in the 2P10 : 6pdm group showed low levels of seroconversion (HI titre ≤ 80) in four of the six quail. In this group, one bird that never shed detectable virus had low HI levels.

DISCUSSION

H9N2 viruses in South-east Asia continue to play a pivotal role in the emergence and maintenance of other avian influenza viruses that affect poultry and are a threat to human health. H9N2 viruses have been the donors of broad-host-range genes through reassortment leading to the emergence of zoonotic viruses like the H5N1, H7N9 and H10N8 strains in China and, more recently, the introduction of Eurasian-lineage H5N8 into the USA and Canada, and subsequent reassortments generating H5N2 and H5N1 mixed-lineage viruses (Eurasian–American) (Chen *et al.*, 2014; Guan *et al.*, 1999; Lee *et al.*, 2015; Liu *et al.*, 2013; Pasick *et al.*, 2015; Wu *et al.*, 2013; Yu *et al.*, 2011). Many of the recent H9N2 isolates from poultry in Asia and the Middle East possess hallmarks of human adaptation and have demonstrated the capacity to occasionally infect humans and swine. The typical mild nature of human H9N2 infections reported to date (Chen *et al.*, 2011; Kimble *et al.*, 2011) suggests that many occurrences may go unreported. Indeed, human serological studies suggest that human exposure is high in poultry workers and in individuals in contact with poultry where H9N2 viruses are present. Studies in Cambodia, Egypt, India, Nigeria and Vietnam have revealed significant exposure of humans to H9N2 viruses (Blair *et al.*, 2013; Huang *et al.*, 2013; Liu *et al.*, 2009; Okoye *et al.*, 2013; Pawar *et al.*, 2012; Uyeki *et al.*, 2012; Zhou *et al.*, 2014).

Table 2. Seroconversion in pigs and quail after exposure to different viruses

Virus group	Pigs: NP ELISA-positive serum*				Quail: HI titres 21 days p.i.†	
	DI 14 days p.i.	DC 14 days p.i.	DI 20 days p.i.	DC 20 days p.i.	DI	DC
2P10 : 6pdm	2/4	0/6	4/4	2/6	6/6 (226)‡	4/6
1P10 : 7pdm	2/5	4/6	5/5	3/6	5/6 (57)	0/6
2P10 : 6WF10	2/5	0/6	3/5	0/6	6/6 (1140)	3/3 (320)
H1N1pdm	3/4	6/6	4/4	6/6	NA	NA
WF10	NA	NA	NA	NA	6/6 (507)	5/5 (735)

NA, Not applicable.

*Number of seropositive pigs out of total number of inoculated (DI) or contact (DC) pigs at 14 and 20 days p.i., using a commercially available kit.

†Number of seropositive quail out of total number of DI or DC birds at 21 days p.i.

‡Numbers in parentheses are geometric mean titres of seropositive samples ± 40.

In this study, we tested the replication and transmission of reassortant H9:pdm viruses having ferret-adaptive mutations in pigs and quail. We reported previously that three amino acid changes in the surface genes of an avian H9N2 virus conferred efficient airborne transmission of a reassortant H9N2 : H3N2 virus in the ferret model (Sorrell *et al.*, 2009). We further showed that, on the H1N1pdm backbone, the two changes in the HA alone were sufficient for airborne transmission in ferrets without further adaptation (Kimble *et al.*, 2011). In this study, we sought to determine whether the ferret-adaptive mutations would restrict the host range of these viruses in other natural hosts of influenza.

Swine carry both avian (α-2,3 sialic acid)- and human (α-2,6 sialic acid)-like influenza receptors in their respiratory tract (Ito *et al.*, 1998; Kimble *et al.*, 2010; Wan & Perez, 2006). This allows for infection with both avian and human viruses and creates an ideal environment where two different viruses may reassort and lead to novel genotypes. Similar to our results in ferrets, both the 1P10 : 7pdm and 2P10 : 6pdm viruses replicated and were transmitted to contact pigs, with the number of transmission events (number of DC pigs that became virus positive) higher in the 1P10 : 7pdm (H9N1) group compared with the 2P10 : 6pdm (H9N2) group. In a similar study, Qiao *et al.* (2012) tested alternative H9N1 and H9N2 viruses having H1N1pdm internal gene segment in pigs and chickens and found that the H9N1 reassortant virus with a wholly avian-origin HA gene was also more efficient in replication and transmission in swine compared with a reassortant H9N2, but not in chickens. It is important to emphasize that the studies in that report were consistent with the notion that the H9 HA and N1 NA combination favours the transmission of these viruses in ferrets and pigs but not in chickens or quail. This leaves the possibility of a swine H9N2 : H1N1pdm reassortant adapting to the pig respiratory tract with the potential to transmit to humans. In a report by He *et al.* (2014) where H9N2 : H1N1pdm reassortants were tested in the guinea pig model, the results were somewhat different, with the (H9N2)2 : 6pdm reassortants transmitting more efficiently than the (H9N1)1 : 7pdm, although the (H9N1)1 : 7pdm viruses displayed enhanced pathogenicity. The animal origin of the H9N2 viruses (avian and swine) as well as different lineages (G1 and Y280) may account for these differences. A recent report from Li *et al.* (2014) showed that some naturally occurring H9N2 isolates have the capacity for respiratory droplet transmission in ferrets without adaptation. At least one of these isolates was transmitted with apparently higher efficiency, although the mean peak titres were lower than those observed for the H9N2 and H9N1 avian/human reassortants presented previously. The HA of the natural isolates from the study by Li *et al.* (2014) have in common with our ferret-adapted P10 H9 the I155T mutation, which favours binding to α-2,6 sialic acid, which may favour replication in mammals. However, P10 HA also differed from the viruses described by Li *et al.* (2014) at 21 other amino acid positions (compared with sites with consensus amino acids). It remains to be determined whether wholly avian H9N2 viruses from the Li *et al.* (2014) study are compatible for replication and transmission in pigs. It also remains to be determined whether the viruses that showed improved replication and transmission in ferrets in the Li *et al.* (2014) report will have a similar phenotype in pigs. Further detailed sequence analyses of viruses should shed light on the adaptive changes that take place during initial rounds of replication in different hosts.

A recent report concluded that the H1N1pdm matrix (M) and NA genes cooperate to allow improved replication and transmission of a triple reassortant swine virus in pigs (Ma *et al.*, 2012). Another study showed that the inclusion of the H1N1pdm M and NA genes in the background of a laboratory-adapted A/Puerto Rico/8/1934 (PR8) virus increased the replication and transmission of the resultant reassortant virus compared with the wt PR8 virus in a guinea pig model (Campbell *et al.*, 2014). Our present results in the pig as well as our previous finding in the ferret support this notion, as the efficiency of transmission was increased with the 1P10 : 7pdm virus in both ferrets and pigs.

Quail are another important agricultural species that are susceptible to infection with many influenza subtypes (Makarova *et al.*, 2003; Thontiravong *et al.*, 2012b) and could act as mixing vessels for reassortment (Perez *et al.*, 2003a, b; Thontiravong *et al.*, 2012a). In the quail study, our results showed that reassortant viruses with both ferret-adapted H9 surface genes (HA and NA) on a wt H9 or H1N1pdm backbone (2P10 : 6WF10 and 2P10 : 6pdm, respectively) replicated and were transmitted in quail. The role of the internal gene constellation appeared to be an important factor in the replication and transmission phenotype observed in quail. The 2P10 : 6pdm virus having ferret-adapted surface genes on an H1N1pdm backbone replicated in the quail and was transmitted to 50 % of the infected birds. Replacing the H1N1pdm internal genes with the avian WF10 backbone increased the transmission efficiency of the 2P10 : 6WF10 virus to 100 %, similar to that of wt WF10. This observation reflects the adaptation of the avian- and mammalian-origin backbones for their respective hosts. The importance of the origin of internal genes in the transmission event was also observed in the pigs. In the 2P10 : 6WF10 virus, despite having surface genes that allowed transmission in pigs, the presence of an avian-origin internal gene constellation obliterated replication and transmission in pigs. In the quail study, we also found that the molecular changes due to mammalian adaptation did not affect the ability of the virus to replicate when both ferret-adapted surface genes were present. It has been reported that a functional balance between HA and NA activity is important for virus replication and transmission (Sun *et al.*, 2013; Yen *et al.*, 2011). An imbalance caused by an avian-origin HA and a mammalian-origin NA may account for the lack of proper replication and transmission

observed in quail with the 1P10 : 7pdm virus. This poor replication phenotype of an H9N1 reassortant has been reported previously in chickens (Qiao *et al.*, 2012). Amino acids at positions 106 and 248 of the N1 NA of H1N1pdm viruses have been reported to play a role in the low-pH stability of H1N1pdm viruses (Takahashi *et al.*, 2013). This low-pH stability has been shown to be important for N2 NA replication in ducks (Takahashi *et al.*, 2003). A valine-to-isoleucine substitution at position 106 and asparagine-to-aspartic acid at position 248 increased the replication of A/California/04/9009 virus by 10-fold in Madin–Darby canine kidney (MDCK) cells. The N1 NA used in this study had V106 and N248, which may have contributed to the poor replication observed in quail. Further tests to confirm the effects of these mutations are needed but are beyond the scope of the present report.

This study highlighted the potential role that pigs and quail may play as amplifiers of reassortant influenza viruses. It also underscores the potential threat that H9N2 viruses could pose to humans as a future pandemic subtype. Swine can be host to both H9N2 and H1N1pdm viruses, and both viruses have a history of reassortment in swine (Howard *et al.*, 2011; Vijaykrishna *et al.*, 2010; Yu *et al.*, 2011). Pigs could also serve as a suitable environment for adaptation to the mammalian host of any potential reassortant virus. Similarly, quail can be infected with H9 viruses, and H1N1pdm virus has been shown to reassort experimentally in quail following co-infection with other avian influenza viruses (Thontiravong *et al.*, 2012a). The results in this report showed that quail are susceptible to and can transmit reassortant H9N2:H1N1pdm viruses and that transmission of these viruses depends on the compatibility of the surface and internal gene segments. These findings emphasize the need for continued surveillance of influenza in swine and quail and the need for additional research on how H9N2 influenza virus behaves in both species and how hosts can affect reassortment.

METHODS

Viruses. Viruses were generated by reverse genetics using previously described plasmids (Kimble *et al.*, 2011; Sorrell *et al.*, 2009). Plasmids containing the genes for A/Netherlands/602/2009 (H1N1) (H1N1pdm), A/guinea fowl/Hong Kong/WF10/1999 (H9N2) (WF10) and the ferret-adapted surface genes from the A/ferret/Maryland/P10_UMD/2008 (H9N2) (P10) virus were used. Five viruses were prepared (Table 1): the wt H1N1pdm and WF10 viruses, the P10 HA and NA on the WF10 backbone (2P10 : 6WF10), the P10 HA with the seven other genes from H1N1pdm (1P10 : 7pdm), and the P10 HA and NA with the six remaining genes from H1N1pdm (2P10 : 6pdm).

Animal studies. Swine studies were performed in large-animal Biosafetly Level 3 for agricultural hazards (BSL3-Ag) facilities of the National Animal Disease Center (NADC), Ames, IA, USA, following protocols approved by the NADC and the University of Maryland Institutional Animal Care and Use Committees (IACUC). The study was done on 3-week-old crossbred pigs obtained from a high-health herd free of swine IAV and porcine reproductive and respiratory syndrome virus. NP-blocking ELISA (IDEXX Laboratories) was used to confirm the absence of antibodies to IAV. Prior to the start of the study, pigs were treated with ceftiofur crystalline-free acid (Pfizer Animal Health) and enrofloxacin (Bayer Animal Health) to reduce bacterial contaminants. The pigs were divided into five groups (n=15 per group) and inoculated with 2 ml intratracheally and 1 ml intranasally (10^5 $TCID_{50}$ ml^{-1}) of 2P10 : 6pdm, 1P10 : 7pdm, H1N1pdm or 2P10 : 6WF10 virus, or mock inoculated with PBS. Inoculation was performed under anaesthesia, using an intramuscular injection of a cocktail of ketamine [8 mg (kg body weight)$^{-1}$], xylazine (4 mg kg^{-1}) and tiletamine-zolazepam [Telazol; 6 mg (kg body weight)$^{-1}$] (Fort Dodge Animal Health). At 2 days p.i., six naive pigs for each virus group were introduced to the 15 inoculated pigs as direct contacts, except in the PBS control group. Nasal swabs (Fisherbrand Dacron swabs; Fisher Scientific) were collected in 2 ml minimal essential medium on 2, 4, 6, 8, 10 and 12 days p.i. On days 3 and 5 p.i., five pigs from each group were swabbed, bled and humanely euthanized with a lethal dose of pentobarbital (Fatal Plus; Vortech Pharmaceuticals). The lungs were scored for macroscopic lesions, followed by collection of BALF for virus titration. Nasal swabs were filtered and subjected to virus isolation by inoculating 200 µl aliquots and 200 µl serum-free Opti-MEM (Gibco, Life Technologies) supplemented with 1 µg tosylsulfonyl phenylalanyl chloromethyl ketone (TPCK)-trypsin ml^{-1} and antibiotics onto confluent PBS-washed MDCK cells in 24-well plates. Virus isolation-positive nasal swab and BALF samples were then titrated in MDCK cells as described previously (Kitikoon *et al.*, 2006), and $TCID_{50}$ titres were calculated according to the method of Reed & Muench (1938).

Quail studies were conducted in a BSL3+ facility at the Department of Veterinary Medicine, University of Maryland, under protocols approved by the IACUC. Four-week-old Japanese quail obtained from B&D Game Farm (Harrah, OK, USA) were kept under observation for 1 week prior to the start of the experiment. NP-blocking ELISA (Synbiotics Co.) was used to confirm the absence of antibodies to influenza virus prior to infection. Quail were randomly divided into four groups (n=12 per group) and housed in HEPA-filtered isolators. Each quail was infected with 1 ml containing 10^6 $TCID_{50}$ ml^{-1} of the respective virus through the nares, trachea and cloaca (0.25 ml administered via the trachea and nares and 0.5 ml via the cloaca). A negative-control group of six quail received 1 ml PBS through the same routes. Naive quail (n=6 per group) were introduced as DC quail at 1 day p.i. Tracheal and cloacal swabs were collected from all quail daily up to 14 days p.i. Swabs were suspended in 1 ml 3.7 % brain–heart infusion medium (Becton Dickinson) containing 10 000 U penicillin, 10 mg streptomycin and 25 µl amphotericin B, and stored at −80 °C until used in virus titrations. At 4 and 6 days p.i., three DI quail per group were euthanized (two quail in the control group) and lung tissue was collected for viral load titration. The left lung lobe was homogenized in 0.5 ml PBS using 3 mm tungsten carbide beads (Qiagen) in a Tissuelyser LT (Qiagen) at 50 cycles min^{-1} for 10 min. Samples were then clarified by centrifugation at 1000 ***g*** for 10 min and stored at −80 °C until used. Swabs and lung homogenate samples were titrated in MDCK cells, as described above.

Serological analysis. Pig serum samples collected at 14 and 20 days p.i. were tested for anti-NP antibodies by ELISA as described above. Quail serum samples collected at 21 days p.i. were tested by HI assay. HI assays were performed following treatment of the samples with receptor-destroying enzyme (Denka Seiken) and heat inactivation at 56 °C for 30 min to remove non-specific HA inhibitors and natural serum agglutinins. HI assays were performed with 8 haemagglutination units of each virus antigen and 0.5 % chicken red blood cells according to standard techniques (WHO, 2002). Negative and positive controls were included in the assay. Seroconversion was considered positive when HI titres to homologous virus were $\geq$40.

Statistical analysis. Statistical analyses were performed using GraphPad Prism software version 4.00 (GraphPad Software). Comparison between two treatment means was done using Student's *t*-test, whilst multiple mean comparisons was done using two-way ANOVA followed by Bonferroni's multiple comparison test. $P<0.05$ was considered significant.

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