The neuraminidase and matrix genes of the 2009 pandemic influenza H1N1 virus cooperate functionally to facilitate efficient replication and transmissibility in pigs

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The 2009 pandemic H1N1 virus (pH1N1) contains neuraminidase (NA) and matrix (M) genes from Eurasian avian-like swine influenza viruses (SIVs), with the remaining six genes from North American triple-reassortant SIVs. To characterize the role of the pH1N1 NA and M genes in pathogenesis and transmission, their impact was evaluated in the background of an H1N1 triple-reassortant (tr1930) SIV in which the HA (H3) and NA (N2) of influenza A/swine/Texas/4199-2/98 virus were replaced with those from the classical H1N1 A/swine/Iowa/15/30 (1930) virus. The laboratory-adapted 1930 virus did not shed nor transmit in pigs, but tr1930 was able to shed in infected pigs. The NA, M or both genes of the tr1930 virus were then substituted by those of pH1N1. The resulting virus with both NA and M from pH1N1 grew to significantly higher titre in cell cultures than the viruses with single NA or M from pH1N1. In a pig model, only the virus containing both NA and M from pH1N1 was transmitted to and infected sentinels, whereas the viruses with single NA or M from pH1N1 did not. These results demonstrate that the right combination of NA and M genes is critical for the replication and transmissibility of influenza viruses in pigs.

INTRODUCTION

Although the 2009 influenza pandemic was relatively mild compared with the last three pandemics in human history, the pandemic H1N1 virus (pH1N1) affected more than 200 countries and territories, causing infection in hundreds of millions of people and more than 18 000 deaths throughout the world by August 2010 (World Health Organization, 2010). One of the unique hallmarks of this pandemic was that pH1N1 spread very quickly throughout the world. As a result, the World Health Organization declared the start of the 2009 pandemic 6 weeks after the first human case was identified in Mexico in April 2009. Another unusual feature was that pH1N1 caused a higher morbidity and fatality in young healthy adults than the seasonal influenza viruses (Chowell *et al.*, 2009). Equally important and unique was the ability of pH1N1 to cross species barriers, as it was detected in different animal species including dogs, turkeys, cats, swine, ferrets and other wildlife (Berhane *et al.*, 2010; Dundon *et al.*, 2010; Pasma & Joseph, 2010; Schrenzel *et al.*, 2011; Sponseller *et al.*, 2010; Swenson *et al.*, 2010). This has raised concerns that reassortments might occur between pH1N1 and other influenza viruses, including highly pathogenic H5N1 avian influenza viruses (Kimble *et al.*, 2011; Schrauwen *et al.*, 2011).

pH1N1 has been determined to be a reassortant virus between North American triple-reassortant (tr) and Eurasian swine influenza viruses (SIVs) (Smith *et al.*, 2009). Six genes

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of pH1N1 – the polymerase (PB1, PB2 and PA), haemagglutinin (HA), nucleoprotein (NP) and non-structural (NS) genes – are derived from North American trSIVs and two genes – the neuraminidase (NA) and matrix (M) genes – are from the Eurasian avian-like SIVs. Since 1998, trSIVs have circulated in pigs in North America and have become the major type of influenza virus circulating in US pigs. TrSIV has PA and PB2 genes derived from avian influenza virus, PB1 from human influenza virus and NP, M and NS from the classical H1N1 SIV (Vincent *et al.*, 2008). These six internal genes found in the North American trSIVs have been designated the triple-reassortant internal gene cassette (TRIG), which can accept different HA and NA combinations and may endow a selective advantage to swine viruses possessing them (Ma *et al.*, 2009). North American trSIVs, including H1 and H3 subtypes, have been documented to infect humans sporadically (Cox *et al.*, 2011; Olsen *et al.*, 2006; Robinson *et al.*, 2007; Shinde *et al.*, 2009). However, these viruses have never obtained human-to-human transmission ability that would allow them to be maintained in humans.

In this study, we showed that the TRIG six internal genes conferred transmissibility in pigs to a laboratory-adapted H1N1 SIV inefficient in pig transmission. Thus, a reassortant SIV (tr1930) containing the six internal genes from the North American trSIV H3N2 influenza A/swine/Texas/4199-2/98 (TX98) virus and the HA and NA genes from the laboratory-adapted SIV influenza A/swine/Iowa/15/30 (1930) H1N1 virus was transmitted efficiently in pigs, whilst the parental 1930 virus was not. We also showed that single substitution of the NA or M gene of pH1N1 into tr1930 virus prevented transmissibility in pigs, but that when both NA and M genes of the pH1N1 virus were included, transmission was preserved.

RESULTS

Characterization of wild-type 1930 and tr1930 viruses in cell cultures and pigs

The wild-type 1930 and tr1930 (1930HANA/TX98) viruses were generated using reverse genetics (Fig. 1a). Plaque assays showed that both wild-type 1930 and tr1930 viruses formed plaques of a similar size (Fig. 1b). Interestingly, the wild-type 1930 virus grew to higher titres than tr1930; a significant difference was observed between viruses ($P<0.05$) at both 24 and 48 h post-infection (p.i.) (Fig. 1c).

Pigs inoculated with wild-type 1930 or tr1930 virus had fever from day 1 p.i., but the controls did not. There were no significant differences in body temperature between the inoculated groups (data not shown), and no clinical symptoms such as coughing, sneezing and lethargy were observed in either of the inoculated groups. Wild-type 1930 and tr1930 caused severe macroscopic lung lesions (plum-coloured, consolidated areas) (Table 1), and no significant differences were observed in lung lesions

between the two groups on days 3 and 5 p.i. The microscopic score (0–3), indicative of the extent of damage to lung architecture, was 2.30–3.00 on days 3 and 5 p.i. in both inoculated groups, and no significant differences were observed between the two groups on these days (Table 1). No macroscopic and microscopic lung lesions were found in the control pigs. Both wild-type 1930 and tr1930 viruses replicated to similar titres in infected pig lungs (ranging from $10^{4.46}$ to $10^{5.78}$ TCID$_{50}$ ml^{-1}). No nasal shedding of virus was detected from pigs inoculated with the wild-type 1930 virus, whereas shedding virus ($10^{3.67}$–$10^{5.12}$ TCID$_{50}$ ml^{-1}) was detected from nasal swab samples collected from pigs infected with tr1930 on days 3 (30 %, 3/10) and 5 (80 %, 4/5) p.i. (Table 1). These results indicated that the TRIG conferred efficient nasal shedding in pigs to the laboratory-adapted 1930 virus.

Generation and characterization of reassortant viruses

Three reassortant viruses with single NA (tr1930/CA09NA), single M (tr1930/CA09M) or both NA and M (tr1930/CA09NAM) from pH1N1 were generated as depicted in Fig. 1(a). The parental (tr1930) and three reassortant viruses were amplified in Madin–Darby canine kidney (MDCK) cells, titrated and stored at $-80\,^{\circ}$C until used for *in vitro* and *in vivo* studies. The HA, NA and M genes of the stock viruses were sequenced and no mutations were found. Plaque assays showed that the two single-reassortant and the tr1930/CA09NAM viruses formed plaques of similar size that were comparable to the wild-type 1930 and tr1930 viruses (Fig. 1b). The tr1930/CA09NAM virus grew to similar titres to the wild-type 1930 virus, and both reached significantly higher titres ($P<0.001$) than the reassortant viruses with single NA (tr1930/CA09NA) or single M (tr1930/CA09M) from pH1N1 (Fig. 1c). The tr1930 virus grew to significantly higher titres ($P<0.05$) than the reassortant tr1930/CA09NA with single NA from pH1N1 (Fig. 1c). A significant difference in virus replication was observed between the tr1930 and tr1930/CA09NAM viruses ($P<0.05$) at 36 and 48 h p.i., and between the tr1930 and reassortant tr1930/CA09M with only M from pH1N1 ($P<0.01$) at 12 h p.i. (Fig. 1c).

Pathogenicity and transmissibility of the tr1930 and reassortant viruses in pigs

Pigs infected with the two single-reassortant, tr1930 or tr1930/CA09NAM viruses had a fever that started on day 1 p.i. and lasted to the end of experiment. No significant differences were found in body temperature among each inoculated group from days 1 to 7 p.i. None of the infected pigs showed obvious clinical signs. The two single-reassortant, tr1930/CA09NAM and tr1930 viruses caused severe macroscopic lung lesions, whereas no lung lesions were found in the control pigs (Table 2). There were no significant differences in lung lesions among the four inoculated groups on days 3, 5 and 7 p.i. The microscopic score (0–3) was between 1.40 and 2.60 on days 3, 5 and 7

1: 1930; 2: tr1930; 3: tr1930/CA09NA; 4: tr1930/CA09M; 5: tr1930/CA09NAM
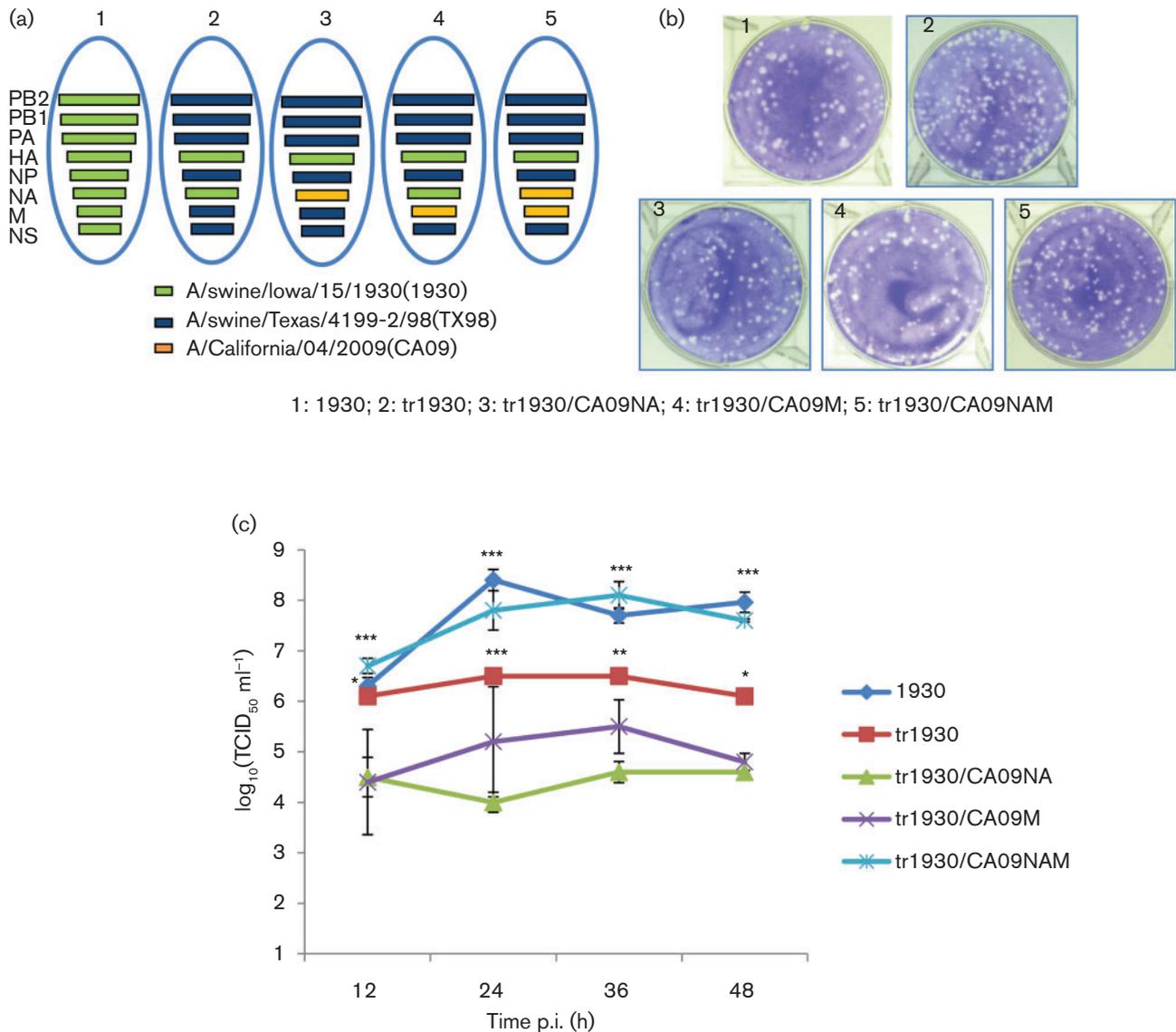
Fig. 1. Genotype, plaque morphology and growth dynamics in MDCK cells of influenza viruses. (a) Virus genotypes. Green and blue bars represent genes from A/swine/Iowa/15/30 (1930) and A/swine/Texas/4199-2/98 (TX98), respectively, and orange bars indicate genes derived from A/California/04/2009 (CA09). (b) Plaque assays of the parental and reassortant viruses in MDCK cells at 2 days p.i. (c) Growth dynamics of the parental and reassortant viruses in MDCK cells infected with the indicated viruses at an m.o.i. of 0.01. Each data point on the curve indicates the mean ± SEM of three independent experiments. *$P<0.05$; **$P<0.01$; ***$P<0.001$.

p.i. in all four inoculated groups compared with a score of 0.00–0.10 in the control pigs. There was a significant difference ($P<0.05$) in histopathological lung damage between the tr1930-inoculated group and the tr1930/CA09NAM-inoculated group on day 3 p.i.; no significant differences were observed among the other challenged groups on days 3, 5 and 7 p.i. (Table 2). All infected pigs had variable degrees of damage, ranging from mild to moderate bronchointerstitial pneumonia, atelectasis, acute to subacute bronchiolitis with epithelial necrosis, and variable lymphocytic cuffing of bronchioles on days 3, 5 and 7 p.i. Some control pigs on day 7 p.i. had incidental

non-influenza-associated lung lesions (Table 2), but were negative for SIV infection.

The single-reassortant, tr1930 and tr1930/CA09NAM viruses were able to replicate in the pig lungs, with virus titres reaching approximately $10^{2.70}$–$10^{5.80}$ TCID$_{50}$ ml^{-1} in the bronchioalveolar fluid (BALF) on days 3 and 5 p.i. (Fig. 2). The tr1930 and tr1930/CA09NAM viruses replicated to higher titres in pig lungs than the two single-reassortant viruses on days 3 and 5 p.i., and grew to a significantly higher titre ($P<0.05$) than the reassortant tr1930/CA09M virus on these two days (Fig. 2). On day 7 p.i., virus was

Table 1. Macroscopic and microscopic lung lesions, and virus titres in BALF and nasal swabs and percentage of nasal shedding in pigs inoculated with 1930 and tr1930 viruses or mock-infected

Time p.i.	Group	Macroscopic lung lesions (%)	Microscopic lung lesion score (0–3)*	Virus titre in BALF ($TCID_{50}$ ml^{-1})†	Positive (%) in NS (virus titre)‡
3 days	1930	19.40 ± 1.40	2.70 ± 0.30	5.78 ± 0.43	0 (<1)
	tr1930	17.20 ± 4.06	2.82 ± 0.17	5.08 ± 0.24	30 (3.67 ± 0.88)
	Control	0.00	0.00	<1	0 (<1)
5 days	1930	10.75 ± 2.30	3.00 ± 0.00	4.46 ± 0.32	0 (<1)
	tr1930	18.22 ± 3.18	2.30 ± 0.19	5.21 ± 0.36	80 (5.12 ± 0.28)
	Control	0.00	0.00	<1	0 (<1)

*Mean $\pm$ SEM.
†Numbers are $\log_{10}$(geometric mean) $\pm$ SEM of BALF $TCID_{50}$ ml^{-1}.
‡Numbers in parentheses are $\log_{10}$ (geometric mean) $\pm$ SEM of nasal swab (NS) $TCID_{50}$ ml^{-1}.

detected in the lungs of all five pigs inoculated with tr1930 and in three of five pigs inoculated with the tr1930/CA09NAM virus; no virus was detected in the lungs of pigs inoculated with tr1930/CA09M or tr1930/CA09NA (Fig. 2). Virus nasal shedding was detected from pigs inoculated with the tr1930 virus on days 3 (20 %, 3/15), 5 (80 %, 8/10) and 7 (100 %, 5/5) p.i., and from those infected with tr1930/CA09NAM on days 3 (53 %, 8/15), 5 (100 %, 10/10) and 7 (80 %, 4/5) p.i. (Table 3). No virus was detected in nasal swabs from pigs inoculated with the two single-reassortant viruses on these days (Table 3). Interestingly, a large amount of viral antigen was detected in the bronchiolar epithelium by immunohistochemical (IHC) staining in pigs infected with the tr1930 or tr1930/CA09NAM virus on day 5 p.i., but only very low levels of viral antigen were found in pigs infected with the reassortant tr1930/CA09NA virus, and no viral antigen was found in pigs infected with the reassortant tr1930/CA09M virus on day 5 p.i. (Fig. 3a–e).

In the contact groups, several contact pigs had a fever on the tested days p.c. in the tr1930 group (20 %, 1 day p.c.; 60 %, 3 days p.c.) and tr1930/CA09NAM group (20 %, 1 day p.c.; 20 %, 2 days p.c.; 80 %, 3 days p.c.; 60 %, 4 days p.c.); none had a fever in the two single-reassortant virus contact groups. All five sentinels that were co-housed with

pigs inoculated with the tr1930 or tr1930/CA09NAM virus shed virus from the nasal cavity (Table 3). A significant amount of virus (ranging from 10^4 to 10^6 $TCID_{50}$ ml^{-1}) was detected in the lungs of these pigs (Fig. 2), although only limited macroscopic and no microscopic lung lesions were observed in these sentinel animals. In the groups challenged with the two single-reassortant viruses, no virus was detected from nasal swabs (Table 3) or in BALF samples collected from the contact animals (Fig. 2); these samples were also negative using an M gene-specific real-time RT-PCR (data not shown) (Ma *et al.*, 2010b; Richt *et al.*, 2004). Viral antigen was detected in the lungs of contact pigs that were co-housed with animals infected with the tr1930 or tr1930/CA09NAM virus at 4 days p.c., while no viral antigen was found in contact pigs that were co-housed with animals infected with the two single-reassortant viruses (Fig. 3f–i). These results indicated that a combination of NA and M genes from pH1N1 is critical for efficient virus replication and transmission to sentinel pigs, whereas the introduction of single pH1N1 NA and M genes is inefficient.

DISCUSSION

pH1N1 originated from North American trSIVs, which obtained the NA and M genes from Eurasian avian-like

Table 2. Macroscopic and histopathological lung lesions of inoculated and control pigs on days 3, 5 and 7 p.i.

Virus	Lung lesion score (%)*			Histopathological score (0–3)*		
	3 days p.i.	5 days p.i.	7 days p.i.	3 days p.i.	5 days p.i.	7 days p.i.
tr1930	15.60 ± 4.56	16.57 ± 3.62	11.34 ± 1.65	2.60 ± 0.19†	2.10 ± 0.19	1.40 ± 0.19
tr1930/CA09NA	25.14 ± 12.57	22.57 ± 7.25	7.46 ± 3.18	2.30 ± 0.25	2.60 ± 0.19	1.80 ± 0.25
tr1930/CA09M	18.37 ± 4.80	22.03 ± 7.14	10.20 ± 4.98	2.50 ± 0.16	2.10 ± 0.40	1.90 ± 0.24
tr1930/CA09NAM	16.29 ± 3.82	16.11 ± 4.60	8.20 ± 2.08	1.70 ± 0.20†	1.90 ± 0.24	1.60 ± 0.19
Control	0	0	0	0	0	0.10 ± 0.10

*Values are means $\pm$ SEM.
†Significant difference between the two groups ($P < 0.05$).
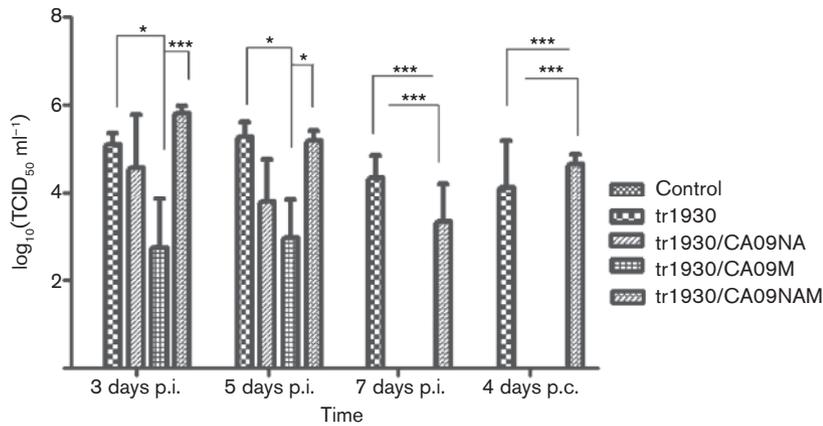
Fig. 2. Virus titres of BALF samples collected from lungs of contact or infected pigs inoculated with reassortant influenza viruses on the indicated days p.i. or post-contact (p.c.). Results are shown as means ± SEM. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

SIVs. North American trH1 and trH3 SIVs have caused sporadic infection in humans in North America, most often following direct exposure of humans to pigs (Cox et al., 2011; Olsen et al., 2006; Robinson et al., 2007; Shinde et al., 2009). However, these viruses have not established efficient human-to-human transmission. To date, it is still unknown why pH1N1 is transmitted and spreads so efficiently. In this study, we generated a North American trH1N1 virus as a parental virus to create various reassortant viruses in order to study the roles of NA and M from pH1N1 in pathogenicity and transmissibility in a pig model. First, we demonstrated that the TRIG cassette of North American trSIVs conferred enhanced transmissibility to the laboratory-adapted classical 1930 SIV, which is otherwise very limited or non-transmissible in this host (Ma et al., 2011). This was supported by the fact that: (i) the wild-type 1930 virus was not shed through the nasal cavity of infected pigs; (ii) the tr1930 virus was shed efficiently from infected pigs, and also transmitted to and infected sentinel animals, and (iii) the pathogenicity and transmissibility in pigs of the tr1930 virus was similar to that reported for trH1N1 SIVs (Ma et al., 2010c; Vincent et al., 2009). Our results also support the notion that the TRIG cassette confers a transmission advantage to influenza viruses, regardless of the surface genes. Furthermore, we showed that efficient replication and transmission of a

tr1930/CA09NAM virus in pigs required both NA and M genes from pH1N1; introduction of the single NA or M gene from pH1N1 into the tr1930 H1N1 virus resulted in decreased replication in MDCK cells and pigs, and complete loss of transmissibility in pigs, indicating that a combination of NA and M genes from pH1N1 is critical for efficient virus replication and transmissibility of the tr1930/CA09NAM virus in pigs following intratracheal inoculation.

It is important to note the differences in virus replication and transmission between the reassortant viruses with the single NA or M gene from pH1N1 and the parental tr1930 or the tr1930/CA09NAM virus with both NA and M from the pH1N1. The reassortant virus with the single M or NA was cleared earlier and was not transmissible in pigs compared with the tr1930 and tr1930/CA09NAM viruses. These findings were confirmed in contact pigs by virus isolation and IHC assays: no virus or viral antigen was detected in contact pigs co-housed with the single-reassortant virus group, and very weak or no viral antigen was found at 5 days p.i. in the lungs of pigs infected with single-reassortant viruses. These results indicated that compatible NA and M genes are critical for efficient replication and transmission of the tr1930 and tr1930/CA09NAM viruses.

Table 3. Percentage of virus nasal shedding and virus titres in nasal swabs of infected and contact pigs

Results are shown as the percentage of positive pigs out of total pig numbers on days 3, 5 and 7 p.i. or day 4 p.c. Numbers in parentheses are $\log_{10}$ (geometric means) ± SEM of $TCID_{50}$ ml^{-1}. Contact pigs were co-housed with infected animals for 4 days. NA, Not applicable (there were no contact pigs in the control group).

Virus	Infected pigs			Contact pigs
	3 days p.i.	**5 days p.i.**	**7 days p.i.**	**4 days p.c.**
tr1930	20 (3.68 ± 0.73)	80 (4.55 ± 0.36)	100 (4.23 ± 0.51)	100 (6.03 ± 0.41)
tr1930/CA09NA	0 (<1)	0 (<1)	0 (<1)	0 (<1)
tr1930/CA09M	0 (<1)	0 (<1)	0 (<1)	0 (<1)
tr1930/CA09NAM	53 (4.06 ± 0.36)	100 (4.33 ± 0.19)	80 (4.03 ± 0.51)	100 (4.60 ± 0.40)
Control	0 (<1)	0 (<1)	0 (<1)	NA
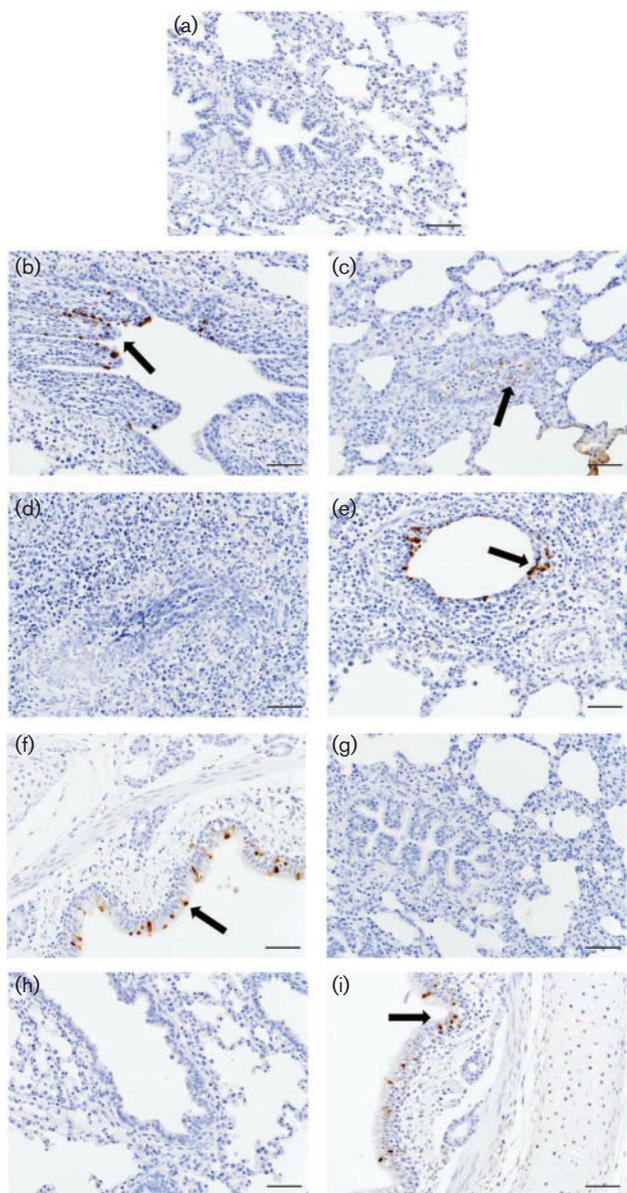
Fig. 3. IHC staining of microscopic lung sections from pigs infected with parental and reassortant viruses at day 5 p.i. and from contact pigs on day 4 p.c. No positive reaction was seen in lung sections of control pigs (a) or pigs infected with reassortant virus tr1930/CA09M (d). A mild positive reaction (arrow) was seen in lung sections of pigs infected with reassortant tr1930/CA09NA virus (c). A moderate to strong positive reaction (arrow) was seen in lung sections of pigs infected with tr1930 (b) and tr1930/CA09NAM (e). No positive reaction was seen in lung sections from contact pigs in the two single-reassortant virus groups, tr1930/CA09NA (g) and tr1930/CA09M (h). A moderate positive reaction (arrow) was seen in lung sections from contact pigs in the tr1930 (f) and tr1930/CA09NAM (i) groups. An anti-influenza A mAb against NP (Thermo Scientific) was used for the IHC staining. Bars, 50 μm.

Previous studies showed that interaction of M1 with both HA and NA is critical in triggering the budding process, resulting in the formation and release of virus particles. Both HA and NA associate with M1 via their transmembrane domain (Ali et al., 2000; Barman et al., 2001). When the NA sequence of the 1930 and pH1N1 viruses was compared at the amino acid level (87.8 % identity), major differences were found in the transmembrane domain (75.9 % identity; 7 aa differences: I7V, L9M, I10T, V11I, I13M, T14A and S15N), which interacts with the M1 protein required for viral assembly and budding. There were 12 aa differences (95.2 % identity) in the M1 protein and 13 aa differences (86.6 % identity) in the M2 protein between the TX98 and pH1N1 viruses. The amino acids that are critical in affecting the interaction between NA and M1 remain unknown. How this interaction between surface proteins (HA and NA) and M1 or M2 affects virus transmissibility needs to be elucidated in future studies.

A balance between HA and NA activities is required for efficient virus replication (Wagner et al., 2002; Yen et al., 2011) and is important for improving viral fitness and transmission (Kimble et al., 2011). A recent study showed that HA/NA balance confers respiratory-droplet transmissibility of pH1N1 in ferrets, and the NA of pH1N1 had a significantly higher enzyme activity than that of SIVs (Yen et al., 2011). In the present study, the transmissibility of the tr1930 virus could be attributed partially to the balance between HA and NA of this virus, as introduction of NA from pH1N1 into tr1930 resulted in decreased replication and loss of transmissibility in pigs. Interestingly, the loss of transmissibility could be restored by introduction of the matching M from pH1N1. The single-reassortant tr1930/CA09M virus with a balanced HA and NA from the 1930 virus, however, did not show transmissibility, suggesting that this was due to the introduction of the M gene from pH1N1, which probably leads to a mismatch between the NA and M genes. This finding supports the argument that an optimal genetic constellation and balanced HA/NA are critical factors for efficient viral transmission (Yen et al., 2011). Another recent study showed that the M gene of pH1N1 in the genetic background of the A/Puerto Rico/8/34 (H1N1) or A/swine/Texas/1998 (H3N2) virus is critical for its transmission efficiency in the guinea pig model (Chou et al., 2011). The latter finding fits current field situations where triple-reassortant H3N2 SIVs carrying only the M gene of the pH1N1 have been reported to infect humans in the USA (Centers for Disease Control and Prevention, 2012). A recent study using a ferret model showed that both NA and M genes contribute to transmissibility, aerosol release and morphology of the 2009 pandemic H1N1 influenza virus (Lakdawala et al., 2011), which is consistent with the present study. The controversial findings on the role of NA and M from pH1N1 on transmissibility are probably due to the use of different virus strains, different inoculation routes and different animal models (ferret/guinea pig/pig) used for these studies.

Taken together, we demonstrated that a combination of NA and M genes from pH1N1 virus was critical for replication and transmissibility of a North American triple-reassortant H1N1 virus in pigs. Furthermore, we also showed the impact of the TRIG cassette, which conferred efficient transmissibility of a triple-reassortant virus with HA and NA genes from a classical H1N1 SIV. Our study highlights the importance of an optimal genetic constellation for influenza virus replication and transmissibility in a natural host model.

METHODS

Cells. Human embryonic kidney 293T cells were maintained in Opti-modified minimal Eagle's medium (MEM) supplemented with 10 % FCS (HyClone) and 1 % antibiotics (Invitrogen). MDCK cells were maintained in Dulbecco's MEM with 5 % FCS, L-glutamine, MEM vitamins (Invitrogen) and 1 % antibiotics. Cells were inoculated with the respective viruses in MEM infecting medium containing 0.3 % BSA (Sigma), 1 μg TPCK-treated trypsin (Sigma) ml^{-1} and 1 % antibiotics. Ten-day-old embryonated chicken eggs were used for virus amplification.

Plasmid construction and virus generation. The full-length NA and M genes of pH1N1 A/California/04/2009 (CA09) were amplified using influenza virus universal primers, as published previously (Hoffmann *et al.*, 2001), and cloned into the *Bsm*BI sites of the pHW2000 vector. The resulting plasmids (pHW-CA-NA and pHW-CA-M) were confirmed by sequencing. The wild-type 1930 virus was rescued using reverse genetics, as described previously (Ma *et al.*, 2010a). Two plasmids (pDZ-HA and pDZ-NA) from the 1930 reverse genetics (Ma *et al.*, 2010a) and six internal gene plasmids (pHW-Sw-PB2, pHW-Sw-PB1, pHW-Sw-PA, pHW-Sw-NP, pHW-Sw-M and pHW-Sw-NS) from the TX98 reverse genetics (Solórzano *et al.*, 2005) were used to generate the tr1930 H1N1 (1930HANA/TX98) virus. Plasmids pHW-CA-NA and pHW-CA-M were used to substitute pDZ-NA and pHW-Sw-M to rescue the reassortant tr1930/CA09NA, tr1930/CA09M and tr1930/CA09NAM viruses, as depicted in Fig. 1(a).

Viral growth. To analyse virus growth dynamics, confluent MDCK cells were infected with each virus at an m.o.i. of 0.01 and incubated at 37 °C in MEM infecting medium. The supernatants from infected cells were collected at different time points and virus titres were determined as TCID$_{50}$ ml^{-1}. Plaque assays were performed to compare the sizes of plaques formed by each virus.

Pig experiments. Four-week-old porcine reproductive and respiratory syndrome virus- and SIV-seronegative pigs were used in this study, and all pig experiments were approved by the Institutional Animal Care and Use Committee at Kansas State University. Two pig experiments were conducted, using an inoculation protocol described previously (Richt *et al.*, 2003).

In the first experiment, 30 pigs were divided into three groups of ten. Pigs from each group were inoculated intratracheally with 10^6 TCID$_{50}$ wild-type 1930 or tr1930, or with virus-free MEM (control). Five inoculated and five control pigs were necropsied on days 3 and 5 p.i. Nasal swabs were taken from infected and control pigs on days 0, 3 and 5 p.i., placed in 2 ml MEM and stored at −80 °C. Blood was collected from all pigs on day 0 and at the time of necropsy. BALF was obtained by lavage of each lung with 50 ml MEM. The viral load in BALF and nasal swabs was determined in a 96-well plate, as described previously (Richt *et al.*, 2003). Lungs were removed *in toto* during necropsy. A single experienced veterinarian recorded the percentage of gross lesions on each lobe and a mean value for the seven lobes was calculated for each animal (Richt *et al.*, 2003). For histopathological examination, tissue samples from the trachea, the right cardiac lung lobe and other lobes if affected were fixed in 10 % buffered formalin, processed and stained with haematoxylin and eosin. IHC staining was performed using anti-influenza A mAb against NP. Lung sections were examined by a veterinary pathologist in a blinded fashion and given a score of 0–3 to reflect the severity of bronchial epithelial injury, as described previously (Ma *et al.*, 2007; Richt *et al.*, 2003).

In a second experiment, 95 pigs were divided into five groups (20 pigs in each infected group and 15 pigs in the control group). Fifteen pigs from each group were inoculated intratracheally with 10^6 TCID$_{50}$ reassortant virus (tr1930, tr1930/CA09NA, tr1930/CA09M or tr1930/CA09NAM) or virus-free MEM. Five contact pigs were added to each infected group on day 2 p.i. to investigate viral transmission. Five inoculated pigs from each group and five control pigs were necropsied on days 3, 5 and 7 p.i. and five contact pigs were euthanized on day 4 p.c. Nasal swabs were taken from infected and control pigs on days 0, 3, 5 and 7 p.i. and from contact pigs on days 0 and 4 p.c. All other procedures were as in the first experiment.

Statistical analysis. Virus titres, body temperature and macroscopic and microscopic pneumonia scores were analysed by analysis of variance in GraphPad Prism version 5.0 (GraphPad Software); a *P* value of 0.05 or less 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. Pairwise mean comparisons between inoculated and control groups were made using Student's *t*-test.

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REFERENCES

Ali, A., Avalos, R. T., Ponimaskin, E. & Nayak, D. P. (2000). Influenza virus assembly: effect of influenza virus glycoproteins on the membrane association of M1 protein. *J Virol* **74**, 8709–8719.

Barman, S., Ali, A., Hui, E. K., Adhikary, L. & Nayak, D. P. (2001). Transport of viral proteins to the apical membranes and interaction of matrix protein with glycoproteins in the assembly of influenza viruses. *Virus Res* **77**, 61–69.

Berhane, Y., Ojkic, D., Neufeld, J., Leith, M., Hisanaga, T., Kehler, H., Ferencz, A., Wojcinski, H., Cottam-Birt, C. & other authors (2010). Molecular characterization of pandemic H1N1 influenza viruses isolated from turkeys and pathogenicity of a human pH1N1 isolate in turkeys. *Avian Dis* **54**, 1275–1285.

Centers for Disease Control and Prevention (2012). Update: influenza A (H3N2)v transmission and guidelines – five states, 2011. *MMWR Morb Mortal Wkly Rep* **60**, 1741–1744.

Chou, Y. Y., Albrecht, R. A., Pica, N., Lowen, A. C., Richt, J. A., García-Sastre, A., Palese, P. & Hai, R. (2011). The M segment of the 2009 new pandemic H1N1 influenza virus is critical for its high transmission efficiency in the guinea pig model. *J Virol* **85**, 11235–11241.

Chowell, G., Bertozzi, S. M., Colchero, M. A., Lopez-Gatell, H., Alpuche-Aranda, C., Hernandez, M. & Miller, M. A. (2009). Severe respiratory disease concurrent with the circulation of H1N1 influenza. *N Engl J Med* **361**, 674–679.

Cox, C. M., Neises, D., Garten, R. J., Bryant, B., Hesse, R. A., Anderson, G. A., Trevino-Garrison, I., Shu, B., Lindstrom, S. & other authors (2011). Swine influenza virus A (H3N2) infection in human, Kansas, USA, 2009. *Emerg Infect Dis* **17**, 1143–1144.

Dundon, W. G., De Benedictis, P., Viale, E. & Capua, I. (2010). Serologic evidence of pandemic (H1N1) 2009 infection in dogs, Italy. *Emerg Infect Dis* **16**, 2019–2021.

Hoffmann, E., Stech, J., Guan, Y., Webster, R. G. & Perez, D. R. (2001). Universal primer set for the full-length amplification of all influenza A viruses. *Arch Virol* **146**, 2275–2289.

Kimble, J. B., Sorrell, E., Shao, H., Martin, P. L. & Perez, D. R. (2011). Compatibility of H9N2 avian influenza surface genes and 2009 pandemic H1N1 internal genes for transmission in the ferret model. *Proc Natl Acad Sci U S A* **108**, 12084–12088.

Lakdawala, S. S., Lamirande, E. W., Suguitan, A. L., Jr, Wang, W., Santos, C. P., Vogel, L., Matsuoka, Y., Lindsley, W. G., Jin, H. & Subbarao, K. (2011). Eurasian-origin gene segments contribute to the transmissibility, aerosol release, and morphology of the 2009 pandemic H1N1 influenza virus. *PLoS Pathog* **7**, e1002443.

Ma, W., Vincent, A. L., Gramer, M. R., Brockwell, C. B., Lager, K. M., Janke, B. H., Gauger, P. C., Patnayak, D. P., Webby, R. J. & Richt, J. A. (2007). Identification of H2N3 influenza A viruses from swine in the United States. *Proc Natl Acad Sci U S A* **104**, 20949–20954.

Ma, W., Kahn, R. E. & Richt, J. A. (2009). The pig as a mixing vessel for influenza viruses: human and veterinary implications. *J Mol Genet Med* **3**, 158–166.

Ma, W., Lager, K. M., Lekcharoensuk, P., Ulery, E. S., Janke, B. H., Solórzano, A., Webby, R. J., García-Sastre, A. & Richt, J. A. (2010a). Viral reassortment and transmission after co-infection of pigs with classical H1N1 and triple-reassortant H3N2 swine influenza viruses. *J Gen Virol* **91**, 2314–2321.

Ma, W., Oberst, R., Li, X., Clouser, D., Hesse, R., Rowland, R. & Richt, J. A. (2010b). Rapid detection of the pandemic 2009 H1N1 virus M gene by real-time and gel-based RT-PCR assays. *Influenza Other Respir Viruses* **4**, 397–403.

Ma, W., Vincent, A. L., Lager, K. M., Janke, B. H., Henry, S. C., Rowland, R. R., Hesse, R. A. & Richt, J. A. (2010c). Identification and characterization of a highly virulent triple reassortant H1N1 swine influenza virus in the United States. *Virus Genes* **40**, 28–36.

Ma, W., Lager, K. M., Li, X., Janke, B. H., Mosier, D. A., Painter, L. E., Ulery, E. S., Ma, J., Lekcharoensuk, P. & other authors (2011). Pathogenicity of swine influenza viruses possessing an avian or swine-origin PB2 polymerase gene evaluated in mouse and pig models. *Virology* **410**, 1–6.

Olsen, C. W., Karasin, A. I., Carman, S., Li, Y., Bastien, N., Ojkic, D., Alves, D., Charbonneau, G., Henning, B. M. & other authors (2006). Triple reassortant H3N2 influenza A viruses, Canada, 2005. *Emerg Infect Dis* **12**, 1132–1135.

Pasma, T. & Joseph, T. (2010). Pandemic (H1N1) 2009 infection in swine herds, Manitoba, Canada. *Emerg Infect Dis* **16**, 706–708.

Richt, J. A., Lager, K. M., Janke, B. H., Woods, R. D., Webster, R. G. & Webby, R. J. (2003). Pathogenic and antigenic properties of phylogenetically distinct reassortant H3N2 swine influenza viruses cocirculating in the United States. *J Clin Microbiol* **41**, 3198–3205.

Richt, J. A., Lager, K. M., Clouser, D. F., Spackman, E., Suarez, D. L. & Yoon, K.-J. (2004). Real-time reverse transcription-polymerase chain reaction assays for the detection and differentiation of North American swine influenza viruses. *J Vet Diagn Invest* **16**, 367–373.

Robinson, J. L., Lee, B. E., Patel, J., Bastien, N., Grimsrud, K., Seal, R. F., King, R., Marshall, F. & Li, Y. (2007). Swine influenza (H3N2) infection in a child and possible community transmission, Canada. *Emerg Infect Dis* **13**, 1865–1870.

Schrauwen, E. J., Herfst, S., Chutinimitkul, S., Bestebroer, T. M., Rimmelzwaan, G. F., Osterhaus, A. D., Kuiken, T. & Fouchier, R. A. (2011). Possible increased pathogenicity of pandemic (H1N1) 2009 influenza virus upon reassortment. *Emerg Infect Dis* **17**, 200–208.

Schrenzel, M. D., Tucker, T. A., Stalis, I. H., Kagan, R. A., Burns, R. P., Denison, A. M., Drew, C. P., Paddock, C. D. & Rideout, B. A. (2011). Pandemic (H1N1) 2009 virus in 3 wildlife species, San Diego, California, USA. *Emerg Infect Dis* **17**, 747–749.

Shinde, V., Bridges, C. B., Uyeki, T. M., Shu, B., Balish, A., Xu, X., Lindstrom, S., Gubareva, L. V., Deyde, V. & other authors (2009). Triple-reassortant swine influenza A (H1) in humans in the United States, 2005-2009. *N Engl J Med* **360**, 2616–2625.

Smith, G. J., Vijaykrishna, D., Bahl, J., Lycett, S. J., Worobey, M., Pybus, O. G., Ma, S. K., Cheung, C. L., Raghwani, J. & other authors (2009). Origins and evolutionary genomics of the 2009 swine-origin H1N1 influenza A epidemic. *Nature* **459**, 1122–1125.

Solórzano, A., Webby, R. J., Lager, K. M., Janke, B. H., García-Sastre, A. & Richt, J. A. (2005). Mutations in the NS1 protein of swine influenza virus impair anti-interferon activity and confer attenuation in pigs. *J Virol* **79**, 7535–7543.

Sponseller, B. A., Strait, E., Jergens, A., Trujillo, J., Harmon, K., Koster, L., Jenkins-Moore, M., Killian, M., Swenson, S. & other authors (2010). Influenza A pandemic (H1N1) 2009 virus infection in domestic cat. *Emerg Infect Dis* **16**, 534–537.

Swenson, S. L., Koster, L. G., Jenkins-Moore, M., Killian, M. L., DeBess, E. E., Baker, R. J., Mulrooney, D., Weiss, R., Galeota, J. & Bredthauer, A. (2010). Natural cases of 2009 pandemic H1N1 influenza A virus in pet ferrets. *J Vet Diagn Invest* **22**, 784–788.

Vincent, A. L., Ma, W., Lager, K. M., Janke, B. H. & Richt, J. A. (2008). Swine influenza viruses a North American perspective. *Adv Virus Res* **72**, 127–154.

Vincent, A. L., Swenson, S. L., Lager, K. M., Gauger, P. C., Loiacono, C. & Zhang, Y. (2009). Characterization of an influenza A virus isolated from pigs during an outbreak of respiratory disease in swine and people during a county fair in the United States. *Vet Microbiol* **137**, 51–59.

Wagner, R., Matrosovich, M. & Klenk, H. D. (2002). Functional balance between haemagglutinin and neuraminidase in influenza virus infections. *Rev Med Virol* **12**, 159–166.

World Health Organization (2010). Pandemic (H1N1) 2009 – update 112. http://www.who.int/csr/don/2010_08_06/en/index.html

Yen, H. L., Liang, C. H., Wu, C. Y., Forrest, H. L., Ferguson, A., Choy, K. T., Jones, J., Wong, D. D., Cheung, P. P. & other authors (2011). Hemagglutinin-neuraminidase balance confers respiratory-droplet transmissibility of the pandemic H1N1 influenza virus in ferrets. *Proc Natl Acad Sci U S A* **108**, 14264–14269.