Short Communication

1918 and 2009 H1N1 influenza viruses are not pathogenic in birds

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The susceptibility of chickens to both 1918 and 2009 H1N1 influenza virus was evaluated. The intravenous pathogenicity index of 1918 and 2009 H1N1 viruses in chickens was 0. Chickens did not develop clinical signs following experimental inoculation simulating natural infection. No gross pathological changes were observed in any tissues of chickens between 2 and 18 days post-infection (p.i.) and viral RNA was not detected by real-time RT-PCR in mucosal secretions or tissues. Seroconversion was not detected in any of the chickens following inoculation with H1N1 2009 virus, whereas half the chickens developed influenza-specific antibodies at 28 days p.i. with 1918 influenza, suggesting limited infection. Viral RNA was detected by real-time RT-PCR in mallard ducks following inoculation with 1918 influenza virus at 3 days p.i. in cloacal swabs, but not in tissues, and all ducks seroconverted by 28 days p.i. Both 1918 and 2009 H1N1 influenza viruses behave as LPAI in gallinaceous poultry.

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The 1918 pandemic influenza virus is highly transmissible and has caused severe disease, leading to death in humans (Johnson & Mueller, 2002) as well as several other animal species such as mice (Tumpey *et al.*, 2005), ferrets (Tumpey *et al.*, 2005) and non-human primates (Kobasa *et al.*, 2007). Recently, it was shown that pigs can be infected with the 1918 influenza virus without a lethal outcome of the infection (Weingartl *et al.*, 2009). The origin of the 1918 pandemic influenza virus is unknown. However, genetic sequence analysis suggests that it is avian-like (Taubenberger *et al.*, 2005), raising the question of how virulent this virus would be in bird species. In April 2009, a reassortant H1N1 virus related to swine influenza viruses was identified as the cause of influenza like disease in humans [Novel Swine-Origin Influenza A (H1N1) Virus Investigation Team]. Understanding how human pandemic viruses behave in domestic animal species is critical for surveillance programmes as well as comprehending the biology of the virus. Chickens and ducks are susceptible to avian influenza especially highly pathogenic H5 and H7 isolates. Several strains of highly pathogenic H5N1 virus induce severe disease in mice (Gubareva *et al.*, 1998), ferrets (Zitzow *et al.*, 2002) and macaques (Rimmelzwaan *et al.*, 2001) and highly pathogenic H5N1 human infections can result in a case fatality rate as high as 80 % (Kandun *et al.*, 2008), depending on the isolate and quality of living conditions. Fortunately, efficient transmission similar to either 1918 or 2009 H1N1 viruses between humans has not yet been demonstrated for H5N1 (Tran *et al.*, 2004). To aid in understanding the possible origin of 1918 influenza, and to understand whether determinants for avian tropism still remain in either the 1918 or 2009 H1N1 viruses, chickens were experimentally infected with both viruses and ducks with the 1918 virus. The 1918 influenza virus [haemagglutinin (HA) South Carolina] was reconstructed by reverse genetics as previously described (Tumpey *et al.*, 2005), and all virus work, including virus rescue, was performed in the BSL4 facilities at the Canadian National Centre for Foreign Animal Disease (NCFAD) in Winnipeg. The rescued virus was sequenced and evaluated in mice challenge

experiments to confirm its high pathogenic phenotype (Weingartl *et al.*, 2009). In addition, the 1918 virus was able to replicate in and kill embryonated chicken eggs as previously described (Tumpey *et al.*, 2005). The 2009 H1N1 virus was isolated from clinical samples from Mexico at the National Microbiology Laboratory, Public Health Agency of Canada by Dr Yan Li.

To characterize the 1918 and 2009 H1N1 viruses, both viruses were pathotyped using the intravenous pathogenicity index (IVPI) according to the World Organization for Animal Health (OIE, 2008). The results of the IVPI challenge with either the 1918 or 2009 H1N1 influenza in chickens was 0 since no clinical disease or death was observed in any chickens, indicating that both the 1918 and 2009 H1N1 viruses are not pathogenic in chickens. These results are in agreement with other studies in which human H1N1 viruses, including a 1918 HA recombinant virus was injected in chickens using the intravenous route and did not cause death (Tumpey *et al.*, 2004).

Twenty 40-day-old specific pathogen-free leghorn chickens and 40-day-old mallard ducks were acclimatized for 1 week. Following acclimatization, chickens were infected with 10^5 p.f.u. of either 1918 or 2009 influenza and ducks were infected with 1918 influenza virus in a 1 ml total volume applied to the cloaca, trachea, nares and eyes using a previously demonstrated technique (Pasick *et al.*, 2007). All animals were handled and cared for in accordance with the Canadian Council on Animal Care guidelines. Following challenge, birds were monitored for clinical signs twice each day. Chickens and ducks did not develop any clinical signs of disease during the entire experiment. Cloacal and oropharyngeal swabs were collected from each bird on each of days 2, 3, 4 and 7 post-inoculation. Oral and cloacal swabs from chickens were determined negative at all time points tested for influenza virus by real-time RT-PCR of the M1 gene of influenza A virus segment 7 (Spackman *et al.*, 2002) for 1918-inoculated chickens and for H1N1 2009-inoculated chickens using the above assay with updated primers M+25 – 5′-AGATGAGTCYTCT-AACCGAGGTCG-3′ and M−124 – 5′-TGCAAARACA-YYTTCMAGTCTCTG-3′ for 2009 H1N1. In mallard ducks, at 3 days post-infection (p.i.) 8 of 17 cloacal swabs were positive by real-time RT-PCR, following inoculation with 1918 influenza virus (Table 1). Two chickens and ducks were assessed for gross pathology and virus replication in tissues on days 2, 3, 4 and 7. No macroscopic lesions were observed in tissues from any of the chickens or ducks examined at any time point. The lung, trachea, thymus and intestine from all chickens and ducks were assessed for the presence of viral RNA at 2, 3, 4 and 7 days p.i., and all were negative by real-time RT-PCR. These results indicate 1918 influenza virus did not replicate efficiently in ducks and probably the level of replication was very low, below the detectable limit in chickens inoculated with either 1918 or 2009 H1N1.

At 28 days p.i., the remaining chickens and ducks were euthanized and bled. The sera were evaluated for anti-influenza antibodies by employing a virus neutralization assay by using a constant virus/varying serum method with 1918 influenza and a competitive ELISA for the detection of antibodies against influenza A nucleoprotein (Zhou *et al.*, 1998). Only 6 of 12 chickens inoculated with 1918 influenza had antibody responses by either the virus neutralization or the competitive ELISA assays (Table 2), whereas chickens that were inoculated with 2009 H1N1 did not seroconvert. The absence of seroconversion in all chickens confirms the lack of replication of 2009 H1N1 in chickens and the lack of efficient 1918 influenza virus replication in chickens, since it would be expected that all animals would seroconvert if efficient replication had occurred. In contrast, all 12 ducks had antibody responses detected by the competitive ELISA assay. The detection of viral RNA in only 47 % of ducks at 3 days p.i. indicated that the level of replication in most ducks was below the detection limit.

Table 1. Quantification of influenza viral RNA in cloacal swabs from ducks following 1918 influenza virus inoculation

Cloacal swabs were collected 3 days p.i. and influenza-specific viral RNA was quantified by real-time RT-PCR.

Cloacal swab	Cycle threshold value	RNA copies ml^{-1} (log_{10})
1	>35	–
2	>35	–
3	33	3.7
4	>35	–
5	>35	–
6	31	4.2
7	24	6.4
8	22	7.1
9	28	5.2
10	34	3.5
11	>35	–
12	33	3.5
13	>35	–
14	>35	–
15	25	6.4
16	>35	–
17	>35	–

In gallinaceous poultry, H1N1 avian influenza virus infections may cause a drop in egg production and these viruses are classified as low pathogenic (Suarez *et al.*, 2002). An H1N1 isolate from a poultry market in China was only recovered from two of nine trachea homogenates and not from cloacal swabs from experimentally inoculated poultry, illustrating that viral replication of low pathogenic avian H1N1 influenza virus is difficult to detect (Liu *et al.*, 2003). Our results are in agreement with a recent study that demonstrated that chickens could not be infected with 2009 H1N1 in a transmission study (Lange *et al.*, 2009). Our results indicate that despite its high virulence in mammals, the 1918 influenza virus shows only limited

Table 2. Influenza antibody responses following 1918 influenza virus inoculation in chickens and ducks

Twenty-eight days following infection with 1918 influenza virus, chicken and duck sera were evaluated for anti-influenza A antibodies by virus neutralization (VN) and a competitive ELISA assay. Serum neutralization values are the reciprocal dilution where neutralization activity was observed. Sera were determined to be either '+' positive or '–' negative for influenza nucleoprotein by competitive ELISA.

	VN	ELISA
Chicken		
1	–	–
2	40	+
3	–	–
4	80	+
5	–	–
6	–	–
7	320	+
8	320	+
9	–	–
10	–	–
11	1280	–
12	2560	+
Duck		
1	10	+
2	320	+
3	80	+
4	160	+
5	80	+
6	20	+
7	160	+
8	160	+
9	320	+
10	160	+
11	320	+

replication in experimentally inoculated chickens as well as mallard ducks and it behaves as a low pathogenic avian influenza virus by the OIE criteria (OIE, 2008). The 1918 influenza virus used in this study has receptor binding preference for the α-2,6 sialic acid receptor considered mammalian and not for the α-2,3 sialic acid receptor considered to be the primary avian receptor (Glaser *et al.*, 2005). However, chickens display both α-2,3 and α-2,6 sialic acid receptors (Gambaryan *et al.*, 2002) and avian influenza viruses isolated from chickens with α-2,6 sialic acid receptor specificity have been described, indicating that receptor specificity is probably not the only reason for 1918 virus inefficient replication in these species (Wan & Perez, 2007). Other characteristics of the virus might contribute to viral attenuation. Consistent with this notion, changing the receptor binding specificity of the 1918 virus HA to the avian α-2,3 sialic acid receptor prevented transmission, but not virus replication and consequent lethality in ferrets (Tumpey *et al.*, 2007). However, it did not change the virulence or pathogenesis of 1918 influenza virus in mice (Qi *et al.*, 2009). In any case, our results argue that the 1918 influenza virus, despite its high virulence in mice, ferrets, macaques and humans and its genotypic similarities to avian influenza viruses, replicates poorly in chickens and mallard ducks and therefore was unlikely to have made the direct jump from domestic chicken or mallard duck to humans. The 1918 influenza might then have originated from an 'unknown' avian or other source (Reid *et al.*, 2004) before probably acquiring several adaptive changes through mutation or even reassortment processes with other influenza viruses. As indicated with the present epidemic of a 2009 H1N1 virus transmitting efficiently in humans and apparently having genes derived from swine influenza viruses, swine could also potentially be a source for pandemic influenza viruses. Thus, despite the genotypic similarities between the 1918 virus and avian influenza viruses, this highly pathogenic pandemic human virus appears to be phenotypically closer to mammalian influenza viruses than to avian influenza viruses, as indicated by its replication deficiency in chickens and mallard ducks.

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