Comparison of the pathogenic potential of HPAI H5N6 and H5N8 viruses isolated in South Korea during the 2016–2017 winter season

Young-Il Kim, Kwang-Min Yu, Eun-Ha Kim, Se-Mi Kim, and Young-Ki Choi

Abstract

Highly pathogenic avian influenza (HPAI) A(H5N6) and A(H5N8) virus infections resulted in the culling of more than 37 million poultry in the Republic of Korea during the 2016/17 winter season. Here we characterize two representative viruses, A/Environment/Korea/W541/2016 [Em/W541(H5N6)] and A/Common Teal/Korea/W555/2017 [CT/W555 (H5N8)], and evaluate their zoonotic potential in various animal models. Both Em/W541(H5N6) and CT/W555(H5N8) are novel reassortants derived from various gene pools of wild bird viruses present in migratory waterfowl arising from eastern China. Despite strong preferential binding to avian virus–type receptors, the viruses were able to grow in human respiratory tract tissues. Em/W541(H5N6) was found to be highly pathogenic in both chickens and ducks, while CT/W555(H5N8) caused lethal infections in chickens but did not induce remarkable clinical illness in ducks. In mice, both viruses appeared to be moderately pathogenic and displayed limited tissue tropism relative to HPAI H5N1 viruses. Em/W541(H5N6) replicated to moderate levels in the upper respiratory tract of ferrets and was detected in the lungs, brain, spleen, liver, and colon. Unexpectedly, two of three ferrets in direct contact with Em/W541(H5N6)-infected animals shed virus and seroconverted at 14 dpi. CT/W555(H5N8) was less pathogenic than the H5N6 virus in ferrets and no transmission was detected. Given the co-circulation of different, phenotypically distinct, subtypes of HPAI H5Nx viruses for the first time in South Korea, detailed virologic investigations are imperative given the capacity of these viruses to evolve and cause human infections.

Introduction

In the Republic of Korea, the H5N6 virus was first found in late October of 2016 in fecal specimens from migratory wild birds and went on to cause poultry outbreaks in mid- November 2016. Outbreaks in domestic poultry were spatially and temporarily associated with die-offs of wild birds, leading to speculation that migratory waterfowl were the source of infection. During the 2016/17 outbreak, additional novel H5N8 viruses were isolated from fecal specimens of wild birds in the Gyeonggi Province of central South Korea and subsequently caused devastating outbreaks in domestic poultry. Genetic characterization of EM/W541 (H5N6) and CT /W555 (H5N8) showed that both are novel viral reassortants of clade 2.3.4.4 HPAI H5Nx and co-circulating low pathogenic avian influenza (LPAI) viruses.

The continued presence of the H5N6 and H5N8 viruses in poultry and wild birds has raised questions as to their immediate public health threat. Considering this and the co-circulation of two different HPAI subtypes for the first time in South Korea, we undertook a detailed assessment of the risk posed by H5N6 and H5N8 viruses.

Results

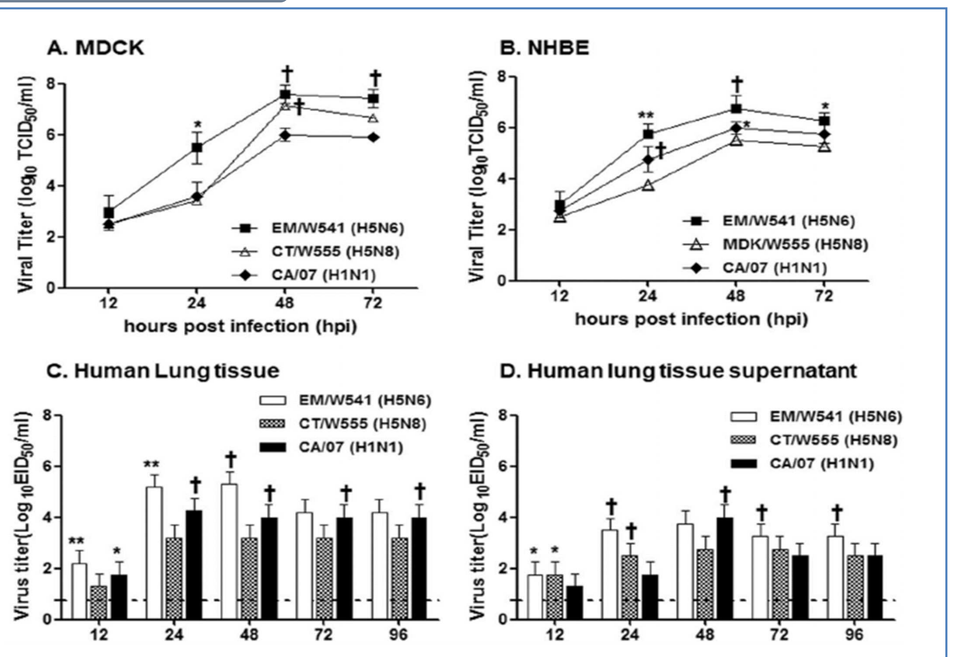

Fig. 1 Growth kinetics and attachment of viruses in human lung tissues ex vivo.

Replication of two Em/W541(H5N6) and CT/W555(H5N8) viruses were monitored in MDCK cells (a), Normal Human Bronchial Epithelial (NHBE) cells (b), Human lung tissue explants and corresponding culture supernatants (c and d, respectively) starting at 12 and 24 hpi intervals thereafter. The titers shown are means ± SD from three independently performed experiments.

Table 1 Biological properties of HPAI H5 viruses

Virus Name	H5 Clade	Biological characteristics						Transmission	
		EID_{50}	$TCID_{50}$	Ck LD_{50}	Dk LD_{50}	IVPI	MLD_{50}	Chicken	Duck
Em/541 (H5N6)	2.3.4.4	9.2	7.8	3.2	5.5	2.7	5.5	6/6	6/6
CT/555 (H5N8)	2.3.4.4	8.9	6.5	4.7	≥6.0	2.9	5.2	6/6	6/6
MDk/W452 (H5N8)	2.3.4.4	8.9	6.9	2.5	≥6.0	3	5.8	6/6	6/6
BDk/Gochang1 (H5N8)*	2.3.4.6	N/A	N/A	N/A	N/A	3*	N/A	N/A	N/A

All values are expressed as log10: EID_{50},; $TCID_{50}$; Ck LD_{50}; Dk LD_{50}; IVPI determined in chickens; MLD_{50} in mice N/A not applicable.

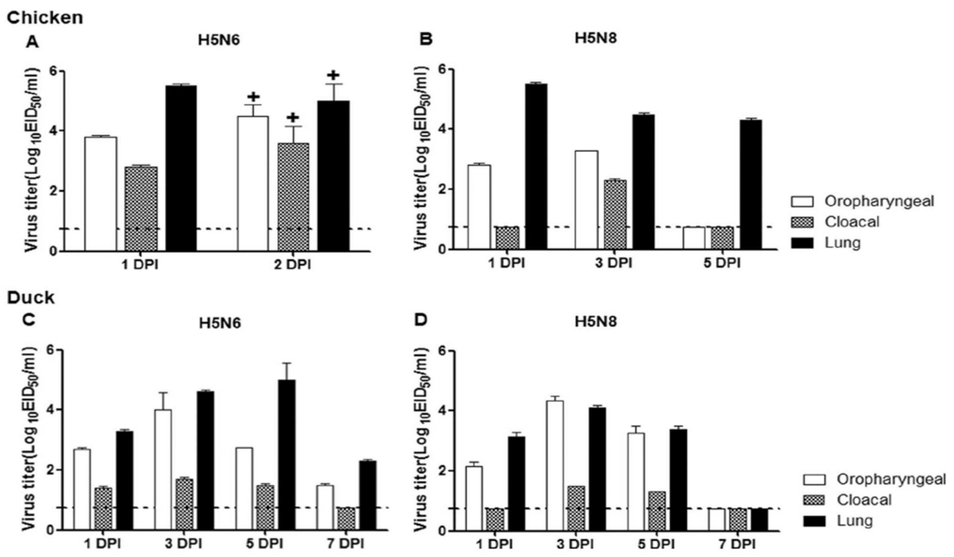

Fig. 2 Replication of Em/W541(H5N6) and CT/W555(H5N8) in avian models.

Virus replication was examined in chickens and ducks that had been experimentally inoculated via the intranasal route with 10^6 EID_{50}/ml of each virus. Mean viral titers (log_{10} EID_{50}/ml) are shown for each group of birds. The limit of virus detection was 0.7 log_{10} EID_{50}/ml

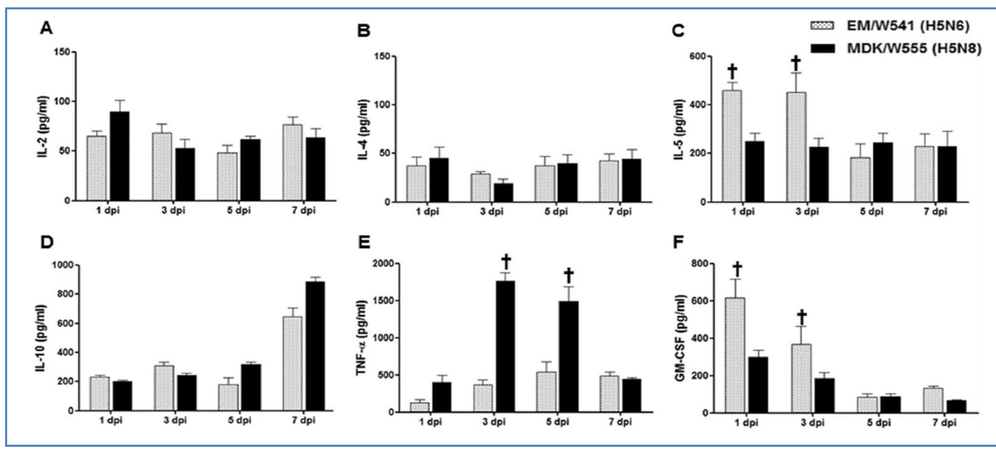

Fig. 3 Cytokine and chemokine responses in the lungs of infected mice.

Concentrations of various cytokines/chemokines in BAL fluid from mice at 1, 3, 5, and 7 dpi were measured by protein analysis with the Luminex™ Instrumentation Systems multiplex array reader. The values shown are means ± SD from BAL fluid of three mice per time point tested.

Table 2 Viral tissue titers in experimentally inoculated animals

Tissue[a]	Em/W541(H5N6)								CT/W555(H5N8)							
	Chicken		Duck		Mice		Ferret		Chicken		Duck		Mice		Ferret	
	1d[b]	2d	3d	5d	3d	5d	3d	5d	3d	5d	3d	5d	3d	5d	3d	5d
Lung	5.5±0.1[c]	5.0±0.2	4.6±0.4	5.0±0.5	4.2±0.3	5.2±0.3	6.0±0.2	5.5±0.3	5.5±0.5[b]	4.5±0.2	4.2±0.2	3.5±0.3	3.5±0.3	4.3±0.2	3.5±0.3	3.0±0.2
Brain	2.5±0.4	2.0±0.4	3.8±0.1	3.2±0.4	-	-	3.2±0.1	1.2±0.4	3.3±0.3	-	-	-	0.8±0.5	1.8±0.3	1.0±0.3	-
Kidney	2.5±0.3	3.0±0.4	2.8±0.4	4.5±0.1	1.0±0.1	-	-	-	5.3±0.3	3.3±0.2	4.3±0.2	3.3±0.1	1.2±0.5	1.0±0.5	-	-
Spleen	2.5±0.2	2.3±0.3	1.7±0.4	-	2.7±0.5	-	1.0±0.2	2.0±0.1	3.8±0.4	-	3.5±0.3	1.5±0.2	1.5±0.1	-	-	-
Heart	3.5±0.3	2.5±0.1	4.4±0.4	5.4±0.4	4.2±0.2	2.7±0.5	-	-	5.5±0.3	3.5±0.1	3.8±0.2	1.8±0.3	1.2±0.2	1.2±0.3	-	-
Liver	3.0±0.2	2.8±0.5	1.5±0.1	-	2.2±0.4	-	-	-	1.8[d]	-	4.2±0.2	2.7±0.3	1.2±0.3	1.5±0.2	1.0±0.2	1.0±0.2
Colon	4.5±0.1	4.75±0.2	2.1±0.4	2.7±0.2	-	-	-	-	4.5±0.3	3.75±0.3	2.5±0.1	1.3±0.3	0.8±0.4	1.5±0.3	1.3±0.3	1.0±0.5

Dashed lines indicate negative for virus detection (lower limit = 0.7 $log_{10}EID_{50}$/g)

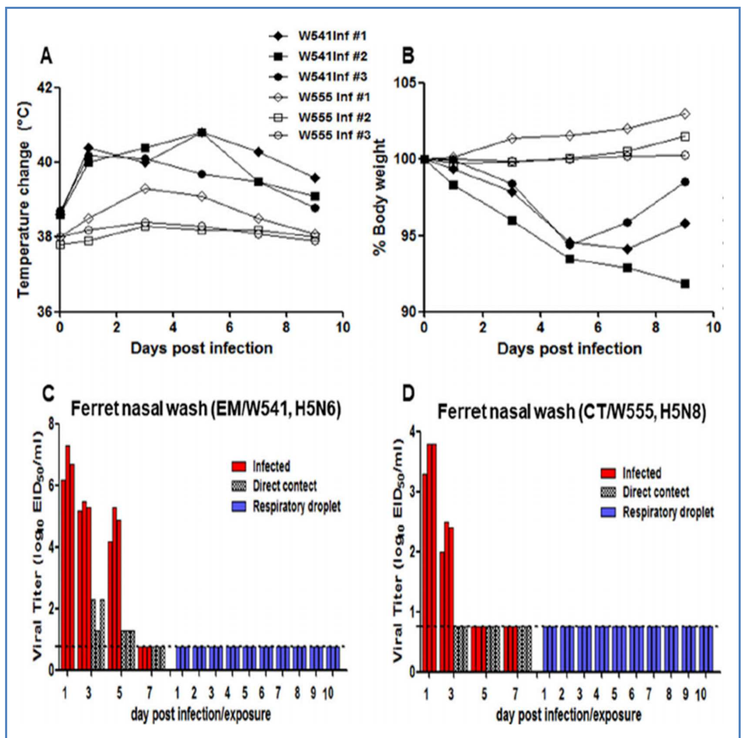

Fig. 5 Replication of Em/W541(H5N6) and CT/W555(H5N8) in ferrets experimentally inoculated via the intranasal route with 106 EID50/ml of each virus.

Table 2 Viral tissue titers in experimentally inoculated animals (Ferrets)

Virus	Exposure	Nasal wash titer (log10 EID50/ml)				HI titer
		1 dpi	3 dpi	5 dpi	7 dpi	
Em/W541 (H5N6)	Inoculated	6.2	5.2	4.2	-	320
	Direct contact	1.3	1.3	-	-	<10
	RD contact	-	-	-	-	<10
	Inoculated	7.3	5.5	5.3	-	640
	Direct contact	2.3	1.3	1.3	-	80
	RD contact	-	-	-	-	<10
	Inoculated	6.7	5.3	4.9	-	320
	Direct contact	2.3	1.3	1.3	-	80
	RD contact	-	-	-	-	<10
CT/W555 (H5N8)	Inoculated	3.3	2.0	-	-	80
	Direct contact	-	-	-	-	<10
	RD contact	-	-	-	-	<10
	Inoculated	3.8	2.5	-	-	160
	Direct contact	-	-	-	-	<10
	RD contact	-	-	-	-	<10
	Inoculated	3.8	2.4	-	-	80
	Direct contact	-	-	-	-	<10
	RD contact	-	-	-	-	<10

Summary & Discussion

1. **The H5N6 virus exhibited high pathogenicity in avian hosts, including chickens where it had 100% mortality, a 2-day mean survival time, and broad tissue dissemination.**
2. **The NS1 gene of the H5N6 virus has 80 to 84 residue deletions compared to the H5N8 virus, which deletion of the NA stalk region is a major virulence determinant that kill infected birds with H5N6 .**
3. **Of note, Em/W541 (H5N6) showed more potential to infect mammalian hosts than the H5N8 virus as inferred from viral replication in human *ex vivo* lung tissue and infection of ferrets.**
4. **Although the receptor-binding specificity of the Korean H5N6 viruses still showed a preference for the α-2,3 sialic acid avian receptor, the virus could replicate in the upper respiratory tract of ferrets at a rate much higher than the H5N8 virus (peak titers, 6.0 VS 2.5 $log_{10}EID_{50}$/ml in H5N6 and H5N8, respectively) as well as in human NHBE cells and ex vivo lung tissues.**
5. **Supporting an elevated zoonotic risk of Em/W541 (H5N6) over CT/W555 (H5N8) was the finding that only the former virus was transmitted to direct contact ferrets where it was shed for 3 to 5 days; a property not shared with previously circulating HPAI H5N1 and H5N8 viruses in Korea.**
6. **While the H5N6 and H5N8 viruses had similar MLD_{50} values, the H5N6 virus spread more systematically in both mice and ferrets and was detected in brain, spleen, heart, kidney and/or liver.**
7. **Although the H5N6 and H5N8 viruses were less pathogenic than the HPAI H5N1 virus, both viruses are pathogenic enough to cause upregulation of pro-inflammatory cytokines and mortality in infected mice.**

Reference

1. Richard, M., Herfst, S. & van den Brand, J. M. et al. **Low virulence and lack of airborne transmission of the Dutch highly pathogenic avian influenza virus H5N8 in ferrets.** PloS One 10, e0129827 (2015)
2. Yang, H., Carney, P. J. &Mishin, V. P. et al. **Molecular characterizations of surface proteins hemagglutinin and neuraminidase from recent h5nx avian influenza viruses.** J. Virol. 90, 5770–5784 (2016)
3. Gu, M., Liu, W., Cao, Y. & Peng, D. et al. **Novel reassortant highly pathogenic avian influenza (H5N5) viruses in domestic ducks, China.** Emerg. Infect. Dis. 17, 1060–1063 (2011)
4. Lee, Y.-J., Kang, H.-M. & Lee, E.-K. et al. **Novel reassortant influenza A (H5N8) viruses, South Korea, 2014.** Emerg. Infect. Dis. 20, 1087 (2014)
5. Bouwstra, R., Koch, G. & Heutink, R. et al. **Phylogenetic analysis of highly pathogenic avian influenza A (H5N8) virus outbreak strains provides evidence for four separate introductions and one between-poultry farm transmission in the Netherlands, November 2014.** Eur. Surveill. 20, 21174 (2015)