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Experimental inoculation of a crow derived influenza A (H5N1) virus in chickens and its pathological and genetic characterization

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ABSTRACT

We report the infectivity of a crow derived influenza A (H5N1) virus (A/crow/India/01TR01/2012) in chickens and its pathological and genetic characterization. Histopathological changes and immunohistochemistry staining of internal organs and skeletal muscle were consistent with influenza A virus infection. Real time RT-PCR and virus isolation results demonstrated the systemic spread of the virus in chickens with 100% mortality. Comparatively higher level of virus shedding was detected in oropharyngeal swab (7.63×10^6 viral RNA copy) than in cloacal swab (6.66×10^6 viral RNA copy). Concentrations of viral antigen in kidney, lungs, brain, spleen and large intestine were higher compared to pancreas and skeletal muscle. No genetic change was observed on interspecies transmission of the virus. The study revealed that the crow derived H5N1 virus is able to kill the poultry, underlining the need for close monitoring of presence of virus in poultry near crow roosting areas so that further transmission to other avian and mammalian hosts can be prevented.

Key words: Chicken, Crow, H5N1 subtype, Influenza A virus

Influenza A (H5N1) virus is a threat to poultry industry worldwide. Since late 2003, the virus has spread to over 60 countries in Asia, Europe and Africa resulting in loss of millions of poultry due to death or culling to control the disease. Occasionally, the virus has jumped from poultry to mammals including humans thereby revealing its pandemic potential (Peiris *et al*. 2007). Land-based wild birds including house sparrows, European starlings and Carneux pigeons may also play a role in the ecology of H5N1 virus and could contribute to the spread and interspecies transmission of virus (Boon *et al*. 2007). The virus is transmitted to crows and H5N1 virus was isolated from crows in many Asian countries including Afghanistan, Bangladesh, India, Japan, Nepal and Pakistan (Tanimura *et al*. 2006, Nagarajan *et al*. 2010, Siddique *et al*. 2012, Khan *et al*. 2014).

In India, crow mortalities were reported from Asom, Bihar, Jharkhand, Maharashtra, Odisha and Uttar Pradesh and H5N1 virus was isolated from dead birds (http://www.oie.int/animal-health-in-the-world/official-disease-status/). However, there is no report of poultry mortalities near the crow roosting sites in India.

Crows are ubiquitous in India and they migrate a few kilometres (up to 20 km) away from the roost in search of food and return to the roost before night (http://www.nonnativespecies.org/factsheet/downloadFactsheet.cfm?speciesId=924). They remain close to the human habitat and poultry chain for their food. Therefore, they could play an important role in the ecology and epidemiology of influenza virus H5N1. However, data describing the susceptibility of chickens to crow derived H5N1 viruses are limited.

In this report, we present the gross and histologic lesions, and the immunohistochemical distribution of virus in chickens infected with a crow H5N1 virus. We assess the virus shedding in different swabs and carried out genetic characterization of the isolated virus to identify changes, if any, during interspecies transmission.

MATERIALS AND METHODS

Virus: Influenza A (H5N1) virus (A/crow/India/01TR01/2012) isolated from a dead house crow (*Corvus splendens*) in Odisha was used in the study. The virus was isolated from tracheal tissues by inoculating in the allantoic cavity of 10-days-old specific pathogen free (SPF) embryonated chicken eggs (OIE 2012). The infectivity titre (embryo infective dose 50 (EID_{50})) of the virus was determined by standard method (Reed and Muench 1938). The harvested allantoic fluid was clarified by centrifugation, aliquoted, and stored at –80°C for further use. All experiments with

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the H5N1 virus were performed in the containment laboratory (BSL-3) of ICAR-National Institute of High Security Animal Diseases (NIHSAD), Bhopal.

Animal experiment: To investigate the replication and tissue tropism of the virus in chickens, 6-week-old SPF white leghorn chickens (8 birds) were inoculated via intranasal route with 0.1 ml (10^6 EID_{50}) of H5N1 virus. All the birds were monitored daily for clinical signs/death. For monitoring viral shedding, oropharyngeal and cloacal swabs from infected birds were collected. Brain, trachea, lungs, heart, liver, spleen, kidney, pancreas, small intestine, large intestine and skeletal muscle were collected for virus isolation following death. Six SPF chickens were kept as negative control in the study. The animal experiment was carried out in the ABSL-3 containment animal wing of ICAR-NIHSAD, Bhopal as per the guidelines of the Institutional Animal Ethics Committee.

Virus isolation: Virus isolation from swabs/tissues was performed in 9- to 11-day-old SPF embryonated chicken eggs following standard procedures (OIE 2012). The allantoic fluid collected from dead embryos was screened by haemagglutination (HA) test (OIE 2012). The samples negative in HA were subsequently given one more blind passage in SPF eggs before declaring negative. The samples, which gave no HA titer after second passage were declared negative for virus isolation.

RNA extraction and real time RT-PCR: Viral RNA was extracted from the swab and tissues using viral RNA mini kit and Trizol reagent, respectively, following manufacturers' protocol. Cloning and *in vitro* transcription (IVT) of full length matrix gene and its quantification was carried out as per Behera *et al*. (2015). A series of 10-fold serial dilution of the quantified IVT RNA standards was prepared in RNase-free water and 1µl of each dilution was used as template for preparation of standard curve for quantitative analysis. One step real time RT-PCR assay was performed using one-step quantitative RT-PCR system to detect and quantify the presence of influenza A virus genome using primers and probes against matrix gene as described previously (Nagarajan *et al*. 2010). The standard curve was prepared using real time PCR system II software version 1.5 in plotting the Cp values against each standard of known concentration and by extrapolating the linear regression line of this curve. Data analysis for estimated viral copies was performed using the second derivative method of the instrument extrapolating the standard curves.

RT-PCR and sequencing: The viral RNA was reverse transcribed with influenza A virus universal primer (Hoffmann *et al*. 2001) by using AMV reverse transcriptase. PCR amplification was performed with a set of segment-specific primers using Platinum *Taq* high fidelity DNA polymerase as per Tosh *et al*. (2011). The PCR amplified products were purified using gel extraction kit and sequenced using specific primers with terminator cycle sequencing kit, version 3.1 in genetic analyzer.

Histopathology and immunohistochemistry: Tissue sections collected from dead birds at necropsy were fixed in 10% neutral buffered formalin (NBF), routinely processed, embedded in paraffin, sectioned at approximately 5µm, mounted on poly L-lysine coated glass slides and routinely stained by Haematoxylin-eosin (HE) (Perkins and Swayne 2003). Another set of formalin-fixed, paraffin embedded sections after deparaffinization, rehydration and treatment with proteinase-K solution for antigen retrieval, were stained by immunoperoxidase technique (Haines *et al*. 1993). Polyclonal serum raised in chickens against A/chicken/India/03CL488/2011 (H5N1) was used as the primary antibody to detect the antigens. Antigen-antibody reaction was detected by incubating with HRPO conjugated anti-chicken antibody for 60 min at 37°C followed by washing and addition of AEC (3-amino 9-ethylcarbazole)-H_2O_2 as chromogen substrate and allowing to develop colour for 5 min at room temperature. The sections were then counterstained with Mayer's haematoxylin and sealed with aqueous clear mount. Positive results appeared as reddish staining at the site of binding.

Statistical analysis: Student t-test was performed using the software program SPSS 16.0; $P<0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

All the 8 chickens inoculated with A/crow/India/01TR01/2012 (H5N1) virus showed depression, prostration, ruffled feathers, ocular and nasal discharge with respiratory signs such as gasping on one day post infection (dpi) and died on 2 dpi. Mean body temperature of the birds on 1 dpi showed an elevation (42.8°C) as compared to before virus inoculation (41.6°C) which decreased further to 41.1°C on 2 dpi before death. All the necropsied birds revealed predominant vascular and necrotic gross lesions such as meningeal congestion and haemorrhages in brain, edema and congestion of lungs and kidneys, reddish demarcated multiple foci scattered on pancreatic surface, haemorrhages on serosal surface of gizzard and proventriculus, petechial haemorrhages on the epicardial fat, distended intestines with excessive fluid contents along with petechial haemorrhages on mucosa visible from serosal surface (Figs 1A-D). The nasal sinuses and pharynx had excessive mucus accumulation.

The virus caused foci of acute necrosis and haemorrhage in multiple tissues. The cerebral cortex showed diffusely distributed degenerating neurons with satellitosis and prominent glial nodule (Fig. 2A). In lungs, air capillary congestion and haemorrhages along with interstitial infiltration of inflammatory cells and swelling of endothelial cells was prominent change in all the birds (Fig. 2B). Liver revealed sinusoidal congestion and vacuolar degeneration of hepatocytes (Fig. 2C). Pancreatic parenchyma had necrotic foci of acinar cells without involving Islet of Langerhans. Kidneys showed interstitial congestion and haemorrhages and degeneration of proximal convoluted tubular epithelium (Fig. 2D). The congestion and haemorrhages were also seen in lamina propria of other epithelial tissues. Immunohistochemistry of tissues

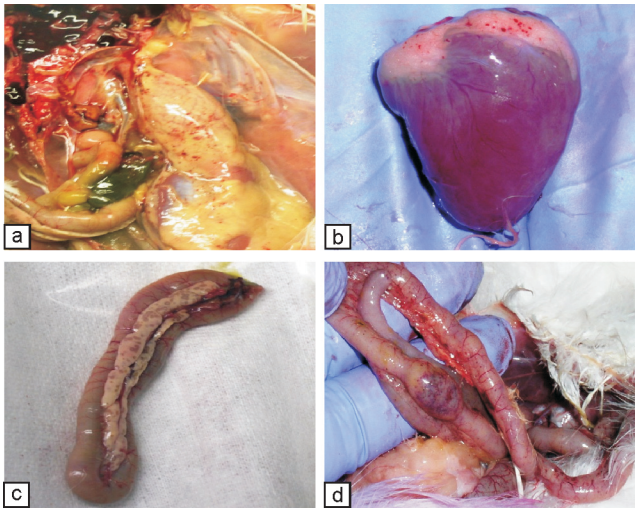

Fig. 1. (A-D). Gross lesions in selected organs from infected chickens that died on 2 dpi. A, Haemorrhages on serosal surface of proventriculus; B, Petechial haemorrhages on epicardial fat; C, Multiple demarcated reddish foci on pancreatic surface, and D, Haemorrhages on mucosal surface of small intestine.

demonstrated presence of influenza virus antigen in different magnitudes with highest scoring observed in brain, lung, heart, pancreas and kidney followed by trachea, liver and intestine (Table 2). In brain focal staining of glial cells with staining of neuronal cell processes, cytoplasm and nuclei observed in the cerebrum (Fig. 2E). In the trachea, the ciliated epithelia showed positive staining for influenza virus antigen. Distribution of viral antigens was more pronounced in lung tissues with staining of capillary endothelial cells and epithelial cells of the bronchioles/air sacs (Fig. 2F). Antigen staining was present at varying intensity in a wide range of other tissues including myocardiocytes, hepatocytes and Kupffer cells, pancreatic acinar epithelium and renal tubular epithelium (Figs 2G, I, J). In the sections of duodenum, the ganglionic cells within the submucosa and myenteric plexus/auerbach's plexus stained positively for viral antigen (Fig. 2H). The above clinical findings of chickens were comparable with those previously reported for other HPAI H5N1 viruses in chickens indicating that crow derived H5N1 virus is highly pathogenic to chickens (Perkins and Swayne 2003, Yuan *et al*. 2014).

Virus shedding was demonstrated by virus isolation in oropharyngeal and cloacal swabs collected on different days of virus inoculation. It was seen that the virus shedding into oropharyngeal swab were higher than those into the cloacal swab ($P<0.05$). Shedding of virus into oropharyngeal swab was significantly higher on 2 dpi than 1 dpi ($P<0.01$) as revealed by the viral RNA copy numbers (Table 1). All the inoculated birds shed infectious virus until 2 dpi, with maximum viral RNA copy number detected in oropharyngeal swabs (7.63×10^6 viral RNA copy) than cloacal swabs (6.66×10^6 viral RNA copy). Earlier study also revealed maximal shedding of virus into oropharyngeal swab than cloacal in chickens inoculated with HPAI H5N1 viruses isolated from different species of birds (Jeong *et al.* 2009, Forrest *et al*. 2010). Virus was isolated from brain, trachea, lungs, heart, liver, spleen, small intestine, pancreas, large intestine, kidney, skeletal muscle collected from the infected chickens that died on 2 dpi (data not shown). Viral RNA was also detected in all the tissues as revealed by real time RT PCR assay (Fig. 3). Even though all the tissues

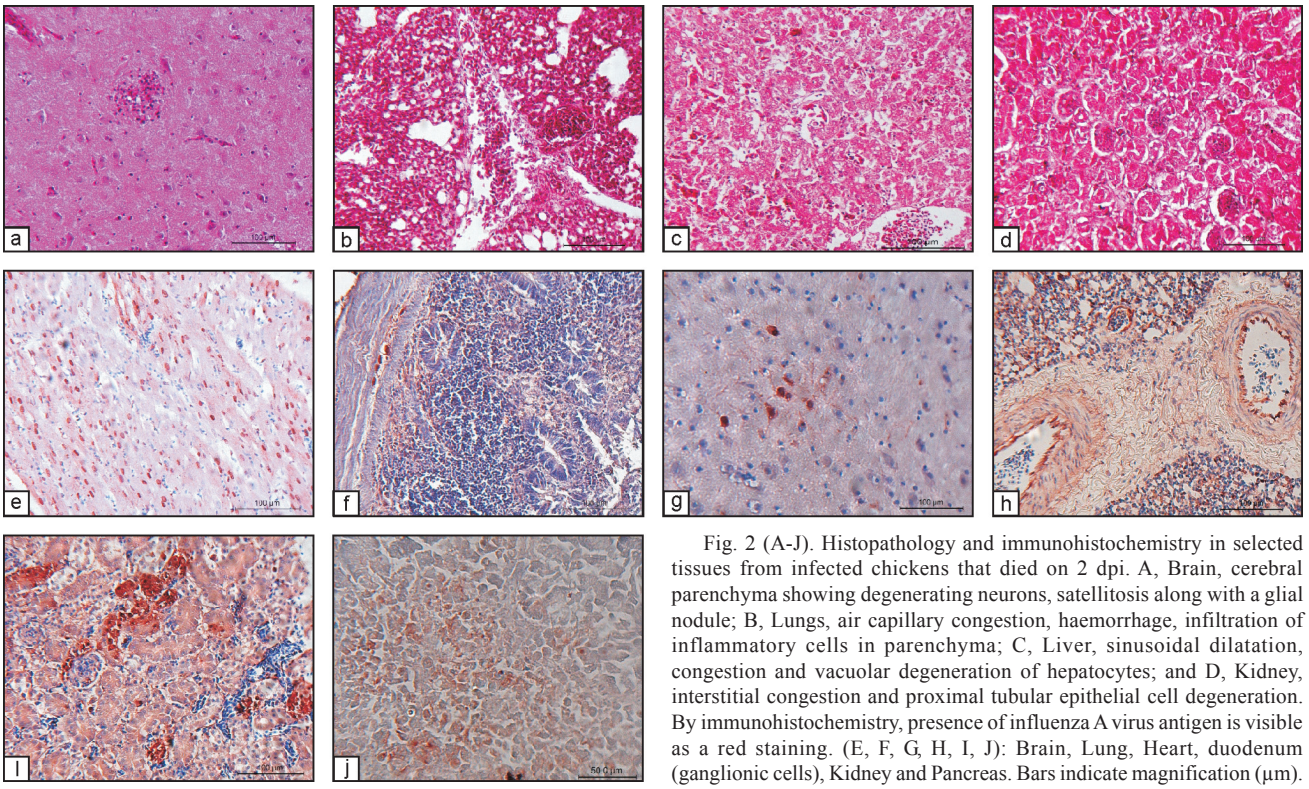

Fig. 2 (A-J). Histopathology and immunohistochemistry in selected tissues from infected chickens that died on 2 dpi. A, Brain, cerebral parenchyma showing degenerating neurons, satellitosis along with a glial nodule; B, Lungs, air capillary congestion, haemorrhage, infiltration of inflammatory cells in parenchyma; C, Liver, sinusoidal dilatation, congestion and vacuolar degeneration of hepatocytes; and D, Kidney, interstitial congestion and proximal tubular epithelial cell degeneration. By immunohistochemistry, presence of influenza A virus antigen is visible as a red staining. (E, F, G, H, I, J): Brain, Lung, Heart, duodenum (ganglionic cells), Kidney and Pancreas. Bars indicate magnification (µm).

Table 1. Isolation and quantification of virus in oropharyngeal and cloacal swabs of chickens on different dpi infected with A/crow/India/01TR01/2012 via intranasal route

Bird No.	Swab	1 dpi		2 dpi	
		Virus isolation*	Realtime RT PCR	Virus isolation	Realtime RT PCR
CK-11	Oropharyngeal	+	4.97×10^3 #	+	4.53×10^5
	Cloacal	-	0	+	2.15×10^5
CK-12	Oropharyngeal	-	4.32×10^3	+	7.09×10^5
	Cloacal	-	0	+	1.92×10^4
CK-13	Oropharyngeal	-	3.33×10^3	+	7.72×10^6
	Cloacal	-	0	+	7.03×10^5
CK-14	Oropharyngeal	+	1.32×10^4	+	3.52×10^6
	Cloacal	+	6.38×10^3	+	4.87×10^5
CK-15	Oropharyngeal	-	8.16×10^2	+	1.71×10^6
	Cloacal	-	0	+	1.27×10^6
CK-16	Oropharyngeal	+	5.24×10^3	+	4.62×10^6
	Cloacal	-	0	+	1.94×10^5
CK-17	Oropharyngeal	+	2.52×10^3	+	5.66×10^6
	Cloacal	-	1.43×10^3	+	4.04×10^6
CK-18	Oropharyngeal	+	4.48×10^3	+	7.63×10^6
	Cloacal	-	0	+	6.66×10^6

*Confirmed by HA test; #viral RNA copy number per ml of swab suspension.

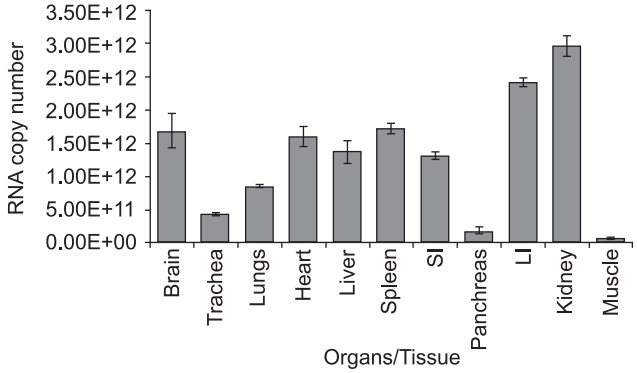

Fig. 3. Real time RT-PCR quantification of viral RNA copy number in different organs/tissues of chickens infected with A/crow/India/01TR01/2012 via intranasal route. (SI, small intestine and LI, large intestine).

had high viral RNA load (8.20×10^{10} to 2.96×10^{12} viral RNA copy number), kidneys had significantly higher virus concentration than other tissues ($P < 0.05$) (Fig. 3), which is correlated with antigen detection in formalin-fixed tissues from kidney (Table 2).

Table 2. Viral antigen distribution by immunohistochemistry in different organs

Organ	Antigen distribution	Antigen positive cells
Brain	+++	Neurons, glial cells and endothelial cells
Lung	+++	Endothelial cells, air capillary epithelium and macrophages
Trachea	++	Lining epithelial cells
Heart	+++	Myocradiocytes
Pancreas	+++	Acinar cells
Liver	++	Hepatocytes and Kupffer cells
Intestines	++	Ganglionic cells and epithelial cells
Kidney	+++	Proximal convoluted epithelial cells

++, common; +++, widespread.

To find out genetic changes, if any, the H5N1 virus isolated from the lung tissues of the chickens that died on 2 dpi with A/crow/India/01TR01/2012(H5N1) was sequenced. The genome sequence of the PB2, HA, NA, M, NS segments were determined and compared with the parent

Table 3. Comparison of specific amino acid residues of selected proteins of influenza A virus isolated from chickens following inoculation with A/crow/India/01TR01/2012 (H5N1)

Virus	Amino acids at positions of H5N1 viruses in the proteins													
	PB2		HA		NA				M2					NS1
	627	701	222	224	119	275	293	295	26	27	30	31	34	92
01TR01/12*	E	D	Q	G	E	H	R	N	L	V	A	S	G	D
chicken isolate#	-	-	-	-	-	-	-	-	-	-	-	-	-	-

* A/crow/India/01TR01/2012 (H5N1); #virus isolated from chicken following inoculation of A/crow/India/01TR01/2012 (H5N1).

virus (GenBank Accession Nos. KM872062-KM872069) (Table 3). Results indicated no change in any of the five genes including receptor binding residue at position 222Q and 224G of HA, which is an avian-type receptor specificity or the amino acid positions 627E and 701D in the PB2 protein and 92D in the NS1 protein, which are associated with increased virulence of virus to mammals (Neumann *et al*. 2010). No change was observed in the NA and M2 proteins that reduces the susceptibility of the virus to anti-influenza drugs.

In conclusion, the crow derived H5N1 virus, like other HPAI viruses, spreads systemically in chickens leading to high pathogenicity. The infected chickens shed infectious virus via oral and cloacal route, which could initiate fresh infection. No change was observed in the genetic makeup of the virus after inoculation of the virus to chickens. However, absence of mortality in domestic poultry near crow dying areas in India, which has also been reported in Bangladesh (Khan *et al*. 2014), could be due to low/no shedding of infectious virus to the environment, which needs separate study.

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REFERENCES

Behera P, Nagarajan S, Murugkar H V, Kalaiyarasu S, Prakash A, Gothalwal R, Dubey S C, Kulkarni D D and Tosh C. 2015. siRNAs targeting PB2 and NP genes potentially inhibit replication of highly pathogenic H5N1 avian influenza virus. *Journal of Biosciences* **40**: 233–40.

Boon A C, Sandbulte M R, Seiler P, Webby R J, Songserm T, Guan Y and Webster R G. 2007. Role of terrestrial wild birds in ecology of influenza A virus (H5N1). *Emerging Infectious Diseases* **13**:1720–24.

Forrest H L, Kim J K and Webster R G. 2010. Virus shedding and potential for interspecies waterborne transmission of highly pathogenic H5N1 influenza virus in sparrows and chickens. *Journal of Virology* **84**: 3718–20.

Haines D M, Waters E H and Clark E G. 1993. Immunohistochemical detection of swine influenza A virus in formalin-fixed and paraffin-embedded tissues. *Canadian Journal of Veterinary Research* **57**:33–36.

Hoffmann E, Stech J, Guan Y, Webster R G and Perez D R. 2001. Universal primer set for the full-length amplification of all influenza A viruses. *Archives of Virology* **146**: 2275–89.

Jeong O M, Kim M C, Kim M J, Kang H M, Kim H R, Kim Y J, Joh S J, Kwon J H and Lee Y J. 2009. Experimental infection of chickens, ducks and quails with the highly pathogenic H5N1 avian influenza virus. *Journal of Veterinary Science* **10**: 53–60.

Khan S U, Berman L, Haider N, Gerloff N, Rahman M Z, Shu B, Rahman M, Dey T K, Davis T C, Das B C, Balish A, Islam A, Teifke J P, Zeidner N, Lindstrom S, Klimov A, Donis R O, Luby S P, Shivaprasad H L and Mikolon A B. 2014. Investigating a crow die-off in January-February 2011 during the introduction of a new clade of highly pathogenic avian influenza virus H5N1 into Bangladesh. *Archives of Virology* **159**: 509–18.

Nagarajan S, Tosh C, Murugkar H V, Venkatesh G, Katare M, Jain R, Behera P, Khandia R, Tripathi S, Kulkarni D D and Dubey S C. 2010. Isolation and molecular characterization of a H5N1 virus isolated from a Jungle crow (*Corvus macrorhynchos*) in India. *Virus Genes* **41**:30–36.

Neumann G, Chen H, Gao G F, Shu Y and Kawaoka Y. 2010. H5N1 influenza viruses: outbreaks and biological properties. *Cell Research* **20**: 51–61.

OIE. 2012. Manual of diagnosis tests and vaccines for terrestrial animals. *Avian Influenza.* Chapter **2.3.4**. Paris, France.

Peiris J S M, de Jong M D and Guan Y. 2007. Avian influenza virus (H5N1): a threat to human health. *Clinical Microbiology Reviews* **20**: 243–67.

Perkins L E L and Swayne D E. 2003. Varied pathogenicity of a Hong Kong–origin H5N1 avian influenza virus in four lasserine species and budgerigars. *Veterinary Pathology* **40**: 14–24.

Reed L J and Muench H. 1938. A simple method of estimating fifty percent endpoints. *American Journal of Hygiene* **27**: 493–97.

Siddique N, Naeem K, Abbas M A, Ahmed Z and Malik S A. 2012. Sequence and phylogenetic analysis of highly pathogenic avian influenza H5N1 viruses isolated during 2006–2008 outbreaks in Pakistan reveals genetic diversity. *Virology Journal* **9**: 300.

Tanimura N, Tsukamoto K, Okamatsu M, Mase M, Imada T and Nakamura K. 2006. Pathology of fatal highly pathogenic H5N1 avian influenza virus infection in large-billed crows (*Corvus macrorhynchos*) during the 2004 outbreak in Japan. *Veterinary Pathology* **43**: 500–09.

Tosh C, Murugkar H V, Nagarajan S, Tripathi S, Katare M, Jain R, Khandia R, Syed Z, Behera P, Patil S, Kulkarni D D and Dubey S C. 2011. Emergence of amantadine-resistant avian influenza H5N1 virus in India. *Virus Genes* **42**: 10–15.

Yuan R, Cui J, Zhang S, Cao L, Liu X, Kang Y, Song Y, Gong L, Jiao P and Liao M. 2014. Pathogenicity and transmission of H5N1 avian influenza viruses in different birds. *Veterinary Microbiology* **168**: 50–59.