Quantification of avian influenza virus in tissues of mute swans using TaqMan real time qRT-PCR

K. Rosenbergova[1], P. Lany[1], Z. Pospisil[1], O. Kubicek[2], V. Celer[1], D. Molinkova[1]

[1]Faculty of Veterinary Medicine, University of Veterinary and Pharmaceutical Science Brno, Czech Republic

[2]National Institute for Nuclear, Chemical and Biological Protection, Kamenna, Czech Republic

ABSTRACT: This study reports on the first quantification of avian influenza virus in the organs of mute swans that died during the epizootic of avian influenza (H5N1) between January and April 2006 in the Czech Republic. The quantitative real-time Reverse Transcriptase PCR (qRT-PCR) assay based on a TaqMan probe was developed for a rapid detection and quantification of avian influenza virus RNA in clinical samples collected from mute swans. Conserved regions in the matrix protein gene of avian influenza virus served as targets for the primers and TaqMan probe design. A recombinant plasmid containing the matrix protein gene amplicon was constructed for a quantitative assay of copy numbers of the target gene. Quantification of avian influenza virus RNA was accomplished using a standard curve generated from ten-fold serial dilutions of recombinant plasmid DNA in the range of 10^2 to 10^8 copies/µl. Avian influenza virus A/Cygnus olor/Brno-cz/2006 was adapted to grow in VERO cells. In the same passage of cell cultivation, the concentration of viral RNA was determined to be 1.01×10^7 copies/ml and $TCID_{50}$ was $10^{4.2}$/ml. From these values the ratio of one RNA copy to 0.00157 virion capable of VERO cells infection was calculated. This ratio was used to estimate the virus concentrations in the tissues of dead mute swans.

Keywords: avian influenza virus; qRT-PCR; TagMan probe; $TCID_{50}$; mute swan

Avian influenza viruses belong to the influenza virus A genus of the Ortho-myxoviridae family and are negative-stranded, segmented RNA viruses. In influenza A viruses, 16 haemagglutinin subtypes (Fouchier et al., 2005) and nine neuraminidase subtypes are currently known. All the subtypes have been found in water birds, which are a natural reservoir of avian influenza virus, and particularly in those of the Anseriformes and Charadriformes families (Brown and Stallknecht, 2008). Transmission of avian influenza to domestic bird flocks and their conversion to highly virulent forms often result in great economic losses (Alexander, 2007; Senne, 2007) and also involve a potential risk of transmission to the human population (Wong and Yuen, 2006). The causative agents of highly pathogenic avian influenza (HPAI) of poultry are most often viruses with H5 or H7 haemagglutinin. Viruses of avian origin are also responsible for diseases in both wild animals, such as seals (Geraci et al., 1982) or whales (Hinshaw et al., 1986), and domestic animals, such as horses (Guo et al., 1992), pigs (Scholtissek et al., 1983; Guan et al., 1996; Karasin et al., 2000), minks (Englund et al., 1999), tigers and leopards (Amonsin et al., 2006; Keawcharoen et al., 2004) or cats (Kuiken et al., 2004; Songsermn et al., 2006).

The first case of HPAI in wild birds caused by the H5N1 subtype was recorded at the end of 2002 in Hong Kong. The virus was isolated from water birds, namely, geese, ducks and swans, flamingos living in captivity, and in other wild birds (Ellis et

Supported by the State Office for Nuclear Safety of the Czech Republic (Grant No. 78/06).

al., 2004). Since then, in Asia, the HPAI virus H5N1 has been the cause of disease and death in a number of wild birds. In the spring of 2005 this virus was transmitted to the population of wild birds on the lake Qinghai in northern China (Liu et al., 2005; Chen et al., 2006), causing the death of thousands of birds (Zhou et al., 2006). The most affected species was bar-headed goose *(Anser indicus)*. During the summer of 2005 the H5N1 virus was transmitted by migrating water birds to Europe, particularly to Rusia, Ukraine and Turkey (OIE, 2005a). In the winter months of 2006, the virus gradually spread to central, western and northern Europe (OIE, 2005b), including the Czech Republic (Nagy et al., 2007). The virus was also detected in the Middle East and in the north of Africa.

In Europe the infected wild water birds were from the order Anseriformes, namely the tufted duck (*Aythya fuligula*), Canada goose (*Branta canadensis*), mallard (*Anas platyrhynchos*), whooper swan (*Cygnus cygnus)* and mute swan (*Cygnus olor*). In that period in the Czech Republic, 14 cases of avian influenza H5N1 were detected and only the mute swan was involved (Nagy et al., 2007). It remains to be found out whether the mute swan is an exceptionally susceptible species of water birds, or whether factors other than infection with H5N1 avian influenza virus are also responsible, and whether the swan may jeopardize other wild animals by shedding the H5N1 virus in the environment. The aim of this study was to determine the amount of avian influenza virus in the organs available from the mute swans that had died of H5N1 avian influenza in the Czech Republic.

MATERIAL AND METHODS

Swan tissue samples

Tissue samples (liver, lung, trachea, intestine, brain and muscle) from mute swans (*Cygnus olor*) that died in the Czech Republic during the epizootic of avian influenza (HPAI H5N1) from January to April 2006 were used. The samples obtained from the National Reference Laboratory for Newcastle Disease and Avian Influenza, State Veterinary Institute in Prague, Czech Republic, were used to quantify viral RNA by the TagMan qRT-PCR system (Table 1). All experimental work with tissue samples and H5N1 virus was carried out in a biosafety level 3 laboratory.

Virus isolation

Avian influenza virus was isolated from swan liver tissue, using 9- to 11-day-old specific-pathogen-free (SPF) embryonated chick eggs. For each liver tissue sample, three chick embryos were inoculated into the allantoic sac, each with 200 µl of 10% liver tissue suspension in minimum essential medium with antibiotics. The eggs were incubated at 36°C for three days and observed daily. Allantoic fluid collected from both the embryos that died between 24–48 hours and the surviving embryos was tested for the presence of hemagglutinating virus by a standard procedure (OIE, 2005a,b).

VERO cells

The African green monkey kidney cell line (VERO) was obtained from the Veterinary Research Institute, Brno, Czech Republic. The cells were propagated in RPMI 1640 medium (Cambrex), supplemented with 1×10^2 IU/ml penicillin, 1×10^2 IU/ml streptomycin and 5% foetal calf serum (FCS), in tissue culture flasks at 36°C for 24 hours; then the medium was replaced with fresh growth medium.

Adaptation of avian influenza virus to VERO cells

Sample of liver was cultivated in embryonated chick eggs and isolated strain of avian influenza virus was adapted to VERO cells by six passages as follows: a 200-µl amount of allantoic fluid with avian influenza virus was inoculated in each culture tube with VERO cells with remove growth medium and allowed to absorb for 30 min at 36°C. Then 5-ml RPMI 1640 medium without FCS was added and the cultures were incubated at 36°C. Reincubation of the cells followed at weekly intervals or when a 50% cytopathic effect was observed. Virus was subsequently propagated by inoculating the infected cells in fresh VERO cultures.

Viral RNA extraction

Total RNA was isolated using an *RNeasy Mini Kit* (Quiagen) according to the manufacturer's instructions. Briefly, either a 200-µl amount of 10%

Table 1. Influenza A viruses and other avian pathogens tested by the qRT-PCR specificity assay

Isolate	HA subtype	Results of qRT-PCR
A/Larus/Slovakia 36/77	H3N6	positive
A/Duck/Czechoslovakia/56	H4N6	positive
A/Chicken/Scotland/59	H5N1	positive
A/Turkey/Canada 6213/66	H5N1	positive
A/Fowl plaque/Brescia/1902	H7N1	positive
A/Turkey/Oregon/71	H7N3	positive
A/Turkey/England/63	H7N3	positive
A/Fowl plaque/Steele	H7N7	positive
A/Swine/Gent/1/84	H3N2	positive
A/Swine/England/117316/86	H1N1	positive
A/Swine/Scotland/410440/94	H1N2	positive
A/Swine/Finistere/2899/82	H1N1	positive
A/Equine 1/Prague/56	H7N7	positive
A/Equine 2/New Market/2/93	H3N8	positive
A/New Caledonia/20/99	H1N1	positive
NDV (Komarov/45/LK)	NA	negative
NDV (APMV1/chicken/Japan/Sato/30)	NA	negative
NDV (CZ 3898/96)	NA	negative
IBDV	NA	negative

NA = not applicable; NDV = Newcastle disease virus; IBDV = infectious bursal disease virus

tissue suspension in PBS or a 200-µl homogenate of VERO cells with avian influenza virus was mixed with 350-µl RLT buffer and 350-µl ethanol (70%). Subsequently, the entire sample was applied to the *RNeasy* spin column and centrifuged at 15 000 g for 1 min. The isolated RNA was eluted in 50 µl nuclease-free water.

Specific matrix protein gene-based quantitative Real-time Reverse Transcriptase (qRT-PCR) development

The matrix protein gene sequence (no. AF457712) obtained from the GenBank was used for qRT-PCR development. Two primers and a probe based on a conserved region of the matrix protein gene of influenza A virus were designed and used to carry out a specific qRT-PCR. The sequence of a sense primer was 5'-CTT CTA ACC GAG GTC GAA ACG TA-3', the sequence of an antisense primer was 5'TGA CAG GAT TGG TCT TGT CTT TAG C 3' and the probe was 5' [FAM] CAA AGC CGA GAT CGC GCA GAG ACT [Tamra] 3'. The probe was labelled at the 5'end with 6-carboxyfluorescein (FAM) reporter dye and, at the 3'end, with 6-carboxytetramethylrhodamine (Tamra) quencher dye. A Light Cycler RNA Amplification kit for a one-step real time RT-PCR (Roche Diagnostic) was used with a 20-µl mixture under the following conditions: 0.4 µl kit-supplied RT-PCR Enzyme Mix, 10pmol of each primer, 0.3 µM probe, 400µM (each) dNTP and 4mM $MgCl_2$. Reverse transcription was performed with 3 µl of total RNA at 55°C for 30 min, followed by a short incubation at 95°C for 30 s to inactivate reverse transcriptase. The PCR amplification programme consisted of 55 cycles of 95°C for 5 s, followed by 60°C for 30 s. All temperature transition rates were set at a maximum transition rate of 20. Fluorescence data was acquired at the end of each annealing step. The reactions were carried out in a Rotor Gene thermocycler (Corbet Research) and data was analyzed using Rotor-Gene 6 software.

Specificity of qRT-PCR

Specificity of the matrix protein gene- based qRT-PCR was verified using available influenza virus isolates of avian, equine, swine and human origin as well as other avian pathogens (Table 1).

Standard curve preparation

A *Taq* polymerase-amplified PCR product of the matrix protein gene sequence (985 bp) was purified using a QIAquick gel extraction kit (Quiagen) and cloned into the plasmid vector pTrcHis TOPO® using a pTrcHis TOPO® TA Expression Kit (Invitrogen). The recombinant plasmid was propagated and purified from *E.coli* TOP10F' competent cells. Plasmid DNA was purified using a *Plasmid Mini Kit* (Quiagen) in accordance with the kit instructions, and DNA concentration was measured by absorbance at 260 nm. A standard curve was generated from ten-fold serial dilutions of the recombinant plasmid DNA. The standard curve was used for the quantification of influenza A virus RNA in tissue samples and infected VERO cells.

Comparison of viral RNA quantification and $TCID_{50}$

Growth medium at 24 hours of VERO cell infection was collected and used to assess the following:

(a) **Quantity of viral RNA** by means of qRT-PCR.

(b) **$TCID_{50}$**. Serial ten-fold dilutions of virus in the growth medium were prepared. A 200-µl amount of each dilution was placed in each of five culture tubes with VERO cells with remove growth medium and allowed to absorb for 30 min at 36°C. Then 2-ml RPMI 1640 medium without FCS was added and the cultures were incubated at 36°C until finally assessed at seven days. The cultures were observed daily and both positive and negative results were recorded. The $TCID_{50}$ was calculated from the scores by the method of Reed and Muench (1938).

The data from both assessments (a, b) ware used to calculate the amount of potentially live avian influenza virus in the swan tissue samples tested.

RESULTS

Specificity of qRT-PCR

The matrix protein primer and probe set was tested with RNA obtained from avian-origin influenza virus isolates representing different HA subtypes and isolates of swine, equine and human origin. The matrix protein set was able to detect all tested influenza A viruses. With other avian pathogens no signal amplification was observed (Table 2).

Standard curve

Serial ten-fold dilutions of recombinant plasmid DNA were used to produce a standard curve for quantitative analysis. The standard curve was gen-

Table 2. Quantification of viral RNA in tested tissue samples of swans

Swan No.	RNA copies per g of tissue					
	liver	lung	trachea	muscle	intestine	brain
1	4.6×10^8	3.4×10^9	2.3×10^9	NT	NT	NT
2	3.4×10^7	4.0×10^8	1.8×10^8	NT	NT	NT
3	4.4×10^8	2.6×10^9	3.3×10^9	NT	NT	NT
4	1.5×10^9	8.8×10^9	1.1×10^{10}	NT	NT	NT
5	1.8×10^9	2.2×10^{10}	NT	NT	1.2×10^{10}	NT
6	3.0×10^8	NT	NT	8.41×10^7	NT	NT
7	6.7×10^6	NT	NT	4.2×10^5	NT	8.1×10^5

NT = not tested

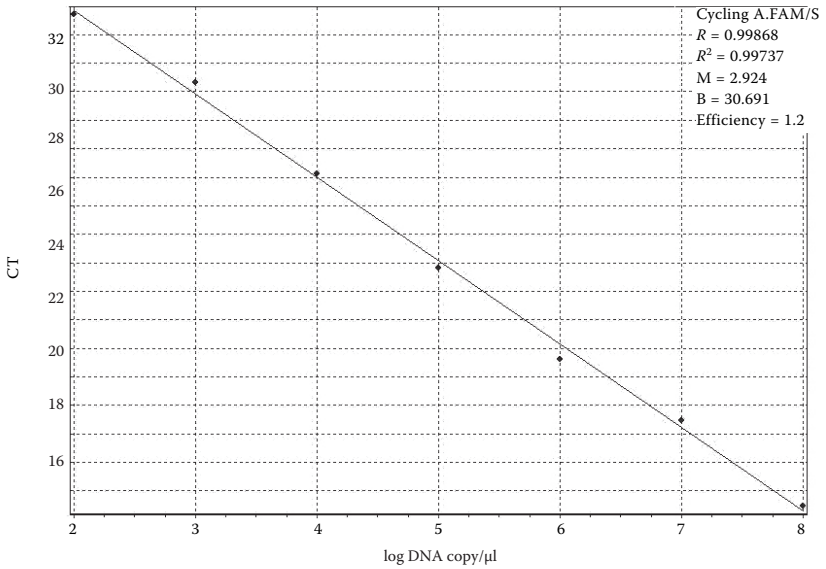

Figure 1. Standard curve generated from 10-fold serial dilutions of recombinant plasmid DNA. The standard curve was achieved by plotting the log concentration of copy numbers against C_T values

erated by amplification of the diluted recombinant plasmid in the range of 10^1 to 10^8 copies per 1 µl, and the log concentration of plasmid DNA copies was plotted against the measured threshold cycle (C_T) values. The threshold of detection was determined as 10^2 molecules. Figure 1 presents a linear correlation between the logarithmic number of plasmid DNA copies and the C_T. The regression coefficient (R^2) between of the copies of plasmid DNA and C_T was 0.999.

Quantification of RNA in tissue samples

All tissue samples were tested by the TaqMan qRT-PCR system. Positive amplification by qRT-PCR was observed in all tested samples. Quantification of viral RNA is summarized in Table 1.

Comparison of viral RNA quantification and $TCID_{50}$

Virus viability was determined on the basis of the following comparisons: the concentration of viral RNA was found to be 1.01×10^7 copies per ml and $TCID_{50}$ was $10^{4.2}$/ml. From these values, the ratio of one RNA copy to 0.00157 virion capable of VERO cell infection was obtained. When the amount of RNA in a tissue sample was multiplied by this ratio, the concentration of live influenza A virus in that tissue was estimated (Table 3).

Table 3. Estimated concentrations of live influenza A virions in tissue samples tested

Swan No.	Virus concentration expressed as number of infectious virions per g of tissue					
	liver	lung	trachea	muscle	intestine	brain
1	7.2×10^5	5.3×10^6	3.6×10^6	NT	NT	NT
2	5.4×10^4	6.3×10^5	2.9×10^5	NT	NT	NT
3	7.0×10^5	4.0×10^6	5.1×10^6	NT	NT	NT
4	2.4×10^6	1.4×10^7	1.7×10^7	NT	NT	NT
5	2.8×10^6	3.5×10^7	NT	NT	1.9×10^7	NT
6	4.7×10^5	NT	NT	1.3×10^5	NT	NT
7	1.1×10^4	NT	NT	6.7×10^2	NT	1.3×10^3

NT = not tested

DISCUSSION

In the years 2005 and 2006, an epizootic of avian influenza due to the HPAI H5N1 Asian virus line occurred in Europe and caused the death of many wild birds from the orders Charadriformes and Anseriformes; with the mute swan population being most affected (Brown and Stallknecht, 2008). In the Czech Republic the mute swan was also infected (Nagy et al., 2007) and, in this study, we made an attempt to estimate the amount of live avian influenza virus in organs available from the swans that had died of H5N1 avian influenza infection.

Under experimental conditions, the pathogenesis of H5N1 avian influenza can be studied by infecting birds with various doses of the virus by either oral or respiratory routes, and observing the development and duration of clinical signs, evaluating histopathological findings and assessing concentrations of the virus in various organs (parenchymatous organs, brain, muscle, etc.) (Lee et al., 2005; Kishida et al., 2005; Swayne, 2006; Zhou et al., 2006). By monitoring the time dynamics of virus excretion from the digestive or respiratory systems and assessing its concentration, risk factors for the spread of infection to other birds can be estimated. At the present moment an experimental infection of swans or related bird species is not feasible in the Czech Republic for both technical and administrative reasons.

An assessment of live H5N1 virus concentrations in dead swan tissues by direct methods, i.e., cultivation in chick embryos or cell cultures, may not always reflect the real values, because virus viability in tissues decreases with the time interval following death. In this study, time between the death of swans and the finding of their bodies and collection of tissues and their storage at –80°C was not known. As detectable concentrations of influenza viruses in tissue decrease more rapidly than their RNA content (Johansen et al., 2002), the detection of RNA in organs may often be the only source of information on viral infection. The proportion of viral RNA to live virus in a tissue cannot be assessed without comparison with an experimental infection. In another system, such as cell culture, this proportion may be similar. For this purpose, a strain of A/Cygnus olor/Brno-cz/1/2006 virus was isolated from swan liver tissue and subsequently adapted to a VERO cell line. The number of RNA copies can be assessed by the quantitative real-time Reverse Transcriptase PCR assay (qRT-PCR). This method has previously been used to determine the amount of avian influenza virus (Lee and Suarez, 2004), as well as other avian viral pathogens (Markowski-Grimsrud et al., 2002; Peters et al., 2005; Islam et al., 2006; Callison et al., 2007). A comparison of the number of viral RNA copies detected by qRT-PCR with that of infectious virions in a VERO cell line, gave a ratio of one RNA copy to 0.00157 $TCID_{50}$. Similar results have been reported by Di Trani et al. (2006) who, using the qRT-PCR assay, could detect up to 0.001 $TCID_{50}$ of the reference virus – an equivalent to 0.08 EID_{50}. The ratio calculated by us was used to estimate the amount of virus originally present in swan tissues and, to be able to compare the amount of virus in swan tissues with those in the organs of swans and other water and gallinaceous avian species reported in the literature, we converted the $TCID_{50}$ obtained to EID_{50} values.

There is no accessible literature information about the HPAI virus (H5N1) concentration in tissues of naturally infected swans. We could compare our results only with data of experimentally infected chickens, ducks and geese, where the concentration of the virus was estimated by cultivation in chicken embryos and $EID_{50.}$

In the brain tissue of experimentally infected chicks, the virus concentration was found to be $10^{6.0}$–$10^{8.1}$ EID_{50}, in those of ducks and geese it was $10^{5.6}$–$10^{7.4}$ EID_{50} and $10^{5.0}$–$10^{6.9}$ EID_{50}, respectively (Zhou et al., 2006). In the brain tissue of experimentally infected ducks, the virus concentration was $10^{6.5}$ EID_{50} in those that died showing brain involvement, and up to $10^{3.6}$ EID_{50} in ducks without clinical signs of the disease (Kishida et al., 2005). The virus concentration in the brain tissue of the examined swan was estimated by us $10^{5.0}$ EID_{50} and this made us assume that, at that virus concentration, the birds' brains may have been affected.

In experimentally infected ducks, the concentration of virus in liver tissue was $10^{2.7}$–$10^{4.0}$ EID_{50}, but the majority of birds were free from clinical signs (Kishida et al., 2005) Our estimate showed a higher concentration ($10^{5.9}$–$10^{8.3}$ EID_{50}) of virus in the liver, and therefore the affected liver may have been one of the causes of swan death.

In experiments with muscle infection in chickens, the concentration of HPAI virus H5N1 virus in the tissue ranged from $10^{2.3}$–$10^{6.8}$ EID_{50}, according to the virus strain used for infection (Swayne, 2006). The muscle tissue of the swans in this study showed values of virus $10^{4.7}$–$10^{7.0}$ EID_{50}, approximately similar to those of the chickens, and indicated an increased vireamia level at the time of bird death.

The lungs of ducks experimentally infected with avian influenza H5N1 virus showed virus concentration in the range of $10^{7.3}$–$10^{8.2}$ EID_{50}, in chickens this was $10^{6.5}$–$10^{7.5}$ EID_{50}, while geese had a concentration of $10^{4.5}$–$10^{5.5}$ EID_{50} (Zhou et al., 2006). In the experiments with a South-Korean H5N1, the virus concentration in lung tissue was $10^{8.5}$–$10^{9.5}$ EID_{50} in chickens infected intranasally, resulting in 100% mortality, while in ducks it was only about $10^{5.0}$ EID_{50} and the birds were free from clinical signs (Lee et al., 2005). Similar findings were made in ducks infected with a Japanese H5N1 virus, in which virus concentrations in the lungs and trachea ranged from $10^{3.3}$–$10^{6.3}$ EID_{50}, and the majority of birds were free from clinical signs (Kishida et al., 2005). In this study the virus concentrations estimate in swan lung tissues were between $10^{7.7}$ and $10^{9.4}$ EID_{50}, i.e., values close to those detected in the chickens that died. In other studies, however, pathological findings in swan lungs did not indicate any severe pulmonary lesions (Teifke et al., 2007), only occasional congestion or even less frequent pulmonary oedema (Palmai et al., 2007).

The studies focused on the route of exit in experimentally infected ducks showed that the H5N1 virus isolated between 1997 and 2001 was transmitted by droppings, while the virus detected from 2002 to 2004 was spread by the respiratory route (Sturm-Ramirez et al., 2005). The south-Korean H5N1 virus was found in the airways and cloacae at concentrations of $10^{6.5}$–$10^{7.0}$ EID_{50} in chickens and $10^{2.0}$ EID_{50} in ducks (Lee et al., 2005). The Japanese H5N1 virus was found in the large intestine at concentrations of $10^{3.0}$–$105^{.3}$ EID_{50} (Kishida et al., 2005). In the swans examined in this study, virus concentrations were estimated $10^{7.3}$–$10^{9.4}$ EID_{50} in the trachea and $10^{9.2}$ EID_{50} in the intestine, which is opposed to the view of Sturm-Ramirez et al. (2005) that H5N1 viruses occurring from the year 2002 on were spread predominantly by the respiratory route.

The results of this study show that, in contrast to duck organs, H5N1 virus multiplies in swan organs to high concentrations, as it does in chickens. In the range of susceptibility to avian influenza infection, swans appear to be sensitive like chickens and therefore can be considered an indicator of the H5N1 virus presence in the wild bird populations of the area. The high concentrations of virus in the trachea and intestine also suggest that swans can be the source of infection for birds and mammals including humans (Anonymous, 2006).

REFERENCES

Alexander D.J. (2007): Summary of avian influenza activity in Europe, Asia, Africa, and Australasia, 2002–2006. Avian Diseases, 51, 1661–1666.

Amonsin A., Payungporn S., Theamboonlers A., Thanawongnuwech R., Suradhat S., Pariyothorn N., Tantilertcharoen R., Damrongwantanapokin S., Buranathai C., Chaisingh A., Songserm T., Poovorawan Y. (2006): Genetic characterization of H5N1 influenza A viruses isolated from zoo tigers in Thailand. Virology, 344, 480–491.

Anonymous (2006): Weekly Epidemiological Record, 81, 173–188.

Brown J.D., Stallknecht D.E.(2008): Wild bird surveillance for the avian influenza virus. Methods in Molecular and Cellular Biology, 436, 85–97.

Callison S.A., Riblet S.M., Oldoni I., Sun S., Zavala G., Williams S., Resurreccion R.S., Spackman E., Garcia M. (2007): Development and validation of a real-time Taqman PCR assay for the detection and quantitation of infectious laryngotracheitis virus in poultry. Journal of Virological Methods, 139, 31–38.

Chen H., Li Y., Li Z., Shi J., Shinya K., Deng G., Qi Q., Tian G., Fan S., Zhao H., Sun Y., Kawaoka Y. (2006): Properties and dissemination of H5N1 viruses isolated during an influenza outbreak in migratory waterfowl in western China. Journal of Virology, 80, 5976–5983.

Di Trani L., Bedini B., Donatelli I., Campitelli L., Chiappini B., De Marco M.A., Delogu M., Buonavoglia C., Vaccari G. (2006): A sensitive one-step real-time PCR for detection of avian influenza viruses using a MGB probe and an internal positive control. BMC Infectious Disease, 6, 87.

Ellis T.M., Bousfield R.B., Bissett L.A., Dyrting K.C., Luk G.S., Tsim S.T., Sturm-Ramirez K., Webster R.G., Guan Y., Malik Peiris J.S. (2004): Investigation of outbreaks of highly pathogenic H5N1 avian influenza in waterfowl and wild birds in Hong Kong in late 2002. Avian Pathology, 33, 492–505.

Englund L., Klingeborn B. (1999): A summary of studies on influenza virus, H10N4 and H10N7, of avian origin in mink. In: Symposium on Animal Influenza Viruses. European Society for Veterinary Virology, Gent, 15.

Fouchier R.A., Munster V., Wallensten A., Bestebroer T.M., Herfst S., Smith D., Rimmelzwaan G.F., Olsen B., Osterhaus A.D. (2005): Characterization of a novel influenza A virus hemagglutinin subtype (H16) obtained from black-headed gulls. Journal of Virology, 79, 2814–2822.

Geraci J.R., St. Aubin D.J., Barker I.K., Webster R.G., Hinshaw V.S., Bean W.J., Ruhnke H.L., Prescott J.H.,

Early G., Baker A.S., Madoff S., Schooley R.T. (1982): Mass mortality of harbor seals: pneumonia associated with influenza A virus. Science, 215, 1129–1131.

Guan Y., Shortridge K.F., Krauss S.P., Li H., Kawaoka Y., Webster R.G. (1996): Emergence of avian H1N1 influenza viruses in pigs in China. Journal of Virology, 70, 8041–8046.

Guo Y., Wang M., Kawaoka Y., Gorman O., Ito T., Saito T., Webster R.G. (1992): Characterization of a new avian-like influenza A virus form horses in China. Virology, 188, 245–255.

Hinshaw V.S., Bean W.J., Geraci J.R., Fiorelli P., Early G., Webster R.G. (1986): Characterization of two influenza A viruses from a pilot whale. Journal of Virology, 58, 655–656.

Islam A., Cheetham B.F., Mahony T.J., Young P.L., Walkden-Brown S.W. (2006): Absolute quantitation of Marek's disease virus and Herpesvirus of turkeys in chicken lymphocyte, feather tip and dust samples using real-time PCR. Journal of Virological Methods, 132, 127–134.

Johansen C.A., Hall R.A., van den Hurk A.F., Ritchie S. A., Mackenzie J.S. (2002): Detection and stability of Japanese encephalitis virus RNA and virus viability in dead infected mosquitoes under different storage conditions. The American Journal of Tropical Medicine and Hygiene, 67, 656–661.

Karasin A.I., Brown I.H., Carman S., Olsen C.W. (2000): Isolation and characterization of H4N6 avian influenza viruses from pigs with pneumonia in Canada. Journal of Virology, 74, 9322–9327.

Keawcharoen J., Oraveerakul K., Kuiken T., Fouchier R.A., Amonsin A., Payungporn S., Noppornpanth S., Wattanodorn S., Theambooniers A., Tantilertcharoen R., Pattanarangsan R., Arya N., Ratanakorn P., Osterhaus D.M., Poovorawan Y. (2004): Avian influenza H5N1 in tigers and leopards. Emerging Infectious Diseases, 10, 2189–2191.

Kishida N., Sakoda Y., Isoda N., Matsuda K., Eto M., Sunaga Y., Umemura T., Kida H. (2005): Pathogenicity of H5 influenza viruses for ducks. Archives of Virology, 150, 1383–1392.

Kuiken T., Rimmelzwaan G., van Riel D., van Amerongen G., Baars M., Fouchier R., Osterhaus A. (2004): Avian H5N1 influenza in cats. Science, 306, 241.

Lee C.W., Suarez D.L. (2004): Application of real-time RT-PCR for the quantitation and competitive replication study of H5 and H7 subtype avian influenza virus. Journal of Virological Methods,119, 151–158.

Lee C.W., Suarez D.L., Tumpey T.M., Sung H.W., Kwon Y.K., Lee Y.J., Choi J.G., Joh S.J., Kim M.C., Lee E.K., Park J.M., Lu X.H., Katz J.M., Spackman E., Swayne D.E., Kim J.H. (2005): Characterization of highly pathogenic H5N1 avian influenza A viruses isolated from South Korea. Journal of Virology, 79, 3692–3702.

Liu J., Xiao H., Lei F., Zhu Q., Qin K., Zhang X.W., Zhang X.L., Zhao D., Wang G., Feng Y., Ma J., Liu W., Wang J., Gao G.F. (2005): Highly pathogenic H5N1 influenza virus infection in migratory birds. Science, 309, 1206.

Markowski-Grimsrud C.J., Miller M.M., Schat K.A. (2002): Development of strain-specific real-time PCR and RT-PCR assays for quantitation of chicken anemia virus. Journal of Virological Methods, 101, 135–147.

Nagy A., Machova J., Hornickova J., Tomci M., Nagl I., Horyna B., Holko I. (2007): Highly pathogenic avian influenza virus subtype H5N1 in Mute swans in the Czech Republic. Veterinary Microbiology, 120, 9–16.

OIE (2005a): World organisation for animal health. Update on highly pathogenic avian influenza in animals (type H5 and H7). Available from http://www.oie.int/downld/AVIAN%20INFLUENZA/A2005_AI.php.

OIE (2005a): World organisation for animal health. Update on highly pathogenic avian influenza in animals (type H5 and H7). Available from http://www.oie.int/downld/AVIAN%20INFLUENZA/A2006_AI.php.

Palmai N., Erdelyi K., Balint A., Marton L., Dan A., Deim Z., Ursu K., Londt B.Z., Brown I.H., Glavits R.(2007): Pathobiology of highly pathogenic avian influenza virus (H5N1) infection in mute swans (*Cygnus olor*). Avian Pathology, 36, 245–249.

Peters M.A., Lin T.L., Wu C.C. (2005): Real-time RT-PCR differentiation and quantitation of infectious bursal disease virus strains using dual-labeled fluorescent probes. Journal of Virological Methods, 127, 87–95.

Reed L.J., Muench H. (1938): A simple method for estimating fifty percent endpoints. American Journal of Hygiene, 27, 493.

Scholtissek C., Burger H., Kistner O., Shortridge K.F. (1985): The nucleoprotein as a possible major factor in determining host specificity of influenza H3N2 viruses. Virology, 147, 287–294.

Senne D.A. (2007): Avian influenza in North and South America, 2002–2005. Avian Disease, 51, 167–173.

Songsermn T., Amonsin A., Jam-on R., Sae-Heng N., Meemak N., Pariyothorn N., Payungporn S., Theamboonlers A., Poovorawan Y. (2006): Avian influenza H5N1 in naturally infected domestic cat. Emerging infectious diseases, 12, 681–683.

Sturm-Ramirez K.M., Hulse-Post D.J., Govorkova E.A., Humberd J., Seiler P., Puthavathana P., Buranathai C., Nguyen T.D., Chaisingh A., Long H.T., Naipospos T.S.P., Chen H., Ellis T.M., Guan Y., Peiris J.S.M., Webster R.G. (2005): Are ducks contributing to the ende-

micity of highly pathogenic H5N1 influenza virus in Asia? Journal of Virology, 79, 11269–11279.

Swayne D.E. (2006): Microassay for measuring thermal inactivation of H5N1 high pathogenicity avian influenza virus in naturally infected chicken meat. International Journal of Food Microbiology,108, 268–271.

Teifke J.P., Klopfleisch R., Globig A., Starick E., Hoffmann B., Wolf P.U., Beer M., Mettenleiter T.C., Harder T.C. (2007): Pathology of natural infections by H5N1 highly pathogenic avian influenza virus in mute (*Cygnus olor*) and whooper (*Cygnus cygnus*) swans. Veterinary Pathology, 44, 137–143.

Wong S.S., Yuen K.Y. (2006): Avian influenza virus infections in humans. Review. Chest, 129, 156–168.

Zhou J.Y., Shen H.G., Chen H.X., Tong G.Z., Liao M., Yang H.C., Liu J.X. (2006): Characterization of a highly pathogenic H5N1 influenza virus derived from barheaded geese in China. The Journal of General Virology, 87, 1823–1833.

Received: 2009–02–09
Accepted: 2009–10–02

Corresponding Author:

Mgr. Katerina Rosenbergova, University of Veterinary and Pharmaceutical Science, Faculty of Veterinary Medicine, Department of Infectious Diseases and Epidemiology, Palackeho 1–3, 612 42 Brno, Czech Republic
Tel. +420 723 872 071, Fax + 420 549 248 841, E-mail: rosenbergovak@vfu.cz