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Pathogenicity Evaluation of Low Pathogenic Avian Influenza (H9N2) Virus Isolated from Layer Flocks in Malaysia in Specific-pathogen-free Chickens

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ABSTRACT

Infection with the low pathogenic avian influenza (LPAI) H9N2 virus has been reported worldwide and poses a health risk to poultry as well as to global health due to its ability to re-assort with other avian influenza viruses. Besides, the silent spread of the H9N2 infection causes significant economic damage to the poultry industry. Recently, Malaysia reported major outbreaks of LPAI H9N2 in commercial layer chicken flocks. Genome sequence analysis indicated that the predominant LPAI H9N2 viruses are of the Y280/BJ94-like lineages. However, the pathogenicity of the virus has not been evaluated. This study determines the pathogenicity of LPAI H9N2 strain UPM994/2018, previously isolated from commercial layer chickens, in one-week-old specific-pathogen-free (SPF) chickens. Clinical signs such as ruffled feathers, mild tracheal rales, facial edema, sero-nasal discharge, and diarrhea were observed from days 6 to 10 post-inoculation (PI). However, no mortality was recorded. Based on a real-time polymerase chain reaction assay, the viruses can be detected in the lungs, trachea, and kidneys of the inoculated chickens on the second day,

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increased until day 10, then declined at day 16 PI. However, swab samples collected from the oropharyngeal and cloacal regions remain positive from day 2 to day 14 PI, with the highest viral load detected at day 10 PI. In conclusion, although the virus is an LPAI, it is pathogenic in SPF chickens, causing respiratory, gastrointestinal, and renal-associated illnesses.

Keywords: Clinical sign, LPAI H9N2 virus, pathogenicity, SPF chicken, viral load

INTRODUCTION

The disease caused by avian influenza viruses (AIVs) may vary depending on the host species, virus subtypes, and lineage. Based on the requirements of the World Organization for Animal Health (WOAH) and the amino acid sequences of the HA gene cleavage site, AIVs can be divided into low pathogenic avian influenza (LPAI) and highly pathogenic avian influenza (HPAI) (Umar et al., 2017). The increase in the virulence and pathogenicity of AIV is associated with polygenic traits. However, hemagglutinin (HA) is the dominant gene in determining the virus pathogenicity (Stech & Mettenleiter, 2013). Nevertheless, molecular determinants of all eight segments, especially mutations of the polymerase genes complex, are associated with high virulence of AIV (Sun et al., 2020). The HPAI viruses encode polybasic cleavage sites at the HA gene that are cleaved by a family of subcellular-like proteases, such as furin and proprotein convertase, which are expressed abundantly in different tissues (Matrosovich et al., 2001).

Hence, infection with HPAIV is associated with systemic disease with high mortality in experimental inoculation of chickens (Mo et al., 2021). All HPAIVs discovered so far are H5 and H7 subtypes; however, not all H5 or H7 viruses are highly pathogenic. However, due to antigenic shift and drift, LPAI viruses can be transformed into HPAIV (Su et al., 2015). On the other hand, LPAIV, with a monobasic cleavage site at the HA gene, can activate soluble trypsin-like proteases, which are present only in cells of the mucosae of the respiratory and intestinal tracts. Hence, experimental infection with the LPAI virus in SPF chickens is associated with mild or asymptomatic disease (Umar et al., 2017). In addition, in an experimental infection study by Gharaibeh (2008), SPF chickens show milder pathogenic lesions compared to broiler chickens. However, the reasons for this were not specified clearly.

Field H9N2 infections in poultry have been reported to show varying degrees of clinical manifestations depending on the presence of host species, virus strains, secondary respiratory pathogens, and immunological factors (Aamir et al., 2007; Wang et al., 2015). Sinuses inflammation, nasal and ocular secretions, severe respiratory symptoms, and low mortality were observed in most reported cases. However, mortality may vary depending on the pathogenicity of the virus and conditions in the field. Experimental studies after H9N2 infection reported mortality

rates of 5, 20, and 65% in commercial broiler chickens (Gharaibeh, 2008; Nili & Asasi, 2003) and, in some cases, of up to 80% in commercial broiler chickens due to concurrent bacterial infections (Guo et al., 2000). A review study by Umar et al. (2017) reported that experimental infection of SPF chickens with LPAI H9N2 is normally associated with mild clinical manifestations and low mortality. However, concurrent experimental infections of H9N2 with other pathogens such as Newcastle disease virus, Infectious bronchitis virus, *Mycoplasma*, and *Escherichia coli* may increase the severity of H9N2 infection (Umar et al., 2017).

H9N2 is also a primary pathogen in field outbreaks in layer and breeder chickens. Severe respiratory signs, drop in egg production by 10 to 27%, and mortality has been observed (Awuni et al., 2019). In most cases, the infected layer and breeder flocks were prone to secondary bacterial infections that may aggravate the severity of the infection (Awuni et al., 2019; Lai et al., 2021). In another study, Hassan and Abdul-Careem (2020) reported that the H9N2 virus could infect the infundibulum of chickens, which eventually affects the proper function of oviducts in layers and breeder chickens. Furthermore, virus binding ability depends upon the virus inoculation route (Pantin-Jackwood et al., 2012; Umar et al., 2017). Thus, all these factors may influence the outcome of H9N2 infection in chickens, including the molecular determinants that have been found to govern the pathogenicity of AIV in chickens.

In 2018, major outbreaks of LPAI H9N2 were reported in commercial chickens in Peninsular Malaysia, leading to massive economic burdens (Syamsiah et al., 2019). Infection with LPAI H9N2 in long-lived birds, namely layers and breeder, is associated with a drop in egg production and quantity and quality of fertile eggs. In addition, the infected birds may die due to secondary bacterial infection. Sequence analysis of the HA gene indicated that the predominant virus isolated during the outbreaks is of the Y280/BJ94 lineage (Gunasekara, 2021; Syamsiah et al., 2019). The complete genome sequence of two Malaysian LPAI H9N2 strains isolated from 2018 to 2019 indicates the virus is a novel reassorted virus of the reassort lineage originating from China (Gunasekara, 2021). Sequence analysis of the HA cleavage site of H9N2 strain UPM994/2018 revealed the presence of a dibasic cleavage site at position 333-PSRSSR-GLF-341 (data not shown). This study determined the pathogenicity of one of the genome-sequenced LPAI H9N2 viruses, A/chicken/Malaysia/UPM994/2018, in SPF chickens.

MATERIALS AND METHODS

Ethical Statement

The live animal trial of this study was conducted according to the guidelines of the institutional animal care and use committee (IACUC), which Universiti Putra Malaysia (UPM) approved under UPM/IACUC/AUP-R055/2020.

Virus Preparation and EID_{50} Measurements

The virus used in this study, A/chicken/Malaysia/UPM994/2018, was isolated from a breeder farm in Negeri Sembilan in 2018 (Gunasekara, 2021). The infected flocks had a history of depressed birds, with mortality ranging from 2 to 4 % per week. Upon post-mortem examination, the affected birds had pulmonary edema with fibrinonecrotic exudate deposition in the trachea and bronchi. The reproductive organs were congested with the presence of egg-yolk peritonitis. According to the WOAH Terrestrial Manual, the virus was propagated in embryonated SPF chicken eggs (WOAH, 2023). Briefly, the allantoic fluid of the inoculated eggs was harvested and centrifuged for 30 min at 1,200 × *g*, and the supernatant was collected and stored at -80°C.

The virus stock was thawed and diluted from 10^{-1} to 10^{-10} in sterile phosphate-buffered saline (PBS, Invitrogen, USA). Five eggs were inoculated with 100 µl of each dilution via the allantoic route and incubated at 37°C for 4 days. Another 5 eggs were inoculated with sterile PBS as the negative control. All the eggs were monitored daily for embryo mortality. Eggs demonstrating embryonic mortality in less than 24 hr were discarded and not considered. The virus titer in embryo infectious dose 50 (EID_{50}) was calculated using the Reed and Muench (1938) method as the reciprocal of the highest dilution that gave HA-positive results for 50% of the inoculated eggs. The HA assay was done in V-bottom 96-well plates with 1% chicken red blood cells.

SPF Chicken

SPF embryonated chicken eggs (Malaysian Vaccines and Pharmaceuticals, Malaysia) were hatched in the Laboratory of Vaccine and Biomolecules, Institute of Bioscience (IBS), UPM. The hatched chicks were transferred to the experimental animal house facility at Biologics Laboratory, IBS, UPM, where they were housed in clean stainless steel bird cages and fed pelleted chicken feed. Water was provided ad libitum, and the drinkers were cleaned daily.

Pathogenicity Study

Seven-day-old SPF birds were randomly allocated into two groups: Group 1 consisted of 48 birds for the infected, and Group 2 consisted of 24 as the control. Before virus inoculation, a blood sample from eight birds was collected randomly to confirm their freedom from H9N2. Each chicken in the infected group was inoculated via the intraocular route with 0.1 ml of 10^7 EID_{50} of H9N2 strain UPM994/2018. For the control group, 0.1 ml of sterilized PBS was inoculated. After inoculation, the chickens in each group were observed daily, and body weight gain was recorded. Three chickens in the control group and six in the infected group were randomly selected and euthanized on days 2, 3, 5, 7, 10, 12, 14, and 16 PI accordingly.

At post-mortem examination, tissue samples of the trachea, lung, gastrointestinal tract, spleen, kidneys, and brain were collected and divided into two parts. Oropharyngeal and cloacal swabs were also collected from these birds prior to

the necropsy. For each tissue sample, a portion was kept in 10% neutral buffered formalin (Sigma-Aldrich, Singapore) for histopathological examination and a portion in 3 ml of PBS for molecular detection.

The body weight of the chickens was measured, and the following equation was used to calculate the percentage of growth retardation rate of the SPF chickens.

$$\text{Retardation rate (\%)} = \frac{\text{Mean weight gain (Control, g)} - \text{Mean weight gain (Infected, g)}}{\text{Mean weight gain (Control, g)}} \times 100\%$$

RNA Extraction

Tissue samples from the kidneys, lungs, and trachea were homogenized with a sterile 1x PBS solution. After centrifugation for 1,200 x *g* (Eppendorf 5810R Centrifuge, Germany) for 15 min at 4°C, the supernatant was passed through a 0.45 µM Minisart® syringe filter (Sartorius AG, Germany) for the RNA extraction. Total RNA was extracted from the supernatant using TRIzol™ Reagent (Invitrogen, USA) according to the manufacturer's instructions. Viral RNA was extracted from the oropharyngeal and cloacal swabs using the innuPREP Virus RNA Kit (Analytik Jena, Germany). The extracted RNA was subjected to a quantitative real-time-polymerase chain reaction (qRT-PCR) to detect virus copy numbers (VCNs).

Clinical Signs, Gross, and Histopathologic Examination

Infected chickens were monitored daily for the presence of respiratory and gastrointestinal distress. Necropsy was performed on chickens on days 2, 3, 5, 7, 10, 12, 14, and 16 PI. Tissues from the respiratory system and gastrointestinal tracts, spleen, kidneys, and brain were collected and kept in 10% buffered formalin for 24 hr. The tissues were processed, embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H&E) (Feldman & Wolfe, 2014). The prepared slides were viewed under the microscope (Leica® Biosystems, USA) to evaluate lesions.

Quantitative Real-time PCR

A TaqMan real-time RT-PCR assay was conducted using a designed and optimized forward primer, 5'-TGCAGCGTAGACGTTTTGTC-3', reverse primer 5'-CAAGCGCACCAGTTGAGTAA-3', and probe 5'-(6-FAM)-TAAATGGGAATGGAGACCCA-(MGB)-3', which could amplify the 153 bp fragment of the M gene. The TaqMan real-time PCR reaction mixture was prepared according to the manufacturer's instructions using a one-step SensiFAST™ Probe No-ROX Kit (Bioline, Germany). The cycling condition was programmed in a CFX96 real-time system (Bio-Rad, USA) at 45°C for 10 min for cDNA synthesis, 95°C for 2 min, followed by 40 cycles at 95°C for 5 s and 60°C for 20 s.

Virus stock RNA amplification was performed by 10-fold serial dilution starting

from 100 to 0.01 ng/µl per reaction to create a standard curve as a positive control. All dilutions were performed in triplicate. The amount of viral shedding was expressed as VCN, where the absolute quantification of the number of viral RNA copies in the swabs was based on quantification cycle (Cq) values for each sample and the qPCR standard curve. The number of viral RNA copies was calculated with the following formula:

$$\text{Number of copies} = \frac{(\text{Concentration in g}) \times (6.023 \times 10^{23})}{330 \times \text{Total amplicon length}}$$

Statistical Analyses

The results of the VCNs of the swab and tissue samples were analyzed using one-way analysis of variance (ANOVA) with Turkey as the post hoc test ($p<0.05$) using SAS 9.1 software. Statistically significant differences in the VCNs were determined by comparing the groups at the given time ($p<0.05$). All quantitative data were expressed as mean ± standard error.

RESULTS

Clinical Signs

The H9N2-infected chickens showed varying clinical signs, from moderate to mild, or the absence of clinical signs in some infected birds. No mortality was recorded in the infected group. Meanwhile, the control group remained healthy throughout the study period. Generally, clinical signs were first recorded at day 6 PI, where two of the six infected birds presented mild diarrhea and ruffled feathers (Table 1). Clinical signs such as depression, sneezing, gasping, ocular discharge, and a slightly swollen face were observed in 2 out of the 6 chickens on days 7 and 10 PI. After day 10 PI, the clinical signs subsided and disappeared on day 12 PI.

On days 3, 5, 7, 10, 12, 14, and 16 PI, birds in the infected group exhibited 7.68, 12.57, 13.3, 11.5, 9.6, 6.7, and 7.5% retardation of growth, respectively, with the highest retardation rate on day 7 PI (Table 2). The body weights of the chickens in the infected and control groups were significantly different ($p<0.01$) from day 5 to day 16 PI onwards.

Table 1
Clinical signs in specific-pathogen-free chickens following inoculation with H9N2 at different days post-inoculation

Clinical signs	Number of chickens showing clinical signs on different days post inoculation													
	2	3	4	5	6	7	8	9	10	11	12	13	14	16
RFD	0/6	0/6	0/6	0/6	1/6	2/6	2/6	2/6	2/6	0/6	0/6	0/6	0/6	0/6
G	0/6	0/6	0/6	0/6	0/6	1/6	1/6	2/6	2/6	0/6	0/6	0/6	0/6	0/6
SN	0/6	0/6	0/6	0/6	0/6	1/6	2/6	2/6	2/6	0/6	0/6	0/6	0/6	0/6
SF	0/6	0/6	0/6	0/6	0/6	0/6	0/6	1/6	1/6	0/6	0/6	0/6	0/6	0/6
WF	0/6	0/6	0/6	0/6	0/6	0/6	1/6	1/6	1/6	0/6	0/6	0/6	0/6	0/6

Note. RED = Ruffled feathers and depression; G = Gasping; SN = Sneezing; ND = Nasal discharge and lacrimation; SF = Swollen face; WF = Watery faeces

Table 2
Growth retardation of specific-pathogen-free chickens after H9N2 inoculation

Days post inoculation (dpi)	Mean weight (g)±SD		Retardation (%)
	Group 1	Group 2	
3	41±3.34	44.33±2.16	7.68
5	45.17±1.16	51.67±3.20	12.57*
7	52.83±1.94	61±3.34	13.3*
10	71.33±3.5	80.67±3.66	11.5*
12	82.33±3.38	91.17±2.78	9.6*
14	94.67±2.25	101.5±2.88	6.7*
16	101.83±1.60	110.17±3.97	7.5*

Note. Groups 1 = Infected; Group 2 = Control; * =The body weight of the chickens in the infected and control groups were significantly different (p<0.01) from day 5 to day 16 post-inoculation onwards

Macroscopic Lesions

Some infected birds, especially cachexic birds, had prominent keel bones at necropsy. Generally, mild gross lesions were observed in H9N2-infected SPF chickens between days 7 to 10 PI. The kidneys were enlarged in one out of six chickens on day 7 PI and two out of six chickens on day 10 PI, respectively. The intestinal and trachea mucosae were mildly hyperemic in H9N2-infected birds. Mild congestion in the lungs was exhibited on days 7 and 10 PI. No gross lesions were observed on day 10 PI afterward (data not shown).

Microscopic Lesions

The tracheas revealed an increased number of goblet cells on day 3 PI. Lymphoid cell infiltration of the lamina propria, epithelial cell desquamation, hyperplasia of epithelial and congested blood vessels was observed at days 7 and 10 PI (Figure 1). In addition, detachment of the trachea's cilia lining with fibrin-heterophilic exudates inside the tracheal lumen was observed on day 10 PI. The tracheal lesions subsided on day 14 PI.

The histopathological changes in the lungs were seen between days 2 to 16 PI, with varying degrees of (mild to moderate) congestion, hemorrhages, edema, and infiltration of lymphocytes into the submucosa of secondary bronchi (Figure 2). Fibrin exudates and focal infiltration of lymphocytes was observed at days 5 to 10 PI, and congestion and hemorrhages of the parabronchi were seen at day 16 PI (Figure 2).

In the case of the kidney, varying degrees of (mild to moderate) congestion, hemorrhage, degeneration, and multifocal necrosis of the renal tubules were seen from day 2 to day 16 PI (Figure 3). Focal infiltration of lymphocytes was also noticed in the renal interstitial tissue. By day 10 PI, cystic tubules containing cellular and hyaline casts and foci of mononuclear leukocyte and macrophage infiltration were prominent, mostly in the medullary region. Mild edema fluid accumulation was detected in the renal interstitial tissue (Figure 3).

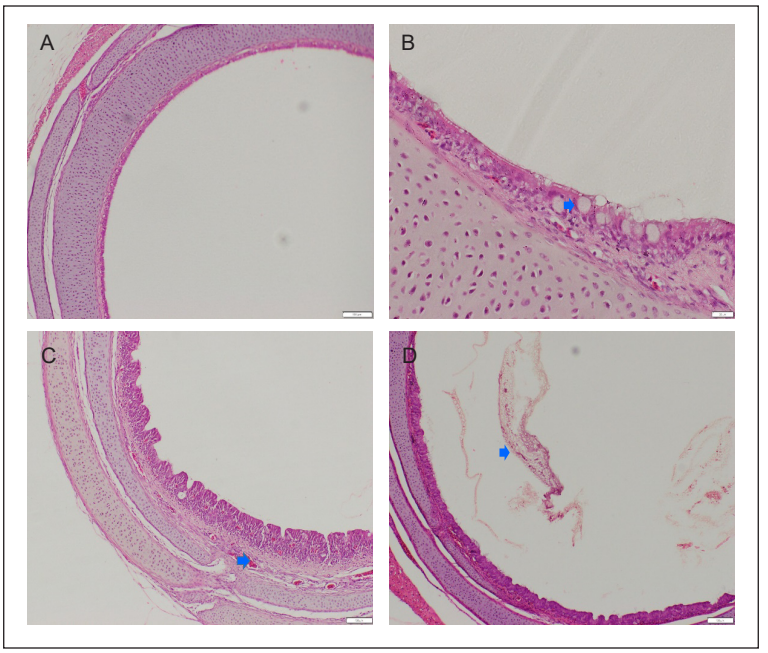

Figure 1. Microscopic lesions in tracheas of H9N2 infected chickens with hematoxylin and eosin staining
Note. A = Normal trachea; B = Increased number of goblet cells on day 3 post-inoculation (PI) (arrows); C = Hyperplasia of epithelium with diffuse loss of cilia, lymphocytic infiltration on day 7 PI; D = Total detachment of the cilia lining on day 10 PI; Scale bars A, C, and D = 100 µm, B = 20 µm

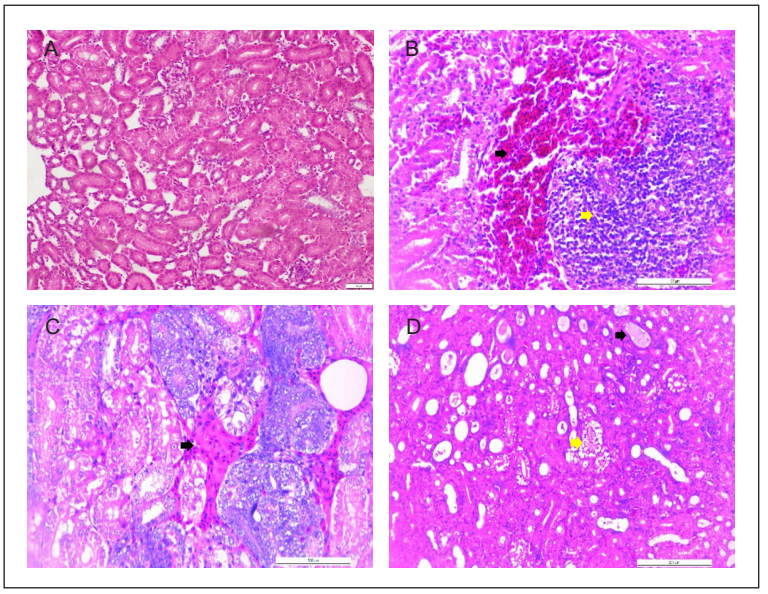

Figure 2. Microscopic lesions in kidneys of H9N2 infected chickens with hematoxylin and eosin staining
Note. A = Normal kidney control; B = Hemorrhages (black arrows) and granulocytic leukocytes dense infiltration and disruption of the tubulointerstitial tissue on day 3 PI (yellow arrow); C = Oedema fluid and cell infiltration (black arrow); D = Mild hyaline cast deposit in the tubular lumen (black arrow), and vacuolation of the cell due to degeneration (10 PI) (yellow arrow); Scale bars B = 20 µm, A and D =50 µm, C = 100 µm

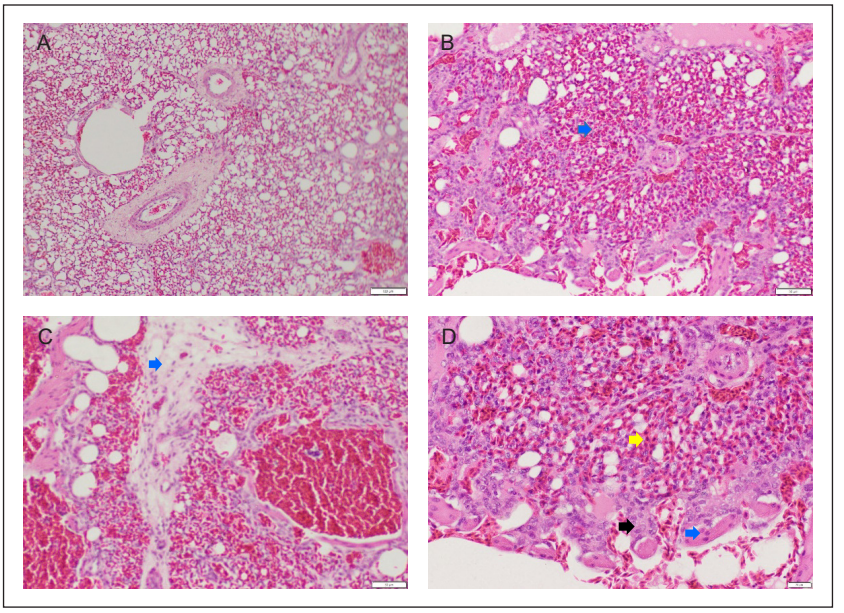

Figure 3. Microscopic lesions in lungs of H9N2 infected chickens with hematoxylin and eosin staining
Note. A = Normal lung control; B = Congested lung on day 5 PI (arrow); C = Edematous lung with accumulation of edema fluid on day 7 PI (arrow); D = Mononuclear cell infiltration (black arrow), hemorrhages (yellow arrow), and presence of edema fluid on 10 PI (blue arrow); Scale bars A = 100 µm, B, C, and D = 50 µm

Mild depletion of lymphoid cells was seen in the spleen at day 14 PI. Mild mucus cell hyperplasia was observed in the intestine, but most changes were carefully observed to differentiate from the control group chickens. No pathological lesions were observed in brain tissue. As expected, no detectable lesions were observed in the control group of chickens. All the microscopic lesions observed in the lung and trachea are summarized in Table 3.

Table 3
Microscopic lesions observed in H9N2 infected specific-pathogen-free chickens

Histopathological changes	2	5	7	10	14	16
Congestion[A]	3/3	3/3	1/3	2/3	2/3	2/3
Haemorrages[AB]	3/3	3/3	2/3	3/3	1/3	1/3
Edema[AB]	0/3	0/3	3/3	3/3	0/3	0/3
Mono nuclear cell infiltration[AB]	0/3	3/3	3/3	3/3	2/3	2/3
Necrosis[B]	0/3	2/3	3/3	2/3	1/3	1/3
Tubular degeneration[B]	0/3	0/3	3/3	3/3	1/3	1/3
Eosinophilic materials inside the lumen[B]	0/3	0/3	0/3	3/3	0/3	0/3
Perirenal haemorrages[B]	0/3	0/3	2/3	0/3	1/3	1/3

Note. A = Lung; B = Kidney

Standard Curve

The standard curve was constructed based on a 10-fold dilution of a known RNA concentration of H9N2 UPM944/2018 using the Bio-Rad CFX manager 3.1 software (Bio-Rad, USA). The standard curve y-intercept is 58.298, and the slope of the standard curve is -3.334. The calculated coefficient of the standard curve is 99.6%, and the real-time PCR efficiency is 99.5% (Figure 4). The graph shows the linear relationship of quantification threshold (ct) versus virus $\log_{10}$ virus copy.

Viral Copy Number

The virus shedding of the H9N2-infected chickens from the cloacal and oropharyngeal swabs was measured using TaqMan-based real-time PCR. The produced viral copy number was calculated as $\log_{10}$ at each time point, and the mean significant difference between the groups was analyzed using one-way ANOVA and Tukey's post hoc test. As shown in Table 4, all the infected chickens from day 2 to 14 PI were detected positive for H9N2. However, the amount of viral load varies according to different time points.

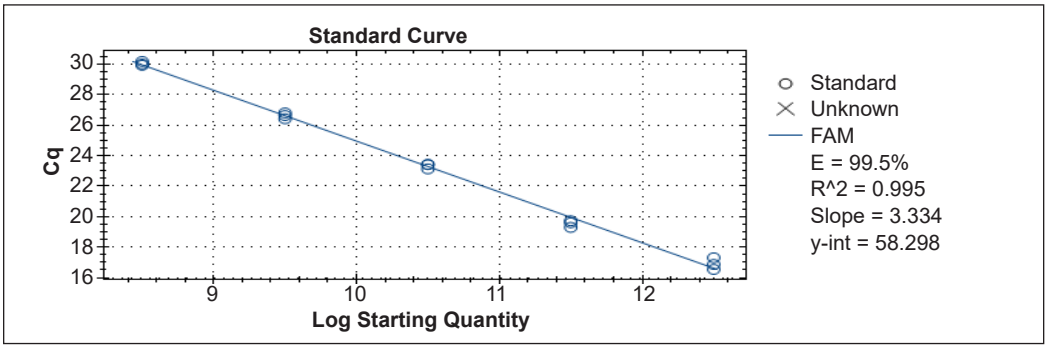

Figure 4. Standard curve of real-time polymerase chain reaction detection of H9N2 virus

Table 4
Virus shedding from oropharyngeal and cloacal swabs of H9N2 infected specific-pathogen-free chickens at different time points

Time points (dpi)	Oropharyngeal swab		Cloacal swab	
	Number of positive/ Total samples	Virus copy number, $\log_{10}$ (means ± SE)	Number of positive/ Total samples	Virus copy number, $\log_{10}$ (means ± SE)
2	6/6	11.00 ± 0.01^{aA}	6/6	10.05 ± 0.12^{bB}
3	6/6	11.10 ± 0.07^{aA}	6/6	9.55 ± 0.06^{cB}
5	6/6	9.39 ± 0.04^{cB}	6/6	$9.71\pm0.11b^{cA}$
7	6/6	9.99 ± 0.04^{b}	6/6	9.97 ± 0.05^{b}
10	6/6	11.24 ± 0.09^{aA}	6/6	10.96 ± 0.09^{aB}
12	6/6	9.99 ± 0.14^{b}	6/6	10.05 ± 0.06^{b}
14	6/6	9.36 ± 0.08^{c}	6/6	9.16 ± 0.06^{d}
16	0/6	ND	0/6	ND

Note. * The values with different superscripts (a, b, c, and d) within a column in each group are significantly different ($p<0.05$). The values with different superscripts (A, B) compared the effect between oropharyngeal and cloacal swabs, and each row is significantly different ($p<0.05$); ND = None detected

The viral load in cloacal and oropharyngeal swab samples showed a declining pattern with low detection at day 3 and day 5 PI, respectively, while the highest viral loads in both samples were detected at day 10 PI. On the last day of the sampling at day 16 PI, no virus load was detected from the oropharyngeal and cloacal swab samples (Table 4). The virus copy number in the oropharyngeal of challenged SPF chickens is significantly higher (p<0.05) than in the cloacal of challenged SPF chickens at days 2, 3, and 10 PI. In contrast, the value in the oropharyngeal of SPF chickens was lower than in the cloacal of challenged SPF at day 5 (p<0.05).

The virus copy numbers in the oropharyngeal were significantly reduced on days 5 and 14 compared to those on other days in the challenged study (p<0.05). The highest virus copy number (p<0.05) was recorded in the oropharyngeal of H9N2-challenged SPF chickens at days 2, 3, and 10 PI. However, no significant changes were shown among virus copy numbers of oropharyngeal of challenged SPF chickens at day 2, 3, and 10 PI. Meanwhile, the highest virus copy number (p<0.05) was recorded in the cloacal route at day 10 PI.

Viral load detection was detected in both renal and respiratory tissues of the H9N2-infected chickens throughout the study. However, on day 5 PI, the mean viral load was significantly higher (p<0.05) in respiratory tissue compared to kidney tissue. In contrast, the viral load detection was significantly (p<0.05) elevated in renal tissue compared to respiratory tissues at day 7, 10, and 14 PI (Table 5).

DISCUSSION

The H9N2 virus is widespread in selected Asian, European, and African countries (Peacock et al., 2019). H9N2 viruses detected in Eurasian poultry can be classified into three well-defined poultry lineages: G1, Y280/BJ94, and Y439 (Korean-like) (Guan et al., 2000; Matrosovich et al., 2001; Peacock et al., 2019). Among the three lineages, the Y280/BJ94-like viruses are

Table 5
Detection of virus copy numbers from the respiratory tract and kidneys of H9N2 infected specific-pathogen-free chickens at different time points

Time points (dpi)	Lung/Trachea		Kidney	
	Number of positive/ Total samples	Virus copy number, $\log_{10}$ (means ± SE)	Number of positive/ Total samples	Virus copy number, $\log_{10}$ (means ± SE)
2	6/6	10.20±0.06^{b}	6/6	9.94±0.06^{b}
5	6/6	10.35±0.09^{a}	6/6	10.01±0.04^{b}
7	6/6	10.29±0.03^{b}	6/6	11.05±0.34^{a}
10	6/6	10.62±0.08^{c}	6/6	11.48±0.11^{a}
14	6/6	10.28±0.15^{b}	6/6	10.74±0.06^{a}
16	6/6	10.27±0.06	6/6	10.44±0.06

Note. The values with different superscripts (a, b, and c) compared the effect between respiratory and kidneys in each column are significantly different (p<0.05)

predominantly found in Asian countries, resulting in severe respiratory signs, drops in egg production, and mortality. In addition, the H9N2 viruses from G1 and Y280/BJ94 can also infect humans (Lai et al., 2021).

Several Malaysian H9N2 viruses have been characterized by sequencing analysis (Syamsiah et al., 2019). Gunasekara (2021) sequenced the genomes of two Malaysian H9N2 viruses of the Y280/BJ94 lineage, UPM994/2018 and UPM2033/2019, isolated from breeder and layer flocks, respectively. In this study, the pathogenicity of one of the isolates (UPM994/2018) were carried out in SPF chickens. In addition, the ability of this virus to replicate in the respiratory tract and the kidneys was evaluated using a TaqMan real-time quantitative PCR assay designed against the H9N2 M gene. Previous studies have used real-time PCR based on the M gene to detect and quantify H9N2 viral load (Cattoli et al., 2004; Spackman et al., 2005; Yang et al., 2018).

One-week-old SPF chickens inoculated intraocularly with the Malaysian LPAI H9N2 UPM994/2018 exhibited mild to moderate clinical signs starting from day 6 PI that subsided gradually, and by day 12 PI, most of the chickens were healthy. The infected chickens exhibited signs of depression, ruffled feathers, sneezing, gasping, ocular discharge, a swollen face, and mild watery diarrhea. Bijanzad et al. (2013) reported similar observations of a generalized clinical illness associated with respiratory distress in SPF chickens infected with H9N2. The post-mortem examination also recorded swollen and pale kidneys and mild hemorrhages in the lungs of the infected chickens. These gross lesions are in agreement with the results of Hablolvarid et al. (2004).

In addition, at day 7 PI, the infected chickens demonstrated a 13% growth retardation that is statistically significant ($p<0.01$) compared to the control group. Bijanzad et al. (2013) reported a similar observation of significant weight loss in SPF chickens after infection with the H9N2 virus. The incubation period of LPAI H9N2 may vary from a few days to 2 weeks (3 to 14 days) depending on various factors associated with the different strains of the H9N2 virus, host, age, and environment (Bóna et al., 2023).

Histopathology lesions in H9N2-infected chickens in this study include tracheitis, pneumonia, and tubulointerstitial nephritis. The qPCR approach detected the virus in the respiratory tissues and kidneys. Previous findings have shown that the Y280/BJ94 and G1 lineages of H9N2 viruses replicate efficiently in the lungs and multiple organs, including the kidneys of SPF chickens, following intranasal inoculation (Song et al., 2019). Gu et al. (2021) reported the mechanism underlying the cytokine-storm network and how it causes pathological damage to the host after infection with the influenza A virus. Therefore, in this study, histopathological changes can be associated with the release of cytokines in response to inflammation (Mahana et al., 2019) and appear beyond the incubation period of LPAI H9N2 viruses.

Although the SPF chickens did not display any clinical manifestations by day 12 PI, the persistence of the virus in kidney and lung tissues was detected throughout the study period. Mosleh et al. (2009) observed that virus persistence is longer in the kidney tissues of broilers experimentally infected via intranasal inoculation. Therefore, it seems that it would be necessary to collect samples from the kidneys to detect the persistence of the virus in this organ. Furthermore, virus shedding from the cloaca and oropharyngeal persisted throughout the study period in all infected chickens from day 2 to day 14. A similar finding was reported by Iqbal et al. (2013), where the presence of the virus in both the oropharyngeal and cloaca began on day 3 PI and resolved by day 11 or 15 PI following intranasal inoculation of Pakistan isolated H9N2 viruses in both broiler and layer chickens experimentally.

The detection of viruses in organs other than the respiratory tracts, such as the kidney, is probably associated with molecular characteristics of the virus, including a dibasic cleavage site at position 333-PSRSSR-GLF-341 of HA gene cleavage locations. Previous studies have indicated that dibasic cleavage motif like RSSR is associated with systemic infections in poultry (Baron et al., 2013; Thuy et al., 2016). In addition, LPAI H9N2 viruses with dibasic HA1/HA2 cleavage sites have been isolated from Japan, Vietnam, Indonesia, and China (Mase et al., 2007; Thuy et al., 2016).

Our experiment detected the highest viral load in oropharyngeal swabs compared to cloacal swabs on days 2, 3, and 10 PI. However, the highest viral load from cloacal swabs was detected on day 5 PI, suggesting that the Malaysian isolate could be shed via the oropharyngeal and cloacal routes. These findings corroborate those of the Chinese isolate belonging to the Y280 (Song et al., 2019). In addition, since the virus replicates in the kidneys, this may contribute significantly to virus shedding in the cloacal. Previous studies have shown that the gastrointestinal route and the urinary tract are involved in both virus replication and excretion (Slemons & Swayne, 1990).

Although clinical signs were observed in a few H9N2 infected birds from day 6 to day 10 PI, all the infected chickens continuously shed the virus via the oropharyngeal and cloacal routes until day 14 and stopped at day 16 PI, suggesting the silent shedding of the virus from the infected birds. Song et al. (2019) also reported a similar finding during the H9N2 pathogenicity study on SPF chickens in China. The real-time PCR measurement based on viral copy is a measurement that quantifies the virus copy numbers. Based on the virus copy numbers, we cannot determine the spread of infection, which can only be addressed by a virus transmission sentinel study. Future research is, therefore, required to measure the virus transmission sentinel study to determine the spread of infection. Although, a pathogenicity study is not a routine step in a virus characterization study. However, it is an important step in identifying suitable challenge viruses for vaccine efficacy study

since some layer and breeder farms in Malaysia vaccinate against H9N2.

The isolation of novel reassorted AIVs has been reported in terrestrial birds such as chickens and ducks as well as in non-terrestrial birds such as migratory birds and waterfowl (Li et al., 2020). In addition, Youk et al. (2020) showed that the live bird market (LBM) serves as an evolutionary epicenter for the emergence of the reassorted novel H9N2 viruses. Mixed infections of low and highly pathogenic subtypes of H9 and other pathogenic subtypes, such as H5 and H7 viruses in live birds, may result in genetic reassortments of genes from different influenza subtypes, which can lead to a complete change in the antigenic structure and emergence of new viruses. Hence, detecting this virus and its spread across the poultry population in Malaysia requires continuous monitoring to identify the circulating H9N2 strains. In addition, surveillance for the H9N2 viruses in other poultry birds, such as ducks and quails, and non-poultry birds located in hotspot areas, such as live bird markets and bird sanctuaries, is essential to determining the genetic profiles of the viruses in these avian species. Rapid detection and continuous virus monitoring are crucial for implementing warning systems to prevent and control the disease effectively.

CONCLUSION

Detecting and characterizing H9N2 viruses are paramount for developing proper control and prevention measures. The characterized Malaysian LPAI H9N2 isolate is pathogenic to SPF chickens, generally causing a mild to moderate illness and lesions associated with the respiratory and gastrointestinal tracts and the kidneys, with a gradual recovery, and the clinical signs subsided and disappeared by day 12 PI. However, virus shedding persisted until the last sampling day, day 16 PI.

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REFERENCES

Aamir, U. B., Wernery, U., Ilyushina, N., & Webster, R. G. (2007). Characterization of avian H9N2 influenza viruses from United Arab Emirates 2000 to 2003. *Virology*, *361*(1), 45–55. https://doi.org/10.1016/j.virol.2006.10.037

Awuni, J. A., Bianco, A., Dogbey, O. J., Fusaro, A., Yingar, D. T., Salviato, A., Ababio, P. T., Milani, A., Bonfante, F., & Monne, I. (2019). Avian influenza H9N2 subtype in Ghana: Virus characterization and evidence of co-infection. *Avian Pathology*, *48*(5), 470–476. https://doi.org/10.1080/03079457.2019.1624687

Baron, J., Tarnow, C., Mayoli-Nüssle, D., Schilling, E., Meyer, D., Hammami, M., Schwalm, F., Steinmetzer, T., Guan, Y., Garten, W., Klenk, H.-D., & Böttcher-Friebertshäuser, E. (2013). Matriptase, HAT, and TMPRSS2 activate the hemagglutinin of H9N2 influenza A viruses. *Journal of Virology*, *87*(3), 1811–1820. https://doi.org/10.1128/jvi.02320-12

Bijanzad, P., Momayez, R., Fard, M. H. B., Hablolvarid, M. H., Mahmoodzadeh, M., Moghaddam, A. R. J., Kaboli, K., Azizpour,

A., & Eshratabadi, F. (2013). Study on clinical aspects of SPF chickens infected with H9N2 subtype of avian influenza virus. *Annals of Biological Research*, *4*(3), 81–85.

Bóna, M., Kiss, I., Dénes, L., Szilasi, A., & Mándoki, M. (2023). Tissue tropism of H9N2 low-pathogenic avian influenza virus in broiler chickens by immunohistochemistry. *Animals*, *13*(6), 1052. https://doi.org/10.3390/ani13061052

Cattoli, G., Drago, A., Maniero, S., Toffan, A., Bertoli, E., Fassina, S., Terregino, C., Robbi, C., Vicenzoni, G., & Capua, I. (2004). Comparison of three rapid detection systems for type A influenza virus on tracheal swabs of experimentally and naturally infected birds. *Avian Pathology*, *33*(4), 432–437. https://doi.org/10.1080/03079450410001724058

Feldman, A. T., & Wolfe, D. (2014). Tissue processing and hematoxylin and eosin staining. In C. Day (Ed.), *Histopathology: Methods in molecular biology* (Vol 1180, pp. 31-43). Humana Press. https://doi.org/10.1007/978-1-4939-1050-2_3

Gharaibeh, S. (2008). Pathogenicity of an avian influenza virus serotype H9N2 in chickens. *Avian Diseases*, *52*(1), 106–110. https://doi.org/10.1637/8108-090907-reg

Gu, Y., Zuo, X., Zhang, S., Ouyang, Z., Jiang, S., Wang, F., & Wang, G. (2021). The mechanism behind influenza virus cytokine storm. *Viruses*, *13*(7), 1362. https://doi.org/10.3390/v13071362

Guan, Y., Shortridge, K. F., Krauss, S., Chin, P. S., Dyrting, K. C., Ellis, T. M., Webster, R. G., & Peiris, M. (2000). H9N2 influenza viruses possessing H5N1-like internal genomes continue to circulate in poultry in southeastern China. *Journal of Virology*, *74*(20), 9372–9380. https://doi.org/10.1128/jvi.74.20.9372-9380.2000

Gunasekara, E. (2021). *Genome characterisation and pathogenicity study of low pathogenic avian influenza virus subtype H9N2 isolated in Malaysia* [Unpublished Master's thesis]. Universiti of Putra Malaysia.

Guo, Y. J., Krauss, S., Senne, D. A., Mo, I. P., Lo, K. S., Xiong, X. P., Norwood, M., Shortridge, K. F., Webster, R. G., & Guan, Y. (2000). Characterization of the pathogenicity of members of the newly established H9N2 influenza virus lineages in Asia. *Virology*, *267*(2), 279–288. https://doi.org/10.1006/viro.1999.0115

Hablolvarid, M. H., Haghdost, S., I, Pourbakhsh, S. A., & Gholami, M. R. (2004). Histopathological study of intratracheally inoculated A/Chicken/Iran/259/1998 (H9N2) influenza virus in chicken. *Archives of Razi*, *58*(1), 51–62. https://doi.org/10.22092/ARI.2004.103825

Hassan, M. S. H., & Abdul-Careem, M. F. (2020). Avian viruses that impact table egg production. *Animals*, *10*(10), 1747. https://doi.org/10.3390/ani10101747

Iqbal, M., Yaqub, T., Mukhtar, N., Shabbir, M. Z., & McCauley, J. W. (2013). Infectivity and transmissibility of H9N2 avian influenza virus in chickens and wild terrestrial birds. *Veterinary Research*, *44*, 100. https://doi.org/10.1186/1297-9716-44-100

Lai, V. D., Kim, J, W., Choi, Y, Y., Kim, J.-J., So, H.-H., & Mo, J. (2021). First report of field cases of Y280-like LPAI H9N2 in South Korean poultry farms: Pathological findings and genetic characterization. *Avian Pathology*, *50*(4), 327–328. https://doi.org/10.1080/03079457.2021.1929833

Li, X., Sun, J., Lv, X., Wang, Y., Li, Y., Li, M., Liu, W., Zhi, M., Yang, X., Fu, T., Ma, P., Li, Y., Zhou, X., Li, Y., Yang, G., Chen, G., Zhang, J., Zheng, H., Zhang, G., Chai, H. (2020). Novel reassortant avian influenza A (H9N2) virus isolate in migratory waterfowl in Hubei Province, China. *Frontiers in Microbiology*, *11*, 220. https://doi.org/10.3389/fmicb.2020.00220

Mahana, O., Arafa, A. S., Erfan, A., Hussein, H. A., & Shalaby, M. A. (2019). Pathological changes, shedding pattern and cytokines responses in

chicks infected with avian influenza-H9N2 and/or infectious bronchitis viruses. *Virus Disease*, *30*, 279–287. https://doi.org/10.1007/s13337-018-00506-1

Mase, M., Eto, M., IMai, K., Tsukamoto, K., & Yamaguchi, S. (2007). Characterization of H9N2 influenza A viruses isolated from chicken products imported into Japan from China. *Epidemiology and Infection*, *135*(3), 386–391. https://doi.org/10.1017/S0950268806006728

Matrosovich, M. N., Krauss, S., & Webster, R. G. (2001). H9N2 influenza A viruses from poultry in Asia have human virus-like receptor specificity. *Virology*, *162*(281), 156–162. https://doi.org/10.1006/viro.2000.0799

Mo, J., Youk, S., Pantin-Jackwood, M. J., Suarez, D. L., Lee, D. H., Killian, M. L., Bergeson, N. H., & Spackman, E. (2021). The pathogenicity and transmission of live bird market H2N2 avian influenza viruses in chickens, Pekin ducks, and guinea fowl. *Veterinary Microbiology*, *260*, 109180. https://doi.org/10.1016/j.vetmic.2021.109180

Mosleh, N., Dadras, H., & Mohammadi, A. (2009). Evaluation of H9N2 avian influenza virus dissemination in various organs of experimentally infected broiler chickens using RT-PCR. *Iranian Journal of Veterinary Research*, *10*(1), 12–20. https://doi.org/10.22099/IJVR.2009.1083

Nili, H., & Asasi, K. (2003). Avian influenza (H9N2) outbreak in Iran. *Avian Diseases*, *47*(S3), 828–831. https://doi.org/10.1637/0005-2086-47.s3.828

Pantin-Jackwood, M. J., Smith, D. M., Wasilenko, J. L., & Spackman, E. (2012). Low pathogenicity avian influenza viruses infect chicken layers by different routes of inoculation. *Avian Diseases*, *56*(2), 276–281. https://doi.org/10.1637/9950-092711-Reg.1

Peacock, T. P., James, J., Sealy, J. E., & Iqbal, M. (2019). A global perspective on H9N2 avian influenza virus. *Viruses*, *11*(7), 620. https://doi.org/10.3390/v11070620

Reed, L. J., & Muench, H. (1938). A simple method of estimating fifty percent endpoints. *American Journal of Epidemiology*, *27*(3), 493–497. https://doi.org/10.1093/oxfordjournals.aje.a118408

Slemons, R. D., & Swayne, D. E. (1990). Replication of a waterfowl-origin influenza virus in the kidney and intestine of chickens. *Avian Diseases*, *34*(2), 277–284. https://doi.org/10.2307/1591409

Song, Y., Zhang, Y., Chen, L., Zhang, B., Zhang, M., Wang, J., Jiang, Y., Yang, C., & Jiang, T. (2019). Genetic characteristics and pathogenicity analysis in chickens and mice of three H9N2 avian influenza viruses. *Viruses*, *11*(12), 1127. https://doi.org/10.3390/v11121127

Spackman, E., Senne, D. A., Bulaga, L. L., Myers, T. J., Perdue, M. L., Garber, L. P., Lohman, K., Baum, L. T., & Suarez, D. L. (2005). Development of real-time RT-PCR for the detection of avian influenza virus. *Avian Diseases*, *49*(2), 313. https://doi.org/10.1637/7272-090704R.1

Stech, J., & Mettenleiter, T. C. (2013). Virulence determinants of high-pathogenic avian influenza viruses in gallinaceous poultry. *Future Virology*, *8*(5), 459–468. https://doi.org/10.2217/fvl.13.27

Su, S., Bi, Y., Wong, G., Gray, G. C., Gao, G. F., & Li, S. (2015). Epidemiology, evolution, and recent outbreaks of avian influenza virus in China. *Journal of Virology*, *89*(17), 8671–8676. https://doi.org/10.1128/jvi.01034-15

Sun, Y., Hu, Z., Zhang, X., Chen, M., Wang, Z., Xu, G., Bi, Y., Tong, Q., Wang, M., Sun, H., Pu, J., Iqbal, M., & Liu, J. (2020). An R195K mutation in the PA-X protein increases the virulence and transmission of influenza A virus in mammalian hosts. *Journal of Virology*, *94*(11), e01817-19. https://doi.org/10.1128/jvi.01817-19

Syamsiah, A. S., Leow, B. L., Faizul Fikri, M. Y., Muhammad Redzwan, S., Ong, G, H., & Faizah Hanim, M. S. (2019). Genetic analysis of H9N2 avian influenza viruses isolated from chickens in Malaysia from 2015-2018. *Malaysian Journal of Veterinary Research*, *10*(2), 79–92.

Thuy, D. M., Peacock, T. P., Bich, V. T. N., Fabrizio, T., Hoang, D. N., Tho, N. D., Diep, N. T., Nguyen, M., Hoa, L. N. M., Trang, H. T. T., Choisy, M., Inui, K., Newman, S., vu Trung, N., van Doorn, R., To, T. L., Iqbal, M., & Bryant, J. E. (2016). Prevalence and diversity of H9N2 avian influenza in chickens of Northern Vietnam, 2014. *Infection, Genetics and Evolution*, *44*, 530–540. https://doi.org/10.1016/j.meegid.2016.06.038

Umar, S., Guerin, J. L., & Ducatez, M. F. (2017). Low pathogenic avian influenza and coinfecting pathogens: A review of experimental infections in avian models. *Avian Diseases*, *61*(1), 3–15. https://doi.org/10.1637/11514-101316-Review

Wang, J., Tang, C., Wang, Q., Li, R., Chen, Z., Han, X., Wang, J., & Xu, X. (2015). Apoptosis induction and release of inflammatory cytokines in the oviduct of egg-laying hens experimentally infected with H9N2 avian influenza virus. *Veterinary Microbiology*, *177*(3–4), 302–314. https://doi.org/10.1016/j.vetmic.2015.04.005

World Organization for Animal Health. (2023). *Avian influenza (including infection with high pathogenicity avian influenza viruses)*. https://www.woah.org/fileadmin/Home/eng/Health_standards/tahm/3.03.04_AI.pdf

Yang, Y., Li, S., Wong, G., Ma, S., Xu, Z., Zhao, X., Li, H., Xu, W., Zheng, H., Lin, J., Zhao, Q., Liu, W., Liu, Y., Gao, G. F., & Bi, Y. (2018). Development of a quadruple qRT-PCR assay for simultaneous identification of highly and low pathogenic H7N9 avian influenza viruses and characterization against oseltamivir resistance. *BMC Infectious Diseases*, *18*, 406. https://doi.org/10.1186/s12879-018-3302-7

Youk, S.-S., Lee, D.-H., Jeong, J.-H., Pantin-Jackwood, M. J., Song, C.-S., & Swayne, D. E. (2020). Live bird markets as evolutionary epicentres of H9N2 low pathogenicity avian influenza viruses in Korea. *Emerging Microbes and Infections*, *9*(1), 616–627. https://doi.org/10.1080/22221751.2020.1738903