Evaluation of a High-Pathogenicity H5N1 Avian Influenza A Virus Isolated from Duck Meat


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**Evaluation of a High-Pathogenicity H5N1 Avian Influenza A Virus Isolated from Duck Meat**


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**SUMMARY.** The introduction of an influenza A virus possessing a novel hemagglutinin (HA) into an immunologically naive human population has the potential to cause severe disease and death. Such was the case in 1997 in Hong Kong, where H5N1 influenza was transmitted to humans from infected poultry. Because H5N1 viruses are still isolated from domestic poultry in southern China, there needs to be continued surveillance of poultry and characterization of virus subtypes and variants. This study provides molecular characterization and evaluation of pathogenesis of a recent H5N1 virus isolated from duck meat that had been imported to South Korea from China. The HA gene of A/Duck/Anyang/AVL-1/01 (H5N1) isolate was found to be closely related to the Hong Kong/97 H5N1 viruses. This virus also contained multiple basic amino acids adjacent to the cleavage site between HA1 and HA2, characteristic of high-pathogenicity avian influenza viruses (HPAI). The pathogenesis of this virus was characterized in chickens, ducks, and mice. The DK/Anyang/AVL-1/01 isolate replicated well in all species and resulted in 100% and 22% lethality for chickens and mice, respectively. No clinical signs of disease were observed in DK/Anyang/AVL-1/01-inoculated ducks, but high titers of infectious virus could be detected in multiple tissues and oropharyngeal swabs. The presence of an H5N1 influenza virus in ducks bearing a HA gene that is highly similar to those of the pathogenic 1997 human/poultry H5N1 viruses raises the possibility of reintroduction of HPAI to chickens and humans.

**RESUMEN.** Evaluación de un virus altamente patógeno H5N1 de influenza aviar A, a partir de carne de pato.

La introducción de un virus de influenza tipo A con una hemoaglutinina nueva en una población humana susceptible fue capaz de ocasionar una enfermedad severa y muerte. Este fue el caso en Hong Kong en 1997, el cual el virus de influenza H5N1 fue transmitido a humanos a partir de aves domésticas. Debido a que los virus de influenza H5N1 aún se aíslan a partir de aves domésticas en el sur de China, existe la necesidad de continuar con la supervisión de las aves domésticas y la caracterización de los subtipos del virus y las variantes. Se presenta la caracterización molecular y la evaluación de la patogenicidad de un virus H5N1 aislado recientemente a partir de carne de pavo importada a Corea del Sur, proveniente de China. Se observó una estrecha relación entre el gen de hemoaglutinina del aislamiento A/Pato/Anyang/AVL-1/01 (H5N1) y de los virus Hong Kong/97 H5N1. Este virus contenía igualmente aminoácidos básicos múltiples en el sitio de división entre las proteínas HA1 y HA2, característico de los virus de influenza aviar de patogenicidad alta. Se caracterizó la patogenicidad del virus en pollos, patos y ratones. El aislamiento Pato/Anyang/AVL-1/01 se replicó bien en todas las especies resultando en mortalidades del 100% y 22% para los pollos y los ratones, respectivamente. No se observaron signos clínicos de la enfermedad en los patos inoculados con el aislamiento Pato/Anyang/AVL-1/01, sin embargo, se pudieron detectar títulos de virus infecciosos altos en múltiples tejidos y en hisopos orofaríngeos. La presencia en patos de un virus...
de influenza aviar H5N1 con el gen de hemoaglutinina similar a los genes observados en los virus patógenos H5N1 en humanos y en aves domésticas en 1997, aumentan la posibilidad de una nueva introducción de virus de influenza aviar de patogenicidad alta en pollos y en humanos.

Key words: avian influenza, orthomyxovirus, pathogenicity, poultry
Abbreviations: AI = avian influenza; DK = duck; ELD<sub>50</sub> = 50% egg lethal dose; HA = hemagglutinin; HP = high pathogenicity; IN = intranasal; IV = intravenous; p.i. = postinoculation

Although domestic chickens and turkeys are not natural hosts for avian influenza viruses, these birds can become infected with H5 and H7 influenza subtypes. This can result in virus adaptation to a new host and the emergence of a virulent virus referred to as high-pathogenicity avian influenza (HPAI) (9). Such an event was demonstrated in 1997 in Hong Kong where domestic chickens became infected with a H5N1 virus and eventually transmitted the virus to humans (10,11). Six deaths were recorded from 18 confirmed hospitalized cases, and this was the first report of a purely avian influenza virus causing respiratory disease and death in humans.

A variety of animal models have provided useful information into the characterization of H5N1 influenza virus isolates. Domestic chickens are highly susceptible to such isolates and are used to determine whether an avian virus is high pathogenicity (HP) for poultry (13). The pathogenesis of avian influenza (AI) viruses in ducks is less understood. However, the available surveillance data suggest that domestic ducks are susceptible to influenza A virus infections (1,14), and virus shedding can be detected in H5N1-infected ducks up to 5 days postinfection (8). Most influenza A virus subtypes replicate in the cells lining the intestinal tracts of ducks, but infection in these species generally does not produce clinical signs (1,14). The BALB/c mouse model is recognized as the most relevant small animal model for the study of H5N1 influenza virus pathogenesis and immunity (4,7). Such viruses were shown to replicate efficiently in the lungs of mice without prior adaptation, but clear differences in lethality were observed (3,4,5,6,7).

This paper reports the recovery of a HPAI (H5N1) influenza virus from domestic duck meat. The isolate, designated A/Duck/Anyang/AVL-1/01 (DK/Anyang/AVL-1/01), was typed as an influenza A H5N1 virus. The purpose of the present study is to provide molecular characterization and identify the pathogenic properties of DK/Anyang/AVL-1/01 in chickens, ducks, and mice. A more detailed report of this study has been published (12).

**MATERIALS AND METHODS**

*Virus isolation, identification, and sequencing of influenza genes.* An influenza A virus was isolated from imported Cherry Valley Pekin duck (*Anas platyrhynchos*) meat, which had been processed at a food factory in Shanghai, Mainland China. Each tested meat sample was frozen and thawed three times, and the extracted fluid was collected and clarified by low-speed centrifugation. The supernatant was inoculated into the allantoic cavity of embryonated chicken eggs. Inoculated eggs were incubated at 37°C for 30 hr, after which allantoic fluids from dead eggs were harvested and tested for hemagglutination activity. DK/Anyang/AVL-1/01 virus was typed as an influenza A H5N1 virus by means of a hemagglutination-inhibition and neuraminidase-inhibition tests with a panel of antisera (provided by Office Internationale des Epizooties Reference Laboratory, Veterinary Laboratory Agency, U.K.). RNA from the isolate sequenced in this study was extracted with Trizol LS reagent (Life Technologies, Rockville, MD) from infectious egg allantoic fluid prior to reverse transcriptase/polymerase chain reaction (RT/PCR) amplification as previously described (10). Briefly, the RT/PCR amplification was performed with the OneStep RT/PCR kit (Qiagen, Valencia, CA) and the nonstructural (NS), matrix (M), and nucleoprotein (NP) gene segments were amplified with primers to conserved sequences present on the 5' and 3' end of each viral segment. The hemagglutinin (HA), neuraminidase (NA), polymerase B<sub>1</sub> (PB<sub>1</sub>), polymerase B<sub>2</sub> (PB<sub>2</sub>), and polymerase A (PA) genes were also RT/PCR amplified with specific primers from the noncoding sequence of each gene segment. For all eight viral genes, the full coding sequence was amplified.

The sequencing information was compiled with the Seqman program (DNASTAR, Madison, WI), and the nucleotide sequences were compared initially with the Megalign program (DNASTAR, Madison, WI) using the clustal alignment algorithm. Pairwise sequence
alignments were also performed in the Megalign program to determine sequence similarity between DK/Anyang/AVL-1/01 and other published sequences for each gene segment.

**Experiment 1.** Four-week-old white Plymouth Rock (WPR) chickens were used in pathogenicity studies using established procedures (13,2). Eight chickens were inoculated by the intravenous (IV) route with 0.2 ml of a 1:10 dilution of allantoic fluid containing 10^8.0 50% egg lethal dose (ELD50) of DK/Anyang/AVL-1/01 virus. The pathogenicity test also included 11 chickens inoculated intranasally (IN) with 10^6.0 ELD50 of the same virus. Three chickens were euthanatized on day 3 postinoculation (p.i.), and brain, lung, kidney, and skeletal muscle tissues were collected for determination of infectious virus titers in eggs (10).

**Experiment 2.** Two-week-old Pekin white ducks (*Anas platyrhyncos*) (Privett hatchery, Portales, NM) (eight per group) were inoculated IN with 10^6.0 ELD50 of DK/Anyang/AVL-1/01 virus administered in a volume of 0.1 ml. In addition, four control ducks were inoculated with 0.1 ml sterile allantoic fluid and served as the mock-infected controls. Ducks were observed daily for clinical signs of disease. Oropharyngeal swabs were collected from four ducks each day from 1 to 7 days p.i. Two ducks were euthanatized and necropsied at 2, 4, 7, and 14 days p.i. Gross lesions were recorded, and tissues (brain, lung, kidney, skeletal muscle from proximal shank) were collected separately from each duck for virus isolation.

**Experiment 3.** Male BALB/c mice, 6 to 8 weeks old (Simonsen Laboratories, Gilroy, CA), were anesthetized with ketamine-xylazine (1.98 and 0.198 mg per mouse, respectively). A total of 13 mice were inoculated IN (50 µl) with 10^6.0 ELD50 of DK/Anyang/AVL-1/01 virus diluted in phosphate-buffered saline (PBS). An additional group of 12 mice were inoculated with diluent PBS and served as mock-infected controls. Nine mice per group were monitored daily for death for 14 days p.i. Four mice from each group were euthanatized on day 4 p.i., and whole lungs, kidneys, brains, and tracheas (5 mm in length) were collected and homogenized in 1 ml of cold PBS. The solid debris was removed by brief centrifugation before homogenates were titrated for virus infectivity in eggs from initial dilutions of 1:10 (lung and trachea) or 1:2 (kidney and brain). The limit of virus detection was 10^{1.2} ELD50/ml for lung and trachea and 10^{0.8} ELD50/ml for other tissues.

**RESULTS**

Comparisons of the nucleotide sequence of the HA gene of DK/Anyang/AVL-1/01 with those of other 1997 H5N1 influenza viruses revealed that the HA gene clustered with the H5 Goose/Guangdong/1/96 lineage and the Hong Kong/97 chicken and human isolates. The HA gene had the highest sequence similarity (98.4%) with the 1999 Hong Kong isolate, A/Environmental/Hong Kong/437-6/99 virus that was associated with geese entering Hong Kong for slaughter in March 1999 (3). DK/Anyang/AVL-1/01 virus contained the characteristic multiple basic amino acids at the cleavage site of HA, with the insertion of four basic amino acids in addition to three other basic amino acids. Phylogenetic analysis of the neuraminidase gene also showed the DK/Anyang/AVL-1/01 isolate clustered with the Goose/Guangdong/1/96 virus lineage but not to later viruses from this lineage. For the internal genes, the matrix and nucleoprotein genes did cluster loosely with the Goose/Guangdong/1/96 lineage of viruses, although the highest nucleotide sequence similarity was only 97% and 97.4%, respectively, with this group of viruses. The nonstructural and all three polymerase genes did not cluster closely with any other H5N1 influenza viruses.

As seen in Table 1, IV or IN inoculation with DK/Anyang/AVL-1/01 virus caused 100% (eight of eight dead) mortality of young chickens, and disease signs observed were typical of those seen in chickens infected with H5N1 Hong Kong/97 viruses (10). Gross lesions observed included severe pulmonary edema, necrosis of the comb, and edema of the brain. The mean death times were 2.8–3 days following both routes of inoculation. Tissues from six chickens that died or were euthanatized on days 2 and 3 days p.i. were evaluated for infectious virus content. Titration of tissues revealed high titers (6.0 to 6.7 log_{10} ELD50/gm of tissue) of infectious virus.
from brain, lung (Table 1), kidney, and thigh muscle, with lower titers in the breast muscle (5.3 to 5.5 log_{10} ELD_{50}/gm). In contrast to chickens, no clinical signs of disease were observed in DK/Anyang/AVL-1/01-inoculated Pekin white ducks. However, infectious virus could be detected in oropharyngeal swabs and lung tissue from four of four ducks up to 4 days postinfection (Table 1). Interestingly, infectious virus ranging from 2.7 to 3.5 log_{10} EID_{50}/gm of tissue could also be detected in brain and skeletal muscle tissue on days 2 and 4 p.i. Infectious virus was not detectable in any tissues beyond day 5 p.i.

To determine the pathogenicity of DK/Anyang/AVL-1/01 virus in a mammalian host, BALB/c mice were inoculated IN, and mortality and virus replication in tissues were determined. The DK/Anyang/AVL-1/01-infected mice showed moderate signs of illness such as ruffled fur and hunched posture and began to lose weight 2 days after infection. However, the majority (seven of nine), infected mice began to recover and gain weight after day 4 p.i. DK/Anyang/AVL-1/01 virus replicated to high titers in lung tissue on day 4 p.i. (Table 1); however, virus was not detected in the brain or kidney tissues from four mice tested.

**DISCUSSION**

In Southern China, Hong Kong/97 H5N1-like viruses continue to circulate throughout poultry (3). In this study, we provided molecular characterization and pathogenesis of a recent H5N1 virus isolated from meat of domestic duck origin and helped define the host range of this virus. The DK/Anyang/AVL-1/01 isolate possesses several genes that are highly similar to the 1996 Goose/Guangdong/1/96 virus, believed to be the HA donor of the Hong Kong/97 chicken and human isolates. Such Hong Kong/97 H5N1-like viruses replicate in mice without prior adaptation. We and others have previously demonstrated that the H5N1 Hong Kong/97 chicken and human isolates differed from other HPAI H5 viruses in their high pathogenicity for mice (3,4,5,6,7). The molecular basis for the lethal phenotype in mice has not been completely elucidated, but it is most likely specified by multiple genes (5,6,7). Like the Hong Kong/97 H5N1 viruses, DK/Anyang/AVL-1/01 (H5N1) isolate infected the respiratory tract of mice without the requirement of mouse adaptation and induced some lethality. Because domestic duck meat was the source of the Dk/Anyang/AVL-1/01 virus, we also determined the pathogenicity in this species and examined whether skeletal muscle could support virus replication. We found that although a high inoculation titer of DK/Anyang/AVL-1/01 virus did not induce clinical signs in 4-week-old Pekin ducks, it was apparent that this H5N1 isolate replicated in multiple tissues, including the skeletal muscle tissue. Infectious virus was cleared from all tissues by days 5 to 7 p.i. These findings, along with the continued circulation of Hong Kong/97 H5N1-like viruses in poultry, have highlighted the need for continued virological surveillance in southern China.

**REFERENCES**


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