Research Notes

The susceptibility of magpies to a highly pathogenic avian influenza virus subtype H5N1


*National Veterinary Research and Quarantine Service, Anyang, Kyonggi 430-824, Korea; and †College of Veterinary Medicine, Seoul National University, Gwanak 599, Seoul, Korea

ABSTRACT Korean wild magpies (Pica pica sericea) were intranasally inoculated with highly pathogenic avian influenza (A/Chicken/Korea/ES/03 virus) (H5N1), which was classified as clade 2.5. We estimated viral replication, death after infection, and histology and immunohistochemistry. This species was highly susceptible to severe infection; 100% of birds died within 5 to 8 d. The virus was detected from oropharyngeal (1 to 5 d postinfection) and cloacal (3 to 5 d postinfection) swabs from infected magpies. At necropsy, the prominent lesions were coalescing necrosis of the pancreas with enlargement of livers and spleens. Microscopically, pancreas, brain, heart, adrenal gland, and kidney were most consistently affected with necrotic and inflammatory changes, and viral antigen was frequently demonstrated in the parenchyma of these organs. As a result, Korean wild magpies were very susceptible to avian influenza (H5N1) virus.

Key words: avian influenza, highly pathogenic avian influenza, wild bird, Korean magpie

INTRODUCTION

Avian influenza (AI) is caused by segmented, negative-strand RNA viruses of the influenzavirus A genus of the Orthomyxoviridae family (Swayne and Halvorson, 2008). Low pathogenicity AI viruses have been isolated from numerous wild and domestic avian species (Stallknecht and Brown, 2008; Swayne and Halvorson, 2008), and wild waterfowl are regarded as the primordial reservoir hosts of these viruses (Webster et al., 1992). However, high-pathogenicity AI (HPAI) viruses arise from mutation of low-pathogenicity AI viruses as they circulate in poultry (Rohm et al., 1995). These HPAI viruses produce a severe, systemic disease with near 100% mortality in chickens, turkeys, and other gallinaceous birds (Perkins and Swayne, 2003) but usually do not cause infection, clinical disease, or death in domestic waterfowl or wild birds, especially aquatic birds of the order Anseriformes (ducks, geese, and swans) (Nettles et al., 1985; Alexander et al., 1986; Capua et al., 2000).

Since the isolation of H5N1 HPAI virus in 1996 from a domestic goose in Guangdong Province, China, descendants of this virus have developed varying abilities to infect and cause disease in domestic ducks and wild aquatic birds under natural and experimental settings (Chen et al., 2004; Sturm-Ramirez et al., 2004; Brown et al., 2006, 2008; Hulse-Post et al., 2007). In experimental infections, H5N1 HPAI viruses isolated between 1997 and 2001 in Hong Kong produced high mortality in gallinaceous birds but only asymptomatic infections in domestic ducks or captive-raised aquatic birds. However, the diversity of avian species for which H5N1 HPAI viruses are potentially infective, pathogenic, and lethal changed dramatically in late 2002, as shown by virus isolation from a variety of dead waterfowl in Pенfold Park and Kowloon Park in Hong Kong, as well as from various waterfowl (ducks, geese, and swans), flamingos (Phoenicopterus ruber), and, more recently, tree sparrows (Passer montanus) in China (Ellis et al., 2004; Kou et al., 2005; Chen et al., 2006). Similarly, during the HPAI epidemic in Korea between 2003 and 2004, there were 2 natural infections with H5N1 HPAI virus in the magpies that had pathologically specific lesions characterized by severely necrotized pancreatitis and nonpurulent meningoencephalitis (Kwon et al., 2005).

Land-based wild bird populations might be vulnerable to HPAI (H5N1) infection and could contribute to the spread and interspecies transmission of the viruses (Webster et al., 2002). Most terrestrial birds including magpies are important hosts in influenza (H5N1) ecology because many of them intermingle freely with wild and poultry flocks (Boon et al., 2007). However, experimental data describing magpies’ susceptibility to H5N1 HPAI virus infection or their potential to transmit the viruses are limited.
This study was initiated to determine the susceptibility of magpies to intranasal inoculation with the A/Chicken/Korea/ES/03 (H5N1) HPAI virus and to describe the duration and routes of viral shedding from the birds, the pathologic lesions, and the distribution of virus in the species.

**MATERIALS AND METHODS**

**Virus**

The A/Chicken/Korea/ES/03 (H5N1) (Ck/Korea/03) virus was isolated from the broiler breeder infected with HPAI virus in the middle of December 2003 (Avian Disease Division, National Veterinary Research and Quarantine Service, Kyunggi, South Korea). It was propagated in specific-pathogen-free embryonated chicken eggs. The virus was titrated to determine the median egg infectious dose by the Reed and Muench method (Reed and Muench, 1938) and was used as the inoculum.

**Experimental Infection of Wild Korean Magpies**

Twelve Korean wild magpies that were captured by using wire traps containing food at the Cheonan City, Chungchon Nam Province, which had not been affected with an H5N1 epidemic, were used for this study. That is why we did not test whether the magpies were seronegative or seropositive. After the name tag was put on the leg of each magpie, 9 magpies were intranasally inoculated with Ck/Korea/03 virus at a dose of \(10^{6.6}\) median egg infectious dose/mL without treatment of anesthesia. The control group contained 3 birds inoculated with 0.1 mL of the sham inoculum of PBS. The control birds were euthanized on the same day when the last virus-inoculated bird died. All of the birds were monitored on a daily basis for clinical signs including feed and water consumption, respiratory and nervous signs, diarrhea, moribund, and mortality, and oropharyngeal and cloacal swabs were collected on 1, 2, 3, 5, and 7 DPI postinoculation (DPI). To isolate the virus, oropharyngeal and cloacal swabs were diluted or homogenized in sterile PBS containing 1% gentamicin, and each supernatant was titrated in chicken embryo fibroblast cells using the Reed and Muench method.

All of the birds were housed in an isolator (Three-Shine, Seoul, Korea) that was ventilated under negative pressure with high efficiency particulate absorbing-filtered air. All experiments were performed in biosafety level 3 containment facilities at the National Veterinary Research and Quarantine Service.

**Histopathology and Immunohistochemistry**

Tissue samples collected at necropsy from the inoculated and control birds were fixed in 10% neutral-buffered formalin solution for 24 h, routinely processed, and embedded in paraffin blocks. Sections were 5 µm and were stained with hematoxylin and eosin. Duplicate sections were stained with a mouse-derived monoclonal antibody specific for a type A influenza virus nucleoprotein (H16-L10-4; kindly donated by the Commonwealth Scientific and Industrial Research Organisation, Clayton South, Victoria, Australia) as the primary antibody. All reactions were carried out in an automated immunohistochemistry instrument (Ventana ES, Ventana Medical Systems, Oro Valley, AZ). Antigen-antibody reactions were revealed with standardized development times by the instrument, using the avidin-biotin method with copper enhancement and diaminobenzidine as the substrate. The tissue sections were counterstained with Gill’s No. 3 hematoxylin (Sigma, St. Louis, MO).

**RESULTS**

**Sham-Inoculated Controls**

There was no morbidity or mortality observed in sham birds and these birds lacked evidence of infection with AI virus at the beginning of the study and at 8 DPI, and no AI virus was detected from oropharyngeal or cloacal swabs collected during the study. Histologically, random lymphoid nodules were occasionally observed in the liver and small intestine. Infrequently, nonspecific immunohistochemical staining, which was restricted to cytoplasmic granules of a few individual cells, was observed in the lymphoid tissues, especially the spleen and liver. Immunohistochemical staining of this nature has been previously interpreted as nonspecific staining of mast cell granules.

**Virus Isolation**

We tested the virus titers in cloacal and oropharyngeal swabs taken from the intranasally inoculated birds on 1, 2, 3, 5, and 7 DPI. The birds shed virus from their oropharynx and cloaca and the virus was already detected on the first day postinoculation from the oropharynx. Virus shedding from the oropharyngeal swabs was detected between 1, 2, 3, and 5 DPI, not on 7 DPI, whereas reisolation of the virus from the cloacal swabs was on only 2 d (3 and 5 DPI), not on 1, 2, and 7 DPI, shown in Table 1. Oropharyngeal swab titers ranged from \(10^{2.2}\) tissue culture infectious doses (TCID<sub>50</sub>/mL on 2 DPI to \(10^{4.3}\) TCID<sub>50</sub>/mL on 5 DPI, but virus shedding titers from cloacal swabs were between \(10^{2.5}\) and \(10^{1.2}\) TCID<sub>50</sub>/mL on 5 DPI. As results, viral shedding was more frequent and higher from the oropharynx (1 to 5 DPI) than the cloaca (3 and 5 DPI). Our results indicated that Ck/Korea/03 virus may replicate in the respiratory tract and is more likely to be transmitted through direct contact than through the fecal-oral route.
Clinical Signs (Mortality) and Gross Lesions

The mortality after virus exposure to the Ck/Korea/03 H5N1 HPAI virus is shown in Table 1. Clinically, on 5 DPI, mortality suddenly occurred in 5 inoculated magpies without any symptoms. Most of the dead birds were shedding the virus from oropharyngeal swabs on 3 DPI. After that day, intranasal inoculation produced other clinical signs, typically listlessness, ruffled feathers, mild watery diarrhea, and neurological signs such as incoordination (Figure 1) and head tremors, which terminated in death in all remnant magpies until 8 DPI. At necropsy, all infected magpies had multiple, 0.5 to 20 mm, white necrotic foci with mildly to moderately coalescent hemorrhage in the pancreas, mild to moderate hepatomegaly with friable parenchyma, and congestion; however, the magpies that died on 5 DPI were grossly more severe than those that were dead between 6 and 8 DPI. They were also dehydrated with mild to moderate dilation of the small intestine, had empty gastrointestinal tracts, and had white discolored feathers around the cloaca; these were most prominent in the magpies that survived much longer. In addition, the kidney was moderately enlarged and congested.

Histology and Immunohistochemistry

Histological lesions and the corresponding viral antigen were distributed among multiple tissues in the magpies, and although the experimental period increased, the severity of histological lesions became weak gradually and the distribution of AI viral antigen was restrictive (Table 2). In this species, the most significant lesions were found in the pancreas, brain, adrenal gland, kidney, and liver. The pancreata had moderate to severe, multifocal to confluent acinar necrosis with associated viral antigen in degenerative and necrotic cells (Figure 2). The brain had focally extensive neuronal degeneration and necrosis and vacuolation of the neuropil in the cerebellum, cerebrum, and medulla. Slight lymphocytic perivascular cuffs, mild perivasculares edema, and randomly scattered gliosis were also observed in the cerebrum and cerebellum. Viral antigen was detected in neurons, glial cells, neurophilis, ependymal cells, epithelium of choroid plexi, and cerebellar Purkinje cells and neurons and dendrites of the molecular layer (Figure 3). The corticotrophic cells and less frequently the chromaffin cells of the adrenal gland had moderate to severe, multifocal to confluent areas of cytoplasmic vacuolar degeneration to coagulative necrosis. Mildly focal coagulative necrosis of hepatocytes with minimal lymphocytic infiltration was identified. The heart had random, slight myocardial degeneration to necrosis with mild congestion. Multiple degeneration and necrosis of tubular epitheliums in the kidney were also present. In the respiratory tracts, variable epitheliums including the trachea, bronchiole, and air sac had mild to moderate vacuolar degeneration or necrosis, or both. Viral antigen was associated with the histological lesions, commonly in corticotrophic and chromaffin cells in the adrenal glands (Figure 4), hepatocytes, cardiac myofibers (Figure 5), tubular epitheliums of the kidney, histiocytes in the lungs, and mucosal epithelia cells of the trachea, bronchioles (Figure 6), and air sacs.

In the absence of histological lesions, viral antigen was infrequently identified in the parasympathetic ganglia within the submucosal and myenteric plexus of the small and large intestines, cryptic epithelium of small intestine, and seminiferous tubular epithelium in the testis (Figure 7).

Table 1. Mortality and virological data in magpies after intranasal inoculation with A/Chicken/Korea/ES/03 (H5N1) virus

<table>
<thead>
<tr>
<th>Bird</th>
<th>MDT(^1) (d)</th>
<th>Sample</th>
<th>1 DPI(^2)</th>
<th>2 DPI</th>
<th>3 DPI</th>
<th>5 DPI</th>
<th>7 DPI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Magpies</td>
<td>5.8</td>
<td>Oropharyngeal swab</td>
<td>1/9 (2.4)(^4)</td>
<td>2/9 (2.2 to 2.8)</td>
<td>5/9 (2.4 to 3.4)</td>
<td>1/4 (4.3)</td>
<td>0/1 (0)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cloacal swab</td>
<td>0/9 (0)</td>
<td>0/9 (0)</td>
<td>4/9 (2.4 to 4.2)</td>
<td>2/4 (2.5 to 2.8)</td>
<td>0/1 (0)</td>
</tr>
</tbody>
</table>

\(^1\)MDT = mean death time.
\(^2\)DPI = day postinoculation.
\(^3\)Number of dead/total birds.
\(^4\)Number of positive birds/tested birds (titer range tissue culture infectious doses/mL).
DISCUSSION

All magpies became infected after experimental inoculation and shed the virus, mainly from the oropharynx and to a lesser extent from the digestive tract. Similarly, in the field case associated with clade 2.5 H5N1 HPAI virus in South Korea during the epidemic HPAI outbreaks during early December 2003 to late March 2004, two mortality events were identified in the Korean wild magpies (Kwon et al., 2005).

Intranasal inoculation of the Ck/Korea/03 virus produced fatal infections in all magpies within 8 d of inoculation. The broad distribution of necrotic and inflammatory lesions with associated AI viral antigen staining suggested similar pathogenesis for disseminated infections as reported in gallinaceous birds and some other waterfowl infected with various HPAI viruses (Kobayashi et al., 1996; Perkins and Swayne 2001, 2002, 2003). The current data were also consistent with field observations and other experimental studies that wild birds such as water fowl and terrestrial birds were highly susceptible to infection and pathological effects of H5N1 HPAI viruses. These infections could be lethal resulting from multiorgan virus replication and lesions in neuronal, parenchymal, and epithelial cells (Brown et al., 2006; Terregino et al., 2006; Nagy et al., 2007; Palmai et al., 2007; Teifke et al., 2007; Keawcharoen et al., 2008; Konar and Olsen, 2008).

In chickens and other gallinaceous species, vascular damage including severe pulmonary edema, congestion, and microthrombosis, as well as viral antigen in the vascular endothelium, have been commonly reported and may be responsible for fatal outcomes as early as 1 to 2 d after inoculation (Swayne, 2007). However, such widespread vascular damage and viral antigen in the endothelium rarely had been shown in wild birds, especially domestic Pekin or mallard-type ducks infected with HPAI viruses (Palmai et al., 2007; Pantin-Jackwood and Swayne, 2007; Brown et al., 2008; Kalthoff et al., 2008). Consistently, in the present study, the dead magpies rarely had vascular damages with antigen detected in the endothelium of capillaries, small arteries, and veins in various visceral organs including lungs, intestine, heart, and brain. Those results might indicate why clinical signs including mortality with nervous abnormality in the magpies were observed as late as 5 DPI.

An important question concerning passerine birds including magpies is whether they can serve as intermediate hosts or reservoirs for AI (H5N1) viruses and transmit them to domestic birds and mammals. Magpies are a very adaptable bird found in the northern hemisphere ranging from northeastern Asia to North America (Lee et al., 2003). The Korean magpie (Pica pica sericea), a medium-sized passerine bird from the family Corvidae, is a common resident breeder evenly distributed in urban, rural, and open landscapes. It prefers nesting near towns and villages (recently domestic poultry farms and orchards) and they have omnivorous feeding

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1-2</td>
<td>2</td>
<td>+++</td>
<td></td>
<td></td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>3-4</td>
<td>3</td>
<td>+++</td>
<td></td>
<td></td>
<td>+++</td>
<td>+</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>5-6</td>
<td>4</td>
<td>+++</td>
<td></td>
<td></td>
<td>+++</td>
<td>+</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>7-8</td>
<td>5</td>
<td>+++</td>
<td></td>
<td></td>
<td>+++</td>
<td>+</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
</tbody>
</table>

1= no staining; ± = minimal; + = mild; ++ = moderate; +++ = severe.

Table 2. Average distribution of avian influenza viral antigen in the tissues from magpies exposed to A/Chicken/Korea/ES/03 (H5N1) virus

1 = day postinoculation.
habits. Magpies are susceptible to AI viruses (Kwon et al., 2005; Boon et al., 2007). A previous study reported that although sparrows did not transmit to sentinel contact birds, this species could act as an intermediate host and potentially transmit to both poultry and mammals. Similarly, our results indicated that magpies may become efficient intermediate hosts in the ecology of influenza (H5N1) viruses at early stage of infection because viral shedding is enough from the oropharynx and digestive tracts before starting severe clinical signs, but magpies do serve as a reservoir for prolonged shedding of highly pathogenic influenza (H5N1) viruses.

REFERENCES


