An Outbreak of Highly Pathogenic Avian Influenza Subtype H5N1 in Broiler Breeders, Korea

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ABSTRACT. Highly pathogenic avian influenza (HPAI) was diagnosed in broiler breeders, submitted to the National Veterinary Research and Quarantine Service in South Korea. Grossly, the dead breeders had lesions consistent with HPAI, including pancreatic mottling, splenomegaly, pulmonary edema and congestion, and hemorrhages in the mucosa of the proventriculus, gizzard and small intestine, and on the serosal surface. Microscopically, there were necrotized hepatitis and pancreatitis, lymphocytic meningencephalitis, myocarditis, and interstitial pneumonia. Influenza viral antigen was demonstrated in areas closely associated with histopathologic lesions. The H5N1 virus was isolated from cecal tonsils, feces, trachea, and kidney of the chickens. The isolated virus was identified as the highly pathogenic H5N1, with a hemagglutinin proteolytic cleavage site deduced amino acid sequences of QREKRKKR/GLFGAGLFGAIAG. In order to determine the pathogenicity of the isolate, eight 6-week-old specific pathogen free chickens were inoculated intravenously with the virus, and all the birds died within 24 hr after inoculation. This is the first report of an outbreak of HPAI in the chickens in South Korea.

KEY WORDS: broiler breeder, highly pathogenic avian influenza, H5N1, South Korea.

Avian Influenza (AI) is an infectious disease caused by viruses of the Influenza A genus of the Orthomyxoviridae family [18]. The AI virus (AIV) is distributed throughout the world in many domestic birds, including chickens, turkeys, quails, geese, and ducks, and in wild waterfowls, gulls and shorebirds [1, 17]. Poultry are not natural hosts for this virus [4], but infections with AIVs produces a variety of syndromes ranging from asymptomatic, to respiratory disease with low mortality, to highly pathogenic with high mortality [17]. The viruses are classified into subtypes based on the antigenic differences between their two surface glycoproteins, haemagglutinin (HA) and neuraminidase (NA) [18]. Fifteen HA subtypes (H1-H15) and 9 NA subtypes (N1-N9) have been identified for influenza A viruses [8, 13, 20]. Viruses of all HA and NA subtypes have been recovered from birds, but only two HA subtypes (H5 and H7) can have virus isolates that are highly pathogenic (HP) AI in for poultry [18].

AIVs of the H5 subtype have only been isolated from avian species and humans [3, 16]. Most H5 AIVs from wild aquatic birds and domestic ducks are of low pathogenicity [6]. However, in the poultry such as chickens and turkeys, some H5 AIVs have caused severe systemic disease with high mortality and are classified as highly pathogenic AI viruses [2, 7]. Epidemiological and molecular evidence of the first outbreak of H5N1 AIV (H5N1) in humans occurred in Hong Kong during 1997, and this epidemiologic and molecular evidence suggested that poultry were the source of the HPAI virus for humans [16]. In many of the cases of HPAI in chickens and turkeys, clinical signs were not observed because the viruses caused peracute death. However, sometimes typical clinical signs of HPAI are observed in chickens, including decreased egg production, respiratory signs, cyanosis of the unfeathered skin, particularly the combs and wattles, diarrhea, and nervous disorders, especially if the clinical disease is less fulminating [18].

In South Korea, a low pathogenicity AI outbreak was reported in 1996, and H9N2 AIV was isolated from several broiler breeder flocks [9]. Later, H5N1AIV (DK/Anyang/AVL-1/01), classified as HPAIV, was also isolated from frozen duck meat that had been imported to South Korea from China in 2001 [19]. However, prior to this outbreak, HPAI had not been reported previously in poultry in South Korea. Therefore, in the present study, we first describe of clinical and pathological findings observed in the broiler breeders infected with HPAI.

Case history: On December 10, 2003, a private practitioner submitted 6 dead broiler breeders (46-week-old) to the Avian Disease Division, National Veterinary Research and Quarantine Service, South Korea from a farm exhibiting high mortality with decreased egg production. A diagnosis of HPAI was made on December 15, 2003.

The mortality at the breeders farm, which housed 24,000 birds, began on December 6. Prior to submission of samples for diagnosis, over 9,000 birds died from the infection. When visiting to the farm on December 12, few chickens were found on the floor. The clinical signs reported by farmer and practitioner were a sudden increase in mortality with pronounced depression, decreased feed consumption, a precipitous drop in egg production (over 60%), and mild
respiratory signs.

To control the spread of AIVs, all broiler breeders on the farm and their eggs stored at the hatchery were buried immediately. Movement restrictions were applied to all domestic birds and birds products within a 10 km radius of the outbreak farm.

**Gross pathology:** On post mortem examination, the chickens most frequently had necrotized wattles and combs with ruffled feathers. General congestion in most veins of the abdominal viscera was common. The tracheal mucosa was edematous, hemorrhagic with mucopurulent exudates. Lungs were congested and/or had hemorrhages with edema. The livers of the birds were severely enlarged and very friable. Petechial hemorrhages were found on the epicardium, coronary fat pad, adipose tissues of the abdomen, and in mucosa of the proventriculus and duodenum. Ovarian follicles were flaccid, hemorrhagic, and wrinkled due to rupture of theca wall (Fig. 1). White necrotic foci with hemorrhages occurred in the spleen. In addition, fibrinous inflammation was found in the coelomic cavity, and egg yolk peritonitis was observed due to yolk escaping into the abdominal cavity.

**Histopathology and immunohistochemistry:** The most prominent microscopic lesions were found in the liver, lungs, pancreas, intestine, and brain. There were multiple confluent foci of coagulative necrosis with mild inflammatory cellular responses in multiple organs, including liver, heart, lungs, pancreas and brain. Many small lymphocytes, and a few macrophages, were found in the portal triads of the liver. In the pancreas, multifocal necrotic foci of acinar epitheliums were extensively present with mildly heterophilic infiltration. There was diffuse necrosis of the epithelium in the trachea and moderate mononuclear cell infiltration with edema in the submucosa. The lungs had bronchointerstitial pneumonia consisting of fibrinous thrombi in the small vessels, fibrino-puruleant exudates in the tertiary bronchi and atria, and edema in the interlobular space. Focal myocardial necrosis with hemorrhages and mononuclear cellular infiltration were observed. Severely lymphocytic depletions and necrosis were typically found in spleen and gut associated lymphoid tissues including the esophageal-proventricular junction, Peyer’s patches of the small intestine, and cecal tonsils. The brain had randomly scattered foci of malacia with gliosis (Fig. 2), and perivascular edema.

Paraffin embedded sections were immunohistochemically stained with a mouse-derived monoclonal antibody (kindly donated from Dr. Selleck CSIRO, Australia) as the primary antibody specific for a type A influenza virus nucleoprotein [14]. The influenza viral antigen was demonstrated within the tracheal epithelium, pulmonary endothelial cells, cardiac myocytes, renal tubular epithelial cells, and pancreatic acinar epithelium (Fig. 3), and in the neurons, purkinje cells and ependymal cells of the brain, including the cerebral hemispheres, medulla, and pons.

**Virology:** Virus isolation was attempted on the tissues of the cecal tonsils and kidneys and on feces by inoculation of 10% homogenates into the allantonic cavity of 9-day-old, embryonating specific pathogen free (SPF) chicken eggs, as described previously [15]. All the embryos died within 24 hours.

![Fig. 1. Severely flaccid and wrinkled by rupture of the theca wall of ovarian follicles from the broiler breeder.](image)
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hr post inoculation. Allantoic fluids from the dead embryonated eggs were harvested and tested using an agar gel precipitin (AGP) test and hemagglutination activity. Type A influenza viruses were identified by using AGP test. Also, hemagglutinating viruses were identified and typed as the influenza H5N1 virus by means of hemagglutination inhibition and NA inhibition tests with a panel of antisera (provided by OIE Reference Laboratory, Veterinary Agency, Surrey, United Kingdom).

To analyze HA cleavage site sequences, RNA was extracted from virus-containing allantoic fluid, and this was followed by cDNA synthesis and polymerase chain reaction (PCR), as described earlier [11]. The amino acid sequence of the HA cleavage site deduced from the polymerase chain reaction product (290 bp) was QREKRKKR/GLFGAIAG.

Six-week-old SPF chickens were used in pathogenicity tests using the OIE procedure [11]. Eight chickens were inoculated by the intravenous route (IV) with 0.2 ml of a 1:10 dilution of bacterial free, allantonic fluids of the isolate. The mortality rate of all the infected chickens infected was 100% within 24 hr after inoculation. The clinical signs and lesions observed were similar to those seen in the submitted broiler breeders.

The results of a pathogenicity test by intravenous inoculation on SPF chickens showed that the isolated H5N1 subtype caused a high (100%) mortality. A sequence analysis revealed that the virus possessed a series of four basic amino acids at the cleavage site (RKKRGLFG). In addition, the signs of disease that were observed in the broiler breeders on a gross and histopathological level were very similar to those cases reportedly caused by HPAI viruses. Thus, the isolated virus was defined as being highly pathogenic.

Although H5N1 viruses have been responsible for outbreaks of HPAI in many Asian countries, this is the first report of HPAI in domestic poultry in South Korea. The HA cleavage site of the isolated AIVs were identical to the virus (DK/Anyang/AVL-1/01) isolated from the imported China duck meat in 2001 [19]. Based on the results of a phylogenetic analysis of the eight viral genes, the H5N1 poultry isolates from the South Korea were of avian origin and contained hemagglutinin and neuraminidase genes of the A/goose/Guangdong/1/96 (Gs/Gd) lineage. In addition, the topology of the phylogenetic tree clearly differentiates the Korean isolates from the Vietnamese and Thai isolates that have been reported to infect humans [10].

Although HPAI has occurred in many countries and has been extensively studied for many years, a basic understanding of how the HPAIV enters a new country, especially a peninsula like Korea, has not yet been clearly obtained. There are several possible mechanisms for the introduction of an AIV to domestic poultry in South Korea. These mechanisms of introduction may include infected migratory waterfowl and shorebirds, import of infected raw poultry products (including duck meat), import of contaminated equipment or supplies, such as egg crates or chick boxes, and legal or illegal importation of live infected domestic poultry, exotic captive birds, and other animals. In 2001,

Fig. 2. Extensively focal malacia with gliosis of the cerebrum. Hematoxylin & Eosin stain.
H5N1 HPAIV was detected in raw frozen duck meat imported from China [19], but at that time, no outbreaks of H5N1 occurred in South Korea. Additional work is on going to determine the source of the introduction of the HPAIV into South Korea.

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