<Research Note>

Characterization of Avian Encephalomyelitis Outbreaks Occurred in South Korea from 2006 to 2013

Hye-Ryoung Kim, Yong-Kuk Kwon and Hee-Soo Lee

Avian Disease Division, Animal and Plant Quarantine Agency, South Korea

Running Title:  Avian encephalomyelitis outbreaks in South Korea

Correspondence: Dr. Hye-Ryoung Kim,
Avian Disease Division, Animal and Plant Quarantine Agency, 175 Anyangro Manangu,
Anyangsi, Gyeonggido 430-757, South Korea.
(E-mail: dvmkim77@korea.kr)
Abstract

This study was conducted to characterize avian encephalomyelitis (AE) viruses obtained from various flocks of breeder and commercial chickens in South Korea. Young chicken less than 4 weeks old showed neurological sign were diagnosed as typical AE infection between 2006 and 2011. In 2013, idiopathic AE occurred on the unvaccinated 79 day-old chickens that had clinical signs of ataxia and paralysis. Phylogenetic analysis of viral protein 2 genes of AE viruses showed that all AE field viruses tested were genetically similar to vaccine strain [Calnek 1143]. In the embryo-inoculation test via the yolk sac, only one field strain and one commercial vaccine were embryo-adapted. The results indicated that the AE outbreaks in South Korea were caused by strains genetically similar to vaccine strain indicating possibility of vaccine breakdown or persistence in the chicken population.

Keyword: avian encephalomyelitis, live vaccine, RT-PCR, viral encephalitis
Introduction

Avian encephalomyelitis (AE) is an infectious disease caused by the avian encephalomyelitis virus (AEV), a member of the Picornaviridae. Similar to other picornaviruses, the open reading frame of AEV can be divided into three regions, P1, P2 and P3. The P1 region encodes viral structural proteins VP4, VP2, VP3 and VP1. VP4 and VP 2 proteins are assembled into the particle in the form of precursor VP0 protein (Marvil et al., 1999).

In young chicks, 1-4 weeks of age, AE is characterized by neurologic signs such as ataxia, paralysis and rapid tremors of the head and neck (Jungherr and Minard, 1942). In natural outbreaks the disease usually appears in 1-2-week-old chickens with a morbidity rate of 40-60% and an average mortality of 25% (Swayne et al., 1998).

The first outbreak of this disease was observed in the USA in 1930, and AEV is now a worldwide problem; most flocks are susceptible unless vaccinated. In South Korea, AE was initially reported in 1973 and like in many other countries it is believed to be widely distributed (Choi et al., 1973). Since 2000, the AE has been occurring regularly in South Korea with sharp periodic increase in 2002-2003, 2006-2007 and 2010. There was no recorded AE outbreak in 2012, but AE reoccurred in 2013 (Figure 1). Vaccine strain [Calnek 1143] was first introduced in South Korea in 1986, and numerous commercially available vaccines have been used within the poultry (breeder and layer) industry to control the outbreak of AE.

The aim of this study was to determine genetic relationship between Korean AE virus genomes detected in breeder and commercial chicken flocks and to assess the AEV field strains susceptibility in embryo-adaptation test. In addition, we describe a
unique outbreak of AE in 79-day-old layers affected by neurological symptoms in South Korea in 2013.

Materials and Methods

Samples

Brains and cecal tonsil samples from cases of commercial chickens suspected of AE were inspected in this study. The chickens, submitted to the Animal and Plant Quarantine Agency for disease diagnosis between 2006 and 2011, were less than 1 month of age showing neurological symptoms. In addition, five AE positive samples were obtained from a survey of the avian diseases in broiler breeder farms located in several provinces of Korea in 2009. The flocks showed no clinical sign. The seven dead 79-day-old chickens were submitted and examined in 2013. Disease history was indicating signs of depression, lateral recumbency, lameness, ataxia, and tremor, observed first in 55-day-old chickens and persisted for a period of 38 days. As a result, an average of 15 chickens was culled daily. Mortality was approximately 0.4 % in the flock.

Necropsy and histopathology

Diagnostic postmortem examination was performed and tissues and swab samples were collected for histopathology, bacteriology and virology. For histopathological analysis, the brain, heart, liver, spleen, pancreas, proventriculus and duodenum were collected. Samples were fixed in 10 % neutral buffered formalin, and sections of the tissues were prepared using standard methods.

Bacteriology
Tracheal and synovial swabs were collected during necropsy, streaked on blood agar plate, McConkey agar and pleura-pneumonia-like organism agar plates and incubated.

**Mycoplasma serology**

Ten blood samples were collected from sick flock reared together with the dead chickens. The sera were diluted and tested using enzyme-linked immunosorbent assay (ELISA) against Mycoplasma. The BioChek Mycoplasma synoviae antibody kits (CK115, Biochek, Holland) were used in this study, according to the manufacturer's instructions. Absorbance of controls and test samples was measured at 405 nm (SpectraMax PLUS 384, Molecular Devices Corp., CA, USA).

**Virus detection**

RNA was extracted from brain and cecal tonsil samples, pooled from each flock, using the Viral Gene-Spin Viral DNA/RNA Extraction Kit (Intron Biotechnology, Seongnam, South Korea). Polymerase chain reaction was conducted using the Maxime RT-PCR Premix Kit (Intron Biotechnology, Seongnam, South Korea) amplifying a 619 base pair (bp) segment of the VP2 gene of AEV as described previously (Xie et al., 2005). The cycling conditions were comprised an initial reverse transcription (RT) step of 45°C for 30 min, following by denaturation at 94°C for 5 min, and 35 cycles of 94°C for 1 min, annealing at 62°C for 1 min and extension at 72°C for 1 min, with a final extension step at 72°C for 7 min and a 4°C hold. The RT-PCR products were visualized on a 1% agarose gel, stained with ethidium bromide, and exposed to ultraviolet light, and the image captured.

**Sequence and Phylogenetic Analysis**

The RT-PCR products were sequenced at COSMO (Seoul, South Korea) with an ABI
3730 XL DNA sequencer (Applied Biosystems, CA, USA) and the sequences were analyzed using Vector NTI (Invitrogen Ltd., Paisley, United Kingdom) and BioEdit (http://www.mbio.ncsu.edu/bioedit/bioedit.html). The phylogenetic trees were generated by the neighbor-joining method using the MEGA 4.0 software (Tamura et al., 2007) with 1,000 bootstrap replications. The AEV sequences generated in this study were submitted to GenBank and are deposited under accession numbers KF923352 to KF923364.

**Embryo-adaptation test**

The embryo adaptation test has been developed to determine the AE susceptibility or immunity status of a breeder flock (Sumner et al., 1957). If 70%-100% of the embryos are without lesions, the breeder flock is considered to be adequately immune (Swayne et al., 1998). Samples of pooled brain from less than 1 month-old chicks, and pooled brain and cecal tonsil from 79 day-old pullet and broiler breeder were processed for embryo-adaptation test. Homogenized samples were passed through a 0.45-μm filter and used to inoculate into the yolk sac of 6-day-old specific pathogen-free embryonated chicken eggs (Hy-Vac, Adel, IA). Two commercial vaccines (MA and JA) of the same strain [Calnek 1143] were also used in this test. The embryos examined for gross lesions such as paralysis, muscle atrophy and death at 12 day per inoculation. The brains from the inoculated embryos were pooled and tested using the AEV RT-PCR.

**Results and Discussion**

Six cases (06D79, 06D87, 06D88, 08D397, 10Q145 and 11Q353), which were
collected during the period of 2006-2011 were all diagnosed as the typical AE (Table 1). The birds were less than 1 month of age and showed neurological signs, such as lack of coordination and tremors. Histopathological examination and reverse transcription-polymerase chain reaction (RT-PCR) of the brains of the affected chickens also confirmed the AE finding. These cases were showed moderate to severe nonpurulent encephalomyelitis in the brains and the lymphoid follicles in the proventriculus or pancreas in the histopathological investigation.

Investigation into the disease of the 79-day-old hens affected by stunted growth and lameness was revealed no gross lesions in the postmortem examination. The bacterial culture and serological tests failed to detect a pathogen relating to the neurological signs and bacterial arthritis of the flock. The allantoic fluids were harvested from the eggs, inoculated with brains tissues of the hens, found to be negative for hemagglutinating activity, ruling out Newcastle disease and avian influenza causing neurological symptom. The brains of the clinically affected chickens showed moderate encephalomyelitis in the histopathological investigation (Figure 2). However, no abnormalities were observed in the proventriculus, duodenum or pancreas, in which lymphoid follicles would be observed, if young chicks were infected with AEV. The RT-PCR examination of a 619 bp band containing the VP2 gene of AEV was performed to confirm AE. Five AE strains (CN JS, HR, JB, OS and SJ) from breeder chickens vaccinated or not were detected and analyzed to genetic study, together with seven strains obtained from clinical cases (Table 1).

So far, complete sequence of only 4 AEV strains (Calnek, van Rokel, L2Z and 204C) have been known. However, after RT-PCR method (Xie et al., 2005) targeting to the VP2 gene to
detect AEV was developed, many VP2 gene reference sequences (such as AV1775 strain and VR strain) could be obtained to make the phylogenetic tree for genetic analysis from NCBI. Phylogenetic analysis of VP2 genes of the AEVs clustered the twelve Korean field strains (GenBank accession numbers: KF923352 - KF923364) with Calnek strain regardless of age, species and clinical signs of affected birds. The nucleotide sequence of the Korean AEV strains were 99.7% - 100% similar to that of the Calnek strain, and the amino acid sequence was 100% identical (data not shown). The Korean AEV strains were closer to the Chinese strains L2Z (95.4% nt, 97.6% aa), VR (94.6% nt, 97.1% aa) and Van Reokel strain than the AV1775/07 strain (82.8% nt, 100% aa) isolated from a pheasant in United Kingdom (Welchman et al., 2009) (Figure 3).

In the embryo–adaptation test, four clinical cases (06D79, 06D87, 06D88 and 13AD56) and the MA vaccine [Myelovax (Merial SAS, France; strain: Calnek1143)] did not showed any gross abnormalities in the inoculated embryos (Table 1). All of the embryos’ brains were examined for the presence of AEV using RT-PCR, and AEV was not identified, except for this MA vaccine. These results showed that these field strains were the non-adapted strains of AEV. However, 08D397 strain and the JA commercial vaccine [PoulShot® AE (CAVAC, Korea, strain: Calnek1143)] were showed embryo death, muscular dystrophy and rigidity of the leg on the 7th and 11th incubation day, respectively, meaning the embryo-adaptation. Field strain would occasionally adapt and induce lesions after several serial passages, and the adapted vaccine strain has lost their enterotropic properties and could not infect via the oral route (Calnek, 1998). Glisson (Glisson, 1997) reported that the embryo-adapted vaccines were probably responsible for the 1% - 4% incidence of clinical AE seen in some vaccinated flocks.
The present report included the outbreak AE in 79 day-old layer showing neurologic signs. This is an unusual finding because AEV seldom results in disease in birds older than 4 weeks of age (Swayne et al., 1998). A few reports suggested that no viral antigen was found in central nervous system of two-year-old hens infected orally with field strains of AEV (Miyamae, 1981) and Infection at 28 days caused no clinical signs (Cheville, 1970). Infection of AE in birds more than 8 weeks of age rarely occurred. However, VN antibodies were detected in experimental infection induced by intracerebral inoculation of AEV in adult birds (Calnek et al., 1960) and Peckham described brain lesions typical of AE in chickens that were approximately 3 or 4 months of age with ocular lesions and clinical symptoms, although there was no demonstration about other recognized causes of encephalitis (Peckham, 1957).

In summary, twelve AE field strains identified in South Korea between 2006 and 2013 could not be genetically differentiated from vaccine strain (Calnek 1143). Four AE isolates and one commercial vaccine could not be propagated in embryos, but another vaccine has adapted to embryo and it could be responsible for the recent field outbreak in South Korea. So, the embryo-susceptibility test could be useful to evaluate AE vaccine and to control AE outbreak. We report that the flock was naturally infected by AE virus at growing age (79 day-old) leading to the clinical signs and mortality which persisted for 38 days. It is likely that virus was circulating in the house and the flock was infected via continuous horizontal transmission, although the source of the original entry of AEV is unknown.

Acknowledgements
The authors thank Hyuk-Man Kwon for excellent technical assistance. This work was supported by a grant from the National Animal Disease Control Project and the GSP Project [213005-04-1-SBA10] of the Ministry of Agriculture, Food and Rural Affairs of Korea.
References


**Figure legends**

Figure 1. Number of chickens infected with Avian Encephalomyelitis (AE) in South Korea between 2000 and 2013. This graph was obtained from the Korea Animal Health Integrated System developed and operated by the Animal and Plant Quarantine Agency.

Figure 2. Histopathological findings of the brain from 79 day-old pullets. A, moderate mononuclear cellular infiltration in the perivascular areas and molecular layers of cerebellum (bar: 100 μm); B, central chromatolysis of a neuron in the white matter of cerebellum (bar: 50 μm); C, multifocal hemorrhages in the neuropile of medullar oblongata (bar: 100 μm). Black arrows indicate respective pathological lesions.

Figure 3. Phylogenetic diagram of VP2 genes among avian encephalomyelitis virus (AEV) strains detected in Korea. The numbers above and below the branches indicate neighbor-joining distances with 1000 bootstrap replicates. Bootstrap value of 90-100% means that the phylogeny is highly resolved. AEV strains isolated at cases showing neurological sign are highlighted by star and the vaccine strains (Calnek and van Reoekel) are indicated in green.
Table 1. History of Avian Encephalomyelitis cases diagnosed from various flock from 2006 to 2013 in South Korea

<table>
<thead>
<tr>
<th>Year</th>
<th>Strains</th>
<th>Species</th>
<th>Ages(^a)</th>
<th>Vaccination(^b)</th>
<th>Clinical signs</th>
<th>Embryo lesion(^c)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2006</td>
<td>06D79</td>
<td>layer</td>
<td>29D</td>
<td>N</td>
<td>Neurological sign</td>
<td>No</td>
</tr>
<tr>
<td>2006</td>
<td>06D87</td>
<td>broiler</td>
<td>14D</td>
<td>N</td>
<td>Neurological sign</td>
<td>No</td>
</tr>
<tr>
<td>2006</td>
<td>06D88</td>
<td>broiler</td>
<td>11D</td>
<td>N</td>
<td>Neurological sign</td>
<td>No</td>
</tr>
<tr>
<td>2008</td>
<td>08D397</td>
<td>broiler</td>
<td>21D</td>
<td>N</td>
<td>Neurological sign</td>
<td>Death(3/4)</td>
</tr>
<tr>
<td>2010</td>
<td>10Q145</td>
<td>broiler</td>
<td>13D</td>
<td>N</td>
<td>Neurological sign</td>
<td>nt</td>
</tr>
<tr>
<td>2011</td>
<td>11Q353</td>
<td>broiler</td>
<td>4D</td>
<td>N</td>
<td>Neurological sign</td>
<td>nt</td>
</tr>
<tr>
<td>2013</td>
<td>13AD56</td>
<td>layer</td>
<td>79D</td>
<td>N</td>
<td>Neurological sign</td>
<td>No</td>
</tr>
<tr>
<td>2009</td>
<td>CN JS</td>
<td>broiler breeder</td>
<td>22W</td>
<td>nk</td>
<td>No</td>
<td>nt</td>
</tr>
<tr>
<td>2009</td>
<td>HR</td>
<td>broiler breeder</td>
<td>14W</td>
<td>nk</td>
<td>No</td>
<td>nt</td>
</tr>
<tr>
<td>2009</td>
<td>JB</td>
<td>broiler breeder</td>
<td>35W</td>
<td>Y</td>
<td>No</td>
<td>nt</td>
</tr>
<tr>
<td>2009</td>
<td>OS</td>
<td>broiler breeder</td>
<td>16W</td>
<td>nk</td>
<td>No</td>
<td>nt</td>
</tr>
<tr>
<td>2009</td>
<td>SJ</td>
<td>broiler breeder</td>
<td>18W</td>
<td>Y</td>
<td>No</td>
<td>nt</td>
</tr>
<tr>
<td>Vaccine</td>
<td>JA (Calnek1143)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Death(2/3)</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>MA (Calnek1143)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

\(^a\)Ages: D, days old; W, weeks old

\(^b\)Vaccination: nk, not known; N, no; Y, yes

\(^c\)Embryo lesion was obtained from inoculation experiment into yolk sac of six day-old embryo: nt, not tested,

The numbers in the parenthesis correspond to the number of dead embryos of the number of inoculated embryos.
Fig. 1. Number of chickens infected with Avian Encephalomyelitis (AE) in South Korea between 2000 and 2013. This graph was obtained from the Korea Animal Health Integrated System developed and operated by the Animal and Plant Quarantine Agency.
Fig. 2. Histopathological findings of the brain from 79 day-old pullets. A, moderate mononuclear cellular infiltration in the perivascular areas and molecular layers of cerebellum (bar: 100 μm); B, central chromatolysis of a neuron in the white matter of cerebellum (bar: 50 μm); C, multifocal hemorrhages in the neuropile of medullar oblongata (bar: 100 μm). Black arrows indicate respective pathological lesions.
Fig. 3. Phylogenetic diagram of VP2 genes among Avian encephalomyelitis virus (AEV) strains detected in Korea. The numbers above and below the branches indicate neighbor-joining distances with 1000 bootstrap replicates. Bootstrap value of 90-100% means that the phylogeny is highly resolved. AEV strains isolated at cases showing neurological sign are highlighted by star and the vaccine strains (Calnek and van Reokel) are indicated in green.