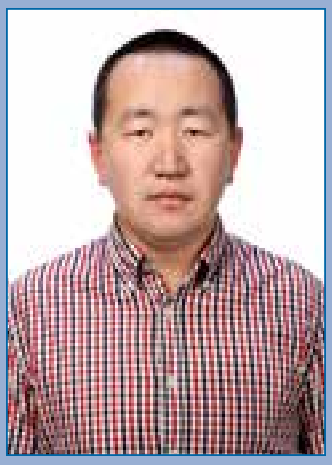

Erdene-Ochir

Erdene-Ochir TO has degree in veterinary medicine from the Mongolian State University of Agriculture and studied for his PhD degree at Konkuk University of the Republic of Korea. Currently, he is working as a researcher at the State Central Veterinary Laboratory on some avian influenza projects, particularly on surveillance of avian influenza in wild birds (SCVL) of Mongolia.

This research was supported with a grant from the International Society for Infectious Diseases (ISID).

ISID Grant Report

Studies on characteristics and distribution of avian influenza viruses in wild birds of Mongolia and genetic comparison avian influenza viruses isolated from North East Asia including Mongolia and Korea

Tseren-Ochir ERDENE-OCHIR[1]

[1] *State Central Veterinary Laboratory, Mongolia*

Introduction

Avian influenza (AI) represents one of the greatest concerns for domestic (poultry) and wild birds and a listed disease of the World Organisation for Animal Health (OIE) that has become a disease of great importance both for animal and human health Webster, Krauss et al. (2007).

Poultry is the second most widely eaten meat in the world, accounting for about 30% of meat production worldwide, after pork at 38%. WHO and OIE warn all countries to implement activities to combat and prevent from the HPAI without delay. Although wild birds are the recognized source and reservoir for all 144 subtypes of influenza A, the majority of possible combinations have been isolated specifically from species in the order Anseriformes (e.g. ducks, geese and swans) and Charadriiformes (e.g. gulls, terns and shorebirds) (Webster, Bean et al. 1992).

Since its emergence in 1997 and re-emergence in 2002 and 2003, HPAI caused by H5N1 virus has been spreading to poultry farms from the South Asian countries through migratory birds. A new occurrence of morbidity and infections of AI to wild birds, domestic birds and humans have been registered worldwide. Wild birds mortality associated with HPAI H5N1 virus infected many wild bird species at two waterfowl parks in Hong Kong was reported in 2002 (Ellis et al., 2004) and continued through 2005. More significant outbreaks in wild birds were reported at Lake Qinghai in China (Chen, Smith et al. 2005) and the current distribution suggests movement of this virus via migratory birds, so the World Health Organization (WHO) and the World Organisation for Animal Health (OIE) regularly warn all countries to implement activities to combat and prevent from the HPAI without delay.

Mongolia was the second country to report wild birds infected with the H5N1 virus after the Qhinghai Lake outbreak of China in August, 2005 (OIE, 10/Aug/2005) and to date eight HPAI H5N1 outbreaks have been reported in wild bird since that time (Erdene-Ochir et al., 2012) and thousands of birds died of the disease.

A combination of geographical and other several features such as the rich bird fauna and bird migration routes make Mongolia an ideal location for conservation of wild birds and understanding the epidemiology of avian influenza viruses in wild birds.(Erdene-Ochir et al., 2010). A further advantage inherent in studies of avian influenza virus in wild birds in Mongolia is the opportunity for the conservation of wild bird populations and to study early detection of virus in wild bird populations. There are four main migration routes passing through Mongolia: the East-Asia/Australia Flyway, Central-Asia/India Flyway, West-Asia/Africa Flyway and the Mediterranean/Black-see Flyway between their Arctic breeding grounds and the wintering grounds in the south (Minister of Nature, 2009). This combination of geographical features makes Mongolia a hot location for avian influenza viruses in wild birds. To date there are 16 H and 9 N subtypes are known, for a total of 144 possible different influenza subtypes, each with potentially different host susceptibility (Causey and Edwards, 2008).

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The goal of this project was to study epidemiology of AI viruses in wild birds, and relate the findings to the avian influenza outbreak dynamics in in the world. The result useful to minimizing HPAI risk in wild birds by early detection and better understanding the epidemiology of AI and to developing an appropriate strategy sensible to human and animal health, a survey of avian influenza virus presence in wild birds in North-East Asia including Mongolia, is proposed.

Based on the results, prevalence and evolution of influenza virus will be discussed. The data will be used in reducing influenza risk and the early detection of emerging viruses with pandemic potential and developing strategies to combat new pandemic in both wild and domestic bird populations.

Materials and Methods

The project carried out during a year. Field study (sample collection) conducted in eastern and central region of Mongolia covered about 10 lakes in Mongolia in 2013 and 2014. Research methodologies included sample collection, virus isolation and identification, bird species identification, molecular and phylogenetic analyses, and data analyses described below. It was done according to OIE, WHO and FAO manuals. The surveillance scheme was designed to target important lakes and routes of migratory birds in Mongolia. The sample size estimated 2000, based on the study period and diagnostic capacity of SCVL.

Bird Observation

Observation, looking through binoculars and other common methods were used to define the bird species and population numbers, and detect suspected AI and dead birds.

Sample Collection

Fresh fecal samples (n=2023) from wild birds were collected from September to October in 2013 and from May to July 2014 survey for AIV in Mongolia in 2013. Fecal samples were collected after observing defecation. The sample collection sites include major wild bird habitats and outbreak sites of H5N1 HPAI in wild birds in Mongolia from 2005 to 2011..

Sample storage

- All samples transported from field to the Animal Health Center of SCVL in a refrigerated container and stored at 4°C until assayed.
- After inoculation all samples were stored at -70°C.

Testing

The samples tested for AI virus detection and subtyping according to the standard methodology, recommended by OIE in BSL III laboratory of State Central Veterinary Laboratory.

Virus Isolation and Identification

Each sample was suspended in an antibiotic solution and centrifuged at 2,300 × g for 15 min at 4°C. Each supernatant was inoculated into the allantoic cavity of 10-d-old embryonated hen's SPF eggs and incubated at 37°C for 4 to 5 days. Allantoic fluid from the incubated eggs were harvested and centrifuged for purification. The presence of virus was determined by an HA assay, and detected viruses determinated AIV and NDV by Real-time PCR and subtyped by reverse transcription-PCR using influenza-specific primers.

Bird Species

A barcoding system for mitochondrial DNA of bird feces was used to determine host species in this study. Mitochondrial DNA was extracted from HA assay and RT-PCR fecal samples by using a DNA Stool Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. Universal and modified primers were used to amplify the gene encoding mitochondrial

cytochrome oxidase gene subunit present in host feces. The generated PCR products were sequenced and identified using information contained on the Barcode of Life Data Systems web site (www.boldsystems.org/views/login.php).

Molecular and Phylogenetic Analyses:

Viral genes were sequenced and analyzed as described previously. Briefly, viral RNA was extracted from the allantoic fluid of embryonated eggs by using a RNeasy Mini Kit (Qiagen). Segments of the Surface and Internal genes were amplified with gene-specific universal primers. (Table 3) Polymerase chain reaction products were purified from agarose gels by using a Qiaquick Gel Extraction Kit (Qiagen), and sequencing of the PCR product performed at State Central Veterinary Laboratory with an ABI 3730 XL DNA sequencer (Applied Biosystems).

Assembly of the sequencing contigs and translation of the collated nucleotide sequences into a deduced amino acid sequence was performed using the VectorNTI Advance program (Invitrogen, Carlsbad, CA). The sequence data was aligned using the AlignX multiple sequence alignment in the VectorNTI Advance program. A phylogenetic tree was constructed using the neighbour-joining method within Clustal X version 1.83, with 1,000 bootstrapping replicates. The final phylogenetic tree was constructed using the neighbour-joining method within MEGA6 software.

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Results and Discussion

The samples were tested for AI virus detection and subtyping according to the standard methodology, recommended by OIE in BSL III laboratory of SCVL.

Briefly, virus isolation was carried out from five fresh dropped samples from the same location that were pooled and suspended in a 1% antibiotic PBS pH 7.2 and inoculated into embryonated hen's eggs. Allantoic fluid was harvested and presence of virus was determined by a hemagglutinin (HA) assay. Consequently, positive pooled samples were performed individually for virus isolation to obtain the real prevalence.

For the identification of AIV, RNA was extracted from individual HA-positive allantoic fluid using the RNeasy kit (Qiagen, Valencia, California, USA) according to the manufacturer's instructions and matrix gene of AIV was determinates by Real-time PCR(11).

For molecular characterization, RNA was amplified by Two-step RT-PCR with a gene-specific universal primer set (12) for segments of the genes encoding polymerase subunit PB2 and PB1, polymerase acidic (PA), HA, NP, NA, matrix glycoprotein (M), and nonstructural protein (NS) and further sequenced with an ABI Prism 3730xl genetic analyzer (Applied Biosystems, Foster City, CA).

We isolated HPAIV (n=1) named as A/whooper-swan/Mongolia/W243/2013(H5N1) from whooper-swan (Cygnus cygnus) fecal samples at Uvs Lake in western part of Mongolia.

Also we isolated 11 LPAIVs from wild bird fecal samples and the subtypes were H3N8 (n=5), H4N6 (n=2), H11N8 (n=2), H12N2 and H3N2. The viruses were related with the Euro-Asia lineage.

No.	Date of Sampling	Location	Species	Sample ID	Subtype H	N
1	2013.09.20	Bayank-hongor	Pallas's Gull/ *Laurs ichthyaetus*	C-97	H4	N6

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2	2013.09.23	Arkhangai	Whooper Swan *Cygnus cygnus*	C-160	H3	N2
3	2013.09.23	Khuvsgul	Falcated duck/*Anas falcata*	C-177	H3	N8
4	2013.09.23	Khusgul	Falcated duck/*Anas falcata*	C-180	H3	N8
5	2014.05.20	Khentii	Eurasian wigeon *Anas Penelope*	W-8	H4	N6
6	2014.05.21	Khentii	Eurasian wigeon *Anas Penelope*	W-80	H3	N8
7	2014.05.21	Khentii	Eurasian wigeon *Anas Penelope*	W-99	H3	N8
8	**2013.09.29**	**Uvs**	**Whooper Swan *Cygnus cygnus***	**E-243**	**H5**	**N1**
9	2013.09.29	Uvs	Whooper Swan *Cygnus cygnus*	E-266	H12	N2
10	2013.09.29	Uvs	Gadwall/ *Anas strepera*	E-288	H3	N8
11	2014.05.26	Sukhbaatar	Mallard/ *Anas platyrhynchos*	W-281	H11	N8
12	2014.05.26	Sukhbaatar	Mallard/ *Anas platyrhynchos*	W-288	H11	N8
		Total				**12**

Conclusions

In Mongolia, all previous H5N1 isolates were obtained from tissue samples of dead or ill wild birds and the last case reported in wild bird populations were ill whooper-swans in Apr 2011. Since that time any HPAI/H5N1 cases with mortality were not reported. Interestingly, more than 2 years later in 2013, the first isolates obtained from fecal samples and phylogenetic analysis revealed that the HA genes of the isolates were also classified into clade 2.3.2.1 and homology were closely related (100%) with recent (2009/2010) Mongolian HPAI/H5N1 isolates that had recently been circulating in Eastern Asia. Also genetic characterization of the HA gene revealed the cleavage site of the isolates (PQRERRRKRGLF) consistent with HPAI viruses. However, we did not find any birds clinically ill or dead in the sampling sites. That result suggests 2.3.2.1 clade HPAI/H5N1 is still circulating in wild bird populations without any clinical signs or mortality. Also in recent years, poultry is increasing due to several changes. It indicates that the role of the migratory birds in Mongolia in the AIV mutation should be clarified and also domestic birds are a potential source of infection. Therefore, it is necessary to continue the research on avian influenza in wild and domestic birds in Mongolia and it could be helpful for a better understanding of HPAI evolution, epidemiology, pathogenicity and influence in public health contexts.

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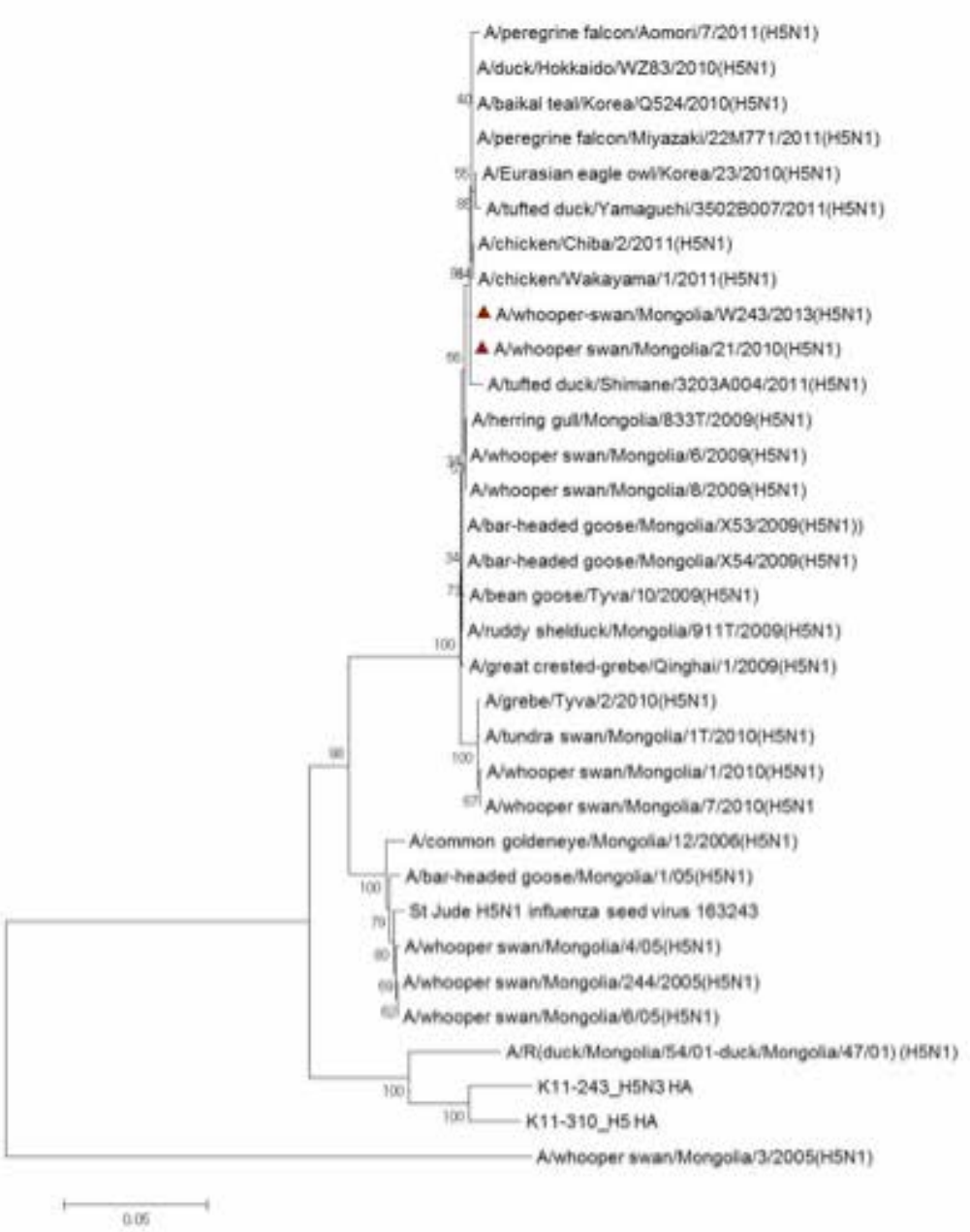

FIGURE 1. Comparative analysis of highly pathogenic avian influenza H5N1 virus isolated from North East Asia includes Mongolia and Korea based on Phylogenetic tree of the HA gene sequence.

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