

Characterization of low pathogenicity avian influenza viruses isolated from wild birds in Mongolia from 2009 to 2018

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Abstract

Background: Since 2005, highly pathogenic avian influenza A H5N1 viruses (Goose/Guangdong/1/96-H5 lineage) have spread from Asia to Africa and Europe, infecting poultry, humans and wild birds. Subsequently, avian influenza surveillance has increased worldwide. Mongolia is a good location to study influenza viruses in wild birds within Asia because the country has very low densities of domestic poultry and supports large concentrations of migratory water birds. We conducted avian influenza surveillance in Mongolia over two time periods from 2009 to 2018, utilizing environmental fecal sampling.

Methods: Fresh fecal samples were collected in areas where wild water birds, including orders Anseriformes and Charadriiformes, congregated. Hemagglutinin (HA) and neuraminidase (NA) subtypes of positive samples were identified through viral isolation or molecular assays, with pathogenicity determined by HA subtype or sequencing the HA cleavage site.

Results: A total of 10,222 samples were collected and tested for avian influenza virus. Of these, 7,025 fecal samples were collected from 2009-2013, and 3,197 fecal samples were collected from 2016-2018 period. Testing revealed 175 (1.7%) positive samples for low pathogenicity influenza A, including 118 samples from 2009-2013 (1.7%) and 57 samples from 2016-2018 (1.8%). Over the period and locations of surveillance no influenza A viruses were detected in association with a wild bird mortality event. HA and NA subtyping of all positives identified eleven subtypes of HA (H1-H8; H10-H12) and nine subtypes of NA (N1-N9) in 29 different combinations. Within periods, viruses were detected more frequently during the fall season (August to October, 2.5%; 95% CI 2.1-2.9%; 146/5,750) than the early summer (April to July, 0.6%; 95% CI 0.4-0.9%; 29/4,472).

Conclusion: Mongolia is an important location for wild birds and is positioned as a crossroad of multiple migratory bird flyways. Breeding birds from Mongolia and further north pass through the country to wintering areas as widespread as southern Africa, the Indian Subcontinent, Southeast Asia and Australasia. Our work demonstrates the feasibility of using an affordable environmental fecal sampling approach for avian influenza surveillance and contributes to understanding the prevalence and ecology of low pathogenicity avian influenza viruses in this important location, where birds from multiple flyways mix.

Background

Influenza A viruses are pathogens of global concern in both human and veterinary medicine (1–3). Avian influenza virus (AIV) subtypes are generally classified into two groups based on their pathogenicity in domestic poultry: high pathogenicity avian influenza viruses (HPAIV) and low pathogenicity avian influenza viruses (LPAIV). Subtypes are characterized based on the antigenic profile of their surface proteins hemagglutinin (HA, 16 subtypes, H1–H16) and neuraminidase (NA, nine subtypes, N1–N9) (4–7). Highly pathogenic strains can cause high rates of mortality in domestic poultry flocks and can lead to large outbreaks with severe economic consequences in affected countries (8). In the spring of 1996, domestic geese in Guangdong, China were affected by HPAI associated with hemorrhagic and neurological disease. The causal agent was identified as an HPAI virus strain designated as A/goose/Guangdong/1/1996 (H5N1) (9). In late 2002 and early 2003, A/goose/Guangdong/1/1996 (H5N1) HPAI viruses were associated with the mortality of wild and captive water birds in Hong Kong parklands, representing the first reported detections of HPAI in wild birds since 1961 (10, 11). In 2005, HPAI viruses were associated with an outbreak among migratory birds on Lake Qinghai, China, in May and June, during which more than six thousand birds were died (12). Since 2005, surveillance of wild birds for AIV has increased worldwide, tracking the spread of the

A/goose/Guangdong/1/1996 (H5N1) HPAIV among domestic and wild birds in Asia, Europe, Africa and North America (13).

Within Asia, Mongolia is a particularly ideal location to study the dynamics of wild bird influenza viruses, in part because the country has very low densities of domestic poultry (14). Mongolia also supports large populations of wild birds from two major migratory flyways and has important breeding, molting and pre-migratory staging areas (15, 16). The relative absence of poultry within the country provides a near unique opportunity to study the epidemiology of the virus in the absence of domestic influence (17, 18). The migration of wild birds contributes to the global movement of these viruses (19). Therefore, AIV surveillance is important to enhance our understanding of the spread of influenza viruses around the world and especially the opportunity for exposure of domestic poultry and transmission from poultry to humans, as well as to identify potential exposure of wild birds to highly pathogenic influenza viruses from poultry. In addition, AIV surveillance in wild birds aids our understanding of the dynamics and viral ecology in the natural hosts, capturing the viral diversity in these reservoirs, which can improve our understanding of influenza A virus risk in other species (20). We report the detection of 175 LPAIVs isolated from wild birds in Mongolia. The surveillance was conducted throughout Mongolia in two time periods representing eight years in the period 2009–2018 (Fig. 1).

Methods

Study area and period

Lying between latitudes 42°N and 51°N, Mongolia is a vast land-locked country extending east-west across 2,500 km of Central Asia between Russia and China, with an approximate area of 1.5 million km². Although a country of climatic and geographic extremes (21), much of the land area consists of open steppe, transitioning to taiga forest in the north and the Gobi Desert in the south and west. Wetlands and lakes of variable size fleck the landscape, with approximately 3,000 rivers stretching over 67,000 km in the north (21). Sampling sites were generally selected based on known water bird congregation points. During the 2009–2013 period, samples were collected annually at a total of 34 locations throughout the country and in spring, summer and autumn. Whereas during the 2016–2018, samples were collected at a total of 25 locations across Mongolia during the spring and fall seasons each year (Fig. 1).

Fecal Sample Collection

Fecal samples were collected from three ecogeographic regions, steppe (eastern region), mountain steppe (central region), and desert steppe (western region). Before fresh fecal sample collection, we observed and identified bird species throughout the water body and then selected potential sampling areas. Fresh fecal samples were collected where waterbirds were observed congregating or roosting. Fecal sampling focused on single-species or single-genus flocks to enable the identification of the species being sampled. Between 2009 and 2013, fecal sampling was focused on the order Anseriformes (particularly dabbling ducks and shelducks), and host species identity of all AIV positive samples was confirmed using DNA barcoding (22). Sampling from 2016–2018 was targeted on orders Anseriformes and Charadriiformes, and species identification was based on field observation. In addition, we collected small numbers of samples from order Gruiformes (n = 10, cranes) in 2016–2018. Samples were stored individually in 2.0 ml cryovials containing viral transport media (VTM) between 2009–2013, and between 2016–2018 samples were stored in 2.0 ml VTM and 2.0 ml Trizol (23). Samples were initially maintained at 4°C, then frozen in liquid nitrogen within four hours of collection. Care was taken to maintain the cold-chain for specimens throughout transport, and once in the lab, all samples were stored at -70°C or below until processed.

Laboratory Protocols For 2009–2013 Surveillance

Specimens were inoculated into 9–10 day old embryonated specific-pathogen-free eggs and incubated at 37°C for three days. The allantoic fluid harvested from egg cultures were then tested by hemagglutinin assay (24). Positive samples in HA were extracted with viral RNA extraction kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. One step RT-PCR was performed using SuperScript® III One Step RT-PCR systems with Platinum® Taq polymerase (Invitrogen) using primers designed for the conserved sequence for the HA2 coding region (25) and NA gene (26). Conventional RT-PCR was performed in GeneAmp PCR systems 9700 (Applied Biosystems). Correct size bands were excised and purified using a gel purification kit and a DNA purification kit (Qiagen, Hilden, Germany). Sequencing was performed using DNA Analyzer 3730xl (Applied Biosystems), and the HA and NA subtypes were confirmed and identified using the NCBI Blast tool. Pathogenicity of the isolates were determined by studying the sequence at the HA cleavage site.

Laboratory Protocols For 2016–2018 Surveillance

At the State Central Veterinary Laboratory, RNA was extracted from the Trizol sample using the RNA MiniPrep Kit (Sigma-Aldrich), and cDNA was transcribed using the SuperScript III First Strand cDNA Synthesis System (Invitrogen). Avian influenza RNA was detected with two assays. All samples collected from 2016–2018 were screened for the M gene (27) with a universal control, and samples collected in 2017–2018 were additionally screened for the Orthomyxoviridae PB1 gene with a specific Influenza A control (28). Each test batch included distilled water as a negative control. PCR products were visualized using 1.5% agarose gels. Correct size bands were excised, cloned and sequenced by Sanger dideoxy sequencing. Samples with genetic sequences confirmed as Influenza A using the NCBI Blast tool were then subtyped by reverse transcription-PCR (RT-PCR). An assay targeting the HA1 coding region was used to identify H1-H16 hemagglutinin subtypes (29), and an assay targeting the NA gene was used to identify N1-N9 neuraminidase subtypes with distilled water as a negative control (30). The RT-PCR product was visualized on a 2% agarose gel with a 100 bp DNA ladder (Thermo Fisher Scientific), and the length of the product was used to identify a specific HA or NA subtype. Low pathogenicity was determined based on the absence of H5 or H7 subtypes.

Results

A total of 10,222 fecal samples were collected from wild birds in Mongolia and tested for avian influenza virus. Of these, 7,025 samples were collected from 2009–2013, and 3,197 samples were collected from 2016–2018. A total of 175 samples (1.7%) were positive for influenza A viruses, and the annual proportion was between 0.5% and 3.9% (Table 1). Eleven subtypes of HA (H1-H8; H10-H12) and nine subtypes of NA (N1-N9) were identified in 29 different combinations. The most common subtypes of HA were H3 and H4, followed by H2, H10 and H1. For the NA subtypes, N8 and N6 were the most common, followed by N1, N2, N5 and N7. Subtyped cases were most often found in Anseriformes (n = 28), and the lowest number of subtypes were identified from Charadriiformes (n = 3). The most frequently detected subtype combinations in wild birds were H3N8 (n = 69; 39.4%), H4N6 (n = 31; 17.7%) and H10N7 (n = 9; 5.1%). The H4N6 subtype was detected in all years, and H3N8 in all but one of the years (Table 1). In contrast, the majority of subtypes were found in only a single year (n = 19). The highest number of subtypes were identified in the eastern region with 27 subtypes detected compared to nine in the central and four in the western regions (Supplementary Table 1). No avian influenza viruses were detected in association with a wild bird mortality event.

Table 1
 Number of viruses and subtype combinations detected in wild birds in Mongolia, 2009–2018.

No. of samples		1,001	1,612	2,400	810	1,202	1,170	1,062	965
Proportion of positives		2.0%	1.4%	1.1%	2.0%	2.8%	3.9%	0.6%	0.5%
Subtype	Total detection years	2009	2010	2011	2012	2013	2016	2017	2018
Anseriformes	H1N1	3	5		1	2			
	H2N2	2				1		1	
	H2N3	1	1						
	H2N5	1				6			
	H3N1	2	1	3					
	H3N2	3		2	1	2			
	H3N3	1		1					
	H3N5	1				3			
	H3N6	2	1			1			
	H3N8	7	9	1	13	6	9	27	4
	H3N9	1			1				
	H4N3	1	1						
	H4N6	8	4	3	7	2	2	8	4
	H5N1	1				1			
	H5N3	1		2					
	H6N2	2				1	3		
	H6N6	1		1					
	H7N1	1	4						
	H7N3	1	1						
	H7N7	1	1						
	H8N4	1	1						
	H8N6	1	1						
	H10N7	2	1				8		
	H10N8	2	3	2					
	H11N2	1			1				
	H11N9	1			1				

	No. of samples	1,001	1,612	2,400	810	1,202	1,170	1,062	965
	H12N1	1				2			
	H12N5	1				1			
	Subtotal	20	23	26	14	30	46	5	5
Charadriiformes	H2N2	1						1	
	H2N5	1			1				
	H2N6	1				3			
	Subtotal	0	0	0	1	3	0	1	0
Unknown	H3N1	1			1				
	Subtotal	0	0	0	1	0	0	0	0
	Total viruses	20	23	26	16	33	46	6	5
	No. of subtypes	7	11	6	10	12	4	3	2

Fecal samples were collected throughout eastern (67.8%), central (22.2%) and western (16%) regions of Mongolia. Avian influenza viruses were detected relatively more frequently in the central (3.2%; 95% CI 2.5–3.9%; 72/2,264) region compared to the eastern (1.5%; 95% CI 1.2–1.8%; 93/6,319) and the western (0.6%; 95% CI 0.2–1.1%; 10/1,639) regions (Fig. 1). The highest proportion of influenza virus detections between 2009–2013 was found in September (4.1%) and October (3.3%), and the lowest in May (0.0%). In contrast, in 2016–2018, the highest proportion was in September (3.3%) and August (2.5%), and the lowest in April and October (0.0%). However across both surveillance periods, the fall season, including the months of August, September and October, tended to have more positives (2.5%; 95% CI 2.1–2.9%; 146/5,750) than the early summer (0.6%; 95% CI 0.4–0.9; 29/4,472), which included the months of April, May, June and July (Fig. 2).

Among the positives, Anseriformes [tufted duck (*Aythya fuligula*), Stejneger's scoter (*Melanitta stejnegeri*), mallard (*Anas platyrhynchos*), ruddy shelduck (*Tadorna ferruginea*), common shelduck (*Tadorna tadorna*), common teal (*Anas crecca*), northern pintail (*Anas acuta*), northern shoveler (*Anas clypeata*), Eurasian wigeon (*Anas penelope*), eastern spot-billed duck (*Anas poecilorhyncha*), common goldeneye (*Bucephala clangula*), tundra swan (*Cygnus columbianus*), whooper swan (*Cygnus cygnus*), red-crested pochard (*Netta rufina*) and unidentified (*Anas spp* and *Anser spp*)] hosted 96.6% of all isolated viruses. Another 3.4% of isolates were obtained from Charadriiformes [spotted redshank (*Tringa erythropus*)] and unidentified *Larus spp.*] and unknown species (Supplementary Table 1).

Discussion

It is generally accepted that wild birds represent the principal natural host of LPAI viruses (1), with isolations documented from at least 105 wild bird species from 26 different taxonomic families (31). In this study, the proportion of samples positive for AIV was relatively low (1.7%) and consistent across the different sampling periods and methodologies. This low proportion is consistent with previous studies that have been conducted in wild birds in Mongolia (17, 32). In our surveillance of Anseriformes and Charadriiformes orders an absence of AIV-linked wild bird mortality suggests detected viruses were LPAI. Many Anseriformes and Charadriiformes are known to make regular

long-distance migrations (16, 33), and migratory birds play important roles in the geographic spread of AIV (18, 19, 31, 32). Numerous surveillance studies of wild ducks in the northern hemisphere have revealed higher LPAIV prevalence in juvenile birds with a peak in early autumn, when the birds start migrating south (34). In North America, the prevalence falls from more than 60% in early autumn in ducks sampled at marshalling sites close to the Canadian breeding areas to 0.4–2% at the wintering grounds in the southern United States of America (USA) and ~0.25% when the ducks return to the northern breeding grounds in spring (1). Similar patterns have been observed in Northern Europe, although LPAIV detection during the spring migration can be significantly higher (35–37). Surveillance of the nesting grounds of ducks in Alaska and Siberia before winter migration revealed the presence of LPAIV in 2.5–8% of birds (38). During this study, we were not able to estimate ages of sampling target species. However, the proportion of AIV in autumn surveillance (2.5%) was significantly higher than spring surveillance (0.6%). This result may be explained by the presence of large numbers of immunologically naïve juvenile birds from late summer onwards, which are more likely to be infected with LPAIVs.

Globally, a total of 112 AIV subtypes have been identified in wild birds, of which 49 have also been found in domestic species (2). Multiple AIV subtypes have been detected from wild birds in East Asia (2, 39, 40). In our study, 11 HA subtypes and nine NA subtypes in 29 different combinations were detected in wild birds. The H1-H8, H10-H13 and H16 subtypes had already been isolated from wild birds in Mongolia (17, 32, 41–44); H13 and H16 were not isolated. Among the subtypes, the most frequently isolated were H3N8 (39.4%), H4N6 (17.1%) and H10N7 (5.1%); these results are similar to influenza virus surveillance conducted in Japan, South Korea, North America and European studies (6, 32, 35, 45, 46). This finding indicates that AIVs have become mixed among different migratory bird species in overlapping flyways, driving the spread of the virus over long distances within Asia and between continents (47). Dabbling ducks of the *Anas* genus have been found to be infected with influenza viruses more frequently than other birds, including diving ducks (31). However, we also found AIVs in diving species and waders during the 2009–2013 with species identification confirmed using DNA barcoding. H1N1, H2N5, H2N6, H4N6, H7N1 and H10N8 subtypes were found in diving ducks and waders, and all of these subtypes were also found in dabbling ducks except H2N6, which was only detected in a sample from a spotted redshank (Supplementary Table 1).

Conclusions

Mongolia is an important location in Asia for both bird habitat and migration, and our eight-year study identified 175 LPAIVs of 29 subtypes from 10,222 samples and revealed geographic, taxonomic, and seasonal patterns, as well as inter-annual variation in prevalence. While no highly pathogenic avian influenza viruses were found during this period, detection in wild birds can help address threats to domestic animals and human health. This effort demonstrates the feasibility of surveillance and contributes to understanding the prevalence and ecology of LPAIV in wild birds. Continued seasonal surveillance will provide additional information on the prevalence and virus circulation in the region. To understand global patterns of LPAI viruses in wild birds, it will be crucial to integrate virus and host ecology with long term surveillance studies, like this one, to provide more insight on the year-round perpetuation of influenza viruses in wild birds (31). In addition, long term surveillance efforts are important for providing ecological data on infections and for modeling disease spread for more precise risk analyses (48).

Abbreviations

AIV avian influenza virus

HA hemagglutinin

HPAIV high pathogenicity avian influenza viruses

LPAIV low pathogenicity avian influenza viruses

NA neuraminidase

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable

Availability of data and materials

All data generated or analyzed during this study are included in this published article [and its supplementary information files]. Additionally, 2016-2018 surveillance datasets generated and analyzed during the current study are available in the USAID Data Development Library (DDL) repository, <https://data.usaid.gov/d/tqea-hwmmr> (49).

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

MG, AEF, JAKM, CKJ, ES and SHO developed the surveillance and sampling design. AB, MG, LJ, UA, DP, JMT, MS, BK and TS conducted the field work to collect the fecal samples. JSMP, CYHL and UA performed the laboratory assays to identify AIVs, determine subtypes, and analyze their pathogenicity. AB, MG, AEF, BD, BB, CYHL and SHO analyzed and interpreted the fecal wild bird surveillance data. All authors read and approved the final manuscript.

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Figures

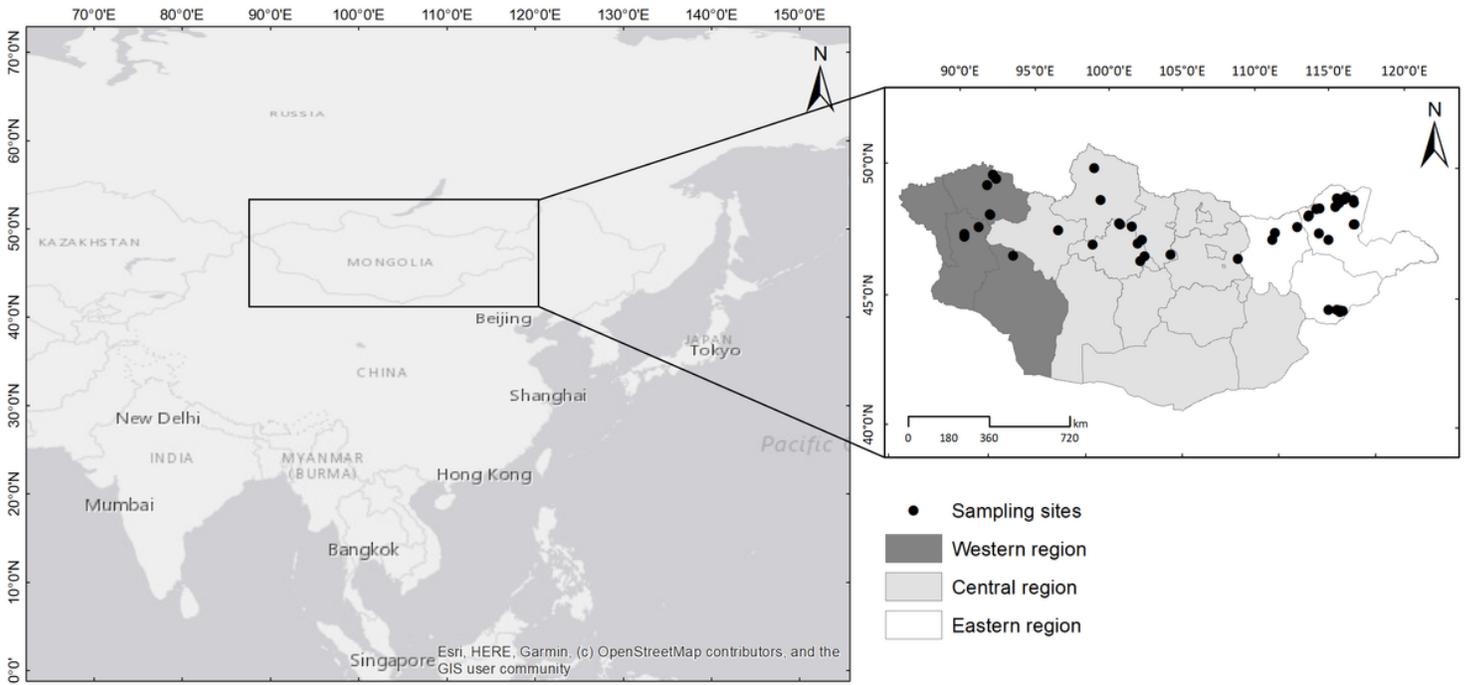


Figure 1

Locations of sampling site of avian influenza surveillance in Mongolia, 2009-2018.

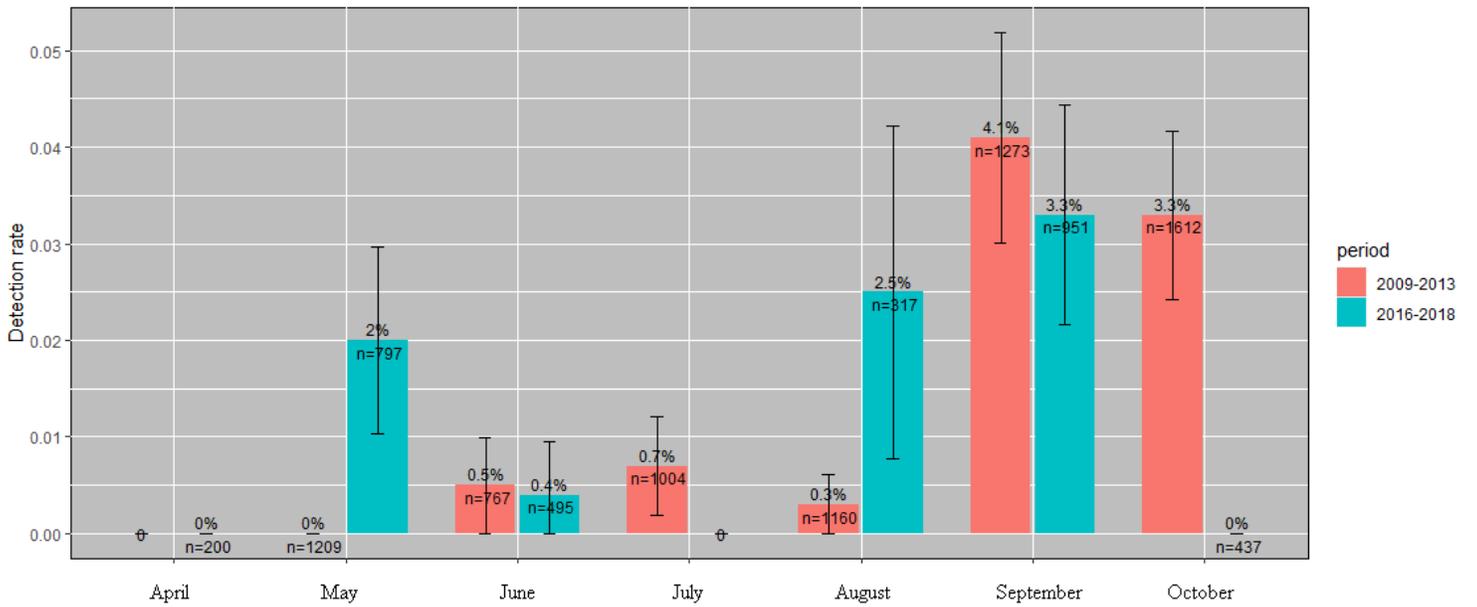


Figure 2

Detection prevalence of AIVs with standard deviations in wild birds by month in Mongolia from 2009-2018.

Supplementary Files

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- [SupplementaryTable1.pdf](#)