NARRATIVE REPORT
WILDLIFE CONSERVATION SOCIETY – GLOBAL AVIAN INFLUENZA NETWORK FOR SURVEILLANCE (GAINS)

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GAINS Activities
Quarter 9 – Months 25-27, April 1-June 30, 2008

Highlights for this quarter:

1) Two international training workshops for Asian wild bird surveillance (Page 7);

2) Publication of a study showing the existence of a separate cluster of AI viruses circulating within South America only, not linked to transference of AI strains by migratory birds of the western hemisphere (Appendix II);

3) Publication of a study that maps H5N1 "hotspots" and shows that high numbers of people, ducks, and rice farms can be stronger predictors of the emergence of AI than high numbers of domestic chickens, supporting evidence that certain domestic duck production methods are a major risk factor for HPAI outbreaks (Appendix III);

4) Completion of a study applying isotope analyses to answer questions about long distance bird migrations via Asian pathways at the broadest scale to date, to help advance our understanding of the potential spread of AI (Page 5);

5) Completion of comprehensive field expeditions in the Ukraine with almost 4000 wild bird samples collected for analysis at the Friedrich-Loeffler Institute (German Federal Animal Health laboratories) as an in-kind GAINS collaboration (Page 14);

6) Deployment of 37 satellite transmitters on migratory waterfowl in southern Africa to support the launch of the first locally managed, large-scale technological approach to studying bird migrations in the region (Page 17);

7) Linking of the GAINS database and mapping system to Google Scholar, Avibase, Wikipedia, and other similar programs to give users easy access to background information (Page 25);

8) More than 5000 wild birds sampled globally this quarter, in natural habitats and markets, for AI surveillance.

Executive Summary

The aim of the Wild Bird Global Avian Influenza Network for Surveillance (GAINS) project is to expand operational field capabilities, improve the understanding of viral strains and transmission of influenza viruses in wild birds, and to disseminate information to all levels of governments, international organizations, the private sector and the general public. GAINS is an initiative of the Wildlife Conservation Society (WCS) with collaborating partner institutions around the world. Please refer to Appendix I for a table summarizing recent activities and GAINS project partners listed by country. Through GAINS, WCS and its partners have collected tens of thousands of samples for Highly Pathogenic Avian Influenza (HPAI) H5N1 analysis, and are making available census data from more than 105 million bird observations via an open access database and mapping system (http://www.GAINS.org). GAINS project staff and partners have also trained thousands of individuals worldwide in wild bird handling, sampling, and data collection for the purpose of understanding and controlling the spread of HPAI H5N1.

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Appendix I. Summary of GAINS partners and countries

Appendix II. Advance copy of an article in press (not for distribution to the public). Pereda, A.J., M. Uhart et al. 2008. “Avian influenza virus isolated in wild waterfowl in Argentina: Evidence of a potentially unique phylogenetic lineage in South America.” *Virology* (doi:10.1016/j.virol.2008.06.010).

Appendix III. Gilbert, Marius, X. Xiao et al. 2008. “Mapping H5N1 highly pathogenic avian influenza risk in Southeast Asia.” *Proceedings of the National Academy of Sciences* 105(12) pp. 4769–4774.

INTRODUCTION

Since 1989, when WCS Field Veterinarians pioneered the field of conservation health, WCS has used a collaborative approach to address the complexities of maintaining ecosystem health. The United States Agency for International Development (USAID) and the Centers for Disease Control and Prevention (CDC), among others described below, provided funding to WCS to administer the wild bird Global Avian Influenza Network for Surveillance (GAINS) project that was launched in 2006. GAINS is a smart and targeted investment in the US government's fight against highly pathogenic H5N1 avian influenza, since, for example, wild birds can serve as sentinels for early detection of the virus' presence.

Under the GAINS project, WCS unites a network of US-based and international partner institutions, including governments, NGO's and universities. These partners work together to improve our understanding of the dynamics of avian influenza, to evaluate disease risks, and to use timely information to help protect people, wildlife, and domestic animals from the threats posed by HPAI H5N1. Across the globe, field surveillance for avian influenza in wild birds is underway.

A primary purpose of GAINS is to share international disease information through an interactive, publicly accessible web-based database, which is available online at www.GAINS.org. GAINS surveillance includes systematic monitoring of wild birds along major global flyways, with information on over 105,000,000 bird observations already entered into the mapping and database systems.

ASIA AND THE NEAR EAST

WCS institutional collaborations for GAINS in Asia include the private sector (Cargill Inc., the largest distributor of meat and poultry products in the US) and US-based and international agencies such as the United Nations Food and Agriculture Organization (FAO), and the National Institutes of Health (NIH) via the University of California Los Angeles Center for Rapid Influenza Surveillance and Research (CRISAR) and the University of Minnesota Center of Excellence for Influenza Research and Surveillance (MCEIRS) programs. These funding partnerships have made possible WCS contracts and collaborations for GAINS with top research institutions and laboratories in the US and abroad, including the University of California Davis, the Center for Tropical Disease in Los Angeles, the University of Minnesota, and a host of foreign laboratories that contribute in-kind services and materials. Over $1.5 million has been leveraged through the five largest funding partnerships for GAINS in Asia, for work in Indonesia, Laos PDR, Cambodia and Mongolia.

All data collected through these partnerships are incorporated into the growing GAINS database (please see the Cross-Cutting Activities section of this report for more details). Specifics of our many regional and local partnerships with researchers, universities and NGOs are also mentioned in individual country sections.

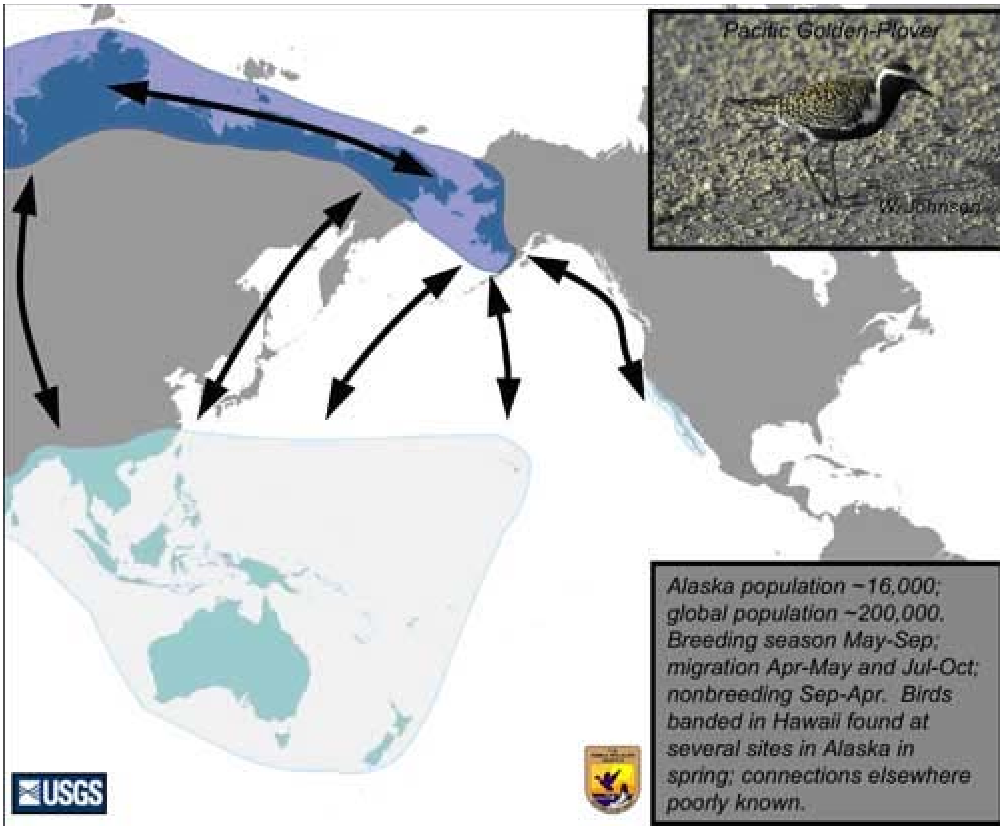
Mongolia

Stable isotopes reveal migratory bird movements.

Information on migratory bird flyways is critically needed to advance our understanding of the potential role of wild birds in the cross-continental spread of HPAI H5N1 and other diseases. Ongoing GAINS activities around the world, such as bird marking and satellite telemetry, shed light on these questions. But WCS and its partners have also been seeking to advance the science we use to study bird movements. This quarter marked the completion of a new GAINS-funded study analyzing stable-hydrogen isotopes in feather and claw tissues to reveal the migratory movements of wild birds captured in northern Mongolia.

Most elements on earth have equal numbers of protons and neutrons, but a few elements, known as isotopes, have unequal numbers ("stable" isotopes are non-radioactive). Scientists are beginning to take advantage of natural abundances of stable isotopes in the environment to help reconstruct ecological processes like migrations. Stable isotopes in the food and water that birds ingest become incorporated into stable organic compounds in feather and claw tissues, permanently capturing the isotopic record of the place (the particular habitat, latitude or continental region) where the tissue was formed. The abundance of hydrogen stable isotopes in a bird's feather thus serves as a biogeochemical "signature" of where a bird spent a season, regardless of where it was captured.

In this study -- the first of its scope for Asia -- the researchers identified approximate locations where 27 bird species had molted or grown new feathers. The species were sampled at seven sites in northern Mongolia in 2007, and included Pacific golden plovers (*Pluvialis fulva*), Mongolian gulls *(Larus vegae mongolicus*), swan geese (*Anser cygnoides*), curlew sandpipers (*Caladris ferruginea*), bar-headed geese (*Anser indicus*) and whooper swans (*Cygnus cygnus*) among others. Most of the study results were consistent with what is known about the biology of the species and their northern and southern migrations; this demonstrates the effectiveness of hydrogen-stable isotope analysis for obtaining population- and species-level data on approximate distances traveled by wild Asian birds. The full 61-page report may be downloaded at www.GAINS.org.



Alaska population ~16,000; global population ~200,000. Breeding season May-Sep; migration Apr-May and Jul-Oct; nonbreeding Sep-Apr. Birds banded in Hawaii found at several sites in Alaska in spring; connections elsewhere poorly known.

Breeding and wintering distribution of the Pacific golden plover, with possible migration pathways in Asia and North America marked with arrows (Map courtesy of USGS). The GAINS study using stable isotopes in feathers and claws concurs with previously published data based on banding studies.

Surveillance

During this quarter, the field team in Mongolia continued to conduct live bird surveys and shoreline mortality transects of birds during the spring migration at Erhel Nuur and Khunt Nuur, where all previous HPAI H5N1 outbreaks in Mongolia have occurred. Birds captured included a pale martin (*Riparia diluta*), possibly the first record of the species in Mongolia.

The team is preparing for the summer avian influenza surveillance expedition, which will include nearly three times the number of field personnel as compared to last year. Six international bird experts will participate, along with eight field assistants (students and post graduates in biology and veterinary medicine), two translators, and six logistical assistants. Preparations required the importation of equipment from the USA weighing more than one metric ton. WCS is grateful to the USAID mission in Ulaanbaatar for help obtaining duty exemption for the shipment.

The WCS team is also building on the successful creation last year of a framework for a national-level color banding scheme for migratory birds in Mongolia. This year's banding program will expand to include more neck collars and more species, such as whooper swan (*Cygnus cygnus*), bean goose (*Anser fabalis*), bar-headed goose (*Anser indicus*), graylag goose (*Anser anser*), and the endangered swan goose (*Anser cygnoides*). The team procured 22,000 numbered metal bird rings for migratory and demographic studies for the coming season. On the tags, bird watchers are asked to alert the Institute of Biology at the Mongolian Academy of Sciences, which was recently nominated as the coordinating body for the bird marking project.

Ms. Losloo Jambal, WCS Mongolia, captures a bar-headed goose for the color-marking project studying migratory birds and the potential role they may play in the spread of AI. (Photo: M. Gilbert)

Training and Outreach

Two highly successful training events were held in Mongolia this quarter. First, Drs. Martin Gilbert and Enktuvshin Shilegdamba of WCS and Dr. Phillip Round, an ornithologist with the NGO Wetlands Trust and Mahidol University, Bangkok, Thailand, co-hosted “The First Mongolian Bird Marking Workshop” at Ulaanbaatar’s National University.

Nearly 60 local and international students, researchers and professionals from the health and biology sectors attended. Topics included an introduction to why birds are marked, safety and welfare considerations, a review of current legislation, and data collection and standardized marking and capturing techniques. Attendees had an opportunity to apply many of the themes presented in the workshop classrooms when they inaugurated the first official season of the newly created Mongolian bird marking project.

Attendees of the "First Bird Marking Workshop" held in Mongolia, organized by Dr. Martin Gilbert (center, bottom row) and the WCS team in Mongolia. (Photo: M. Gilbert)

During a practical at the WCS training workshop in Mongolia in June 2008, attendees learn techniques for systematic bird observations. (Photo: M. Gilbert)

The WCS team in Mongolia also led a two-day “Introductory Training to Bird Identification and Avian Influenza Surveillance.” The 38 trainees included WCS AI surveillance team members, and participants from the Veterinary Departments in Hovsgol and Bulgan provinces, the National Environment Office, the Mongolian Academy of Sciences, the Veterinary Research Institute of Mongolia and the University of Iowa. The field practical included bird identification, mortality surveillance and GPS navigation. The first waterbird sighted by the team during the fieldwork practical was a bean goose (*Anser fabalis*) that had been marked one year earlier by the WCS team (July 2007).

Dr. Amanda Fine continues to brief the national USAID mission on avian influenza and results of surveillance at monthly Chief of Party and USAID contractors meetings, chaired in Ulaanbaatar and attended by the US Ambassador and the USAID Chief of Party, Barry Primm.

> *“It was impressive to see people coming together from all over Asia and to watch them learning as a team during the workshop. Now we are really getting a critical mass of participants in GAINS… and it seemed like a great omen when the first bird the team spotted during the field training session happened to be a bean goose we had color-marked for GAINS last season!”*
>
> *-- Dr. Martin Gilbert, WCS Field Veterinarian*

Cambodia

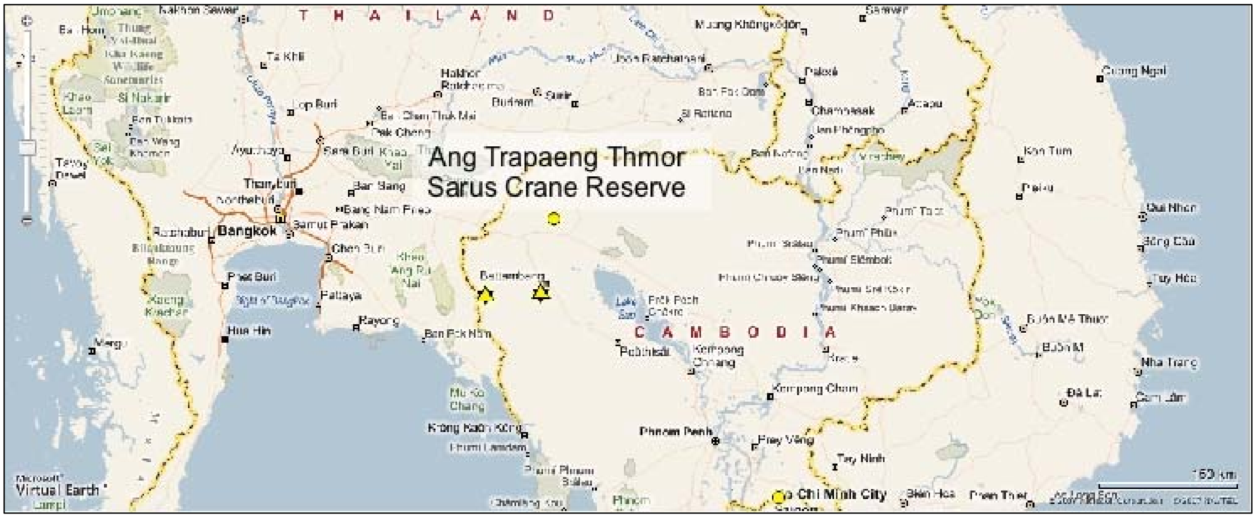
Surveillance

A total of 4,977 samples collected during Quarter 8 from nearly 2,300 birds of 94 species (including waterbirds, and perching birds of the Passeriforme order) were tested for influenza A virus using RT-PCR methods at the laboratory at University of California, Davis.

Of the 2,300 birds, 346 were found positive for influenza A virus. While the viruses are likely to be of low pathogenic subtypes, which do not currently present a public health threat, the test results are of critical importance to our understanding of the epidemiology of AI in wild birds. Asia is a “hotspot” for human and poultry cases of avian influenza globally, but with few exceptions, investigations of avian influenza viruses among wild birds have been limited to countries with temperate climates, primarily in the western hemisphere. GAINS surveillance helps create avian influenza baselines for wild birds in tropical climate countries like Cambodia and thus provides a key piece of the AI puzzle globally.

In addition, one sample collected from an apparently healthy greater painted snipe (*Rostratula benghalensis*) tested positive for influenza subtype H5. The bird was captured live at

Ang Trapaeng Thmor Sarus Crane Reserve in Banteay Meanchey province last quarter. The WCS team immediately informed the government of Cambodia about the test result and advised that further testing will be conducted to determine the pathogenicity of the viral strain. The team emphasized to the Minister of Agriculture, Forestry & Fisheries of Cambodia that an H5 positive is not unusual in wild birds, that the result most likely represents a low pathogenic strain of AI, and that pending further diagnostics on the sample, no action would be required at present. Viral isolation and further subtyping are being conducted at the World Organization for Animal Health (OIE) Reference Laboratory for Newcastle Disease and Avian Influenza in Padova, Italy.



WISDOM Map Explorer 3.0 (www.GAINS.org) depicts the location of the reserve where a bird tested positive for influenza subtype H5. Determination of the subtype and pathogenicity of the virus is pending.

Greater painted snipe that tested positive for the H5 virus in northwestern Cambodia. (Photo: R. Thompson)

WCS staff and AI surveillance partners from University of California, Los Angeles (UCLA) and National Institutes of Health (NIH) are promoting the organization of national-level systems for effective response to the detection of AI in wild birds. They helped implement such a system in Cambodia, which enabled the coordinated handling of the H5 positive result mentioned above. A formalized communication protocol ensured rapid and nearly simultaneous dissemination of information from WCS staff who received the laboratory result to the appropriate point persons in the Cambodian government and to researchers at UCLA and NIH.

This quarter, 1,180 new samples from nearly 700 free-ranging and captive wild birds were collected for testing under ongoing collaborative partnerships with NIH, FAO and the National Veterinary Research Institute in Phnom Penh. The team continued conducting routine market surveillance throughout the quarter, sampling in provinces such as Battambang, Kandal, Kampong Thom, Kep and Phenom Penh.

Collaborations

WCS Cambodia staff contacted the USAID Country Coordinator for Avian Influenza Behavior Change Communication about incorporating USAID Academy for Educational Development training materials into WCS outreach activities in local communities and markets where WCS surveys birds for avian influenza. The coordinator provided educational materials, written in the Khmer language for a range of ages, about how to prevent the spread of avian influenza in Cambodia.

In May, Dr. Robert Thomson and Ms. Angela Yang (WCS Cambodia) attended the USAID Avian and Human Influenza (AHI) Partner meeting, organized by Kate Crawford, USAID Cambodia. WCS staff updated the USAID Avian Influenza Task Force on GAINS activities in Cambodia, and discussed how AI surveillance "platforms" or models that have been developed through GAINS might be applied to other disease monitoring efforts beyond avian influenza and Highly Pathogenic H5N1.

Indonesia

Surveillance

A total of 330 samples were collected from Cemara Beach in Jambi, Sumatra and Surabaya, Java, from species that included common redshanks (*Tringa totanus*), Mongolian plovers (*Charadrius mongolus*) and terek sandpipers (*Xenus cinereus*). All samples are being tested by real-time PCR at the Disease Investigation Center (DIC) in Wates, Jakarta, for presence of the matrix gene for avian influenza virus. Results are pending.

The matrix gene is shared by all influenza A viruses, including low pathogenic viruses that cause infection with minor illness, and highly pathogenic viruses that cause severe disease and death – such as the H5N1 subtype. A positive sample in the screening for the matrix gene thus alerts us to the presence of one of the influenza A viral subtypes, but further testing is required to determine the subtype, and therefore whether it is high or low pathogenicity.

WCS Indonesia team member Mr. I Londo Febrianto observed nearly 1,400 birds of over 25 species in the Serangan district in Bali in June; the species diversity evident at this site make it likely that the team will return for bird surveillance missions later in the year.

Marked bird re-sightings reported via the GAINS project continue to provide new knowledge about poorly understood flyways of migratory species. The WCS Indonesia team noted two interesting re-sightings of birds they had marked under the framework of the Australasian Wader Studies Group (AWSG), which monitors shorebirds in the East Asian Australasian Flyway:

- A greater sand plover (*Charadrius leschenaultia*) originally flagged in Sumatra, Indonesia, was sighted in Phetchaburi Province, Thailand, in April (a distance of approximately 1681 km).
- An Asian dowitcher (*Limnodromus semipalmatus*) originally flagged in Sumatra was sighted in Mai Po, Hong Kong (approximately 3105 km away).

Even basic information about birds passing through a certain site on their way from point A to point B cannot be taken for granted. That is because different populations (or even age classes within a population) of a single species may follow different routes to arrive at the same place. For instance, in 2006 the WCS team re-sighted adult red-necked stints (*Calidris ruficollis*) that were flagged in southeast Australia, and pass through eastern Mongolia on their way across Asia. However, juveniles of the same species appear to follow the coastline instead, and red-necked stints from outside southeast Australia take a different route.

Training and Outreach

GAINS field staff continue to actively engage local communities as the teams conduct AI surveillance work. This quarter, in the Sumatran village of Cemara, approximately 50 children and 15 adults participated in educational activities regarding bird identification and ecology.

WCS Indonesia staff member Uut Yuwana (left) teaches local children about birds at an AI surveillance site in Jambi, Sumatra (Photo: J. Philippa)

Vietnam

Surveillance

The veterinary reference laboratory in Ho Chi Minh City reported results from 666 AI surveillance samples from 333 birds collected by GAINS staff during Quarter 8. All samples from-free ranging birds tested negative to the matrix gene for avian influenza virus by RT-PCR.

Of the 442 samples from 221 market birds collected last quarter in Dong Thap province, one cinnamon bittern (*Ixobrychus cinnamomeus*) tested positive for the presence of avian influenza, but not to the H5 subtype. Further testing to subtype the virus is underway.

Conclusive results are still pending on the 889 samples from domestic ducks and wild birds collected by Dr. Thomas Dietsch of UCLA's Center for Tropical Research last quarter in Nam Dinh province. To facilitate sub-typing of samples found positive for type A influenza, the Center for Tropical Research is employing new protocols that maximize the analytical effectiveness of its High Resolution Melting (HRM) Quantitative PCR (QPCR) technology. (Please see the Quarter 8 report for further details on this technology.)

Collaborations

The WCS team in Vietnam has been building relationships with individual veterinarians, biologists and researchers to facilitate collaborative AI surveillance work in the country, and also seeking partnerships with other institutions working on AI and infectious disease monitoring. For example:

- Google.org staff, while exploring opportunities in Southeast Asia for targeted investment in preventing the emergence of dangerous pathogens, met with WCS to learn about GAINS activities in the region and to brainstorm about future collaborations.
- FAO and consultants with the World Bank in Hanoi have asked WCS staff to serve as consultants in a new initiative to conduct wild bird AI surveillance work and to implement an AI outbreak response system for farms.
- The Oxford Wellcome Trust at the National Institute of Infectious and Tropical Diseases of Bach Mai Hospital in Hanoi is interested in collaborating to conduct additional H5N1 surveillance under the GAINS project.

Training and Outreach

In June, WCS field veterinarian Dr. Joost Philippa participated in the "Avian Influenza Research to Policy" international workshop in Hanoi. Organized jointly by FAO, USAID and the United States Department of Agriculture (USDA), the workshop addressed the latest research findings on Highly Pathogenic Avian Influenza and key research gaps that governments should be aware of in forming policy relevant to AI.

Dr. Dietsch (UCLA Center for Tropical Research) was invited by the Vietnam Department of Animal Health (VDAH) to participate in a two-day training workshop organized by USDA in early April.

Philippines

Through collaboration with the University of Kansas (UK) Biodiversity Research Center, AI surveillance for the GAINS project will include laboratory testing of samples that have been collected by UK researchers under other funding sources for field projects in Asia, Africa and South America. Test results are now pending at the USDA Southeast Poultry Research Laboratory on over 300 samples for AI surveillance that were collected from Mt. Labo on Luzon Island and Zamboanga Peninsula on Mindanao Island in the Philippines.

Afghanistan

The WCS Afghanistan team, led by Dr. Stephane Ostrowski, continued to monitor for the presence of avian influenza in local bird markets in Kol-e Hashamat Khan, Kabul. Monitoring includes ornithological surveys as well as social surveys about the nature of interactions between domestic poultry and wild birds. Results are pending. During the coming quarter, the WCS Afghanistan team will conduct surveillance in Dasht e-Nawar, Ghazni Province, on molting waterbirds.

Ukraine

All 6 scheduled field expeditions for AI surveillance in Ukraine have been successfully completed. A total of 3,989 samples from 34 species of waterbirds were collected and are being analyzed at Friedrich-Loeffler Institute for Animal Health in Germany. The site chosen for surveillance, the Sivash Lake region of the Black Sea, has a history of HPAI, beginning with an H5N1 outbreak in the winter of 2005-2006. A previous analysis of temporal patterns of disease events during the outbreak, which was conducted by GAINS partner National Ecological Center of Ukraine, suggested involvement of waterfowl in the onset of some HPAI cases. The current study being supported by GAINS explores in further depth those potential connections between waterbirds and HPAI outbreaks in the region. Laboratory analysis of samples is pending, but details of the field work appear below.

The field site is one of the most important congregation sites for waterbirds in southern Europe. In particular, eastern Sivash is an important staging area for arctic and boreal species, many of which use the Mediterranean Flyway in both spring and autumn. The first field expeditions focused on migratory waders such as dunlin (*Calidris alpina*), curlew sandpiper (*Calidris ferruginea*), broad-billed sandpiper (*Limicola falcinellus*), little stint (*Calidris minuta*), and grey plover (*Pluvialis squatarola*). The birds were captured using mist nets on calm, moonless nights, with surveillance team members checking the nets throughout the night.

Next, the team sought to sample colonially breeding water birds and had good results sampling chicks on or near nests. Species included great cormorant (*Phalacrocorax carbo*), yellow-legged gull (*Larus michahellis*) and Mediterranean gull (*Larus melanocephalus*).

GAINS team members setting up mist nets in Ukraine to capture waders. (Photo: S. Khomenko)

Sampling great cormorant chicks at the Chongar Islands colony. (Photo: S. Khomenko)

In January of 2008, an outbreak of HPAI H5N1 occurred at a small poultry farm in Crimea, immediately followed by more reported cases in Ukraine and Turkey. This raised concerns among officials about the possibility of disease introduction in the Southern regions of Ukraine. Because permissions to capture ducks in the Askania-Nova Biosphere Reserve were not given under those circumstances, the surveillance team redoubled efforts in collecting fecal samples from wintering waterfowl, as fecal samples do not require capturing and handling birds. They collected 809 fecal samples from 7 species, swabbing fresh droppings immediately after a concentration of birds was observed at a site. Birds sampled in this manner included 175 mallard ducks (*Anas platyrhynchos*), 60 ruddy shelducks (*Tadorna ferruginea*), 32 mute swans (*Cygnus olor*), 30 common shelducks (*Tadorna tadorna*) and 26 whooper swans (*Cygnus cygnus*). The samples will provide material to assess the role of these common wintering species in the epidemiology of AI in the area.

AFRICA

Southern Africa (Botswana, Mozambique, Zimbabwe, South Africa)

Surveillance

GAINS subawardee Percy FitzPatrick Institute of African Ornithology has now sampled more than 3,000 birds in surveillance expeditions in Botswana, Mozambique, Zimbabwe and South Africa. Preliminary results from a series of 300 samples tested at Onderstepoort Veterinary Institute (OVI) yielded 23 “suspect positive” samples for avian influenza, and 7 of these tested “suspect positive” for the H5 subtype using the RT-PCR assay. No H7 positives were detected. Final laboratory results from these and nearly 2,200 additional viral samples are still pending.

The team witnessed a rare occurrence during a surveillance mission in June at Lake Ngami in Botswana, when raptors attacked birds suspended in the team’s mist nets. The team hypothesizes that the seasonal dry-down of the lake has resulted in a concentration of predators and they anticipate that count data collected will provide insights into this largely unstudied phenomenon. Birds the team sampled that are rare for the region include a fulvous whistling duck (*Dendrocygna bicolor*) and a marabou stork (*Leptoptilos crumeniferus)*.

In Mozambique, the team captured 60 birds at Lake Chulai, despite high levels of human disturbance. In Zimbabwe, field work scheduled for late June was postponed due to political unrest in the country, as foreign NGOs were grounded by the Mugabe administration during elections.

GAINS team member Mr. Ngoni Chiweshi inspects red-billed teal captured in the team’s walk-in trap on the shore of Lake Manyame, Zimbabwe. (Photo: G. Cumming)

Dr. Graeme Cumming releases a satellite GPS tagged red-billed teal in Strandfontein, Cape Town, South Africa. (Photo: G. Cumming)

Migration studies

GAINS has deployed 37 satellite GPS transmitter units (out of the 44 targeted) as part of ongoing bird migration studies to elucidate potential regional and global movement of AI. This quarter, 8 red-billed teal (*Anas erythrorhyncha*) and 4 Egyptian geese (*Alopochen aegyptiacus*) in Zimbabwe were fitted with transmitters. At the Barberspan Bird Sanctuary in South Africa, 7 Egyptian geese (*Alopochen aegyptiacus*) were fitted with transmitters.

The first long-distance movement was recorded by the deployed transmitters in early June, when a red-billed teal (*Anas erythrorhyncha)* traveled ~560 km from Lake Manyame in Zimbabwe to the Kafue Flats in Zambia, where it has remained.

This information is significant because a past surveillance mission led by the French NGO CIRAD (a GAINS collaborator) detected a high incidence of low-pathogenic avian influenza at the Flats. The WCS team will continue to monitor such connections between Kafue Flats in Zambia and migratory birds of the study site in Zimbabwe.

Training and Outreach

The GAINS team continues to train a diverse group of people in sample collection protocols for AI field surveillance, including members of the Department of Veterinary Services in Harare, interns from the South African Department of Environmental Affairs and Tourism (DEAT), and the Pretoria Zoo. Dr. Graeme Cumming presented his AI mapping work at an international scientific meeting in Stockholm on the topic of "Resilience, adaptation and transformation in turbulent times."

Nigeria

Nearly 1,100 birds including 667 wild and 419 domestic were sampled at the Dagona Waterfowl Sanctuary in Shugum wetland area in northern Nigera's Hadejia-Nguru Wetlands. The surveillance team was led by Dr. Tim Dodman and with participation by AP Leventis Ornithological Research Institute (APLORI) and three Swedish bird experts. The team sampled wild species including cattle egret (*Bubulcus ibis*), spur-winged goose (*Plectropterus gambensis*) and African jacana (*Actophilornis africana*). Samples have been sent to Luxembourg for analysis as per the Letter of Agreement signed last quarter, and results are pending.

Egypt

A total of 663 wild birds of 38 species were sampled at sites that included Lake Manzala, Ismailia, Sharqeya, Qualybia, Giza and Fayoum. The team was led by Dr. Luay of the Ministry of State for Environmental Affairs and Dutch ornithologist Dr. Andre Duiven, who sampled wild cattle egrets (*Bubulcus ibis*), turtle doves (*Streptopelia turtur*), house sparrows (*Passer domesticus*), and Mediterranean gulls (*Larus melanocephalus*). Laboratory results are pending.

LATIN AMERICA AND THE CARIBBEAN

Based in Argentina, Dr. Marcela Uhart oversees 23 Principal Investigators and WCS staff in their AI surveillance, collaboration and training activities in Latin America. Dr. Uhart and her teams facilitate more than 20 collaborative relationships with governments and other research partners to implement field sampling, AI early warning networks, and related training in Latin America.

Argentina

Surveillance

Dr. Uhart co-authored a paper in the upcoming issue of the journal *Virology* on avian influenza viruses found in wild birds in Argentina. (A pre-publication PDF version of the paper, not for distribution to the general public, is attached to this report as Appendix II). The study, supported by GAINS, is the first to begin to demonstrate the existence of a separate geographic circulation of avian influenza viruses in the western southern hemisphere, which is not linked to that of North America. To date, South American strains of AI have been poorly studied; the GAINS project is helping to fill those important gaps in our knowledge.

Two rosy-billed pochards (*Netta peposaca*), out of 226 birds sampled by WCS staff in northern Santa Fe province in Quarter 8, tested positive for an avian influenza virus using reverse transcription polymerase chain reaction (RT-PCR) analysis. The reference laboratory at the National Institute of Agriculture (INTA) has confirmed that the birds testing positive were not carrying H5 or H7 viruses (both of which are fairly common in wild birds in their low pathogenic forms). Further testing is underway to determine the viral subtype and whether they are of low or high pathogenicity.

In Quarter 9 the WCS team sampled 99 hunter-killed wild ducks in wetlands in Santa Fe province, Argentina. Samples are being tested for avian influenza using RT-PCR at the INTA reference laboratory. Species sampled included rosy-billed pochard (*Netta peposaca*), fulvous whistling duck (*Dendrocygna bicolor*), white-faced whistling duck (*Dendrocygna viduata*), Brazillian duck (*Amazonetta brasiliensis*), silver teal (*Anas versicolor*), and ringed teal (*Callonetta leucophrys*). Results are pending.

The last surveillance mission for the quarter scheduled for mid June was postponed due to civil unrest in Argentina, which resulted in numerous road blocks in rural areas as well as fuel shortages. The team will carry on the planned surveillance during next quarter.

Training and Outreach

WCS field veterinarians Drs. Hebe Ferreyra and Marcelo Romano continue to train veterinary students and young veterinarians in avian influenza surveillance methods as part of their ongoing field work. In Quarter 9 trainees included students from Esperanza University and veterinarians from Tandil University and Salvador University, Buenos Aires.

Dr. Hebe Ferreyra (right) instructs students during AI surveillance activities in Santa Fe wetlands, Argentina. (Photo: V. Rago)

In May, WCS staff member Dr. Pablo Beldomenico traveled to the National Wildlife Research Center of the United States Department of Agriculture (USDA) in Fort Collins, Colorado, to present an overview of the GAINS initiative and specific AI surveillance and training activities in South America.

At the conference *Intergovernmental and Interagency Collaboration for Pandemic and Avian Influenza Preparedness in the Americas*, held at the Naval Medical Research Center Detachment in Lima, Peru, Dr. Marcela Uhart was invited to present on “Wildlife Health: Surveillance at the Animal/Human Interface,” with emphasis on avian influenza surveillance and the GAINS project.

> *“Training is a key part of everything we do.”*
>
> -- Dr. Marcela Uhart, WCS Field Veterinarian

Bolivia

Drs. Glenda Ayala and Carla Barriga participated in an annual Andean flamingo (*Phoenicopterus andinus*) bird banding event organized by the Bolivian NGO BIOTA in Laguna Colorada. They capitalized on the opportunity to conduct their second round of sampling flamingos for avian influenza and other diseases (please see the quarterly report from April-June 2007 for details of the first sampling trip). A total of 194 chicks were sampled at the site in Quarter 9. Results from the Bolivian laboratory analyzing the samples (LIDIVECO) are pending.

Brazil

The WCS Brazil team is now leveraging funds at the national level to augment avian influenza preparedness and surveillance activities in the country. The "One World, One Health" WCS Brazil Fund (supported by Cargill, Inc.) approved five proposals for funding, and two of them were for avian influenza related activities:

- Monitoring of avian influenza and Newcastle disease in the Brazilian Amazon region ($30,000 USD)
- Monitoring of migratory birds and their relation with sylvan birds and aviculture focusing on the prevention of avian influenza ($25,890 USD).

Field activities are expected to begin in fall 2008.

Colombia

Collaborations

With nearly 10 formal partnerships signed or being finalized with local NGOs and regional government agencies, the WCS team in Colombia serves as the major catalyst for building avian influenza surveillance capacity in Colombia. In addition to the multi-year financial commitments being made on the part of participating government and NGO organizations, personnel for field and laboratory assistance will be provided to facilitate GAINS activities in Colombia once the agreements are finalized. (For more details on the partner institutions and specific proposed activities please see our Quarter 8 report). During Quarter 9, the team strengthened its collaborative ties with groups with waterbird survey experience, extensive local veterinary expertise, and/or ecological field work expertise in the Cauca River Valley, where the AI surveillance efforts will focus beginning in Quarter 10.

Training and Outreach

The WCS Colombia team was invited to provide training in AI monitoring techniques to staff from the Regional Autonomous Corporation of the province of Nariño (the state park service for that region). The invitation followed a seminar organized by the Universidad de Nariño with a local NGO in southwest Colombia, where the WCS team presented the GAINS initiative for Colombia.

Peru

GAINS collaborators in Peru, including the Universidad Peruana Cayetano Heredia and Texas A&M University, continue to study infectious diseases present in the Peruvian wild bird trade. In Quarter 9 twelve sets of samples were tested corresponding to 91 wild birds sampled in markets in Lima, Pucallpa and Iquitos in 2007. All samples tested negative for avian influenza.

From April to June 2008, markets in Lima, Iquitos, and Pucallpa were opportunistically monitored. In May, following long permit delays, the team was able to sample 13 market-confiscated birds from a single confiscation event in Lima. Federal monitoring of illegal trade has been augmented somewhat in recent months; the researchers rely on their strong contacts with government officials to turn these confiscations into opportunities for avian influenza sampling. Increased federal monitoring also has the effect of decreasing the numbers of birds visible in public markets. However, the overall trade continues unabated, as becomes obvious when federal officials are absent for even a brief holiday. To date the team has sampled 212 of the proposed 300 birds.

Caribbean

St. Martin, Anguilla, Antigua, St. Kitts, and Trinidad

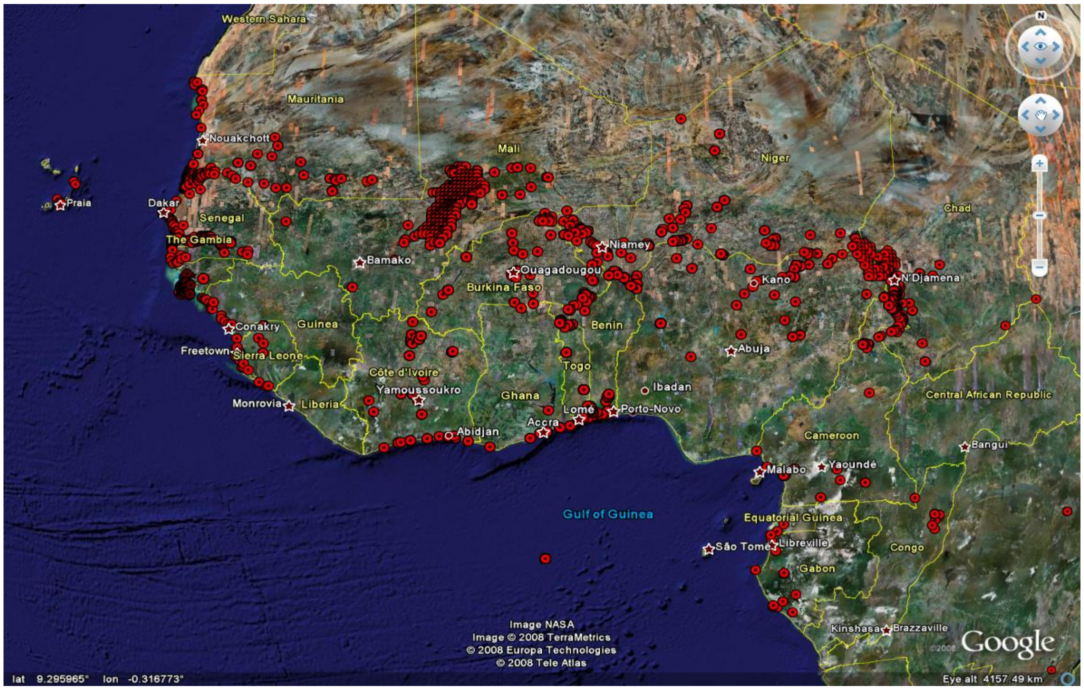
The samples from 588 waterbirds representing 25 species collected in Quarter 8 are being analyzed by the United States Department of Agriculture (USDA) Southeast Poultry Research Laboratory in Athens, Georgia. Results are pending. (Please refer to our Quarter 8 report for details on specific sampling activities and local collaborations in St. Martin, Anguilla, Antigua, St. Kitts and Trinidad.)

GAINS CROSS-CUTTING ACTIVITIES

African Waterbird Census Database

WCS partner Wetlands International continues working on the web-based International Waterbird Census (IWC) database application for GAINS. The waterbird census is a global scale program for counting water birds at wintering sites, but until now the data has not been made available in a standardized, searchable electronic form. When complete, the African waterbird census database will be linked to the global GAINS database of bird observations, which now contains more than 105,000,000 individual records.

A total of 6,390 African waterbird observations have been added to the database for the period 2005-2007. The prior period (1999-2004) includes more than 200,000 bird observations, but still contains many inconsistencies which must be "cleaned" and all the count sites must be georeferenced. The team is currently conducting this work for nearly 2000 bird count sites in 20 African countries including Mauritania, Senegal, Niger, Mali, Burkina Faso, Nigeria, Cape Verde, Guinea Bissau, Guinea, The Gambia, Sierra Leone, Ivory Cost, Ghana, Togo, Benin, Cameroon, Chad, Equatorial Guinea, Gabon and Congo. Nearly 95.8% of the bird observation sites are now linked to proper georeferenced coordinates.



Georeferenced observation data for African waterbirds.

Visualizing and mapping risk of avian influenza outbreaks

WCS partners at University of New Hampshire (UNH) Institute for the Study of Earth, Oceans and Space are helping GAINS to meet its objective to predict "hot spots" and timing associated with outbreaks and movements of highly pathogenic H5N1. They continue to enrich the functionality and performance of the websites and data sets they are developing to enable GAINS to model H5N1 globally. In Quarter 9 the team refined the global, georeferenced field photo library and the Moderate Resolution Imaging Spectroradiometer (MODIS) data browser.

The latter can now display high resolution satellite images (e.g., Landsat images from Google maps), as well as lower resolution satellite products (e.g. vegetation indices), and both can be linked to enable the user to use fine resolution images to interpret information in lower resolution images. This is an important advance because it can substantially increase the quantity of information suitable for risk analysis studies. MODIS images have been shown to be useful in studying the ecology of and risk factors associated with HPAI.

Training and Outreach

A fundamental barrier to advancing our understanding and analysis of HPAI spatial and temporal dynamics in Asia is that numerous data sets relevant to transmission and spread of H5N1 at different spatial scales have been collected over the years, but they are scattered among various locations and reside with individual investigators. This quarter, the National Institutes of Health (NIH) awarded the UNH group $150,000 to organize two international workshops on community-based synthesis, analysis and modeling of HPAI H5N1 in Asia. The first workshop will establish a network for data sharing and synthesis in Asia, and the second will establish spatial epidemiological modeling capacity in Asia. The goal is to enable participants to map risk probability over space and time and help Asian countries target their resources for surveillance and preparedness. Dr. Xiangming Xiao of UNH leads the efforts, with a scientific organizing committee that includes Drs. William Karesh (WCS), Jan Slingenbergh and Scott Newman (FAO, Italy), John Takekawa (USGS), and Marius Gilbert (UNH and Universite´ Libre, Brussels, Belgium). The two 5-day workshops will be held in Bangkok, Thailand in late fall 2008 and summer 2009.

Dr. Xiao and his colleagues at UNH made important contributions in a study recently published in the journal *Proceedings of the National Academy of Sciences* (attached as Appendix III). The article analyzes the relationship between the H5N1 outbreaks in several Asian countries and 5 environmental variables including elevation, human population, chicken numbers, duck numbers, and rice cropping intensity. The researchers' models suggest that risk of H5N1 outbreak is more highly associated with duck abundance, human population, and rice cropping intensity than with numbers of domestic chickens.

Mapping wetlands that are potential reservoirs for avian influenza

WCS partner Wetlands International, South America Program, continues to improve the database for the Neotropical Waterbird Census (NWC) for the International Waterbird Census (IWC). A new web-based system is being introduced to overcome the data standardization and quality limitations of the existing database. This quarter, the team began analyzing entries from 1990-2006 on 25 selected indicator bird species, to identify and describe wetland sites that could be possible reservoirs for avian influenza.

GAINS Technology

WISDOM (Wildlife Information System for Disease Observation and Monitoring) Map Explorer) (WME)

WME Version 3.0 was released this quarter, providing sophisticated web-based mapping of AI in wild birds using NAVTEQ imagery. In a map session, users can:

- Display data from the WISDOM database in a spatial format that is filtered based on specific criteria
- Overlay that data with both pre-configured and external layers of data (such as poultry density) as well as with files uploaded from the user's desktop
- Annotate each map session with labels, drawing shapes, lines, etc. (e.g. draw a "buffer zone" around a location where positive samples have been found as part of disease control planning efforts)
- Save and load various map sessions
- Collaborate with colleagues by emailing map sessions
- Export data on maps into KML format to be used in desktop GIS tools such as Google Earth
- View reports to gather more detail about locations and map sessions
- Zoom immediately to specific locations identified by lat./long. or a place name stored in the Virtual Earth library
- Load map states (filter criteria, pan, and zoom) through the URL
- View migration flyways for many taxa using the "Show Flyway" function
- Cluster points within a specified geographical unit when viewing the map at specific scales, improving scalability, speed of loading, and legibility of symbols.

Screenshot of the WISDOM Map Explorer (www.GAINS.org)

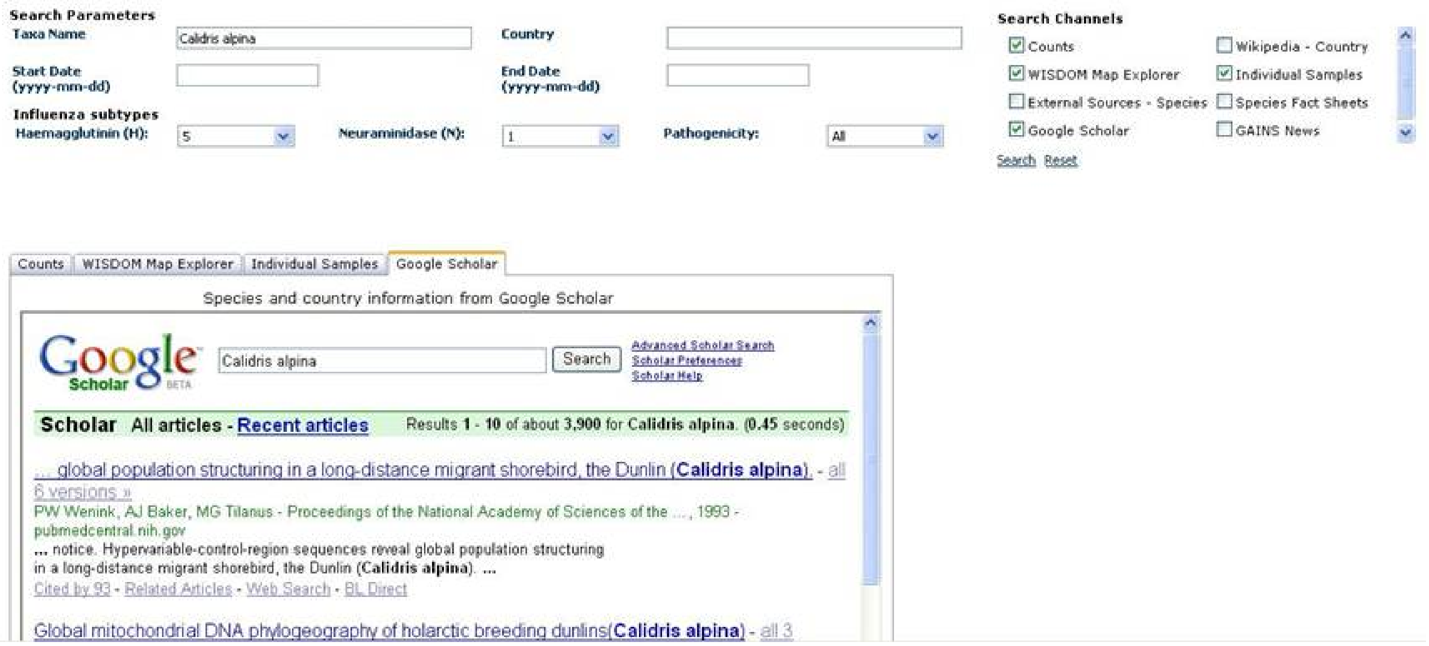
Current Criteria:
Between 5/11/1998 and 7/22/2008 Country: All, Species: All, H: 5, N: 1, Pathogenicity: all,

Site:Kota Bungan-E Kalimantan
Has Counts:No
Has Samples:Yes

Latitude: 0, Longitude: 72

WISDOM Search

WISDOM Search is a powerful tool allowing users to combine data and documents from the database with information from external sources, such as ITIS, Avibase, and Google Scholar. WISDOM Search links to the WISDOM Map Explorer, and can be used to generate map views.



Taxa Name: Caldris alpina
Country:
Start Date (yyyy-mm-dd):
End Date (yyyy-mm-dd):
Haemagglutinin (H): 5
Neuraminidase (N): 1
Pathogenicity: All

☑ Counts
☑ WISDOM Map Explorer
☐ External Sources - Species
☑ Google Scholar
☐ Wikipedia - Country
☑ Individual Samples
☐ Species Fact Sheets
☐ GAINS News

Species and country information from Google Scholar

Calidris alpina

Results **1** - **10** of about **3,900** for **Calidris alpina**. (**0.45** seconds)

... global population structuring in a long-distance migrant shorebird, the Dunlin (**Calidris alpina**). - all 6 versions »
PW Wenink, AJ Baker, MG Tilanus - Proceedings of the National Academy of Sciences of the ..., 1993 - pubmedcentral.nih.gov
... notice. Hypervariable-control-region sequences reveal global population structuring in a long-distance migrant shorebird, the Dunlin (**Calidris alpina**). ...
Cited by 93 - Related Articles - Web Search - BL Direct

Global mitochondrial DNA phylogeography of holarctic breeding dunlins(**Calidris alpina**) - all 3

Above, a WISDOM data search links to additional information on a bird species

Keyhole Markup Language (KML) Export

Data in the database (through WISDOM Map Explorer searches, or for specified criteria) can be exported into KML format. KML files can be imported into desktop GIS tools, such as Google Earth. Through the KML export, a user can access the data offline and through various tools. A library of KML export links has been created that allows users to download KML files without setting criteria through the Map Explorer.

www.GAINS.org Website Usage

The GAINS web site was visited 6,225 times by 2,822 individuals from 119 countries and territories during Quarter 9 (an increase from last quarter) and users spent an average of 9 minutes on the site during each visit.

Appendix I

Partial list of contributing WCS partners and subawardees in the GAINS project

1. United States Agency for International Development (USAID)
2. United States Department of Health and Human Services, Centers for Disease Control and Prevention (CDC)
3. United States Department of Defense
4. United States Department of Agriculture, South East Regional Poultry Laboratory (SEPRL)
5. United States Department of the Interior, United States Geological Survey (USGS)
6. Texas A&M University
7. Freidrich Loeffler Institute, Germany
8. Centre de Cooperation Internationale en Recherche Agronomique pour le Developpement (CIRAD)
9. United Nations Food and Agriculture Organization (FAO)
10. Centro de Investigación de Enfermedades Tropicales (CIDEIM), Peru
11. Centro de Investigación de Enfermedades Tropicales (CIDEIM), Colombia
12. Naval Medical Research Unit - Cairo, Egypt
13. Naval Medical Research Center Detachment, Peru
14. Woodland Park Zoo
15. Instituto Nacional de Tecnología Agropecuaria (INTA Castelar), Argentina
16. Instituto Colombiano Agropecuario (ICA), Colombia
17. Cargill, Inc.
18. National Institutes of Health (NIH)
19. Australasian Wader Study Group (AWSG)
20. Tanzania Bird Atlas
21. IDEXX via University of Georgia and SERPL
22. Environmental Protection in the Caribbean (EPIC)
23. National Aviary, United States
24. National Ecological Center of Ukraine
25. University of Cape Town through Percy FitzPatrick Institute of African Ornithology
26. The Regents of the University of California on behalf of The Center for Tropical Research at UCLA
27. Wetlands International
28. The University of Kansas Center for Research, Inc. on behalf of the National History Museum and Biodiversity Research Center, University of Kansas

Summary of Selected Activities by Country

Country or State	Collaborator(s)	Activities in 2007 - 2008	Planned Activities
Afghanistan	WCS/DoD-NAMRU/FAO	Ongoing wild bird surveillance and training of local biologists and veterinarians	Sampling for AI in waterbirds, market wild birds, bridge species
Argentina	WCS/Wetlands Int. Argentina	Ongoing wild bird surveillance and training; courses organized by GAINS with USDA support	Wild waterbird sampling, training in AI surveillance and preparedness
Bolivia	WCS	Monitoring for AI in flamingo populations in Laguna Colorada	Wild waterbird/market sampling, training
Botswana	Percy FitzPatrick Ornithological Institute	Wild bird surveillance including sampling at Lake Ngami	Surveillance/migratory satellite telemetry
Brazil	Cargill, Inc./ FAO/GAINS/CIRAD	New projects to monitor wild birds for AI in the Amazon region and to examine interactions between migratory wild and domestic birds with a focus on AI prevention	Surveys and sampling for AI, training activities
Cambodia	WCS/UC Davis/FAO/AWSG/ USAID	Surveys and sampling of waterbirds, market wild birds, bridge species	Surveys/sampling of waterbirds, market wild birds, bridge species; education in AI preparedness
Cameroon	WCS/UCLA/Birdlife	Sampling domestic and wild birds of 65 species for AI testing	Fieldwork complete
Caribbean region (including Anguilla, Antigua, St. Kitts, St. Martin and St. Kitts)	EPIC (Environmental Protection in the Caribbean)	Sampling over 500 waterbirds from 5 countries, Training local biologists	Fieldwork complete
Chile	USDA-APHIS/WCS/SAG	Training in AI surveillance methods to personnel from the Chilean Agriculture and Livestock Service (SAG)	Surveys and sampling; adding existing AI surveillance data from Chile to GAINS
Colombia	WCS	Creating a national level AI monitoring program for wild waterbirds with local NGOs and government	AI surveillance in the Valle del Cauca area
Ecuador	Charles Darwin Research Station	Sampling of waterfowl for AI; training of biologists	Fieldwork complete
Egypt	Wetlands International/NCE	Waterbird surveys and training courses in collaboration with Nature Conservation Egypt	Surveys and sampling of waterbirds and bridge species

Falkland Islands	Falklands Conservation/WCS	Sampling waterbirds, training vets and technicians, mortality surveillance	Mortality surveillance and live wild bird sampling
Gabon	WCS	Census and mortality surveillance in waterbirds	Census and mortality surveillance in waterbirds
Ghana	University of Kansas (UK)	Analysis of samples previously collected by UK	Global risk map analysis with UNH
India	Wetlands International India	Census and AI sampling of waterbirds, bridge species; 4 training courses in 2007-08	Fieldwork and training complete
Indonesia	WCS/Wetlands Int. Indonesia/Cargill/AWSG	Census and AI sampling of market wild birds, waterbirds, bridge species, training gov't and NGO staff; local education	Census and AI sampling of market wild birds, waterbirds, bridge species
Kazakhstan	National Aviary/ Wetlands International/ FAO	Census and AI sampling of waterbirds, bridge species; placing satellite transmitters to monitor migratory movements	Census and AI sampling of waterbirds, bridge species; additional transmitters
Laos PDR	NIH/UMN	Funding from NIH via UMN supporting sampling plans	AI surveillance
Mongolia	WCS/FAO/USGS/NIH/UMN/AWSG	Census and AI sampling of waterbirds, bridge species; bird marking migration studies; training courses; stable isotope migration study	Census and AI sampling of waterbirds, bridge species, migration studies, training
Mozambique	Percy FitzPatrick Ornithological Institute	Census and AI sampling of waterbirds, bridge species	Census and AI sampling of waterbirds, bridge species; satellite - telemetry of waterbirds
Nigeria	WCS/CIRAD/Wetlands International	AI sampling of waterbirds, bridge species	AI sampling of waterbirds, bridge species
Peru	Cayetano Heredia University/DoD-NMRCD/Texas A&M	Wild bird and market surveillance	Waterbird sampling in new wetland sites; monitor market trade in wild birds
Philippines	University of Kansas (UK)	Analysis of samples previously collected by UK	Global risk map analysis with UNH
Republic of Congo	WCS	Census and mortality surveillance of wild birds	Census and mortality surveillance
Russia	Wetlands International/USGS	Waterbirds sampling for AI	Transmitters for migration studies
South Africa	Percy FitzPatrick Ornithological Institute	Census and AI sampling of waterbirds, environmental sampling, risk analysis and mapping	Census and AI sampling of waterbirds, bridge species; transmitters
South Korea	WCS, FAO, National Agriculture Department of South Korea	AI outbreak investigation	Fieldwork complete
Sudan	WCS, Wetlands International	Building collaborative relationships with government agencies and NGOs	Waterfowl sampling and censuses

Tanzania	Tanzania Bird Atlas	Census and AI sampling of waterbirds, bridge species, scavenger species	Census/sampling of waterbirds, bridge spp., transmitters for birds that fly the Nile-Rift Valley pathway
Ukraine	Wetlands International Black Sea Program; Friedrich-Loffler Institute, Germany	AI sampling of waterbirds	Fieldwork complete
Zimbabwe	Percy FitzPatrick Ornithological Institute	AI sampling of waterbirds, bridge species	AI sampling of waterbirds, bridge spp.
CONTRIBUTING WILD BIRD DATA TO www.GAINS.org	**Organization**	**Species groups**	**Supporting Agencies**
Burkina Faso	CIRAD	Anatidae, Waders	FAO
Chad	CIRAD/Wetlands Int.	Anatidae, Doves	FAO
Egypt	CIRAD/Wetlands Int.	Waders, Cormorants, Cattle Egrets	FAO
Ethiopia	CIRAD	Anatidae, Waders	FAO
Iran	Wetlands Int.	Anatidae	FAO
Jordan	CIRAD	Waders	FAO
Kazakhstan	CIRAD/Wetlands Int.	Waterbirds	FAO
Kenya	CIRAD/Wetlands Int.	Anatidae, Waders, Flamingo	FAO
Malawi	CIRAD/Wetlands Int.	Anatidae, Waders, Rails	FAO
Mali	CIRAD	Anatidae, Waders, Rails	FAO
Mauritania	CIRAD	Anatidae, Waders, Gulls	FAO
Morocco	CIRAD	Anatidae, Waders	FAO
Niger	CIRAD	Waterbirds	FAO
Nigeria	CIRAD/Wetlands Int	Waterbirds	FAO/WCS
Romania	CIRAD	Anatidae, Waders, Sparrows	FAO
Senegal	CIRAD	Anatidae, Waders	FAO
Sudan	CIRAD/Wetlands Int.	Anatidae, Waders	FAO
Tunisia	CIRAD/Wetlands Int.	Anatidae, Waders	FAO
Turkey	Wetlands Int.	Anatidae	FAO/WCS
Zambia	CIRAD	Anatidae, Waders	FAO

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Avian influenza virus isolated in wild waterfowl in Argentina: Evidence of a potentially unique phylogenetic lineage in South America

Ariel J. Pereda [a,*], Marcela Uhart [b], Alberto A. Perez [c], María E. Zaccagnini [d], Luciano La Sala [b,e], Julieta Decarre [d], Andrea Goijman [d], Laura Solari [d], Romina Suarez [d], María I. Craig [a], Ariel Vagnozzi [f], Agustina Rimondi [a], Guido König [g], María V. Terrera [c], Analía Kaloghlian [c], Haichen Song [h,i], Erin M. Sorrell [h,i], Daniel R. Perez [h,i]

[a] *Instituto de Virología, CICVyA, Instituto Nacional de Tecnología Agropecuaria (INTA), C.C. 25, Castelar (1712), Buenos Aires, Argentina*
[b] *Field Veterinary Program-GAINS Wildlife Conservation Society (WCS), Estivariz 197, Puerto Madryn (9120), Chubut, Argentina*
[c] *Servicio Nacional de Sanidad y Calidad Agroalimentaria (SENASA), Av. Paseo Colon 367, Buenos Aires (1063), Argentina*
[d] *Instituto de Recursos Biológicos, CIRN, INTA, C.C. 25, Castelar (1712), Buenos Aires, Argentina*
[e] *Centro de Estudios Parasitológicos y Vectores CONICET, Calle 2 Nro 584, La Plata (1900), Buenos Aires, Argentina*
[f] *Laboratorio de Sanidad Aviar, EEA C. del Uruguay, INTA, Ruta Prov. 39 km 143,5, Concepción del Uruguay (3260), Entre Ríos, Argentina*
[g] *Instituto de Biotecnología, CICVyA, INTA, C.C. 25, Castelar (1712), Buenos Aires, Argentina*
[h] *Department of Veterinary Medicine, University of Maryland, USA*
[i] *Virginia-Maryland Regional College of Veterinary Medicine, 8075 Greenmead Drive, University of Maryland, College Park, MD 20742-3711, USA*

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ABSTRACT

Avian influenza (AI) viruses have been sporadically isolated in South America. The most recent reports are from an outbreak in commercial poultry in Chile in 2002 and its putative ancestor from a wild bird in Bolivia in 2001. Extensive surveillance in wild birds was carried out in Argentina during 2006–2007. Using RRT-PCR, 12 AI positive detections were made from cloacal swabs. One of those positive samples yielded an AI virus isolated from a wild kelp gull (*Larus dominicanus*) captured in the South Atlantic coastline of Argentina. Further characterization by nucleotide sequencing reveals that it belongs to the H13N9 subtype. Phylogenetic analysis of the 8 viral genes suggests that the 6 internal genes are related to the isolates from Chile and Bolivia. The analysis also indicates that a cluster of phylogenetically related AI viruses from South America may have evolved independently, with minimal gene exchange, from influenza viruses in other latitudes. The data produced from our investigations are valuable contributions to the study of AI viruses in South America.

Introduction

Natural infections with influenza A viruses occur in a variety of avian and mammalian hosts. Influenza A viruses of avian origin have been implicated in outbreaks of influenza in mammals, such as seals, whales, pigs, horses, mustelids, felines and humans as well as in domestic poultry species (Gauthier-Clerc et al., 2007; Ito et al., 1995; Webster et al., 1992). Influenza A viruses are classified on the basis of two glycoproteins expressed on the virus surface, the haemagglutinin (HA) and the neuraminidase (NA). To date, 16 HA (H1–H16) and 9 NA (N1–N9) antigenic subtypes have been detected in avian species and can be found in multiple combinations (Capua and Alexander, 2006; Olsen et al., 2006). In wild aquatic birds, most avian influenza (AI) infections are either subclinical or accompanied by mild clinical signs, usually caused by low pathogenicity AI strains (LPAI). Occasionally, highly pathogenic avian influenza strains (HPAI) emerge in nature and cause severe outbreaks with high mortality rates (Donis et al., 1989; Gauthier-Clerc et al., 2007).

The low virulence of AI subtypes in wild aquatic birds is thought to be the result of co-adaptation and evolution, which has allowed these viruses to find an optimal balance between high levels of replication and few pathological effects for the host (Gauthier-Clerc et al., 2007; Sharp et al., 1997; Widjaja et al., 2004). LPAI viruses have been isolated from at

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[*] Corresponding author. Laboratorio de Aves y Porcinos, Instituto de Virología, CICVyA, INTA – Casilla de Correo 25, Castelar, CP 1712, Provincia de Buenos Aires, Argentina. Fax: +54 11 4621 1743.
E-mail address: apereda@cnia.inta.gov.ar (A.J. Pereda).

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least 105 different wild bird species representing 26 families. It is widely accepted that birds of wetlands and aquatic environments such as the Anseriformes and Charadriiformes constitute the major natural reservoirs of influenza A viruses (Alexander, 2000; Olsen et al., 2006; Rappole and Hubalek, 2006). A great deal of information exists on the ecology of LPAI viruses in these reservoirs from North America and Eurasia. Surveillance studies of wild ducks in the Northern Hemisphere have revealed a seasonal infection pattern of influenza A viruses (Olsen et al., 2006). In contrast, little information is available about the presence, movement, ecology and phylogenetic relationships of LPAI viruses in wild birds in the Southern Hemisphere, particularly in South America (Donis et al., 1989; Krauss et al., 2004; Olsen et al., 2006). Waterfowl are possible reservoirs of influenza virus in South America, since there are in this region the Anatidae counterparts of the Northern Hemisphere species (De La Peña and Rumboll, 2001; Narosky, 2003). Early surveillance efforts focusing on marine birds of South America revealed negative serologic results for influenza A virus though no viral isolation was attempted (Karesh et al., 1999; Padilla et al., 2003; Travis et al., 2006; Uhart et al., 2003). Interestingly, besides historical references of HPAI outbreaks in the early 20th century the only other HPAI virus outbreak in South America was reported in 2002 from commercial poultry in Chile. Additionally, a LPAI virus H7 strain was isolated in Bolivia from an indigenous wild duck (Cinnamon Teal — *Anas cyanoptera*), which was phylogenetically related to the Chilean H7 HPAI strains (Spackman et al., 2006, 2007; Suarez et al., 2004).

Susceptibility of South American waterfowl to the Asian HPAI H5N1 strains was confirmed during a HPAI outbreak in two nature parks in Hong Kong in late 2002, when 11 Anseriformes species, indigenous to South America, were infected and died (Ellis et al., 2004). The unprecedented emergence and spread of the HPAI H5N1 virus during 2003–2004 in East and Southeast Asia and its subsequent expansion to the Middle East, Europe and Africa has placed AI on top of the priority list of potential transboundary diseases (Alexander, 2000). Whether the broad scale spread of HPAI H5N1 is due to Anatidae migrations (Gilbert et al., 2006) or human activity involved in movement of infected domestic poultry, there is certainly an urgent need to establish worldwide epidemiological efforts to track the movement and ecology of this and many other AI subtypes (Gauthier-Clerc et al., 2007). International organizations involved in human and animal health have promoted collaboration and coordinated efforts to intensify AI surveillance programs across the globe. In Argentina, the National Agriculture Technology Institute (INTA) and the National Animal Health Service (SENASA), in collaboration with the Wildlife Conservation Society (WCS) have co-organized a long-term surveillance program in resident and migratory wetland bird populations. These surveillance efforts are aimed at elucidating the presence and ecology of influenza A viruses in wild aquatic birds and to provide an early warning system for the potential introduction of HPAI strains in densely populated poultry areas (DPPAs) in Argentina. The present study reveals preliminary results obtained from the initial surveillance activities performed during 2006–2007 on hunter-killed waterfowl and captured live birds in Argentina. Using RRT-PCR, 12 AI positive samples were detected from 2895 samples. After blind passages in 10-day-old SPF chicken eggs one positive remained. The virus identified in this report, A/Kelp Gull/Argentina/LDC4/06 (H13N9), represents the first AI isolate from indigenous wild birds captured in Argentina. Sequence and phylogenetic analysis revealed similarities with other AI viruses isolated in South America, and suggest the existence of a population of AI viruses in South America that evolved independently from viruses in other latitudes.

Materials and methods

Geographic locations of sampling sites in Argentina

The targeted geographic locations included in this study are considered potential risk areas for avian influenza infections and outbreaks (Fig. 1). The sampling locations consist of vast wetlands located along important migratory bird pathways and in close proximity to Densely Populated Poultry Areas (DPPAs), which comprise approximately 70% of the poultry production in Argentina. These wetland locations serve as reservoirs for multiple populations of resident waterfowl species, particularly ducks (*Netta peposaca*, *Amazonetta brasiliensis*, *Anas versicolor*, etc.). From October to March (Austral spring and summer seasons), a large number of gregarious migratory Charadriiformes use these wetlands as feeding grounds or stopover sites, interacting with resident waterfowl populations. In addition, agro-ecosystems, including rice plantations, receive significant numbers of Nearctic waders during December and January. These habitats are extensive along the Argentine Atlantic coast and in addition, vast fresh water wetlands, consisting of lagoons, forest islands and marshes are interconnected via the Paraná and Uruguay rivers, two of the major river systems in South America. Contrary to the Northern Hemisphere, South American duck populations have a more homogeneous distribution of the number of birds during the year, as winter migrations are not as critical (López-Lanús and Blanco, 2005; Olsen et al., 2006; Gilbert et al., 2006). From May to August, the wetlands are used for game bird hunting focusing mainly on ducks, tinamous and wild pigeons.

Two major geographic areas were used as sampling sites (Fig. 1).

Area 1 includes four districts of Entre Ríos province (Gualeguay, Paraná, La Paz and Victoria), one district in Corrientes province (Esquina) and one district in Santa Fe province (San Javier). Samples were collected from private hunting lodges and national reserves, strategically set within the described wetlands. Each of these 6 districts covers 15,000 hectares. In area 2 samples were obtained from a variety of seabirds (gulls, penguins, cormorants, terns and shorebirds) captured along the Argentine Atlantic coast from the provinces of Buenos Aires to Tierra del Fuego. This particular seashore area covers approximately 5000 km from parallel 36°S to 55°S.

Sample collection

Trained biologists and veterinarians carried out the sampling activities. Each bird was initially identified by species, sex, age category (adult, juvenile or fledgling) and weight (Tables 1A and 1B). Following identification swabs were collected. Cloacal swabs, 2895 in total, were collected from wild waterfowl using single-use polyester sterile swabs and then stored separately into single plastic cryo-tubes, containing 2 ml of Phosphate Buffer Solution (PBS) with 50% glycerol and Penicillin 10,000 IU/ml, Streptomycin 5000 μg/ml, Gentamicin Sulfate 1000 μg/ml, Kanamycin sulfate 700 μg/ml and Anphotericin B 10 μg/ml (Sigma Chemical Co™, St. Louis, MO, USA). Samples were either refrigerated at 4 °C for less than 48 h before they were processed for molecular diagnosis and virus isolation or, if immediate processing was not available, they were frozen in liquid nitrogen.

AI detection

Viral RNA was extracted from 200 μl of PBS suspension from cloacal swabs using the Total RNA Isolation Chemistry Starter Kit (Applied Biosystems™, Foster City, CA, USA) in accordance with the manufacturer's instructions. RNA was eluted in a final volume of 100 μl and stored at −80 °C. Viral cDNA was prepared using 30 μl of viral RNA and random hexamers in a final volume of 60 μl as per manufacturer's directions using the High Capacity cDNA Archive kit™ (Applied Biosystems™, Foster City, CA, USA). The cDNA was tested for AI by real-time reverse transcription PCR (RRT-PCR) using TaqMan Universal PCR Master Mix™ (Applied Biosystems™, Foster City, CA, USA) directed to the matrix (M) gene, which detects all type A influenza viruses as previously reported (Spackman et al., 2002). The PCR reaction was performed in an ABI Prism 7500 SDS apparatus (Applied Biosystem™, Foster City, CA, USA).

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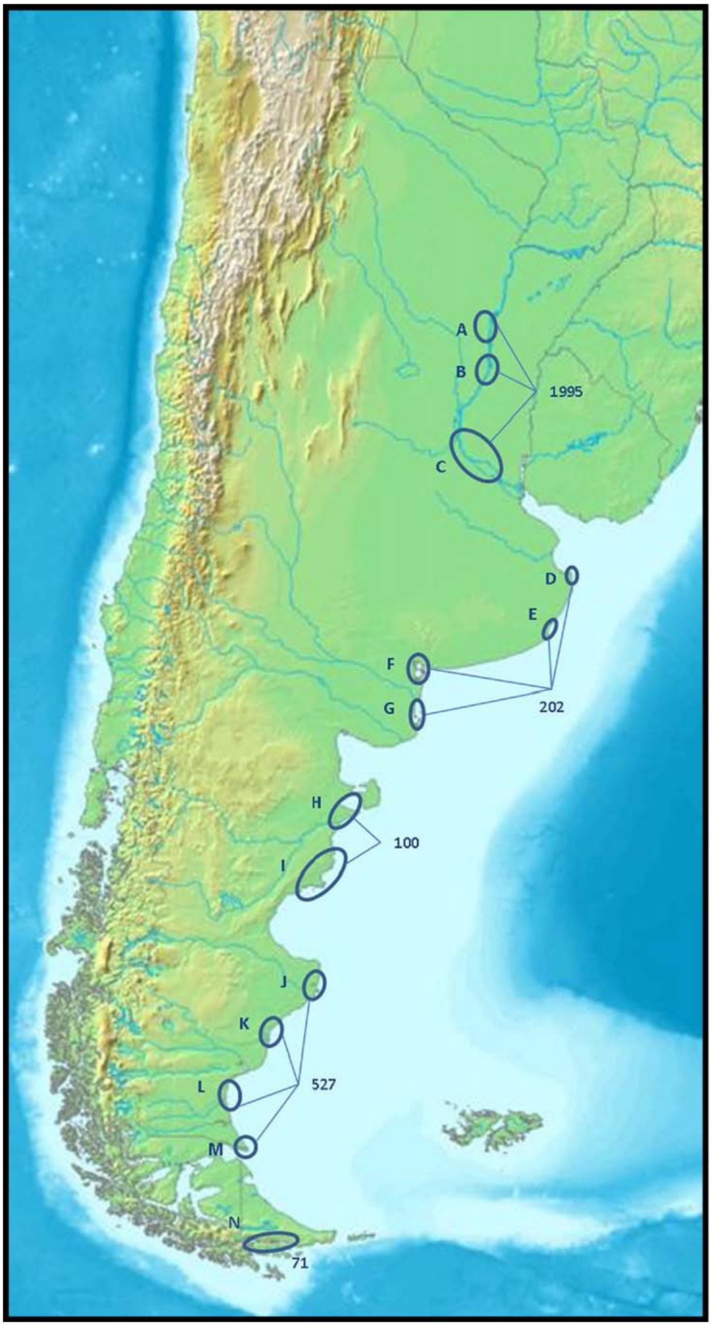

#	Location	Province	Positive RRT-PCR
A	Esquina	Corrientes	3
B	San Javier La Paz	Santa Fe Entre Ríos	5 2
C	Victoria	Entre Ríos	1
D	Pta. Rasa	Buenos Aires	0
E	Mar Chiquita	Buenos Aires	0
F	Bahía Blanca	Buenos Aires	1
G	Bahía San Blas	Buenos Aires	0
H	Pta Loma – Pta. León	Chubut	0
I	Pta Tombo - Cb. Dos Bahías - Bahía Bustamante	Chubut	0
J	Puerto deseado	Santa Cruz	0
K	Bahía San Julián	Santa Cruz	0
L	Monte León	Santa Cruz	0
M	Cabo Vírgenes	Santa Cruz	0
N	I. Conejo, I. Martillo, La Turbera	Tierra del Fuego	0

Fig. 1. Geographical location of the areas under surveillance. The numbers correspond to the quantity of samples analyzed from each area. The associated table indicates the locations individualized from A to N, with the positive RRT-PCR results obtained from each of them.

Virus isolation and characterization

All swab samples determined positive by RRT-PCR were processed for virus isolation in specific pathogen free (SPF) embryonated chicken eggs. Briefly, 200 µl of PBS suspension from the cloacal swab samples were used for viral isolation in the allantoic cavity of 9–11 day-old SPF chicken embryonated eggs, in accordance to standardized protocols described in the OIE (2000) and to Argentine regulations.

Genetic analysis and phylogenetic characterization

The genome segments of AI were amplified by RT-PCR as described by Hoffmann et al. (2001) and directly sequenced with the BigDye terminator kit (Applied Biosystems™, Foster City, CA, USA) on an ABI 3730 (Applied Biosystems™, Foster City, CA, USA). Regions corresponding to the entire open reading frame of HA, NP, NA, M1 and NS, and partial sequences of PB2, PB1 and PA were obtained (primer sequences available upon request). Genomic information was derived from overlapping sequences covered by forward and reverse primers. At least two independent RT-PCR reactions were produced for each gene and used for sequencing. Sequences are available through GenBank, accession nos. EU523136 through EU523143. Nucleotide BLAST analysis (at http://www.ncbi.nlm.nih.gov/blast/Blast.cgi) was initially used to identify the most closely related influenza A virus gene for each respective segment. Then, all available isolates obtained were included in a multiple alignment by CLUSTAL X Version 1.8.3 program (Thompson et al., 1994), and the percent identities were calculated. The same reference isolates were then used for phylogenetic analysis. The phylogenetic trees, calculated by neighbor-joining method, were computed with the DNADIST and NEIGHBOR modules

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Table 1A

Details of birds sampled in the present study, indicating the scientific and common name, the order and family and the number of birds sampled

Scientific name	Common name	Samples[a]	Order	Family
Amazonetta brasiliensis	Brazilian duck	143	Anseriformes	Anatidae
Anas bahamensis	White-cheeked pintail	5	Anseriformes	Anatidae
Anas flavirostris	Speckled teal	16	Anseriformes	Anatidae
Anas platalea	Red shoveler	9	Anseriformes	Anatidae
Anas sibilatrix	Southern wigeon	1	Anseriformes	Anatidae
Anas versicolor	Silver teal	140	Anseriformes	Anatidae
Callonetta leucophrys	Ringed teal	103	Anseriformes	Anatidae
Coscoroba coscoroba	Coscoroba swan	1	Anseriformes	Anatidae
Dendrocygna bicolor	Fulvous whistling duck	139	Anseriformes	Anatidae
Dendrocygna viduata	White-faced whistling duck	53	Anseriformes	Anatidae
Heteronetta atricapilla	Black-headed duck	1	Anseriformes	Anatidae
Netta peposaca	Rosy-billed pochard	1242	Anseriformes	Anatidae
Oxyura dominica	Masked duck	1	Anseriformes	Anatidae
Sarkidiornis melanotos	Comb duck	1	Anseriformes	Anatidae
Anas georgica	Brown pintail	4	Anseriformes	Anatidae
Dendrocygna autumnalis	Black-bellied whistling duck	1	Anseriformes,	Anatidae
Calidris fuscicollis	White-rumped sandpiper	9	Charadriiformes	Scolopacidae
Calidris melanotos	Pectoral sandpiper	16	Charadriiformes	Scolopacidae
Charadrius collaris	Collared plover	5	Charadriiformes	Charadriidae
Larus atlanticus	Olrog's gull	73	Charadriiformes	Laridae
Larus dominicanus	Kelp gull	162	Charadriiformes	Laridae
Larus scoresbii	Dolphin gull	15	Charadriiformes	Laridae
Nycticryphes semicollaris	South American painted snipe	2	Charadriiformes	Rostratulidae
Pluvialis dominica	American golden plover	3	Charadriiformes	Charadriidae
Rynchops niger	Black skimmer	6	Charadriiformes	Rhynchopidae
Thalasseus s. eurygnatha	Cayenne tern	17	Charadriiformes	Sternidae
Sterna hirundinacea	South American tern	91	Charadriiformes	Sternidae
Thalasseus maximus	Royal tern	36	Charadriiformes	Sternidae
Thalasseus sandvicensis	Sandwich tern	42	Charadriiformes	Sternidae
Tryngites subruficollis	Buff-breasted sandpiper	8	Charadriiformes	Scolopacidae
Ardea cocoi	White-necked heron	1	Ciconiiformes	Ardeidae
Ardea alba	Great egret or white heron	1	Ciconiiformes	Ardeidae
Phimosus infuscatus	Bare-faced ibis	1	Ciconiiformes	Threskiornithidae
Plegadis chihi	White-faced ibis	5	Ciconiiformes	Threskiornithidae
Columba maculosa	Spotted-winged pigeon	3	Columbiformes	Columbidae
Columbina picui	Picui ground-dove	11	Columbiformes	Columbidae
Fulica leucoptera	White-winged coot	16	Gruiformes	Rallidae
Fulica rufifrons	Red-fronted coot	1	Gruiformes	Rallidae
Furnarius rufus	Rufous hornero	3	Passeriformes	Furnariidae
Mimus saturninus	Chalk-browed mockingbird	2	Passeriformes	Mimidae
Molothrus bonariensis	Shiny Cowbird	2	Passeriformes	Icteridae
Polioptila dumicola	Masked gnatcatcher	2	Passeriformes	Sylviidae
Progne modesta	Southern martin	19	Passeriformes	Hirundinidae
Saltator coerulescens	Greyish saltator	1	Passeriformes	Thraupidae
Synallaxis frontalis	Sooty-fronted spinetail	2	Passeriformes	Furnariidae
Taraba major	Great antshrike	1	Passeriformes	Thamnophilidae
Turdus amaurochalinus	Creamy-bellied thrush	10	Passeriformes	Turdidae
Turdus rufiventris	Rufous-bellied thrush	2	Passeriformes	Turdidae
Zonotrichia capensis	Rufous-collared sparrow	1	Passeriformes	Emberizidae
Phalacrocorax albiventer	King cormorant	17	Pelecaniformes	Phalacrocoracidae
Phalacrocorax atriceps	Imperial cormorant	78	Pelecaniformes	Phalacrocoracidae
Phalacrocorax magellanicus	Rock cormorant	1	Pelecaniformes	Phalacrocoracidae
Phalacrocorax brasilianus	Neotropic cormorant	1	Pelecaniformes	Phalacrocoracidae
Macronectes giganteus	Southern giant petrel	18	Procellariiformes	Procellariidae
Spheniscus magellanicus	Magellanic penguin	349	Sphenisciformes	Spheniscidae
Nothura maculosa	Spotted tinamou	2	Tinamiformes	Tinamidae

[a] Number of samples obtained.

of the PHYLIP package (Felsenstein, 1989). Bootstrapping values (1000 replicates) were calculated with the modules SEQBOOT, DNADIST, NEIGHBOR and CONSENSE. Branches with bootstrapping values ≥70 were considered significant, corresponding to a confidence interval ≥95% (Hillis and Bull, 1993). For visualization and printing of the trees, the TREEVIEW program, Version 1.6.6 (Page, 1996) was used.

Results

Isolation of a H13N9 type A influenza virus from a Kelp Gull

From March 2006 to December 2007, 2895 cloacal swabs were collected from wild waterfowl from different locations (as described previously in Materials and methods). Our sampling and processing

Table 1B

Table summarizing the number of samples for each order and the percentage from the total of samples obtained

Order	Samples[a]	Percentage
Anseriformes	1860	64,2
Charadriiformes	485	16,8
Ciconiiformes	8	0,3
Columbiformes	14	0,5
Gruiformes	17	0,6
Passeriformes	45	1,6
Pelecaniformes	97	3,4
Procellariiformes	18	0,6
Sphenisciformes	349	12,1
Tinamiformes	2	0,1
TOTAL	2895	100.0

[a] Number of samples obtained.

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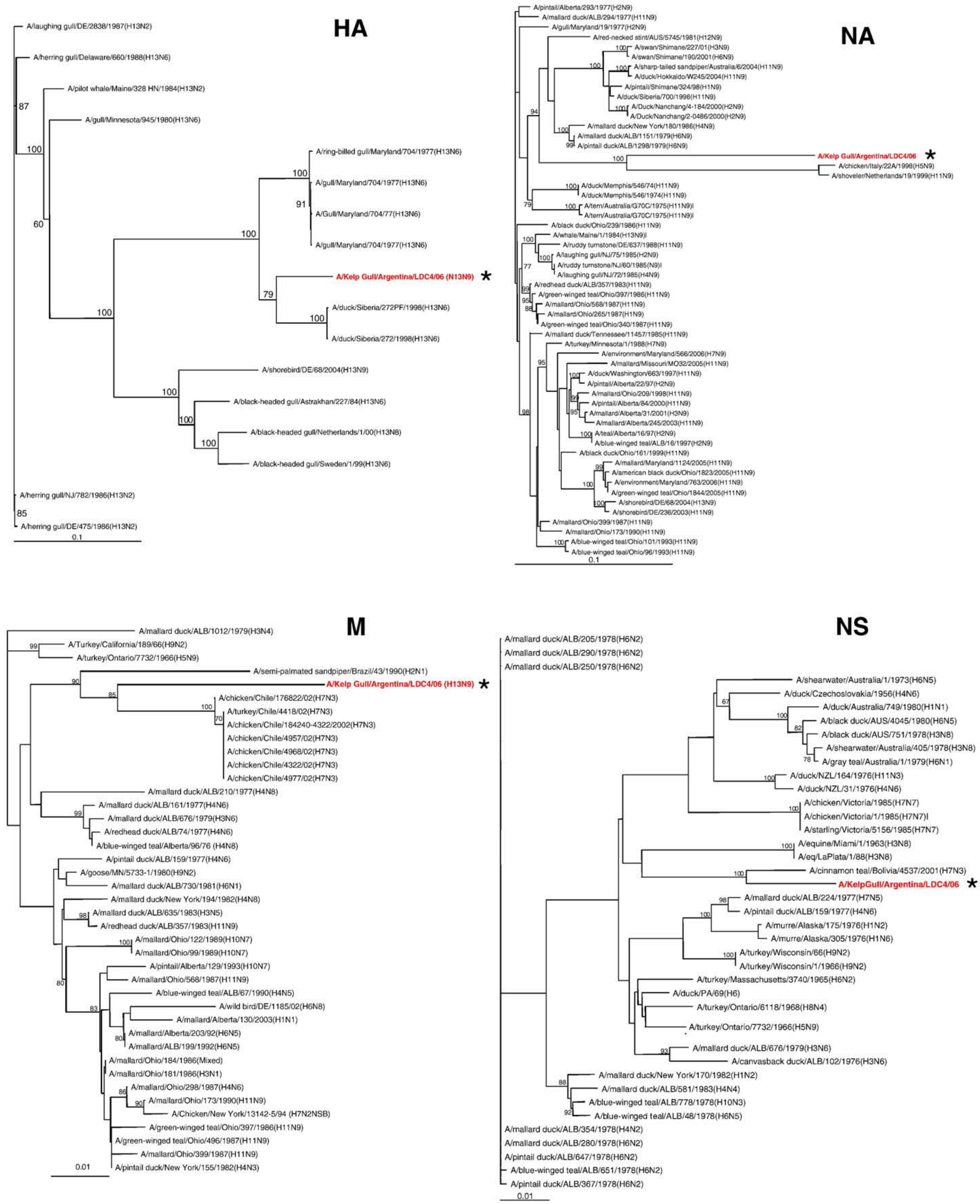

Fig. 2. Phylogenetic trees of individual gene segments (indicated in the upper-right side of each tree). All trees were constructed with PHYLIP package (Felsenstein, 1989) using Neighbor Joining with 1000 bootstrap replicates. Bootstrap values above 60% are detailed. To easily identify the Argentinean isolate an asterisk (*) was used.

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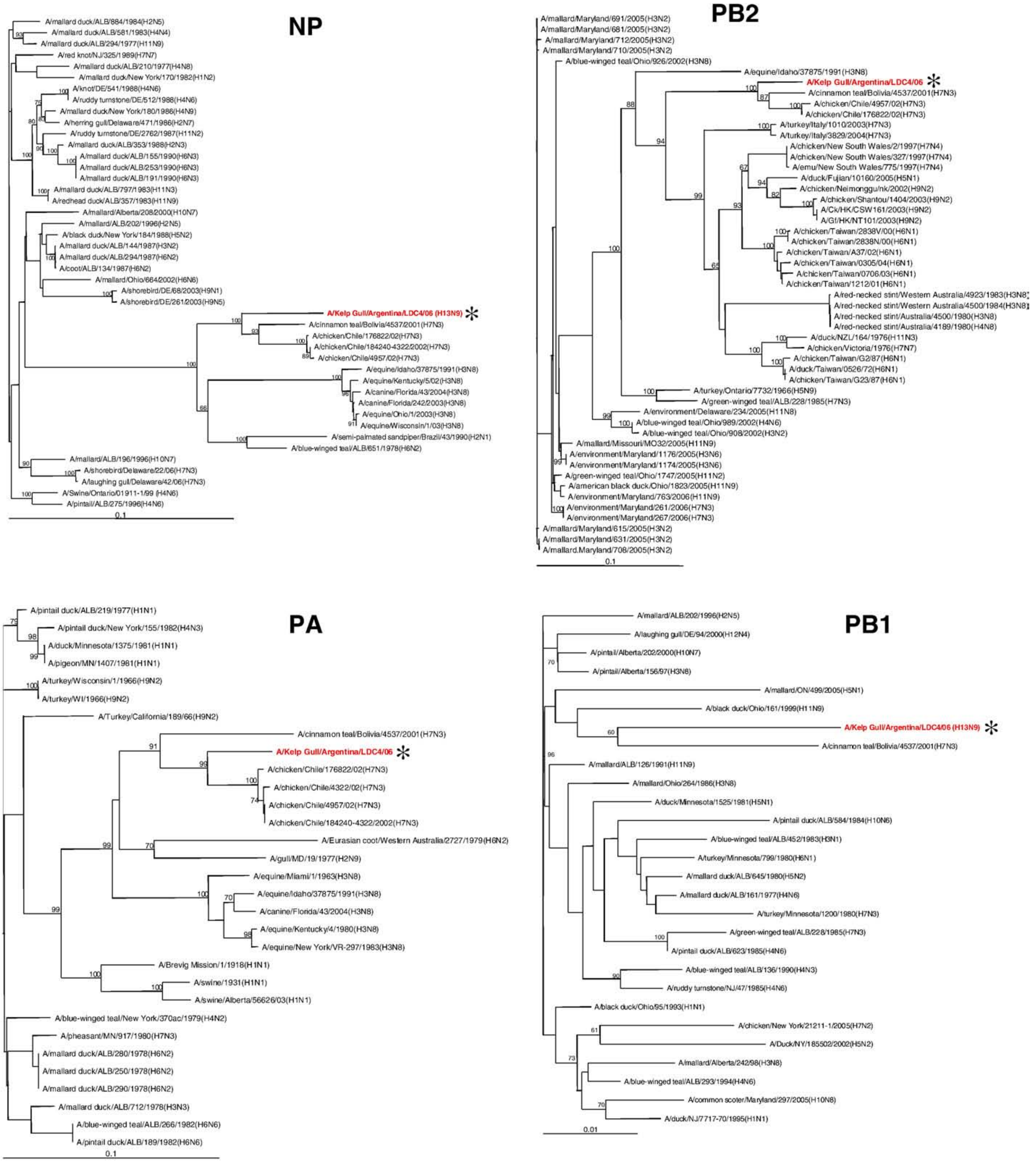

Fig. 2 (*continued*).

procedures revealed the presence of 12 AI positive samples by RRT-PCR, using the matrix gene as the target for amplification. Additional RRT-PCR tests ruled out the presence of H5 and H7 using a protocol kindly provided by Dr. William Dundon at the Istituto Zooprofilattico Sperimentale delle Venezie (IZSVE, 2006). Eleven of the twelve RRT-PCR positives samples corresponded to Area 1 and one to Area 2 (Fig. 1). Efforts to grow the RRT-PCR positive samples in SPF embryonated chicken eggs resulted in only one positive isolation from Area 2. This virus was isolated from a free-ranging Kelp Gull (*Larus dominicanus*) captured in the estuary of Bahía Blanca, in the southern part of Buenos Aires province (40° 33′S, 62° 16′W). Initial molecular characterization of this isolate revealed that it is an influenza type A virus of the H13N9 subtype and was named A/Kelp Gull/Argentina/LDC4/06 (H13N9). The Kelp Gull is mainly a coastal gull, which breeds on shores and islands throughout much of the southern hemisphere. *L. dominicanus* is a subspecies found mostly in

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South America, parts of Australia and New Zealand (where it is also known as the Southern Black-backed Gull or Karoro). It is the southern equivalent of the northern hemisphere's Lesser Black-backed Gull and is similar in size and wingspan. Our study suggests that the Kelp Gull of the Argentine Atlantic coast is a potential reservoir of avian influenza viruses in South America. To our knowledge this is the first avian influenza virus isolated from gulls in South America.

The detection rate using RRT-PCR was 0.41%, whereas isolation in SPF eggs yielded an overall virus prevalence of 0.035%. This and previous studies suggest that it is not possible to rely singly on RRT-PCR or virus isolation to perform thorough characterizations of avian influenza viruses in wild birds (Runstadler et al. 2007). The H13N9 virus isolated in this study was obtained after a second blind passage in chicken embryos producing HA titers between 1/64 and 1/128 and a virus titer of 10^5 EID50/ml (mean egg infectious dose). Thus, it is tempting to speculate that failure to amplify some of our RRT-PCR positives in SPF chicken eggs maybe due to the inability of the latter to act as a good substrate for virus growth.

Sequencing and phylogenetic characterization of the H13N9 from Kelp Gull suggest the presence of influenza genes unique to the Southern Hemisphere

In order to better characterize the H13N9 virus isolated from Kelp Gull, we performed sequencing and phylogenetic analysis for the 8 genomic segments. Based on the phylogenetic analysis performed in this study (Fig. 2), the PB2, PB1, PA, NP, M1 and NS genes are most closely related, phylogenetically, to the AI isolates collected during an outbreak in commercial poultry in Chile in 2002 and the putative ancestor isolated from a Cinnamon Teal in Bolivia (Table 2). In addition, the NP and M genes are more distantly related to an AI virus isolated from a shorebird in Brazil in 1990, A/semi-palmated sandpiper/Brazil/43/1990 (H2N1) (Fig. 2). The analysis also indicates that the internal genes from the South American isolates are grouped in their own cluster with considerable bootstrap values (Fig. 2). The PA, NP and NS genes also showed phylogenetic relationship with influenza viruses isolated from equine and canine species; most notably the NS tree shows a close relationship with an Argentinean equine isolate. Spackman et al. (2006) previously described a similar relationship in the NS gene for the Bolivian isolate. This data suggests the potential for occasional introduction of AI genes from birds into the horse population in South America.

With respect to genes that code for surface proteins, the closest HA gene relative is A/duck/Siberia/272PF/1998 (H13N6) while bootstrap values indicate a more distant relationship to H13 viruses isolated from gulls in Maryland, USA in 1977. Interestingly, our kelp gull isolate is less phylogenetically related to more recent H13 viruses isolated in North America or Europe. Thus, it is tempting to speculate that H13 viruses in South America may have evolved independently from other H13 viruses in other parts of the world. Analysis of the deduced HA protein sequence shows a cleavage site consistent with a low pathogenic AI (VPAISNRGLF) with little variation among viruses of the H13 subtype (Table 3). Interestingly, the N9 NA gene is more closely related to European isolates than to North American isolates. The two most phylogenetically related NA genes are from influenza viruses isolated during a poultry outbreak in Italy and from a wild duck captured in the Netherlands (Fig. 2). The NA phylogenetic tree shows that these isolates are grouped in a different cluster from others N9 subtype viruses and supported with high bootstrap values, indicating a phylogenetically distinct lineage group of N9 NA genes. However, the nucleotide distance between the European and the Argentinean isolates is remarkably high (22%) to support a unique lineage group and additional N9 sequences from this cluster would be necessary to better explain its evolutionary pattern.

Table 2
Type A Influenza virus isolates most related to A/KelpGull/Argentina/LDC4/06 by individual gene segment

Gene segment	Most related virus isolate	Percentage of identity (percentage sequenced)
4 – HA	A/duck/Siberia/272/1998 (H13N6)	91 (100)
6 – NA	A/shoveler/Netherlands/19/1999(H11N9)	78 (94)
8 – NS	A/cinnamon teal/Bolivia/4537/2001 (H7N3)	97 (100)
7 – M	A/chicken/Chile/184240-4322/2002(H7N3)	96 (50)
3 – PA	A/chicken/Chile/184240-4322/2002(H7N3)	95 (26)
2 – PB1	A/cinnamon teal/Bolivia/4537/2001 (H7N3)	94 (28)
1 – PB2	A/chicken/Chile/176822/02(H7N3)	94 (29)
5 – NP	A/cinnamon teal/Bolivia/4537/2001 (H7N3)	94 (100)

Table 3
Amino acidic sequence pattern of the cleavage site of all available H13 HA subtype Influenza viruses

A/duck/Siberia/272/1998(H13N6)	VPAISNRGLF
A/gull/Maryland/704/1977(H13N6)	VPAISNRGLF
A/black-headed gull/Netherlands/1/00(H13N8)	VPAISKRGLF
A/black-headed gull/Sweden/1/99(H13N6)	VPAISNRGLF
A/herring gull/DE/475/1986(H13N2)	VPATSNRGLF
A/shorebird/DE/68/2004(H13N9)	VPAIASRGLF
A/herring gull/NJ/782/1986(H13N2)	VPATSNRGLF
A/laughing gull/DE/2838/1987(H13N2)	VPSTSNRGLF
A/herring gull/Delaware/660/1988(H13N6)	VPATSNRGLF
A/gull/Maryland/704/1977(H13N6)	VPAISNRGFF
A/gull/Minnesota/945/1980(H13N6)	VPATSNRGLF
A/Gull/Maryland/704/77(H13N6)	VPAISNRGLF
A/ring-billed gull/Maryland/704/1977(H13N6)	VPAISNRGLF
A/pilot whale/Maine/328 HN/1984(H13N2)	VPAISNRGLF
A/Kelp Gull/Argentina/LDC4/06 (H13N9)	**VPAISNRGLF**

Discussion

As noted previously (Spackman et al., 2006, 2007), AI surveillance in wild birds in South America has been minimal. In this study we attempted to provide a better understanding of the ecology of AI in wild birds in Argentina. National Veterinary Services in South American countries are routinely screening commercial and backyard poultry farms for the presence of AI. These efforts are targeted to prevent the introduction of AI in domestic poultry. However, in order to provide a better and more credible risk analysis of the potential introduction of AI to commercial and/or backyard poultry it is necessary to monitor the presence and prevalence of these viruses in wild bird populations (Munster et al., 2006). One H7N3 HPAI outbreak in commercial poultry was reported in Chile in 2002 (Suarez et al., 2004). The source of the virus was postulated to be wild waterfowl due to the low infectivity of this virus strain in chickens. Interestingly a closely related LPAI H7 virus was obtained from a Cinnamon Teal in Bolivia collected only a few months before the HPAI outbreak. However, this virus was only characterized recently (Spackman et al., 2006, 2007) highlighting the importance of timely wild bird surveillance and virus characterization.

The present study reveals data obtained from the initial, inter-institutional AI surveillance effort performed in Argentina during 2006–2007 on hunter-killed waterfowl and captured live birds. As a result, we report for the first time an isolation of a H13N9 AI virus from a kelp gull in Argentina. As past surveillance efforts have shown, a majority of the H13 viruses isolated to date have been from different gull species and this case is not an exception (Fouchier et al., 2005). However, our phylogenetic analysis suggests that the H13 virus isolated in Argentina is evolving independently from other H13 viruses. More importantly, our analysis indicates that the Kelp Gull N9 gene belongs to a phylogenetic cluster that until recently contained NAs of purely Eurasian origin, leading us to believe that this lineage spreads beyond Eurasia. However, additional N9 gene sequences from this poorly defined lineage are required to draw proper conclusions based on the high nucleotide distance observed between the European and Argentinean isolates. It is also interesting to note that

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the internal genome segments (PB2, PB1, PA, NP, M, and NS) of this isolate are closely related to those viruses previously isolated from the Chilean outbreak, and the putative ancestor from a wild bird captured in Bolivia. This relation suggests a pattern of genetic evolution with little exchange of segments with North American isolates, despite sharing multiple trans-hemispheric migratory routes. As noted by others (Spackman et al., 2006) the NS genes from these South American isolates are related to equine viruses; however it is not a gene derived from the equine lineage due the great nucleotide divergence observed. Our data coupled with the fact that the kelp gull (*L. dominicanus*) is a nonmigratory bird, supports the notion of a particular, and yet poorly understood, ecology of AI viruses in South America. In this regard, this study certainly sheds more attention and awareness regarding the presence and molecular characteristics of AI viruses in South America.

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Mapping H5N1 highly pathogenic avian influenza risk in Southeast Asia

Marius Gilbert[*,†], Xiangming Xiao[‡], Dirk U. Pfeiffer[§], M. Epprecht[¶], Stephen Boles[‡], Christina Czarnecki[‡], Prasit Chaitaweesub[‖], Wantanee Kalpravidh[**], Phan Q. Minh[††], M. J. Otte[‡‡], Vincent Martin[‡‡], and Jan Slingenbergh[‡‡]

[*]Biological Control and Spatial Ecology, Université Libre de Bruxelles, CP160/12, Avenue FD Roosevelt 50, B-1050 Brussels, Belgium; [‡]Institute for the Study of Earth, Oceans, and Space, University of New Hampshire, 39 College Road, Durham, NH 03824; [§]Epidemiology Division, Department of Veterinary Clinical Sciences, The Royal Veterinary College, University of London, London AL9 7TA, United Kingdom; [¶]Swiss National Center of Competence in Research North–South, 3012 Berne, Switzerland; [‖]Department of Livestock Development, Ministry of Agriculture and Cooperatives, Bangkok 10400, Thailand; [**]Regional Office for Asia and the Pacific, Food and Agriculture Organization of the United Nations, Bangkok 10200, Thailand; [††]Department of Animal Health, Ministry of Agriculture and Rural Development, Hanoi, Vietnam; and [‡‡]Food and Agriculture Organization of the United Nations, Viale delle Terme di Caracalla, 00100 Rome, Italy

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The highly pathogenic avian influenza (HPAI) H5N1 virus that emerged in southern China in the mid-1990s has in recent years evolved into the first HPAI panzootic. In many countries where the virus was detected, the virus was successfully controlled, whereas other countries face periodic reoccurrence despite significant control efforts. A central question is to understand the factors favoring the continuing reoccurrence of the virus. The abundance of domestic ducks, in particular free-grazing ducks feeding in intensive rice cropping areas, has been identified as one such risk factor based on separate studies carried out in Thailand and Vietnam. In addition, recent extensive progress was made in the spatial prediction of rice cropping intensity obtained through satellite imagery processing. This article analyses the statistical association between the recorded HPAI H5N1 virus presence and a set of five key environmental variables comprising elevation, human population, chicken numbers, duck numbers, and rice cropping intensity for three synchronous epidemic waves in Thailand and Vietnam. A consistent pattern emerges suggesting risk to be associated with duck abundance, human population, and rice cropping intensity in contrast to a relatively low association with chicken numbers. A statistical risk model based on the second epidemic wave data in Thailand is found to maintain its predictive power when extrapolated to Vietnam, which supports its application to other countries with similar agro-ecological conditions such as Laos or Cambodia. The model's potential application to mapping HPAI H5N1 disease risk in Indonesia is discussed.

animal husbandry | epidemiology | remote sensing | spatial modeling

Highly pathogenic avian influenza (HPAI) H5N1 virus emerged in Southern China in the mid-1990s (1), but the first large-scale epizootic took place in the winter of 2003/2004 in East and Southeast Asia (2). The virus persisted in the region until the winter of 2005/2006 when it spread westward across the Palearctic zoogeographical region (as of August 6, 2007, 60 countries have reported the virus; ref. 3). The impact in the affected countries comprises human disease and death (194 people of 321 cases as of August 16, 2007; ref. 4 and www.who.int/csr/disease/avian_influenza/country/en), mortality in poultry and birds culled to halt the spread of disease, and the loss of local and international trade of poultry and poultry products. In Southeast Asia alone, it has been estimated that HPAI H5N1 virus outbreaks caused the death of 140 million domestic birds with economic losses at ≈$10 billion (5). Importantly, reducing virus circulation in the poultry sector is the best way to prevent human infections and a possible mutation in a form that could pass between humans (6).

The rapid spread of HPAI H5N1 virus results from its ability to transmit through both human and bird host contact systems (7). However, even if HPAI H5N1 virus has been introduced, local development vary greatly as the virus does not become established, spread, and persist everywhere equally. Virus establishment is influenced by the extent of surveillance and early detection, and therefore it is subject to an unknown degree of underreporting bias. Once established, HPAI H5N1 virus spread is believed to be influenced primarily by local trade patterns, density of wet markets, poultry production structure, and disease control and preventive efforts. Persistence is thought to be mediated by domestic ducks because of their potential role as virus reservoir (8–10), but the large live poultry markets also probably contribute (11). However, even when the main risk factors associated with local introduction, spread, and persistence are broadly known, it remains challenging to quantify their relative importance and contribution and to define HPAI H5N1 virus outbreak risk in space and time (location-sensitive "hot spots" and time-sensitive "hot times").

With the panzootic originating and persisting in East and Southeast Asia, it is of interest to concentrate on this region to quantify how established risk factors are associated with disease patterns. Vietnam and Thailand are interesting in that regard because they were both subject to several epidemic waves, applied differential control strategies, succeeded in temporarily controlling the virus, and were faced with periodic reoccurrences (Fig. 1). Vietnam undertook massive repeated vaccination campaigns in combination with other control measures. Thailand has not implemented vaccination and placed emphasis on early detection, prevention of movements in high-risk areas, including premovement testing, and transformation of the free-ranging duck production sector (12). It is difficult to assess whether the reoccurrences resulted from local persistence of the virus or new introductions, but recent results suggest that Southeast Asia may constitute a regional "evolutionary sink" for HPAI H5N1 virus (13), supporting the idea that the region faces periodic reintroductions.

In previous work, it was demonstrated that the risk of HPAI H5N1 virus presence was associated with free-ranging duck numbers in Thailand (10) and the local abundance of both duck and geese in Vietnam (14), in addition to other risk factors such as chicken numbers, human population, and topographical features. Both studies also found evidences of a relationship between HPAI H5N1 virus presence and rice production. Free-ranging duck production in Thailand is largely confined within areas with double

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[†]To whom correspondence should be addressed. E-mail: mgilbert@ulb.ac.be.

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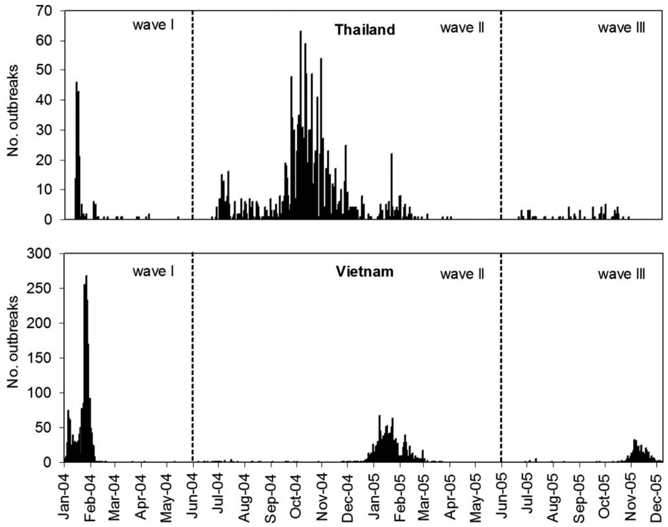

Fig. 1. Temporal distribution of daily HPAI H5N1 virus records reported in Thailand (*Upper*) and Vietnam (*Lower*).

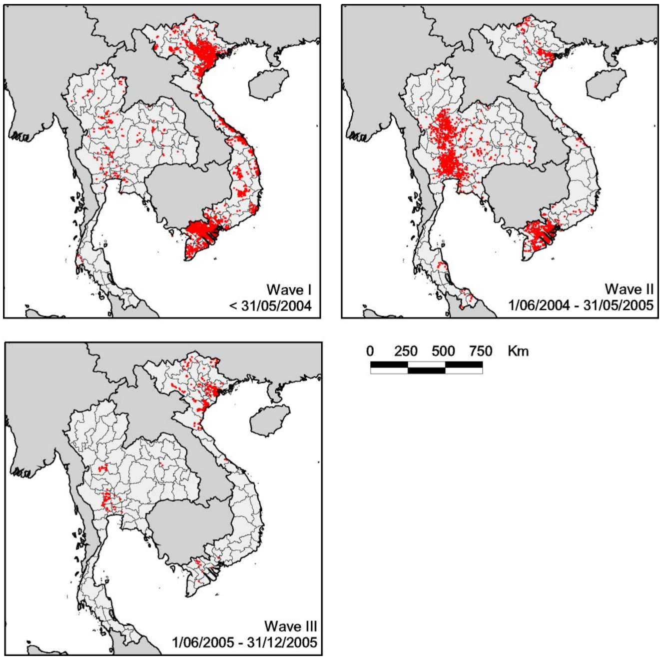

Fig. 2. Spatial distribution of HPAI H5N1 virus records reported during the three main epidemic waves in Thailand and Vietnam.

or triple rice cultivation per year because the rice grain left in the field after harvest provides a low-cost source of feed for duck production (15). Similar coupled production systems associating free-ranging ducks and multiple-rice agriculture are observed across Southeast Asia, including Vietnam (e.g., ref. 16) and Indonesia. Rice paddy fields are an important habitat for free-ranging ducks, but also for wild waterfowl exploiting the same food resource in the wintering season, and thus they may form a critical risk factor in HPAI H5N1 virus introduction, persistence, and spread. In recent years, substantial progress has been achieved in methods for predicting rice crop distributions. It is now possible to routinely map and monitor rice paddy agriculture (17, 18) and cropping intensity in Asia, using images from the Moderate Resolution Imaging Spectroradiometer (MODIS) sensor onboard the National Aeronautics and Space Administration Terra satellite. The satellite image-based algorithms permit the production of maps of cropping intensity, cropping calendar (planting and harvesting dates), and irrigation practices at moderate spatial resolution (250–500 m) and in near-real-time fashion.

This article has three main objectives: first, to compare risk factors associated with HPAI H5N1 virus presence in Thailand and Vietnam during the three epidemic waves that took place between early 2004 and end 2005 by using the same set of risk factors (elevation, human population, and chicken and duck density); second, to evaluate and compare the added value of rice cropping variables for predicting HPAI H5N1 virus presence in the two countries; and third, to evaluate the predictive power of an HPAI H5N1 virus risk map for Southeast Asia, by developing a model for Thailand, testing it for Vietnam, and applying it to the region.

Results

The three H5N1 epidemic waves that have affected Thailand and Vietnam show distinct temporal and spatial distributions (Figs. 1 and 2). The spatial pattern of wave II in Thailand is explored in further detail in Gilbert *et al.* (10) and Tiensin *et al.* (19), and the temporal and spatial patterns of waves I–III in Vietnam are examined in Pfeiffer *et al.* (14). However, the results obtained from the statistical analysis of outbreak distributions in relation to risk factors are more consistent than one would expect on the basis of the contrasted spatial patterns between waves (Table 1). The variables showing the highest level of consistency in their relationship with HPAI H5N1 virus presence are human population, rice cropping intensity, and, to a somewhat lesser extent, duck numbers, all of which were found to be significantly associated with virus presence in both countries. Chicken numbers failed to show as a significant predictor of disease presence, except in wave III in Vietnam. The latter is probably because wave III primarily affected the Red River delta where chickens are far more abundant than in the rest of the country. If one considers only the northern part of Vietnam in the analysis [supporting information (SI) Table S1], chicken number is no longer a highly significant predictor of HPAI H5N1 virus presence. The number of ducks was positively associated with HPAI H5N1 virus presence in Thailand waves I and II and Vietnam wave II. Elevation was negatively related to virus presence only in Thailand during waves I and II, although the significance of the change in −2 log likelihood (LL) upon removal is close to 0.05 for Vietnam wave I. All significant variables showed consistency in similar sign in all models where they were significantly related to HPAI H5N1 virus presence. The predictive power of the models, as measured by the area under the curve (AUC) (Table 1), receiver-operating characteristic (ROC) curves (Fig. S1) and Cohen's kappa is moderate, with the AUC value ranging from 0.66 to 0.88.

In complement with the results presented in Table 1, we verified that the significance of cropping intensity did not result from the fact that free-grazing ducks and farmed ducks were pooled into a single category. In Thailand, the data on free-grazing ducks and farmed ducks were available separately and allowed testing models where they replaced the duck/geese variables. The results showed that (*i*) the free-grazing duck variable is significantly associated with HPAI H5N1 virus presence (change in −2LL upon removal is 8.56, 58.49, and 3.90 for waves, I, II, and III, respectively), and (*ii*) cropping intensity maintains its significance in waves I and II in the presence of the free-grazing duck variable (change in −2LL upon removal is 3.28, 40.83, and 1.85 for waves, I, II, and III, respectively). Farmed ducks were in no instance found to be significantly associated with virus presence.

Despite their moderate predictive power, these five variables appear to provide relatively robust risk prediction. The model

Table 1. Summary results of the autologistic regression models for the three main HPAI H5N1 virus epidemic waves in Thailand and Vietnam between 2004 and 2005

Wave	Cst	Alt	Hpop	Ch	Du	CropMean	ArT	AUC, ± SD	kappa, ± SD	Pseudo-R2, ± SD
Thailand I	−1.05	0.00103* 2.31† $P = 0.129$‡	2.2510^{-5} 5.46 $P = 0.0194$	3.3910^{-6} 2.98 $P = 0.08411$	2.0310^{-5} 7.14 $P = 0.00753$	0.556 4.13 $P = 0.0422$	247 22.4 $P < 0.001$	0.66 ± 0.023	0.26 ± 0.043	0.062 ± 0.019
Thailand II	−1.143	−0.00219 25.7 $P < 0.001$	1.0610^{-5} 5.92 $P = 0.0150$	1.8810^{-7} 1.07 $P = 0.301$	3.0410^{-5} 44.7 $P < 0.001$	0.964 46.8 $P < 0.001$	88.6 65.8 $P < 0.001$	0.79 ± 0.0073	0.49 ± 0.018	0.19 ± 0.012
Thailand III	0.0634	−0.0128 19.3 $P < 0.001$	5.9910^{-5} 2.99 $P = 0.084$	1.6910^{-6} 1.49 $P = 0.222$	5.5110^{-5} 5.04 $P = 0.024$	0.777 2.03 $P = 0.154$	1486 5.58 $P = 0.0181$	0.88 ± 0.034	0.67 ± 0.073	0.40 ± 0.079
Vietnam I	−1.74	−0.00021 3.83 $P = 0.0503$	9.1910^{-5} 90.0 $P < 0.001$	4.7110^{-6} 3.56 $P = 0.0591$	6.0910^{-6} 2.49 $P = 0.115$	0.188 5.73 $P = 0.0167$	63.1 808 $P < 0.001$	0.69 ± 0.0049	0.27 ± 0.009	0.078 ± 0.0042
Vietnam II	−2.67	0.000328 1.39 $P = 0.238$	9.2210^{-5} 19.8 $P < 0.001$	-2.1110^{-6} 1.45 $P = 0.229$	2.8510^{-5} 12.1 $P < 0.001$	0.730 19.3 $P < 0.001$	160 312 $P < 0.001$	0.77 ± 0.0085	0.41 ± 0.018	0.17 ± 0.013
Vietnam III	−1.58	0.000408 1.31 $P = 0.253$	-3.9010^{-5} 3.55 $P = 0.0595$	3.4310^{-5} 14.1 $P < 0.001$	$6.4\ \ 10^{-6}$ 1.20 $P = 0.274$	0.497 4.36 $P = 0.0367$	245 198 $P < 0.001$	0.66 ± 0.018	0.25 ± 0.031	0.055 ± 0.014

All values result from 500 models including all positive and an equivalent number of bootstrap-selected negatives. Alt, Hpop, Ch, Du and CropMean are the average altitude, human population, chicken number, duck number, and mean number of rice crop harvested 500-m pixels, respectively, in each subdistrict (Thailand) or commune (Vietnam). ArT is the autoregressive term.

*Average coefficient.

†Average change in −2LL upon variable removal.

‡Significance of the change.

with best-fit parameters based on Thailand wave II data applied to predicting HPAI H5N1 virus presence in other epidemic waves provides very close predictive power to that of each wave's best-fit model (Fig. 3). In Thailand, the wave II model predicts wave I and wave III risk with an AUC of 0.66 and 0.74, respectively, which are comparable with the 0.66 and 0.88 of each best-fit model. More remarkably, the Thailand wave II model predicts Vietnam waves I, II, and III with AUC values of 0.66, 0.74, and 0.61, respectively, which are comparable with 0.69, 0.77, and 0.66 of each best-fit model. This result implies that spatial and temporal extrapolation of the model from Thailand wave II to other areas and other years is possible at relatively low cost in terms of loss of predictive power. On average, the AUC of the Thailand wave II model accounted for 93.5% of the AUC of the best-fit models.

Discussion

The results presented here highlight three main findings: (*i*) although large differences may have resulted from the contrasting and dynamic disease management in Thailand and Vietnam, there is a common, consistent risk pattern reemerging for the three waves observed in Thailand and Vietnam; (*ii*) cropping intensity is consistently associated with HPAI H5N1 virus presence in Thailand and Vietnam; and (*iii*) the model of HPAI H5N1 virus risk developed in Thailand with the data from the second epidemic wave maintains its predictive power when applied to other epidemic waves or other regions, indicating that the model predictions can be extrapolated in space and time.

Disease management in the two countries changed markedly over the 3 years, and the results of the risk factor analysis need to be considered by taking account of these changes. During the first wave, there was undoubtedly a problem with disease reporting because the highest priority for the two countries was to curb the epidemics in an emergency situation, involving logistically demanding culling and depopulation. These operations took most of the resources, distracting attention from disease detection and notification. At the same time, the public and farmers were not yet well informed on risky practices of HPAI transmission, which may have assisted the spread of the disease and could explain some of the findings obtained for wave I. In Thailand, outbreaks were scattered and less numerous than during Vietnam wave I, and underreporting may explain part of this difference. With human population density as the top predictor of wave I HPAI H5N1 presence in Vietnam this suggests a relatively large contribution of human-related transmission during that wave. The relatively lower importance of duck and cropping intensity, both variables interpreted here as defining the ecological niche of the virus, is then not surprising as it may have been hidden by human-mediated transmission or may also result from reporting bias.

By the time the second wave emerged, disease surveillance systems were fully in place. Thailand implemented its x-ray surveys involving the participation of several hundred thousand inspectors searching door to door for evidence of HPAI presence (19). The second wave epidemic started earlier in Thailand and was concentrated to areas where free-grazing ducks are raised. In Vietnam, the epidemic wave was concentrated in the Mekong delta where domestic ducks are most abundant. At the time of the second wave, people and farmers were much better informed about the risk of human-mediated transmission of HPAI. The second-wave epidemic was significant for both countries, highlighting that control efforts were still insufficient. In both countries, the number of ducks and cropping intensity constitute the most important predictors during the second wave, followed by human population. Although the role of ducks as a reservoir of virus may already have played a role during wave I in Thailand, the results suggest it played a more prominent role during the second wave.

Thailand and Vietnam both undertook major efforts to step up control efforts. Thailand targeted domestic ducks by implementing systematic annual testing, imposing premovement testing, and initiating the transformation of the free-ranging duck sector into farms. Vietnam undertook massive vaccination campaigns, targeted also at domestic duck populations. Both strategies were apparently very effective in that the third wave that started in July 2005 involved a very low number of outbreaks in Thailand

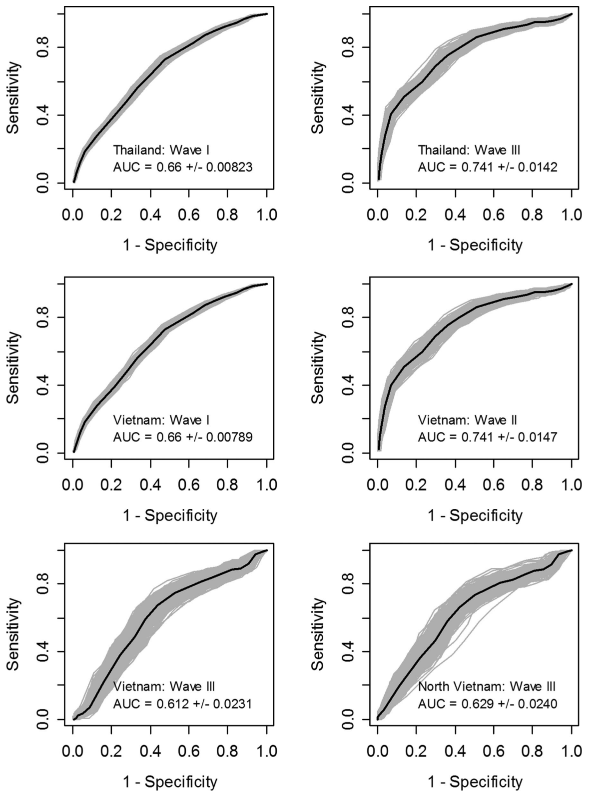

Fig. 3. ROC curves of the predictive power of the Thailand wave II multiple logistic regression model on the presence/absence of HPAI H5N1 virus at the subdistrict (Thailand) or commune (Vietnam) level (gray areas, 500bootstrap ROC curves; black lines, average ROC curve).

and was confined mostly to the northern part of the country in Vietnam. With control measures targeted at ducks, which decouples the linkage between free-grazing ducks and paddy rice fields, ducks and rice cropping intensity became less strong predictors of the HPAI H5N1 presence in wave III.

The statistical relationship between ducks and HPAI H5N1 virus presence and the role of ducks as reservoir has been discussed elsewhere (10, 14, 20–22). However, we found a strong and consistently positive association with rice cropping intensity across epidemic waves and countries. Rice descriptors may form a better risk predictor than free-grazing duck number numbers for causal reasons. For example, the x-ray survey that provided the duck data was carried out at the village level, and flocks were assigned to the village where the duck raisers lived. However, free-ranging duck flocks are moved around to multiple, adjacent, and nonadjacent districts during their life span (21). The added value of a rice variable over a free-ranging duck variable may therefore ensue from the fact that the rice descriptor defines more precisely where the ducks are moved, fed, and kept during high-risk periods with extensive opportunities for virus release and exposure. Moreover it is possible that rice paddy fields form a temporary habitat of other bird species also feeding on leftover rice grains that may become infected and initiate a local outbreak. Finally, rice fields are frequently flooded, and water presence improves viral persistence in the environment as opposed to dry soils.

Given the role of free-grazing duck as a strong driver of the spatial distribution of HPAI risk in Thailand and Vietnam, demographics and seasonality of the free-grazing duck production sector may also help in the understanding of the temporal variability in HPAI H5N1 virus prevalence. Exploratory results obtained from Thailand suggest that this may indeed be the case. Most duck restocking in the form of hatching and subsequent release in nurseries takes place in July/August, so that the rapidly growing young ducks benefit from the forage at the peak of the monsoon-associated rice harvest in November/December/January. Juvenile ducks are known to be of particular importance in avian influenza ecology (23, 24), and because of these demographics, the peak in the proportion of juveniles in the flocks occurs in September/October; at the time when the peak of the Thailand second wave outbreak took place. So far this evidence is circumstantial, but it suggests the need for the collection of comprehensive data quantifying duck production in space and time in all duck–rice areas where HPAI H5N1 virus persistence risk is to be quantified.

A traditional problem with risk distribution maps predicted by statistical models, based on linking the presence/absence of a disease or species to a series of predictors, is that they often lose much of their predictive power when extrapolated outside of the spatial range of their training data, which makes external validation difficult. It is thus quite remarkable that this model based on Thailand data loses so little predictive power when validated in Vietnam, which gives confidence to extrapolating the model to adjacent areas. Of course, the model has only moderate predictive power, but it is largely compensated for by its extrapolation ability.

It thus appeared justified to produce risk maps for adjacent regions with similar farming conditions, such as Vietnam, Laos, or Cambodia (Fig. 4). In the latter two countries, the model predicts only restricted HPAI H5N1 virus spread in Laos, a higher risk in the areas surrounding the Tonle Sap Lake in Cambodia, and, even more so, in the transfrontier area of the Mekong delta. A risk map based on the same model was also produced for Indonesia (Fig. 4), but it needs to be interpreted cautiously, for several reasons. First, comparatively higher disease circulation is taking place in Indonesia as compared with Thailand and Vietnam. This assumption is based on regular reports of human cases (4) reflecting disease circulation in poultry and results from participatory disease surveys. Observations in the field suggest a much higher relative contribution of people and chickens to disease spread and persistence, and with it, a relatively lower importance of duck–rice contribution to the spatial definition of HPAI risk. At present, these observations are particularly difficult to evaluate because of the scarce and patchy data on HPAI H5N1 virus distribution in Indonesia. Second, eco-climatic conditions differ from those in the Mekong countries. Precipitation is less seasonal and more evenly distributed across the monthly calendar. As a result, the divide between rain-fed rice crop areas with a single rice crop per year and areas with irrigated rice and two or three rice crops per annum is less clear. The mapping of rice cropping intensity is also complicated by the local relief of volcano landscapes that results in small paddy fields asynchronously planted at any time of the year (25). Hence, although free-grazing ducks are known to be associated with rice production, the scale at which this relationship applies, and its spatial and temporal characteristics may differ from those in the Mekong countries. For the above reasons, HPAI H5N1 data for Indonesia for 2003–2005 were not used to evaluate the model quantitatively, but are provided for illustrative purpose (Fig. 4).

An applied result of this study is that the distribution of rice cropping intensity can readily be established at any time and be used to complement traditional duck census data. Remote sensing data are available at a much higher spatial and temporal resolution than traditional censuses, thus allowing fine-scale risk mapping. So the prospect of fine-scale spatiotemporal prediction of duck production and the associated HPAI risk is to be considered as a potential medium-term output still requiring significant development and understanding of spatiotemporal relationships between duck and crop farming. Areas of first interest include Thailand and Vietnam, where detailed data are available for performing follow-up analyses, in particular to

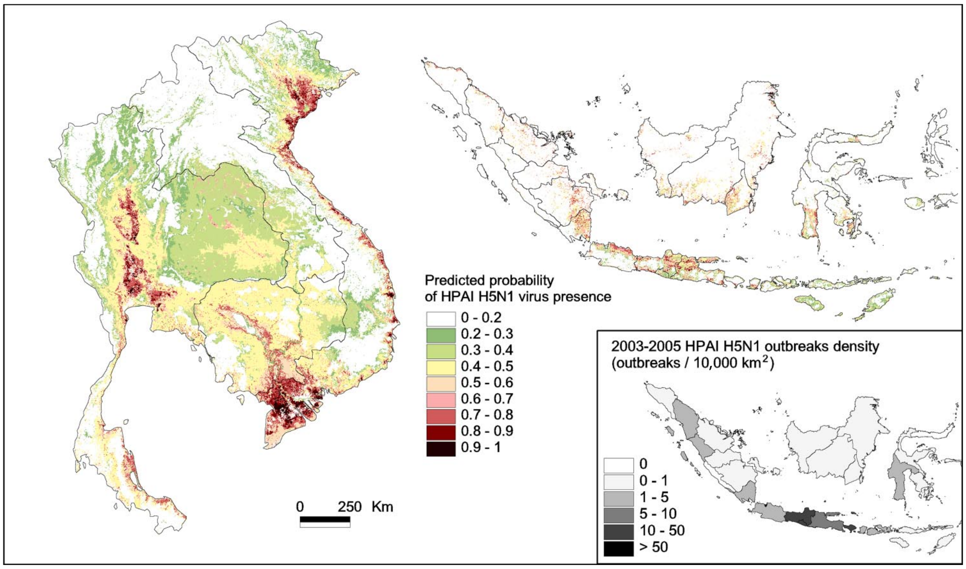

Fig. 4. Predictions of HPAI H5N1 virus relative risk of presence in Thailand, Laos, Cambodia, and Vietnam based on the Thailand wave II model using the following parameters: Logit(p) = $-0.903 - 3.11\ 10^{-3}$ Alt $-\ 1.62\ 10^{-4}$ HpopDn $-\ 2.47\ 10^{-5}$ ChDn $+\ 5.40\ 10^{-4}$ DuDn $+\ 0.968$ CropI, where Alt, HpopDn, ChDn, DuDn, and CropI are the elevation, human population density, chicken density, duck density, and annual number of rice crop harvest, respectively, in each 500-m pixel. (*Inset*) Shown is the density of HPAI H5N1 outbreaks recorded in Indonesia in 2003–2005 expressed as number of outbreaks per 10,000 km^2.

contrast the Mekong and Red River deltas. But this recommendation extends to other countries, even outside of Asia, where duck production may also be an important driver of HPAI H5N1 virus persistence. For example, preliminary unpublished reports indicate that several tens of millions of ducks and geese are concentrated in the Nile Delta of Egypt; similarly, the Hadejia-Jama'are river system in Kano (Nigeria) is an important area for duck production.

One should also acknowledge that considerable variability remains unexplained by our model, given that duck distribution is only one of the drivers of HPAI disease risk and should be considered in conjunction with other factors such as the diverse production systems for terrestrial poultry, role of wet markets, contacts with migratory and resident avifauna, and environmental conditions affecting persistence of the virus outside the host in the environment (26).

Materials and Methods

Data. Data on HPAI H5N1 virus outbreaks in Thailand included laboratory-confirmed cases recorded between January 23, 2004 and December 31, 2005, compiled by the Department of Livestock Development, Ministry of Agriculture and Cooperatives, Bangkok, Thailand. Data on HPAI H5N1 virus outbreaks in Vietnam were collated by the Vietnam government's Department of Animal Health between January 10, 2004 and December 31, 2005. Data on Indonesia were provided by the Directorate General of Livestock Services in the Ministry of Agriculture, Jakarta, Indonesia, but these data were used only for illustrative purpose.

All epidemiological data were grouped into three main epidemic waves (Fig. 1). HPAI H5N1 virus struck the two countries for the first time during the winter of 2003/2004, with the first cases being officially notified in December (Vietnam) and January (Thailand). Both countries established surveillance and control systems, and this first wave of outbreaks was followed by a period with virtually no new cases during May and June. New cases started to emerge again in July. The countries were better prepared at the onset of this second wave that reached its highest incidence in October 2004 (Thailand) and January 2005 (Vietnam). In 2005, both countries succeeded in controlling the disease, again with no outbreaks during May and June, but the virus again re-emerged in July. When this third wave started, Thailand and Vietnam had been investing significant effort into halting the disease, with early detection, control of duck movements, and premovement testing in Thailand and massive vaccination campaigns combined with trade restrictions in Vietnam. Therefore, it appears justified to group the epidemiological data into three epidemic waves. The first wave (termed wave I) pools the data from January 2004 to May 2004, the second wave (wave II) pools the data from June 2004 to May 2005, and the third wave (wave III) pools the data from June 2005 to December 2005.

This work aimed to limit the number of risk factors to a few carefully chosen variables scrutinized for their quantitative association with HPAI H5N1 virus presence risk in space and time for each epidemic wave and country. Some variables available for Thailand were not available for Vietnam, and vice versa, prompting us to restrict the study to the variables available in both countries. All data were collated at the subdistrict level in Thailand (median area: 40.4 km^2) and commune level in Vietnam (median area: 14.4 km^2).

Poultry census data for Thailand were collected from October to mid-November 2004 during the x-ray survey organized by the Department of Livestock Development (Bangkok, Thailand), involving the participation of several hundreds of thousands inspectors searching door to door for evidence of HPAI presence. These inspectors collected detailed information on domestic poultry numbers and species in each and every farm and household. For the present study, five poultry variables were extracted from the x-ray survey database: farm duck numbers, which includes meat and layer ducks raised in farms, free-grazing ducks, geese, native chickens, and chickens used in industrial production (broilers and layers). Poultry data for Vietnam were extracted from the 2001 Agricultural Census database (General Statistics Office of Vietnam), where two poultry categories could be extracted at the commune level: (*i*) chickens and (*ii*) ducks plus geese. To obtain an identical variable applicable to both countries, native and farm chicken data from Thailand were pooled into a single chicken variable. Similarly, ducks, free-grazing ducks, and geese were pooled into a single duck and geese variable. We chose to test chicken numbers in the model because most recorded outbreaks were observed in chicken flocks. The duck and geese number was taken as a variable because previous findings identified ducks as an important risk factors, and recent results highlighted domestic waterfowl as the foremost host species among poultry, with a much higher virus prevalence on a year-round basis than in chickens (22).

Additional variables obtained at the subdistrict (Thailand) and commune (Vietnam) levels comprise: human population (27, 28) and average elevation [90-m resolution Digital Terrain Model from the Shuttle Radar Topography Mission data, STRM V3 (ref. 29 and http://srtm.csi.cgiar.org)]. Human population was chosen because it is an indicator of trade-related viral traffic and, possibly, because it creates a bias in reporting. Elevation was included because it was found to be a significant risk predictor in both Thailand (10) and Vietnam (14), is easily obtained for any country, and is considered to be a surrogate indicator of other unmeasured variables related to HPAI risk. High-elevation areas have higher slopes and land cover dominated by forests and permanent vegetation. In contrast, flat plains, deltas, and coastal areas are dominated by agriculture and a mixture of intensive uses of the land. In addition, wetlands, rivers, canals, ponds, and irrigation networks are concentrated in those lowlands.

Satellite-based mapping algorithm using data from the MODIS of the Terra satellite allows identification and tracking of image pixels that experienced flooding and rice transplanting over time, based on temporal profile analysis of the Normalized Different Vegetation Index, Enhanced Vegetation Index, and Land Surface Water Index (17, 18). The method permits an estimation of the cropping intensity (number of rice cropping in a year) within individual 500-m pixels. This variable estimated for 2004 was aggregated by averaging the pixel values within each subdistrict and commune of Thailand and Vietnam, respectively. This variable was incorporated in the analysis because of preliminary results indicating that the presence of rice paddy fields is associated with HPAI H5N1 virus presence risk (10, 14), and that free-grazing ducks are found in much higher numbers where cropping intensity is high (15).

Analyses. We used a multiple logistic regression framework to relate HPAI H5N1 presence to the predictors by pooling the data by epidemic wave and country, converting the number of outbreaks into presence or absence. The model was built by using altitude, human population, chicken, ducks and geese, and mean rice cropping intensity as predictors of HPAI H5N1 presence for each of the three synchronous epidemic waves in Thailand and Vietnam at the subdistrict and commune levels, respectively.

An autologistic approach was used to account for spatial autocorrelation in the response variable, by forcing an additional covariate, termed autoregressive term, into the multiple logistic regression model (30). The autoregressive term accounts for spatial dependency in the response variable and is estimated by averaging the presence/absence among a set of neighbors defined by the limit of autocorrelation, weighted by the inverse of the Euclidean distance (30). The extent of the autocorrelation in the response variable is obtained from the range of the spatial correlogram $\rho(h)$ of the response variable, here HPAI H5N1 virus presence or absence.

Another problem that arises when applying logistic regression models to disease data are that low prevalence values for the response variable ($<10\%$) tend to bias model performance metrics (31). For each model, we selected all HPAI H5N1 virus-present subdistricts or communes and a randomly selected equivalent number of negative samples. We then bootstrapped this operation 500 times. For each model and each set of negatives, we estimated the coefficient of each variable, the change in -2LL of each variable upon removal, the AUC of the ROC plots, Cohen's κ index, and pseudo-R^2 as indicators of model performance. We then averaged these estimates over the 500 bootstraps and estimated the significance of the average change in -2LL of each variable upon removal. The individual variable coefficient and change in -2LL upon removal were estimated based on an autologistic model, and the AUC, ROC curve, Cohen's κ, and pseudo-R^2 were estimated based on the same model without the autoregressive term.

Our second objective was to test the performance of a model built for a particular time period and region for predicting HPAI H5N1 virus presence for a different time period and region, i.e., to measure the ability of a model to extrapolate risk predictions in space and time. The parameters of the best-fit model for Thailand wave II were used to predict HPAI H5N1 virus presence in Thailand waves I and III and Vietnam waves I, II, and III. The model performance was estimated by using the AUC and ROC plot, by bootstrapping data to force an equivalent number of positive and negatives in the test sets.

Finally, we used the model based on Thailand wave II HPAI data to predict HPAI H5N1 virus presence probability for the whole of the Mekong region and for Indonesia, at a spatial resolution of 500 m, using topographic data from the STRM V3 (29, 32), human population data from the Global Rural-Urban Mapping Project (33, 34), and poultry population data from the Global Livestock Production and Health Atlas database (35). Poultry data were included for Cambodia (chicken: province level; ducks: province level; except for the provinces Ratana Kiri and Svay Rieng for which the information was missing and replaced by the national average duck density), Laos (chickens: province level; ducks: province level), and Indonesia [chicken: Province level, ducks: district level for Java and Sumatra (36), province level elsewhere], and 500-m rice cropping intensity predictions.

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