

Avian influenza in ocean going birds of NL: A threat to the province's poultry industry?

Agriculture and Agrifoods Research and Development Program

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Submitted by: Dr. Hugh Whitney

Chief Veterinary Officer

Forestry and Agrifoods Agency

Executive Summary

Avian influenza (AI) is a viral disease of commercial poultry that has caused severe economic and trade disruptions in Canada and abroad. The primary source of the avian influenza viruses is wild birds, with waterfowl (ducks, geese) considered to be the most important reservoir. Most recently, British Columbia saw an outbreak of highly pathogenic avian influenza (HPAI) H5N2 in December 2014 and non-zoonotic H5N1 in February, 2015. As this province receives millions of wild birds from Eurasia as normal migrants or, occasionally, as accidentals, it was decided that research and surveillance should be directed at answering the questions of whether AI viruses normally cross the North Atlantic into this province and if so, what viruses and which bird species are involved.

Through the direct support of research into the presence and variety of AI viruses in this province, we are better able to advise the poultry community on how to prevent entry of these viruses into their premises. In the event that an AI outbreak occurs in this province, the act of having performed advance research will 1) assist in identifying the source of the virus and 2) also provide a source of scientific expertise to help provide valuable information during the eradication of the disease. The budget awarded to the AI effort in 2014/15 from the Agriculture and Agrifoods Research and Development Program was \$64,833.00, but the contribution from various sources in the past 10 years puts the total funding amount over 1 million dollars.

This year, it was determined that the application of high-throughput sequencing has demonstrated great promise to fully sequence genomes of multiple viruses more quickly at a lower cost than traditional Sanger sequencing. Also, it was determined that wild birds that have previously been infected with AI pass maternal antibodies onto their embryos. Sequencing is still on-going from samples collected last year to meet additional project goals.

Background

Avian influenza (AI) is a viral disease of commercial poultry that has caused severe economic and trade disruptions in Canada (e.g. British Columbia in 2004, 2005, 2009 and 2014/15, Saskatchewan in 2007, Manitoba in 2010) and abroad. Most recently, British Columbia saw an outbreak of highly pathogenic avian influenza (HPAI) H5N2 in December 2014 and non-zoonotic H5N1 in February, 2015. This outbreak affected 13 premises which led to the destruction of more than 240,000 commercial birds. During this outbreak, wild bird surveillance was enhanced to determine if the AI variant causing the outbreak was related to the highly virulent strain circulating in Eurasia. This puts further emphasis on the importance of wild bird surveillance. Due to this ongoing threat, industry

and government have invested significant time and money into the development, implementation and monitoring of biosecurity infrastructure and protocols.

The primary source of the avian influenza viruses is wild birds, with waterfowl (ducks, geese) considered to be the most important reservoir. However, it is becoming clear that other migratory birds such as gulls and seabirds are also important in avian influenza virus movements. Though there are many types of AI virus, one in particular has caused global concern in both the poultry and human health fields, highly pathogenic H5N1 (HPH5N1). This virus was first detected in Hong Kong in 1997 and re-emerged in 2003 with outbreaks in poultry and wild bird populations, as well as to a limited extent in human populations. Since that time it has spread across Eurasia and into Africa with total bird mortalities (due to infections and slaughter for control purposes) in the 100s of millions. The virus continues to re-emerge in affected areas. At the current time, other strains of great concern are H7N9, which is circulating in China and causing economic and public health threats, and H5N8, which has caused significant mortalities in South Korea in both wild birds and farm operations.

A long standing question had been whether these HPAI viruses (or any other future HPAI) would cross into North America. The two most likely entry points would be across the Bering Strait (Russia to Alaska) or across the North Atlantic Ocean. The United States Geological Services (USGS) states "it is widely believed that if HPAI were introduced via North American migratory birds, Alaska is most likely the location for the initial introduction". The H5N2 virus discovered in the BC farms is related to the Eurasian HPH5N1 and Korean H5N8 viruses, and there have now also been discoveries of related H5N8 and H5N2 viruses in dead wild birds in several Pacific states of the U.S.

As this province receives millions of wild birds from Eurasia as normal migrants or, occasionally, as accidentals, it was decided that research and surveillance should be directed at answering the questions of whether AI viruses normally cross the North Atlantic into this province and if so, what viruses and which bird species are involved.

Rationale for Investigation

Through the direct support of research into the presence and variety of AI viruses in this province, we are better able to advise the poultry community on how to prevent entry of these viruses into their premises. We have been focusing on three major groups of wild birds: seabirds, gulls and waterfowl. It is seabirds and gulls that are most likely to directly transport viruses from Eurasia to Atlantic Canada, but waterfowl are also important as possible amplifiers of novel viruses and for possible transfer to poultry. This is allowing us to determine if there is crossover of viruses between gulls and ducks, as well as to see how duck viruses in this province compare to those from elsewhere in North America.

In the event that an AI outbreak occurs in this province, the act of having performed advance research will 1) assist in identifying the source of the virus and 2) also provide a source of scientific expertise to help provide valuable information during the eradication of the disease. To further explain, the fact of having done advance research will establish a library of AI viruses known to be circulating in this province. These viruses are usually associated with a host species. Therefore virus found on a commercial premise may be matched with virus in the library suggesting a putative source host species. In the case of managing an outbreak, the fact of having invested in local expertise will assure the presence of laboratory and academic support to help answer further questions related to the virus' origin.

The public health community is also extremely interested in the influenza research that is carried out here as they spend a lot of time with influenza preparedness and need to know what viruses are circulating in the province. This research also has relevance for wildlife management. In addition to waterfowl, some of our target seabird species and known hosts of AI are murre, which are hunted in the province.

Funding and Partnerships

The total budget awarded from the Provincial Agricultural Research & Development Program for the 2014/15 fiscal year was \$64,833, broken down by:

Temporary Salary	\$27,500
Professional Services	\$15,333
Supplies	\$22,000

The total contribution by Agriculture Research Initiative and Provincial Agriculture Research & Development Program since the commencement of this research project has been:

Year	Amount
2014/15	\$64,833.00
2013/14	\$109,500.00
2012/13	\$87,500.00
2011/12	\$205,000.00
2010/11	\$85,000.00

Additional funding has been awarded to this project and is detailed in the table below:

Source	Amount	Year	Purpose
Animal Health Division	\$27,500.00	2014/15	Supplement salary
NSERC	\$10,000.00	2015	Andrew Lang: Discovery Grant
	\$10,000.00	2014	Andrew Lang: Discovery Grant
	\$10,000.00	2013	Andrew Lang: Discovery Grant
	\$4500.00	2013	Natasha Walzthoni fellowship
	\$10,000.00	2012	Andrew Lang: Discovery Grant
	\$4500.00	2012	Natasha Walzthoni fellowship
	\$10,000.00	2011	Andrew Lang: Discovery Grant
	\$17,500.00	2011	Michelle Wille stipend
	\$10,000.00	2010	Andrew Lang: Discovery Grant
	\$17,500.00	2010	Michelle Wille stipend
	\$10,000.00	2009	Andrew Lang: Discovery Grant
Environment Canada	\$12000.00	2015/16	Sheena Roul fellowship
	\$8000.00	2014/15	Sheena Roul fellowship
	\$15,000.00	2013/14	Core EC operating funds
	\$18,000.00	2012/13	Core EC operating funds
	\$31,000.00	2011/12	STAGE (Strategic Technology Applications of Genomics in the Environment)
	\$55,000.00	2010/11	Lab set-up at MUN
	\$19,000.00	2010/11	STAGE
	\$17,727.00	2009/10	STAGE
	\$12,000.00	2008/09	STAGE
Memorial University	\$9000.00	2015	Jessica Benkaroun
	\$7000.00	2015	Sheena Roul fellowship
	\$7000.00	2014/15	Ashley Kroyer fellowship
	\$9000.00	2014	Yanyan Huang fellowship
	\$4500.00	2014	Ashley Kroyer summer fellowship
	\$9000.00	2014	Jessica Benkaroun
	\$7000.00	2014	Sheena Roul fellowship
	\$9000.00	2013	Yanyan Huang fellowship
	\$500.00	2012/13	Natasha Walzthoni: Honours research
	\$9000.00	2012	Yanyan Huang fellowship
	\$9000.00	2011	Yanyan Huang fellowship
	\$3,000.00	2010	Michelle Wille merit fellowship
	\$500.00	2009/10	Ashley Dobbin: Honours research
	\$500.00	2009	Alissa Granter: Honours research
Chinese Fellowship	\$20,000.00	2014	Yanyan Huang fellowship
	\$20,000.00	2013	Yanyan Huang fellowship
	\$20,000.00	2012	Yanyan Huang fellowship
	\$20,000.00	2011	Yanyan Huang fellowship

Methods and Implementation

This study has been ongoing since 2006 and has involved the sampling of wild birds (either dead or alive) and screening for AI virus, H5/H7 detection, culturing of viruses and genome sequencing. Cultured viruses were used for further experiments to determine the properties of the viruses that determine the species of birds they are able to infect.

The sites for sampling vary but have included Witless Bay Ecological Reserve (Gull Island and Great Island), Robin Hood Bay, St. John's Harbour, Quidi Vidi Lake (and other sites in and around the city), Conception Bay (Kelly's, Little Bell and Carbonear Islands), Cape St. Mary's, Middle Lawn Island (Burin), Cabot Island, Baccalieu Island, Funk Island and the Gannet Islands (Labrador). Sampling of dead birds is opportunistic and includes any wild birds submitted to the Animal Health Division for postmortem examination and mass die-offs of wild birds as had happened due to the spring ice entrapments (murre), starvation events (gulls), or sanctioned culling (gulls at Robin Hood Bay landfill).

The essential scientific approach of this research entails sampling for the presence of AIV from available wild birds. From there, the samples were processed by polymerase chain reaction (PCR) to determine if they are positive for AI. The PCR is a technique used to amplify a section of RNA, generating numerous copies of that RNA sequence used to identify the virus. From there the viral genomes were sequenced (In Torrent) at the Lang Laboratory (MUN), which characterizes them genetically and enables comparison among viruses. The Lang Laboratory is also equipped to culture AI viruses using the traditional egg system, wild bird eggs and tissue cultures, which is a recent addition to the project.

Results and Discussion

1) Development of a high-throughput sequencing method for AIV genome sequence

Ion torrent high-throughput sequencing technology was applied to AI virus genome sequences to improve the characterization of the diversity and evolutionary dynamics of AI viruses in wild birds. This method allowed determination of the full genomic composition of identified AI viruses and characterization of multiple viruses at one time. During the trial, only partial sequences of some gene segments of some viruses were able to be obtained. Therefore, some optimization in the procedure is required.

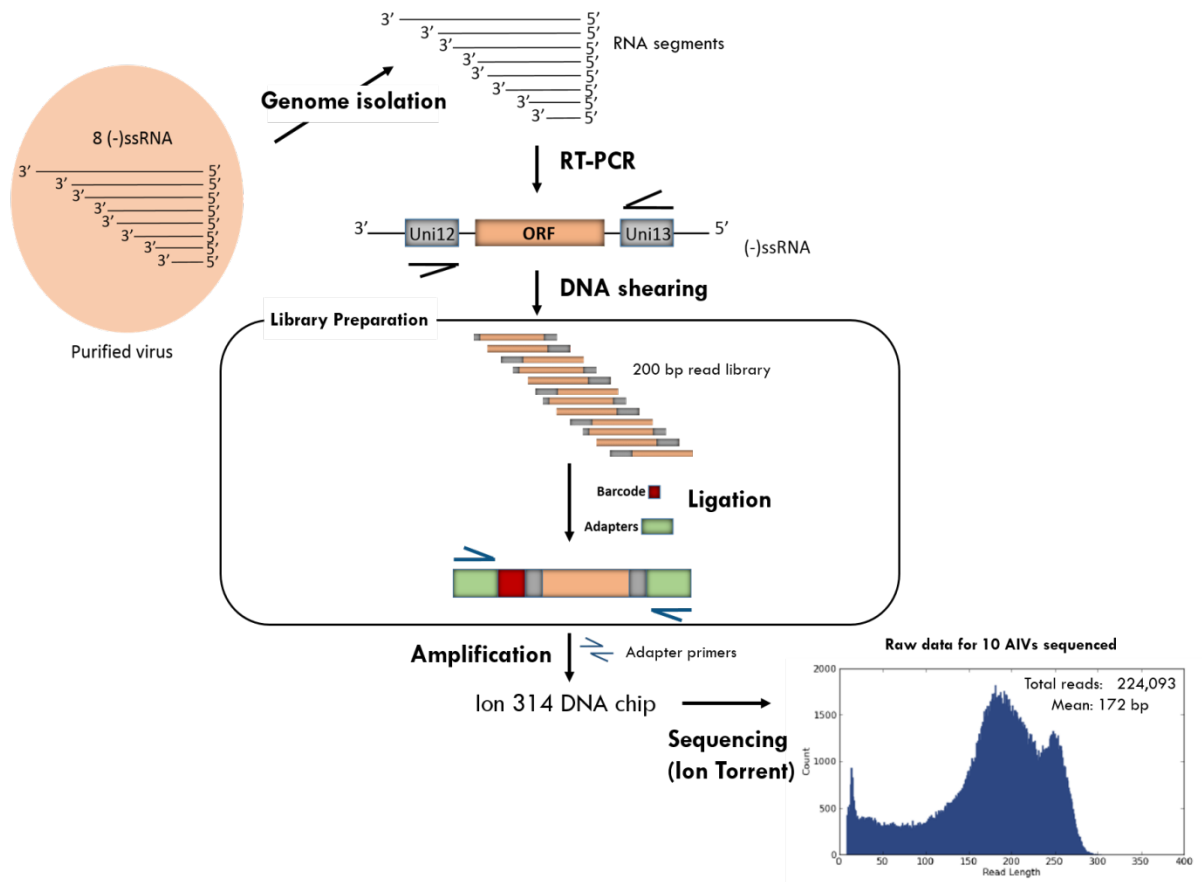


Figure 1: Ion Torrent high-throughput sequencing workflow for multiple AIV genomes.

In comparison to traditional Sanger sequencing, the application of high-throughput sequencing has demonstrated great promise to fully sequence genomes of multiple viruses more quickly at a lower cost.

2) AIVs evolution study through experimental reassortment and mutation assays.

During this project, reassortment and mutation have been investigated in wild birds. Common Murre, Herring Gull and Atlantic Puffin eggs have been collected over the past two summers and co-infection has been performed using two or three different AI virus isolates. Two days post-infection, the allantoic fluids were harvested and stored. Allantoic fluids were used in cell culturing in order to isolate and characterize single viruses originated from reassortment experiments. The use of high-throughput sequencing will then determine the genetic sequences of each reassortant viruses.

Co-infection
Murre virus X Gull virus X Duck virus

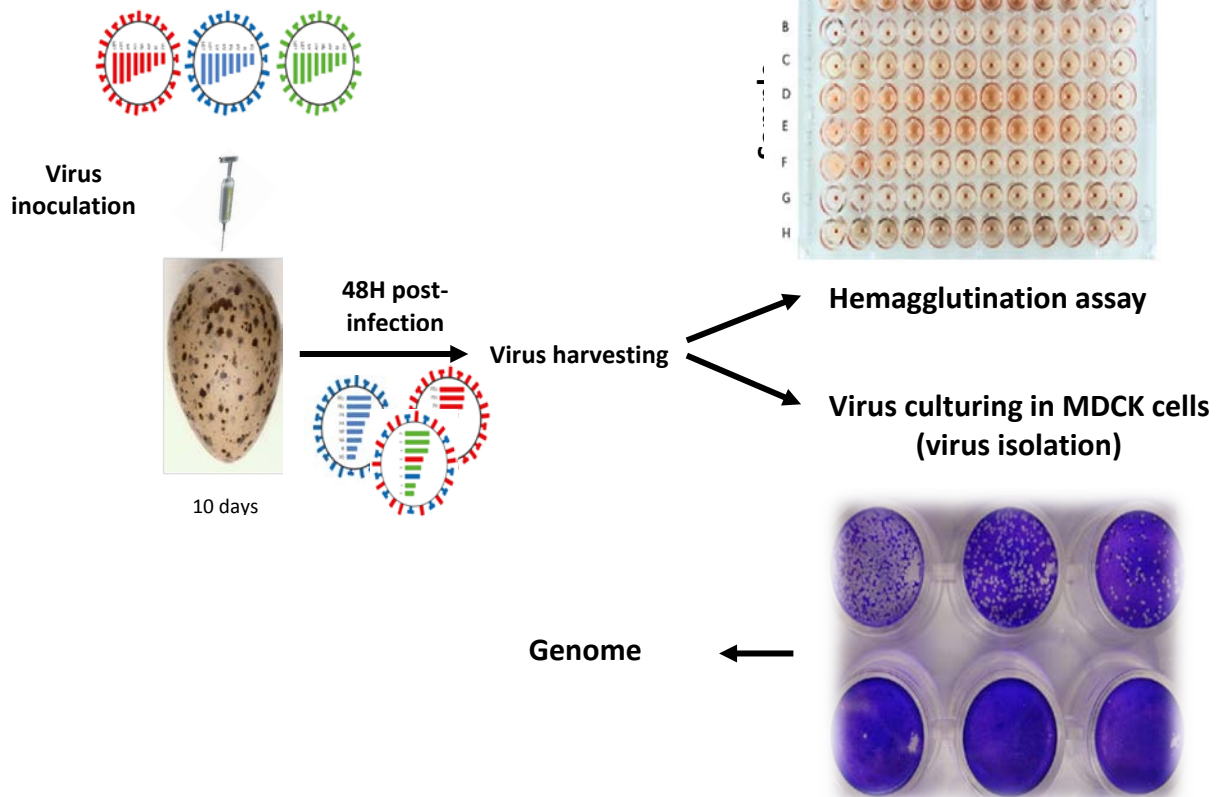


Figure 2: Diagrammatic representation of the reassortment assay process.

3) Detection of AIV virus from environmental samples

Last summer, water samples from ponds occupied by waterfowls were collected and tested for the presence of AI virus. Positive samples were identified by RT-PCR, cloned and characterized by sequencing. Positive samples were detected by RT-PCR, but after sequencing, the results were highly similar to previous work. At this point, we are not sure if we are dealing with true sequences from the environment or with contaminants from past RT-PCR sequencing.

In addition, wild bird eggs were also tested for the presence of maternal antibodies specific to AI virus. Extractions of egg yolks were tested and 40% were found to have maternal antibodies (a high prevalence). This shows that female adults previously infected with AIV have transmitted protection through specific antibodies against AI virus to the embryo.

4) Determination of the non-coding region sequences of the avian influenza virus genomes

Genomic non-coding regions of AI virus isolates are known to be important for virus replication of wild bird isolates. These regions were investigated to highlight variation and specificity that could be involved in host restriction, virus adaptation and evolution. We obtained the identification of gene's non-coding regions of murre, duck, and gull viruses. We are still in the process to complete the identification.

Communications and Outreach

Alissa Granter

Granter, A., Wille, M., Robertson, G. J., Whitney, H., Ojkic, D. & Lang, A. S. (2010). The genome sequence of an H11N2 avian influenza virus from a Thick-billed Murre (*Uria lomvia*) shows marine-specific and regional patterns of relationships to other viruses. *Virus Genes*, *41*, 224-230.

Michelle Wille

Wille, M., Whitney, H., Robertson, G. J., Ojkic, D. & Lang, A. S. (2011). Reassortment of American and Eurasian genes in an influenza A virus (H13N2) isolated from a Great Black-backed Gull (*Larus marinus*), a species demonstrated to move between these regions. *Archives of Virology*, *156*, 107-115.

Wille, M., Robertson, G. J., Whitney, H., Bishop, M. A., Runstadler, A. & Lang, A. S. (2011). Extensive geographic mosaicism in avian influenza viruses from gulls in northern hemisphere. *PLoS ONE*, *6*(6), 1-10.

Wille, M., Huang, Y., Robertson, G. J., Ryan, P., Wilhelm, S. I., Fifield, D., Bond, A. L., Granter, A., Munro, H., Buxton, R., Jones, I. L., Fitzsimmons, M. G., Burke, C., McFarlane-Tranquilla, L., Rector, M., Takahashi, L., Kouwenberg, A., Storey, A., Walsh, C., Hedd, A., Montevicchi, W. A., Runstadler, J. A., Ojkic, D., Whitney, H. & Lang, A. S. (2014). Evolution of seabirds in Newfoundland and Labrador, Canada, as hosts of influenza A viruses. *Journal of Wildlife Diseases*, *50*(1), 98-103.

Yanyan Huang

Huang, Y., Wille, M., Dobbin, A., Robertson, G. J., Ryan, P., Ojkic, D., Whitney, H. & Lang, A. S. (2013). A 4-year study of avian influenza virus prevalence and subtype diversity in ducks of Newfoundland, Canada. *Canadian Journal of Microbiology*, *59*, 701-708.

Huang, Y., Wille, M., Dobbin, A., Walzthoni, N., Roberston, G. J., Ojkic, D., Whitney, H. & Lang, A. S. (2014). Genetic structure of avian influenza viruses from ducks of the Atlantic flyway of North America. *PLoS ONE*, 9(1), e86999.

Huang, Y., Robertson, G. J., Ojkic, D., Whitney, H. & Lang, A. S. (2014). Diverse inter-continental and host lineage reassortant avian influenza A viruses in pelagic seabirds. *Infection, Genetics and Evolution*, 22, 103-111.

Huang Y., M. Wille, J. Benkaroun, H. Munro, A.L. Bond, D.A. Fifield D, G.J. Robertson, D. Ojkic, H. Whitney, & A.S. Lang. (2014). Perpetuation and reassortment of gull influenza A viruses in Atlantic North America. *Virology*, 456-457, 353-363.

Additional Publications

Parmley, E. J., Bastein, N., Booth, T. F., Bowes, V., Buck, P. A., Breault, A., Caswell, D., Daoust, P. Y., Davies, J. C., Elahi, S. M., Fortin, M., Kibenge, F., King, R., Li, Y., North, N., Ojkic, D., Pasick, J., Pryor, S. P., Robinson, J., Rodrigues, J., Whitney, H., Zimmer, P. & Leighton, F. A. (2008). Wild bird influenza survey, Canada, 2005. *Emerging Infectious Diseases*, 14(1), 84-87.

Posters (2014/15 only)

Benkaroun, J., Y. Fu, Y. Huang, G.J. Robertson, D. Ojkic, H. Whitney & A.S. Lang. Application of the Ion Torrent high throughput sequencer to genetic analysis of avian influenza A viruses. International Union of Microbiological Societies, Montreal, Canada, 7/2014.

Huang, Y., G.J. Robertson, D. Ojkic, H. Whitney & A.S. Lang. Diverse inter-continental and host lineage reassortant avian influenza A viruses in pelagic seabirds. BIT's 5th Annual World DNA and Genome Day, Dalian, China, 4/2014.

Conclusion and Future Recommendations

The AI project has been an on-going effort since 2008 because of the increasingly importance in the virus to poultry farms and human health. Wild bird research and surveillance has been increasing in other parts of Canada and the world and in the case of managing an outbreak, the fact of having invested in local expertise will assure the presence of laboratory and academic support to help answer further questions related to the virus' origin.