

# Ecology of avian influenza virus in non-commercial backyard poultry

Trends in seroprevalence to Influenza A virus in non-commercial backyard poultry in the Bay of Plenty and South Wairarapa (2006-CO125)

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# Summary final report

Title: Ecology of avian influenza virus in non-commercial backyard poultry

Business/Institution: National Centre for Biosecurity and Infectious Disease - Wallaceville

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## GOAL

To study the ecology of avian influenza viruses in non-commercial backyard poultry in New Zealand.

## CONTEXT OF THE PROJECT

This project is part of a larger study designed to study risk factors and potential transmission pathways that may contribute to the spread of influenza A viruses between wild birds, farmed birds and, farming families. This report will focus on the poultry testing component of the study, the outcome of the human study and the risk factor questionnaires are covered in separate reports.

There have been no outbreaks of avian influenza in poultry in New Zealand. However, non-pathogenic avian influenza viruses have been detected in healthy wild mallard ducks including low-pathogenic notifiable avian influenza virus (LPNAI) H5 and H7 (Stanislawek pers. comm. 2008). It is not known if non-pathogenic avian influenza viruses have been transmitted from wild ducks to backyard poultry in New Zealand. The aim of the current study was to determine if non-pathogenic avian influenza viruses were present in the non-commercial backyard poultry in areas where wild ducks are in close proximity.

## APPROACH

Farms with non-commercial backyard poultry located in the Bay of Plenty or Wairarapa were recruited. These were either within 15 km from Maketu Estuary or Waihi Estuary in the Bay of Plenty region or from Lake Wairarapa in the Wellington region. A cross-sectional study was conducted on 12 farms from each location between September and November 2006. The birds were individually identified using a labelled leg band. A blood sample, a tracheal swab and a cloacal swab were collected from each bird. Serological assays were conducted to detect antibody to AI viruses in serum samples. A real-time reverse transcriptase PCR (RT-PCR) was used to detect influenza virus in swabs. Ten of the 12 farms in each location were followed up at bimonthly intervals for 12 months to study the seasonal trend of the influenza viruses in backyard poultry.

## OUTCOME

The presence of antibodies to influenza A virus(es) in serum collected from poultry (ducks and chickens) was identified by ELISA. ELISA positive sera, (89/1083 from chickens and 31/85 from ducks) were tested by the haemagglutination inhibition test against antigens to subtype H5 and H7 viruses. All were negative for these subtypes. A total of 2336 cloacal/tracheal swabs were tested using real-time RT-PCR. There were 10 real-time RT-PCR samples with c.t. values that required further testing. All but one was found to be negative on re-test. No virus was isolated when the swab was subjected to virus isolation. The seroprevalence in chickens sampled at bimonthly intervals over the twelve month period ranged from 0-13.7 percent in Bay of Plenty and from 3.2-26.5 percent in South Wairarapa. In the Bay of Plenty, there were significantly more serum samples with positive titres in May

2007 ( $p < 0.05$ ) than in other months. In South Wairarapa, there was a wider peak of seroprevalence with significantly more serum samples having positive titres in February, April and June ( $p < 0.05$ ) than in August, October and December 2007. The intra-flock seroprevalence in chickens ranged from 0-57.1 percent in May 2007 in Bay of Plenty, and from 0-50 percent for a similar period in South Wairarapa.

There was a large discrepancy between ELISA positives and agar gel diffusion confirmative assay. New sensitive confirmative serology assays are required to verify ELISA results (refer to testing algorithms in Appendix 1).

## SUMMARY

The goal of the project was to study the ecology of influenza viruses in non-commercial backyard poultry in New Zealand. This is part of a larger project designed to identify risk factors for the transmission of influenza viruses from wild birds to poultry and potentially, to humans. The work outlined in this report aimed to assess whether or not non-pathogenic influenza viruses were present in backyard poultry located in close proximity to wild ducks. Farms with non-commercial backyard chickens located within 15 km from Maketu Estuary or Waihi Estuary in the Bay of Plenty region and from Lake Wairarapa in the Wellington region were recruited. These regions were selected because influenza A viruses had previously been isolated from healthy mallard ducks caught in the locality.

A cross-sectional study was conducted in 12 farms in each location between September and November 2006. Ten of the 12 farms in each location were visited at bimonthly intervals for 12 months and samples (cloacal/tracheal swabs and serum) were collected from individually tagged poultry. Serological assays were conducted to detect the presence of antibody to influenza viruses in serum samples. A real-time reverse transcriptase PCR (RT-PCR) was used to detect influenza virus RNA in swabs.

The presence of antibodies to influenza A virus(es) in serum collected from poultry (ducks and chickens) was identified by ELISA. ELISA positive sera, 89/1083 from chickens and 31/85 from ducks were tested by the haemagglutination inhibition test against antigens to subtype H5 and H7 viruses. All were negative for these subtypes. A total of 2336 cloacal/tracheal swabs were tested using real-time RT-PCR. There were 10 real-time RT-PCR samples with Ct values that required further testing. All but one was found to be negative on re-test. No virus was isolated when the swab was subjected to virus isolation. The seroprevalence in chickens sampled at bimonthly intervals over the twelve month period ranged from 0-13.7 percent in Bay of Plenty and from 3.2-26.5 percent in South Wairarapa. In the Bay of Plenty, there were significantly more serum samples with positive titres in May 2007 ( $p < 0.05$ ) than in other months. In South Wairarapa, there was a wider peak of seroprevalence with significantly more serum samples having positive titres in February, April and June ( $p < 0.05$ ) than in August, October and December 2007. The intra-flock seroprevalence in chickens ranged from 0-57.1 percent in May 2007 in Bay of Plenty, and from 0-50 percent for a similar period in South Wairarapa.

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The Avian Influenza Project Team. A consultation report on risk targeted study of avian influenza zoonosis. A milestone report for C0125/2006 Biosecurity operational research—*Avian influenza type A in non-commercial birds and pigs* (BNZ-751), 7 April 2007

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# 1. Introduction

There have been no outbreaks of avian influenza in poultry in New Zealand. However, non-pathogenic avian influenza viruses have been detected in healthy wild mallard ducks including low-pathogenic notifiable avian influenza virus (LPNAI) H5 and H7 (Stanislawek pers. comm. 2008). It is not known if non-pathogenic avian influenza viruses have been transmitted from wild ducks to backyard poultry in New Zealand. The aim of the current study was to develop a better understanding of the ecology of avian influenza viruses in New Zealand by studying non-commercial backyard poultry flocks in areas where wild ducks are in close proximity.

This is a joint research project between IDC, MAF Biosecurity New Zealand, ESR and AgResearch. The study provides an opportunity for multi-institutional collaboration on topics of animal and public health interests.

## 2. Materials and methods

### 2.1. SELECTION OF STUDY FARMS

Information about farms with backyard poultry, including distance from the major water sources in South Wairarapa and Bay of Plenty were obtained from AgriBase® administrated byASUREQuality. These areas were selected because non-pathogenic avian influenza viruses had previously been isolated from wild ducks in these areas.

Suitable study farms located within 15 km from Maketu Estuary or Waihi Estuary in the Bay of Plenty region and from Lake Wairarapa in the Wellington region were identified. Selection criteria for the survey included evidence of owning more than five chickens as registered on AgriBase® database and proximity to the major water sources, identified from the AgriBase®. Farms registered as having more than five chickens on AgriBase® were contacted by phone to update farm information and to brief them about the study. A Farming Community Household Study Questionnaire (Appendix 2) and a brief description of the study were posted to farmers who had expressed an interest in the study. We received 27 and 31 copies of the filled questionnaire from farms at South Wairarapa and Bay of Plenty, respectively. We then selected 12 study farms in each location for the initial cross-sectional study to get baseline data. Farm selection was based on farmers' willingness to participate in the study, identified risk factors for exposure to avian influenza viruses, including the number of chickens, sighting of wild birds on the farm and the presence of multiple avian species etc.

### 2.2. SAMPLE COLLECTION

#### 2.2.1 Cross-sectional study

During mid-September to mid-November 2006, we visited 12 farms in South Wairarapa and 12 farms in Bay of Plenty. A blood sample, a tracheal/throat swab (transferred to an individual vial of 1.5 ml of virus transport medium, virus transport media (VTM) and a cloacal swab (transferred to an individual vial of 1.5 ml VTM) were collected from each bird. All birds sampled for the study were individually identified by double banding the legs. A total of 309 chickens, 54 ducks, 2 geese, 2 turkeys and 4 guinea fowl were sampled (Table 1). All procedures involving the experimental use of birds were approved by the Wallaceville Animal Ethics Committee, Upper Hutt.

## 2.2.2 Longitudinal study

After examining the serological results of the cross-sectional study and risk factors identified in the questionnaire, we selected 10 farms in the South Wairarapa and 10 farms in the Bay of Plenty for a 12-month longitudinal study (February 2007 to January 2008), to study the seasonal influence on the prevalence of AI in the backyard poultry. The 20 farms were visited at bi-monthly intervals to collect samples from birds, namely February, April, June, August, October and December 2007 in South Wairarapa, and March, May, July September, November 2007 and January 2008 in Bay of Plenty. The number and species of birds in the coop available for sampling varied at each collection. In South Wairarapa, Farm SW10 was not sampled on one of the six collections, while farms SW05 and SW06 were not sampled on two occasions. In Bay of Plenty, farm BP07 and BP08 were not sampled for the last two and three collections, respectively. The birds were not available for testing at these times.

## 2.3. SAMPLE TESTING

### 2.3.1 Serology assays

#### 1. ELISAs

Antibodies to influenza A viruses in chicken and turkey sera were tested by IDEXX ELISA, Avian Influenza Virus Antibody Test Kit (Flockchek, IDEXX Laboratories, Westbrook Maine, USA), according to manufacturer's instructions. The IDEXX ELISA was designed to measure the relative level of antibody to AI in chicken serum. Briefly, viral antigen was coated on 96-well plates. Upon incubation of the test sample in the coated well, antibody specific to AI formed a complex with the coated viral antigens. After washing the unbound material from wells, a conjugate was added which binds to any attached antibody in the wells. Unbound conjugate was washed away and enzyme substrate was added. Subsequent colour development was directly related to the amount of antibody to AI present in the test sample. The Positive Control in the kit was standardised and represented significant antibody levels to AI in chicken serum. The relative level of antibody in the unknown was determined by calculating the sample to positive (S/P) ratio. S/P ratios greater than 0.5 were considered positive and indicated exposure to AI.

Antibodies to influenza A viruses in duck, goose and guinea fowl sera were tested by Pourquier ELISA, Avian Influenza A Blocking ELISA (Institute Pourquier, Montpellier, France), according to manufacturer's instructions. Briefly, nucleoprotein (NP) of influenza virus was coated on 96-well plates. Upon incubation of the test sample in the coated well, antibody specific to NP formed a complex that masked the coated NP antibody binding sites. After washing, an anti-NP monoclonal antibody conjugate was added. In presence of NP specific antibodies in the test sample, the NP sites were "masked" and the conjugate could not bind to the corresponding epitope. In the contrast, the conjugate bound to the NP antigen if the tested sample did not contain any specific NP antibodies. Unbound conjugate was washed away and enzyme substrate was added. The intensity of the colour was an inverse measure of the proportion of NP antibodies present in the test sample. The cut-off was determined by using a Negative Control serum supplied with the kit, which did not induce any extinction and had to be added to each microplate. The relative level of antibody in the unknown was determined by calculating percentage of inhibition of the test sample as compared to the negative control. Samples with a percentage of inhibition lower than 35 percent were considered positive and indicated exposure to AI.

#### 2. Agar gel immunodiffusion (AGID) test

IDEXX ELISA positive chicken sera were further tested using AGID test for antibodies against the nucleocapsid and matrix antigens of the influenza A viruses at IDC (BNZ).

### 3. Haemagglutination inhibition (HI) test

The IDEXX and Pourquier ELISA positive sera were tested by HI for antibodies against H5 and H7 antigens at IDC (BNZ).

## 2.3.2 Virology assays

### 1. RNA purification

RNA was purified from pools of three individual VTM extracts of tracheal/throat swabs or three cloacal swabs at AgResearch using QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions at AgResearch. RNA from a preparation of H4N6 (A/duck/NZ/6/9) was also purified as a positive control. The titre of the H4N6 was 108.2 median egg infectious doses (EID50) (provided by W. Stanislawek, IDC, BNZ).

### 2. Real-time RT-PCR

Influenza viral RNA was assayed by a real-time reverse transcriptase-PCR (Spackman et al, 2002) at AgResearch targeting the matrix gene of influenza virus A using SuperScript III Platinum One-Step Quantitative RT-PCR System (Invitrogen Corp, Carlsbad CA, USA) at AgResearch.

The sensitivity of the real-time RT-PCR at AgResearch was evaluated. The H4N6 virus was serial 10-fold diluted in AI-free allantoic fluid. RNA was then purified as described above from the diluted virus, and these RNA samples were subjected to real-time RT-PCR in duplicates to determine its detection sensitivity. The lowest detection level was 101 EID50/ml of AI H4N6 diluted in allantoic fluid (Ct = 34.4).

For a result with a Ct value higher than Ct=34, the sample is forwarded to IDC for PCR confirmatory test as well as viral isolation.

### 3. Virus isolation in embryonated SPF eggs

Swab samples with Ct values less than 40 by Real-time RT-PCR were subjected to virus isolation in embryonated SPF eggs at IDC, MAF

## 2.4. STATISTICAL ANALYSIS

Analysis of the differences in intra-flock and inter-flock seroprevalence at different sampling time-points at the two study locations in the longitudinal study were conducted using Fisher's exact test. Data was analysed using Minitab® v15.1.0.0 (Minitab Inc. State College, PA, USA). A p-value of < 0.05 was considered significant.

## 3. Results

### 3.1. CROSS-SECTIONAL STUDY

#### 3.1.1 Farm information

A total of 24 farms, 12 at South Wairarapa and 12 at Bay of Plenty, were selected for the cross-sectional study. The selected farms were located between 0.9 and 12.1 km away from major water bodies; with a median distance of 8.4 km. Information collected from the questionnaires indicated that wild waterfowl were seen on the pasture of 21 of the 24 farms. Wild waterfowl were seen in the waterways of 18 of 24 farms. The number of chickens in each farm varied from 4 to 35, with a median number of 16. Five farms had multiple bird species, such as chickens, ducks, geese, turkeys, guinea fowl, pheasants, peacocks and pigeons.

#### 3.1.2 Serology

A total of 309 chicken sera were tested by IDEXX ELISA between September – November 2006 and 11 were positive (Table 1). The seroprevalence in chickens for the Bay of Plenty and South Wairarapa was 4.4 percent (6/136) and 2.9 percent (5/173), respectively. The positive samples from chickens were collected from 2 farms in the Bay of Plenty and 3 farms in South Wairarapa.

A total of 54 duck sera were tested by Pourquier ELISA and 16 duck sera were positive. The positive duck sera were collected from 3 farms in South Wairarapa (Table 2).

All ELISA positive sera were tested by HI and were negative for antibodies to avian influenza virus subtype H5 and H7.

#### 3.1.3 Virology

A total of 371 tracheal/throat swabs and 371 cloacal swabs were tested by real-time RT-PCR for the presence of AI viruses. The sensitivity of the real-time RT-PCR was investigated using a preparation of H4N6. No AI viral RNA positive sample was identified by the real time RT-PCR from swabs collected from birds at South Wairarapa and Bay of Plenty between September and November 2006.

To confirm the negative real-time RT-PCR results tested at AgResearch, a batch of 24 RNA samples, purified from tracheal and cloacal swabs collected from 36 ducks in farm SW06 in September 06, were re-tested by real-time RT-PCR at IDC, and no positive samples were identified.

### 3.2. LONGITUDINAL STUDY

#### 3.2.1 Detection of antibodies to influenza A virus

Continuous bimonthly sampling took place in seven and eight of the ten study farms in South Wairarapa and Bay of Plenty, respectively (Table 3 & 4). The number of AI antibody positive samples over the entire longitudinal sampling period were 18/521 and 71/562 in Bay of Plenty and South Wairarapa, respectively. The seroprevalence in chickens at bimonthly intervals sampled over the twelve month period ranged from 0 percent to 13.7 percent in Bay of Plenty and from 3.2 percent to 26.5 percent in South Wairarapa (Tables 3 & 4, Figures 1 & 2). In Bay of Plenty, the seroprevalence in chickens in May 2007 was significantly higher ( $p <$

0.05) than those collected in other months (Figure 1). In South Wairarapa, however, there was a wider peak of seroprevalence (Figure 2); the seroprevalence in months of February, April and June was significantly higher ( $p < 0.05$ ) than that of August, October and December 2007.

In the Bay of Plenty, the number of seropositive flocks in May (7/10) was also significantly higher ( $p < 0.05$ ) than that of other months; while in South Wairarapa the number of positive flocks ranged from 30 percent (3/10) in October 2007 to 90 percent (9/10) in April 2007, with no significant differences (Tables 3 & 4). The intra-flock seroprevalence in chickens ranged from 0 percent (BP05: 0/14) to 57.1 percent (BP06: 4/7) in late autumn 2007 in Bay of Plenty, and ranged from 0 percent (SW07: 0/12) to 50 percent (SW09: 4/8) for a similar period in South Wairarapa (Figure 3 & 4).

When the serological results were combined according to seasons, the seroprevalence of AI antibody in South Wairarapa was significantly higher ( $p < 0.05$ ) than that in Bay of Plenty in all seasons.

When the serological findings for individual chickens were assessed, there were only six chickens, all in South Wairarapa, that were seropositive at three or more collections. One of the six chickens was not available for all sampling points. The relative AI antibody levels, as indicated by the sample to positive control ratio, fluctuated in a manner consistent with the overall seasonal trend (Figure 5).

Ducks were also available for sampling in farms BP01 and SW06 during the longitudinal study. In BP01, three to five duck samples were collected at each of the six visits and no seropositive sample was identified. In SW06, the seroprevalence in ducks were 33.3 percent (5/15), 37.5 percent (6/16), 36.4 percent (4/11) and 28.6 percent (2/7) in April, June, and October and December 2007, respectively; the ducks were not available for sampling in February and August 2007.

The ELISA positive chicken samples were further tested by AGID. Only 2 of 88 ELISA positive samples were also positive by AGID. All of the ELISA positive bird samples were tested negative for antibodies to H5 and H7 by HI.

### 3.2.2 Virology

Four swabs, one duck and two chicken tracheal samples, and one chicken cloacal sample collected in South Wairarapa in December 2007, were tested with Ct values greater than 35 for influenza A virus by a real-time RT-PCR. Six chicken swabs, two cloacal and four tracheal samples collected in Bay of Plenty in January 2008, were tested with Ct values greater than 35 for influenza A virus by a real-time RT-PCR. These samples with Ct values greater than 35 were also re-tested by a real time RT-PCR at IDC. On the re-test only a single chicken tracheal sample had a Ct value greater than 31. This sample was subjected to virus isolation in embryonated eggs with a negative result.

## 4. Discussion

From the initial cross-sectional study (Sept-Nov 2006), a low seroprevalence of avian influenza virus antibodies was identified in backyard chickens in South Wairarapa and Bay of Plenty, 2.9 percent (5/173) and 4.4 percent (6/136) respectively. No influenza A virus was detected from the collected tracheal and cloacal swabs, and H5 and H7 subtypes viruses were ruled out in the seropositive birds using an HI test. Findings illustrate that healthy backyard chickens had been exposed to influenza viruses but the source of the virus was not clear. The farms selected for the study were in close proximity to major water sources where wild birds including mallard ducks congregated. Avian influenza viruses, of a number of subtypes, had previously been isolated from wild mallard ducks from these locations (Stanislawek et al, 2002). In the study, 21 of the 24 farm questionnaires recorded the presence of wild waterfowl on the pasture, and 18 of the 24 had recorded wild waterfowl in the waterways, indicating the potential for virus introduction from wild waterfowl.

There appears to be a seasonal pattern of avian influenza virus shedding in wild ducks. Worldwide virus is more frequently isolated from juvenile ducks, peaking in late summer and early autumn (Hinshaw et al, 1980; Stanislawek et al, 2002). In the current study, the seroprevalence in backyard chickens peaked in the late autumn and early winter months, indicating exposure to influenza viruses in the preceding months (Summer), a period that coincides with the previously reported active months of influenza virus circulation in wild waterfowl in New Zealand (Stanislawek et al, 2002).

There is limited published data on the duration of the antibody response in chickens following natural exposure to avirulent avian influenza viruses. In the Bay of Plenty, 13/13 seroconverted chickens in May all became negative at the July 2007 sampling. In South Wairarapa, of the 13 seroconverted chickens in April, eight, four and one became negative in June, August and October, respectively. Therefore, in the majority of seroconverted chickens significant antibody levels were not detectable at the next sampling event two months later, indicating that positive levels are generally detectable for about one to three months. Further analysis of this aspect is being explored. However, the serological assay used in the current study detects antibodies reactive to type A-specific antigens shared by all influenza A viruses. The antibody level detected by the assay could be elicited by exposure to more than one strain of influenza A virus, pointing to a need for caution when interpreting antibody response duration.

There was a large discrepancy in the detection of antibody to influenza viruses between IDEXX ELISA and AGID. In 115 IDEXX ELISA positive samples only 2 tested positive by AGID. Other researchers have found that ELISA tests tend to be more sensitive than AGID (Meulemans et al, 1987; Snyder et al, 1985). The differences in reagents, especially AI antigens used in the different assays may also play a role in detection sensitivity.

In summary, we have demonstrated for the first time that healthy backyard chickens and ducks in Bay of Plenty and South Wairarapa have antibodies to influenza A virus. The presence of antibodies as determined by ELISA is likely to indicate exposure to these viruses but the source of the virus and the subtype of the virus(es) involved has not been determined. The number of birds with antibody to influenza peaked in late autumn and early winter, suggesting exposure to influenza viruses in the preceding months (summer). This seasonal pattern coincides with that reported for influenza A viruses in wild mallard ducks in New Zealand (Stanislawek et al, 2002). No influenza virus was isolated during the study which meant specific HI assays requiring virus could not be developed, and so the seroprevalence to particular subtypes of influenza virus could not be determined. This capability would have

provided us with more information on the ecology of circulating strains of influenza viruses in the areas under study. Exposure to H5 and H7 subtype viruses was however ruled out in all seropositive birds using present capability at IDC (Wallaceville).

The project was a collaborative research effort by medical and veterinary scientists based at the National Centre for Biosecurity and Infectious Diseases (NCBID). There is a need to develop further serological typing capability to allow confirmation of ELISA seropositive samples and sub-typing of positive sera possible. The laboratory findings reported here will be used in further analyses to understand the persistence of a detectable antibody response, spatial and temporal trends, transmission pathways and risk factors for avian influenza in the rural backyard poultry setting.

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