Australian Government

Australian Centre for International Agricultural Research

Final report

project

The epidemiology, pathogenesis and control of highly pathogenic avian influenza (HPAI) in ducks in Indonesia and Vietnam

project number	AH/2004/040
date published	November 2011
prepared by	Associate Professor Joanne Meers, University of Queensland
co-authors/ contributors/ collaborators	Dr Joerg Henning Dr John Bingham Dr Ngo Thanh Long Dr Nguyen Truc Ha Dr Walujo Priyono Dr Hendra Wibawa
approved by	Dr Doug Gray, Research Program Manager for Animal Health, ACIAR
final report number	FR2011-30
ISBN	978 1 921962 23 3
published by	ACIAR GPO Box 1571 Canberra ACT 2601 Australia

Contents

1 Acknowledgments

We sincerely thank the farmers in Indonesia and Viet Nam for their cooperation during the field studies. Without their willingness to be involved in the study, we could not have conducted these activities. We also thank the many field veterinarians, para-veterinarians, and district livestock officers who helped in both countries with sample collection, questionnaire interviews and disease outbreak investigations. We express our thanks to the head and staff of all the district livestock services involved in the project in Indonesia, including those from Batang, Pemalang, Brebes, Kendal, Klaten, Purworejo, Bantul, Kulon Progo, Sleman and Magelang. In Viet Nam we sincerely thank the heads and staff of all the provincial Sub-Departments of Animal Health and District Veterinary Stations who were involved in the project.

We are very grateful for the work of all the laboratory staff at DIC in Wates, Bbalitvet in Bogor and RAHO-IV in Ho Chi Minh City. We also thank the staff in both countries who conducted many hours of data entry for the field studies, including Tri Widayati and Ika Wahyu Setyowati SE.

In Indonesia, we particularly thank the project leader Dr Bagoes Poermadjaja for facilitating the work in Indonesia, the Directors of DIC Wates, (Dr Akhmad Junaidi and Dr Isep Sulaiman (deceased)) and the Directors of Bbalitvet, Bogor for their support for the project. In Viet Nam, we thank the directors of RAHO-IV (Dr Donh Manh Hoa and Dr Nguyen Xuan Binh) for their support.

At AAHL, we wish to thank Dr Peter Daniels and Dr Deborah Middleton for their support. We also thank the many staff at AAHL who helped with animal infection trials and with laboratory activities, including Paul Selleck, Kelly Davies, Vicky Stevens, Jianning Wang, Wendy Ha, Som Walker, Sue Lowther, Jean Payne, Jenni Harper and Anna Axell.

At the University of Queensland, we thank Dr John Morton for his epidemiology advice, Dr Zuhara Bensink for her organizational skills and staff of the Research Projects Office and the School of Veterinary Science for their administrative assistance.

We are grateful for the assistance and support provided by the ACIAR country offices in both Indonesia and Viet Nam

We apologise for the omission of many other contributors whose names have not been mentioned.

2 Executive summary

H5N1 highly pathogenic avian influenza (HPAI) emerged in Asia in the late 1990s, causing massive outbreaks of disease in poultry, deaths in humans and worldwide concern that the virus could lead to a global influenza pandemic. Because of the central role ducks play in the maintenance and evolution of other avian influenza viruses, it was considered likely that they played a similar role with the H5N1 virus. However, there was limited evidence to support this assumption, particularly from field studies. Considering the seriousness of the situation, and to provide a knowledge-based approach to control of the disease in poultry and prevention of further deaths in humans, an understanding of the biology and epidemiology of the virus in ducks was essential.

The project had substantial scientific achievements from a series of field and experimental activities. In both Indonesia and Viet Nam, our field studies of smallholder duck farms and chickens in- contact with the duck flocks showed that ducks were more likely than chickens to have been exposed the virus, to harbour the virus, and to survive the virus infection. In Indonesia, 35% (34/96) of the study farms experienced a HPAI disease outbreak during the course of a 12-month longitudinal field study. Most chickens in the outbreaks succumbed to the disease, whereas most ducks showed minimal clinical signs and survived the infection. Experimental studies (using Indonesian virus isolates) confirmed that ducks had an asymptomatic infection and that ducks can shed the virus up to 8 days post-infection, indicating they may be an effective maintenance host. However, replication of virus in ducks was limited, and shedding of virus was intermittent and at low concentration suggesting that ducks are not a very effective species for transmission of the virus. In contrast, infection in chickens was rapidly fatal, virus replication was extensive, and virus shedding was extremely high for the short period of time before death, indicating very efficient transmission of virus.

The project identified management factors that reduced the risk of a HPAI disease outbreak or H5N1 infection occurring, including completing a full course of vaccination, confining duck flocks over-night on the farm, preventing ducks scavenging around neighbouring houses, and avoiding consumption of carcasses of dead birds. The field studies of vaccinated ducks and chickens in Viet Nam showed that many birds failed to develop appropriate antibody responses. However, despite this lack of response, there were no disease outbreaks in our study villages in Viet Nam, confirming the risk factor analysis that vaccination can be an effective control approach for HPAI.

The project had major scientific impacts, with eight articles published or currently in press in international journals, and more than 20 conference presentations. These publications made a substantial contribution to scientific literature on H5N1 HPAI. The project impacted on the design of other research projects on avian influenza in both Indonesia and Viet Nam. The project also had capacity impacts in both partner countries, with three postgraduate students awarded John Allwright Fellowships and a fourth student aligned to the project undertaking a masters degree in Indonesia based on project research. In addition, informal training courses were conducted on epidemiology, study design, data management and analysis, sample collection and molecular diagnostics, which increased the level of expertise in these areas in the partner institutions.

The risk factors determined in the project (outlined above) can be used to provide recommendations to farmers in order to reduce the likelihood of HPAI infection and disease in their duck flocks. The project showed that ducks are likely maintenance hosts of the virus but that chickens are more likely to transmit the virus at high concentration to other birds and to humans. Further studies should focus on the mechanisms of virus persistence in populations, on the routes of transmission of virus from each species and on the mechanisms underlying the different replication sites and replication dynamics in both species.

3 Background

Highly pathogenic avian influenza (HPAI) caused by an H5N1 virus continues to be a serious concern to both human and animal health in the Asian region. The H5N1 virus first emerged in Hong Kong in 1997 causing deaths in poultry and humans. The disease outbreak was controlled following closure of wet markets, stringent movement controls and culling of domestic poultry. However, the virus re-emerged in 2003, and spread quite rapidly through south-east Asia, causing devastating losses of chickens throughout the region and a substantial number of human infections and deaths.

Avian influenza viruses vary greatly in virulence, ranging from viruses that cause no disease at all to viruses that cause explosive epidemics in chickens, with 100% mortality rates. The major determinants of virulence relate to the type of hemagglutinin (H) and neuraminidase (N) glycoprotein spikes that the virus possesses, and the amino acid sequence at key sites on these glycoproteins. There are 14 different types of H and 9 different types of N, and only certain combinations of these glycoproteins are known to cause severe disease in poultry. The H5N1 virus that emerged in Asia between 2001-2004 was recognised to be highly pathogenic for chickens and in some studies was also shown to cause disease signs in ducks.

This finding of pathogenicity in ducks was unusual because avian influenza viruses generally cause no or very mild disease in ducks, even viruses that are highly pathogenic for chickens. However, there were inconsistent reports regarding the pathogenicity for ducks of the H5N1 viruses that emerged in Asia between 2001-2004. Some investigators reported clinical signs of disease in H5N1-infected ducks on commercial farms (Kwon et al, 2005), for example some studies showed severe neurological dysfunction and death in experimentally-infected ducks (Sturm-Ramirez et al, 2004). However, other studies reported that ducks infected with H5N1 virus isolates did not show severe clinical disease and the birds were able to shed virus over an extended period of time (Hulse-Post et al. 2005).

Although different mammalian species are host to their own influenza viruses (e.g. human influenza viruses, equine influenza viruses, swine influenza viruses), all of which show some degree of host-specificity, birds are thought to be the original source of all influenza viruses. In particular, ducks and other waterfowl are considered to be the major reservoir of influenza viruses, and all H and N types have been detected in ducks. New strains of human influenza virus are thought to arise by various reassortment processes, with waterfowl providing the new H and N genes and other species such as pigs potentially providing the mixing vessel for the reassortment event.

One of the major concerns regarding H5N1 HPAI virus is its ability to infect humans and to cause fatal disease in this host. As of August 2011, there have been 565 cases of H5N1 avian influenza virus infection of humans, with 331 deaths (WHO, 2011). Given the widespread nature of the virus and the density of human population in countries where it is endemic, the relatively small number of human infections suggests that humans are not readily infected with the virus. The risk factors for human infection have not been clearly defined, but it appears that human infection must require exposure to a large concentration of virus through contact with infected birds, and perhaps may involve a degree of genetic susceptibility in the host (Forrest and Webster, 2010). However, despite the relatively small number of human cases, the high case fatality rate (proportion of cases that die) of currently 59% (WHO, 2011) means that this virus is of major global concern. In addition to the direct effect of the virus in humans, there was worldwide anxiety that the highly pathogenic avian virus would either mutate to become more readily transmissible amongst humans or that it would recombine with a human influenza virus to produce a highly virulent human virus. With either scenario, there was danger that a highly virulent virus could be produced that spreads easily in humans, causing high mortalities in human populations around the world. Although the emergence of the H1N1 “swine”

influenza virus in 2009 took the focus away from H5N1 HPAI virus, the possibility remains that this virus could be the source of a major human influenza pandemic of the scale not observed since 1918.

Role of ducks in the maintenance and transmission of H5N1 HPAI virus

Although it is not clear whether waterfowl were the initial source of the H5N1 virus that emerged in Hong Kong in 1997, it seems likely that they play a central role in the maintenance of the virus in poultry populations and are probably responsible for transmission of the virus to chickens. However, there is only limited information to support these statements. There have been a number of studies reporting experimental infection of ducks and chickens, and a similar number that have investigated the use of vaccines to prevent infection in these species. However, there are very few reports of field studies that have investigated H5N1 infection dynamics in ducks, particularly the patterns of infection over time.

Mixed poultry farming is very common throughout Asia, particularly in the village sector and in small-holder farms. Chickens and ducks are two of the most important livestock species in Indonesia and Viet Nam. In 2009, Viet Nam and Indonesia were the second and fourth largest producers of ducks in the world, with estimated duck populations of 84 million and 42 million, respectively (FAO, 2011). Most ducks in these countries are reared extensively, and this is considered to be a key factor in the transmission of H5N1 HPAI viruses. There are two extensive duck management systems used by small-holders in Indonesia and Viet Nam: the home-based system and the fully mobile herding systems. In the former system, ducks are allowed to leave the farm and scavenge during the day, but are confined overnight on the farm or in the village vicinity; hence these duck flocks remain “stationary”. In contrast, under the mobile herding or “moving” duck management system, ducks are transported throughout the country to scavenging areas and kept on or near the scavenging locations overnight, with the owners and their flocks often spending prolonged periods away from the village-farm. In both duck production systems, there is opportunity for ducks to come into contact with chickens. Many small-holder farmers do not segregate their ducks and chickens, and with extensive grazing of ducks there is also opportunity to come into contact with other farmers’ poultry.

The control of H5N1 HPAI requires thorough understanding of the behaviour of the virus in the field, particularly in small-holder farms where there is ready mixing of different poultry species and generally limited attention to biosecurity measures. In addition, knowledge of the pathogenicity of the virus in ducks, including the duration of virus excretion and the tissue tropism (tissues in which the virus preferentially replicates) of the virus, is necessary to determine the risk of transmission to chickens and humans and the mechanisms of virus persistence in the field. Thus, in order to develop control strategies, an improved understanding of both the epidemiology and pathogenesis of the disease is crucial, from both economic and public health perspectives. It is likely that different control strategies will be required in various countries because of differences in management systems, environment, and breed of ducks. The findings from one country cannot necessarily be transferred to another and country-specific data are required in order to determine the most suitable approaches for the individual country or region. Thus, a clear understanding of the mechanisms of maintenance and spread of H5N1 virus in the field is required to determine the most efficient control method for each country and the most effective way to break the cycle of transmission to humans.

4 Objectives

The key aim of this research project was to better understand the role that domestic ducks play in the transmission and maintenance of HPAI H5N1 virus in Indonesia and Viet Nam. It was recognised that this knowledge would allow better management and containment of outbreaks of HPAI, which would ultimately reduce the risks to human health and lead to more informed decisions on control of the disease in poultry.

The project had five objectives:

1. To understand the epidemiology of HPAI H5N1 virus (e.g. seroprevalence, duration of virus shedding) in small-holder duck production systems in Indonesia and Viet Nam.
2. To understand the role of ducks as maintenance hosts and amplifiers of H5N1 virus during and after infection.
3. To understand the pathogenesis of HPAI H5N1 virus infection in ducks.
4. To evaluate the efficacy of current vaccines in ducks and determine the possible role of vaccination in reducing virus load and shedding in ducks.
5. To develop recommendations for effective monitoring of infection in ducks that will assist in making policy for control of HPAI.

The objectives were achieved through a combination of field and experimental activities conducted in Indonesia, Viet Nam and Australia. Field studies addressed Objectives 1 and 2. Longitudinal surveys were conducted on ducks in small-holder farms in Indonesia and Viet Nam, following selection of appropriate field sites and calculation of sample sizes and sample strategy. In these field surveys, the prevalence of H5N1 antibody H5N1 (which indicates previous exposure to the virus) and presence of H5N1 virus (which indicates current infection with the virus) in ducks and in-contact chickens were determined to provide the data for Objective 1. For Objective 2, the impact of physical association between ducks and chickens in spreading HPAI virus was assessed through analysis of the data collected in the longitudinal surveys, including the serological, virological and questionnaire data.

A series of experimental trials were conducted to address Objectives 2, 3 and 4. The studies were performed at the Australian Animal Health Laboratory (AAHL), Geelong, Australia and at Balitvet, Bogor, Indonesia. Samples collected in the field studies above provided H5N1 virus isolates, which were used to experimentally infect ducks and chickens in the secure facilities at AAHL and Balitvet. The viruses were well-characterised both genetically and antigenically. Following experimental challenge, samples were collected at defined time points post-inoculation to assess the presence of virus in various tissues and excretions/secretions of the infected ducks and chickens. The titre of H5 antibody in serum of ducks was also measured at defined time points post inoculation. Following euthanasia of the experimentally-infected birds, a full post-mortem examination was conducted, including histopathological investigation. For objective 4, groups of vaccinated ducks were included in the challenge trials and their responses compared to those of unvaccinated birds. In addition, field data from vaccinated birds contributed to understanding the effects of H5N1 vaccination in ducks.

To achieve Objective 5, the information gained from the previous four objectives was assimilated and transformed into various media formats appropriate for different audiences.

5 Methodology

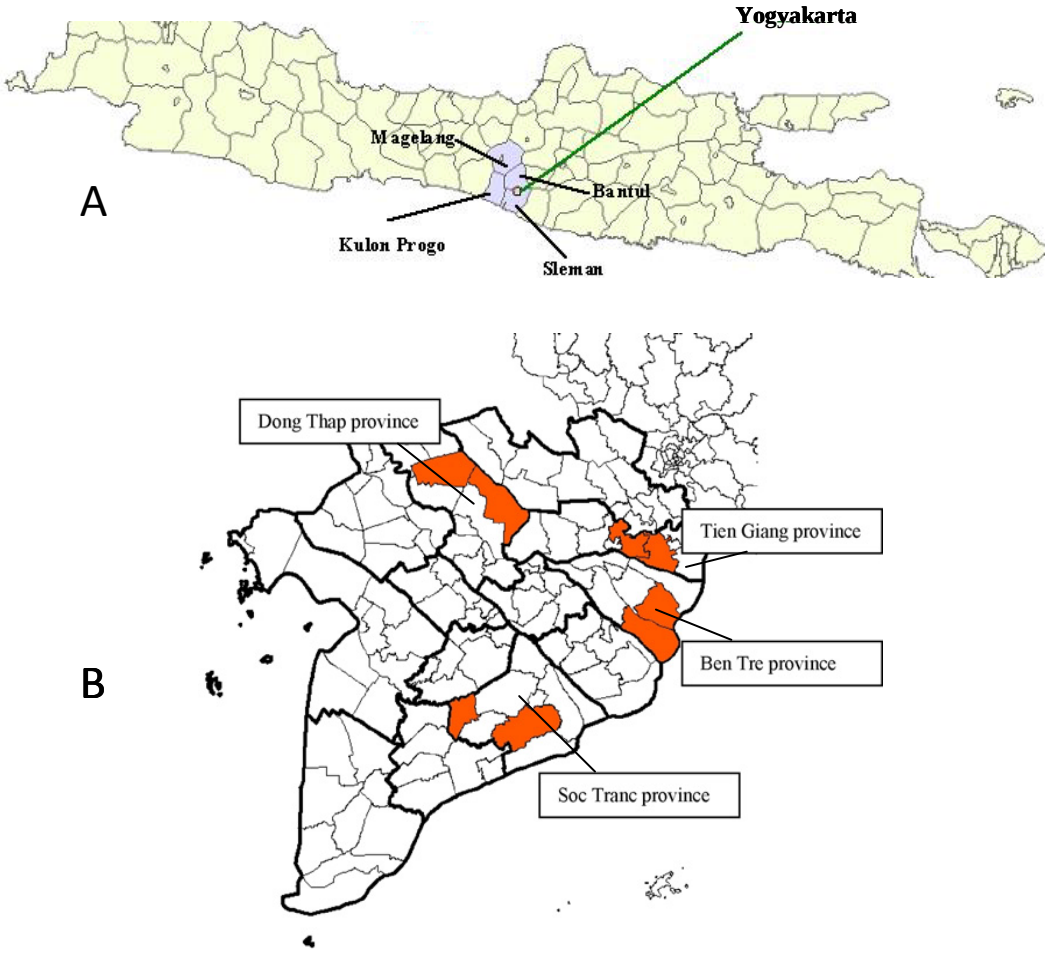
There were six research institutions involved in the project, comprising the University of Queensland, Brisbane and CSIRO's Australian Animal Health Laboratory (AAHL), Geelong in Australia; the Disease Investigation Centre (BBVET), Wates and the Research Institute for Veterinary Science (Bbalitvet), Bogor in Indonesia; the Regional Animal Health Office VI (RAHO-VI), Ho Chi Minh City and the National Institute of Veterinary Research (NIVR), Hanoi in Viet Nam. In the project establishment phase, it was decided that the field activities would be based at BPPV-IV Wates in Indonesia and at RAHO-VI Ho Chi Minh City in Viet Nam, while the experimental activities would be based at Balitvet Bogor in Indonesia, NIVR Hanoi in Viet Nam and at AAHL in Australia.

Field activities

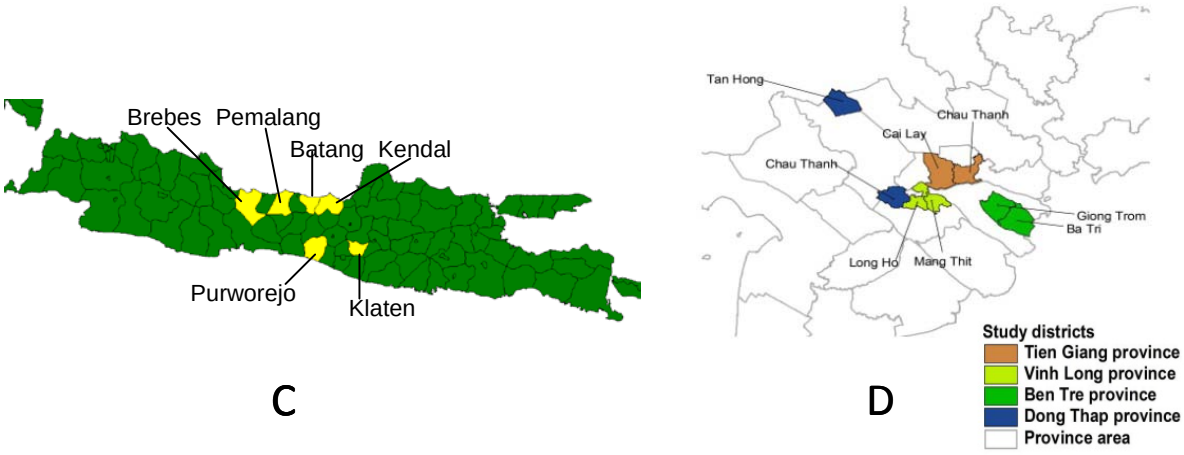
Epidemiological studies were conducted in Indonesia and Viet Nam. The design and overall supervision of these studies was performed by Dr Joerg Henning from the University of Queensland, with major inputs and advice from epidemiologists, laboratory and field staff in both countries. In particular, there was epidemiological participation from Drs Putut D.P. and Didik Yulianto (Indonesia) and Drs Nguyen Truc Ha and Le Tri Vu (Viet Nam), laboratory and/or field participation from Drs Hendra Wibawa, Tri Bhakti Usman, Walujo Prijono, Sri Handayani, Verawati and Rama Dharmawan (Indonesia) and Ngo Thanh Long, Pham Phuong Vu (Viet Nam) with overall management of the field activities by Drs Akhmad Junaidi and Isep Sulaiman (dec) (Indonesia) and Drs Dong Manh Hoa and Nguyen Xuan Binh (Viet Nam)

The major activity was a 12-month longitudinal survey of small-holder 'stationary' duck farms in central Java, Indonesia and in the Mekong Delta, Viet Nam. The 'stationary' duck production system was defined as one in which the ducks may be allowed to leave the farm during the day to graze and scavenge in rice paddies, but they return at night for housing within the farm. During a 12-month extension to the project, a second series of longitudinal studies was completed in each country, focused on the 'moving' duck production system, i.e. duck flocks that are moved long distances away from the home farm, often following the pattern of recent rice harvests, and remain for extended periods away from the farm. In addition, characteristics of external risk factors related to HPAI occurrence in moving duck flocks was also investigated in the extension period. Interviews were conducted with people associated with the moving duck flock management. These included owners of the rice paddies on which the moving duck flocks grazed, the people who supplied transport to the moving duck flocks and the owners of hatcheries which provided ducklings to the flocks or purchased eggs from the flocks.

The locations of the longitudinal studies conducted in 'stationary' and 'moving' duck flocks is shown in the figures below. In addition to these planned longitudinal studies, an 'opportunistic' case-control study was conducted in Viet Nam to define risk factors associated with disease outbreaks of HPAI in the Mekong Delta.



Sites for the longitudinal field studies in 'stationary' duck flocks. A: In Indonesia, four districts were selected in Central Java province (Magelang, Bantul, Sleman, Kulon Progo). B: In Viet Nam, two districts were selected in each of four provinces (Dong Thap, Tien Giang, Ben Tre, Soc Trang)



Sites for the longitudinal field studies in 'moving' duck flocks. C: In Indonesia, six districts were selected in Java (Brebes, Pemalang, Batang, Kendal, Klaten, Purworejo).D: In Viet Nam, two districts were selected in each of four provinces (Tien Giang, Vinh Long, Ben Tre, Dong Thap)

(i) Prevalence and incidence of HPAI in 'stationary' duck flocks

[Henning J, Wibawa H, Morton J, Usman TB, Junaidi A, Meers J. (2010) Scavenging ducks and transmission of highly pathogenic avian influenza, Java, Indonesia. *Emerging Infectious Diseases* 16(8):1244-50.

Henning J, Henning KA, Morton JM, Long NT, Ha NT, Vu le T, Vu PP, Hoa DM, Meers J.(2011) Highly Pathogenic Avian Influenza (H5N1) in ducks and in-contact chickens in backyard and smallholder commercial duck farms in Viet Nam. *Preventive Veterinary Medicine*. 101(3-4):229-40.

Henning J, Morton J, Wibawa H, Yulianto D, Usman TB, Priyono W, Junaidi A, Meers J. (2011) Focussing not on HPAI outbreaks: incidence and risk factors for H5 infection of clinically healthy ducks flocks. *Preventive Veterinary Medicine* (submitted)]

The specific sites within each of these areas were selected following detailed discussions held in each country to determine the most appropriate locations to conduct these longitudinal studies. A number of criteria were considered before selection of the sites:

(1) The area should be a high risk areas for HPAI infection as indicated by occurrence of HPAI outbreaks in the past,

(2) A large population and high density of ducks in the area

(3) The presence habitats with high wild bird abundance, promoting contact between wild birds and farmed ducks.

(4) Availability of experienced veterinary or para-vet field staff to collect data and samples in the area

(5) Reasonable proximity to the diagnostic laboratory to allow for timely transport of samples from the field to the laboratory

The overall study design for the longitudinal study on 'stationary' duck flocks was similar in both countries, i.e. small-holder duck farms were selected for inclusion in the study, then monitored at 2-monthly intervals over a period of 13 months (7 sampling occasions). The monitoring included collection of samples for serological and virological analysis and a questionnaire interview conducted with the farmer. There were some differences in details in the study design in each country, because of differences in expected prevalence of HPAI infection, and differences in the costs of diagnostic testing and travel in each country. Thus the study design for each country will be reported separately.

Indonesia:

In Indonesia, four districts of Central Java (Sleman, Magelang, Bantul, and Kulon Progo) were monitored (see figure). Sample size calculations were based on DIC surveillance data collected in Central Java in 2006; 13 (4.7%) of 278 cloacal swabs from ducks were positive for H5 viral RNA on real-time reverse transcription–PCR (RT-PCR). On the basis of an expected true bird-level virus prevalence of 5%, a precision of the estimate of ±1.5 % and a 95% confidence interval (CI), a total of 811 ducks had to be sampled. Ninety-six duck farms were enrolled in the study and a total of 960 ducks (10 ducks per farm) and 480 in-contact chickens (5 chickens per farm) were sampled during each of 7 visits over 13 months (initial visit plus 6 bimonthly visits).

A multistage sampling strategy was used, with stratification by district and a 3-level sampling process that involved villages, duck farms, and birds. A sampling frame was prepared by agriculture extension officers who listed all villages in the selected districts, including the total number of duck farms within each village. We selected 4 villages within each district using probability proportional to size sampling. Field veterinarians then prepared a second sampling frame containing the names of all duck farmers within the 16 villages selected and the number of ducks kept by each farmer. From this sampling frame, 6 duck farms per village were selected by using simple random sampling. Farms with <10 ducks were excluded (at least 10 ducks per farm were required for sampling) as were farms with >700 ducks (these were considered to be large commercial farms and not appropriate for this study of small-holder farms). Random numbers for village and duck

farm selection procedures were produced in STATA version 10.0 (StataCorp, College Station, TX, USA).

Four veterinarians from the DIC were trained in the use of data collection tools and interviewing techniques. Field visits were conducted once every 2 months from March 2007 through March 2008; duck owners were interviewed and swab and blood samples from birds were obtained during each visit. On the first visit, birds were selected for the study. The duck owner enclosed all ducks in a pen and selected the first 10 ducks that could be caught. If available,5 chickens kept on the same farm were also selected in the same manner. Wing tags or leg bands were attached to each selected duck and chicken. Blood samples were collected from the wing vein of each bird, and an oropharyngeal swab and a cloacal swab were collected from each bird and placed into a single tube containing virus transport media (Universal Viral Transport 3mL; Becton Dickinson, Franklin Lakes, NJ, USA). Duck owners confirmed that none of the ducks and chickens sampled had been vaccinated against HPAI before the study and that none were vaccinated during the study. Serum samples were tested for antibodies to avian influenza (H5) by using the hemagglutination inhibition (HI) test according to methods recommended by the World Organisation for Animal Health (OIE, 2009). Antigen and control antiserum were supplied from Pusat Vetenerinaria Farma (Surabaya, Indonesia). The antigen was derived from an HPAI (H5N1) chicken isolate obtained in 2004 in Indonesia (A/chicken/Pare/East Java/2004). This antigen is commonly used for HI tests to detect antibodies to avian influenza (H5N1) at all veterinary diagnostic laboratories in Indonesia. A titer $>2^4$ against 4 hemagglutinating units of antigen was considered positive. Real-time reverse transcriptase polymerase chain reaction (rRT-PCR) was used to test the combined oropharyngeal and cloacal swabs of individual birds in pools of 5 for subtypeH5 virus RNA. Sequencing was conducted on the H5 RTPCR–positive samples to confirm the HPAI multiple basic amino acid motif at the cleavage site of the hemagglutinin gene and to determine whether the neuraminidase gene of the isolate belonged to the N1 subtype.

Duck farmers involved in the study were asked to immediately report sickness or deaths of birds to the DIC. Compensation was paid to duck farmers to encourage reporting. Upon notification, veterinarians conducted an outbreak investigation at the reported farm by using a predesigned questionnaire. Clinical signs were recorded and carcasses were collected for post-mortem examination. Blood and swab samples from clinically normal birds from the same farm were obtained on the day of the investigation. Blood samples were tested for antibodies to avian influenza (H5) as already discussed; swab samples from carcasses (combining lung, heart, liver, spleen, pancreas,and intestinal tissues) and from live birds were processed by virus isolation in embryonated eggs. Two passages of virus isolation were conducted, and allantoic fluid was tested for H5 antigen of avian influenza by using the HI test. An HPAI outbreak was defined as >1 bird dying within a few days of each other from HPAI (i.e., positive by subtypeH5 virus isolation or RT-PCR).

For both ducks and in-contact chickens, bird-level seroprevalence (proportion of study birds with H5 antibodies) and flock-level seroprevalence (in which at least 1 study bird had antibodies) were calculated for each of the 7 sampling periods and pooled across the entire study period. Virus prevalence was calculated only at flock level (proportion of flock visits in which at least 1 pool of swab samples from the farm was positive for H5 RNA) for the entire study period. The multistage sampling strategy was accounted for in the data analyses by using survey commands in STATA version 10.0 (StataCorp); districts were treated as strata; villages were specified as primary, and farms as secondary, sampling units. For bimonthly bird-level prevalences, and for bird- and flock level prevalences over the entire study period, sampling weights were the inverse of the product of the proportion of villages in the district that were sampled and the proportion of duck farms in the village that were sampled. The finite population correction factor for primary sampling units was the total number of villages in the district. Finite population correction accounted for reduction in variance associated with sampling without replacement. For bird-level seroprevalence calculations over the entire study period, repeated measurements within

the same birds were accounted for by specifying the individual bird as the third level of sampling and incorporating the number of duck farms per village as the finite population correction factor for secondary sampling units. For the bimonthly flock-level seroprevalence, only primary sampling units with their finite population correction factor were specified in the analyses. Sampling weights for bimonthly flock-level seroprevalence were the inverse of the proportion of villages in the sampled district. Logistic regression models accounting for 3levels of clustering (birds within farms within villages) were used to compare the odds of birds having titers positive for avian influenza (H5) between ducks and in-contact chickens. For flock-level comparisons, logistic regression models accounting for 2 levels of clustering (farms within villages)were used to compare the odds of flocks having at least 1bird with antibodies to avian influenza (H5) between duck and in-contact chicken flocks, and for duck flocks between sampling months. Logistic regression accounting for 2 levels of clustering was also used to evaluate whether the odds of duck (or chicken) flocks being seropositive were independent of the results of the other species at the same farm and sampling. All logistic regression models also accounted for sampling weights and incorporated finite population correction. Adjusted Wald tests were used to assess the overall effect of sampling month. After fitting the logistic regression models taking the survey sampling design into account, we applied the F-adjusted mean residual goodness-of-fit test.

Incidence study: Incidence rates were calculated at bird-level and at flock-level, as follows: Bird-level incidence rate for each flock sampling = Number of retested birds initially seronegative that seroconverted on all farms at the next flock-sampling / (Sum of days between flock-samplings for birds retested seronegative at the next flock-sampling + ½ Sum of days between flock-samplings for birds that seroconverted at the next flock-sampling).

Flock-level incidence rate for each flock-sampling = Number of retested flocks initially seronegative where≥1 birds seroconverted at the next flock -sampling / (Sum of days between flock-samplings for flocks retested where all tested birds were seronegative at the next flock-sampling + ½ Sum of days between flock-samplings for flocks initially seronegative where≥1 birds seroconverted at the next flock -sampling). The flock-level incidence rate over the entire study period was also calculated as a pooled incidence rate: Flock-level incidence rate over the entire study period = Sum of retested flocks initially seronegative where≥1 birds seroconverted across all flock -samplings/Sum of all flock-days at risk across all flock-samplings (i.e. the denominator of the flock-level incidence rate calculation summarized for all flock-samplings).

Flock-level incidence rate for each sampling was multiplied by 1,000 to obtain 1,000-bird days at risk and further multiplied with 365.25 to obtain 1000-bird-years at risk. The standard errors for the incidence rate were calculated as SE(p)=√Number of cases/Bird -days-at risk^2 to produce 95% confidence intervals.

A logistic General Estimation Equation (GEE) model with an exchangeable correlation structure was used for the risk factor analysis. Flock-level incidence risk was used as a dichotomized outcome variable. A total of 129 dichotomous and categorical risk factors were derived from the questionnaire data. Initially an univariate analysis was conducted to identify risk factors variables to be included in a multivariable model at a p-value cut-off of 0.2. The multivariable model was build with a backward elimination procedure, hence the maximum model was fit and then risk factor variables were sequential removed at each step until all variables had p-values <0.05. If flocks were positive at a preceding sampling they were not used for the next sampling, because only flocks that changed their status from negative to positive between flock-sampling could be used. To avoid losing flock-sampling periods from the analyses flock-sampling as a categorical variable was fitted to all univariate models and to the multivariable model.

Viet Nam:

Two districts within each of four provinces (Dong Thap, Tien Giang, Ben Tre, Soc Trang) located in the northern part of the Mekong Delta of Viet Nam were selected for inclusion in the study (see figure). Sample size calculations were performed using WinPepi version6.8 (Abramson, 2004) The expected bird-level prevalence for H5 virus was based on results of PCR analysis of swab samples collected by the RAHO VI during surveillance work in five provinces of the Mekong Delta of Viet Nam in January 2007. Of 395 individual birds (H5 vaccinated and unvaccinated ducks), 25 (4.9%) had tested PCR positive. Assuming a true bird-level H5 virus prevalence of 5%, a required precision of ±2% and 95% confidence for the estimate from each visit, a sample size of 457 ducks was required. As no information on the extent of clustering of H5 virus infection by farm was available, the sample size was not adjusted to account for clustering within farm; hence this estimated sample size was viewed as an underestimate. The costs of diagnostic testing and travel limited the number of farms that could be visited. However, it was possible to sample a total of 560 ducks and 240 in-contact chickens (7 ducks and 3 in-contact chickens in each of 80 farms) at each of 7 bi-monthly visits.

A sampling frame, based on records from the 2006 HPAI vaccination campaign, was created by staff of the provincial sub-Department of Animal Health (DAH), in cooperation with veterinarians of the District Veterinary Stations (DVS) and para-veterinarians working in the districts and villages. Farms with >1000 ducks or < 12 ducks were ineligible for selection. A combined list of all villages in the two selected districts per province was created and. a total of 16 study villages across the four provinces were selected for inclusion in the study. Five farms in each village were then selected by simple random sampling without replacement using computer-generated random numbers.

At the time of the study, HPAI vaccination was conducted routinely in ducks and chickens in Viet Nam: therefore in addition to vaccinated birds, a sample of unvaccinated birds was selected and vaccination was withheld from them during the study. On each farm, a total of 10 birds was selected: 7 ducks (4 sentinels and 3 vaccinated ducks) and 3 chickens (2 sentinels and 1 vaccinated chicken) that were in contact with the ducks (including scavenging together or using common areas). Study birds were individually identified with numbered wing tags with different colours for vaccinated and unvaccinated birds, or with numbered adjustable leg bands

Visits were conducted in May, July, September and November 2007, and January, March and May 2008. At each visit, a blood sample and oropharyngeal and cloacal swabs were collected from each study bird. Both swabs from the same bird were placed into one tube containing virus transport medium. Serum samples were tested for H5 antibody using the hemagglutination inhibition (HI) test and antigen prepared at AAHL from a chicken southern Viet Nam in 2004. Real-time reverse transcriptase PCR was used to detect H5 virus RNA from pooled oropharyngeal and cloacal swabs. From each set of combined swabs in virus transport medium, pools of swab media from five individual birds were tested.

The data analysis in Viet Nam was conducted using the same methodology as in Indonesia (above) with some modifications. For Viet Nam, bird-level seroprevalence was calculated separately for unvaccinated and vaccinated ducks and chickens for each visit, and pooled over the whole study period. Titres in vaccinated birds were only analysed for birds more than 3 weeks post vaccination to allow the development of an immune response to the vaccine virus. In addition, bird-level prevalence of positive HI titres for vaccinated ducks and in-contact chickens was calculated for different time periods post-vaccination for each sampling (>3-6 weeks, >6-12 weeks, >12 weeks). For the bird-level prevalence estimates for the entire study period in Viet Nam, we were not able to include finite population correction for the SSU because some farms were replaced with neighbouring farms when all birds of the initially selected farm had been sold, thus for some villages the number of farms selected over the entire study period was greater then the number of farms specified at the beginning of the study as being present in the village.

Furthermore, in Viet Nam logistic regression models were not only used to compare the odds of birds having positive HI titres between species, but also comparisons were made between different age groups (≤90 days, 91-360 days, >360 days) and between genders.

In both countries, baseline questionnaires were used to record information on the flock structure, flock management, feeding, housing, scavenging practices, the health status of ducks and mortalities due to HPAI and other diseases. Questionnaire data were analysed in SPSS PASW 18.0 (SPSS Inc.) and descriptive results such as proportions, percentages, means, standard deviations and minimum and maximums and ranges were produced.

Also in both countries, shorter bi-monthly questionnaires were used to monitor changes in flock management between visits. This information will be used to identify management risk factor associated with duck flocks becoming H5 seropositive.

(ii) Prevalence of HPAI in 'moving' duck flocks

During the initial field studies on 'stationary' duck flocks in Indonesia and Viet Nam, the project team became aware of a second production system for farming ducks in both countries – the nomadic or 'moving' production system. Although detailed information on this production system was not available in either country, it was clear that many characteristics of this system might pose a high risk for the maintenance and transmission of H5N1 virus. Therefore, longitudinal studies to determine H5N1 antibody prevalence and incidence rates, to determine H5N1 virus prevalence, to describe HPAI vaccination patterns and to describe risk factors associated with HPAI infection in moving ducks were designed.

In Indonesia, 9 moving duck flocks in each of six districts on Java (Brebes, Pemalang, Batang, Kendal, Klaten, Purworejo) were included in the study (see figure), giving a total of 54 flocks. Fifteen birds were selected from each flock, comprising 10 unvaccinated sentinel birds and 5 birds to be vaccinated against HPAI. The ducks were wing-tagged and leg-banded to allow repeated sampling.

In Viet Nam, 6 moving duck flocks from each of 2 districts in each of 4 provinces (Tien Giang, Vinh Long, Ben Tre, Dong Thap) of the northern Mekong Delta region were included in the study, giving a total of 48 flocks. Twenty birds were selected from each flock, comprising 10 unvaccinated sentinel birds and 10 birds to be vaccinated against HPAI. Birds were marked as in Indonesia (above).

The duck flocks in each country were monitored at monthly intervals over a period of 6 months. At the first visit, a baseline questionnaire was completed by the farmer, to describe the flock's management over the previous 12 months and to specify details of the planned management over the following 12 months. At subsequent visits, a monthly questionnaire was completed, recording changes to the flock structure, movements of the flock, scavenging locations, health status of the ducks and production parameters since the previous visit. At each visit, blood samples and cloacal and pharyngeal swabs were collected from the marked birds.

In the laboratory, serum samples were tested using the HI test to detect antibody against H5N1 virus, and swabs were tested for the presence of H5N1 virus RNA using real-time reverse transcriptase PCR. Any swabs positive for H5 RNA were subjected to virus isolation in embryonated eggs.

(iii) Studies of external HPAI risk factors relating to moving duck management

Characteristics of 'external risk factors' related to HPAI occurrence in moving duck flocks was also investigated. Interviews were conducted with people associated with the moving duck flocks that were monitored in the study. These interviews comprised a questionnaire that included questions connected to potential biosecurity risks. The interviews were conducted with owners of the rice paddies on which the moving duck flocks grazed, the people who supplied transport to the moving duck flocks and the owners of hatcheries which provided ducklings to the flocks or purchased eggs from the flocks. Descriptive statistics such as proportions, percentages, means, standard deviations and minimum and maximums and ranges will be used to describe the management factors related to the moving duck management. Hazards that might be able to increase the risk of HPAI infection of moving duck flocks will be highlighted. The data analysis is currently underway.

(iv) Analysis of risk factors in the major Mekong Delta HPAI outbreak of 2006 & 2007 – a case-control study – Viet Nam

[Henning KA, Henning J, Morton J, Long NT, Ha NT, Meers J. (2009) Farm- and flock-level risk factors associated with Highly Pathogenic Avian Influenza outbreaks on small holder duck and chicken farms in the Mekong Delta of Viet Nam. *Preventive Veterinary Medicine*. 91:179-88]

In Viet Nam, a large-scale vaccination program together with other control measures such as movement control of live birds, breeding restrictions in small holder households and education campaigns was associated with 11 consecutive months in 2006 with no HPAI disease outbreaks in poultry and no human cases of H5N1 infection. However in early December 2006, HPAI re-emerged in the Mekong Delta of Viet Nam and resulted in another wave of outbreaks in poultry which lasted until late January 2007. This event coincided with the commencement of the project's field activities in that country, so a decision was made to conduct a retrospective matched case–control study soon after the epidemic. Although not planned in the original project proposal, it was felt that the study might be able to provide valuable information on farm- and flock-level risk factors for HPAI H5N1disease outbreaks on small-holder poultry farms in Viet Nam.

The retrospective matched risk-based case–control study was conducted with the flock as the unit of analysis. The individual farm was the primary sampling unit for case and control farms; case and control flocks were selected from within study farms. For every case flock (flocks that experienced an HPAI H5N1 outbreak), one matched control flock (flocks on control farms i.e. farms that had poultry but that did not experience an HPAI H5N1 outbreak at that time) was selected from each of two control farms. Case and control flocks were matched on the time of outbreak occurrence, on farm location and on species. Data collection was carried out between the 2nd of February2007 and the 14th of March 2007. A flock was defined as a group of ducks or chickens of the same breed from the same farm that are managed together.

Case farms were selected from those meeting the following outbreak criteria:

1. The farm was a small holder poultry farm stocking ducks (including Muscovy ducks) and/or chickens located in the Mekong Delta of Viet Nam.
2. The HPAI outbreak on the farm had to have occurred between the 6th of December 2006 and the 31st of January 2007.
3. HPAI H5N1 had to be confirmed with a positive real-time PCR test from samples obtained from affected birds on the farm.
4. Clinical signs which are consistent with HPAI H5N1 had to be present in affected birds on the farm during the outbreak.

Twenty-four case farms were selected from the 99 farms in 7 provinces of the Mekong Delta Region that had recorded HPAI outbreaks between the 6th of December 2006 and the 31st of January 2007. Within each province, case farms were selected randomly without replacement from a list of all outbreak farms. The number of case farms selected within each province was determined based on accessibility of farms, with less case farms selected in provinces where access was most time consuming and expensive. The selected farms were visited and owners were questioned to confirm that the farm met the selection criteria and to confirm that the owner was willing to participate in the study

For each case farm, two matched control farms were selected. Control farms were selected from those meeting the following criteria:

1. The farm was located the same village as the corresponding case farm.
2. There was no reported HPAI outbreak occurring on the farm 2 weeks before or 2 weeks after the first day of the outbreak (outbreak date) on the corresponding case farm.
3. The same species class of bird (ducks/Muscovy or chickens) had to be present on the control farms as on the corresponding case farm from 2 weeks before until 2 weeks after the outbreak date in the case farm.
4. The crude mortality risk in all flocks from 2 weeks before until 2 weeks after this outbreak date was less than 5%.

Within each village, potential control farms were randomly selected from a list provided by the District Veterinary Station (DVS) by drawing numbers from a hat without replacement. Owners of selected farms were then questioned to determine whether the farm met the selection criteria and to confirm that the owner was willing to participate in the study.

A questionnaire containing 67 mostly closed questions was developed in English.. Information on household demographics, farm characteristics, husbandry practices, trading practices, poultry health, vaccination, biosecurity measures carried out on the farm and integrated poultry and fish production was collected using the questionnaire. The same questionnaire was used for both case and control farms, except for collection of additional information on the case farms that related specifically to the outbreaks. Interviews were conducted by 5 trained veterinarians from the RAHO VI and VII within the Department of Animal Health and 2 veterinarians from the University of Queensland who were assisted by translators Interviews were conducted in Vietnamese and answers were recorded on printed copies of the questionnaire in English. On average, each interview took 40 min.

Statistical analyses were conducted with the flock as the unit of analysis, using Stata 10. Exact stratified logistic regression models were used to assess putative risk factors associated with a flock having or not having a HPAI outbreak. Potential risk factors evaluated included a wide range of management factors that could be associated with the spread and maintenance of HPAI. Those biologically plausible risk factors associated with flock HPAI status at $P \leq 0.25$ from the univariable exact logistic regression models and with at least 55 flocks with exposure data were then selected for inclusion in the multivariable modelling process.

Experimental activities

(i) Characterisation of Indonesian H5N1 virus isolates

[Wibawa H, Henning J, Wong F, Selleck P, Junaidi A, Bingham J, Daniels P, Meers J. (2011) A molecular and antigenic survey of H5N1 highly pathogenic avian influenza virus isolates from smallholder duck farms in Central Java, Indonesia during 2007-2008. *Virology Journal* 2011, 8:425]

Virus isolates collected during the field studies in Indonesia were analysed genetically and antigenically by Hendra Wibawa and his colleagues at DIC Wates, AAHL and UQ. Virus isolation was attempted from oropharyngeal and cloacal swabs collected from ducks and in-contact chickens during the longitudinal survey of 'stationary' duck flocks (described above). From each bird, the two swabs were placed into a single tube containing 3 ml viral transport medium. In addition, samples were collected during the investigation of disease outbreaks on the study farms. Oropharyngeal and cloacal swabs were collected from decayed carcasses, while fresh carcasses were transferred to the veterinary diagnostic laboratory at Disease Investigation Centre (DIC) Regional IV Wates, Indonesia, for post-mortem examination and collection of tissue samples. Healthy marked birds from the outbreak study farms were also swabbed during these disease investigations.

Molecular and virological testing was conducted in the DIC Wates. Swab media sub-samples from the survey were combined in pools of five by species and tested for the presence of viral RNA using real-time reverse transcription polymerase chain reaction (rRT-PCR) assays for type A influenza and H5 subtype as previously described (Heine et al, 2007). Virus isolation in specific-antibody-negative (SAN) embryonated chicken eggs was performed on rRT-PCR positive or indeterminate swabs collected in the longitudinal survey and on swabs and tissue samples collected during disease investigations. The H5 virus then was confirmed by haemagglutination inhibition (HI) assay with H5-specific antiserum using standard methods (OIE, 2009).

Equal numbers of virus isolates from chickens (n=50) and ducks (n=50) were selected from 132 isolate samples collected over the study period of 13 months and sent to the Australian Animal Health Laboratory (AAHL), Geelong, for molecular and antigenic characterization. These viruses were propagated in specific pathogen-free (SPF) embryonated chicken eggs within microbiological physical containment level 3 facilities at AAHL. Allantoic fluid was collected and tested for haemagglutination of chicken red blood cells (RBC), followed by rRT-PCRs for influenza type A and H5 subtype virus, respectively.

Eighty-four samples were found to have viable H5 subtype virus and they were subjected to molecular characterization. Of these 84 viruses, 8 were isolated from dead ducks, 46 from dead chickens, and 28 and 2 were isolated from live ducks and live chickens, respectively. Seventy-six (90.5%) viruses were isolated from live or dead ducks or chickens during the investigation of disease outbreaks, while the remaining 8 (9.5%) viruses were isolated from live ducks during the bi-monthly survey.

Sequencing of the HA gene was conducted on all 84 virus isolates, while a subset of 24 isolates were selected for NA gene sequencing based on characteristics of their HA amino acid sequence and position in the HA phylogenetic tree. Viral RNA was extracted from allantoic fluids using Rneasy® Mini Kit (Qiagen, Australia) as per manufacturer's protocol. One-step RT-PCR reaction were performed with Super-Script™ III Reverse Transcriptase (Invitrogen, Australia) using respective primers for HA and NA, to obtain overlapping fragments that span the entire coding sequence of each gene. PCR products were extracted from an agarose gel using QIAquick Gel Extraction Kit (Qiagen, Australia), and each purified amplicon was used directly for cycle sequencing using BigDye Terminator® v3.1 Sequencing Kit (Applied Biosystems). Post sequencing products were purified using BigDye XT Terminator® Purification Kit (Applied Biosystems) prior to running on the ABI PRISM 3130*xl* Genetic Analyzer (Applied Biosystems).

Virus gene sequences were aligned using ClustalW program. Multiple sequence alignments of 1683 and 1353 nucleotide lengths of the HA and NA coding sequences respectively, were used for phlylogenetic analysis, which was conducted using the MEGA v4.0 software using the Neighbour-Joining (NJ) method with 2000 bootstrap replicates and the Tamura-Nei 93 (TN93) nucleotide substitution model.

Potential positive (diversifying) and negative (purifying) selection affecting the HA gene were detected by three codon-based maximum-likelihood methods, single likelihood ancestor counting (SLAC), fixed effects likelihood (FEL), and internal fixed effects likelihood method (IFEL), using the web interface of the HY-PHY package (www.datamonkey.org).

The 24 isolates that were selected for NA sequencing were further characterized for their antigenic reactivity based on the HI test using a panel of chicken sera produced from three different clade 2.1.3 antigens and one clade 1 antigen.

(ii) Experimental infection studies and investigation of the use of feathers for diagnosis

[Bingham J, Green DJ, Lowther S, Klippel J, Burggraaf S, Anderson DE, Wibawa H, Hoa DM, Long NT, Vu PP, Middleton DJ, Daniels PW. (2009) Infection studies with two highly pathogenic avian influenza strains (Vietnamese and Indonesian) in Pekin ducks (Anas platyrhynchos), with particular reference to clinical disease, tissue tropism and viral shedding. *Avian Pathology*. 38(4):267-78.

Wibawa H, Bingham J, Nuradji H, Lowther S, Payne J, Rookes J, Junaidi A, Middleton D, Henning J, Meers J. The pathogenesis of two distinct clades of Indonesian H5N1 avian influenza viruses in chickens and ducks. (in preparation)]

A series of experimental infection studies (challenge trials) was conducted at the Australian Animal Health Laboratory, Geelong and at Bbalitvet, Bogor under the supervision of Dr John Bingham. The majority of these trails were conducted by two John Allwright Fellowship holders (PhD students) from Indonesia – Hendra Wibawa from DIC-Wates and Harimurti Nuradji from Balitvet, Bogor.

- *Challenge studies at AAHL*

Three challenge studies were conducted using two Indonesian H5N1 HPAI virus isolates (A/duck/Sleman/BBVW-1003-34368/2007 – clade 2.1.1 and A/duck/Sleman/BBVW-598-32226/2007 - clade 2.1.3) and one Vietnamese H5N1 HPAI virus isolate (A/Muscovy duck/Vietnam/453/2004 - clade 1), propagated in SPF embryonated chicken eggs. The viruses were inoculated into 7-week-old Pekin ducks (*Anas platyrhyncos*) and 3-week-old broiler chickens (*Gallus domesticus*), which were housed separately (according to species) in the PC3 facilities at AAHL and provided with commercial pellets and water *ad libitum*. The birds were inoculated by the intra-nasal, oral and ocular routes with between $10^{6.0}$ and $10^{8.4}$ EID_{50}. Fourteen birds of each species were used in the trials using Indonesian isolates and five of each species were inoculated with the Vietnamese virus. Birds were examined daily following inoculation. Oropharyngeal and cloacal swabs, blood samples and 1-4 immature and mature contour feathers were collected daily from the inoculated birds. Post-mortem examination was conducted following euthanasia and samples of body tissues, flight and tail feathers and skin samples from 7 different regions of the body. All experimental procedures were conducted with the approval of AAHL's Animal Ethics Committee.

Virus isolation in embryonated eggs was attempted from all swab samples. The inoculated eggs were incubated for 5 days at 37^0 C and the allantoic fluid tested using haemagglutination (HA) test with 0.5% chicken red blood cells. Virus titration in Vero cells was also conducted on oropharyngeal and cloacal swabs and feathers. Feather samples were initially ground using mortar and pestle with sea sand. Briefly, samples were titrated using in four replicates in 96-well plates, incubated at 37^0C for 5 days and the titre was calculated using standard formula. Swabs and feather samples were also tested using

Anigen rapid test kits (Anigen® AIV Ag and H5 AIV Ag), kindly provided by Animal Genetics Inc. Hwasung, South Korea through Life Bioscience Pty Ltd, Oakleigh, Victoria.

Histological and immunohistochemical examination of tissue and skin sections was conducted to assess tissue morphology and presence of viral antigen, respectively.

- *Challenge study at Bbalitvet*

One challenge study using native chickens was conducted at Bbalitvet. Ten 6-week-old and ten 16-week-old native backyard chickens (*Gallus-gallus bankiva*) were obtained from Indonesian Research Institute for Animal Production, Ministry of Agriculture. Chickens were housed in two separate isolation units (Montair Andersen B.V. HM 1500, Sevenum, The Netherlands) according to age group. The chickens were inoculated by the intra-nasal, oral and ocular routes with totals of 0.2 ml of diluted infective allantoic fluid containing $10^{6.8}$ EID_{50} ($10^{6.0}$ $TCID_{50}$). Chickens were monitored daily throughout the experiment for clinical sign and mortality. Oropharyngeal and cloacal swabs were collected daily during the experiment including the days prior to challenge. Mature, immature, flight and tail feathers and tissues (skin, brain, lung, heart and spleen) were collected from dead or euthanized chickens. The swabs and feathers were each placed in 2 ml transport medium. The internal organ and skin samples were split for processing, with half being stored fresh for virus isolation and half being fixed in 10% neutral buffered formalin, sectioned, and stained with haematoxylin and eosin for histological examination and immunohistochemically stained for the detection of influenza virus antigens in the tissues.

Virus isolation was conducted in embryonated chicken eggs and virus titration in Vero cells using standard procedures. Each sample was inoculated into three 9 to 11-day old embryonated chicken eggs, which were incubated at 37^0C for 5 days and allantoic fluids were tested with HA assay. Virus titration was conducted using serial tenfold dilutions of each sample from 10^{-1} to 10^{-8}, inoculated into Vero cells in 96 wells flat-bottomed tissue culture plate andincubated at 37^0C in a humidified CO_2 incubator with 5% CO_2. Cells were monitored daily up to 5 days to evaluate the cytopathic effect. The titre was calculated by using standard formula.

Before being processed, tissues were thawed, weighed and homogenized using a tissue grinder, and made into 10% homogenates in phosphate buffered saline (PBS). Feathers (2 feathers) were ground with mortar and pestle in PBS, with sterile sea sand (Merck) added to assist homogenization. HA and HI tests were performed according to standard procedures (OIE, 2009). Inactivated H5N1 HPAI virus ((A/duck/Sleman/BBVW-598-32226/2007) was used as the antigen. HI titres ≥4 (2^4=16) were considered as positive.

Rapid antigen detection tests (Anigen® AIV Ag, Animal Genetics Inc. Hwasung, South Korea) were conducted according to the company's instructions with minor modifications. Briefly, four to five drops (approximately 125µL) of sample were added into the sample hole of the kit with the dropper provided. The test result was read after 15 minutes.

6 Achievements against activities and outputs/milestones

Objective 1: To understand the epidemiology of HPAI H5N1 virus in small-holder duck production systems in Indonesia and Viet Nam.

no.	activity	outputs/ milestones	completion date	comments
1.1	Conduct longitudinal surveys in small holder farms ('stationary' duck flocks) in Indonesia and Viet Nam (PC)	Outputs: The role of ducks in the persistence of HPAI in small holders assessed. The impact of physical association between ducks and chickens in spreading HPAI determined.	August 2008	In Indonesia, serological results indicated that about 20% of the duck flocks and 2% of the in-contact chicken flocks had been exposed to HPAI virus. In about 16% of flock-visits ducks were positive while chicken at the same sampling were H5 antibody negative, indicating that ducks were exposed to H5 and might have been carriers of the virus. Disease outbreaks were common with nearly half of the 80 monitored flocks experiencing at least one outbreak over the 12-month study period. PCR results indicated that virus shedding was increased during outbreak periods. In Viet Nam, although serological results indicated vaccination responses were generally poor, no mortality due to HPAI was reported in any of the study villages suggesting that the flock level of protection was adequate. Flock-level virus prevalence was low with only 0.2% of flocks having birds that were shedding H5 virus over the study period. These results from Viet Nam suggest that despite not achieving protective levels of immunity in all birds, vaccination against HPAI can decrease the likelihood of disease outbreaks and reduce mortality in the presence of active exposure to the virus.
1.2	Conduct longitudinal studies on nomadic ('moving') duck flocks in Indonesia and Viet Nam (PC)	Outputs: The role of nomadic ducks in the spread of HPAI infection assessed	Ongoing	Databases were created, data entry was completed and cross-checked for completeness and errors and coded for analysis. Descriptive and univariate analysis to describe the prevalence of infection has been completed. Preliminary analysis indicated that the overall crude bird-level HPAI H5 antibody prevalence (HI titre≥16) was about 15.5% in Viet Nam and 5.3% in Indonesia. Data analysis in both countries is continuing after the official completion of the project.
1.3	Conduct surveys with people involved in nomadic duck management in Indonesia and Viet Nam (PC)	Outputs: Biosecurity risks associated with the moving duck management are evaluated in these studies.	Ongoing	"External risk factors" related to moving duck management of monitored study flocks were explored through interviews of people associated with 'moving' duck flock production system. Databases were created and data entry completed. Data analysis is continuing after the official completion of the project in both countries.

PC = partner country, A = Australia

Objective 2: To understand the role of ducks as maintenance hosts and amplifiers of H5N1 virus during and after infection …

no.	activity	outputs/ milestones	completion date	comments
2.1	Challenge trials conducted in ducks using H5N1 viruses isolated in Viet Nam and Indonesia. (A)	Output: The duration of shedding of HPAI and amount of virus produced in infected ducks quantified. Characteristics of the antibody response in infected ducks determined.	January 2010	Two H5N1 virus isolates from the longitudinal field study in Indonesia were selected for the challenge trials. Groups of 14 chickens and 14 ducks were challenged with one subclade 2.1.1. virus (isolated from a dead duck) and one 2.1.3 virus (isolated from a healthy duck) and the birds were monitored for clinical signs, virus shedding and tissue distribution of virus. Both virus isolates were highly pathogenic in chickens, but not able to cause apparent disease in the experimentally-infected ducks. In chickens, both of the virus isolates replicated in most body tissues and were shed orally and cloacally at high titres; conversely, their replication in ducks was limited and they were shed intermittently from the oral route only, and at very low concentrations. The levels of replication and shedding of the 2.1.1 virus in ducks were lower than those of the 2.1.3 virus. No evidence of long-term infection or sustained virus shedding in ducks was observed.
2.2		Output: H5N1 viruses isolated and characterised.	2009	Approximately 100 virus isolates from Indonesia have been isolated. The nucleotide sequences of haemagglutinin (HA) and neuraminidase (NA) genes of selected isolates were analysed and antigenic mapping using haemagglutination inhibition data was performed. Of 84 virus isolates analysed, 80 belonged to HA subclade 2.1.3, one belonged to subclade 2.1.1 and three belonged to the Indonesia/6/05 H5N1-like virus subclade. The 24 selected isolates analysed by HI tests (using 4 reference antisera) were antigenically similar even though some of these isolates were from distinct genetic subclades. None of these isolates had the antigenic pattern of the antigenic variant virus, implying that there have been no significant mutations in the HA epitopes of these H5N1 isolates.
2.3	Conduct challenge trials to examine the long-term virus shedding patterns in ducks (A)	Output: the ability of ducks to maintain long-term infection and to be carrier hosts of H5n1 virus assessed	ongoing	These trials form part of the PhD research of one of the JAF students associated with the project. His candidature continued past the end-date of the project.

PC = partner country, A = Australia

Objective 3: To understand the pathogenesis of HPAI H5N1 virus infection in ducks

no.	activity	outputs/ milestones	completion date	comments
3.1	Challenge trials conducted in ducks using H5N1 viruses isolated in Viet Nam and Indonesia. (A)	Outputs: The pathogenic effects of Vietnamese and Indonesian H5N1 viruses on ducks determined	December 2010	The distribution and titre of virus in a range of tissues of experimentally-infected ducks and chickens has been assessed, following the challenge trials described above in Objective 2. Chickens were euthanized when they showed moderate to severe clinical signs, whereas 3 ducks were sacrificed at each sampling point (days 2, 4, & 7) and 5 ducks were kept until 18 days post inoculation (p.i.). Chickens inoculated with 2.1.1 and 2.1.3 virus isolates were severely depressed, lethargic and died by 30 and 24 hours p.i., respectively. Virus was detected by immunostaining or virus isolation from lung, heart, pancreas, skeletal muscle, spleen and brain. Ducks showed no apparent clinical signs and no increase in temperature. Small amounts of antigen were detected in the epithelieum of air sacs and paranasal sinuses of some ducks. Virus was isolated (at lower titres) from heart, lung, pancreas and spleen of some ducks up to 4 days p.i., but not from the brain of any ducks and not from any tissues after 4 days p.i. Work on this objective continues through the PhD research activities of two JAF students.

PC = partner country, A = Australia

Objective 4: To evaluate the efficacy of current vaccines in ducks and determine the possible role of vaccination in reducing virus load and shedding in ducks

no.	activity	outputs/ milestones	completion date	comments
4.1	Challenge trials conducted in vaccinated ducks using H5N1 viruses isolated in Viet Nam and Indonesia. (A)	Outputs: The potential for vaccine to reduce viral load and duration of shedding in ducks evaluated.		Preliminary work on this objective focused on the development and refinement of the challenge model, which was essential before vaccination trials could be conducted. This was based on the challenge trials outlined in objectives 2 & 3 above. During the course of the project, a decision was made by the project team that the remaining work in this objective would not be pursued. This decision was based on consideration of the large number of laboratory-based H5N1 vaccine trials that have been conducted and published by other researchers around the world since the commencement of the project. The novelty of this aspect of the project was therefore considerably reduced and the need to conduct the work was considered highly questionable.

4.2	Evaluation of vaccination responses in ducks vaccinated in field studies	Outputs: antibody responses in vaccinated ducks described	December 2010	On each farm included in the longitudinal field study on 'stationary' duck flocks in Viet Nam, the sampled birds comprised 7 ducks (4 unvaccinated and 3 vaccinated) and 3 chickens (2 unvaccinated and 1 vaccinated) that were in close contact with the ducks. Only 54.3% (95% CI: 39.2, 69.3) of vaccinated ducks and 55.5% (95% CI: 46.8, 64.2) of vaccinated in-contact chickens had H5 antibodies at more than 3 weeks post-vaccination. At about 40% and 48% of flock- visits, less than 50% of sampled vaccinated ducks and chickens, respectively, had positive antibody titres. Despite these moderate responses post-vaccination, flocks were not affected by HPAI outbreaks during our study period.

PC = partner country, A = Australia

Objective 5: To develop recommendations for effective monitoring of infection in ducks that will assist in making policy for control of HPAI

no.	**activity**	**outputs/ milestones**	**completion date**	**comments**
5.1	Develop recommendations for the control and surveillance of HPAI infections in ducks and in other poultry associated with duck production (PC & A)	Outputs: Peer-reviewed journal articles published Conference and meeting presentations to present and discuss research findings In-country meetings held mid-2010 to present study results to decision makers in each partner country	Journal articles and conference presentations on-going. Project meetings held June 2010	The findings of the project were readily available to decision makers in both partner countries throughout the course of the project, through journal articles, conference and meeting presentations, and the final project meetings. Implementation of massive vaccination programs and controlled culling of infected birds occurred soon after the start of the project and to some extent negated the weight of this objective.

PC = partner country, A = Australia

7 Key results and discussion

As with the methodology section, the results and discussion is divided into two sections – the field activities of the project in Indonesia and Viet Nam, and the experimental activities conducted predominantly at AAHL, Geelong.

A: Field activities

(i) Prevalence and incidence of HPAI in 'stationary' duck flocks

[Henning J, Wibawa H, Morton J, Usman TB, Junaidi A, Meers J. (2010) Scavenging ducks and transmission of highly pathogenic avian influenza, Java, Indonesia. *Emerging Infectious Diseases* 16(8):1244-50.

Henning J, Henning KA, Morton JM, Long NT, Ha NT, Vu le T, Vu PP, Hoa DM, Meers J.(2011) Highly Pathogenic Avian Influenza (H5N1) in ducks and in-contact chickens in backyard and smallholder commercial duck farms in Viet Nam. *Preventive Veterinary Medicine*. 101(3-4):229-40.

Henning J, Morton J, Wibawa H, Yulianto D, Usman TB, Priyono W, Junaidi A, Meers J. (2011) Focussing not on HPAI outbreaks: incidence and risk factors for H5 infection of clinically healthy ducks flocks. *Preventive Veterinary Medicine* (submitted)

Henning J, Wibawa H, Yulianto D, Usman TB, Junaidi A, Meers J. (2011) The management of smallholder duck flocks in Central Java, Indonesia, and potential hazards promoting the spread of Highly Pathogenic Avian Influenza virus. *Worlds Poultry Science Journal* (submitted)]

Indonesia

From March 2007 until March 2008, a total of 8,993 serum and swab samples were collected from clinically healthy ducks (n = 6,705) and chickens (n = 2,288). The average flock size for ducks was 53.7 and for in-contact chickens 8.5 birds. Of all combined oropharyngeal and cloacal swab sets from individual birds, 8,900 were analyzed in pools of five by real-time RT PCR and all serum samples were tested for H5 antibodies. In addition, during outbreak investigations, a total of 174 sets of swabs from dead birds and 136 sets of swabs from apparently healthy live birds were collected from the outbreak farms.

Bird-level seroprevalences of H5 antibody titres $\geq 2^4$ for all bird-samplings pooled over the whole study period were 2.6% (95% CI 1.8-3.5) for ducks and 0.5% (95% CI 0.0-0.9) for in-contact chickens. The odds of ducks being H5 seropositive were 5.5 times (95% CI 2.1-14.4) higher than for in-contact chickens. The flock-level seroprevalence of H5 antibodies in ducks over the study period was 19.5% (95% CI 14.3-24.6) for ducks and 2.0% (95% CI 0.1-3.9) for chickens. The odds of duck flocks being seropositive were 12.4 times (95% CI 3.9-40.1) higher than for chicken flocks. Duck flock-level prevalence varied over time from 5.9% to 24.7%; the odds of a duck flock being seropositive differed significantly by month (P=0.05), with higher odds in July 2007 (odds ratio [OR] = 3.1; 95% CI 1.1-9.0), September 2007 (OR = 2.9; 95% CI 1.4-6.3), November 2007 (OR = 3.7; 95% CI 1.7-8.1) 2007, January 2008 (OR = 3.9; 95% CI 1.5-10.0) and March 2008 (OR = 5.3; 95% CI 1.9-14.7) relative to May 2007. In-contact chicken flock-level seroprevalences remained below 6.2% throughout the study. In 21.4% of 501 farm-visits, one or more study ducks were H5 seropositive, when at the same time all of the in-contact study chickens on the same farm were seronegative. Conversely, on only 1.4% of farm visits were one or more study chickens found to be H5 seropositive when all of the study ducks in the farm were seronegative. Seropositivity of ducks provided no indication of seropositivity of chickens on the same farm and vice versa (OR = 3.9, 95% CI 0.4-43.0).

One explanation for the higher seroprevalence in ducks compared to chickens is that the HPAI H5 field virus was circulating more successfully among ducks compared to in-contact chickens because ducks are more likely to harbor and transmit the virus. In

addition, the observed lower proportion of chickens with positive H5 titers was probably influenced by higher HPAI mortalities of chickens, hence resulting in fewer surviving chickens with H5 antibodies.

In flocks where no study birds were detected with antibody, these may have been present in other ducks or chickens in the same flock, and therefore our flock estimates are likely to underestimate the actual true flock prevalence. As we sampled more ducks than chickens in each study flock, the risk of non-detection of infection was higher for chickens. However based on sample size calculations for assessing freedom from disease, this bias is unlikely to explain the differences in flock prevalences of antibodies between ducks and chickens. A further possible explanation for the observed difference in seroprevalences between poultry species is that duck flocks were exposed to HPAI more frequently than chickens. This could be due to grazing of duck flocks in the same rice fields, where potentially other domestic or wild birds have previously grazed.

Birds on 25 of the 96 monitored farms (26.0%) tested H5 viral RNA positive, with 20 farms having test positive birds on only one sampling occasion and five farms having test positive birds on two different sampling occasions. On these 25 farms, 30 flocks tested H5 viral RNA positive over the study period (22 duck and 8 chicken flocks). On only 3 farms, both duck and chicken flocks tested H5 viral RNA positive at the same visit (6 flocks); otherwise, only one flock (either ducks or chickens) was H5 viral RNA positive at any one visit (24 flocks). The flock-prevalence of H5 viral RNA (proportion of flock-visits where at least one study bird was positive) over the entire study period in clinically healthy birds was 2.5% (95% CI 0.9-4.1) for ducks and 1.5% (0.4-2.7) for in-contact chickens.

A total of 35% (34/96) of study farms across all four districts had HPAI outbreaks during the 12-month study period. There was a large increase in the numbers of outbreaks from one outbreak in each of May and June 2007 to seven in both July and September 2007. In 16 of 34 study farms that experienced HPAI outbreaks, combined swab samples were collected from 136 clinically normal birds (109 ducks and 27 chickens) at the same time as samples were collected from dead birds on the same farm. On 68.8% (11/16) of these outbreak farms HPAI H5 virus was isolated from 27.2% (37/136) of clinically normal birds (28 ducks and 9 chickens).

In total, 180 deaths of both marked and unmarked birds (59 ducks and 121 chickens) were assessed virologically. HPAI H5 virus was isolated from 65 of these birds (10 ducks and 55 chickens). Another 14 birds (3 ducks and 11 chickens) had most likely died from HPAI H5 infection based on characteristic clinical signs. Therefore the total number of likely HPAI deaths over the whole study was 79 birds, resulting in a proportional mortality rate for HPAI of 44% (79/180).

Despite high mortality risk in chickens and deaths of some ducks, other birds carrying the virus appeared to be unaffected, indicating that host-specific characteristics of susceptibility might have varied between birds. It is possible that some of these virus-positive healthy birds were in the early stages of infection and were yet to develop clinical signs. However, a small number of chickens in the longitudinal study were H5 antibody positive, providing further evidence that some chickens do survive infection. It is possible that these birds had been infected with low pathogenic Avian Influenza (LPAI) viruses. To our knowledge, the prevalence of LPAI in poultry in Indonesia is unknown. However, H5N1 viruses isolated from dead and live birds in our study were confirmed to be highly pathogenic (discussed in the experimental studies below)

The frequency of occurrence of HPAI outbreaks varied throughout the study period. There was a large increase in outbreaks in July 2007 (the beginning of the dry season), coinciding with a significant increase in the proportion of flocks with seropositive ducks, suggesting that HPAI H5 virus circulated among more duck flocks over this time. It is possible that this time period coincided with particular rice-harvest activities, which allowed intermingling of ducks on the paddy fields and extensive opportunities for virus

release and exposure. It is also possible that other factors, such as the long-distance movement of duck flocks influence outbreak patterns in Indonesia.

Although duck farming has been proposed as a major factor in HPAI maintenance and transmission, few studies have investigated infection patterns over time in poultry farming systems. These results indicate that scavenging ducks may be a source of infection for other poultry and possibly people.

Analysis of management practices: Data from the baseline questionnaire used in the longitudinal study was analysed to identify management practices that could potentially promote the risk for HPAI spread among the duck flocks. The questionnaire recorded information on the flock management, feeding, housing, scavenging practices, the health status of ducks and mortalities due to HPAI and other diseases. The questionnaire contained 64 questions; these questions were multiple choice questions, yes/no questions and open-end questions. The analysis identified a number of potential hazards among the various management practices conducted by the study farmers, some of which are summarised in the table below.

Identified hazards (% of farms where practised)	**Potential impacts on HPAI virus transmission**
Intermingling between ducks and chickens on the same farm was common (48% of farms)	Enhancing interspecies HPAI virus transmission on the same farm
Contact of ducks with neighbours' chickens was assessed to be frequent (44% of farms)	Enhancing interspecies HPAI virus transmission within the village
Ducks often disperse on the way to the scavenging locations (33% of farms)	Enhancing excreted HPAI virus spread on transport routes
Frequent visits of the same rice paddies by various duck flocks are common (88% of farms)	Increasing the excreted HPAI virus concentration in the scavenging locations and enhancing contacts between duck flocks
During scavenging, frequent contact of ducks with other ducks, chickens, people, wild birds occurs (88%, 30%, 80% and 77% of farms respectively)	Enhancing interspecies HPAI virus transmission in scavenging locations
Singing birds often kept as pets on duck farms (17% of farms)	Enhancing interspecies HPAI virus transmission on the same farm
Main predators visiting the scavenging areas were lynx and ferrets (18% of farms)	Predators are susceptible to HPAI infection and might play also a role in the spread of the HPAI virus
Although the majority of duck owners uses rice paddies for scavenging throughout the year (69% of farms), almost a quarter of owners use them only seasonally	Might be related to the seasonal occurrence of HPAI outbreaks
Use of droppings as fertilizer was very important or important for almost 60% of duck farm owners	Highlights the integrated crop-harvest-duck production practiced by small-scale ducks owners

	and enhances HPAI virus spread in the environment
Burning of litter from the enclosures was uncommon (2% of duck farms)	Enhances HPAI virus concentration in the environment
Most important duck health problems specified: deaths from pesticides in the rice paddies, problems that inhibit the ability of ducks to scavenge (e.g. leg injuries) and external parasites	Avian influenza is not considered to be of high importance for duck owners; hence vaccination of ducks against HPAI or preventive culling of infected ducks during outbreaks was not conducted on study farms

This descriptive survey provides scientifically valid data for HPAI risk assessments or further HPAI risk-based investigations.

Incidence analysis: Bird-level incidence remained stable during the first three samplings with a peak being observed in the September 2007 sampling and then decreased to a low in January 2008, followed by a rise again in February 2008. Flock-level incidence rate also was high at the first flock-sampling and peaked in July 2007 and then decreased and remained low until January 2008, before it rose again in February 2008. The pooled incidence rate for the entire study period was 1,673.0 per 1,000 flock-years at risk (95% CI 1304.1, 2041.9).

In the univariate analysis, of 129 risk factors assessed, 16 were significant at $p<0.2$ and were included in the multivariable analysis. Four risk factors were found to be significant in the multivariable model. Ducks scavenging around neighbouring houses within the village increased the risk of the flock developing H5 antibodies. The consumption of carcasses of dead birds also increased this risk (although this practice was only conducted on six out of 310 flock-samplings over the study period). If duck flocks were confined over-night on the farm, the risk of flocks developing H5 antibodies was reduced. Finally, if birds died suddenly on the farm in the preceding two months before the flock-sampling, the risk of duck flocks developing H5 antibodies was also reduced.

The increased risk of flocks becoming H5 seropositive as a result of roaming around neighbours' houses in the village is likely due to increased contact with various other birds, people and other sources of infection that this practice would allow. This highlights the significance of the preventive measure of separating 'stationary' duck flocks from other flocks, not only in their scavenging areas, but also in their village environment.

If birds were confined over night on the farm, the risk of HPAI infection was reduced, most likely because there was less contact with potentially infected birds or other sources of infection. In contrast, confinement of the flock overnight in the rice field had an increased the risk of infection in the univariate analysis, although this risk was not apparent when adjustments were made for other risk factors in the multivariable analysis. This finding again highlights the need to reduce contact with potential sources of the virus, both during the day and night.

The consumption of carcasses of dead birds by the farmer and their family was a significant risk factor for H5 seroconversion of the flock, although this was an uncommon practice on the study farms. The exact cause of death of birds that were consumed is unknown, but sudden deaths had not occurred on any of the farms where carcases were consumed. It's possible that the birds were sick or injured and considered at risk of dying by the farmer, so were slaughtered and consumed. If HPAI-infected birds were slaughtered, their carcasses would have contained high concentrations of virus and through slaughtering and disposal of the remains, the virus might have readily spread across the farm. It appeared that farmers were aware of the risks associated with eating birds that had died suddenly, but when deaths occurred more slowly, the carcasses were considered appropriate to eat. Based on this study, it is important to educate farmers that

the consumption of any carcasses of dead birds will likely increase the risk of HPAI infection in their flock.

Surprisingly, farmers who reported sudden deaths in their flocks in the previous two months were less likely to have flocks that seroconverted. On the 25 farms that reported sudden deaths, the carcasses of the birds were rapidly disposed using a variety of methods (most commonly burial of the carcass). This rapid disposal suggests that sudden deaths made farmers aware of the bio-security risks associated with these carcasses and that they were attempting to prevent further spread of HPAI infection within the flock. This disease control practised by the farmers appears to have been effective and ultimately reduced the risk of H5 seroconversion in their flocks.

This study was the first to estimate incidence rate of H5 virus infection in clinically healthy duck flocks. The four risk factors that were identified should be included in awareness and education programs aiming for change in farmer attitudes and management practices. Based on these results, practical and simple interventions can be adopted.

Viet Nam

A total of 5433 bird-samplings and 555 flock-samplings were completed across the 80 farms over the seven study visits. Vaccination status (vaccinated and date of vaccination or not vaccinated) was recorded for 5409 bird-samplings. A total of 47 initially selected farms were replaced over the study period.

Serology in unvaccinated birds: The bird-level seroprevalence of H5 antibodies in unvaccinated birds for visits pooled across the entire study period was 17.5% (95% CI: 14.1, 20.9) for ducks (including Muscovy, N=2235) and 10.7% (95% CI: 7.4, 14.1) for in-contact chickens (N=1087). The odds of unvaccinated ducks being H5 seropositive were 1.9 (95% CI: 1.1, 3.1) times higher than for unvaccinated chickens. Between May 2007 and May 2008, bird-level seroprevalence peaked in the January 2008 in both ducks and in-contact chickens, with 33.1% of ducks and 22.2% of chickens having titres of 2^4 or higher at that visit. A smaller peak occurred also in July 2007.

The flock-level prevalence of H5 antibodies in unvaccinated birds (proportion of flock-visits with at least one unvaccinated bird having a titre $\geq 2^4$) over the study period was 42.6% (95% CI: 38.0, 47.2) for ducks and 19.0% (95% CI: 13.6, 24.4) for chickens. The flock-level seroprevalences over time largely mirrored the bird-level seroprevalences. The highest proportions of flocks with at least one unvaccinated bird having a H5 antibody titre of $\geq 2^4$ was observed in January 2008 (68.0% of duck flocks, 29.1% of in-contact chicken flocks) and May 2008 (61.2% of duck flocks, 31.8% of in-contact chicken flocks).

The proportion of antibody-positive duck and chicken flocks increased from November and peaked in January, suggesting that H5 virus was circulating during that period. This is consistent with waves of outbreaks peaking between December and March, as demonstrated in other studies (Pfeiffer et al. 2007, Minh et al. 2009). This has been attributed to the increase in poultry movement associated with trade leading up to the Tet festival and with the movement of birds to scavenging areas during the post-harvest period of the rice cycle.

Serology in vaccinated birds: Overall bird-level seroprevalence for H5 antibodies $\geq 2^4$ more than three weeks post-vaccination was 55.3% (95% CI 39.2, 69.3) for ducks and 55.5% (95% CI: 46.8, 64.2) for in-contact chickens over the study period. The odds of being positive did not differ between vaccinated ducks and chickens (OR for ducks relative to chickens: 1.0, 95% CI: 0.5, 1.8). The bird-level seroprevalences over time in vaccinated ducks and in-contact chickens more than three weeks post vaccination were adjusted for sampling weights and clustering by village and farm. Bird-level seroprevalence was very low during the November 2007 visit (22.0% of ducks and 27.7% of in-contact chickens), but peaked at the subsequent visit in January 2008 for both species (76.6% of ducks and 83.5% of in-contact chickens).

The bird-level seroprevalences for different time periods post-vaccination at seven sampling occasions between May 2007 and May 2008 are shown in the table below. The lowest prevalence of positive HI titres in vaccinated ducks was observed in November 2007 for both ducks vaccinated more than twelve weeks (1.4%) and ducks vaccinated three to six weeks (22.2%) before the November sampling. The lowest prevalence of positive HI titres in vaccinated chickens was observed in November 2007.

Bird-level prevalence of positive HI titres (Number of birds) for vaccinated ducks and chickens at different time periods post vaccination.

Species	Period between vaccination and sampling (weeks)	Bird-level prevalence of positive HI titres (N birds)						
		May-07	Jul-07	Sep-07	Nov-07	Jan-08	Mar-08	May-08
Ducks	>3 to 6	49.5 (112)	66.8 (60)	60.0 (15)	22.2 (72)	87.0 (90)	n.a. (0)	80.0 (5)
	>6 to 12	29.7 (81)	30.7 (31)	37.4 (32)	100 (13)	63.0 (97)	72.1 (26)	70.9 (45)
	>12	43.8 (9)	42.6 (112)	52.9 (166)	1.4 (24)	57.2 (36)	63.3 (166)	56.8 (97)
Chickens	>3 to 6	63.2 (7)	50.3 (11)	n.a. (0)	15.7 (18)	86.9 (13)	n.a. (0)	100.0 (1)
	>6 to 12	32.9 (13)	89.7 (12)	44.0 (11)	14.2 (5)	79.5 (50)	50.6 (6)	15.7 (17)
	>12	16. 7 (6)	47.6 (23)	37.0 (22)	54.2 (7)	88.7 (11)	65.6 (64)	39.1 (31)

n.a. – not available

The table below shows the distribution of the prevalences of positive H5 antibody titres in sampled birds (the proportion of birds with positive titres more than three weeks post vaccination) at 307 duck flock-visits and 268 chicken flock-visits. At about 40% and 48% of flock-visits, less than 50% of sampled vaccinated ducks and chickens, respectively, had protective titres.

Distribution of prevalences of positive H5 antibody titres in birds sampled more than three weeks post vaccination at 307 duck flock-visits and 268 chicken flock-visits.

Prevalence of positive titres	Percentage (N) of flock-visits	
	Ducks	Chickens
0%	19.2 (59)	43.7 (117)
>0 to 25%	2.3 (7)	0 (0)
>25 to 50%	18.6 (57)	4.5 (12)
>50 to 75%	18.6 (57)	0.4 (1)
>75%	41.4 (127)	51.5(138)
Total	100 (307)	100 (268)

Many laboratory studies have demonstrated that avian influenza vaccines provide good protection against clinical signs and death along with a reduction in viral shedding. This is supported by some field evidence. However the results described here suggest the possibility of some problems with vaccination in Viet Nam, with many vaccinates failing to develop positive HI antibody titres. Substantial proportions of both ducks and chickens

developed only low titres post vaccination, and only just over half of the vaccinated ducks (55.4%) and chickens (56.8%) developed titres considered to be positive (OIE, 2009). Others have also reported that vaccination in the field resulted in very low or absent antibody titres, compared to much stronger responses following vaccination under laboratory conditions (Cristalli et al., 2007). Under field conditions, many factors can affect the efficacy of vaccination. Inactivated avian influenza vaccines are not thermostable nor is administration simple, since injection is required as opposed to oral or mucosal administration. Thus, a reliable cold chain and skilled vaccinators are required for large-scale vaccination programs, both of which are challenging in a tropical developing country such as Viet Nam. In addition, the response to avian influenza vaccination could be adversely affected by other vaccinations administered to birds at the same time, by immunosuppressive viruses such as IBD, and by maternally derived antibodies. The breed of birds will also affect antibody titres after vaccination in the field. Vaccination-induced antibodies in chickens have been shown to be lower in local breeds than in commercial breeds (van den Berg et al., 2008).

There was a peak in seroprevalence in both vaccinated ducks and chickens in January 2008 following the trough in November 2007. This peak is largely the response to vaccinations conducted from November 2007 until January 2008. However this temporal pattern in vaccinated birds appears to mirror that of unvaccinated birds over the same time, suggesting that exposure to circulating virus may have contributed to the seroprevalence pattern observed in vaccinates.

Disease outbreaks and virus shedding: No HPAI outbreaks or mortalities suspected to be due to HPAI occurred in any monitored flock during the study period. A total of 5430 combined pharyno/tracheal and cloacal swab samples was tested in pools of five by real-time PCR for the H5 gene of the viral RNA. One pooled sample from each of two farms tested H5 positive. The pool from one farm tested H5 virus positive at the second (August 2007) and the pool from the other farm at the last visit (June 2008). Both pools consisted of combined samples from clinically healthy vaccinated ducks and unvaccinated ducks. Therefore the overall H5 virus flock prevalence (proportion of flock-visits with at least one positive duck) over the whole study period was 0.7% (95% CI: 0, 2.1).

Despite the low seroprevalences observed post vaccination, no disease outbreaks due to HPAI H5N1 occurred on any of the study farms. Of particular interest is the lack of chicken outbreaks as infected chickens almost always succumb to the disease. In the present study, 17.5% of unvaccinated ducks and 10.7% of unvaccinated chickens were seropositive without recorded clinical signs of HPAI during the study. These higher bird-level seroprevalence in unvaccinated ducks compared to unvaccinated in-contact chickens may be due to greater durations of infection and shedding of H5 virus in ducks, indicating that ducks are potentially an important source of H5 virus for other bird species.

Even though virus was detected on only two study farms, it is likely that virus was circulating in at least some study farms during the observation period indicated by the high H5 seroprevalence in unvaccinated birds and flocks. The sensitivity and specificity of the HI test used to measure seropositivity were estimated to be 99% and 90%, respectively. Considering the high specificity of the HI test, it is unlikely that the high seroprevalences observed in this study are due to false positive results. The continuing occurrence of disease outbreaks during the study period in other parts of Viet Nam also suggests that there was virus circulation in the field. Thus, the presence of H5 antibodies in substantial proportions of unvaccinated ducks and chickens was probably due to exposure to field virus. It is possible that the H5 antibodies measured were from exposure to either a low pathogenic avian influenza (LPAI) H5 virus or a HPAI H5 virus of lower pathogenicity, resulting in birds surviving infection and maintaining immunity.

The low apparent prevalence of virus shedding in this study despite high prevalence of antibody titres may be because virus circulated between the bimonthly sampling times. Experimental infections in non-vaccinated ducks have shown that HPAI H5N1 viruses can be shed from 2 to 17 days (Sturm-Ramirez et al., 2004; Hulse-Post et al., 2005) and it is

generally accepted that vaccines against H5N1 reduce the duration of viral shedding post-challenge (Middleton et al., 2007). Thus it is possible that periods of viral infection and shedding on our study farms were missed, at least in ducks. However, considering the high diagnostic sensitivity of the RT-PCR for the H5 gene, we are confident that most H5 viral RNA would have been detected in our swab samples if it was present.

In summary, a significant proportion of birds and flocks in this study in Viet Nam had been exposed to HPAI field virus with distinct temporal peaks in seroprevalence occurring throughout the year. The study highlighted a moderate, but inconsistent H5 antibody response among vaccinated birds, indicating that efficacy of vaccination in the field does not reflect the laboratory vaccination studies for H5N1. Despite this there were no outbreaks on study farms during the study period. Higher bird-level seroprevalence in unvaccinated ducks compared to unvaccinated in-contact chickens observed in our study may be due to greater durations of infection and shedding of H5 virus in ducks. These findings indicate that ducks are potentially an important source of H5 virus for other bird species.

(ii) Prevalence of HPAI in 'moving' duck flocks

a) Pilot studies

Pilot studies were conducted in both Indonesia and Viet Nam to obtain a better understanding of the 'moving' duck flock management system and to assess the feasibility of conducting a more extensive epidemiological study on the prevalence and incidence of HPAI in 'moving' ducks.

In Indonesia, 7 farmers in 2 districts of the Central Java were interviewed. Questionnaires recorded information on management details, movement patterns and potential HPAI risk factors, and topographical maps were used to identify the scavenging locations used by the moving duck farmers over a period of 12 months.

The data showed that travel was usually restricted to a few months per year during the seasonal rice harvests, although there were some regional differences. The ducks in the flocks were mainly layer ducks, no HPAI vaccination was conducted, and payment to the rice-field owner to allow scavenging was not usually required. Farmers often worked together in a co-operative system, each with 5 to 65 duck flock owners. Each of these cooperatives had one coordinator who usually identified new scavenging areas. Members of these cooperatives worked together to save costs when hiring transport or purchasing feed. Several members of each cooperative travelled together and their birds scavenged together at the final destinations.

In Viet Nam, 22 farmers in 4 provinces in the Mekong Delta were interviewed using questionnaires as above. The sale of eggs was the most important purpose for keeping 'moving' ducks, with 70% of farmers saying this reason was 'very important'. The median flock size was 1500 ducks, and the main breeds of ducks were Supermeat (31%), Khaki Campell (25%), Chinese/Peking (25%), and Agriculture (16%). All ducks were vaccinated against HPAI. The duck flocks were transported by boat (62%), car/truck (14%), a combination of boat and truck (14%) or were herded on foot (10%). The total time spent away from the home village ranged from 30-285 days, with a median travel time of 90 days. The median total distance travelled was 80 km. The flocks were moved according to the stage of the post-harvest rice cycle and the scavenging areas were rice fields only (86%) or rice field and waterways (14%). About 41% of moving duck flocks shared scavenging areas with other duck flocks. Confinement over night was always in or near the scavenging areas.

b) Longitudinal studies

Six-month longitudinal studies using repeated sampling of marked birds were conducted in both Indonesia and Viet Nam. Only preliminary analysis of the data collected in these studies was conducted before the cessation of the project.

In Indonesia, the average distance travelled per flock per month was10.5 km (median 4.9 km), ranging from 0 to 133.2 km. In unvaccinated birds, the bird-level H5 antibody prevalence from November 2008 to April 2009 pooled over all samplings for all districts was 0.9%. The bird-level period seroprevalence from November 2008 to April 2009 for all districts was 2.6%. The flock-level H5 seroprevalence from November 2008 to April 2009 pooled over all samplings for all districts was 7.4% and the flock-level period H5 seroprevalence from November 2008 to April 2009 for all districts was 31.5%. There was temporal variation in the proportion of unvaccinated ducks and flocks being seropositive, with a peak in December 2008, and there was variation in the seroprevalence among the six districts in the study (Brebes, Pemalang, Batang, Kendal, Klaten, Purworejo). Only about 30% of vaccinated ducks developed positive titres post vaccination, and antibody titres decreased rapidly within only 1-2 months post vaccination.

In Viet Nam, the bird-level H5 seroprevalence in unvaccinated birds over the study period was 17.7% (95% CI 16.2, 19.1). The flock-level H5 seroprevalence over the study period was 49.0% (95% CI 43.2, 54.8). The H5 viral RNA prevalence at flock-level over the study period was 2.1% (95% CI 0.5, 4.8) for cloacal swabs and 0.4% (95% CI 0, 1.0) for pharyngo-tracheal swabs. Flocks were H5 viral RNA positive in December 2008 (2 flocks) and in February 2009 (4 flocks).

(iii) External HPAI risk factors relating to moving duck management

The questionnaire data from the biosecurity survey of rice paddy owners, transporters of 'moving' duck flocks and hatchery owners was collected and partially entered into databases in both Indonesia and Viet Nam. However, only the data from Indonesia was able to be analysed before completion of the project.

In Indonesia, rice paddy owners (N=121), transporters of moving duck flocks (N=30) and hatchery owners (N=75) were interviewed in 2009 in central Java, using cross-sectional questionnaire surveys.

The majority of rice paddy farmers provided their paddies for scavenging in the period from March to May with a peak in April and then again from August to October with a peak in September. The number of flocks scavenging per paddy per month varied between 3-4 flocks in the peak scavenging months and 5-6 flocks in the months when fewer paddies are available. Usually farmers did not receive any payment from the duck owners. The most important benefit for paddy farmers was the control of snails (69%), followed by the provision of fertiliser from duck faeces (43%). The majority of duck owners disposed carcasses of ducks that died during scavenging by burial (50%), but many owners just threw them into nearby rivers (38%).

About 90% of the duck transporters combined flocks from different farms into one load with a median number of 14 duck farms visited to obtain one load. About 67% of transporters indicated that ducks had contact with each other during transport. A median number of 16 journeys were conducted per year to scavenging locations (median distance travelled 90km). About 65% of transporters also transported duck feed, 14% chickens, 25% other birds, 25% other animals than birds and 39% eggs together with ducks on the same load. The most common deaths during transport were from physical injuries, followed by dehydration or diseases. About 57% of transporters threw ducks that died during transport into rivers or paddies. All transporters removed faeces from the vehicles, but only 13% used disinfectant on the vehicle surfaces.

About 49% of hatcheries produced their own eggs, 52% purchased eggs from 'stationary' duck farms and 25% from 'moving' duck flocks and 18% obtained eggs from professional egg traders. The majority of hatcheries used modern egg incubators (58%), but the use of traditional methods (e.g. oil lamps) (34%) or Muscovy ducks (18%) was also common. Chickens or ducks (mostly free-ranging) were kept at a close distance to 56% of the hatcheries. Although duckling pens were cleaned in 33% of hatcheries, disinfection was only conducted in 12% of hatcheries.

Discussion: HPAI can only be successfully controlled when the complexity of duck production, with all of its facets, is understood. This survey provided new insights into HPAI epidemiology by identifying potential HPAI hazards related to activities conducted not by duck farmers themselves, but by their associates. This can provide the basis for recommendations for effective HPAI control, to prevent the virus entering susceptible duck populations.

(iv) Analysis of risk factors in the major Mekong Delta HPAI outbreak of 2006 & 2007 – a case-control study

[Henning KA, Henning J, Morton J, Long NT, Ha NT, Meers J. (2009) Farm- and flock-level risk factors associated with Highly Pathogenic Avian Influenza outbreaks on small holder duck and chicken farms in the Mekong Delta of Viet Nam. *Preventive Veterinary Medicine*. 91:179-88]

Characteristics of the Study Population: A total of 24 case farms and 48 control farms that met the selection criteria were initially selected and interviewed. However it was ascertained that two case farms did not in fact meet all of the case selection criteria and were therefore omitted, along with their matched control farms. Thus 22 case farms and 44 control farms were enrolled in the analysis.

On the 22 case farms and 44 control farms, there were a combined total of 118 flocks. The number of flocks on a farm ranged from 1 to 4 (median 2; mean 1.8). Some farms kept duck flocks only (N=25), others kept chicken flocks only (N=9) while both chicken and duck flocks were present on 32 farms.

The duck flocks (N=77) were local breeds (N=49), imported breeds including Khaki Campbell, Supermeat, Chinese and Cherry Valley (N=11) and Muscovy (N=12). For 5 of the duck flocks, the breed was not recorded. All Muscovy flocks were less than 25 in number and of mixed ages but with birds in all flocks older than 20 days. All non-Muscovy duck flocks were of one age group, either ducklings (0-20 days), rearers (20-90 days) or adults (>90 days), and ranged from 5 to 1600 in number. The chicken flocks (N=41) on study farms were mainly small (ranging from 6 to78 birds). Chicken flocks consisted of birds of mixed ages; typically ages ranged from a few days to a few years old. The chicken breeds were local breeds (N=16), fighting cock breed (N= 10) and Chinese breed (N=11). In 4 of the chicken flocks, the breed was not recorded.

Univariable Analyses: Of the 32 potential risk factors analysed in the univariable analyses using all flocks, nine were selected for inclusion in the multivariable modelling process. These were: ducks aged 20 to 60 days present on the farm; the size of the flock; geese present on the farm; in-contact ducks present on the farm; nearby outbreaks; the selling of ducks or chickens; farm visited by family and friends; flock vaccination and the presence of other poultry flocks on the farm without any vaccination.

Multivariable Analyses: The results of the multivariable model for all flocks are shown in the table below. The most important risk factors were incomplete vaccination (defined by none or only one vaccination) of flocks, visits by family and friends to the farms and the presence of geese on farms. Overall, the model appeared to fit the observed data very well.

Nested analyses were performed on three subsets of the data (scavenging flocks, confined flocks and flocks fed with supplementary feeds subsets). Of the scavenging subset, sharing of scavenging areas with ducks from other farms was associated with

increased risk of HPAI outbreaks, whilst scavenging in the household garden was protective. Surprisingly, within the confinement subset, the use of disinfectant for cleaning enclosures was associated with increased the risk of outbreaks, as was visiting of confinement areas by wild birds and people. In the supplementary feeding multivariable analysis, neither of the two risk factors included were significant at P<0.05 when fitted together.

Results of the final multivariable model of risk factors associated with HPAI H5N1 outbreak occurrence in the Mekong Delta of Viet Nam between December 2006 and January 2007

Explanatory variable	Category	Number of cases (%)	Number of controls (%)	OR (95%CI)	P
Geese on farm	No	18 (81.8)	41 (93.2)	Reference	0.02
	Yes	4 (18.2)	3 (6.8)	11.5 (1.1- +infinity)	
Farm visited by family and friends	No	16 (72.7)	42 (95.5)	Reference	0.04
	Yes	6 (27.3)	2 (4.5)	8.2 (1.0- +infinity)	
Vaccination status of flock	2 vaccinations	1 (4.6)	16 (38.1)	Reference	<0.01
	1 vaccinations	3 (13.6)	11 (26.2)	20.2 (1.0- +infinity)	
	None	18 (81.8)	15 (35.7)	85.2 (6.5- +infinity)	
	Missing data	0	2		

Discussion : This study was the first report of a detailed farm- and flock-level assessment of risk factors of HPAI H5N1 outbreaks occurring in Viet Nam. The results indicated that the important risk factors associated with occurrence of HPAI H5N1 disease outbreaks were no or incomplete vaccination of birds, presence of geese on farms and visiting of farms by people.

The results from this study provided essential field evidence about vaccine efficacy in poultry flocks, building on evidence from laboratory studies where HPAI H5N1 vaccination provided protection from mortality and development of clinical signs and reduced viral shedding (Tian et al., 2005). The odds of having an outbreak were highest in unvaccinated flocks, intermediate in flocks vaccinated once and lowest in flocks vaccinated twice. This is consistent with other evidence that two vaccinations in ducks and chickens are required to provide long lasting protection (Tian et al., 2005; van der Goot et al., 2007). Overall, the apparently strong protective effect of vaccination supports the belief that the Government-initiated systematic vaccination campaign was partly responsible for the decline in reported poultry outbreaks and human cases in 2006. However the substantial proportion of study flocks with incomplete or no vaccination highlighted inadequacies in the campaign which need addressing in order to obtain the minimum protective cover in at-risk areas. Mathematical models suggest that 90% of a flock needs to be vaccinated to reduce the probability of an outbreak by 50% (Savill et al., 2006) yet in field situations. In our study, no case farm had full vaccination coverage (2 vaccinations 4 weeks apart) in all of their flocks.

A number of reasons were given by farmers for no or incomplete vaccination in their flocks, the most common being that the birds on the farm were too young for vaccination at the time of the campaign or that they had arrived or were hatched on the farm after the vaccination campaign was conducted (54.5% (12/22) of case farms). Considering the varying ages of ducks across farms in any one village, an immunization strategy is

required whereby vaccination is made available throughout the year for use in specified age groups. Such a strategy would be most practical if vaccine was administered by the flock owner.

With the presence of geese on farms being a significant risk factor for an avian influenza outbreak in ducks and chickens, the introduction of vaccination of geese may be a valid implementation for reducing risk of HPAI outbreaks. At the time of this study, geese were not included in the vaccination campaign despite the published evidence that they are susceptible to the H5N1 HPAI virus (Chen et al., 2006) and can be protected by vaccination, although three doses of vaccine are required in geese (Tian et al., 2005). This additional logistical complexity when vaccinating geese reiterates the need for careful planning of an effective vaccination campaign.

The results of this study suggest that across all flocks, scavenging contributes to a relatively small increase of risk of H5N1 outbreaks when compared to the increase in risk due to non-vaccination. However, based on the multivariable analysis of the scavenging subset, it was shown that amongst scavenging flocks those that share scavenging locations with ducks from other farms are at increased risk of an HPAI H5N1 outbreak. This result suggests that a stricter approach to biosecurity within the practice of scavenging could decrease the risk of avian influenza without banning the practice altogether.

Surprisingly, the results of the confinement subset analyses suggested that the use of disinfectants in the confinement area increased the risk of an avian influenza outbreak. It is possible that the disinfectants used, particularly quaternary ammonium compounds, were inactivated by organic material, since it is difficult to clean the enclosures prior to disinfection because they are commonly made of wood, leaves or fish nets with a dirt floor. In addition, contact time, frequency of use and correct dilution will influence the effect of disinfecting an area and farmers may not have been aware of or adhered to recommendations for use of the disinfectant. Alternatively, this association may also be confounded, i.e. use of disinfectant may lead farmers to take short-cuts in other areas of biosecurity.

Flocks on farms visited by family and friends were at increased risk of having a HPAI H5N1 outbreak. Such visits are integral to the social community lifestyle of the people in the Mekong Delta and an implementation of strategies to minimise these movements would be difficult. Risk of introducing HPAI virus could possibly be reduced through use of viricidal footbaths by people entering and leaving farms.

In summary, none or only one vaccination, visits by family and friends to farms, the presence of geese on farms and sharing of scavenging areas with ducks from other farms increased the risk of HPAI H5N1 disease outbreaks in poultry flocks in Viet Nam.

B: Experimental activities

(i) Characterisation of virus isolates from Indonesia and relationship to field data

[Wibawa H, Henning J, Wong F, Selleck P, Junaidi A, Bingham J, Daniels P, Meers J. (2011) A molecular and antigenic survey of H5N1 highly pathogenic avian influenza virus isolates from smallholder duck farms in Central Java, Indonesia during 2007-2008. *Virology Journal* 2011, 8:425]

Because no H5N1 viruses were isolated in Viet Nam during the course of the field studies in that country, the research on characterisation of virus isolates had to focus on the virus isolates collected during the field studies in Indonesia.

Phylogenetic analysis of HA gene: Of the 100 H5N1 virus isolates that were sent to AAHL for genetic and antigenic characterisation, 84 isolates were found to have confirmed viable H5 virus following attempted propagation in embryonated eggs, HI assay using H5-

antisera and RT-PCR for H5 RNA. The phylogenetic analysis of the HA gene of these 84 isolates showed that they all belonged to clade 2.1. The majority of the viruses (80/84) were clustered into the third-order clade 2.1.3, one virus belonged to clade 2.1.1, and three remaining viruses were clustered together into a distinct sublineage, known as Indonesia/6/05 (IDN/6/05)-like viruses. Previous study indicated that IDN/6/05-like viruses have emerged since 2004 and continue to circulate predominantly in poultry in Java (Takano et al, 2009). The virus isolates belonging to clade 2.1.3 were highly related to each other, but could be divided further into three distinct groups. The phylogenetic relationships determined in the study suggested that multiple subclade 2.1.3 H5N1 viruses circulated and continue to be maintained in smallholder backyard farms in Indonesia.

The phylogenetic analysis of the HA gene demonstrated that viruses isolated from ducks were genetically more diverse than those isolated from chickens. All three clades (2.1.1, 2.1.3, IDN/6/05-like) of viruses were identified in ducks, while only clade 2.1.3 viruses were found in chickens.

Phylogenetic analysis of NA gene: For most of the 24 virus isolates selected for NA sequencing, phylogeny of the NA gene corresponded with the HA groupings. However, the placement of the three IDN/6/05-like viruses were clustered into two separate lineages in the NA phylogenetic tree. The 24 selected viruses had a NA nucleotide sequence identity of 96-99%. The highest nucleotide divergence (3%) in the NA gene amongst the study viruses was found on A/Dk/Bantul/BBVW-387-23310/07, which belonged to the IDN/6/05-like virus HA sublineage.

Antigenic analysis: There was no substantial difference in the antigenic patterns of the 24 selected virus isolates determined by HI testing using antisera against 4 different H5N1 viruses, despite variations found in the epitopes of the HA gene of these viruses. The viruses were antigenically most similar to A/ck/Indonesia/Wates1/05, a 2.1.3-subclade virus isolated from the same region as the study viruses. All viruses demonstrated moderate reactivity with antisera to clade 2.1.3 virus Konawe/204O/07 and the clade 1 virus Vietnam/08/04, but only low reactivity to serum from the recognised Indonesian antigenic variant 2.1.3 virus, PWT-WIJ/06.

Epidemiology of H5N1 virus isolates: A total of 132 virus isolates were collected over the course of the longitudinal field study (from March 2007 to March 2008) on 'stationary' duck flocks in Indonesia. These viruses were isolated from 46 of the 96 farms included in the field study, with some farms providing more than one isolate and others only a single isolate. Some farms were virus-positive in both their duck and chicken flocks, whereas other farms were positive in only one species. Some farms (N= 29) had virus-positive birds on only one sampling occasion, while the remaining 17 farms had positive birds on repeated occasions, with the time period between positive virus sampling occasions ranging from 3 days up to 7 months. These results indicate that H5N1 virus may be maintained over long periods at the flock or farm level.

The amino acid sequence of the HA protein was analysed from farms that had provided multiple isolates (either at the same time point or at different sampling occasions). Amino acid diversity was detected among the viruses from 8 of these farms. These genetic variations were detected either in different birds during the same farm outbreak or at different outbreak times. On 6 farms at least two genetic variants were isolated, either in single or in repeated samplings, during HPAI outbreaks occurring over a relatively short time period, whereas on 2 farms different variants were detected at 2-3 sampling occasions separated by 5-6 months. Phylogenetic analysis showed that some of these farms were infected by two different virus clusters within clade 2.1.3, demonstrating that genetically distinct H5N1 viruses could be isolated from the same farm. On the other hand, the same genetic variants could be isolated from HPAI disease outbreaks on different farms, indicating their widespread geographic occurrence.

There are two possible reasons for the occurrence of multiple genetic variants on a single farm, either mutation of existing viruses or introduction of new genetic variants onto the

farm. The data suggested that mutation of existing viruses had probably occurred on at least one of our study farms. Conversely, the finding of multiple genetic variants on a single farm in September 2007 suggested the introduction of different virus variants onto the farm, possibly through contact with HPAI-infected birds from other farms or through contact with contaminated sources such as traders or farm visitors. The spread of HPAI viruses on some farms could be tracked through the presence of different genetic variants. For example, one of the viruses isolated from a dead chicken on one farm had identical HA sequence to 5 viruses isolated from live ducks in a HPAI outbreak on another farm in the same village. Identical viruses were also found on one farm amongst H5N1 isolates derived from 3 live ducks and those derived from 3 dead chickens one week later. This suggested that surviving ducks had maintained the virus and allowed transmission to chickens on the same farm, leading to an outbreak of disease. Overall, the sequence data suggested that it was likely that these viruses originated from similar sources, then spread widely in the study farms.

The temporal distribution of collection of the virus isolates showed peaks in July 2007 and September 2007. When examined at the district level, these peaks were shown to occur predominantly in single districts, suggesting that the epidemics were not widespread. When examined at species level, the data showed that the number of virus isolations from ducks was relatively stable over time, whereas the isolations from chickens tended to occur in epidemics. The data also demonstrated that virus isolations from ducks were often independent from cases in chickens, but isolations from chicken were nearly always associated with isolations from ducks.

Of the 132 virus isolates, 71 were isolated from chickens and 61 were isolated from ducks. Of the duck-derived viruses, 49 (80.3%) were isolated from live birds, whereas only 10 of 71 (14.1%) chicken-derived viruses were isolated from live birds, i.e. the majority of chicken viruses were isolated from dead birds. Alignment of the HA gene of these viruses showed that viruses isolated from dead ducks or live chickens had identical sequences with other isolates from live ducks or dead chickens. Phylogenetic analysis confirmed these findings, indicating that there was no clear correlation between HA sequence variation and pathogenicity. This suggests that there are other factors influencing pathogenicity, in particular the species of bird. The finding that the majority of duck isolates were derived from live birds whereas the majority of chicken isolates were derived from dead birds suggests that the virus might be more readily maintained and shed by ducks, without producing any signs of disease in that species, whereas the virus usually causes fatal disease in chickens.

(ii) Infection studies in ducks and chickens using H5N1 virus isolates from two different sub-clades

[Bingham J, Green DJ, Lowther S, Klippel J, Burggraaf S, Anderson DE, Wibawa H, Hoa DM, Long NT, Vu PP, Middleton DJ, Daniels PW. (2009) Infection studies with two highly pathogenic avian influenza strains (Vietnamese and Indonesian) in Pekin ducks (Anas platyrhynchos), with particular reference to clinical disease, tissue tropism and viral shedding. *Avian Pathology*. 38(4):267-78.

Wibawa H, Bingham J, Nuradji H, Lowther S, Payne J, Rookes J, Junaidi A, Middleton D, Henning J, Meers J. The pathogenesis of two distinct clades of Indonesian H5N1 avian influenza viruses in chickens and ducks. (in preparation)]

Experimental infection studies were conducted in ducks and chickens using two Indonesian H5N1 virus isolates - A/duck/Sleman/BBVW-1003-34368/2007 (abbreviated DK3468), which was a clade 2.1.1 virus isolated from a dead duck; and A/duck/Sleman/BBVW-598-32226/2007 (abbreviated DK32226), which was a clade 2.1.3 virus isolated from a healthy duck during a HPAI outbreak.

Clinical and gross pathological findings: Chickens inoculated with either virus showed mild to severe disease signs one day after challenge. Clinical signs ranged from varying degrees of reduced activity, reduced feeding and drinking, and huddling and recumbency,

reddening and swelling of the face and wattles, and regurgitation of crop contents. Chickens found with clear clinical signs were euthanased immediately, but because of the peracute progression of the disease some chickens were found dead. All chickens had died or been euthanased by 30 hpi (DK34368) or by 24 hpi (DK32226 group). On post-mortem examination varied degrees of oedema was seen in the head, comb, wattles and lungs.

In contrast, ducks inoculated with either virus did not show any clinical signs up to the end of the experiment at 18 days post-inoculation (dpi). They continued to eat, drink, preen, and interact with other birds . There were no gross pathological findings in any of the euthanized ducks. One duck inoculated with DK32226 was found dead at 5 dpi, but this was considered unrelated to H5N1 infection as no virus or viral antigen was detected in laboratory tests.

Virus replication in tissues: The virus load in tissues was measured by titration in Vero cells, and on occasion by virus isolation in embryonated eggs .In chickens inoculated with either virus, infectious virus was detected in many tissues, including brain, heart, lung, spleen, pancreas and skeletal muscle. In contrast, only low concentrations of virus were detected in duck tissues at 2 dpi and 4 dpi, and no virus could be detected at 7 dpi and 18 dpi. With DK34368, virus was detected at 2 and 4 dpi in heart, lung, spleen pancreas and skeletal muscle (4 dpi only), whereas with DK32226 virus was only detected at 4 dpi only, and only in spleen and pancreas.

Histopathology: In chickens the histopathology caused by the two viruses was similar. They caused small foci of acute necrosis and haemorrhage in multiple tissues. While these lesions were most prevalent in the red pulp of the spleen, they also were found sporadically in the parenchyma of the liver, lung and kidney, and in the lamina propria of a variety of epithelial tissues. These necrotic foci were particularly prevalent in lymphoid follicles of the bronchioles, turbinates, proventriculus and intestine.Only one duck had lesions that could be attributable to the virus infection, with mild perivascular cuffing in the brain at 18 dpi.

Immunohistochemistry: Viral antigen was detected in a wide range of tissues of chickens inoculated with both DK34368 and DK32226, in particular brain, lung, heart, kidney, bone, and lymphoid-associated tissues. Viral antigen was also distributed widely in endothelial cells in a variety of tissue types. The concentration of viral antigen was higher in chickens with moderate to severe clinical signs than that in tissues of chickens with milder symptoms. A difference between the two viruses was noted in the distribution of viral antigen in the brain. In DK34368-infected chickens, common to abundant viral antigen was detected in neural tissues, glial nodules, ependymal cells, whereas no or minor amounts of antigen were found in the same tissue types in DK32226-infected chickens. Viral antigen was also observed in spleen, thymus, bursa, gut associated lymphoid tissues (GALT), and in periosteum, endosteum and bone marrow.

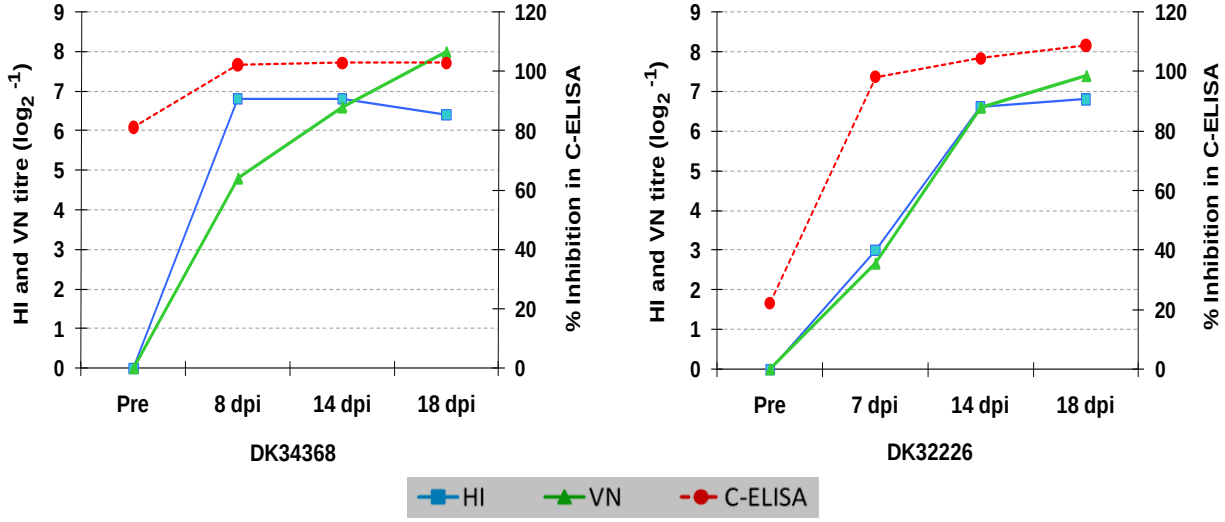
The distribution of viral antigen of both viruses in ducks was very limited and if present, the antigen concentration was much lower than that in chickens. Viral antigen appeared only in the epithelium of air sacs and paranasal sinuses of some, but not all ducks in both virus groups at 2 dpi and 4 dpi. Viral antigen was not detected in any tissues at 7 dpi or 18 dpi. There was no viral antigen detected in any euthanized ducks at the other sampling times (7 dpi and 18 dpi).

Virus shedding: The presence of virus (by virus isolation/titration in eggs/Vero cells) was assessed in oral and cloacal swabs from chickens at 1 dpi and daily from ducks (1-18 dpi). In chickens, virus was isolated (in eggs) from oral and cloacal swabs of all birds inoculated with both viruses at 30 hours post-inoculation (hpi). There was a trend for the titre of virus (in Vero cells) in oral swabs to be higher than that in cloacal swabs, but this difference was only significant for DK34368-infected chickens tested at 24 hpi.

In ducks there was a lower level of virus shed compared to chickens and the shedding was intermittent. Small amounts of viruses were detected in oral swabs of only a

proportion of ducks on days 1 to 8 (DK34368) or days 1 to 5 (DK32226) post-inoculation. Virus was not detected in any cloacal swabs from ducks inoculated with either virus on any day of the study (1-18 dpi).

Serology: H5N1 antibodies were measured in sera of duck that were kept in the study until 18 dpi. using three serology tests (HI test, virus neutralisation test, competitive enzyme-linked immunosorbent assay). All birds were antibody negative in all three tests prior to the infection study apart from 9 of 10 ducks inoculated with DK34368, which showed a variable percentage inhibition in C-ELISA test (see figure below). In ducks inoculated with DK34368, high antibody titres had developed by 8 dpi, with a mean HI titre of 6.8 log2 and mean VN titre of 4.8 log2. At 7 dpi, the antibody titres of ducks inoculated with DK32226 (3 log2 and 2.7 log2 for HI and VN, respectively) were lower than those of DK34368 at 8 dpi. All ducks inoculated with both viruses had high antibody levels at 14 and 18 dpi.



Antibody titres in ducks infected with either DK34368 or DK32226

Abbreviations: haemagglutination inhibition (HI), virus neutralization (VN), competitive enzyme-linked immunosorbent assay (C-ELISA), pre-infection sampling (Pre), day post inoculation (dpi).

Discussion: Severe HPAI clinical signs appeared in all chickens inoculated with either DK34368 or DK32226 viruses. The first deaths in chickens inoculated with DK32226 were at 24 hpi, 6 hours earlier than in chickens inoculated with DK34368 virus. However, a difference in the dose of the virus inoculum (10^8 EID_{50} per bird DK32226; 10^7 EID_{50} per bird DK34368) may explain the slight difference in time to death. A previous infection study in chickens using a clade 2.5 H5N1 virus showed that increasing the titre of virus in the inoculum reduced the mean death time Suzuki et al (2009).

Infectious virus and viral antigen of both viruses was detected in most chicken tissues, suggesting that the viruses could replicate efficiently in wide range of tissue types, and explaining the severe clinical outcomes. In most birds there was an association between the appearance of viral antigen and the identification of lesions in the same tissue. However, this did not occur in all birds, as a large amount of viral antigen was localized in some tissues without any apparent histological lesions. For example, abundant intranuclear and intracytoplasmic antigen was seen in cardiac myocytes of chickens with severe disease, but neither cell degeneration nor inflammatory responses were detected in that tissue. In general, however, where a lesion was found in infected tissues the distribution of viral antigen was usually extensive. Widespread distribution of viral antigen in endothelial cells, often with intense staining, was frequently found in a wide range tissues of chickens inoculated with either virus. The prevalence of positive antigen staining was relatively consistent in the parenchyma of other severe infected tissues

including lung, kidney, spleen, thymus, bursa, and connective tissues. In general, the more severe clinical signs appeared in chickens, the higher the number of cells containing viral antigen.

In contrast with the disease presentation in chickens, DK34368 or DK32226 viruses were not pathogenic to ducks. Despite viral replication being evident in several tissues, ducks clinically did not show any clinical signs during 18 days of observation. The viruses were detected in low quantities (< 3 $\log_{10}$ $TCID_{50}$) in heart, lung, spleen and pancreas of ducks at 2 dpi and 4 dpi and no virus was recovered from brain. DK34368 appeared to replicate more efficiently than DK32226, with virus isolated from more tissues and at higher titre than from DK32226-infected ducks.

Although DK34368 virus was originally isolated from a dead duck, it was not pathogenic for ducks in this study. This could be due the difference between the hosts that were used in this study (Pekin breed) and the one from which the virus was isolated (native, Magelang breed). Alternatively, the HPAI infection in the original bird might have been only secondary to a primary disease, which actually caused the mortality. Other studies have shown that some Eurasian lineage H5N1 viruses could produce severe clinical signs and mortality in experimentally-infected ducks, especially in young birds, and that viral replication occurred in the respiratory tracts and a wide range of tissues, including neurological and cardiovascular systems (Pantin-Jackwood et al, 2007; Swayne, 2007; Kishida, 2005). However, in the current study viral antigen detection of either virus was restricted to epithelium of the paranasal sinuses and air sacs.

Investigating the shedding of virus, chickens shed substantial amount of virus through oral and cloacal routes, particularly birds showing moderate to severe clinical signs. In ducks, intermittent virus shedding in oral swabs was detected up to day 8 (DK34368) or day 5 (DK32226) at very low concentrations, and no shedding was detected in cloacal swabs. The pattern of virus shedding in chickens suggested that viral replication occurs in the epithelial cells of the oral cavity or the respiratory tract as well as the gastrointestinal tract of this species. However the finding that virus was only detected in oral swabs suggests that the viruses replicated predominantly in the respiratory tract or oral cavity. Respiratory epithelial cells of air sacs and paranasal sinuses seem to be preferred sites for replication in ducks. This finding reinforces observations of others and indicates that both oral/tracheal and cloacal sampling should be conducted during HPAI surveillance in ducks.

Despite the lack of clinical signs and the small amount of virus and viral antigen detected in infected ducks, both viruses successfully induced a strong antibody response in the inoculated ducks. Most DK34368-infected ducks developed high antibody titres by 8 dpi. Together with the finding that this group of ducks had detectable cELISA antibodies pre-challenge, this rapid response suggests that these birds may have been exposed to another subtype of AI viruses prior to challenge.

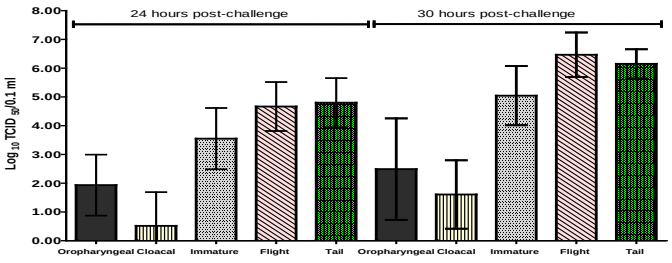
In conclusion, the two viruses used in this study belonged to different H5N1 clades (2.1.1 and 2.1.3) and were isolated from ducks with different presentations (dead versus healthy), the clinical outcomes, pathogenicity and tissue distributions of these viruses were similar. The viruses had a greater ability to replicate in chickens than in ducks, with a high concentration of virus found in a wide range of chicken tissues, but virus was only detected in a limited number of tissues in ducks. As a consequence, a high titre of virus was shed by chickens through both oral and cloacal routes compared to a low and intermittent shedding of virus in only oral swabs from ducks. Thus, HPAI-infected chickens are more likely than ducks to be one of the main sources of virus transmission among poultry and potentially to humans. In contrast, ducks are probably better than chickens in maintaining virus in the poultry population as they can act as silent hosts, but could shed infectious virus for a longer period.

(iii) Investigation of the use of feathers for diagnosis

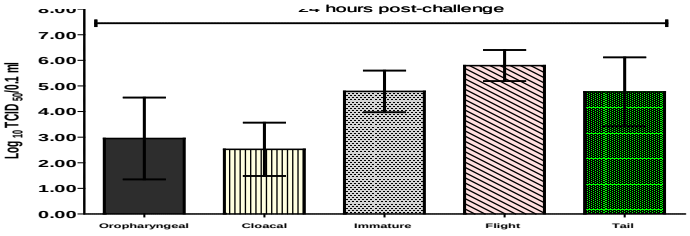
For the feather studies, in addition to using samples from the challenge trials with two Indonesian H5N1 virus isolates described above, samples were collected from a third experimental infection trial using a clade 1 Vietnamese isolate A/Muscovy duck/Vietnam/453/2004 (MD453) and belongs to clade 1.

Virus isolation and titration of swabs and feathers: Oropharyngeal and cloacal swabs and immature and mature feathers were collected 2-3 days pre-challenge, and all samples were negative by virus isolation, haemaglutination (HA) test and virus titration in Vero cells. Following challenge, testing of swabs and feathers (immature, mature, flight and tail feathers), showed that the viral mean titre was considerably higher in feathers than in oropharyngeal and cloacal swabs in all infected chickens (see figure below). The lowest titre was observed in cloacal swabs with titre ranging from $10^{0.4}$ to $10^{2.5}$ $TCID_{50}$/0.1 ml whereas the highest titre was found in either flight ($10^{4.3\text{-}6.5}$ $TCID_{50}$/0.1 ml) or tail ($10^{4.2\text{-}6.2}$ $TCID_{50}$/0.1 ml) feathers.

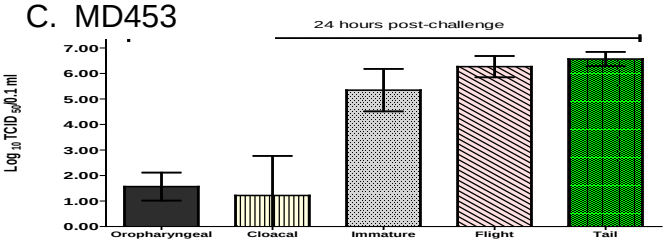
A. DK34368



B. DK32226



C. MD453

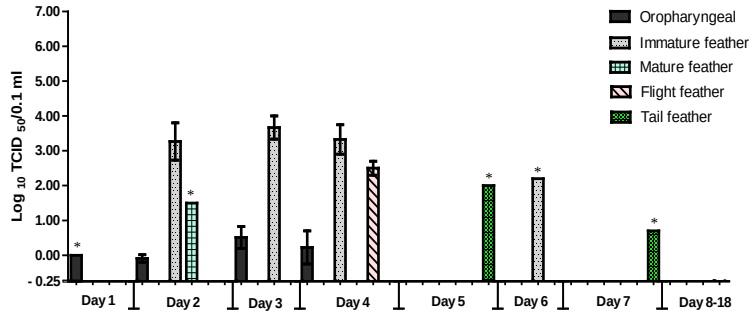


Viral mean titre in oropharyngeal and cloacal swabs and feathers collected from chickens inoculated with DK34368, DK32226 and MD453.

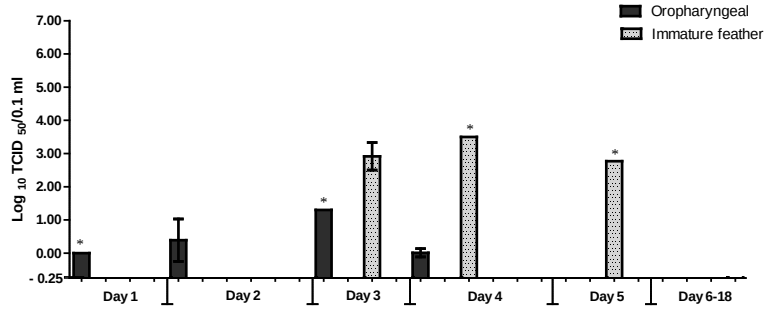
Data were obtained from three separate experiments. Virus titration in Vero cells was started at neat and 1:10 for swabs and feathers respectively. Results are represented as mean ± SD ($Log_{10}TCID_{50}$/0.1 ml of sample).

Similarly, in ducks the viral titre in feathers was much higher than in oropharyngeal and cloacal swabs (see figure below). Unlike in chickens, the highest titre was detected mostly in immature feathers in ducks. Virus could be isolated over a longer period from feathers than from swabs.

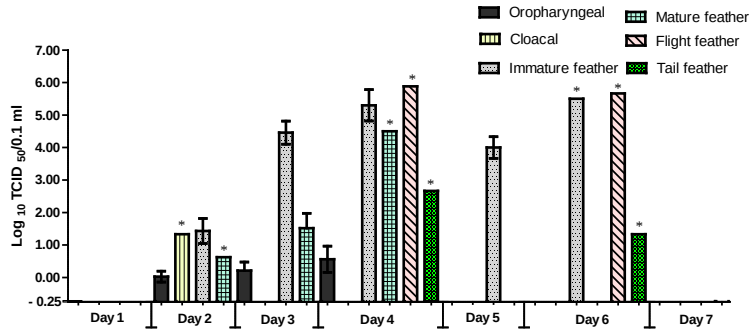
A. DK34368



B. DK32226



C. MD453



Virus mean titre in oropharyngeal and cloacal swabs and feathers of ducks inoculated with DK34368, DK32226 and MD453.

Data were obtained from three separate experiments. Results represented as mean ± SD ($Log_{10}TCID_{50}$/0.1 ml of sample). Virus titration in Vero cells was started at neat and 1:10 for swabs and feathers respectively. * indicates that virus was isolated from only one sample.

Histopathology and immunohistochemistry (IHC): Skin samples collected from 7 different regions on the chickens and ducks (capital tract, cervical tract, interscapular tract, upper major primary covert, pectorosternal tract, femoral tract and upper median tail covert), were stained with H&E and IHC to evaluate the histological changes and to observe the distribution of viral antigen in feather structures and follicles.

Lesions and viral antigen were detected in feathers distributed in skin samples collected from all infected chickens. No difference was observed in the distribution of viral antigen in all skin regions sampled. However, the prevalence of antigen-positive feathers was slightly lower in the capital tract than in the other tracts.

In contrast, lesions and viral antigen were only detected in feathers and skin samples collected from ducks infected with Vietnamese isolate (MD453), and no antigen or lesions were observed in feathers from ducks infected with Indonesian isolates (DK34368 and A/ IND 32226). All skin regions from ducks inoculated with MD453 were positive for viral antigen. The highest prevalence of antigen-positive feathers was detected in upper major primary covert (wing) tract (100%) and the cervical tract (neck) had the lowest prevalence (6% to 26 %).

*Antigen distribution in feathers and skin collected from duck and chicken infected with MD453: V*iral antigen was detected mainly in the pulp of chicken feathers, with lower concentrations found in intermediate and basilar layers of feather epidermis. Antigen was also detected particularly in dermis of feather follicle. In contrast, abundant viral antigen was observed in the outer and intermediate layers of the feather epidermis in ducks, with lower concentrations in feather pulp. A different pattern of viral antigen distribution was found in feather follicle of ducks compared to chickens, with antigen found predominantly in the epidermis. Viral antigen was rarely observed in duck skin.

*Analytical sensitivity of Anigen rapid test kits: A*llantoic fluid infected with isolates DK34368, DK32226 and MD453 was diluted 10-fold from 10^{-1} to 10^{-10} and each dilution was tested with haemagglutination (HA) test, and specific dilutions were tested with Anigen rapid test kits (AIV Ag and H5 AIV Ag test kits). The H5 AIV Ag and the HA test had equivalent sensitivity ($10^{6.7}$ and $10^{7.7}$ $TCID_{50}$/ml), which was 1-2 log lower than that of the AIV Ag kit ($10^{5.0-5.7}$ $TCID_{50}$/ml).

Relative specificity of Anigen rapid test kits: The specificity of the Anigen AIV Ag and H5 AIV Ag tests was assessed using negative samples (swabs and immature feathers collected from ducks and chickens prior to challenge). Virus titration in Vero cells was used as reference standard. No false positive results were detected on cloacal swabs and immature feathers tested with both Anigen AIV Ag and H5 AIV Ag (100% specificity). However, the specificity of both tests on oropharyngeal swabs ranged from only 50% to 71%).

Sensitivity of Anigen rapid test kits with three different virus strains and sample types: The sensitivity of the two Anigen test kits in samples tested from experimentally-infected chickens, varied according to the virus strain and the sample tested (see table below). However, with some exceptions the highest sensitivity in both test kits (AIV and H5 AIV) was found with flight and tail feathers and oropharyngeal swabs. Lower sensitivity was found with immature feathers and cloacal swabs. The exception to this was observed with DK32226 and the H5 AIV test, where the highest sensitivity was detected on cloacal swabs (46.15%), low rates of detection on immature, flight and tail feathers and no positive results were detected on oropharyngeal swabs.

A different pattern of sensitivity was observed in the infected ducks (see table below), although fewer samples were tested compared to chicken samples. Among the three isolates, higher sensitivity was observed on Vietnamese isolate tested with Anigen AIV Ag, with the highest sensitivity (100%) observed on flight and tail feathers. One cloacal swab positive by virus titration was also positive tested with Anigen AIV Ag.

Evaluation three different feather preparation procedures: Three different feather preparation procedures; grinding, bead beating and immersion were assessed, using immature, tail and flight feather samples collected from DK32226-infected chickens. For immature and tail feathers, the bead beating method produced higher virus titres (in Vero cells) than the grinding method whereas for flight feathers, grinding was superior to bead beating. With all types of feathers, immersion produced the lowest titres.

Using Anigen AIV rapid antigen detection test, grinding and bead beating again produced the highest proportion of positive feathers with 100% positivity for immature, tail and flight feathers. The immersion method produced a lower sensitivity (92%) on immature feathers than the other two methods (flight and tail feathers not tested). Only a limited number of samples was tested in the Anigen H5 AIV Ag test, so the results will not be reported.

Relative sensitivity of Anigen rapid test kits on different specimens collected from experimentally-infected chickens

H5N1 HPAI virus	Anigen test	Swab		Feather		
		Oropharyngeal	Cloacal	Immature	Flight	Tail
DK34368	AIV Ag[a]	11/14[c] (78.57%)[*]	0/11 (0)	11/14 (78.57%)	3/3 (100%)	3/3 (100%)
	H5 AIV Ag[a]	12/14 (85.71%)	0/11 (0)	3/14 (21.43%)	2/3 (66.67%)	2/3 (66.67%)
	AIV Ag[b]	8/11 (72.73%)	2/11 (18.18%)	11/11 (100%)	6/6 (100%)	6/6 (100%)
	H5 AIV Ag[b]	5/11 (45.45%)	2/11 (18.18%)	9/11 (81.82%)	6/6 (100%)	6/6 (100%)
DK32226	AIV Ag	12/13 (92.31%)	8/13 (61.54%)	13/13 (100%)	13/13 (100%)	10/10 (100%)
	H5 AIV Ag	0/13 (0)	6/13 (46.15%)	1/13 (7.69%)	2/13 (15.38%)	1/10 (10%)
MD453	AIV Ag	5/5 (100%)	4/5 (80%)	5/5 (100%)	5/5 (100%)	5/5 (100%)
	H5 AIV Ag	5/5 (100%)	0/5 (0)	4/5 (80%)	5/5 (100%)	5/5 (100%)

[a] indicates samples were collected at 24 hours post-challenge, [b] indicates samples were collected at 30 hours post-challenge. [c] number of positive results / total samples tested. * the percentage of specificity.

Relative sensitivity of Anigen rapid test kits on different specimens collected from all infected ducks

H5N1 HPAI virus	Anigen Test	Swab		Feather			
		Oropharyngeal	Cloacal	Immature	Mature	Flight	Tail
DK 34368	AIV Ag	5/12[a] (41.67%)[*]	ns	2/14 (14.29%)	0/1 (0)	ns	1/1 (100%)
	H5 AIV Ag	nt	ns	4/14 (28.57%)	0/1 (0)	ns	0/1 (0)
DK 32226	AIV Ag	6/8 (75%)	ns	2/3 (6.67%)	0/1 (0)	ns	ns
	H5 AIV Ag	nt	ns	0/3 (0)	0/1 (0)	ns	ns
MD453	AIV Ag	8/10 (80%)	1/1 (100%)	10/14 (71.43%)	2/5 (40%)	2/2 (100%)	2/2 (100%)
	H5 AIV Ag	nt	nt	nt	nt	nt	nt

ns: no samples, nt: not tested, [a] positive results/total samples collected, * the percentage of sensitivity.

8 Impacts

8.1 Scientific impacts – now and in 5 years

The project made significant scientific impacts. A total of five journal articles have been published to date, with one further article in press and two submitted to journals. There are an additional five papers in various stages of preparation. Many of these publications were keenly awaited by researchers in both partner countries and elsewhere, because they described studies that had not been undertaken by any other scientists.

The publications produced from this ACIAR project have made a major contribution to scientific literature on H5N1 HPAI. Both the field activities and the experimental activities have been well-designed and rigorously conducted with attention to detail, and these features have been recognised by other researchers in this field.

The project impacted on the design of other research projects on avian influenza. In Viet Nam, the ACIAR project directly influenced research activities undertaken by a NZAID-funded project in the Mekong delta region. The project proposal written by a consultant for NZAID in 2007 was modelled closely on our project design, and used documents supplied by our project team. This allowed the NZAID project to complement rather than compete against the activities of the ACIAR project, as similar approaches were used in data collection, but the work was based in a different area of the Mekong Delta. In Indonesia, a number of studies awaited the preliminary results from our project's longitudinal study before finalising their project designs. These include an ILRI-funded project on HPAI in village chickens based in Yogyakarta and an FAO-funded study on HPAI in ducks in Kalimantan. The project communicated particularly with researchers at FAO, Jakarta and results of our project were presented at FAO on a number of occasions.

Collaborations during and after the project have been extensive. Dr Henning received a UQ early career researcher award to allow a collaborative visit to Dr Mark Stevenson at Massey University, New Zealand, to conduct spatial analysis on the 'moving' duck flock data from Indonesia. He also received a UQ travel award to visit Prof Dirk Pfeiffer in London, to investigate spatio-temporal characteristics and risk factors associated with the 2006/2007 HPAI outbreak in the Mekong Delta, partly based on data collected at the start of the project. Project team members have also been asked to provide expert advice to other ACIAR projects on HPAI in Indonesia.

Project team members have been invited to present the project findings at numerous local and international conferences and meetings (see section 10.2 below for formal conferences presentations). These meetings included epidemiology, virology, biosecurity and poultry conferences, and meetings sponsored by organisations such as FAO, OIE and ILRI.

8.2 Capacity impacts – now and in 5 years

Capacity building was one of the strengths of the project. Three John Allwright Fellowship holders (all from Indonesia) were aligned to the project – Hendra Wibawa (PhD), Harimurti Nuradji (PhD) and Risza Hartawan (MPhil). Mr Wibawa and Mr Nuradji have been based at AAHL, Geelong, and have gained expertise in performing experimental infection trials under high level biosafety conditions, in conducting a range of virological and serological assays and in undertaking molecular biology studies. Mr Hartawan was based at the Queensland Agricultural Biotechnology Centre in a collaborative project with Dr Tim Mahony of the Queensland Department of Employment, Economic Development and Innovation, and gained expertise in molecular biology, cloning and a range of virological assays. All of these students have performed exceptionally well in their research studies.

At the time of writing this report, Mr Hartawan had completed his masterate studies, while Mr Wibawa and Mr Nuradji were both entering the final year of their PhD programs.

During the course of their postgraduate studies in Australia, the students above also undertook various specialised training courses. In 2009, Mr Hartawan completed a one-week training workshop in Canberra in epidemiology conducted by the Australian Biosecurity Cooperative Research Centre for Emerging Infectious Disease. The course focused on the fundamentals of veterinary epidemiology including the design of field studies and data analysis. Mr Wibawa completed a one-week training course in bioinformatics at the Walter and Eliza Hall Institute in Melbourne in December 2010, a 2-week FAO/IAEA-sponsored course on Advanced Bioinformatics and Laboratory Data Management in Vienna in July 2011, and a 2-day course on Publishing with Impact at CSIRO, Clayton, Vic in September 2011. Mr Nuradji joined training courses on avian influenza diagnostics (including serology and molecular assays) conducted at AAHL in 2009.

A fourth postgraduate student in Indonesia is also aligned with the project. Didik Yulianto is completing a Masters program in Veterinary Epidemiology and Poultry Health at Gadja Mada University in Yogyakarta. The program is comprised partly or course-work and partly a research project, which will focus on the longitudinal study conducted in moving duck flocks in central Java. Dr Henning is the Australian supervisor for Mr Yulianto's research project, while the principal supervisor is Prof. Dr. Bambang Sumiarto, SU., MSc of Gadjah Mada University.

In addition to the formal training in research, the project has contributed extensively to informal capacity building in both partner countries. Government veterinarians, para-vets and other field staff were actively trained in questionnaire design, interviewing techniques and sample collection in the field. This allowed the veterinarians to understand the process of scientific data collection and provided them with skills and experiences for further investigations within their own institutes. Staff in both countries were also trained in database entry and data management, and they will be able to bring these skills to other projects in the future, allowing production of high-quality data-sets in the future.

Staff from collaborating institutions in both countries undertook short-term training programs in Australia. Ms Verawati from the molecular diagnostics laboratory at the DIC, Wates, Indonesia completed a 3-week training course from 19 November to 7 December 2007 at AAHL, Geelong. The course covered molecular techniques for HPAI diagnosis, including conventional and real-time PCR and sequencing. Mr Didik Yulianto from the pathology laboratory at DIC Wates completed a 10-day training course at AAHL from 20 to 31 October 2008. The course was partly funded by FAO and also focused on molecular diagnostic methods for HPAI. Two staff members from the RAHO-VI, HCMC, Mr Vu and Mr Phong attended a 3-week training course at AAHL from 27 April to 22 May 2009, to improve their expertise in molecular diagnostics.

Mr Didik Yulianto (DIC, Wates, Indonesia) and Mr Le Tri Vu (RAHO-VI, Viet Nam) undertook a 3-week training course in veterinary epidemiology and data analysis at UQ in 2010. The course was conducted by Dr Joerg Henning and involved an intensive program of lectures, tutorials and exercises. Both trainees worked with the moving duck flock datasets collected in their own countries, and both were supplied with statistical software and textbooks in epidemiology.

8.3 Community impacts – now and in 5 years

The measurement of community impacts from this science-focused project was not within the scope of the current project. Assessment of these economic, social and environmental impacts will more appropriately be considered at the adoption or impact study phase.

However, if the recommendations that were generated by the project are implemented, it is possible to speculate on the potential community impacts that will ensue. The project's findings resulted in conclusions regarding the role that ducks play in maintaining HPAI viruses and identified a number of management factors that are associated with a higher risk of HPAI infection and disease. These findings led to a series of recommendations regarding the management of duck flocks that aim to decrease the incidence of HPAI infection in ducks flocks and to reduce risks to human health. If put into practice, these management changes would contribute to overall control of HPAI disease, resulting in increased incomes to farmers from both chicken and duck production. Importantly, they would potentially lead to reduced risk of H5N1 HPAI infection in humans and decreased mortality figures from this disease.

8.3.1 Economic impacts

8.3.2 Social impacts

8.3.3 Environmental impacts

8.4 Communication and dissemination activities

The project communicated widely in a variety of settings about the activities, findings and conclusions of the research undertaken. Communication to the international scientific community was one of the strengths of the project, with presentations by project staff and postgraduate students at a range of conferences and technical meetings. These meetings included conferences on:

- Epidemiology:
    i. International Society for Veterinary Epidemiology and Economics, Durban, South Africa, 2009;
    ii. Society for Veterinary Medicine and Preventive Medicine, Leipzig, Germany, 2011
- Biosecurity:
    i. Australian Biosecurity CRC for Emerging Infectious Disease Annual Workshop, St Kilda 2007,
    ii. Australian Biosecurity CRC for Emerging Infectious Disease Annual Workshop, Darwin 2009
    iii. Australian Biosecurity CRC for Emerging Infectious Disease Annual Workshop, Fraser Island 2010; the Global Biosecurity Conference, Brisbane, 2010
    iv. Global Biosecurity Conference. 28 February - 3 March 2010. Brisbane, Australia
- Virology:
    i. Australian Virology Group Conference, Lorne, 2009
- Veterinary science:
    i. Australian Veterinary Association Annual Conference, Perth, 2008;
    ii. Australian College of Veterinary Scientists Science Week, Gold Coast, 2009
- Avian influenza:
    i. Research Activities on Avian Influenza & Other Transboundary Diseases in SE Asia, Bangkok, Thailand, 2008;
    ii. FAO Viet Nam Avian Influenza Research Meeting, Hanoi, 2008.

- Poultry:
    i. XXIII World's Poultry Congress, Brisbane, 2008

Project team members also presented the project work at a number of less formal meetings, workshops or seminars, including:

- Meetings organised by government authorities or non-government institutions (Workshop at the Regional Animal Health Office VI, Ho Chi Minh City, October 2009; Workshop at the Disease Investigation Centre, Wates, October 2009;)
- Meetings organised by non-government institutions (Meeting at FAO Avian Influenza Control Programme Indonesia office, Jakarta, February 2009; NZAID Project Technical Review meeting, Can Tho, Viet Nam, September 2009; ILRI meeting on "Development of a National Emerging Diseases Framework for Indonesia: Primary Consultations, Agriculture Sector", Jakarta, October 2009)
- Seminars presented at a variety of institutions (Animal Research Institute, Queensland Department of Employment, Economic Development and Innovation, Yeerongpilly, January 2008; Virology Department, Faculty of Veterinary Sciences, Universidad Autonoma de Yucatan, Merida, Mexico, May 2009; Australian Animal Health Laboratory, Geelong, student seminar series, multiple years).

The project was featured in both ACIAR publications and public media, including:

- ACIAR Partners Magazine, article entitled "Understanding bird flu" by K. McGhee. July-Oct 2007. p4-9
- The University of Queensland Vet Connect newsletter, article entitled "Investigations into the maintenance and transmission of Highly Pathogenic Avian Influenza (HPAI) H5N1 in domestic duck populations in the Mekong Delta of Viet Nam and in Central Java of Indonesia" by J. Henning. 2009
- ACIAR newsletter Viet Nam, September 2008.
- ABC Rural report on HPAI research conducted by ACIAR project in Indonesia (http://www.abc.net.au/rural/content/2008/s2562108.htm)
- Indonesian newspapers articles about duck research conducted in ACIAR project (May 2009)
- ProMed information about research in ACIAR project on 10th August 2008 (http://apex.oracle.com/pls/otn/f?p=2400:1001:674479108843189::NO::F2400_P1001_BACK_PAGE,F2400_P1001_PUB_MAIL_ID:10001,73482)

The project produced a range of extension materials to increase awareness about avian influenza in general and to specifically inform stakeholders and the public about the project's activities. The materials were distributed to farmers who had participated in the field studies, to field veterinarians, paravets and extension workers in both partner countries, to government officials and to selected representatives of international aid organisations, researchers working on HPAI and to universities. These extension materials included:

- Desk calendars (15 cm x 22 cm, 7-pages, double-sided) and wall calendars (50 cm x 75 cm, 4-sheets) were produced in 2008 in Indonesia. The calendars featured photos of duck farms, project staff, sample collection methods and biosecurity techniques. Two hundred copies of the desk calendar and 500 copies of the wall calendar were distributed.
- Desk and wall calendars (as above) were produced in Viet Nam in 2009. Three hundred copies of the desk calendar and 400 copies of the wall calendar were distributed.

9 Conclusions and recommendations

9.1 Conclusions

The project made substantial contributions to knowledge on the biology of H5N1 avian influenza in two broad areas – the behaviour of the virus in the field and the nature of the pathology of the virus in both ducks and chickens. In both areas, the project team was considered leaders by the international scientific community. We were the first to conduct longitudinal field studies to investigate the prevalence and incidence of the virus in ducks. We were the first to conduct a detailed molecular and antigenic analysis of virus isolates collected from a field study and to relate the molecular virological findings to results from the field study. Finally, we were the first to conduct a detailed series of experimental studies in both ducks and chickens investigating the variation in pathogenicity between avian species and between genetically different virus isolates.

Our field studies of smallholder 'stationary' duck farms in both Indonesia and Viet Nam showed that H5N1 antibody prevalence was higher in individual ducks and duck flocks than in individual chickens and chicken flocks that were in contact with the ducks. In Indonesia, we were able to detect shedding of H5N1 virus from birds on more than a quarter of the 96 smallholder farms involved in the field study. Again, ducks flocks were more likely to be virus-positive than chicken flocks. We suggested that these findings indicate that ducks are more likely than chickens to harbour the virus and perhaps to survive the virus infection and to transmit the virus to other birds. They are possibly more likely to be exposed to the virus through their contact with other duck flocks or wild birds, as a result of their extensive grazing practices, whereas chickens more often scavenge close to the household dwelling and have less frequent contact with large numbers of other birds.

Although no outbreaks of HPAI disease occurred on any of our study farms in Viet Nam, 35% (34/96) of the study farms in Indonesia experienced an outbreak during the course of the 12-month longitudinal study. During these outbreaks, the proportion of birds shedding virus on the farm increased substantially and virus was detected in both sick and apparently healthy birds. Although most chickens involved in the outbreaks succumbed to the disease, the finding of small numbers of antibody-positive chickens suggested that some do survive the infection. Conversely, most ducks show minimal clinical signs of infection, with few deaths occurring in that species.

The incidence analysis from the longitudinal field study in Indonesia revealed four factors that significantly influenced the risk of infection for ducks. Allowing ducks to scavenge around neighbouring houses and consuming carcasses of dead birds both increased the risk of infection and seroconversion, while confining duck flocks over-night on the farm, or experiencing sudden deaths in the flock in the preceding two months both reduced the risk of infection.

The field studies of vaccinated ducks and chickens in Viet Nam showed that many birds failed to develop appropriate antibody responses, with just over half of the vaccinated ducks (55.4%) and chickens (56.8%) developing antibody titres considered to be protective. However, despite this lack of response, there were no disease outbreaks in our study villages in Viet Nam, suggesting either that non-antibody mediated immune mechanisms were protecting the vaccinated birds, or that flock immunity was sufficient to prevent disease outbreaks from occurring. The analysis of risk factors in the Mekong Delta disease outbreaks of 2006-2007 (case-control study) revealed three factors that were significantly associated with risk of a HPAI outbreak, comprising no or incomplete vaccination of birds, presence of geese on farms and visiting of farms by people.

Characterisation of virus isolates from Indonesia demonstrated that while multiple genetic variants of virus could exist on one farm, the same genetic variants could be isolated from different farms, indicating widespread geographic occurrence. The spread of HPAI viruses could be tracked through the presence of different genetic variants, with examples of identical viruses being isolated from dead chickens on one farm and live ducks on another farm in the same village, or a virus from live ducks being identical to that causing deaths in chickens on the same farm one week later.

The experimental studies using Indonesian isolates demonstrated that viruses from different phylogenetic clades, or isolated from ducks with different presentations (dead versus healthy), the clinical outcomes, pathogenicity and tissue distributions of these viruses were similar in both chickens and ducks. The viruses had a greater ability to replicate in chickens than in ducks, with a high concentration of virus found in a wide range of chicken tissues, but virus was only detected in a limited number of tissues in ducks. As a consequence, a high titre of virus was shed by chickens through both oral and cloacal routes compared to a low and intermittent shedding of virus in only oral swabs from ducks. Thus, HPAI-infected chickens are possibly more likely than ducks to be a major source of virus transmission among poultry and potentially to humans. In contrast, ducks are probably better than chickens in maintaining virus in the poultry population as they can act as silent hosts, but could shed infectious virus for a longer period.

9.2 Recommendations

The research of this project improved understanding of a number of aspects of H5N1 HPAI virus. However as with all research, the process of answering some questions raised many more questions about the nature of the virus, its behaviour in the field, its mechanisms of persistence and the approaches that should be used for control.

Both the field and experimental studies (using Indonesian viruses) indicated that ducks can be infected and transmit the virus without showing any clinical signs, and can shed the virus up to 8 days post-infection, indicating they may be an effective maintenance host. However, replication of virus in ducks was limited, and shedding of virus was intermittent and at a very low concentration suggesting that ducks are not a very effective transmission host. In contrast, infection in chickens was rapidly fatal, virus replication was extensive, and virus shedding was extremely high for the short period of time before death, indicating very efficient transmission of virus. Future studies should focus on the mechanisms of virus persistence in populations, whether ducks can continue to shed virus for a longer duration or from a different route than detected in this study.

Through field studies in Viet Nam and Indonesia, the project identified a number of management factors that can influence the risk of a HPAI disease outbreak or infection occurring, respectively. Several of these factors could be used immediately in recommendations provided to farmers to avoid HPAI infection or disease in their flocks, e.g. vaccination (see below), confining duck flocks over-night on the farm, prevention of ducks scavenging around neighbouring houses, and avoiding consumption of carcasses of dead birds. Other factors require further research to determine the mechanisms of risk, e.g. the finding that having geese on the farm or the use of disinfectant for cleaning enclosures were both associated with increased risk of a disease outbreak in Viet Nam.

The work in Viet Nam demonstrated that vaccination can prevent disease outbreaks, even when antibody response of many vaccinated birds appears inadequate. Although our results suggested that low levels of virus were circulating amongst the study farms, no disease outbreaks occurred. The case-control study also demonstrated that no or incomplete vaccination resulted in a significantly higher risk of experiencing a HPAI outbreak. Thus, if circumstances and finances allow, a well-conducted vaccination program based on birds receiving three vaccinations can prevent outbreaks of disease, and reduce the amount of virus circulating on farms, which should lower the risk of transmission to people.

Research questions: What we didn't know at the beginning of the projects	**Conclusions: What we know now as result of the project *and* from other sources**	**Implications: What does this increase in knowledge mean for next users and final beneficiaries of the project?**	**Recommendations: On this basis, what recommendations is the project making for surveillance and control of HPAI in Viet Nam and Indonesia?**
Is there transmission of virus between ducks and chickens in the field?	Yes, as implied from genetic studies of viruses collected from chickens and ducks.	Ducks and chickens need to be considered as parts of a single 'epidemiological unit'.	Biosecurity measures should account for the possible transmission of virus between ducks and chickens.
Are the viruses circulating in ducks the same as the viruses in chickens?	Yes, they are the same viruses, as determined by genetic characterisation.	Vaccines suitable for use against chicken viruses are highly likely to be appropriate for duck viruses.	Vaccine strains for use in both ducks and chickens will be similar.
Is vaccination of ducks effective?	Yes, vaccinated ducks appear to have reduced virus load and vaccinated duck flocks are less likely to suffer from a disease outbreak than unvaccinated flocks.	Vaccination of ducks should reduce likelihood of a disease outbreak and should reduce amount of virus in the environment.	Ducks should be included in area-wide vaccination programs to prevent lost productivity in individual flocks.
Do management practices influence the incidence of AI virus infection in duck flocks?	Yes, confinement of duck flocks at night and less scavenging around neighbouring houses reduces incidence of infection.	Management strategies can reduce likelihood of AI infection in duck flocks.	Duck farmers of both stationary and moving systems should more closely control contact of their birds with other poultry.
Do management practices influence the likelihood of AI disease outbreaks in duck flocks?	Yes, vaccination of duck flocks reduces risk of an outbreak, while having visitors or geese on the farm increases this risk.	Vaccination of ducks can reduce risk of a disease outbreak. More research is required to understand the effect of geese on farms.	Ducks should be included in vaccination programs. Visiting friends and family should be included in general biosecurity measures on a farm.
Are ducks an infection risk to humans?	Yes, but levels of virus excreted by ducks are low and therefore likely to be a lower risk than chickens.	Duck flocks are a lesser but still significant hazard for human health.	The same warnings on close contact and consumption of dead animals apply equally to ducks and chickens.
Are ducks an effective maintenance host for H5N1 viruses?	Yes, our field and experimental studies showed that ducks can be infected and shed virus for up to 8 days, without showing any clinical signs or mortality	Ducks can effectively maintain virus within the flock without showing signs of disease and can therefore provide a source of virus for chickens, which invariably show severe disease signs.	Farmers should be aware that virus can be ‘silently’ circulating amongst their duck flocks without any apparent signs.

10 References

10.1 References cited in report

Chen, H., Smith, G.J.D., Li, K.S., Wang, J., Fan, X.H., Rayner, J.M., Vijaykrishna, D., Zhang, J.X., Zhang, L.J., Guo, C.T., Cheung, C.L., Xu, K.M., Duan, L., Huang, K., Qin, K., Leung, Y.H.C., Wu, W.L., Lu, H.R., Chen, Y., Xia, N.S., Naipospos, T.S.P., Yuen, K.Y., Hassan, S.S., Bahri, S., Nguyen, T.D., Webster, R.G., Peiris, J.S.M., Guan, Y., (2006). Establishment of multiple sublineages of H5N1 influenza virus in Asia: Implications for pandemic control. *Proc. Natl. Acad. Sci. U.S.A* 103, 2845-2850.

Cristalli, A., Terregino, C., Long, H.T., Gilbert, J., Lubroth, J., Capua, I., (2007). Field and laboratory evalutation of a vaccination programme against H5N1 in waterfowl. In: Vaccination: A tool for the control of Avian Influenza, conference proceedings of OIE/FAO/IZSVe scientific conference 20-22 March 2007, Verona, Italy. Available http://www.izsvenezie.it/dnn/Portals/0/AI/Aviflu_07%20Complete.pdf (accessed 03.10.2009)

Food and Agriculture Organisation. FAOSTAT-Agriculture. http://faostat.fao.org/site/573/DesktopDefault.aspx?PageID=573#ancor (accessed 18.11.2011)

Forrest, H.L., Webster RG. (2010) Perspectives on influenza evolution and the role of research. *Animal Health Research Reviews*. 11:3-18

Heine H, Trinidad L, Selleck P, Lowther S (2007) Rapid detection of highly pathogenic avian influenza H5N1 virus by TaqMan reverse transcriptase-polymerase chain reaction *Avian Diseases* , 51:370-372

Hulse-Post, D.J., Sturm-Ramirez, K.M., Humberd, J., Seiler, P., Govorkova, E.A., Krauss, S., Scholtissek, C., Puthavathana, P., Buranathai, C., Nguyen, T.D., Long, H.T., Naipospos, T.S.P., Chen, H., Ellis, T.M., Guan, Y., Peiris, J.S.M., Webster, R.G., (2005). Role of domestic ducks in the propagation and biological evolution of highly pathogenic H5N1 influenza viruses in Asia. *Proc. Natl. Acad. Sci. U.S.A*.102, 10682-10687.

Kishida N, Sakoda Y, Isoda N, Matsuda K, Eto M, Sunaga Y, Umemura T, Kida H: Pathogenicity of H5 influenza viruses for ducks. (2005) *Arch Virol.* 150:1383-1392

Kwon YK, Joh SJ, Kim MC, Sung HW, Lee YJ, Choi JG, Lee EK, Kim JH. (2005) Highly pathogenic avian influenza (H5N1) in the commercial domestic ducks of South Korea. *Avian Pathol* 34(4):367-70.

Middleton, D., Bingham, J., Selleck, P., Lowther, S., Gleeson, L., Lehrbach, P., Robinson, S., Rodenberg, J., Kumar, M., Andrew, M., (2007). Efficacy of inactivated vaccines against H5N1 avian influenza infection in ducks. *Virology* 359, 66-71.

Minh, P.Q., Morris, R.S., Schauer, B., Stevenson, M., Benschop, J., Nam, H.V., Jackson, R., (2009). Spatio-temporal epidemiology of highly pathogenic avian influenza outbreaks in the two deltas of Vietnam during 2003-2007. *Prev.Vet. Med*. 89, 16-24.

OIE, (2009). Manual of Diagnostic Tests and Vaccines for Terrestrial Animals 2009, http://www.oie.int/Eng/Normes/Mmanual/A_summry.htm (accessed 03.10.2009)

Pantin-Jackwood MJ, Swayne DE: Pathobiology of Asian highly pathogenic avian influenza H5N1 virus infections in ducks.(2007) *Avian Dis.* 51:250-259.

Pfeiffer, D.U., Minh, P.Q., Martin, V., Epprecht, M., Otte, M.J., (2007). An analysis of the spatial and temporal patterns of highly pathogenic avian influenza occurrence in Vietnam using national surveillance data. *Vet. J*. 174, 302-309.

Savill, N.J., St Rose, S.G., Keeling, M.J., Woolhouse, M.E.J., (2006). Silent spread of H5N1 in vaccinated poultry. *Nature* 442, 757.

Sturm-Ramirez, K.M., Ellis, T., Bousfield, B., Bissett, L., Dyrting, K., Rehg, J.E., Poon, L., Guan, Y., Peiris, M., Webster, R.G. (2004). Reemerging H5N1 influenza viruses in Hong Kong in 2002 are highly pathogenic to ducks. *J.Virol*. 78, 4892-4901.

Suzuki K, Okada H, Itoh T, Tada T, Mase M, Nakamura K, Kubo M, Tsukamoto K. (2009) Association of increased pathogenicity of Asian H5N1 highly pathogenic avian influenza viruses in chickens with highly efficient viral replication accompanied by early destruction of innate immune responses. *J Virol.* 83:7475-7486.

Swayne DE: Understanding the complex pathobiology of high pathogenicity avian influenza viruses in birds. (2007) *Avian Dis.* 51:242-249.

Takano R, Nidom CA, Kiso M, Muramoto Y, Yamada S, Sakai-Tagawa Y, Macken C, Kawaoka Y: Phylogenetic characterization of H5N1 avian influenza viruses isolated in Indonesia from 2003-2007 (2009). *Virology.* 390:13-21

Tian, G., Zhang, S., Li, Y., Bu, Z., Liu, P., Zhou, J., Li, C., Shi, J., Yu, K., Chen, H., (2005). Protective efficacy in chickens, geese and ducks of an H5N1-inactivated vaccine developed by reverse genetics. *Virology* 341, 153-162.

van den Berg, T., Lambrecht, B., Marche, S., Steensels, M., Van Borm, S., Bublot, M., (2008). Influenza vaccines and vaccination strategies in birds. *Comp. Immunol. Microbiol. Infect Dis.* 31, 121-165.

van der Goot, J.A., van Boven, M., de Jong, M.C., Koch, G., (2007). Effect of Vaccination on transmission of HPAI H5N1: the effect of a single vaccination dose on transmission of highly pathogenic avian influenza H5N1 in Peking ducks. *Avian Dis.* 51(Supp), 323-324.

World Health Organisation (2011) Global Alert and Response: Cumulative Number of Confirmed Human Cases of Avian Influenza A/(H5N1) Reported to WHO, 19 Aug 2011.http://www.who.int/csr/disease/avian_influenza/country/cases_table_2011_08_19/en/index.html (accessed 29.08.11)

10.2 List of publications produced by project

Journal Articles

Henning J, Pfeiffer DU, Vu Le (2009). Spatio-temporal characteristics and risk factors associated with the 2006/2007 Highly Pathogenic Avian Influenza Epidemic in the Mekong Delta of Vietnam. *Veterinary Research*, 40 (3):15.

Henning KA, Henning J, Morton J, Long NT, Ha NT, Meers J. (2009) Farm- and flock-level risk factors associated with Highly Pathogenic Avian Influenza outbreaks on small holder duck and chicken farms in the Mekong Delta of Viet Nam. *Preventive Veterinary Medicine*. 91:179-88

Bingham J, Green DJ, Lowther S, Klippel J, Burggraaf S, Anderson DE, Wibawa H, Hoa DM, Long NT, Vu PP, Middleton DJ, Daniels PW. (2009) Infection studies with two highly pathogenic avian influenza strains (Vietnamese and Indonesian) in Pekin ducks (Anas platyrhynchos), with particular reference to clinical disease, tissue tropism and viral shedding. *Avian Pathology*. 38(4):267-78.

Henning J, Wibawa H, Morton J, Usman TB, Junaidi A, Meers J. (2010) Scavenging ducks and transmission of highly pathogenic avian influenza, Java, Indonesia. *Emerging Infectious Diseases* 16(8):1244-50.

Henning J, Henning KA, Morton JM, Long NT, Ha NT, Vu le T, Vu PP, Hoa DM, Meers J.(2011) Highly Pathogenic Avian Influenza (H5N1) in ducks and in-contact chickens in backyard and smallholder commercial duck farms in Viet Nam. *Preventive Veterinary Medicine*. 101(3-4):229-40.

Wibawa H, Henning J, Wong F, Selleck P, Junaidi A, Bingham J, Daniels P, Meers J. (2011) A molecular and antigenic survey of H5N1 highly pathogenic avian influenza virus isolates from smallholder duck farms in Central Java, Indonesia during 2007-2008. *Virology Journal*, **8**:425

Henning J, Wibawa H, Yulianto D, Usman TB, Junaidi A, Meers J. (2011) The management of smallholder duck flocks in Central Java, Indonesia, and potential hazards promoting the spread of Highly Pathogenic Avian Influenza virus. *Worlds Poultry Science Journal* (submitted)

Henning J, Morton J, Wibawa H, Yulianto D, Usman TB, Priyono W, Junaidi A, Meers J. (2011) Focussing not on HPAI outbreaks: incidence and risk factors for H5 infection of clinically healthy ducks flocks. *Preventive Veterinary Medicine* (submitted)

Wibawa H, Bingham J, Nuradji H, Lowther S, Payne J, Rookes J, Junaidi A, Middleton D, Henning J, Meers J. The pathogenesis of two distinct clades of Indonesian H5N1 avian influenza viruses in chickens and ducks. (in preparation)

Conference proceedings/presentations/papers/posters

Hall K, Meers J, Henning J. The ecology of highly pathogenic avian influenza infection among rural poultry in the Mekong delta of Vietnam. Australian Biosecurity CRC for Emerging Infectious Disease Annual National Workshop. St Kilda, 14-16 November 2007

Henning J, Wibawa H, Purnomo PD, Henning K, Truc Ha, Long NT, Meers J. The epidemiology of highly pathogenic avian influenza (HPAI) in ducks in Indonesia and Vietnam – investigations into the maintenance and transmission of HPAI in backyard and small-scale commercial duck farms. Research Activities on Avian Influenza and Other Transboundary Animal Diseases in South-East Asia Workshop. 21-22 January 2008, Bangkok, Thailand.

Meers J, Henning J, Wibawa , Purnomo P, Henning K, Nguyen Truc Ha, Ngo Thanh Long. Epidemiology of highly pathogenic avian influenza (HPAI) in ducks in Indonesia and Viet Nam. Australian Veterinary Association Annual Conference. 25-30 May 2008. Perth.

Henning J, Henning K, Bingham J, Truc Ha, Long NT, Meers J. The epidemiology of highly pathogenic avian influenza (HPAI) in ducks – investigations into the maintenance and transmission of HPAI in backyard and small-scale commercial duck farms. FAO Viet Nam Avian Influenza Research Meeting. 16-18 June 2008. Hanoi

Henning J, Wibawa H, Purnomo P, Henning K, Ha T, Long NT, Sunn K and Meers J. Maintenance and transmission of highly pathogenic avian influenza in backyard and small-scale commercial poultry production systems in South-east Asia. XXIII World's Poultry Congress, 30 June - 4 July 2008. Brisbane

Henning J, K. Henning, J. Bingham, Nguyen Truc Ha, Ngo Thanh Long, J. Meers. The epidemiology of highly pathogenic avian influenza (HPAI) in ducks – investigations into the maintenance and transmission of HPAI in backyard and small-scale commercial

duck farms. FAO Viet Nam Avian Influenza Research Meeting. 16-18 June 2008. Hanoi

Henning J, H. Wibawa, P. Purnomo, K. Henning, T. Ha T, NT. Long, K. Sunn K and J. Meers. Maintenance and transmission of highly pathogenic avian influenza in backyard and small-scale commercial poultry production systems in South-east Asia. XXIII World's Poultry Congress, 30 June - 4 July 2008. Brisbane

Henning K, J. Henning, J. Morton, Ngo Thanh Long, Nguyen Truc Ha, J. Meers. Risk factors for outbreaks of Highly Pathogenic Avian Influenza on small holder poultry farms - a case-control study investigating the 2006/2207 HPAI epidemic in the Mekong Delta of Viet Nam. Australian Biosecurity -CRC National Workshop, 19-21 May 2009, Darwin.

Henning J, Henning K, Wibawa Le Tri Vu, Yulianto Y, Morton, Meers J (2009) The role of free-ranging duck farming in the spread of highly pathogenic avian influenza (HPAI) in Viet Nam and Indonesia. Australian College of Veterinary Scientists Science Week. 2-4 July 2009. Gold Coast, Australia.

Henning J, Le Tri Vu, Yulianto D, Meers J (2009) The role of moving duck flocks in the spread of highly pathogenic avian influenza virus in Vietnam and Indonesia. Proceedings of the 12th Symposium of the International Society for Veterinary Epidemiology and Economics (ISVEE), Durban, South Africa, August 2009, p30

Henning J, Wibawa H, Henning K, Morton J, Meers J (2009) Prevalences of highly pathogenic avian influenza antibodies on small-scale commercial and backyard farming free-ranging duck enterprises in Southeast Asia. Proceedings of the 12th Symposium of the International Society for Veterinary Epidemiology and Economics (ISVEE), Durban, South Africa, August 2009, p53

Henning J, Henning K, Pfeiffer DU, Morton J, Meers J (2009) Risk factors associated with highly pathogenic avian influenza outbreaks in the Mekong delta of Vietnam in the face of vaccination programs. Proceedings of the 12th Symposium of the International Society for Veterinary Epidemiology and Economics (ISVEE), Durban, South Africa, August 2009, p213

Wibawa H, Wong F, Bingham J, Davies K, Steven V, Pye T, Ha W, Lowther S, Selleck P, Henning J, Junaidi A, Middleton D, Daniels P, Meers J. (2009) Diversity of the hemagglutinin and neuraminidase genes from Indonesian H5N1 highly pathogenic avian influenza (HPAI) virus isolates. Australian Virology Group Conference. 13-17 December 2009. Lorne, VIC, Australia. P2.37

Nuradji H, Lowther S, Wibawa H, Payne J, Rookes J, Junaidi A, Meddleton D, Daniels P, Bingham J, Meers J (2009)The pathogenesis of an Indonesian H5N1 highly pathogenic avian influenza virus infection in chicken feathers. Australian Virology Group Conference. 13-17 December 2009. Lorne, VIC, Australia. P2.23

Meers J, Henning J, Henning K, Wibawa H, Le Tri Vu. (2009) Are scavenging ducks a biosecurity risk for HPAI spread and infection? Global Biosecurity Conference. 28 February - 3 March 2009. Brisbane, Australia. p51-52

Wibawa H, Nuradji H, Henning J, Bingham J, Wong, F, Lowther S, Payne J, Rookes J, Meers J. (2010) Characterisation of Indonesian H5N1 isolates to understand the prolonged infection and spread of H5N1 among domestic poultry. Global Biosecurity Conference. 28 February - 3 March 2010. Brisbane, Australia. p32-33

Hartawan R, Meers J, Mahony T, Robinson K. (2010) Vectoring of avian influenza antigens using a recombinant avian herpes virus vector. Global Biosecurity Conference. 28 February - 3 March 2010. Brisbane, Australia. p143

Nuradji H, Wibawa H, F, Lowther S, Payne J, Rookes J, Bingham J, Meers J. (2010) Validating feathers for the field diagnosis of highly pathogenic avian influenza subtype

H5N1. Global Biosecurity Conference. 28 February - 3 March 2010. Brisbane, Australia. p149

Henning K, Henning J, Meers J. (2010) The role of domestic ducks in the maintenance and transmission of highly pathogenic avian influenza virus H5N1 in the Mekong Delta of Vietnam. Australian Biosecurity –Cooperative Research Centre National Workshop, 11-14 May 2010, Fraser Island, Australia

Henning J, Wibawa H, Yulianto D, Junaidi A, Meers J, (2011). Understanding the management of smallholder, village-based duck flocks in Indonesia –opportunities for Highly Pathogenic Avian Influenza (HPAI) control. Poster presentation. Society for Veterinary Medicine and Preventive Medicine, 2011 Annual Conference, Leipzig, Germany. 23-25 March 2011.

Henning J, Wibawa H, Yulianto D, Junaidi A, Meers J, (2011). Incidence and risk factors of Highly Pathogenic Avian Influenza (HPAI) infection in smallholder, village-based duck flocks in Indonesia. Poster presentation. Society for Veterinary Medicine and Preventive Medicine, 2011 Annual Conference, Leipzig, Germany. 23-25 March 2011.

Henning J, Priyono W, Yulianto D, Junaidi A, Meers J, (2011). The role of rice paddy owners, transporters of ducks and hatcheries in influencing the spread of Highly Pathogenic Avian Influenza (HPAI) virus in Indonesia. Poster presentation. Society for Veterinary Medicine and Preventive Medicine, 2011 Annual Conference, Leipzig, Germany. 23-25 March 2011

11 Appendixes

11.1 Appendix 1: Abbreviations

AAHL	Australian Animal Health Laboratory
AIV	avian influenza virus
Bbalitvet	Research Institute for Veterinary Science, Bogor
BPPV-IV	Disease Investigation Centre, Wates (or DIC)
C-ELISA	competitive enzyme-linked immunosorbent assay
CI	confidence interval
CSIRO	Commonwealth Scientific and Industrial Research Organisation
DAH	Department of Animal Health (Viet Nam)
DIC	see BPPV-IV
DVS	District Veterinary Stations (Viet Nam)
EID_{50}	50% Embryo Infectious Dose
FAO	Food and Agriculture Organisation
GEE	general estimation equation
H	haemagglutinin
HA	haemagglutination (test)
HCMC	Ho Chi Minh City
HI	haemagglutination inhibition (test)
HPAI	Highly pathogenic avian influenza
ILRI	International Livestock Research Institute
JAF	John Allwright Fellowship
LPAI	low pathogenic avian influenza
N	neuraminidase
NZAID	New Zealand Agency for International Development
NIVR	National Institute of Veterinary Research, Hanoi
OIE	World Organisation for Animal Health (Office International des Epizooties)
OR	odds ratio
PCR	polymerase chain reaction
RAHO-VI	Regional Animal Health Office VI, Ho Chi Minh City
RT-PCR	reverse transcription PCR
rRT-PCR	real-time reverse transcription PCR
$TCID_{50}$	50% Tissue Culture Infectious Dose
UQ	University of Queensland
WHO	World Health Organisation