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JAMES COOK UNIVERSITY
AUSTRALIA

RISK OF SPILL-OVER OF DISEASES (IN PARTICULAR AVIAN INFLUENZA) FROM WILD AQUATIC BIRDS IN NORTH QUEENSLAND

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A dissertation submitted in total fulfilment of the requirements of
the degree of Doctor of Philosophy

April 2011

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Preface

Chapters 2-7 were prepared with the aim of being published in scientific journals. Consequently, some repetition is unavoidable. However, the reference style of the whole document follows the thesis guidelines of James Cook University. Reduction of text, inclusion of an abstract and change of referencing style will be required for submission of each chapter to a journal for publication.

Lauren Cook and Gareth Smith followed by Ai Lee Cheam contributed to Chapter 4: Processing field samples for molecular analysis. Daniel Grace along with Ai Lee Cheam optimised and developed the semi-nested Polymerase Chain Reaction technique that was applied to field samples for avian influenza viruses. Stephen Garland tested the level of inhibitors in the field samples for real time reverse transcriptase polymerase chain reactions. Graham Burgess and David Blair helped analyse avian influenza viral gene sequence data. Above all, Graham Burgess designed all primers used for molecular analysis for this chapter.

Ai Lee Cheam contributed to Chapter 5 by molecular testing for Newcastle disease viruses in field samples.

Andrew Greenhill, Robert Hedlefs, Orachun Hayakijkosol, Laurie Reilly and Ai Lee Cheam were predominantly involved with Chapter 6. Andrew initially helped process sick and dead bird tissue samples for bacteriological evaluation and also gave comments on this chapter. Robert helped in serosubtyping of *salmonella* positive samples with the collaboration of the Queensland Health Pathology Laboratory in Brisbane and also extended his editorial support on this chapter. Orachun contributed by performing post-mortems of bird carcasses followed by histopathological and bacteriological testing on tissues. Laurie provided valuable technical support for the processing of tissues for histological evaluation. Ai Lee Cheam performed molecular testing on tissue samples for avian influenza and Newcastle disease viruses.

The whole thesis was supervised by Lee Skerratt, Graham Burgess and Stephen Garland and as such had significant input in the design, execution and analysis of this research project, as well as reviewing the individual chapters of this thesis.

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I am very grateful to Ai Lee Cheam, Lauren Cook, Orachun Hayakijkosol, Gareth Smith, Desniwaty Karo-Karo, Daniel Grace and Anthony Baker who provided their support in field or lab work. This research would not have been completed without their sincere and active involvement.

I would like to give special thanks to Bob Flemming (Director, Billabong Sanctuary) along with his property manager, as well as all of the rangers for their cooperation. Bob allowed me to use his property to sample wild birds for this study. The manager and rangers occasionally helped bait our traps and collected sick and dead birds for the passive disease surveillance program.

I am very appreciative of Steve Pilla and his family for their untiring support and allowing me to sample wild birds on their property (Green Acres Lagoon, Cromarty). Steve happily baited and closed my traps when necessary for two years which was extra-ordinary and an incredible

help for me capture and then sample sufficient numbers of wild birds. I will never forget this lovely family!

I would like to give my sincere thanks to David Roshier and his team who supported sampling and providing samples of wild birds from Cape York. Samples from this site were very valuable for which we were able to check the spatial distribution of avian influenza viruses (in particular) in north Queensland. I also extend my thanks to staff members (particularly, Dan Hogarth) of Biosecurity Queensland, a service of the Department of Employment, Economic Development and Innovation who helped sample some wild birds on the Atherton Tableland. These samples were also valuable to determine the spatial distribution of AIVs in north Queensland.

David Blair, my assigned research mentor, always encouraged me through the length of my project. I received some crucial help from David in analysing avian influenza viral sequence. He also commented on my concluding chapter. Bruce Corney facilitated opportunities for me to get training on avian influenza molecular techniques at his lab in Brisbane and contributed his important comments on the sero-epidemiology chapter.

I also received necessary training on AI serology at the Tropical and Aquatic Animal Health Laboratory, Oonoonba and Animal Disease Surveillance laboratory, Toowoomba with the assistance of Elizabeth Houston (Toowoomba), Glenn Edmunds and Robert Hedlefs (Oonoonba). Robert also extended his support in commenting on my dead bird, Newcastle disease and concluding chapters.

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I thank my colleagues at work for their mental and qualified research support. They include Lisa Elliot, Kelly Hogston, Alanna Cooper, Ana Cano-Gomez, Natasha Williams, Rusaini, Anthony Baker and Orachun Hayakijkosol with whom I shared an office.

I received enormous support from the International Student Centre at James Cook University during my stay in Australia. A big thank to Alex Salvador and Katherine Elliot who took the trouble to ensure I was doing fine with my studies and welfare. Very special thanks to two Australian families (Anne's and Peter's) who gave me mental, welfare and neighbour-hood support. Their unconditional support indirectly helped my study.

It would have been great and exciting for my late parents to see my success. My father was so inspirational and all of my achievements so far have been materialized as a result of his love and support. Unfortunately, he died in April 2010 while I was studying in Australia. Many thanks to my other family members, relatives, friends and well wishers scattered in different parts of the world, who blessed and supported my life choices and managed to be close to me even from far away.

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I am very grateful to the Australian Biosecurity Cooperative Research Centre and the Australian Department of Agriculture, Forestry and Fisheries for providing project financial support. I thank AusAID for providing an Australian Leadership Award and the Australian Biosecurity Cooperative Research Centre for a top-up PhD scholarship. I thank International Society of Veterinary Epidemiology (in particular Bruce Gummow) for its financial support in attending the society conference in 2009 in South Africa. I extend my thanks to the scientific and ethics permit authorities for providing the following project approvals, Eco-access permit No. WISp04374507, Queensland Parks and Wildlife Service, Northern Region and ethics permit No-A 1,175, James Cook University. I would like to extend my

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Abstract

Disease surveillance programs and longitudinal studies are uncommon in wild bird populations across the world. But many wild bird species are important sources of pathogens that are of particular importance to animal and human health, for example, avian influenza viruses.

This project included both active and passive surveillance in order to study the diseases and pathogens (in particular avian influenza) in wild aquatic birds of north Queensland. A three-year longitudinal study was conducted on wild aquatic birds at Billabong Sanctuary from April 2007 to March 2010 while a two-year longitudinal study was performed at Green Acres Lagoon (Cromarty), from December 2007 to 2009. Cross sectional studies were also performed on wild aquatic birds at Cape York and on the Atherton Tableland between 2007 and 2009.

The objective of this project was to determine the level of avian influenza and Newcastle disease viral RNA and avian influenza viral antibody, identify the associated potential risk factors and determine the distribution of avian influenza and Newcastle disease viral subtypes and their phylogenetic relationship with other isolates in Australia and overseas. This study also aimed to identify causes of mortality in wild aquatic birds of north Queensland and explore the connection between mortality in birds and avian influenza.

Birds were sampled quarterly at Billabong Sanctuary and Cromarty and sporadically on Cape York and the Atherton Tableland. Birds were captured mostly using funnel traps. A total of 1,555 live birds were captured and this resulted in the collection of 1,522 serum samples, 1,458 cloacal and 1,368 oropharyngeal swab samples. Tissue samples were obtained from 42 sick and dead birds and 1,157 fresh faecal samples of wild aquatic birds were collected from the environment surrounding water bodies. Samples were evaluated by serological, molecular, bacteriological and histopathological examinations where necessary.

Overall avian influenza viral RNA prevalence was ~1.0% in the samples of wild aquatic birds in north Queensland, whereas the avian influenza viral antibody prevalence was 11 times higher. These findings make biological sense given the fact that avian influenza viral shedding periods are relatively shorter than the presence of avian influenza viral antibodies in the blood.

Multivariate regression analysis was performed to identify potential risk factors for avian influenza antibody levels in wild aquatic birds. The odds ratio of being reactive for avian influenza antibodies was 13.1 (95% Confidence interval 5.9-28.9) for Pacific black ducks (53.7%) compared with plumed whistling ducks (10.1%) (Table 3.12; Chapter 3). This result was also supported by the linear regression analysis (Chapter 3; Table 3.11). An identical species pattern was identified in an unadjusted statistical analysis on the viral RNA data of avian influenza (Chapter 4; Table 4.7).

The odds ratios of being reactive for avian influenza antibodies were 2.9 (95% Confidence interval 1.3-6.6) for adult over ≤ sub-adult ducks (Table 3.10; Chapter 3). A similar age pattern was identified in the linear regression analysis (Table 3.9; Chapter 3). This age pattern might be due to more exposure to infections because of more opportunity in addition to longer lasting avian influenza antibodies in older ducks. A different age pattern was, however, identified in unadjusted analysis using the molecular data (Chapter 3; Table 3.11). This analysis indicated that immature birds were more commonly infected which may be due to the fact that they have more frequent infections because they are immunologically naïve whereas adults are more resistant, particularly to viruses to which they may have previously been exposed.

Avian influenza antibodies were at higher levels during warm wet weather (January-April) compared with warm dry weather (September-December) in linear regression analysis (Coefficient 8.3; 95% Confidence interval 3.0-13.6) (Chapter 3; Table 3.7). The warm wet season might reduce the immune status of birds, thus making them more vulnerable to infection which may in turn increase the levels of avian influenza antibodies.

The surveillance programs demonstrated the presence of low pathogenic avian influenza viral subtypes H6 and H9 in samples collected from wild aquatic birds. One of the H6 viruses was likely to have been newly introduced, probably through migratory species of birds such as the sharptailed sandpiper. This migratory bird regularly travels between Australia and Asia. Hence, there is a possibility of highly pathogenic avian influenza exotic viral subtypes such as H5N1 being introduced into Australia. The second H6 virus had a matrix gene similar to those found associated with Australian H7 subtypes. This would suggest an earlier introduction of a H6 subtype which had an opportunity to reassort with local viruses. The low pathogenic avian influenza viral subtype H9 had a matrix gene similar to that found in Asian H9 viruses. Some H9 viruses have been shown to cause mortality in poultry elsewhere in the world. This subtype has also been isolated from pigs and humans in different countries, which indicates its pandemic potential.

At the time that the H6 and H9 subtypes were detected in samples collected from wild birds in north Queensland the serological study demonstrated periods of infection with H6 and H9 serotypes.

The serological study also demonstrated a constant circulation of H5 and sporadic circulation of H7 subtypes in wild aquatic birds. These viruses are perhaps non-pathogenic as evident in other studies elsewhere in Australia. However, these low pathogenic avian influenza viral subtypes have potential to mutate to virulent types once introduced into commercial poultry.

Overall Newcastle disease viral RNA prevalence was 3.5% at the individual bird level which indicates the presence of Newcastle disease viruses in wild bird populations in north Queensland. The prevalence was significantly higher in plumed whistling ducks. Avirulent Newcastle disease viruses (class-one and class-two Australian type) were identified in samples collected from wild aquatic birds. This indicates that wild birds remain a reservoir of paramyxoviruses that could be transmitted to domestic poultry.

A logistic regression model was performed to identify potential risk factors for the level of Newcastle disease viral RNA prevalence in plumed whistling ducks. The odds of reactor samples were 2.7 (95% Confidence interval 1.5-4.9) times more likely in younger than older ducks (Chapter 5; Table 5.5). A similar age pattern of prevalence was observed in the study of avian influenza. This age susceptibility to infection may be due to the fact that young birds are immunologically naïve.

Only univariate logistic analysis indicated birds caught in the warm wet season (January-April) as being significantly associated with a higher prevalence of Newcastle disease viral RNA. This result virtually correlates with an increase in the numbers of immature birds at that time associated with the breeding season of adult birds.

The above identified risk factors will significantly contribute to the design of a targeted avian influenza and Newcastle disease surveillance program in wild aquatic birds in northern Australia by wildlife authorities.

Morbidity and mortality were sporadic and more commonly observed in chicks and juvenile birds in April than other months of the year. Identified bacterial diseases that could be attributable to causing bird mortality were colibacillosis, pasteurellosis and salmonellosis. The investigation identified *Salmonella enterica serotype virchow* and *Salmonella enterica serotype hvittingfoss* from dead bird samples of an Australian white ibis and two plumed

whistling ducks, respectively. These serotypes have been identified as causing disease in Australians and are therefore relevant to public health. No avian influenza viral RNA was detected from any sick or dead birds by the molecular screening assay. There is an opportunity for establishing a long term passive disease surveillance programme for wild aquatic birds in north Queensland.

The project developed a reliable screening assay "competitive enzyme linked immunosorbent assay" (designated as James Cook University-2) and this assay was used to detect avian influenza viral antibodies from serum samples of wild aquatic birds. A semi-nested PCR approach was designed and applied on direct field reactor samples for amplification and sequencing of different avian influenza viral genes (matrix, haemagglutinin and non-structural protein).

Overall findings therefore suggest that there is an opportunity for establishing a long term active and passive surveillance program for monitoring pathogens and diseases of wild aquatic birds in north Queensland, an important region in Australian biosecurity. This would provide valuable information for risk assessment and mitigation and potentially have a significant benefit for public health and the economy for the region and the nation.

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List of abbreviations

Abbreviations	Elaboration
°C	Degree celsius
AAHL	Australian Animal Health Laboratory
ABI	Applied biosystems
ABTS	2, 2’-Azino-bis: 3-benzthiazoline-6-sulphonic Acid
AF	Allantoic fluid
AGID	Agar gel immunodiffusion
AI	Avian influenza
AIV	Avian influenza virus
ANOVA	Analysis of variance
ASD	Australian shelduck
AWI	Australian white ibis
BHQ	Black hole quencher
BLAST	Basic local alignment search tool
bp	Base pairs
BS	Black swan
BSC	Bush stone curlew
BT	Back titration
BVD	Bovine viral diarrhoea
CC	Cell control
cDNA	Complementary deoxyribonucleic acid
cELISA	Competitive enzyme-linked immunosorbent assay
CI	Confidence interval
CR	Crow
CRBC	Chicken red blood cell
C_T	Threshold
DEEDI	Department of Employment, Economic Development and Innovation
DG	Domestic goose
DM	Dusky moorhen
DMEM	Dulbecco’s modified eagle’s transport medium
DNA	Deoxyribonucleic acid

dNTP	Deoxynucleotidetriphosphate
EDTA	Ethylene diamine tetra acetic acid
ELISA	Enzyme-linked immunosorbent assay
F	Fusion
FAM	5 (6)-Carboxyfluorescein
GPG	Green pygmy goose
GT	Grey teal
H	Haemagglutinin
HA	Haemagglutination
HAU	Haemagglutination units
HD	Hardhead
H and E	Haematoxylin and Eosin
HI	Haemagglutination inhibition
HIT	Haemagglutination inhibition titre
HPAI	Highly pathogenic avian influenza
HRP	Horseradish peroxidase
iELISAs	Indirect enzyme-linked immunosorbent assays
IgG	Immunoglobulin
IPC	Internal positive control
g	Gravity
GBR	Great Barrier Reef
JCU	James Cook University
Kg	Kilogram
LP	Low pathogenic
LPAI	Low pathogenic avian influenza
LRT	Likelihood ratio test
M	Matrix
MAb	Monoclonal antibody
Max	Maximum
MB	Mutton bird
MD	Muscovy duck
MDPI	Mean difference percent inhibition
MEGA	Molecular evolutionary genetic analysis
MG	Magpie goose
Min	Minimum
ml	Millilitre

MS	Microsoft
MSF	Multiple sequences file
N	Neuraminidase
ND	Newcastle disease
NDV	Newcastle disease virus
mM	Micro molar
nm	Nanometre
NP	Nucleoprotein
nQLD	North Queensland
NS	Negative serum control
NSP	Non-structural protein
NSW	New South Wales
OD	Optical density
OR	Odds ratio
PBD	Pacific black duck
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PIG	Pigeon
PM	Post-mortem
PNG	Papua New Guinea
PWD	Plumed whistling duck
RNA	Ribonucleic acid
RNS	Red-necked stint
ROC	Receiver operating characteristic
RP	Rutland Plains
RPM	Rotation per minute
rRT-PCR	Real time reverse transcriptase-polymerase chain reaction
RSD	Radjah shelduck
RT	Reverse transcription
QLD	Queensland
S	Serum
SD	Serial dilution
Se	Standard error
STS	Sharp-tailed sandpiper

TAE	Tris base, acetic acid and ethylene diamine tetra-acetate
TAS	Tasmania
TMB	3, 3' 5, 5'-Tetramethylbenzidine
TS	Test serum
UK	United Kingdom
USA	United States of America
VIC	Victoria
VIF	Variance inflation factors
WA	Western Australia
WAB	Wild aquatic bird
WFH	White faced heron
WHO	World Health Organization
WWD	Wandering whistling duck
XLD	Xylose lysine deoxycholate
µl	Micro litre
µM	Micro molar