Application of qRT-PCR for improved understanding and control of infectious bursal disease in chickens

Thesis

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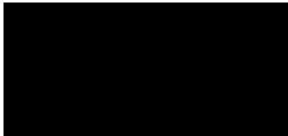
Declaration

I hereby endorse that this work is my own original work. All other resourced I used and any help I received in preparing this thesis, have been kindly acknowledged in this thesis. The materials in this thesis have not been submitted for any other degree, either in full or in partial.



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Dedication

I would like to dedicate this thesis to my beloved son, Oshada Attanayake, daughter, Janani Attanayake and my husband, Wijaya Attanayake for their unconditional love and being part of my life and also to my principal supervisor prof. Stephen Walkden-Brown for his great guidance, patience and support during this study.

Preface

This thesis has been written and structured in journal-article format. I have attempted to minimise the repetition of materials between chapters. However, some repetition remains, particularly in the methodology and introduction sections used in manuscripts.

List of publications for this thesis

Journal articles

1. **Jayasundara, J. M. K. G. K.,** Walkden-Brown, S. W., Margaret E, K., Islam, A. F. F., Renz, K. G., McNally, J., & Hunt, P. (2016). Pathogenicity, tissue distribution, shedding and environmental detection of two strains of IBDV following infection of chickens at 0 and 14 days of age. *Avian Pathology, Accepted for publication*.

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Conference presentations

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2. **Jayasundara, J. M. K. G. K.,** Islam, A. F. M. F., Walkden-Brown, S. W., Katz, E. M., Renz, K., Burgess, S., McNally, J., & Hunt, P. (2015). Infectious bursal disease antibody levels and viral load in bursa, faeces, litter and dust following infection of commercial broiler chickens at 0 and 14 days of age. Proceedings of the Australian Poultry Science Symposium, Sydney, 26: 175-178.

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List of Abbreviation

aa	Amino acids
AEC	Animal Ethic Committee
AI	Avian influenza
Bursa	Bursa of Fabricius
BrL	Brown Leghorn line
CAV	Chicken anaemia virus
cDNA	Complementary DNA
CID_{50}	Median chicken infective dose
CSIRO	Commonwealth Scientific and Industrial Research Organisation
Ct	Critical threshold values
CV	Coefficient of variation
DNA	Deoxyribonucleic Acid
dpe	Days post experiment
dpi	Days post infection
dPCR	Digital PCR
d.o.	Days old
dsRNA	Double stranded RNA
ELISA	Enzyme-Linked Immunosorbent Assay
EID_{50}	Median embryo infective dose
EMA	Ethidium monoazide
FAdV	Fowl adeno virus
hr.	Hours
HEPA	High Efficiency Particulate filtered air
HPAI	Highly pathogenic avian influenza
IBD	Infectious bursal disease
IBDV	Infectious bursal disease virus
ILTV	Infectious laryngotracheitis virus
iNOS	Inducible nitric oxide synthatase
MAb	Maternal antibody
MD	Marek’s disease
MDV	Marek’s disease virus

Min.	Minutes
MVT	Molecular viability test
MW	Molecular weight
NTR	Nontranslated region
OIE	Office International des Epizooties
ORF	Open reading frame
PBS	Phosphate-buffered saline
PC	Positive control
PC2	Physical containment level 2 certified
PCR	Polymerase chain reaction
PVPP	polyvinyl polypyrrolidone
qPCR	Quantitative PCR or real time PCR
qRT-PCR	Real time reverse transcription polymerase chain reaction
RE	Restriction endonuclease
RELP	Restriction fragment length polymorphism analysis
RH	Relative humidity
RNA	Ribonucleic acid
rRNA	Ribosomal RNA
RT-PCR	Reverse transcription PCR
Sec.	Seconds
SPF	Specific pathogen free
$TCID_{50}$	Median tissue culture infective dose
UNE	University of New England
USA	United States of America
VCN	Viral genome RNA copy number
VN	Viral neutralization
vPCR	Viable PCR
vRNA	Viral RNA
vv	Very virulent
WL	White Leghorn

Abstract

The advent of real time polymerase chain reaction test (qPCR) allows greater diagnostic and research capacity especially beneficial in the field of virology. Infectious bursal disease (IBD) is a well-known viral disease of poultry and occurs world-wide despite widespread use of vaccination. In this thesis study, the real time reverse transcription method (qRT-PCR) was used to address to a range of research questions to provide better understanding and control of IBD in chickens in Australia and world-wide.

One experiment was conducted to define the differences between Australian endemic classical and variant infectious bursal disease virus (IBDV) strains by infecting maternal antibody (MAb) free specific pathogen free (SPF) chickens separately with classical strain 06/95 and variant strain 02/95 in isolators. The results revealed that the two IBDV strains did not differ in the degree of bursal atrophy induced, IBDV viral RNA (vRNA) load in bursal and non-bursal lymphoid organs and faecal shedding but variant strain 02/95 induced a greater antibody response to the infection than classical strain 06/95 which was associated with a more rapid decline in IBDV vRNA genome copy number (VCN) in lymphoid organs

Two separate experiments were conducted to investigate the effects of age and presence of IBDV MAb on IBDV infection. MAb-free SPF chickens and MAb-positive commercial chickens were infected independently with variant strain 02/95 at two ages. In SPF chickens, chickens infected on day of hatch (0 d.o.) or 14 days old became infected but infection at 14 days old (d.o.) induced greater bursal atrophy and higher VCN in bursal and non-bursal lymphoid organs than infection on the day of hatch indicating true age susceptibility in the older birds, independent of MAb states. In the experiment with commercial chickens, commercial Ross broilers infected at 16 d.o. had a higher degree of bursal atrophy, IBDV VCN in bursal and non-bursal lymphoid organs and higher active humoral response to infection than those infected at 0 d.o. Chickens infected at 0 d.o. showed no evidence of early bursal atrophy or antibody response to infection, a very low level of IBDV vRNA in bursa and no vRNA in other lymphoid organs. Bursal atrophy and an antibody response were observed at 28 dpi and significant IBDV vRNA load was detected in lymphoid organs at 21 and 28 dpi with faecal shedding at 28 dpi. It could not be ascertained if the later responses were due to cross infection from the older birds, separated only by a wire partition, when MAb levels were reduced, or due to initial

infection with inhibition of virus replication followed by release of inhibition when MAb levels reduced. The results indicated that presence of high MAb titres at hatch blocked or markedly inhibited the pathogenesis of IBD but the reduced MAb titres at 16 d.o. did not prevent rapid IBDV infection and early marked bursal atrophy.

IBDV vRNA was readily detected and quantified in litter and dust samples from isolators and isolation pens. This demonstrates that the use these environmental samples, especially dust, as a diagnostic tool in routine disease monitoring may be feasible. Such testing would have several advantages over diagnostic tests based on tissue samples from individual birds including a single sample representing a population of birds, the sample being non-invasive and easy to collect, not requiring skilled personnel or special equipment and having fewer requirement to maintain cold chain during transportation to the laboratory.

Transmission of IBDV infection by IBDV-contaminated dust was tested by intra-tracheal insufflation of MAb-free SPF chickens with IBDV-contaminated dust collected from isolation pens during active IBDV infection. A marked rise in antibody titres between 7 and 35 dpi revealed active infection following dust insufflation, associated with marked bursal atrophy and high IBDV vRNA load in bursal tissues at 35 dpi. The results showed that IBDV-contaminated dust could be a likely source of infection of IBDV and the role of dust in the epidemiology of IBD requires further investigation.

Three separate chick bioassay experiments were conducted to determine the effects of different temperatures (25-65˚C) and times (5, 10 or 20 days) combinations on inactivation of IBDV in litter. In the first chick bioassay experiment, commercial layer cockerels were exposed to IBDV-contaminated litter at 28-34 days of age, after allowing MAb levels to subside. Chickens exposed to litter kept at 35˚C and above did not seroconvert to IBDV but those exposed to litter kept at 25°C for 5 and 20 days were infected with IBDV based on serological response and bursal atrophy at 35 dpi. Similarly, in the second bioassay experiment, exposure of MAb-free SPF chickens to the same IBDV-contaminated litter kept at 25°C for 5 and 10 days induced bursal atrophy while those exposed to litter kept at 25°C for 10 days showed an antibody response. The results of these two experiments demonstrate that IBDV-contaminated litter showed no seronversion following exposure to litter kept at temperatures at 35˚C and above, but those exposed to litter incubated at 25°C for 20 days could still transmit the disease

successfully to chickens. It could not be definitively concluded that the medium and higher temperatures inactivated the virus because unfortunately the positive control samples (no heat treatment) also did not induce seroconversion or bursal atrophy. In chick bioassay experiment 3, exposure of MAb-free SPF chicks to different temperature-time treated litter samples resulted in no IBDV infection including the unheated positive control litters.

The qRT-PCR analysis of litter samples from chick bioassay experiment 1 showed very low levels of vRNA in unheated positive control litter and no VCN in all other heat treated litter samples including litter treatments for 10 and 20 days at 25°C indicating a poor association between IBDV vRNA in litter and infectivity. This was further supported by results of the second chick bioassay experiment. In that experiment, low levels of vRNA were detected in litter samples prior to heat treatment, but no IBDV vRNA was detected after heat treatment. Again litter kept for 10 days at 25°C was qRT-PCR negative but induced both bursal atrophy and antibody response reinforcing the poor association between IBDV vRNA in litter and infectivity. In chick bioassay experiment 3, pre-heat treated litter had considerably higher IBDV vRNA load than litter in the previous two experiments with loss of vRNA detection following heat treatment. No chickens exposed to the untreated litter or litter given any of the heat treatments seroconverted or exhibited bursal atrophy. These findings taken together indicated that qRT-PCR enumeration of IBDV vRNA in heat-treated litter is not a good measure of the likely infection risk posed by that litter.

The failure to successfully transmit IBDV in litter in most treatments in these three experiments (including all positive controls) may have been as a result of storing the infective litter frozen (all experiments), low levels of infective virus in the litter (first two experiments), inactivation of virus due to desiccation and moderate heating in the litter in isolators prior to collection and storage (third experiment) or that IBDV is more labile in poultry litter than previously thought.