General introduction

Infectious bursal disease (IBD) has had a significant negative impact on the poultry industry worldwide since it was first reported in 1957 in the United States of America (USA) (Rautenschlein & Alkie, 2016). IBD is caused by infectious bursal disease virus (IBDV), a double-stranded RNA virus in the genus *Avibirnavirus* and family *Birnavirideae* (Dobos *et al.*, 1979; Dobos *et al.*, 1995). IBDV targets the surface immunoglobulin M-bearing B lymphocytes of the bursa of Fabricius (bursa) of chickens resulting in subclinical or clinical IBD (Hirai & Calnek, 1979). The losses associated with subclinical IBD are due to prolonged immunosuppression leading to other diseases and vaccination failure (Eterradossi & Saif, 2008). Clinical disease causes economic losses due primarily to mortality and reduced carcass quality (Van Den Berg, 2000). Two IBDV serotypes have been identified. Serotype 1 causes disease in chickens while serotype 2 is apathogenic in chickens (Jackwood *et al.*, 1982). Serotype 1 IBDV field strains vary in pathogenicity and are categorised into three pathotypes namely classical, variant and very virulent (vv) (Eterradossi & Saif, 2008). Classical strains vary in their pathogenicity and may be further classified as pathogenic and immunosuppressive (Ignjatovic *et al.*, 2004). Pathogenic classical and vvIBDV strains cause clinical disease while infection with immunosuppressive classical and variant strains lead to subclinical infection (Hirai *et al.*, 1974; Ignjatovic *et al.*, 2004). Chickens are most susceptible to IBD at 3-6 weeks of age with neonatal and older chickens not exhibiting clinical disease even after infection with pathogenic IBDV strains (Eterradossi & Saif, 2008; Fadly & Nazerian, 1983; Hitchner, 1976). Classical and variant strains predominate in the USA and Australia (Sapats & Ignjatovic, 2002) but the USA and Australian strains are genetically distinct (Sapats & Ignjatovic, 2000; Sapats & Ignjatovic, 2002).

IBDV was first reported in Australia in 1974 (Firth, 1974). Since then, a good understanding at molecular level of Australian IBDV strains has been achieved through gene sequencing, phylogenetic analysis and strain differentiation (Ignjatovic & Sapats, 2002; Ignjatovic *et al.*, 2004; Sapats & Ignjatovic, 2000; Sapats & Ignjatovic, 2002). With the availability of IBDV sequence data (Sapats & Ignjatovic, 2002) and real time reverse transcriptase polymerase chain reaction (qRT-PCR) (Mackay *et al.*, 2002), it is possible to detect and quantify IBDV in various sample types such as host tissues, faecal samples and environmental litter and dust samples. Because this molecular based method

is more accurate and rapid, the disease can be diagnosed within one day (Belak, 2007; Perdue, 2003). This makes it possible to replace the time consuming and labour-intensive classical cell culture and egg inoculation methods to detect viruses with qRT-PCR (Spackman, 2012). Thus, in this thesis study, the application of the qRT-PCR technique to improved understanding and control of IBD in chickens in number of key areas was investigated.

Limited *in vivo* pathotyping studies have been done with Australian endemic classical and variant IBDV strains. One major study investigated clinical signs, bursal atrophy, histopathological changes due to IBDV infection with several Australian endemic strains at 21 d.o. (Ignjatovic *et al.*, 2004) but this did not extend to assessment of IBDV viral RNA (vRNA) load in the bursa and other lymphoid organs over time following the infection, faecal shedding profiles and host humoral immune response related to those. In this thesis, further *in vivo* characterization was performed to define differences between an Australian classical and a variant strain based on bursal atrophy assessed by relative bursal weight, IBDV vRNA load in bursal and non-bursal lymphoid organs following infection, faecal shedding profiles and antibody response to infection. The infection study was conducted in maternal antibody (MAb) free specific pathogen free (SPF) chickens at hatch and the results of this experiment are presented in thesis chapter 3.

Control of IBD is primarily by vaccination. Layer breeders, especially hens, are vaccinated to transfer specific MAb to the next generation to prevent neonatal subclinical infection (Hitchner, 1976). Booster vaccination after MAb decline, to prevent infection later in life, is widely practiced in the poultry industry worldwide to prevent early immunosuppressive infection and later clinical disease between 3 to 6 weeks of age (Lucio & Hitchner, 1979). Therefore, in the field situation, all commercial day old chickens carry MAb against IBDV. For a better understanding of the independent effects of age and presence of MAb on IBDV infection, two separate experiments were conducted. One experiment was carried out in MAb-free SPF chickens with infection at days 0 and 14 of age and the other in MAb-positive commercial chickens with infection at days 0 and 16 of age. The results of these two experiments are presented in thesis chapters 3 and 4 respectively.

With increasing demand for animal protein, the poultry industry has developed rapidly. Today, chicken operations may be very large with tens or hundreds of thousands of chickens on a single site in multiple sheds. Although IBD is controlled through vaccination, disease outbreaks are still reported around the world. When the prevention of IBD fails, there is little early warning of failure until clinical disease occurs. This can be devastating on farms with up to one million chickens present. Effective surveillance strategies have become a key factor in disease control and prevention and this may be applied to IBD as well. Although viral disease surveillance is important for the sustainable poultry production, at the moment, it is not widely practiced mainly due to two practical difficulties. One is that historically isolation and measurement of viruses has been extremely difficult and expensive as viruses do not grow on non-living media and must be grown and titrated in cell culture or chick embryos (Tang *et al.*, 1997). The advent of PCR and qPCR had reduced the time and cost associated with such measurements, but they remain expensive for individual animal tests. The second difficulty relates to sampling procedures to obtain measurements that are representative of very large populations. Current virological methods rely largely on samples collected from individual chickens. This poses a serious challenge in terms of sampling frames for populations of up to 1 million chickens on a farm, and the typical diagnostic samples (e.g. blood, tissues) usually require invasive collection methods and cold chain transportation to the laboratory to avoid spoilage. In response to these issues, an attempt was made to detect and quantify IBDV vRNA in flock-representative environmental litter and dust samples to assess the state of IBDV infection in the flock and feasibility of using poultry litter and dust samples as flock-representative tools for monitoring flock IBDV states. The results of this work are presented in thesis chapter 3 and 4.

IBDV is shed into the environment in faeces (Zhao *et al.*, 2013) and is thought to transmit to other chickens by the oral route (Muller *et al.*, 1979a; Sharma *et al.*, 2000). Faecally shed virus survives in the environment for as long as 122 days in litter (Benton *et al.*, 1967a). Particles of dried faeces are easily aerosolised and become a part of the dust present in poultry houses (Zhao *et al.*, 2014). This dust could be a possible source of infection via respiratory inhalation. To determine this, chickens were infected by intra-tracheal insufflation with known IBDV-contaminated dust samples. The results of this experiment are presented in thesis chapter 3.

The expansion of poultry production requires ever increasing amounts of bedding materials resulting in high prices and low availability for typical bedding materials such as wood shavings and saw dust (Islam *et al.*, 2013). Disposal of large quantities of used litter is also a significant industry problem (Bolan *et al.*, 2010). Re-use of litter/bedding materials for multiple batches of meat chickens could be a solution to these issues. The re-use of litter, with potential carryover of viral pathogens (Islam & Walkden-Brown, 2010) poses a significant risk associated with this strategy. Currently, in Australia the main method for limiting these carryover pathogens in used litter is the heaping of litter between batches, resulting in generation of heat due to bacterial action. This process is known as litter pasteurisation and has been shown, using a chick bioassay, to significantly reduce the load of several key viral pathogens in broiler litter (Islam *et al.*, 2009; Islam *et al.*, 2013; Walkden-Brown *et al.*, 2013a). However, the temperatures achieved within heaped litter vary widely depending on depth within the heap, time after heaping and other factors, typically ranging from 30˚C to 70˚C (Laurenson *et al.*, 2016; Walkden-Brown *et al.*, 2016b). To get a better understanding of temperature-time relationships for inactivation of IBDV in pasteurised litter, three separate experiments were conducted using two different litter sources given a range of heat treatments for different periods. The results of these three experiments are presented in thesis chapter 5.

Chick bioassays to determine the IBDV infective load of pasteurised litter samples measure the proportion of birds that seroconvert within 35 days of exposure to the litter, and are time consuming and a very expensive way to test the level of pathogens in litter. Therefore, an attempt was made to determine whether qRT-PCR enumeration of IBDV vRNA in pasteurised litter was a useful predictor of infective load. This was assessed by testing the association between IBDV vRNA load in heat-treated litter and the IBDV infectivity measured by chick bioassay. The results are presented in thesis chapter 5.

With the above background in mind, this thesis study was designed with the following broad objectives.

1. To compare the level of early immunosuppression and clinical IBD due induced by an Australian classical and an Australian variant IBDV strain *in vivo* by assessing:
    a. The severity of bursal atrophy

   b. the distribution, level and persistence of IBDV vRNA in host lymphoid tissues over the first 28 days post infection;
   c. the shedding profile of IBDV in faeces over the first 28 days post infection; and
   d. the antibody response to infection.
2. To test for a true age effect on susceptibility to IBDV infection with regards to:
   a. timing of disease onset;
   b. the severity of bursal atrophy induced;
   c. viral shedding profile in faeces over the first 28 days post infection;
   d. the persistence of IBDV vRNA in host lymphoid tissues over the first 28 days post infection; and
   e. the antibody response to infection.
3. To determine the combined effects of MAb and chicken age on IBDV infection at hatch and day 16 in commercial chickens by assessing:
   a. timing of disease onset;
   b. the severity of bursal atrophy induced;
   c. virus shedding profile in faeces over the first 28 days post infection;
   d. the persistence of IBDV vRNA in host lymphoid tissues over the first 28 days post infection; and
   e. the antibody response to infection.
4. To determine whether IBDV vRNA can be detected in flock-representative environmental samples such as litter and dust, and the levels of virus measured.
5. To determine if intra-tracheal insufflation with IBDV-contaminated poultry dust will transmit the infection.
6. To determine the rate of IBDV inactivation in litter at various temperatures representative of those found during litter pasteurisation by partial composting.
7. To determine whether qRT-PCR measures of IBDV vRNA can be used to predict the infectivity of IBDV-contaminated pasteurised litter.

Chapter1: Literature review

The review of literature in this thesis is focused on the application of polymerase chain reaction (PCR) methods in the control of poultry viral diseases (Part 1) and on the epidemiology, aetiology, pathogenesis, pathology, prevention and control of infectious bursal disease (IBD) (Part 2).

Part 1: Application of PCR methods for the control of poultry viral diseases

Poultry viruses cause significant losses to world poultry production. Rapid detection of viral pathogens is an important component of controlling outbreaks of viral disease. Detection or identification of viral pathogens is based on direct and indirect methods. Regularly used classical direct identification methods include virus isolation, antigen enzyme linked immunosorbent assay (Ag-ELISA), electron microscopy, agar gel precipitation and immunostaining. Indirect diagnosis methods include virus neutralization, antibody ELISA, haemagglutination and haemagglutination inhibition (Belak & Thoren, 2001). Many of these direct and indirect methods are laborious and time consuming and some do not provide a high level of specificity in pathogen identification (Belak & Ballagi-Pordany, 1993a). PCR diagnostic methods overcome many of these limitations, enabling quick and accurate detection of viruses (Belak, 2007).

The invention of PCR by Kary Mullis and his co-workers in 1983 created a new era for the field of disease diagnosis. It allowed *in vitro* amplification of selected region of a target gene of deoxyribonucleic acid (DNA) that produced large number of copies which could ultimately be visualised in an endpoint assay (Mullis, 1990; Mullis *et al.*, 1987). The ribonucleic acid (RNA) viruses are amplified through complementary DNA (cDNA) produced by using reverse transcriptase enzyme and this form of PCR is called reverse transcription PCR (RT-PCR) (Rappolee *et al.*, 1988). The PCR technique made a significant impact on the field of virology (Mackay, 2004) as it been a very useful economical veterinary viral diagnostic tool since 1988, combining high sensitivity and accuracy with a very short assay time required, enabling quick diagnosis (Belak & Ballagi-Pordany, 1993a; Belak & Thoren, 2001). Currently, conventional end-point PCR

is often replaced by quantitative PCR or real time PCR (qPCR) as it has several advantages over the conventional gel-based PCR (Belak & Thoren, 2001). Conventional and qPCR assays are used to detect nearly all poultry viral disease pathogens (Spackman, 2012) and qPCR has become the most popular detection method for viruses (Mackay, 2004; Spackman, 2012).

1.1 PCR technique

The PCR technology based on *in vitro* amplification of a selected nucleotide sequence of the target organism genome as described above. This technology has developed rapidly since 1983 and numbers of different PCR methods are available today. Frequently used PCR technologies in diagnostic and research laboratories include conventional/end-point PCR (Mullis *et al.*, 1987), qPCR (Spackman, 2012), nested PCR (Kho *et al.*, 2000), multiplex PCR (Chamberlain *et al.*, 1988) and digital PCR (dPCR) (Vogelstein & Kinzler, 1999).These methods are briefly described below.

Conventional/end-point PCR: In this method, *in vitro* amplification of the target sequence is achieved in a single tube providing all necessary reaction components such as DNA template, primers, deoxyribonucleotides, buffer solution, heat-stable DNA polymerase exposed to variable temperatures for specified times to enable denaturation (94-98°C), annealing (50-65°C) and extension (72°C) steps in each amplification cycle. Theoretically the number of amplicons doubles with every cycle until reagents become limiting, so that at the end of a number of thermal cycles (typically 40-45), the PCR reaction tube may contain very large number of amplicon copies. This product then subjected to gel-electrophoresis and staining and the end product visualized as a bands on a gel (Mullis, 1990). For the RNA molecule, amplification is achieved through cDNA as mentioned above (Rappolee *et al.*, 1988). Significant pre- and post-PCR handling is a disadvantage of the conventional PCR method.

qPCR and qRT-PCR: The same basic method is used in qPCR as in conventional PCR but a fluorescent intercalating dye such as SYBR Green or fluorescent-labelled target-specific probe are added to the reaction mix to provide fluorescence-based quantification of the amplicon continuously as it increases through the PCR cycles (Arya *et al.*, 2005). The shape of the amplification curve for each sample enables a critical threshold (Ct) value to be determined, this being the cycle at which this threshold is exceeded. There is a direct association between the C_t and the initial amount of target sequence present in

the sample and this enables quantification of the amount of target in a sample by a range of relative and absolute quantification methods. Absolute quantification involves the inclusion of a set of standards containing known amounts of the target sequence against which the concentration in samples can be estimated. Apart from providing quantification of sample, the qPCR reactions typically occurs in a single tube with real-time measurement of amplification and no post-PCR handling steps. The qRT-PCR is used for RNA viruses, RNA template is converted into a cDNA through reverse transcriptase enzyme activity (Rappolee *et al.*, 1988) and thereafter follows the same principles as in qPCR for amplification and dection of target gene.

Nested PCR: Nested PCR is a modification of conventional PCR method that includes two primer pair sets instead of one. The first primer set produces a comparatively longer end product from the target gene sequence. A shorter sequence within this product then will go through a second amplification using the second primer set as has been used in the detection of paramyxo (Newcastle) viral infection in pigeons (Barbezange & Jestin, 2002). This application increases specificity of the test.

Multiplex PCR: This is another modification of PCR/qPCR in which more than one sequence is targeted in single reaction tube using different primers sets and allowing those to amplify simultaneously (Henegariu *et al.*, 1997).

dPCR: The dPCR is a newer quantification application of the PCR method and it overcomes some of the limitations associated with qPCR. The qPCR assay needs good quality standards for quantification and multiplexing may be difficult with qPCR due to competition for reagents between the different primer sets (Karlen *et al.*, 2007). The dPCR largely eliminates these problems and provides accurate quantification with increased reproducibility supported by reduction in the assay coefficient of variation by as much as 37-86% (Hindson *et al.*, 2013). The dPCR use same reagents as qPCR for the assay but it can directly measure the number of individual target molecules without reference to a standard curve. This is done by separating the total reaction volume (sample) into large number of small reaction volumes (droplets) in a microwell plate (Hindson *et al.*, 2013) or microfluidic chambers (Fan & Quake, 2007). Each portion is so small that it contains no molecule or a single target molecule which allows amplification to end within the individual droplet. At the end of the reaction cycles, the samples which contain the target molecule have bright fluorescence while those that do not have

amplification shows only background fluorescence which is not bright (Hindson *et al.*, 2013). Multiplexing is easier using dPCR as in each compartment, only a single target molecule present, so there is no competition or cross reaction during amplification (Zhong *et al.*, 2011). Absolute quantification is quick and easy as a positive reaction indicates 1 copy of the target in the original template and a negative indicates 0 copies.

1.2 Advantages of PCR-based methods

High sensitivity and specificity: The PCR based laboratory tests can show very high sensitivity with a theoretical limit of detection of one copy of target sequence in the template. Typically however qPCR assays have a limit of detection between 10 and 10^3 copies of a target viral genome. Islam *et al.* (2006) reported lower limits of detection of qPCR assays for serotype 1 Marek's disease virus (MDV1) and herpes virus of turkey (HVT) of 5 and 75 genome copies per reaction. Another reported qPCR assay is able to detect 10 copies of serotype 2 MDV (MDV2) (Renz *et al.*, 2006). The specificity of PCR assays is determined by the design of the primers, and the uniqueness of the target sequence they amplify. The ever increasing amount of viral genomic sequence information available on "GenBank" enables highly effective blast searches and the design of completely specific targets (Belak *et al.*, 2009; Cha & Thilly, 1993).

The analysis of data from the low pathogenic AI H7N2 outbreak in Virginia in 2002 showed diagnostic sensitivity of the qRT-PCR of 85.1% (95% probability interval: 71.9 – 95.7%) whereas the diagnostic specificity was 98.9% (95 % probability interval: 98.0 – 99.5%) (Elvinger *et al.*, 2007).

Using classical methods, the cross-virus neutralization (VN) assay is used to differentiate the two IBDV serotypes and IBDV subtypes within serotype 1 (Jackwood & Saif, 1987; McFerran *et al.*, 1980). However, RT-PCR- restriction endonuclease (RE) and RT-PCR-restriction fragment length polymorphism (RFLP) are able to rapidly detect serotypes and subtypes with in serotype 1 and have become popular techniques for typing and identification of IBDV (Jackwood & Nielsen, 1997; Jackwood & Sommer, 1999). It has been shown that RT-PCR-RE is a simple and sensitive method to detect genetic variations in serologically related IBDV isolates which cannot be identified by classical serological methods (Liu *et al.*, 1994). A number of qRT- PCR based assays are available today to detect, quantify and subtype IBDV strains. The assays are better than classical methods

being more sensitive and specific (Abdul *et al.*, 2013; Ching-Wu *et al.*, 2007). The RT-PCR assays show greater sensitivity and specificity than traditional agar gel immunodiffusion and immunoelectrophoresis methods in the detection of IBDV (Makadiya *et al.*, 2006).

Speedy diagnosis: At present, the PCR methods provide speedy diagnosis of poultry virus infection within hours (hrs.) (Perdue, 2003). PCR robotic machines are available today for DNA/RNA extraction and pipetting. These enable fast detection of viruses (Belak, 2007). In contrast, classical methods for detection of viruses are mainly labour-intensive and time consuming (Spackman, 2012). It is well-known that faster detection of viruses results in faster control of outbreaks.

Detection of IBDV in a tissue specimen takes long time using the classical virus isolation method by embryonated egg inoculation. Usually, it takes about two weeks to detect the virus (Eterradossi & Saif, 2008). In contrast, the reverse transcription qPCR approach (qRT-PCR) of PCR technique able to detect IBDV in infected tissue specimens within one day (Ching-Wu *et al.*, 2007; Jackwood, 2004).

Repeatability and reproducibility: Assessment of repeatability and reproducibility of diagnostic assays is vital during development and validation of assays. Repeatability is usually assessed by running samples in duplicate or triplicate within assays and by running a small number of the same samples or quality controls in each assay to provide an estimate of between assay variations. PCR assays have good repeatability as shown by agreement between duplicates and triplicates (Belak, 2007).

The repeatability and reproducibility of one-step qRT-PCR assay to detect and quantification of AI virus was assessed. Three different concentrations of Ty/214845 AI virus: 100, 1 and 0.01 median tissue culture infective dose ($TCID_{50}$) were used for absolute quantification with known standards to detect the repeatability and reproducibility of the assay. Samples of each dilution were run in triplicate in ten separate assays to calculate repeatability and reproducibility. The reproducibility of the assay was assessed by intra- and inter-assay coefficient of variation (CV) of three dilutions; 100, 1 and 0.01 $TCID_{50}$ of Ty/214845 AI virus. The mean intra-assay CVs were 25, 27 and 30 % while inter-assay CV was 27, 30 and 41% respectively for each dilution (Trani *et al.*, 2006). Mean intra- and inter-assay CV for C_t values and calculated viral copy number

(VCN) for both MDV1 and HVT qPCR assays respectively less than 5% and 20% for C_t and calculated VCN (Islam *et al.*, 2006). For a MDV2 qPCR assay, mean inter- and intra-assay CV for C_t and calculated VCN respectively were less than 3% and less than 21.5% (Renz *et al.*, 2006).

Detection in frozen or stored samples for long time: For the PCR, detection is not based upon the whole genome and small fragments of it may form the template. Primers amplify the target gene sequence regardless of whether it is from live, inactivated, dried, fresh or frozen material (Maurer, 2011; Richards, 1999). However, classical virus isolation methods (cell culture, tissue culture and embryonic egg inoculation) only isolate virus from samples which have infective intact viruses present (Cangelosi & Meschke, 2014). The greater versatility of the PCR method enables detection in a wider range of samples than conventional methods but a disadvantage is that it does not clearly distinguish between infective and inactive virus.

Ability to detect viruses in environmental samples: The versatility of the PCR technique allows detection of poultry viruses in environment samples which are difficult to use for classical virus detection methods as they often need to pass through several pre-treatment steps to remove or reduce contamination before being subject to virus isolation. The ease of detection in environmental samples facilitates efficient and cost-effective flock sampling for detection of viruses. For example, detection of exotic Newcastle disease virus during disease outbreaks (Hietala *et al.*, 2005) and H6N2 influenza virus in commercial quails (Charlton *et al.*, 2009) in air samples collected using wetted-wall cyclone-style air samplers by qRT-PCR. Marek's disease virus is readily detected and quantified in dust samples using qPCR and dust sample testing is used for Marek's Disease surveillance and vaccination decision making in Australia since 2004 (Walkden-Brown *et al.*, 2013b). IBDV has also been detected in indoor air samples using qRT- PCR technique. Samples were collected from three types of bioaerosol samplers at 20 cm above floor level and exhausted air level. IBDV was detected from 5 dpi to 12 dpi at floor level and at 8 to 12 dpi at exhaust air level (Zhao *et al.*, 2013). Findings of those studies support the possibility of qPCR analysis of environmental samples becoming a reliable disease diagnostic or monitoring tool.

Quantification of target: The qPCR approach allows both detection and quantification of target viral genomes even with the very few templates (Mackay, 2004). This

quantification can be achieved in two ways: relative quantification and absolute quantification (Mackay *et al.*, 2002). Relative quantification is simpler and it analyses the amount of target sequence in a given sample relative to another reference sample such as an untreated control. However, in absolute quantification, unknown samples are quantified based on a known standard curve (Freeman *et al.*, 1999). With the droplet digital PCR method, the absolute number of copies of a target of interest can be determined without reference to a standard curve, reducing the number of sample reactions required in an assay (Hindson *et al.*, 2013). Fluorescing droplets or cells indicate amplification a single copy of the target and absence of fluorescence indicates no target in the droplet or cell. The total number of positives gives the total number of target molecules present (Hindson *et al.*, 2013).

Cost effectiveness: In the earlier stages of PCR development and use, the cost associated with PCR diagnosis of an infectious agent was very high compared to conventional methods. It was calculated that the cost per reaction based on cost of PCR reagents, equipment, space, training and labour together was US$ 125 per reaction (Louie *et al.*, 2000). However, increasing demand for the technology and increasing automation have reduced the costs substantially making the costs competitive with conventional methods (Belak, 2007; Kriger *et al.*, 2006; Yang & Rothman, 2004). The cost associated with detection of *Neisseria gonorrhoeae* and *Chlamydia trachomatis* by PCR was estimated at only $9 per reaction (Livengood & Wrenn, 2001). Assaying samples without duplication or triplication significantly reduces cost associated with qPCR without reducing sensitivity as shown with detection of chytridiomycosis. A triplicate chytridiomycosis assay cost $13/sample, but in singlicate assay, the cost reduction per sample was $7.66 (Kriger *et al.*, 2006). Multiplex PCR provides additional economic benefits by allowing detection of several targets simultaneously in one reaction tube (Belak & Hakhverdyan, 2006).

Some pathogens need special media to grow and, in some cases, the growth medium does not have a long shelf life which increases the cost if the diagnostic test is required infrequently. Some samples must be sent to another diagnostic laboratory if special growth conditions are required. The PCR technique has advantages in this as primers and probes can be stored for long time and commercial kits are available for nucleic acid extraction. This eliminates need for maintenance of special culture media and problems

associated with transporting clinical samples while providing cost-effective diagnosis (Tang *et al.*, 1997).

The qPCR allows pathogen enumeration in non-traditional environment samples like poultry house dust (Walkden-Brown *et al.*, 2013b). The one environmental sample may represent an entire flock states and that significantly reduces diagnostic cost associated with flock monitoring (Walkden-Brown *et al.*, 2013b).

1.3 Limitations of PCR-based methods and solutions to them

False positive results: False positive results were a big concern during the early stages of the development of PCR technology due to its extreme sensitivity (Burkardt, 2000). Contamination with as little as one target template could lead to false positive reaction in a PCR assay. It was reported that the results of the first diagnostic PCR were ruined by contamination (Persing, 1991). Contamination could be mainly due to product carryover from positive samples and cross-contamination with PCR products from earlier assays (Belak & Thoren, 2001). This could be through PCR reagents, tubes, pipettes or laboratory surfaces (Yang & Rothman, 2004). Therefore, many precautions have been developed to prevent the contamination during PCR. These include working under laminar flow, use of special tube holders and openers and use of aerosol resistance pipette tips and physical separation of pre- and post- amplification areas (Belak & Ballagi-Pordany, 1993b; Yang & Rothman, 2004). Further reduction of contamination is achieved through enzymatic inactivation of carryover DNA using uracil N-glycosylate (Burkhardt & Muller, 1987).

False positives are less of a problem with qPCR. The qPCR method allows single tube, closed assays which reduces carryover contamination between samples significantly (Schmitt & Henderson, 2005). At the end of the qPCR, reaction tubes are discarded without opening, also reducing carryover contamination (Perdue, 2003).

False negative results: There are several reasons which could cause false negative result in a PCR assay, including human error, technical issues, presence of pathogens below the threshold of detection, mutation and PCR inhibition (Burkardt, 2000; Maurer, 2011). False negative results due to errors with the assay can be identified by including one or more known positive quality controls in each run (Burkardt, 2000). False negatives due to low concentrations of pathogen in the template can be minimized by applying high

quality sample preparation methods. In some cases, pathogens are unable to be detected due to mutations which have been occurred in the nucleotides of target gene. However, if a mutation is suspected, it is possible to check the target gene sequence by gene sequencing and comparing the sequence of the primers with target gene sequence (Burkardt, 2000). PCR inhibitors are the predominant cause of false negative results. Inhibitors suppress both sensitivity and amplification efficiency of assays (Schrader *et al.*, 2012). Today, most of the inhibition which occurs in PCR assays has been overcome by applying proper sample processing and nucleic acid extraction methods, using a suitable DNA polymerase and specific PCR additives (Al-Soud & Radstrom, 2001).

Inability to differentiate inactivated from live infectious agents: The greatest weakness of the PCR technology is its inability to differentiate dead from live infectious agents (Maurer, 2011; Richards, 1999). This is because the PCR test amplifies a selected region of the target genome and not the whole intact genome and because it is measuring presence of nucleic acid and not an infectious organism (Mullis *et al.*, 1987). Therefore, today two approaches have been introduced to overcome the problem associated with distinguish between viable and inactive organisms in PCR assays such as viable PCR (vPCR) and molecular viability testing (MVT) for non-viral cellular pathogens particularly for bacteria (Cangelosi & Meschke, 2014). In vPCR method, cell membrane permeability is used to distinguish live from inactive organisms. In this method, samples are incubated with membrane-impermeative reagent like Propidium monozide (PMA). PMA binds with exposed DNA after photoactivation while damaged membranes or free nucleic acids are not protected and inhibit the amplification (Nocker & Camper, 2009). The vPCR method can apply to detection of bacterial pathogens in environmental samples as well (Fittipaldi *et al.*, 2011).

1.4 Current application of PCR technology for diagnosis of poultry viral diseases

Diagnosis of viruses: The qPCR technology offers rapid viral diagnosis in laboratories usually exceeding the sensitivity and specificity of classical methods (Belak, 2007; Spackman, 2012). Today qPCR technology is used in all most all laboratories to detect poultry viruses and many qPCR assays have been developed for diagnostic purposes at research institutions, reference laboratories and OIE collaborating centres (Belak, 2007).

Recent good example of use of qPCR to control specific one of poultry virus is AI. Remarkable losses to the poultry industry and public health concerns due to AI infection highlighted the importance of a rapid, sensitive and specific diagnostic tool for effective control of AI outbreaks. Novel AI qRT-PCR assays allow detection of the virus within 24 hrs. (Pasick, 2008), which enabled quick action to prevent further spread of the virus (Wright *et al.*, 1995). In 2009, during the swine-origin influenza A H1N1 outbreak, the existing ELISA method was unable to detect the newly emerged influenza virus but qRT-PCR was able to do so and was rapidly used worldwide (Wang & Taubenberger, 2010).

With regard to diagnosis of IBDV, replication of IBDV in cell cultures is time consuming and expensive. Specific pathogen free eggs are required to isolate virus in chick embryos and it takes more than two weeks to obtain a result but PCR assays allow quick and reliable diagnosis. Thus, today most of diagnostic laboratories use qRT-PCR assays to diagnose IBDV and identify IBDV strains (Jackwood, 2004).

Disease monitoring and surveillance: AI is a major re-emerging transboundary animal disease which has received significant attention due to its public health significance and economic losses. Prior to PCR technology, there was a lack of proper surveillance system for early detection of AIV. The PCR technology provided a solution to this problem and today mainly qRT-PCR technique is used in AI surveillance world-wide to combat with the disease threat (Belak, 2007).

During the period of 1999 to 2000, more than 8500 wild bird samples were screened in Northern Europe by conventional RT- PCR (Fouchier *et al.*, 2003). The qRT-PCR technique was successfully used in 2008 with the newly emerged swine-origin influenza A H1N1 outbreaks in USA (Poon *et al.*, 2009). It has been proved in other studies that qRT-PCR could be applied for successful AI surveillance in live bird market surveillance (Spackman *et al.*, 2003), low and highly pathogenic AI eradication programmes (Elvinger *et al.*, 2007) and AI surveys in wild birds (Parmley *et al.*, 2008).

Molecular epidemiology: The PCR technique can be applied to epidemiological problems which cannot be approached by classical methods or are too labour-intensive, expensive and or time consuming (Foxman & Riley, 2001).

Amplicons, the products of PCR assays, provide substrate / material for further sequence studies. This sequence analysis allows the genetic relatedness of the target virus to be

determined. This process is called molecular epidemiology and phylogenetic trees are built to identify the genetic diversity of a virus while tracing its spreads within a country or the world (Belak, 2007).

Retrospective molecular epidemiological analysis is extremely important in the production of commercial vaccines (Balint *et al.*, 2005) as it helps to study the genetic relatedness of vaccine and field strains as shown for IBDV (Jackwood & Sommer, 2005). Vaccination failure may be due to a variety of reasons including antigenically different vaccines and field virus strains. It was reported that during 2007-2009, the H5 subtype of highly pathogenic AI virus circulating in China was not related to the widely used AI vaccines in China (Jiang *et al.*, 2010). IBD outbreaks have occurred despite vaccination with available IBDV viruses in China and a molecular epidemiology study of IBDV in southern China during 2000-2010 showed that all isolated IBDV strains were markedly different from the currently used vaccines against IBDV and those reported IBD outbreaks were due to infection with vvIBDV strains (He *et al.*, 2012).

1.5 Opportunities for wider application of PCR in poultry virology

Increased use of dPCR with multiplexing: The recent development of dPCR with high sensitivity and accuracy exceeds that of qPCR and enabling absolute quantification without external standards as described earlier offer significant improvements over current qPCR methods. A further advantage of the dPCR is multiplex application (Zhong *et al.*, 2011) as the droplet formulation enables quantification of individual different targets without any competition due to the presence of only one target molecule per droplet. These advantages are likely to lead to wider use of dPCR technology to detect and quantify the poultry viral disease in near future as improvements in dPCR technology made it more (Morley, 2014). The extreme sensitivity of dPCR enables it to detect even rare mutations (Taly *et al.*, 2013). This application would help to diagnose newly emerging poultry viral strains and its diversity from existing viral strains in near the future.

Pathogen identification and enumeration in non-traditional samples: PCR technology allows efficient analysis of wide varieties of samples including non-traditional samples which cannot be used, or are difficult to use with, classical method

(Spackman, 2012). A number of scientists have been made significant effort to detect poultry viral pathogens in non-traditional samples such as poultry house dust, litter and air which are non-sterile environmental samples contain mixture of organic and inorganic materials.

Usually, samples are collected at the individual bird level for the disease diagnosis and surveillance at the moment. Sometimes samples are pooled together to assess the flock disease states (Charlton *et al.*, 2009). However, environmental sampling has many advantages during surveillance. It reduces human resources required for sampling, there is no need to have special skill to collect samples, no need to have cold chain during transportation of samples, it greatly reduces sampling time as single sample may represent an entire flock, it significantly reduces the cost associated with assay due to the reduced number of samples required to assess the flock disease states, and vaccine virus and field virus can be measured in the same sample enabling vaccination decisions based on such monitoring (Hietala *et al.*, 2005; Walkden-Brown *et al.*, 2013b).

Feasibility studies were conducted between 2002 and 2011 to assess the suitability of the qPCR method for routine disease monitoring of Marek's disease virus in poultry house dust samples (Walkden-Brown *et al.*, 2013b). It was proved that it is possible with above mentioned advantages over the existing classical methods and it was applied successfully at the field level in Australia to monitor the vaccine and disease states of MDV (Walkden-Brown *et al.*, 2013b). The monitoring system now operates totally in the commercial sector, with the tests conducted by a commercial diagnostic laboratory.

Newcastle disease virus was first detected in contaminated air samples in 1948 by embryonated egg inoculation method (Delay *et al.*, 1948). In 2002 and 2003 Hietala *et al.* (2005) showed that diagnosis of Newcastle disease virus was possible following ''air sampling'' from infected pen using qRT-PCR. Environmental air samples assayed with qRT-PCR provides the effective surveillance method for AI virus monitoring (Charlton *et al.*, 2009).

These studies show that disease surveillance together with environmental samples and qRT-PCR or qPCR can result in an efficient, practical surveillance method, made possible only because of the numerous advantages over traditional diagnostic methods. This application may enable cost-efficient monitoring of both endemic and transboundary diseases. In this study, the feasibility of enumeration of IBDV in environmental samples

is investigated as proof of concept for a potential new surveillance tool to IBDV disease and vaccination monitoring.

Part 2: Infectious bursal disease

IBD is a disease of young chickens with a significant negative economic impact to the poultry industry worldwide due to direct losses associated with mortality and immunosuppression (Eterradossi & Saif, 2008). Therefore, minimising losses due to IBD is a major concern in poultry-producing countries worldwide, because two or more different pathogenic strains of IBDV are endemic to most leading poultry producing countries (Jackwood & Sommer, 2007) and there is evidence of increasing virulence of field IBDV strains over time (Smith *et al.*, 2015).

1.1 Aetiology

IBD is caused by IBDV, a dsRNA virus in the family *Birnaviridae* (Dobos, 1979; Nick *et al.*, 1976; Dobos *et at.*, 1995) as detailed below.

Classification

The *Birnaviridae* family consists of three genera, *Aquabirnavirus, Avibirnavirus* and *Entomobirnavirus* (Dobos & Roberts, 1983). IBDV comes under the genus *Avibirnavirus* (Dobos *et al.*, 1979: Dobos et at., 1995). IBDV is classified as serotype 1 (pathogenic) and serotype 2 (non-pathogenic) based on a viral neutralization tests (VN) (Jackwood *et al.*, 1982; McFerran *et al.*, 1980; McNulty *et al.*, 1979). There is close homology between smaller major structural proteins of the two serotypes. However, two strains differ from each other in neutralizing antibodies induced by one of the larger structural proteins (VP3) which differs between the two serotypes. Epitopes in this protein which are not involved in induction of neutralizing monoclonal antibodies are similar between two serotypes (Becht *et al.*, 1988). Two serotypes cannot be separated using the fluorescent antibody test or ELISA (Eterradossi & Saif, 2008).

Serotype 1 IBDV field strains are further classified into three pathotypes, these being classical or standard, variant (mostly prevalent in Australia and the USA) and very virulent (vv) IBDV strains based on the clinical signs and histopathological lesions included (Hon *et al.*, 2006; Van Den Berg, 2000). Pathogenicity of classical strains varies from classical "Gumboro disease" with up to 25% mortality in SPF chickens, to

subclinical immunosuppressive infection with bursal atrophy and increased susceptibility to secondary infections (Ignjatovic *et al.*, 2004). The vvIBDV strains show the same characteristic IBDV clinical signs and lesions as classical strains but with 50-100% mortality in SPF chickens (Eterradossi & Saif, 2008). Variant strains cause only subclinical disease similar to immunosuppressive classical strains with no mortality or clinical signs but with significant bursal atrophy (Eterradossi & Saif, 2008; Ignjatovic *et al.*, 2004). In general, variant strains cause a greater degree of bursal atrophy during the early stages of infection than classical strains (Hassan & Saif, 1996; Sharma *et al.*, 1989).

1.1.2 Viral structure, genome and virulence

Viral structure: IBDV is non-enveloped, has a single capsid shell and a diameter of about 60 nm with density of 1.32 g/ml (Dobos, 1995; Nick *et al.*, 1976). The hexagonal outline of the virus was observed by immunoelectron microscopy (Almeida & Morris, 1973). The capsid is made of 32 capsomeres and symmetry of the capsid is skewed with a number of T=13 lattice of trimeric subunits (Bottcher *et al.*, 1997; Ozel & Gelderblom, 1985). Four major polypeptides with molecular weights of 110,000, 50,000, 35,000 and 25,000 are associated with capsid structure (Nick *et al.*, 1976). Virions are icosahedral in shape due to their capsid structure (Hirai & Shimakura, 1974).

Viral genome structure: the genome of IBDV consists of double stranded RNA (dsRNA) with two segments, A and B (Dobos, 1979; Muller *et al.*, 1979b; Spies *et al.*, 1987) of molecular weight (MW) respectively 2.2×10^6 and 1.9×10^6 daltons (Muller & Nitschke, 1987b). The dsRNA has a sedimentation coefficient of 14S, buoyant density of 1.62g/ml and some resistance to RNase activity and high melting temperature of 95.5°C. The response to RNase activity of IBDV has been tested with labelled-RNA in 2× saline-sodium citrate buffer by heating and cooling showing potential resistance to RNase (Muller *et al.*, 1979b). These characteristics of the IBDV viral genome are particularly responsible for the hardiness of the virus. Segment A, the largest portion of the genome is 3.4 kb in length encodes viral proteins VP2, VP3 and VP4 in a large open reading frame (ORF) and VP5 in a other small ORF whereas the 2.9 kb-long segment B contains only one ORF encoding VP1 (Kibenge & Dhama, 1997; Kibenge *et al.*, 1997; Lejal *et al.*, 2000b; Muller & Becht, 1982; Mundt *et al.*, 1997; Tacken *et al.*, 2002; Tacken *et al.*, 2000).

Initially four viral proteins were identified as VP1, VP2, VP3 and VP4 (Dobos, 1979; Nick *et al.*, 1976). Another viral protein was identified later and called VP5 (Mundt *et al.*, 1995). Reported molecular weights of five viral proteins are 97, 41, 32, 28, and 21 kDa respectively (Eterradossi & Saif, 2008). It was found that the molecular weights of these proteins did not differ between serotypes 1 and serotype 2 (Becht *et al.*, 1988; Jackwood *et al.*, 1984). The major structural viral proteins of serotype 1 IBDV are VP2 and VP3 contributing 51% and 40% of the capsid respectively while VP1 and VP4 are minor proteins contributing 3% and 6% individually (Dobos *et al.*, 1979). Each viral protein has specific functions within the genome and are listed in Table 1.1.

Virulence determinants: The hypervariable region of the VP2 is responsible for the amino acids (aa) changes associated with differences in virulence with one or more aa changes in this region able to change the virulence of the strain (Rautenschlein & Alkie, 2016). It was determined that aa Gln253, Asp279 and Ala284 of VP2 are responsible for the virulence of virulent IBDV (Brandt *et al.*, 2001). However VP2 is not the sole determinant of virulence in vvIBDV with both segments A and B are responsible for determining the virulence of IBDV (Boot *et al.*, 2005; Escaffre *et al.*, 2013; Liu & Vakharia, 2004).

1.1.3 Resistance to physical and chemical conditions

IBDV is resistant to harsh environmental conditions to heat, UV irradiation and sunlight than other naked viruses such as reoviruses (Petek *et al.*, 1973). This is thought to reflect the viral genomic properties described in section 1.1.2. The virus can survive a wide range of pH values (2-12), is unaffected by many disinfectants including ether and chloroform and the very stable nature of the virus allows it to survive in poultry

Table 1. 1: *Functions of IBDV proteins*

Protein	Functions/ activities
VP1	Putative RNA-dependent RNA polymerase (Hudson *et al.*, 1986; Spies *et al.*, 1987) Facilitate viral RNA replication (Muller & Nitschke, 1987b) In a single-shell capsid and binds to VP3 and keep two genome segments A and B together (Muller & Nitschke, 1987a; Tacken *et al.*, 2002) Contributes to the virulence of vvIBDV with VP2 (Escaffre *et al.*, 2013; Le Nouen *et al.*, 2012)
VP2	One of the major structural protein and forms the outer layer of the capsid (Tacken *et al.*, 2002) Target of neutralizing antibodies (Azad *et al.*, 1987; Becht *et al.*, 1988; Fahey *et al.*, 1989) Induced programmed cell death of infected B lymphocyte precursors which leads to bursal destruction without an inflammatory reaction results in immunosuppression (Fernandez-Arias *et al.*, 1997; Vasconcelos & Lam, 1994) Responsible for pathogenicity/infectivity of IBDV strains (Escaffre *et al.*, 2013; Mundt, 1999; Van Loon *et al.*, 2002)
VP3	Another major structural protein and forms the inner layer of the capsid (Tacken *et al.*, 2002) Group specific antigen which induce neutralizing monoclonal antibody and differ between serotype 1 and 2 (Becht *et al.*, 1988) Important for virion morphogenesis an encapsidation (Tacken *et al.*, 2000) Protect viral genome by suppressing host RNA silencing mechanism (Valli *et al.*, 2012). Ensuring the viability of IBDV replication cycle by preventing programmed cell death through antiapoptotic properties (Busnadiego *et al.*, 2012) Inhibits antiviral innate immunity by blocking binding of viral genomic dsRNA to chicken MDA5 receptors (Ye *et al.*, 2014)
VP4	It is a protease involved in processing of VP2 (Lejal *et al.*, 2000a)
VP5	Inhibition on IBDV induced apoptosis by forming complex with receptor of activated protein kinase C1 and voltage-dependent anion channel 2(Lin *et al.*, 2015) Virus release of virus from infected cells (Lombardo *et al.*, 2000; Wu *et al.*, 2009)

houses for more than 4 months even after proper cleaning and disinfection (Benton *et al.*, 1967a). IBDV in carcasses and litter could be inactivated by composting of carcasses and litter for 14 days with temperatures during composting being slightly above 55°C (Guan *et al.*, 2010). Another study confirmed that composting above 55°C for four weeks inactivated vvIBDV in poultry litter (Crespo *et al.*, 2016). Another further study on the

thermostability of IBDV, but not in litter, showed that IBDV became non-infective (inactivate) above 42°C and not stable (complete degradation of viral structure) above 72°C but unaffected by pH in the range 2-12 (Rani & Kumar, 2015). Those studies revealed that although IBDV is a very hardy virus, it could be inactivated by composting although for comparatively long periods (14-28 days). Such long periods are generally not economically feasible when composting litter to pasteurise it between successive batches of broiler chickens. Understanding the effects of these or slightly higher temperatures in composting litter over more practical shorter periods (5-10 days) would be useful to help to break the IBDV life cycle in carcasses and litter providing greater flexibility in disease control strategies.

1.1.4 Transmission

IBDV is transmitted by the oral route under natural conditions (Sharma *et al.*, 2000) and this has been confirmed by experimental oral infection (Muller *et al.*, 1979a). Ingestion of contaminated water and feed with IBDV contain faeces is thought to be the most common method of horizontal transmission (Rautenschlein & Alkie, 2016). IBDV is highly contagious and usually spread by direct contact between infected and susceptible flocks. Infected chickens with IBDV shed virus through their faeces after day one of infection and are able to transmit the disease for at least 2 weeks after the infection. The extremely stable nature of virus also facilitates indirect transmission of virus via contaminated feed, litter, clothes and or poultry house dust (Benton *et al.*, 1967a). There is no evidence that IBDV is transmitted vertically through eggs or from birds that have recovered from the disease (Eterradossi & Saif, 2008; Fenner *et al.*, 2014; Rautenschlein & Alkie, 2016).

1.2 Pathogenesis

IBDV gains access to the target host cell via various cellular binding sites or receptors including N-glycosylated polypeptides, heat shock proteins, α4β1 integrin or lipid raft endocytic pathways (Rautenschlein & Alkie, 2016). After oral ingestion of the virus, mononuclear phagocytic cells and lymphoid cells of gut mucosa become the primary target cells for viral infection and replication. Virus can be detected in macrophages and lymphoid cells of the cecum four hrs. post infection (PI) and in lymphoid cells of the duodenum and jejunum after 5 hrs. PI. These tissues are the first viral replication sites prior to primary viremia. The virus can be detected in bursa, the main target organ, by

immunofluorescence 11 hrs. PI with the secondary higher viremia associated with clinical disease, lesions and death (Muller *et al.*, 1979a). The virus circulates to the other lymphoid organs from the bursa depending upon the virulence of the strain (Rautenschlein & Alkie, 2016).

1.2.1 Target organ

Serotype 1 IBDV strains target the bursa of chickens (Ivanyi & Morris, 1976). The highly virulent Cu-1 strain caused 100% mortality in experimentally infected four-week-old SPF chickens with high titres of infective virus in the bursa whereas low titres were found in the thymus, spleen and peripheral blood. No clinical disease or mortality was observed in bursectomised four week old SPF chickens when infected with the Cu-1 strain confirming the bursa as the key IBDV target organ (Burkhardt & Muller, 1987; Kaufer & Weiss, 1980).

The surface immunoglobulin M bearing B lymphocytes of bursa are the main target cells of IBDV as shown in an *in vitro* study (Hirai & Calnek, 1979). Another *in vitro* study showed that replication of IBDV occurs in proliferating B cells at an intermediate stage of cellular differentiation but not in either very immature lymphoblasts or competent B cells (Burkhardt & Muller, 1987). Apathogenic serotype 2 IBDV strains do not replicate in lymphoid cells of bursa or other lymphoid cells in chickens (Nieper & Muller, 1996). However, serotype 2 Cu-1 and 23/82 strains propagate well in primary chicken embryo fibroblast and the exact reason for this is not very known (Becht *et al.*, 1988; Nick *et al.*, 1976). However, this could be the reason for non-pathogenic nature of serotype 2 strains.

The severity of the disease is directly correlated with the number of susceptible cells in the bursa (Kaufer & Weiss, 1980). Therefore, the age of highest susceptibility to clinical disease is between three to six weeks of age as the bursa is at its maximum development stage as this age.

1.2.2 IBDV viral RNA load in various tissues and shedding in faeces

Persistence and tissue distribution: In an early virus isolation study, high levels of IBDV were isolated from bursal tissues as early as at 3 dpi followed by declining viral load in bursa over 52 dpi (Winterfield *et al.*, 1972). Another study using qRT-PCR showed similar results with high viral RNA (vRNA) load detected in bursal tissue initially followed by a gradual decline over 28 dpi (Abdul *et al.*, 2013). Lower IBDV and IBDV

vRNA load were detected in non-bursal tissue samples together with a shorter persistence of the virus than in the bursa (Abdul *et al.*, 2013; Winterfield *et al.*, 1972). Similarly in turkeys during subclinical infection with serotypes 1and 2 IBDV, initially higher IBDV vRNA load was detected in bursal tissues followed by reduction with time but IBDV vRNA was not detected in non-bursal tissues with the exception of spleen with serotype 1 infection (Abdul *et al.*, 2014). Serotype 1Australian strain 002/73 was isolated most frequently from bursa and less frequently from thymus, liver, kidney, lung and spleen of infected commercial broilers without maternal antibodies against IBDV without any isolation from pancreatic tissues (Mackenzie & Spradbrow, 1981). IBDV strain FK-78 was isolated from bursal homogenate of SPF chickens up to 10 dpi while virus was recovered from various other tissues including caecum, liver, spleen, thymus, kidney and lung up to 4 dpi (Takase *et al.*, 1982). However in another study IBDV vRNA could be detected up to 42 dpi in bursal tissues (Elankumaran *et al.*, 2002). All of the studies cited indicate that the bursa plays the major role during IBDV infection and is the target organ for the virus as is indicated by the name of the virus and disease it causes. The peak of viral load at 3 dpi is evidence of rapid replication in the target tissues

Immunohistochemical detection of highly pathogenic IBDV strains Ehime/91 and DV86 and moderately pathogenic JI strain showed that pathogenicity of the virus strains were positively associated with the virus antigen distribution in non-bursal lymphopoietic organs (Tanimura *et al.*, 1995). Likewise qRT-PCR analysis of bursal and non-bursal tissues has revealed pathotype differences in IBDV infection with variant strain IN recovered from host tissues earlier than the classical strain STC (Abdul *et al.*, 2013).

Thus the study of tissue distribution and persistence of the IBDV or IBDV vRNA is important to assess in infectivity of the virus, host immune response and its relation to recovery of ongoing infection and differences among pathotypes.

Viral shedding: In SPF chickens infected at one day old with the cell adapted FK-78 strain, IBDV could be isolated from faeces at 2 dpi with highest amount of virus isolated at 4 and 5 dpi followed by a decline to the point where no more virus could be isolated at 12 dpi using virus isolation in chicken embryo fibroblast cells (Takase *et al.*, 1982). In another study, IBDV vRNA was detected in faeces using qRT-PCR at 2 dpi in SPF Ross broiler chickens infected orally at 20 days of age with vaccine strain Gallivac IBD, L258577. Virus continued to be detected at 5 and 8 dpi but not at 12 dpi and was not

detected in exhaled air from infected chickens at any time (Zhao *et al.*, 2013). Determination of faecal shedding profile of IBDV is important as that is the primary route of virus excretion to the environment (Zhao *et al.*, 2013) and proper definition of the shedding profile is an important part of understanding and modelling the epidemiology of the disease.

1.3 Epidemiology and economic importance

1.3.1 Host range and geographical distribution

Host range: Chickens, turkeys and ducks are considered as natural hosts for the IBDV serotype 1 and 2 infection (Eterradossi & Saif, 2008) but chickens are the only host that exhibits clinical disease due to serotype 1 pathogenic IBDV infection (Abdel-Alim & Saif, 2001). However, antibodies against IBDV have been demonstrated in a wide range of wild and migratory birds including quail (Weisman & Hitchner, 1978), pigeons (Kasanga *et al.*, 2008), pied cordon bleus , village weavers (Nawathe *et al.*, 1978) and black ducks (Wilcox *et al.*, 1983) and Antarctic king penguins (Gauthier-Clerc *et al.*, 2002)

Serotype1 IBDV strains are known to infect both commercial and back yard chickens. Both seroconversion against serotype 1 IBDV and detection of virus in bursal tissues have been reported in ducks and turkeys. Clinical signs are not associated with serotype 1 IBDV infection of these species but histopathological lesions have been observed in bursal tissues of experimentally infected 3-6 weeks old turkeys. (Giambrone *et al.*, 1978; Jackwood *et al.*, 1982; McFerran *et al.*, 1980). However, infection of turkeys with vvIBDV does lead to subclinical disease (Razmyar & Peighambari, 2009). Clinical IBD was produced in four-week-old ostriches infected with virulent serotype 1 IBDV strain Faragher 52/70 with gross and histopathological lesions in bursa and thymus (Mendes *et al.*, 2007). Serotype 1 vvIBDV has been detected after an experiment infection in the bursa of quails together with seroconversion and virus shedding in faeces for five days (Van Den Berg *et al.*, 2001). Bursal samples from one dead wild black-billed magpie, two mallard ducks, one bean goose and one white -front goose were positive for IBDV detected by RT-PCR and virus isolation by chicken embryo inoculation. Phylogenetic analysis confirmed that wild birds are infected with vvIBDV and virus isolated from the bursa of a magpie caused 60% mortality and severe bursal atrophy in SPF chickens when experimentally inoculated with that isolate (Jeon *et al.*, 2008).

Serotype 2 IBDV infects turkeys and is distributed in all turkey-producing countries worldwide but is non-pathogenic to turkeys or chicken (Abdul *et al.*, 2014). Sero-conversion against serotype 2 IBDV has been detected in ducks and chickens without any gross or microscopic lesions following natural or experimental infections (Jackwood *et al.*, 1982; McFerran *et al.*, 1980; Richard *et al.*, 1984). Even with the genome reassortant serotype 2 IBDV does not cause mortality, morbidity or gross lesions in turkeys (Jackwood *et al.*, 2016).

Geographical distribution: In 1957, IBD was first reported in the "Gumboro" area of the Delmarva (Delaware-Maryland-Virginia) Peninsula in United States (USA) as a clinical entity in commercial broilers named "avian nephrosis" (Cosgrove, 1962) . The disease had rapidly spread across the USA by 1965 and was effectively brought under control by vaccination in mid-1970s (Lasher & Davis, 1997). New IBDV strains emerged in the mid-1980s in USA when apparent vaccination failure was identified as variant stains that were not protected against by the existing vaccines (Ismail *et al.*, 1990). IBDV classical and variant strains are predominant in USA and Australia (Ignjatovic & Sapats, 2002) but vvIBDV outbreaks were reported in USA in 2008 and 2014 (Crespo *et al.*, 2016; Stoute *et al.*, 2009). The more pathogenic vvIBDV strains emerged in Europe in 1989 (Stuart, 1989). Since then vvIBDV strains have spread rapidly across the world to Asia, the Middle East, South America and Africa with an exception of a few countries including Australia and New Zealand (Banda & Villegas, 2004; Jackwood & Sommer, 2007; Lin *et al.*, 1993; Pitcovski *et al.*, 1998; Rudd *et al.*, 2002; Yuwen *et al.*, 2008; Zierenberg *et al.*, 2000; Zierenberg *et al.*, 2001). IBD is identified as a global threat to the poultry industry according to the survey conducted by Office International des Epizooties (OIE) in 1995 showing that 95% of participant countries confirmed IBDV infection (Eterradossi, 1996). Molecular epidemiology analysis of IBDV in 18 countries from four continents showed that 60% - 76% of isolates were vvIBDV strains (Jackwood & Sommer, 2007).

IBD situation in Australia: IBDV was first reported in Australia in 1974 and two classical IBDV strains, 002/73 and V877 were isolated and characterised at that time (Firth, 1974). Commercial vaccines were made based on these two classical strains and the disease has been successfully controlled through breeder hen vaccination programmes (Sapats & Ignjatovic, 2000).

Australian variant strains emerged in the mid-1990s characterised by failure of protection provided by existing IBDV vaccines based on 002/73 and V877 strains (Sapats & Ignjatovic, 2000). Since the first disease report, a total of 24 Australian IBDV strains have been characterised as classical or variant strains based on restriction fragment length polymorphism analysis (Sapats & Ignjatovic, 2002). Pathogenicity studies on Australian classical strains revealed that these are immunosuppressive and do not cause clinical disease in susceptible chickens (Ignjatovic *et al.*, 2004). Australian classical and variant stains cause subclinical infection and are genetically distinct from other classical, variant and vvIBDV strains found overseas (Ignjatovic & Sapats, 2002; Ignjatovic *et al.*, 2004; Sapats & Ignjatovic, 2002). Classical Australian strains have been isolated in NSW, Queensland, and Victoria while variant strains have been identified in Victoria and South Australia (Ignjatovic & Sapats, 2002). However, recent surveillance has revealed that variant strains are becoming prominent in NSW and displacing existing classical strains. The newer variant isolates from NSW were similar to variant strains identified in South Australia (Sapats & Gould, 2016).

1.3.2 Frequency of occurrence

Although vaccination together with biosecurity measures have been used successfully to limit the occurrence of IBD worldwide, reports of disease outbreaks still occur (Rautenschlein & Alkie, 2016). Detection of antibody against IBDV from 75 poultry farms in Ohio State, USA showed that 58 farms out of 75 (77%) were seropositive for serotype 1 IBDV while 35 farms (47%) had antibody against serotype 2 IBDV (Jackwood & Saif, 1983). A serological survey conducted in Western Australia revealed 10 positive broilers farms out of 22 and 5 broiler breeder farms out of 7 were positive for IBDV antibody and none of the tested farms were vaccinated against IBDV (Wilcox *et al.*, 1980). Phylogenetic analysis of samples from suspected vvIBDV infection in 18 countries (Argentina, Bolivia, Brazil, Venezuela, Colombia, El Salvador, Dominican Republic, Mexico, France, Spain, Denmark, United Kingdom, Israel, Jordan, South Africa, Singapore, Korea and Thailand) on four continents showed that all detected vvIBDVs fell within a single clade with minor non-significant divergence (Jackwood & Sommer, 2007). Molecular analysis of IBDV in the USA revealed that variant strains diverge into four branches. Most variant strains prevalent on the East coast, Southeast and West Coast of USA were in branches 1 and 2 while a few variant stains prevalent in

Central USA, the Mid-West and North Carolina were in branches 3 and 4 (Jackwood & Sommer, 2005).

The frequency of occurrence and severity of IBD in some countries can be assessed from case reports. An average mortality rate of 15.31 ± 1.04% was reported during IBD outbreaks in 50 broiler farms from Mirpur and Kotli district, Pakistan in 1997 and 1998 (Farooq *et al.*, 2003). It was reported that IBD was the most prevalent disease in broilers in Jammu and Kashmir districts, Pakistan based on district veterinary laboratory records between 2008 and 2011 (Ahmad *et al.*, 2012). A pathological study conducted from November 2001 to October 2002 in Sylhet region, Bangladesh revealed that IBD was the main cause of death to poultry being held responsible for 24.26% of cases (Islam *et al.*, 2003). During vvIBDV outbreaks in Ethiopia between November 2007 and April 2008 reported incidence in village chickens in the Bahir Dar district was 38.4% with a 98.6% case fatality rate and in the Farta district 17.4% and 77.7% respectively (Mazengia *et al.*, 2009). The disease emerged in March-April 2002 in the Debye, Zeit district, Ethiopia with both layers and broilers affected with case mortality rates of 25.1 and 56.1 % in each of these groups respectively (Zeleke *et al.*, 2005).

1.3.3 Economic importance

The economic importance of IBD is thought to be due mainly to deaths in chickens aged 3 weeks and older infected with virulent IBDV, and immunosuppression following IBDV infection of all serotypes, resulting in increased susceptibility to various pathogens and reduced vaccination response to a range of poultry viral diseases and IBD itself (Eterradossi & Saif, 2008). For example, low pathogenic H5N2 avian influenza A/Mallard/Pennsylvania/10218/1984 virus was unable to replicate in 3 weeks old SPF chickens. However the virus showed limited replication in 3 week old chickens that had recovered from IBD and after 22 passages in chickens the P22 virus became virulent (Ramirez-Nieto *et al.*, 2010). Further losses also associated with growth retardation and rejection of carcasses due to muscle haemorrhages (Van Den Berg, 2000).

It was found that there was a 14% reduction in income from broiler flocks affected by subclinical IBD due to infection with variant IBDV in Northern Ireland (Mcilroy *et al.*, 1992; Mcilroy *et al.*, 1989). In another study, body weight was reduced by 11% in IBDV infected commercial broilers recovered from classical IBDV infection during first 42

days of their growing period and total profit reduction was 10% due to reduced feed conversion efficiency and weight gain (Van Den Berg *et al.*, 2000).

It was estimated that emerging classical IBDV strains in the poultry industry of New Zealand in 1985 caused USD 10 million annual loss (Christensen, 1985). Emergence of vvIBDV in 1988 in Europe and then in Asia following widespread including to Africa and Middle East, further increased the negative economic impact of the disease worldwide due to high mortality and morbidity (Lasher & Shane, 1994).

1.3.4 Main determinants of the disease

Host species: All breeds of chickens are susceptible to IBD caused by serotype 1 IBDV infection and serotype 2 IBDV strains do not cause disease in chickens (Eterradossi & Saif, 2008). Serotype 1 and 2 IBDV stains infect turkeys and ducks but not are associated with the disease (Giambrone *et al.*, 1978; Jackwood *et al.*, 1982; McFerran *et al.*, 1980; McNulty *et al.*, 1979).

Host genotype: Significant differences in mortality rate were observed in 11 inbred and partially inbred chicken lines such as White Leghorn (WL), WL sublines 151, 72, C, N, 6l, EB and 0 Brown Leghorn (BrL), Road Island Red (RIR) and Light Sussex (LS) inoculated with vvIBDV stain CS89. The BrL showed the highest mortality rate (80%) (Bumstead *et al.*, 1993). Genetic resistance to IBDV infection among commercial chicken lines varies considerably. The highest bursal damage to infection was seen in both BrL and WL lines (Bumstead *et al.*, 1993). Layer type chickens show higher susceptibility to IBDV infection than broiler type chickens (Aricibasi *et al.*, 2010). However, during an IBD field outbreak in Ethiopia, there was a higher case mortality rate in broilers than layers as described under section 1.5.2 (Zeleke *et al.*, 2005). This could be due to vaccination differences as many poultry farmers vaccinate their layers properly due to their long life span compared to broilers. The genetic difference of resistance to IBDV infection is not only due to a single antiviral gene. It may be due a number of antiviral genes including *IFNA, MXI, IFITMI, IFITM3 and IFITM5* (Smith *et al.*, 2015).

Age : There is a narrow range of ages at which chickens are susceptible to clinical IBD with the age of greatest susceptibility being between three and six weeks of age, during the time of greatest bursal development (Eterradossi & Saif, 2008; Hirai & Shimakura, 1974). Neonatal chickens and those above 6 weeks of age do not exhibit clinical signs

and mortality due to IBDV infection even with pathogenic strains (Eterradossi & Saif, 2008; Fadly & Nazerian, 1983; Hitchner, 1976). However, neonatal subclinical infection results in immunosuppression characterised by marked bursal atrophy (Hirai *et al.*, 1979; Kim *et al.*, 1999) and while maternal antibody (MAb) is protective against this immunosuppression an active immune response to infection is induced in both MAb protected and unprotected chicks under 3 weeks of age (Hitchner, 1971). The resistance to clinical disease in adult chickens above 6 weeks of age is thought to be due to bursal regression due to natural involution which greatly reduces the primary site for viral replication (Hitchner, 1976; Ley *et al.*, 1979). Bursal development begins to slow after 6 weeks of age and natural involution starts at 10 weeks of age (Glick, 1956). However, the older age restriction is not absolute and late clinical IBDV infection in older chickens may occur if appropriate target cells remain in the bursa following natural involution (Ley *et al.*, 1979).

Immune status: The immune state of chickens has a significant influence on IBDV infection. Susceptible chickens at hatch are liable to get sub clinical infection. Thus chicks are usually protected in their early life by MAb passed on from breeder hens vaccinated against IBD (Lemiere *et al.*, 2013; Lucio & Hitchner, 1979; Rautenschlein & Alkie, 2016). The extent of protection provided by passive maternal immunity from hens depends mainly on the level of MAb titre in the hens. In hens with high titres, protection can be up to 4 weeks while medium MAb tires protect chicks up to 2 weeks (Al-Natour *et al.*, 2004).

Pathogen factors/ organism factors: Severity of clinical disease in chickens depends on the strain of serotype 1 IBDV (Van Den Berg *et al.*, 2000). Infection with classical pathogenic strains cause clinical disease in susceptible chickens while immunosuppressive classical strains do not cause clinical disease but induce bursal atrophy leading to immunosuppression (Ignjatovic *et al.*, 2004). Infections with variant strains usually do not cause clinical disease in susceptible chickens but cause immunosuppression as a result of bursal damage (Eterradossi & Saif, 2008). The vvIBDV strains cause severe clinical signs and higher mortality (Chettle *et al.*, 1989; Van Den Berg *et al.*, 1991). Mutations occur in hypervariable region of VP2 of IBDV genome occur with high frequency and lead to changes in virulence (Rautenschlein & Alkie, 2016).

Environmental factors: Seasonal changes can affect to the IBD outbreaks and it has been reported that there are significant seasonal effects on IBD severity during outbreaks. Significantly higher losses occurred during winter than spring in one study (19.8 ± 2.1% and 9.0 ± 2.7% respectively) (Farooq *et al.*, 2003). A comparatively high mortality rate due IBD was found in rainy seasons in Bangladesh with 15.6% morality rate while 3.33% and 5.34% in winter and summer respectively (Islam *et al.*, 2003).

1.4 Host immune response to IBDV infection

IBDV infection in susceptible chickens activates both innate and adaptive immune responses (Eterradossi & Saif, 2008; Hitchner, 1976; Rauf *et al.*, 2011a). However, the level of activation of host immune response varies depending on age of infection (Rautenschlein *et al.*, 2007) and the virulence of the strain (Aricibasi *et al.*, 2010; Eldaghayes *et al.*, 2006). Surprisingly the genetic background of the chicken does not appear to have a major effect on the immune response although genetic background is associated with the outcome of the disease (Aricibasi *et al.*, 2010; Eldaghayes *et al.*, 2006).

1.4.1 Innate immunity

The innate or in-born immune response is the first immune response to IBDV infection and it is initiated quickly following IBDV infection but does not provide long lasting immunity as memory cells are not part of this response. There are number of host cells involve in the innate immunity such as natural killer cells, macrophages and mast cells. IBDV can modulate macrophages (Khatri *et al.*, 2005; Rauf *et al.*, 2011a) and mast cell degranulation (Wang *et al.*, 2008) immediately following infection and increases *in vitro* phagocytic activity. Activated macrophages release interleukin, chemokines and high levels of nitric oxide (Khatri & Sharma, 2006; Palmquist *et al.*, 2006; Rauf *et al.*, 2011a). Toll-like receptors in host cells such as TL3 and TL7 play a major role in detecting IBDV nucleic acids (Rauf *et al.*, 2011a) and successively induce antiviral response. (Guo *et al.*, 2012; Rauf *et al.*, 2011a).

1.4.2 Humoral adaptive immunity

Infection with IBDV stimulates production of group and serotype specific antibody to IBDV in host animals (Jackwood *et al.*, 1985). Humoral immunity is the most important component of the protective immune response against IBDV infection (Rautenschlein *et*

al., 2003). The role of antibody in protection against IBDV was well established by studies of passive immunity acquired by the progeny of immunised hens through MAb for first 2-3 weeks (Al-Natour *et al.*, 2004). Thus day-old commercial chicks carry MAb directed against IBDV in their sera. Higher levels of MAb at hatch protect the chicks against neonatal infection and reduced levels of MAb after 2 weeks of age are associated with increased susceptibility (Ahmed & Akhter, 2003; Al-Natour *et al.*, 2004; Hitchner, 1971; Wyeth & Cullen, 1976). Either field or experimental exposure to IBDV and vaccination against IBDV induces production of specific antibody directed against the virus. After exposure to virus or vaccination, anti-IBDV antibody titres are very high with VN assay, usually greater than 1:1000 (Eterradossi & Saif, 2008). No cross-protection against IBDV infection occurs between antibody to serotype 1 and serotype 2 IBDV (Ismail & Saif, 1990; Jackwood *et al.*, 1985). After exposure to either natural IBDV infection or vaccine, B cells start to produce antibody against IBDV and these antibodies then circulate in the host blood stream and help to suppress viral replication by destroying virus during the extracellular phase of infection (Rautenschlein *et al.*, 2003).

1.4.3 Cellular adaptive immunity

Cellular immunity is initiated by IBDV infection and T cells plays a major role in cellular immunity (Kim *et al.*, 2000) as is the case in most viral infections. It has been shown that T cells are resistant to IBDV infection (Hirai & Calnek, 1979; Rauf *et al.*, 2011b). An influx of $CD4^+$ and $CD8^+$ T cells occurs in bursal tissues as early as 4 dpi and peaks at 7 dpi (Kim *et al.*, 2000). Strongly upregulated Th1cytokines, IL-2 and IFN~γ show activation of T cells in cellular immunity (Rauf *et al.*, 2011b). Chickens without functional T cells either because of neonatal thymectomy or Cyclosporin A treatment showed reduced protection against IBDV infection even after immunisation with an inactivated vaccine, demonstration the importance of the cellular immune response (Rautenschlein *et al.*, 2002).

1.5 Immunosuppression induced by IBDV

The immunosuppressive effect of IBDV was identified in early studies (Allan *et al.*, 1972; Faragher *et al.*, 1974). However, the effect of immunosuppression depends on the age at infection. Infection at early life causes significant immunosuppression through reduction of either B cells or their precursor cells (Saif, 1991; Sharma, 1984). The immunosuppressive effect of the virus increases susceptibility to various other diseases

in chickens such as ND (Hirai *et al.*, 1974), inclusion body hepatitis (Fadly *et al.*, 1976), Marek's disease (Sharma, 1984), reovirus (Montgomery & Maslin, 1991), coccidiosis (Anderson *et al.*, 1977; Giambrone *et al.*, 1977a), adeno virus (Rosenberger *et al.*, 1975), infectious laryngotracheitis (Rosenberger & Gelb Jr, 1978), infectious bronchitis, *Myplasma synoviae* and colibacillosis (Giambrone *et al.*, 1977b). Humoral Ab response to vaccines is also suppressed by IBDV-mediated immunosuppression (Hirai *et al.*, 1974).

Both humoral and cellular immune responses are affected. Inhibited humoral antibody production is due to the significant destruction of immunoglobulin producing B cells whereas altered functions of antigen presenting and helper T cells cause suppression of cellular immune response as well (Sharma *et al.*, 2000). Although both humoral and cellular immune responses are suppressed by IBDV, the effect on the cell-mediated immunity is less marked than on humoral immunity (Eterradossi & Saif, 2008).

1.6 Clinical signs of IBD

IBDV has very short incubation period and clinical signs are observed in infected chickens at 2-3 dpi (Eterradossi & Saif, 2008). Clinical signs of IBD were first described in 1962 following infection with classical IBDV strains. The clinical signs, usually seen in young chickens between 3-6 weeks of age, include vent pecking, watery diarrhoea, ruffled feathers, trembling, depression and death in acute disease (Cosgrove, 1962). Mortality commences at 3 dpi and peaks at 4-5 dpi followed by rapid recovery by 5-7 dpi (Eterradossi & Saif, 2008). Clinical signs of IBD vary depending on several factors including age (Fadly *et al.*, 1976; Farooq *et al.*, 2000), immune states (Abdel-Alim & Saif, 2001; Anjum *et al.*, 1993), IBDV strain (Van Den Berg *et al.*, 2000) and chicken breed or strain (Aricibasi *et al.*, 2010; Bumstead *et al.*, 1993).

There are three clinical forms of IBD, the classical form, the variant or immunosuppressive form and the vv form (Van Den Berg *et al.*, 2000). Although vv and classical strains produce similar clinical signs, the clinical signs induced by vvIBDV strains are more acute and mortality rates are higher than those induced by classical strains. With vvIBDV strains mortality rates up to 25% in broilers, 60% in layer pullets and 100% in SPF chickens are seen (Van Den Berg *et al.*, 1991) whereas average mortality rates of around 5% are seen in chickens infected with classical strains

(Cosgrove, 1962). Variant and low pathogenic classical strains cause bursal atrophy and immunosuppression without overt clinical signs (Snyder, 1990).

1.7 Gross Pathology

Gross lesions in birds that have died from IBD include dehydration, haemorrhages in leg, thigh and breast muscles, increased mucus in the intestine and renal changes (Cosgrove, 1962) but the major effects are seen in the bursa, the main target organ.

Infection with virulent classical and vvIBDV strains induces an inflammatory reaction that results in a hypertrophied bursa at 3 dpi. The bursa is double in size and weight at 4 dpi due to oedema and hyperaemia, returns to its normal size at 5 dpi and then atrophies (Cheville, 1967; Helmboldt & Garner, 1964). In contrast, infections with variant stains normally cause mucosal oedema, firmness and rapid atrophy of the bursa without any inflammatory reaction (Dormitorio *et al.*, 2007; Lukert & Mutalib, 2001; Sharma *et al.*, 1989). Australian classical and variant IBDV strains also cause bursal atrophy (Ignjatovic *et al.*, 2004). However, serotype 1 variant IBDV strain IN does cause early inflammation, necrosis and atrophy of the bursa (Hassan *et al.*, 1996). Figure 1.1a shows the inflammatory bursa at 2-3 dpi and 1.1b shows haemorrhages on thigh muscles due to virulent IBDV infection**.**

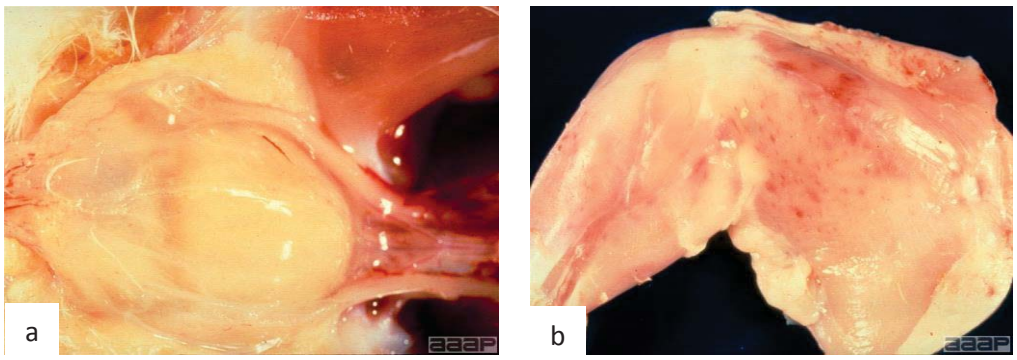

Figure 1. 1: *a) Inflammatory bursa of Fabricius at 2 -3 dpi and b) haemorrhages on thigh muscles due to virulent IBDV infection (Source: American Association of Avian Pathologists, INC. 1985. prepared by P.D. Lukert, University of Georgia, Revised by A. Mutalib, Mississippi State of University.)*

Splenomegaly is associated with acute infection and it can vary from moderate to very severe with uniform grey foci on the surface due to lymphoid necrosis in the germinal follicles and the periarteriolar lymphoid sheath (Cheville, 1967). Although vvIBDV strains cause similar bursal lesions as classical strains, they also induce severe lesions in various other lymphoid organs including the caecal tonsils, thymus, spleen, and bone

marrow with these lesions associated with high mortality rates (Brown *et al.*, 1994; Tanimura *et al.*, 1995). Typically there is severe inflammatory reaction in the bursa followed by rapid bursal atrophy even within 2-3 dpi, accompanied by severe atrophy in thymus. The latter is not associated with infection by either classical or variant strains and it reflects the virulence of the vvIBDV (Tsukamoto *et al.*, 1992).

1.8 Histopathology

IBDV infection induces cellular level lesions mainly in lymphoid organs such as the bursa, spleen, thymus, Harderian gland and caecal tonsils (Cheville, 1967; Helmboldt & Garner, 1964). The lesions associated with the bursa have been extensively described. Degradation and necrosis of B lymphocytes in medullary region of bursal follicles occurs within 24 hrs. after infection. B lymphocytes in the bursal follicles are replaced by mass accumulation of heterophils, pyknotic debris and hyperplastic reticuloendothelial cells sometimes accompanied by haemorrhages at 2 dpi. All bursal follicles are affected by 3dpi (Cheville, 1967; Helmboldt & Garner, 1964). Gross lesions are directly associated with these cellular level changes of IBDV infection. Classical and vvIBDV strains cause increased bursal weight at the same time (at 3 dpi) mainly due to the consequences of inflammation including severe oedema, hyperaemia and marked accumulation of heterophils and reticuloendothelial cells (Eterradossi & Saif, 2008). Inflammation declines at 4 dpi. Necrotic debris in the medulla of bursal follicles and interfollicular connective tissues are cleared by phagocytosis resulting in the appearance of cystic cavities (Cheville, 1967; Naqi & Millar, 1979). No regeneration of bursal follicles occurs up to 12 dpi suggesting that IBDV-induced bursal damage is prolonged (Naqi & Millar, 1979). In contrast, the histopathological lesions in the bursa caused by variant IBDV strains are characterised by rapid degradation of bursal follicles associated with immediate bursal atrophy without an inflammatory reaction (Sharma *et al.*, 1989). Follicular atrophy, mild oedema, some lymphoid necrosis and stromal fibroplasia are observed in infection with Australian IBDV strains with no obvious differences between Australian and overseas IBDV strains (Ignjatovic *et al.*, 2004).

In the spleen, hyperplasia of reticular endothelial cells can be seen around the adenoid sheath arteries in the early phase of infection whereas lymphoid necrosis occurs in the germinal follicles and around the periarteriolar lymphatic sheaths at 3dpi. However, the

spleen recovers rapidly from the infection without any sustained damage (Cheville, 1967; Helmboldt & Garner, 1964).

Lesions can be observed in thymus, caecal tonsils and Haderian glands during the acute phase of infection. However, within a few days of infection the organs return to their normal state (Cheville, 1967; Helmboldt & Garner, 1964). The lesions associated with the thymus depend on the virulence of the viral strain (Inoue *et al.*, 1994). Classical strains cause less damage at the cellular level than do vvIBDV strains with variant strains inducing less severe lesions than classical strains in fewer organs (Cheville, 1967; Helmboldt & Garner, 1964; Inoue *et al.*, 1994; Sharma *et al.*, 1989).

1.9 Diagnosis

Diagnosis of IBD is mainly based on clinical signs, post-mortem findings, and histopathology together with other laboratory tests (Muller *et al.*, 2003). Acute clinical IBD in highly susceptible chickens can be readily identified by its characteristic clinical signs such as quick onset with high morbidity, high mortality at 3-4 dpi followed by rapid recovery by 5-7 days. Visible gross changes in the bursa during post-mortem further help to suspect the IBDV infection (Eterradossi & Saif, 2008) as discussed in Section 2.9. Subclinical disease can be identified by both gross and microscopic lesions in the bursa (Eterradossi & Saif, 2008). However, serology, virus isolation or PCR test are needed for confirmatory diagnosis.

Immunoassay methods such as the agar gel precipitin assay and antigen-capture ELISA assay (AC-ELISA) can be used to detect viral antigens (Muller *et al.*, 2003). Virus isolation can be done by inoculation of antibody-free embryonated chicken eggs at 9-11 days old via chorioallantoic membrane (CAM route) (Hitchner, 1970; McFerran *et al.*, 1980) or by inoculating primary chicken fibroblast cell culture (Begum *et al.*, 2004). At present, qRT-PCR is widely used to detect and quantify the virus because it provides speedy diagnosis with high sensitivity and specificity (Muller *et al.*, 2003; Belak, 2007; Spackman, 2012). At present, various IBDV specific, strain specific qRT-PCR or RT-PCR assays are used in most of research and laboratory diagnosis worldwide and few examples are mentioned here. To detect several strains IBDV simultaneously, strain-specific multiplex RT- PCR assay has been developed by (Kabell *et al.*, 2005; Lin *et al.*, 1994). Differentiation of IBDV into two serotypes is successfully achieved by RT-PCR

(Lin *et al.*, 1994). The PCR test together with RFLP has been used for successful characterization of IBDV strains (Sapats & Ignjatovic, 2002).

ELISA is used to detect specific IBDV antibody in chicken serum (Marquardt *et al.*, 1980). The two IBDV serotypes and various subtypes can be differentiated by VN assay (Jackwood & Saif, 1987).

1.10 Differential diagnosis

The clinical picture of some other poultry diseases can be confused with IBD. Avian coccidiosis, some visceral forms of Newcastle disease, stunting syndrome of poultry, chicken infectious anaemia, mycotoxicoses and nephropathogenic forms of infectious bronchitis are the diseases that have common clinical signs with IBD. However, the presence of inflammatory bursal lesions during acute disease differentiates IBD from other acute diseases. Bursal atrophy during subclinical disease is often confused with atrophy induced by MDV and chicken anaemia virus but histological examination allows IBD to be distinguished Marek's disease and chicken anaemia (Eterradossi & Saif, 2008; Jakowski *et al.*, 1969). Histopathological lesions due to chicken anaemic viral infection are characterised by marked lymphoid cell depletion in bursa, thymus (Sommer & Cardona, 2003) together with greater reduction of granulocytes and thrombocytes in bone marrow (Adair, 2000) which is not associated with IBDV infection. Depletion of bursal follicle is common to both IBDV and MDV infection but MDV infection is associated with lesions in nerve tissues which are typical of MDV (Witter *et al.*, 1968). Bursal atrophy caused by adenovirus infection can also be confused with IBD, but the intranuclear inclusion bodies in liver and pancreas due to the adenovirus allow enable differentiation between the two (Grimes & King, 1977). However, development of molecular era in veterinary diagnosis during past decades facilitates strain specific speedy diagnosis with high sensitivity and accuracy as described in detail in part 1 of this review of the literature.

1.11 prevention and control

Eradication of IBD is difficult due to the extremely stable nature of the virus in the environment. Benton *et al* (1995) reported that IBDV persists on contaminated poultry premises longer than other poultry viruses and showed high resistance to various detergents. Since there is no treatment, prevention of IBD is based on vaccination

together with good biosecurity measures such as all-in and all-out farming systems, proper cleaning and disinfection of poultry pens and equipment, and a gap between depopulation and restocking of poultry batches on a farm (Butcher & Miles, 1995).

Vaccination from serotype 1 IBDV strains is considered as the primary method to control of IBD. Vaccination of breeder chickens with consequent transfer of MAb to the next generation prevents early immunosuppressive infection. This may be followed by schedule of vaccination once MAb protection has waned (Lucio & Hitchner, 1979; Rautenschlein & Alkie, 2016). The reported half-life of IBDV antibodies is between 4 and 5.5 days (Al-Natour *et al.*, 2004; Wyeth & Cullen, 1976). The declining levels of MAb after 2 weeks of age result in an increase in the susceptibility to IBD (Ahmed & Akhter, 2003; Al-Natour *et al.*, 2004; Hitchner, 1971; Wyeth & Cullen, 1976). However, Al-Natour *et al.* (2004) showed that the presence of high MAb levels at hatch can protect chicks up to 4 weeks of age. A general vaccination programme is not applicable for all countries due to variability in levels of MAb, different management systems and presence of vvIBDV. Vaccination of broilers may not be necessary if there is very high level of MAb and low risk of virulent field infection (Fahey *et al.*, 1989). In Australia, breeders are typically vaccinated against IBDV infection at 6 weeks of age with "Bursavac live®" live vaccine followed by subsequent vaccination with killed vaccine Bursavac K® or ALP 000/73 at 10 and 14 weeks of age (personal communication with Dr. Brendan Sharpe). Bursavac live and Bursavac K vaccines contain Australian classical strain V877 and ALP 000/73 contains Australian classical 000/73 strain (Bioproperties Pty, Ltd, Glenorie, NSW, Australia). At present many vaccines are available to protect chickens from IBDV infection including conventional live and inactivated vaccines, genetically engineered live IBDV vaccines, subunit vaccines, IBDV immune complex vaccines, DNA vaccines and live viral vector vaccines (Muller *et al.*, 2012). An example is the recently reported recombinant Rispens/Coa5-IBD vaccine which has potential to protect chickens from infections with virulent IBDV and Marek's disease virus strains (Ishihara *et al.*, 2016). One risk with live IBDV vaccines is genome assortment in the field and these reassorted IBDV in some cases can break through the protection provided by MAb from current breeder hen vaccinations (Jackwood *et al.*, 2016).

1.12 Conclusion

Even with highly efficient modern vaccines, sound management practices and speedy reliable methods of diagnosis, IBDV infection is still a big threat to the world poultry industry. It is probably the well-known immunosuppressive virus which causes the greatest negative impact on the industry. For the disease to be brought under improved control, proper understanding of IBDV strains, their shedding profile and the host immune response is important. The availability of PCR now provides reliable speedy diagnosis. Thus in this thesis study, detailed *in vivo* characterisation, pathogenesis, viral shedding and host immune response to infection of IBDV using Australian classical and variant strains are further studied using qRT-PCR.

The age and immune states of chickens are key factors which determine the level of IBDV infection and disease outcome. Therefore, better understanding of the contribution of those two factors on susceptibility to IBD would help for proper control of the IBD. At the commercial farm level almost all chicks carry MAb in their sera on the day of hatch. In this thesis, the independent effect of age, the presence of MAb and their combined effects are investigated using MAb-free SPF and MAb-positive commercial meat chickens at two different ages (at hatch and early susceptible age).

Disease monitoring can play a key role in a disease control strategy. Practicable disease monitoring system at the field level will help to understand the ongoing disease state at the farm level and which help provide early interventions for control. Monitoring of both vaccine and wild-type strains can assist with vaccination decision making. In this thesis study, the potential of qRT-PCR enumeration of IBDV vRNA in flock-representative dust and litter samples are assessed as a disease monitoring tool.

IBDV is stable virus and can survive in wide range of harsh environmental conditions. IBDV is shed to the environment through faeces and becomes incorporated in bedding material. When faeces get dry, free virus can detach and enter into the air space and can become a part of poultry house dust. Thus poultry house dust could be a possible source of infection. In this study the infectivity of IBDV-contaminated dust administered directly into the trachea are assessed.

Reuse of used litter between multiple batches of chickens is becoming popular now but there is risk associated with carryover pathogens in the litter. In shed litter pasteurisation

of litter by heaping is a common practise to inactivate viruses and other pathogens. However, temperatures within litter heap vary considerably depending on location in the heap and time since heaping. In this study, the effect of various temperature-time combinations, reflective of this variation, on inactivation of IBDV in litter are evaluated.

The currently available methods to assess infective virus load in litter are time consuming and costly. Introducing a quick and less expensive method to evaluate the efficacy of litter pasteurisation treatments would be helpful. In this thesis study, the predictive value of IBDV vRNA load in litter as a measure of the infective states of that litter are assessed by comparison with seroconversion of chickens exposed to the litter.

Chapter 2: General materials and methods

2.1 Animal experiments, management and facilities

2.1.1 Animal experiments

A number of six separate animal experiments were conducted for this thesis. Table 2.1 briefly describes the title, timing, location and chapters related to each experiment.

2.1.2 Source of experimental animals

Specific pathogen free (SPF) white Leghorn layer chickens (Lohmann LSL classic, Valo) sourced from Australian SPF Services Pty Ltd, Woodend Vic., Australia were used in experiments 2, 5 and 6 while SPF eggs from the same source, hatched at UNE were used in experiment 1. Commercial Ross broiler chickens and ISA Brown cockerels from the Country Road Hatchery, Baiada Poultry, Tamworth, New South Wales were used in experiments 3 and 4 respectively.

2.1.3 Handling and management of experimental animals

All experiments met the conditions set by the Animal Ethic Committee (AEC) of UNE and were approved by the AEC. All experimental chickens were well managed under the Australian code of Practice for the Care and Use of Animals for Scientific Purposes 2004, the NSW Animal Research Act 1985 and the NSW Animal Research Regulation 2005.

The cervical dislocation method as described by Zander *et al.* (1997) was used to euthanase the chickens. The legs were held in a fixed position by one hand. The thumb and index finger of the other hand circled the base of the skull and the middle and ring finger under the beak. Cervical dislocation was accomplished by the rapid extension of the arm holding the head with a simultaneous dorsal flexion of the head.

2.1.4 Isolator facility

The UNE isolator facility, which is maintained under the physical containment level 2 certified (PC2) conditions, was used for five experiments. The isolator building contains 24 individual isolators in two rows with 12 isolators in each row (Figure 2.1a) and its own post-mortem facility.

Table 2.1: *The title, timing and location of the experiments conducted during the doctoral research.*

Experiment No	Code	Name of the experiment	Timing	Location	AEC approval number	Chapters
1	LT13-C-KJ 1	*In vivo* characterization of infectious bursal disease variant and classical viral strains in SPF chickens.	12/07/2013 to 09/08/2013	UNE PC2 isolator,W33	AEC13-045	Chapter 3
2	IBDV14-KJ-SPF2	Effect of challenge age on IBDV viral RNA load in tissue and shedding of IBDV in SPF chickens and determination of infectivity of IBDV contaminated dust.	07/10/2014 to 09/12/2014	UNE PC2 isolator,W33	AEC14-074	
3	LT13-C-KJ 2	Determination of shedding profiles of IBDV in commercial broilers challenged at day zero and day 16.	24/09/2013 to 07/11/2013	Isolation pen, Western campus	AEC13-118	Chapter 4
4	LT-C-Bioassay1	Temperature-time relationships for IBDV decay in litter in broiler cockerels.	04/11/2013 to 28/01/2014	UNE PC2 isolator,W33	AEC13-119	Chapter 5
5	LT14-C-Bio2SPF	Temperature-time relationships for IBDV in decay in litter in SPF chickens.	07/10/2014 to 11/11/2014	UNE PC2 isolator,W33	AEC14-074	
6	LT15-C-Bio3SPF	Temperature-time relationships for IBDV in decay in litter in SPF chickens.	02/02/2015 to 09/03/2015	UNE PC2 isolator,W33	AEC15-013	

The size of each isolator is width: 66 cm, length: 210 cm and height: 88 cm. Isolator frames are made with stainless steel and completed with soft plastic bodies in four sides. Gauntlets and gloves were attached at two holes on the front side of the isolator to manipulate the birds with in isolators (Figure 2.1c). There was an access door at the left side of the isolators of the right row and left row it was right. The floor is stainless steel with holes which prevent the accumulation of faeces on the floor through the entire experiments. Faeces accumulated under the floor and water leakage was collected and drained from the isolation unit through a water filled u-tube which was fixed to the each isolation unit. Each isolator unit had its own thermostat control and heat lamps were fixed to it. Chicks at hatch, the isolator temperature was 34°C for first two weeks and reduced by 1°C every second day until it reached 22°C. Figure 2.1b shows experimental birds within isolator and Figure 2.1d shows the external view of the isolator with day-old experimental birds at the beginning of the experiment.

Feed and water were provided *ad libitum* throughout the experimental period. Feed was loaded into the feed hoppers fixed above each unit. The amount and type of required feed was calculated according to the type of birds, age, duration of the experiment and number of experimental animals. Feed was placed into the hopper before the experiment and sealed. The water supply was through low pressure nipple drinkers.

Negative pressure was maintained inside the room housing the isolators while positive pressure was maintained within each isolator unit. Positive pressure prevented entry of outside air into the isolators. Each isolator received temperature-controlled High Efficiency Particulate (HEPA) filtered air through the main air inlet duct and all out-going air was driven through individual scavenger ducts before it entered the main outlet air duct. The outflow of air passed through the HEPA filters again before being released into the environment. Both inflow and outflow of air could be controlled manually to allow control of the pressure within the isolators. The air in each isolator was changed with a fan setting of 12-23 air changes/hour depending on age of the birds. Care was taken to prevent cannibalism by environment enrichment in the form of hanging bunches of string from the roof of the isolators and the provision of litter in some experiments. Feed was laid out on A3 sized paper and additional water was supplied in ice trays to ensure intake of water and feed during the very first few days.

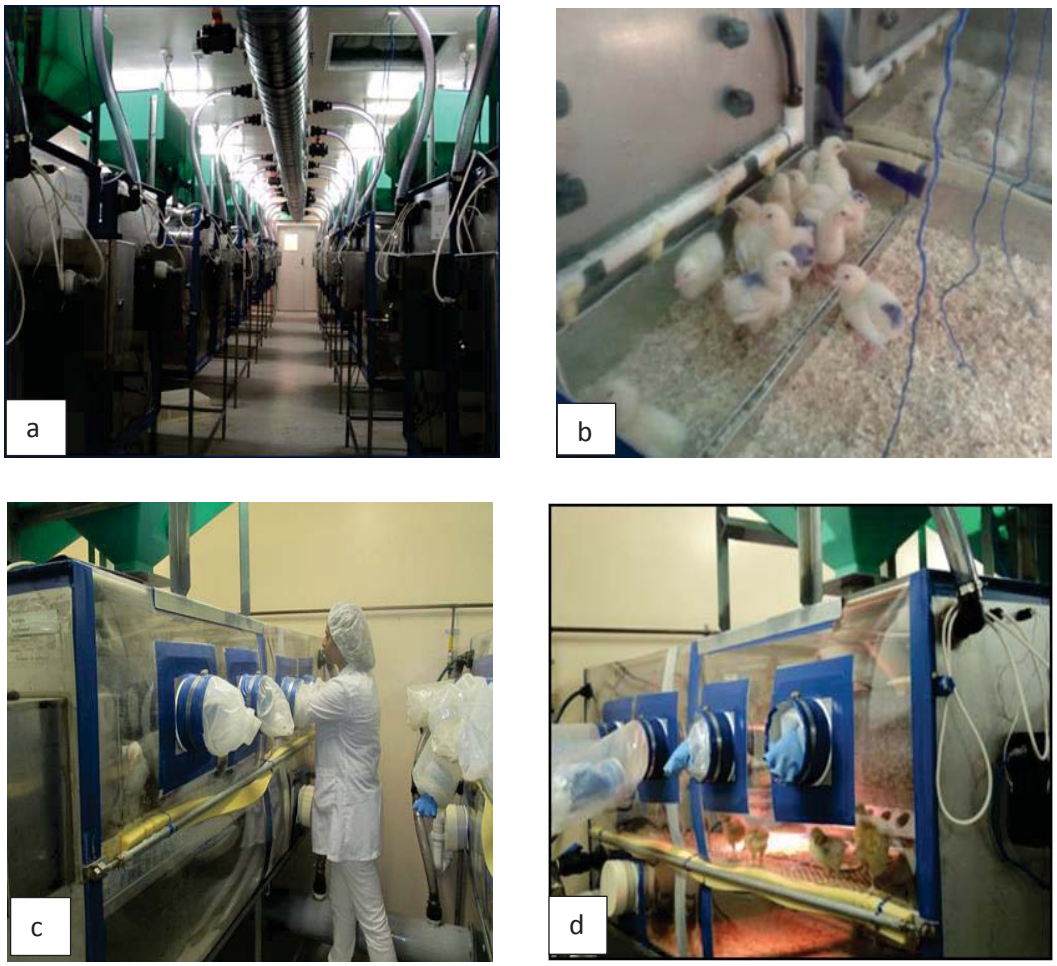

Figure 2. 1: *UNE isolator facility a: 24 isolators aligned into 2 rows b: Experimental birds inside the isolator c:Handling of experimental chicks using gauntlets d: External view of isolator with chicks.*

Care was taken to prevent contamination of the isolators during an experiment and to disinfect the isolators after experiments. All isolators were disassembled after each experiment and all soft bodies (isolator side walls), gauntlets and gloves were disposed. All other surfaces were thoroughly cleaned with detergent followed by soaking in a deep bacteriocidal and virucidal solution (Virkon S® LANXESS, Australia). Prior to each experiment the inside of the building was fumigated with formaldehyde and then ventilated with HEPA-filtered air. There were two change rooms before entering into the isolators. Designated foot wear, head covers and overalls or lab coats were used and clothes changed on entry and exit.

2.1.5 Isolation pens, Western campus

Isolation pens some 20 m apart from each other located at the western campus (Figure 2.2a) were used for determination of shedding profiles of IBDV in commercial broilers infected at 0 days age (d.o.) (day at hatch) and 16 d.o (Experiment 3). These pens were managed similarly to a very small-scale commercial broiler chicken operation with birds were raised on a deep litter system with *ad libitum* feed and water. Infected and control groups were kept in two separate isolation pens with the two age groups were separated by a partition within each pen (Figure 2.2b).

To maintain biosecurity during the experiment each isolation pen was allocated designated equipment and protective clothing and footwear which were put on before entering and when leaving each pen.

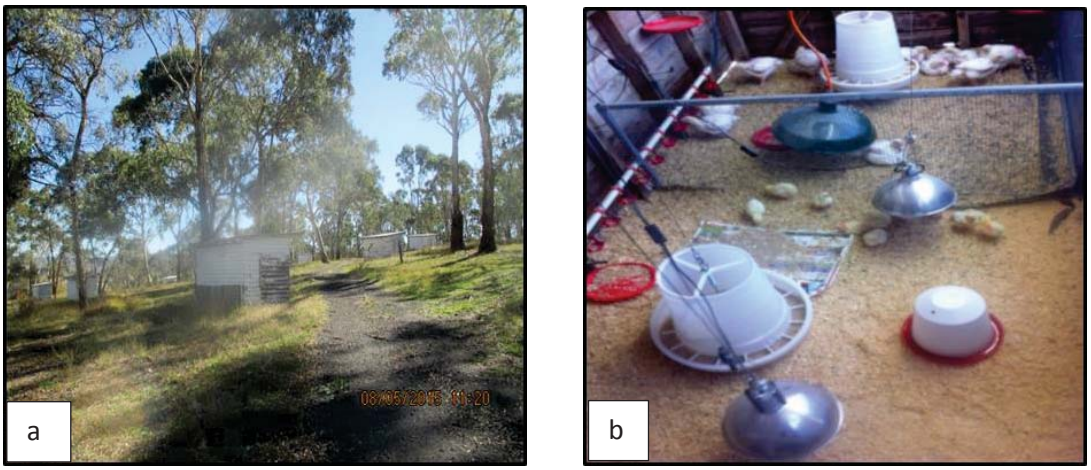

Figure 2. 2a: *Cluster of poultry pens at western campus. 2.2b: Experimental birds inside a pen.*

2.2 IBDV infective materials

Bursal extract, IBDV-contaminated litter and dust samples were used as infective materials for the six animal experiments summarised in Table 2.1. These contained one of two Australian field IBDV strains: serotype 1 Australian classical 06/95 or Australian variant 02/95 (Sapats & Ignjatovic, 2002).

2.2.1 Bursal extracts

Australian serotype 1 classical strain 06/95 and variant strain 02/95 were kindly supplied by Dr. Sandra Sapats of the Commonwealth Scientific and Industrial Research (CSIRO)

Australian Animal Health Laboratory at Geelong, Victoria. Isolates were prepared by filter sterilisation of a 20 % bursal homogenate in phosphate-buffered saline (PBS). Strain 06/95 was isolated from a field outbreak in New South Wales in 1995 and was passaged twice in SPF chickens while strain 02/95 was isolated from Victoria in 1995 and was passaged three times in SPF chickens. The extracts were prepared on 10/08/1998 (06/95) and 15/09/2000 (02/95) with titres of ~10^7 median chicken infective dose (CID_{50})/ml. An inoculum of 0.2 ml of a 1:10 dilution of the virus in PBS was used as the infective dose equating to a dose of ~10^5 CID_{50}/chick.

2.2.2. Infective litter

Infective litter which originated from Experiment 3 was used for Experiments 4 and 5 while litter from Experiment 2 was used for Experiment 6 (See Table 2.1 for details of experiments). Experimental chickens used in Experiment 4, 5 and 6 were directly exposed to the IBDV-contaminated litter which had been incubated at 25-70°C for 0-20 days. In Experiment 4, chickens were reared on wood shavings litter within isolators whereas in Experiments 5 and 6, two plastic "kitty litter trays" containing either clean wood shavings (negative control) or the contaminated litter were placed in each isolator to provide exposure to treated IBDV-contaminated litter as previously described (Islam *et al.*, 2013).

2.2.3 Infective dust

Dust samples which were collected at 7 days post infection (dpi) from experiment 3 were used to infect five 28 d.o. SPF chickens for *in vivo* determination of infectivity of dust. Each experimental chicken received 5 mg of dust by intratracheal insufflation. The dust contained 2.3 × 10^4 IBDV viral RNA (vRNA) copy number (VCN)/mg of dust as quantified by the IBDV real time reverse transcription polymerase chain reaction (qRT-PCR) assay (section 2.7).

2.3 Sampling procedures and data collection

2.3.1 Blood samples

Blood samples for serology were collected by wing stab whereby the cutaneous ulnar vein was pricked with a sterile 21G needle and blood drawn from the surface of the skin over the prick site into a transfer pipette. Samples were allowed to clot and serum was

separated by centrifugation at 1500 × g for 10 minutes (min.) at 4°C. All separated serum samples were stored at -20°C until required for Enzyme Linked Immune-Sorbent Assay (ELISA) to determine the antibody titre against IBDV.

2.3.2 Post-mortem examination

Post-mortem examinations were conducted following standard procedures (Zander *et al.*, 1997). Before opening the carcasses, muscle haemorrhages were detected by removing the skin over the breast and thigh muscles. Once the carcass was opened, all visceral organs were examined to see any gross visible changes. During the post-mortem, the bursa of Fabricius (bursa) and thymus were scored for atrophy by comparison with negative control birds. If the bursa or thymus were severely atrophied, it was assigned a score of three, moderately atrophied assigned a score of two, slightly atrophied a score of one and if no atrophy was visible a score of 0 (Fig. 2.3).

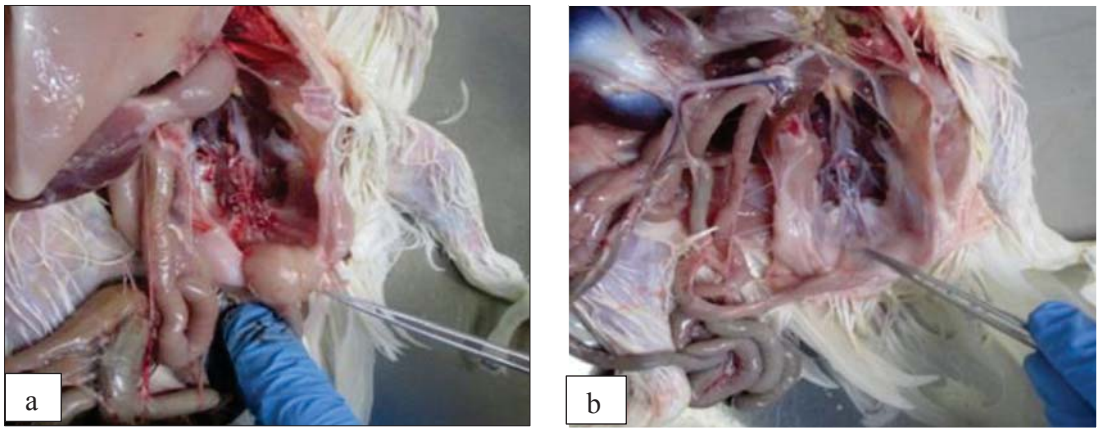

Figure 2. 3: *(a) Physical appearance of the bursa at 28 days of age in an SPF control bird (score 0); (b) severely atrophied bursa (score 3) in a 28 day-old SPF chickens infected with IBDV strain 02/95 at hatch.*

2.3.3 Body weight and relative bursal weight

Body weights of all birds were recorded after euthanasia and bursal weights of all birds were recorded during the post-mortem. Relative bursal weight for each bird was calculated as a percentage of bodyweight.

2.3.4 Organ sampling for qRT-PCR analysis

Bursa, thymus, spleen and caecal tonsils were collected during post-mortems to detect and quantify the IBDV vRNA by qRT-PCR. Whole organs were carefully excised and

rinsed with sterile PBS prior to placement in a storage container followed by storage at -80°C until required for RNA extraction. In Experiment 3, all tissue samples were snap frozen in liquid nitrogen followed by storage at -80°C until used for RNA extraction.

2.3.5 Faecal sample collection

Faecal samples were collected from individual birds for qRT-PCR detection and quantification. In experiment 1, faeces were collected from individually marked birds at regular intervals by placing the chicken in a container inside the isolator and recovering faeces from the container. In experiment 2, faeces were collected into a sterile container from the lower part of the large intestine from euthanized birds. In experiment 3, faeces were collected from individually marked birds at regular intervals by exerting gentle abdominal pressure. All faecal samples were stored at -80°C until required for RNA extraction.

2.3.6 Litter sample collection

Litter samples were collected at eight different spots inside the isolators or pens and mixed thoroughly before placing into zip lock bags at each sampling day for RNA extraction, and detection and quantification of vRNA by qRT-PCR. The samples were stored at -80°C until use.

2.3.7 Dust sample collection

Dust samples were collected from isolators by placing new nylon-anklet-stockings onto the air out-let duct for 48 hours (hrs.). Poultry dust accumulated in the stocking was collected into zip lock bags for subsequent RNA extraction and qRT-PCR detection.

To collect dust from the isolation pens, settle trays were placed in sheds. Dust was collected from the settle trays on each sampling day. All dust samples were stored at -80°C until used for RNA extraction and qRT-PCR analysis.

2.4 Laboratory methods and method development

For this thesis, ELISA and one-step IBDV specific qRT-PCR were conducted as laboratory tests. The ELISA test was carried out to detect antibodies against IBDV while one-step IBDV specific qRT-PCR was used to detect and quantify IBDV vRNA in four different sample types: tissues, faeces, litter and dust.

2.4.1 ELISA

A commercially available ELISA kit (SYNBIOTICS ProFLOCK® infectious bursal disease virus antibody test kit, San Diego, USA) was used to detect antibodies against to IBDV in sera. All serum samples were handled and tested srtictly according to the manufacturer's instruction. Samples were assayed at a standard dilution of 1:50.

2.4.2 RNA extraction from tissue/organ samples

Total RNA from tissues (bursa, spleen, thymus and caecal tonsils) was extracted using the Bioline isolate II RNA Mini Kit (Bioline, Alexandria, NSW, Australia) following the manufacturer's instructions. Thirty mg of tissue sample was wrapped in a piece of sterile aluminium foil and plunged in liquid nitrogen until frozen and then homogenised in liquid nitrogen using a mortar and pestle then the steps as described in the manufacturers protocol were followed. The RNA was eluted in 60 µl RNase-free water and stored at -80°C until used in the one-step IBDV specific qRT-PCR assay.

2.4.3 RNA extraction from dust

To establish an IBDV vRNA extraction method for poultry dust samples, GeneJET viral DNA and RNA Purification kit (Thermo Fisher Scientific Inc., USA) and Bioline isolate II RNA Mini Kit (Alexandria, Australia) were compared. For the Bioline kit, IBDV vRNA was extracted from dust samples by adding 5 mg of poultry dust from infected isolators or isolator pens into a microfuge tube containing 350 µl of lysis buffer and 3.5 µl of 14.3 M 2-mercaptoethanol (Sigma- Aldrich, Castle Hill, NSW, Australia) and then the manufacturer's protocol was followed. For the GeneJET kit, IBDV vRNA was extracted from the same dust samples by adding 5 mg of dust to a microfuge tube with 200 µl of sterile PBS (pH 7.2). The microfuge tube was vortexed vigorously for 5 min. and then RNA was extracted using the manufacturer's protocol. RNA extracted from dust was assayed in duplicate in a single one-step IBDV specific qRT-PCR assay and the results are shown in Table 2.2. Based on the viral genomic copy number (VCN) obtained from the one-step qRT-PCR assay, the Bioline isolate II RNA Mini Kit (Alexandria, Australia) was selected for IBDV vRNA extraction from dust samples as lower the C_t values greater the detection of target nucleic acid in the dust sample.

Table 2. 2: *IBDV qRT-PCR assays of RNA extracted from dust using Bioline and GeneJET kits.*

RNA extraction kit	Source of dust	Elution volume (µl)	Dilution factor for template	Template volume for the reaction (µl)	Source of dust	dpi	C_t value	VCN/mg dust
Bioline isolate 11 RNA kit	Isolation pen	60 in molecular water	1.10	2	Isolation pen	7	32.1	24,552
						14	35.2	2,907
GeneJET DNA and RNA kit	Isolation pen	50 in eluent buffer	1:10	2	Isolation pen	7	36.6	87
						14	-	-

2.4.4 RNA extraction from litter

A protocol for extraction of either DNA or RNA from poultry litter samples developed by project collaborators at CSIRO, Armidale and was used to extract IBDV vRNA (Appendix A). Buffers and other reagents used for the extraction were prepared as described at Appendix B. This method had previously proven to be successful for nucleic acid extraction from DNA and RNA viruses. However, the method is complex and very time consuming. Considering these factors, new methods were tested in this thesis study to develop quicker methods to extract IBDV vRNA from litter samples.

Six alternative methods, described at Appendix C, were evaluated. They included variations in washing, blending and bead beating. Methods 1, 2 and 3 used PBS and samples at different mixing ratios and levels of centrifugation while in methods 4, 5 and 6; overnight incubation with digestion buffer at 50°C and 60°C were tested. The GeneJET viral DNA and RNA Purification kit (Thermo Fisher Scientific Inc., USA) was used to extract IBDV vRNA from litter after all pre-treatment to litter and all extracted RNA samples were diluted 1:10 prior to inclusion in the PCR reaction. Two µl of diluted RNA was used as a template for each extraction method. The Ct values of one-step IBDV specific qRT-PCR assay run and VCN per mg litter are shown in Table 2.3. Based on the IBDV assay results, method 6 (M6) was selected to use for IBDV vRNA from litter samples. However, using this method no IBDV vRNA was detected in litter samples from 3xperiment 3 but re-testing the litter again using M3 gave positive result. Therefore, M3 was selected as the method for IBDV vRNA extraction from litter samples from all experiments.

2.4.5 RNA extraction from faeces

Initially, the commercially available Power Microbiome™ RNA isolation kit (MOBIO Laboratories, Inc., Carishad, CA) which is designed for extraction of RNA from stool samples was used to extract IBDV vRNA from faecal samples. A total of 25 mg of faeces from suspected highly positive samples and negative samples (samples from negative control birds) was used and the extraction was carried out according to the manufacturer's protocol. All standards and positive controls amplified but none of the samples amplified. This indicated that the IBDV assay performed well but the extraction of IBDV vRNA from faecal samples did not.

Thus a method for extraction of IBDV vRNA from faeces was needed to be find out. For that, extraction of IBDV vRNA from faecal samples was attempted by measuring 30 mg from each previously tested faecal samples into microfuge tubes contained 300 µl of sterile PBS (pH 7.2). Samples were vortexed vigorously for 5 min. followed by quick spinning. The volume of 200 µl of supernatant was used to extract RNA using the Thermo Scientific GeneJET viral DNA and RNA Purification kit (Thermo Fisher Scientific Inc., USA) according to the manufacturer's instruction but suspected positives were negative with no amplification curves in qRT-PCR detection.

To overcome the apparent problem associated with the IBDV vRNA extraction from faecal samples, three new methods were tested as described in Appendix D. The same samples were tested with all three methods. All samples were collected from the 16 day old bird group of experiment 3, three samples from 6 dpi and other three samples from 12 dpi were used. All RNA samples from each method were run in duplicate in a single one-step IBDV qRT-PCR assay and the results of the IBDV faecal test run are shown in Table 2.4. Method 3 (M3) was selected based on the IBDV faecal test assay results for the extraction of IBDV vRNA from faecal samples for this thesis study.

2.4.5.1 Test to detect PCR inhibition in extracted faecal samples

Test to detect PCR inhibition in extracted faecal samples was conducted to determine whether there was any inhibition effect from extracted faecal IBDV vRNA samples. This was prompted because of the consistently low levels of IBDV vRNA detected in faeces relative to the very high levels detected in dust samples from the same treatments, particularly in experiment 1.

The approach take was the addition of graded amounts of known IBDV vRNA to either 150 µl of water, or to 150 mg of pooled negative control faeces (uninfected with IBDV), followed by GeneJET RNA extraction from the samples and qRT-PCR quantification of IBDV vRNA. If inhibitors are present in the faeces, less vRNA should be recovered from spiked faecal samples than from water.

A detailed protocol for the faecal spike study is described in Appendix E. Details of the tested samples are shown in Table 2.5. All extracted samples were assayed in duplicate in a single run. The assay results of faecal spike study are shown in Table 2.6 and the fold differences of C_t values and average difference is shown in Table 2.7. Figure 2.4 shows the C_t values in water and faeces plotted against each other. Based on the test result, there seemed to be lower recovery of vRNA at higher concentrations of virus but this did not actually interfere with detection of vRNA as IBDV vRNA was detected in all dilutions tested (Table 2.6). These results are less suggesting of inhibition of the PCR reaction than of reductions in the efficiency of extraction of viral RNA from faecal samples, particularly at high concentrations of virus.

2.5 Final optimization of the faecal extraction method

At a later stage of the study, it was realized that simple wash with PBS had been successful for litter samples could be the best pre-extraction treatment method for faecal samples as well. Therefore, 12 faecal samples were selected from experiment 2 as follows (Table 2.8) to test the suitability of a simple new method 4 (M4).

One hundred and fifty (150) mg of faeces were measured from mentioned samples in Table 2.8 into 12, 1.5 ml microfuge tubes and 1 ml of PBS (pH 7.2) was added to each tube. All tubes were shaken vigorously for 20 min. followed by centrifugation at 4500 × g for 20 min. After centrifugation, 400 µl of supernatant was collected and IBDV vRNA was extracted using the Thermo Scientific GeneJET viral DNA and RNA Purification kit (Thermo Fisher Scientific Inc., USA) following the manufacturer's protocol.

Table 2. 3: *Mean Ct values following IBDV qRT-PCR of RNA samples extracted from litter samples using 6 different methods.*

	Method 1 - 20g/100ml PBS, 15 min Mixing, extract supernatant after spinning					**Method 2 - 20g/100ml PBS, 15 min mixing, extract "soup" without spinning.**					**Method 3 - 5g/45 ml PBS, 20 min mixing, Extract supernatant after 20min spin.**		**Method 4 - 10g/100ml Digestion buffer 60˚C overnight extract supernatant after spinning.**		**Method 5 - 20g/100ml Digestion buffer 50˚C overnight extract "soup" without spinning.**					**Method 6 - 20g/100ml Digestion buffer 50˚C overnight extract supernatant after spinning.**				
	L 1	L2	L3	L4	L5	L1	L2	L3	L4	L5	L 4	L 5	L 1	L 2	L 1	L 2	L 3	L4	L 5	L 1	L 2	L3	L 4	L 5
c_t values			42	36	33	42	41	40	40	38	35	37	40		35		43	41	34	39	39		34	32
VCN /mg					65						1.3	0.13			103				1.7		0.9		0.7	114

Litter samples: L1 Expt. 4. 25°C /5 days litter, L2 Expt. 4. 65°C /5 days litter, L3 Expt. 4 positive control litter, L4 Expt.3 5 dpi litter from 16 d.o.birds, L5 Expt.3 12 dpi litter from 16 d.o.birds (Low VCN was detected as most of tested litter samples contained diluted litter as described in chapter 5).

Table 2. 4: *Mean C_t values, test result and VCN per mg of faces following IBDV qRT-PCR of RNA samples extracted from faeces using 3 different methods.*

	Tested faecal samples	Mean C_t	Results	VCN/mg faeces	Log_{10} VCN + 1/mg faeces
M1 (Wash with PBS with 5 min. vortexing followed by 2 min. centrifugation)	Expt.3-16 old- bird1-12 dpi		Neg	0	0
	Expt.3-16 old- bird2-12 dpi		Neg	0	0
	Expt.3-16 old- bird3-12 dpi		Neg	0	0
	Expt.3-16 old- bird1-6 dpi		Neg	0	0
	Expt.3-16 old- bird2-6 dpi	36.45	Pos	5	1
	Expt.3-16 old- bird3-6 dpi	34.35	Pos	86	2
M2 (15 min. incubation at 50°C with digestion buffer)	Expt.3-16 old- bird1-12 dpi	37.48	Neg	0	0
	Expt.3-16 old- bird2-12 dpi	35.81	Pos	12	1
	Expt.3-16 old- bird3-12 dpi		Neg	0	0
	Expt.3-16 old- bird1-6 dpi	35.11	Pos	16	1
	Expt.3-16 old- bird2-6 dpi	34.26	Pos	202	2
	Expt.3-16 old- bird3-6 dpi		Neg	0	0
M3 (Overnight incubation at 50°C with digestion buffer)	Expt.3- 16 old- bird1-12 dpi		Neg	0	0
	Expt.3- 16 old- bird2-12 dpi	36.29	Pos	47	2
	Expt.3- 16 old- bird3-12 dpi	36.54	Pos	9	1
	Expt.3- 16 old- bird1-6 dpi	35.03	Pos	116	2
	Expt.3- 16 old- bird2-6 dpi	34.09	Pos	228	2
	Expt.3- 16 old- bird3-6 dpi	40.00	Neg	0	0

Table 2. 5: *Sample details of IBDV faecal spike study*

Sample	Description
1	150 mg Faeces (IBDV – ve)
2	150 ml molecular grade water
3	150 mg Faeces (IBDV –ve) + 200 µl of virus equating 10^5 CID_{50}/ml dose
4	150 mg Faeces (IBDV –ve) + 200 µl of virus equating 10^4 CID_{50}/ml dose
5	150 mg Faeces (IBDV –ve) + 200 µl of virus equating 10^3 CID_{50}/ml dose
6	150 mg Faeces (IBDV –ve) + 200 µl of virus equating 10^2 CID_{50}/ml dose
7	150 mg Faeces (IBDV –ve) + 200 µl of virus equating 10^1 CID_{50}/ml dose
8	150 ml molecular grade water (IBDV –ve) + 200 µl of virus equating 10^5 CID_{50}/ml dose
9	150 ml molecular grade water (IBDV –ve) + 200 µl of virus equating 10^4 CID_{50}/ml dose
10	150 ml molecular grade water (IBDV – ve) + 200 µl of virus equating 10^3 CID_{50}/ml dose
11	150 ml molecular grade water (IBDV – ve) + 200 µl of virus equating 10^2 CID_{50}/ml dose
12	150 ml molecular grade water (IBDV – ve) + 200 µl of virus equating 10^1 CID_{50}/ml dose

This method is similar to test M1 (Appendix D) but involved 20 min. mixing in the shaker followed by 20 min. centrifugation.

A comparison of VCN/mg of faeces obtained from the overnight incubation with digestion buffer at 50°**C** (Appendix D: M3) and M4 is shown in Table 2.9. Analysis revealed a significant (P = 0.006) higher $\log_{10}$ VCN/mg faeces using M4 than M3 with respective mean values of 3.5 ± 0.24 and 2.51 ± 0.24. Although the earlier PBS wash method (M1) had not proved successful, the higher PBS volume and greater shaking and centrifugation steps used in M4 may have led to the improved results with this method.

2.6 RNA quantification

The extracted vRNA from all samples were quantified using a NanoDropH ND-1000 UV-Vis spectrophotometer (Nano-DropH Technologies, Wilmington, DE, USA) and stored at -80°C until used for the IBDV qRT-PCR assay.

Table 2. 6: *IBDV qRT-PCR assay details of the spike test for PCR inhibition in extracted faecal samples.*

Sample ID	Extracted RNA concentration (ng/µl)	RNA per reaction	Mean C_t	IBDV test result	No of reps amplifying above threshold	Virus copy number/ reaction	Log10 VCN/ reaction	VCN/mg or ml	Log_{10} VCN/mg or ml
150 mg faeces (-)	119	59		Neg	0	0	0	0	0
150 ml water (-)	2	1		Neg	0	0	0	0	0
Faeces 10^5	173	87	22.5	Pos	2	96,408	4.98	160,679	5.21
Faeces 10^4	105	53	26.1	Pos	2	11,750	4.07	19,584	4.29
Faeces 10^3	167	84	28.9	Pos	2	2,351	3.37	3,918	3.59
Faeces 10^2	152	76	33.0	Pos	2	214	2.33	357	2.55
Faeces 10^1	138	69	36.3	Pos	1	9	0.98	14	1.16
Water 10^5	8	4	19.6	Pos	2	529,765	5.72	882,942	5.95
Water 10^4	10	5	24.1	Pos	2	38,891	4.59	64,819	4.81
Water 10^3	12	6	27.8	Pos	2	4,478	3.65	7,463	3.87
Water 10^2	9	4	31.4	Pos	2	540	2.73	901	2.95
Water 10^1	2	1	35.3	Pos	1	9	1	1.6	0.2

Table 2.7: *Individual and average fold differences of C_t values.*

Virus added (CID_{50})	Faeces (Mean C_t)	Water (Mean C_t)	Difference between 2 C_ts	Fold difference	Value (fold difference)
10^5	22.505	19.58	2.925	2^2.925	7.595
10^4	26.115	24.06	2.055	2^2.055	4.155
10^3	28.87	27.765	1.105	2^1.105	2.071
10^2	32.98	31.39	1.59	2^1.59	3.01
10^1	36.275	35.28	0.995	2^0.995	1.993
Average	29.349	27.615	1.734	2^1.734	3.326

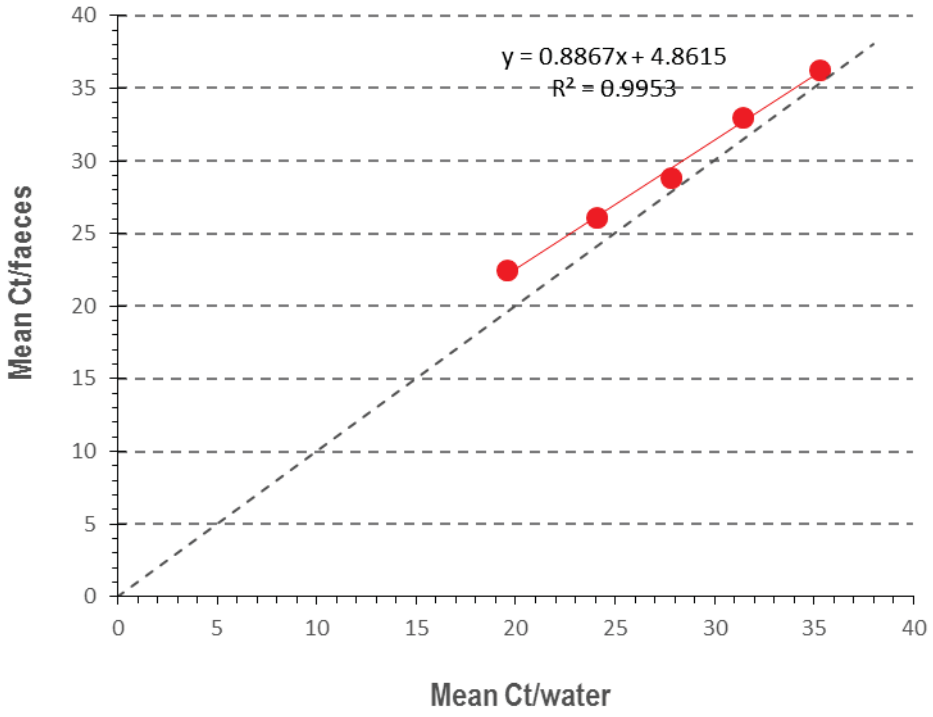

Figure 2. 4: *Scatter plot of mean C_ts of IBDV in water against IBDV in faeces. The dotted-black line represents the predicted linear association if there is no inhibition of the PCR.*

2.7 One-step qRT-PCR

A one-step IBDV specific qRT-PCR assay was used to detect and quntify IBDV vRNA in tissues, faeces, litter and dust samples. This TaqMan® generic IBDV assay was developed and validated at UNE in collaboration with CSIRO, Livestock Production, Armidale. Based on the sequence data for IBDV VP2 as previouly reported (Ignjatovic & Sapats, 2002) primers and probes were designed at the CSIRO. Alignment of sequences of isolates of IBDV from GenBank was performed using Clustal W (version 1.83), seeking for a good well conserved stretch for a probe and flanking primers. Probes

and primers were designed Using Primer3 with a probe GC content between 55-60% and a melting temperature 10˚C higher then primers The best of 3 sets of primers was selected (Table 2.10).

Table 2. 8: ***Faecal samples used in the final test for optimization of RNA extraction.***

Serial No.	Day at infection	DPI	Bird No.
1	Day 0	3	1
2	Day 0	3	2
3	Day 0	3	3
4	Day 0	6	5
5	Day 0	6	6
6	Day 0	6	7
7	Day 14	3	1
8	Day 14	3	2
9	Day 14	3	3
10	Day 14	6	5
11	Day 14	6	6
12	Day 14	6	7

The final volume of reaction mix was 25 µl including 12.5 µl of iTaq universal probe reaction mix (2X) (BIO-RAD, iTaq™ Universal Probes One-Step, Bio-Rad Laboratories, CA), 0.5 µl of iScript reverse transcriptase (BIO-RAD, iTaq™ Universal Probes One-Step, Bio-Rad Laboratories, CA), 10 µM IBDV Forward primer, 10 µM IBDV reverse primer, 10 µM IBDV probe FAM and 5 µl of 1: 10 diluted template (IBDV RNA).

The RNA extracted from different sample types was diluted in molecular grade water in 1:10 before used in the IBDV assay. A Corbett CAS 1200 liquid handling instrument (Corbett Research, Sydney, Australia) was used to prepare all reactions. The prepared reaction tubes were transferred to the Rotor Gene 6000 real-time PCR instrument (Corbett Research, Sydney, Australia) for detection and quantification of IBDV vRNA. The IBDV assay cycle profile consists of 50°C for 10 min., 95°C for 5 min. for initial denaturation followed by 45 cycles involving of denaturation at 95°C for 20 sec., annealing and extension at 60°C for 30 sec. followed by holding at 60°C for 5 min.

All samples were run in duplicate with two positives controls (PC) (Table 2.11) and negative control. A standard curve (Section 2.7.1) was generated for each assay to quantify IBD VCN in tissue, faeces. Dust and litter samples.Samples were randomised across all assays for each experiment to minimise errors within the assays and between assays.

Table 2. 9: *Comparison of IBDV VCN/mg of faeces using faecal digestion buffer overnight incubation method (M3) and new PBS wash (M4).*

	VCN/mg faeces	
Bird ID	**With digestion buffer for overnight incubation (M3)**	**With 1ml PBS after 20 shaking followed by 20 minutes centrifuge (M4)**
D0 challenge-3 dpi-bird1	5,218	986
D0 challenge-3 dpi-bird2	482	13,191
D0 challenge-3 dpi-bird3	1,262	11,890
D0 challenge-6 dpi-bird5	2,200	1,557
D0 challenge-6 dpi-bird6	268	893
D0 challenge-6 dpi-bird7	231	6,820
D14 challenge-3 dpi-bird1	187	4,238
D14 challenge-3 dpi-bird2	9,927	13,359
D14 challenge-3 dpi-bird3	637	8,075
D14 challenge-6 dpi-bird5	15	1,070
D14 challenge-6 dpi-bird6	187	1,569
D14 challenge-6 dpi-bird7	0	4,672

Table 2. 10*: Details of IBDV primers and probes used for the IBDV assay.*

Primers and probe	**Sequence (5' - 3')**	**5' label**	**3' label**	**size**
IBDV2 - Fwd.	GAC CCA GGR GCC ATG AAC TA			20 nt
IBDV2 - Rev.	TGT ACT CYC TKG TTG GCC AG			20 nt
IBDV2 Probe_ FAM	CC AAG ACG GTC CCT CTC ACT CAG GAT	FAM	BHQ1	26 nt

Table 2. 11: *Details of positive controls.*

	Source	Experiment code	DPI	Dilution factor	Final RNA conccentration
PC-1	Bursal tissue : D16 challenge group	LT13-C-KJ 2	7	100	610 ng/µl
PC-2	Bursal tissue : D16 challenge group	LT13-C-KJ 2	14	100	407 ng/µl

2.7.1 Development of the standard curve from bursal samples for the IBDV assay

Absolute quantification was used to quantify the viral RNA and which involves the inclusion of a set of standards containing known amounts of the target sequence against which the concentration in samples can be estimated. Plasmid standards containing cloned target gene sequences are normally used for the absolute quantification. However, due to not enough vaccine standard which were prepared based on plasmid standard to run all samples, it was required to developed new standard curve for the IBDV qRT-PCR assay. Bursal samples from experiment 3 were assayed and two samples were selected to make standard curve for the IBDV assay. Details of the selected samples are shown in Table 2.12.

Table 2. 12: *Details of bursal samples that were used to prepare the standard curve.*

Sample ID	DPI	Mean C_t	log_{10}VCN/mg bursa
B1-16 d.o.-1	7	22.2	7.64
B1-16 d.o.-2	7	26.2	6.03

Two bursal samples were collected at 7 dpi from the 16 day old bird group of experiment 3. The samples were pooled and total vRNA was extracted using the Bioline isolate II RNA Mini Kit (Alexandria, Australia).The vRNA was quantified using the NanoDropH ND-1000 UV-Vis spectrophotometer (Nano-DropH Technologies, Wilmington, DE, USA) and the concentration was 201 ng RNA/µl. Tenfold serial dilutions were prepared from the extracted total vRNA.

Five separate one-step IBDV qRT-PCR assays were run with the tenfold serially diluted samples using IBDV vaccine standard. Figure 2.5 shows the plotted mean C_t values of IBDV vaccine standard and tested new tissue standard against the log_{10} RNA/reaction.

Four standards were selected (10^{-1}, 10^{-3}, 10^{-4} and 10^{-5} dilutions) to use as new standards for the one-step IBDV qRT-PCR assay. The 10^{-2} dilution was removed because of the lowest dilatation 10^{-5} amplified and gave positive result and this enabled a 4 point standard curve.

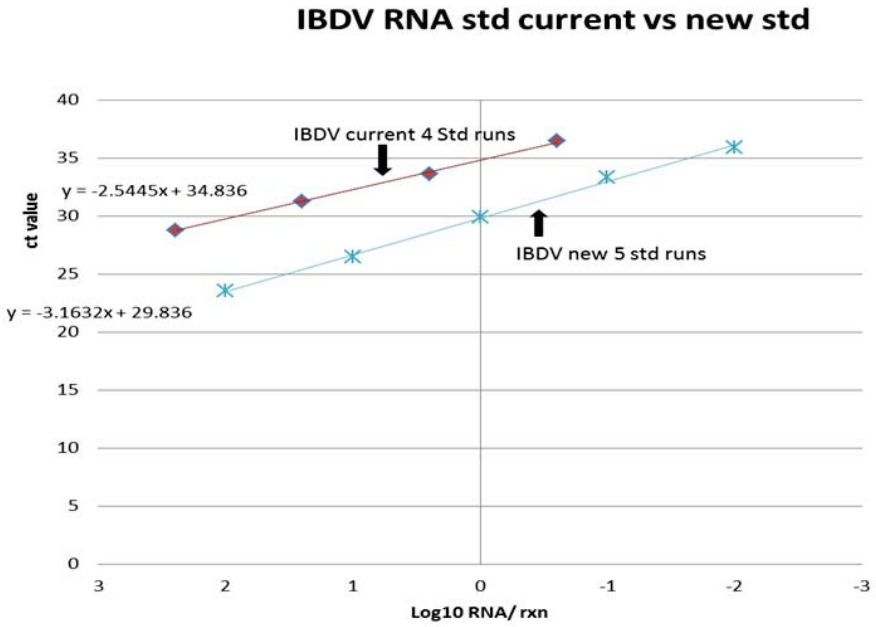

Figure 2. 5: *Current IBDV vaccine standard and new IBDV tissue standard.*

Chapter 3

Pathogenicity, tissue distribution, shedding and environmental detection of two strains of IBDV following infection of chickens at 0 and 14 days of age

Accepted in Journal of Avian Pathology

Chapter 4

Effects of oral infection of commercial meat chickens at days 0 and 16 of age with infectious bursal disease virus on disease outcome and the distribution, shedding and detection in environmental samples of viral genome

Submitted to Australian Veterinary Journal

Chapter 5

Inactivation of IBDV in chicken litter: Temperature – time relationships and prediction using qRT-PCR

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Chapter 6: General discussion and conclusions

This thesis study was conducted to evaluate the application of real-time reverse transcription polymerase chain reaction (qRT-PCR) for improved understanding and control of infectious bursal disease (IBD) in chickens. The experimental results provide strong evidence for the usefulness of qRT-PCR for studies on IBDV viral kinetics and shedding *in vivo* and for measurement of IBDV in environmental samples as a potential monitoring tool, but not as a measure of the IBDV infectivity potential of heat-treated litter samples.

Chapter specific findings have been discussed under each relevant chapter. In this chapter, the findings will be discussed and interpreted in light of the main research objectives outlined in the General Introduction, followed by overall conclusions, implications, limitations and future research.

6.1 Discussion by Research Objectives

6.1.1 Comparison of potential immunosuppressive and clinical outcomes due infection with IBDV Australian classical and variant strains

Neither classical strain 06/95 nor variant strain 02/95 induced overt clinical signs of IBD in any experiment but classical strain 06/95 did induce mild diarrhoea in experiment 1 (Table 2.1). The lack of overt IBD in experiment 1 is expected as IBDV infection of neonates does not result in clinical IBD (Fadly *et al.*, 1976) due to the main target organ being at an immature stage. However, the observed mild clinical signs following infection with classical strain 06/95 are consistent with earlier findings that this strain induces mild clinical signs such as ruffled feathers in chicks inoculated at 21 d.o. (Ignjatovic *et al.*, 2004). In experiment 1, when the two strains were directly compared, they did not differ in the degree of bursal atrophy caused, IBDV vRNA load in lymphoid organs and faecal shedding of IBDV. This finding is in contrast to other recent findings regarding Australian IBDV infection with classical and variant strains. Those results indicate that current variant strains are more virulent than classical strains causing more severe bursal atrophy and higher viral load in spleen (Sapats & Gould, 2016). Several other studies have found that variant strains cause greater early bursal atrophy than classical strains

(Hassan & Saif, 1996; Sharma *et al.*, 1989). In experiment 1 the first sampling day was at 6 days dpi and this may have been too late to detect the early differences between two strains. However, the two strains did differ in antibody response to infection and the rate of decline of IBDV vRNA from lymphoid tissues. The variant strain induced a significantly higher antibody response to infection and this was associated with a more rapid clearance of IBDV vRNA from lymphoid tissues and faeces. The quicker clearance of variant strains from lymphoid tissues is consistent with earlier observations in the USA following infections with classical strain STC and variant strain IN (Abdul *et al.*, 2013).

6.1.2 Age effect on susceptibility to IBDV infection

Greater bursal atrophy and higher IBDV vRNA load in lymphoid tissues in SPF chickens infected at 14 d.o. than those infected at 0 d.o. confirmed the higher susceptibility to infection of the older group. The higher IBDV vRNA in the older group was associated with a slower reduction of IBDV vRNA load in lymphoid organs post infection. However, antibody response or faecal shedding not differs between two age groups. The more rapid reduction of IBDV vRNA load in lymphoid tissues of the younger group could be due to lack of main target cells for the virus to be replicate. Maximum bursal development occurs at 3-6 weeks of age and birds at day on hatch contain a very limited number of target cells in their bursal follicles but chickens at 14 d.o. have a significantly higher number of target cells in their bursal follicles as they approach the most susceptible age for clinical IBD (Eterradossi & Saif, 2008; Hirai & Shimakura, 1974). These findings are consistent with other studies that have showed higher susceptibility to IBDV infection of chickens a few weeks old than neonatal chicks (Abdel-Alim & Saif, 2001; Fadly & Nazerian, 1983).

6.1.3 Combined effects of MAb and age on IBDV infection

Greater degree of bursal atrophy and high IBDV vRNA load in lymphoid tissues were observed in Mab-positive commercial meat chickens infected at 16 d.o. from 7 to 28 dpi in a declining pattern with increasing antibody titres from 7 to 28 dpi. However, commercial chickens infected at 0 d.o. had bursal atrophy and high level of IBDV vRNA in lymphoid organs later at 21 and 28 dpi with tend to increase antibody titres from 21 to 28 dpi. Thus these observations were greatly supported to conclude that commercial chickens infected at 16 d.o had active infection at 7 dpi but chickens infected at 0 d.o. didn't. However, commercial chickens infected at 0 d.o. also had active infection at 21

and 28 dpi. These results indicated that presence of high MAbs titres at hatch was able to protect commercial chickens from early infection but declining titres at 21 dpi were unable to protect chickens from infection. However, age also had some effect on severity of the disease as observed in SPF chickens. Chickens at higher bursal development stage are usually highly susceptible to get infection than chickens at hatch. Results of the current study is consistent with other studies of IBDV infection in MAb-positive chickens (Ahmed & Akhter, 2003; Al-Natour *et al.*, 2004; Hitchner, 1971; Wyeth & Cullen, 1976). In the present study, the observed delayed responses to infection may have been due to cross infection from the older cohort which was only separated by a wire partition.

6.1.4 Determination of IBDV in flock-representative environmental samples

The results of the present study clearly demonstrated that IBDV can readily detect and quantified in flock-representative environmental litter and dust samples. Another study also detected IBDV vRNA in indoor dust samples (Zhao *et al.*, 2013). Disease monitoring can be a very important aspect of disease control but it is not implemented for many diseases in the field level for a range of practical and economic reasons. For some diseases, existing disease diagnostic methods centre on individual bird sampling and to sample a representative number of birds on very big farms is cost prohibitive. Furthermore, individual bird sampling itself involves costs and challenges such as the need to handle individual birds, the technical skills required to take invasive samples from birds, loss of birds if samples require sacrifice and special conditions are usually required to store and transport the samples to the laboratory. Thus disease monitoring under these conditions is too labour-intensive, time consuming and costly to be practical. It is then limited only to some threatened transboundary diseases like avian influenza where government and industry funds for specific surveillance programs make them possible. Routine surveillance is less commonly done for endemic diseases like IBD for the reasons provided above. However, a change in methodology that overcomes all or some of these issues may make it feasible. Using poultry house dust as the sample material has several advantages over the traditional invasive samples such as blood and tissues. These advantages include the sample being non-invasive, there being no need for special techniques or skills for sample collection. In addition, transport of samples to the laboratory is easy as no cold chain is required and laboratory costs are significantly reduced as a single sample potentially represents the status of an entire flock (Walkden-

Brown *et al.*, 2013b). This should encourage further studies into the suitability of dust as an effective disease monitoring tool for IBD as applied in Marek's disease virus (MDV) to detect flock-infection and vaccination status against MDV (Walkden-Brown *et al.*, 2013b).

6.1.5 Determination of infectivity of IBDV-contaminated dust

In this study, IBDV-contaminated dust was shown to be infective and to transmit infection successfully to susceptible chickens following intra-tracheal insufflation. Ingestion of IBDV-contaminated feed and water is thought be the most common method of IBD transmission (Rautenschlein & Alkie, 2016) and oral transmission of the IBDV has been confirmed (Muller *et al.*, 1979a), and indeed was the route of infection used in experiments 1 and 2 (Table 2.1). However, the results of this study suggest that inhalation may be an important route of IBDV infection. This study could not ascertain whether the infection pathway was via respiratory surfaces, or whether virus trapped in mucus was brought up from the respiratory tract and swallowed enabling infection via gastrointestinal surfaces. Irrespective of this, the demonstration that the dust is infective and that it may find its way into the host via the respiratory tract is an important advance in our understanding of IBDV transmission. IBDV is well-known hardy virus and can persist well in the environment (Benton *et al.*, 1967a), so it is perhaps not surprising that virus shed in faeces remains infective when transformed into dust particles. This is a novel finding and further research is warranted into the role of dust, and true respiratory transmission in the epidemiology of the IBD.

6.1.6 Determination of the rate of IBDV inactivation in litter at various temperatures representative of those found during litter pasteurisation by partial composting

The experimental results clearly showed that susceptible chickens exposed to IBDV-contaminated litter held at 25°C for 5, 10 or 20 days became infected with IBDV. Litter exposed to middle and higher temperatures (35-70˚C) did not transmit IBDV but it could not be concluded that virus was inactivated by these treatments as the untreated positive control litters also failed to transmit IBDV. The failure of the positive control samples to transmit IBDV in three experiments calls into question the extent to which IBDV is able to resist adverse environmental conditions. Freezing of litter prior to exposure occurred in all 3 chick bioassay experiments and may have resulted in inactivation and the litter

used in experiments 4 and 5 (Table 2.1) appeared to have low level of of IBDV. In experiment 6, the low relative humidity and warm conditions in the isolators used to generate the infective litter may also have reduced the infectivity of IBDV. As shown by Sobsey and Meschke (2003) drying facilitates inactivation of non-enveloped viruses and IBDV comes under this category.

6.1.7 Determination of qRT-PCR measures of IBDV vRNA can be used to predict the infectivity of IBDV-contaminated pasteurised litter

Prediction of infectivity of IBDV in IBDV-contaminated litter using qRT-PCR was unsuccessful. Although the heat treatments greatly reduced the level of IBDV vRNA detected in experiments 5 and 6 there was no association with infectivity, partly due no doubt to the lack of infectivity observed in these experiments. Mostly there was a lack of infectivity in litters showing significant presence of IBDV vRNA, but in the few litters that did prove to be infective, there was also failure to detect IBDV vRNA presence. There is a need to develop alternative methods to predict infectivity of litter.

6.2 Conclusions and implications

The main conclusions and implications of the work of this thesis are summarised below.

1. Advances in methodology for measurement of IBDV.
    - A new generic IBDV qRT-PCR assay was used and optimised. This can be applied effectively to detect and quantify IBDV vRNA worldwide.
    - Methods for extraction of IBDV vRNA from faecal samples, litter and dust samples were developed and optimised. It was demonstrated that the level of PCR inhibition in samples extracted from faeces was small, and did not explain the low detection rate of IBDV in faeces.
2. There were few differences between the Australian IBDV classical and variant strains used when SPF birds were infected with them on the day of hatch. Both induced marked bursal atrophy. The differences observed were
    - Induction of mild diarrhoea with the classical strain
    - More rapid clearance of the variant strain from non-bursal lymphoid tissues.
    - This faster clearance of the variant strain was associated with a greater and earlier antibody response to infection.

3. A true age effect on susceptibility to infection independent of MAb was observed in SPF chickens with chickens infected with IBDV at 14 d.o. showing greater bursal atrophy and higher IBDV vRNA load in bursal and non-bursal lymphoid organs than those infected at 0 d.o.
4. Presence of MAb against IBDV successfully limited infection at 0 d.o with this effect greatly reduced or lost by 16 d.o. This confirms that existing Australian breeder hen vaccination against IBDV helps to protect young chickens from variant IBDV infection successfully in their first two weeks of life.
5. IBDV vRNA was successfully detected and quantified in flock-representative environmental dust and litter samples with dust having potential as a sample for routine monitoring of IBDV as has been shown for Marek's disease virus.
6. Inhalation of poultry infective dust is a likely source of infection with IBDV.
7. Survival of IBDV in poultry litter at 25°C for 5, 10 and 20 days was demonstrated and is consistent with existing understanding. While there was no transmission of IBDV in litter held at 35-70˚C for 5-20 days an inactivation effect of these temperatures was not definitively demonstrated due to failure of seroconversion in the positive control treatments exposed to unheated litter.
8. Quantification of IBDV vRNA load in pasteurised litter is not a good measure of the infective potential of the litter.

6.3 Limitations of experimental approaches and future research

In this study, 0 d.o and 14 d.o. chickens were used to investigate age susceptibility to IBDV. However, this age range was not sufficient to obtain a fuller picture of age susceptibility to the disease. Thus it would be desirable to test wider range age groups in infection studies using similar measurements as those used in this thesis.

To determine the effects of MAb and its association with age, two groups of commercial broiler chickens infected with IBDV at 0 d.o and 16 d.o. In this design the effects of the presence of MAb and true age susceptibility to IBDV infection are confounded. To get a clearer picture of the effects of MAb alone antibody inoculation studies in SPF chickens of a susceptible age (e.g. 3 weeks) should be undertaken.

IBDV was successfully detected and quantified in IBDV-contaminated dust samples from isolators and isolation pens. Despite the different environmental conditions, IBDV vRNA could be detected successfully in both cases. However, field studies are needed to confirm the feasibility of detection and quantification of IBDV vRNA in IBDV-contaminated dust under commercial conditions. Detection of IBDV vRNA in dust from field samples after live vaccination or disease outbreaks would help to confirm the suitability of dust as a flock-representative diagnostic tool.

Infectivity of IBDV-contaminated poultry house dust was tested by intra-tracheal insufflation in MAb-free SPF chickens at 28 days of age. We confirmed that IBDV-contaminated dust is able to transmit IBDV and induce bursal atrophy. It is likely that inhalation of IBDV-contaminated dust is a source of infection and thus it is important to clarify the role of airborne transmission of IBDV in the epidemiology of IBD.

In this study, IBDV infectivity in litter exposed to a wide range of temperatures (25-65˚C) for 5, 10 or 20 days was tested in three separate experiments. However, we were unable to accurately define the effects of the different temperature-time on inactivation of IBDV as in all three experiments chickens exposed to unheated litter in positive control isolators remained uninfected. Therefore, further studies are needed to confirm temperature-time combinations to inactivate IBDV successfully. In future studies, it is advisable to avoid freeze-thawing of litter sample for this kind of experiments. Litter must be collected from isolation sheds under normal environment conditions during, or shortly after the period of peak viral shedding, while avoiding diluting the litter. The development of appropriate methods to detect the live virus concentration or titre in litter using embryonated egg inoculation or cell culture methods would significantly reduce the problems experienced in our experiments. However, it is always good to use highly susceptible chickens (3-6 weeks old) for this kind of exposure experiments as they are the ones most susceptible to infection. When chickens at 3-6 weeks old are exposed to IBDV-contaminated litter with virulent strains, chickens can show clinical disease within week to exposure, if the litter is infective. This would help to early determination of the infection.

7: References

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Appendices

Appendix A: Original CSIRO method for viral DNA and RNA extraction from litter samples

1. 20 g of litter was measured and placed into 2 L beaker after mixing litter samples thoroughly. Then 300 ml of ice cold TE buffer containing 0.15% Tween 80 was added. Blended the samples on ice using a hand blender on high speed for 2 min. and washed residual sample off the blender using a squeeze bottle of TE/Tween 80 buffer. Then placed the beaker with litter samples on an orbital shaker for 1 hr. at 4°c and set the speed at 8 on a 10 speed shaker.
2. Prepared nylon mesh, funnel and 500 ml collection bottle for the sample.
3. Blended sample again on high for 2 min. and washed blender using squeeze bottle with TE/Tween 80.
4. Further 1 hr. on the orbital shaker at 4°C.
5. Swirled the sample and poured through nylon mesh following squeezing mesh into collection bottle to remove mast of the liquid out.
6. Samples were then placed in a centrifuge tubes and spin at 17500 × *g* for 30 min. at 4°C.
7. Poured off supernatant and kept the pellet. Mixed the pellet with a spatula.
8. Added 2 g of mixed pellet and 6 ml of Qiagen lysis buffer ASL into new 50 ml tube and vortexed thoroughly for mixing.
9. Removed 4 ml of above suspension and place into a bead beating tube containing 2 ml beads ((1mm silica) and beat beads for 5 min.
10. Removed beads and insoluble material by centrifuging 3000 ×*g* for 10 min. at 10°C.
11. Supernatant (Lysate) were collected for nucleic acid extraction.
12. Thermo Scientific GeneJET viral DNA and RNA Purification kit (Thermo Fisher Scientific Inc., USA) was used to extract the nucleic acid following the manufacturer's protocol.
13. Post extraction polyvinyl polypyrrolidone (PVPP) treatment was done as described below. Added 0.01 g PVPP to required number of 1.5 ml tubes.

a. Diluted sample one into ten with reduced TE (40 µl DNA/RNA + 360 µl reduced TE). Added to the PVPP tube and mixed tubes on horizontal shaker for 1 hr. at room temperature.

b. Centrifuged to remove PVPP.

c. Took off supernatant and stored at - 80°C in a new 1.5 ml tube until required for assay.

Appendix B: Buffers and other chemical used for the CSIRO viral DNA and RNA extraction methods

1M Tris-HCl

- Dissolved 121.1 g Tris base in approximately 800 ml Milli Q water
- Adjusted to desired pH with concentrated HCl Adjusted the volume with Milli Q water.
- Autoclaved for sterilization
- Stored up to 6 mon. at 4°C or room temperature.

0.5M EDTA

- Dissolved 186.1 g disodium EDTA dehydrates in 700 ml Milli Q water.
- Adjusted pH to 8
- Made the volume up to 1000 ml
- Autoclaved before use.

1X TE buffer -1 L

10 ml 1M Tris-HCl (pH 8)

- 2 ml 0.5M EDTA (pH 8)
- Added 988 Milli Q water and mix thoroughly
- Autoclaved before use

Reduced TE buffer: used 0.5M Tris- HCl and 0.5M EDTA (pH7.5)

- 20 ml 0.5M Tris-HCl (pH 7.5)
- 200 µl 0.5M EDTA (pH 7.5)
- Added 979.8 ml Milli Q water

- Autoclaved before use.

Appendix C: New methods evaluated for IBDV RNA extraction from litter

Method 1(M 1)

1. 20 g of litter was measured and dissolved in 100 ml of PBS (pH 7.2).
2. Mixed vigoursly for 20 min.
3. 400 µl of liquid was collected without centrifugation to isolate RNA using the Thermo Scientific GeneJET viral DNA and RNA Purification kit (Thermo Fisher Scientific Inc., USA) following manufacturer's protocol.

Method 2 (M 2)

1. 20 g of litter was measured and dissolved in 100 ml of PBS (pH 7.2).
2. Mixed vigoursly for 20 min.
3. Then took off 1000 µl of liquid and centrifuged 6000 × *g* for 1 min.
4. 400 µl of supernatant was collected for RNA extraction using the Thermo Scientific GeneJET viral DNA and RNA Purification kit (Thermo Fisher Scientific Inc., USA) following manufacturer's protocol.

Method 3 (M 3)

1. 5 g of litter was measured and dissolved in 45 ml of PBS (pH 7.2).
2. Mixed vigoursly for 20 min.
3. Centrifuged at 4500 × *g* for 20 min. at 4°C.
4. 400 µl of supernatant was collected for RNA extraction using the Thermo Scientific GeneJET viral DNA and RNA Purification kit (Thermo Fisher Scientific Inc., USA) following manufacturer's protocol.

Method 4 (M 4):

This method was developed by Prof. Parimal Roy (Roy *et al*., 2015) to extract infectious laryngo tracheitis virus from litter samples at the Animal Science molecular laboratory, UNE. Tested for IBDV vRNA extraction for litter samples as well.

1. Prepared litter digestion buffer (200 ml) using 10mM NaCl, 25mM EDTA, 10 mM Tris and 0.33% SDS as described below.

    i. 0.58 g……100mM NaCl
    ii. 0.9306 g……..25mM EDTA
    iii. 0.2152 g ……. 10mM Tris HCl
    iv. 0.66……..SDS (0.33% of final volume)

2. 100 ml of NaCl and 100 ml Tris HCl buffers were prepared severalty using laboratory grade Milli Q water. Then pH was adjusted to 8 for Tris HCl and pooled both NaCl and Tris HCl buffers. Then 0.2152 g of EDTA was added and mixed till dissolved EDTA completely. Finally added 0.66 g of SDS and mixed till dissolved everything. Volume was adjusted up to 200 ml and poured into a sterile autoclavable glass bottle and autoclaved at 120°C for 30 min. Stored at room temperature.
3. Weighed 10 g of litter materials and digested with 100 ml of prepared litter digestion buffer for overnight at 60^0C.
4. After quick vortexed and spinning, 1 ml of supernatant was collected.
5. Added 20 µl of proteinase K which was enclosed in GeneJET kit into 1 ml supernatant and incubated further for 30 min. at 50^0C.
6. 400 µl of supernatant was collected and continued from step 3 onwards of the GeneJET kit (Thermo Fisher Scientific Inc., USA) following manufacturer's instruction for the IBDV RNA extraction.

Method 5 (M 5)

This method is a slight modification of M4 (described above) which was developed by Prof. Parimal Roy to extract infectious laryngo tracheitis virus from litter samples at the Animal Science molecular laboratory, UNE.

1. Prepared litter digestion buffer described as above in M4.
2. Weighed 10 g of litter materials and digested with 100 ml of prepared litter digestion buffer for overnight at 50^0C.
3. 1 ml of liquid was collected.

4. Added 20 µl of proteinase K which was enclosed in GeneJET kit into it and incubated further for 30 min. at 50^0C.
5. 400 µl of liquid was collected and continued from step 3 onwards of the GeneJET kit (Thermo Fisher Scientific Inc., USA) following manufacturer's instruction for the IBDV vRNA extraction.

Method 6 (M 6)

1. Prepared litter digestion buffer as mention in M 4
2. Weighed 10 g of litter materials and digest with 100 ml of prepared digestion buffer for overnight at 50^0C.
3. After quick vortexed and spinning, 1 ml of supernatant was collected.
4. Added 20 µl of proteinase K which was enclosed in GeneJET kit into it. Sample was mixed by quick vortexing.
5. Incubated at 50^0C for 30 min.
6. Centrifuged *at* 6000 ×*g* for 1 min.
7. 400 µl of supernatant was collected and continued from step 3 onwards of the Thermo Scientific GeneJET viral DNA and RNA Purification kit (Thermo Fisher Scientific Inc., USA).

Appendix D: New methods evaluated for IBDV vRNA extraction from faecal samples

Three samples collected at six days post infection (dpi) with IBDV and another three samples from 12dpi were selected from commercial broilers challenged at day 16 of age group (Experiment 3) and subjected to the following methods of RNA extraction.

Method 1 (M1)

1. 150 mg of faeces was weighed out
2. 400 µl of sterile PBS (pH 7.2) was added
3. The mixture was vortexed vigorously for 5 min.
4. Samples were centrifuged at 5000 ×*g* for 2 min.
5. 200 µl of supernatant was collected for IBDV RNA extraction using Thermo Scientific GeneJET viral DNA and RNA Purification kit (Thermo Fisher Scientific Inc., USA) following t the manufacturer's protocol.

Method 2 (M2): Modified Prof. Roy's method

1. Digestion buffer (200 ml) was prepared at the laboratory using 100mM NaCl, 25mM EDTA, 10 mM Tris and 2% SDS as follows.

   0.58g……100mM NaCl
   0.9306g……..25mM EDTA
   0.2152g ……. 10mM Tris HCl
   4g……..SDS (2% of final volume)

2. 100 ml of NaCl and 100 ml of Tris HCl buffers were prepared separately using laboratory grade Milli Q water. The pH was adjusted and mixed till dissolved EDTA completely. Finally, added 4 g of SDS and mixed till dissolved everything. Prepared buffer was Poured into sterile autoclavable glass bottle and autoclaved at 120°C for 30 min. Prepared digestion buffer was stored at room temperature.
3. 150 mg of faeces was weighed out.
4. 1 ml prepared digestion buffer plus 20 µl of proteinase K was added.
5. Samples were vortexed vigorously for 5 min. followed incubation at 50^0 C for 15 min.
6. Samples were vortexed after incubation and centrifuge 6000 × g for 1 min.
7. 400 µl of supernatant was collected to extract IBDV vRNA and proceeded from step three onwards of the Thermo Scientific GeneJET viral DNA and RNA Purification kit (Thermo Fisher Scientific Inc., USA).

Method 3

Method 3 is exactly the same as M2 but instead of 15 minutes incubation samples were incubated overnight at 50° C.

Appendix E: Protocol for IBDV spike study to test for PCR inhibition

1. 150 mg of pooled faeces from Day zero negative control birds of Experiment 2 (Table 2.1) was measured into 6 separate 2 ml RNase/DNase free tubes.
2. One tube with 150 mg of IBDV negative faeces was kept as negative control without adding anything.
3. Volume of 200 µl of IBDV 02/95 variant virus equating 10^5 CID_{50}/ml dose was added to one 2 ml tube with 150 mg IBDV negative faeces.

4. Volume of 200 ul from each tenfold serial dilution: 10^{-1}, $10^{-2,}$ 10^{-3} and 10^{-4} from initial strength stock (10^5 CID_{50}/ml) was added to four 2 ml tubes with 150 mg IBDV negative faeces individually.
5. 150 µl of PCR grade RNase/DNase free molecular water was added to six 2 ml RNase/DNase free tubes separately.
6. One tube with 150 µl of PCR grade RNase/DNase free molecular water was kept as negative control without adding anything.
7. Volume of 200 µl of IBDV 02/95 variant virus equating 10^5 CID_{50}/ml dose was added to one 2 ml tube with 150 µl of molecular water.
8. Volume of 200 ul from each tenfold serial dilution: 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4} from initial strength stock (10^5 CID_{50}/ml) was added to four 2 ml tubes with 150 µl of molecular water respectively.
9. Then 1 ml of digestion buffer as described above in Appendix D was added to all twelve 2 ml tubes.
10. 20 µl of proteinase K which included in the Thermo Scientific GeneJET viral DNA and RNA Purification kit (Thermo Fisher Scientific Inc., USA) was added to all twelve tubes followed by vigorous vortexing for 5 min. and incubate at 50°C for overnight.
11. After incubation, samples were vortexed and centrifuged 4500 $\times g$ for 1 min. after incubation.
12. 400 µl of supernatant was used to extract IBDV vRNA using the Thermo Scientific GeneJET viral DNA and RNA Purification kit (Thermo Fisher Scientific Inc., USA) starting step 3 onwards of the protocol.
13. Extracted IBDV vRNA was quantified using NanoDropH ND-1000 UV-Vis spectrophotometer (Nano-DropH Technologies, Wilmington, DE, USA) and stored - 80°C until assayed.

Appendix F: Faecal extraction method 4 (M4) for optimization of faecal IBDV vRNA extraction

1. 150 mg of faeces was measured and dissolved in 1 ml of PBS (pH 7.2).
2. Mixed vigoursly for 20 min.
3. Centrifuged at 4500 $\times$ g for 20 min. at 4°C.

4. 400 µl of supernatant was collected for RNA extraction using the Thermo Scientific GeneJET viral DNA and RNA Purification kit (Thermo Fisher Scientific Inc., USA) following manufacturer's protocol.