UNIVERSITI PUTRA MALAYSIA

# EXPERIMENTAL INTRAOCULAR INFECTION OF JAPANESE QUAILS (*Coturnix coturnix japonica*) WITH INFECTIOUS BURSAL DISEASE VIRUS (IBDV) INTERMEDIATE STRAIN

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# EXPERIMENTAL INTRAOCULAR INFECTION OF JAPANESE QUAILS (*Coturnix coturnix japonica*) WITH INFECTIOUS BURSAL DISEASE VIRUS (IBDV) INTERMEDIATE STRAIN

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**SITI NOR AZIZAH BINTI MAHAMUD**

A Project paper submitted to the
Faculty of Veterinary Medicine, Universiti Putra Malaysia
In partial fulfilment of the requirement for the
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## CERTIFICATION

It is hereby certified that we have read this project paper entitled "Experimental Intraocular Infection of Japanese Quails (*Coturnix coturnix japonica*) with Infectious Bursal Disease (IBDV) Intermediate strain", by Siti Nor Azizah binti Mahamud and in our opinion it is satisfactory in terms of scope, quality and presentation as partial fulfilment of the requirement for the course VPD4999 – Project.

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**DEDICATION**

This project paper is dedicated to Allah SWT, who had created me and made all things possible,

To my family,

Father

Mother

Brothers, Sisters

Awatif, Amjad, Asyraff

And to all my teachers who have committed themselves towards the noble cause of education.

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LIST OF ABBREVIATION

| | |
|---|---|
| % | Percentage |
| $^0$C | Degree celcius |
| µl | Micrometer |
| ml | Mililiter |
| H&E | Haematoxylin and Eosin |
| IACUC | Institutional Animal Care and Use Committee |
| IBD | Infectious bursal disease |
| IBDV | Infectious bursal disease virus |
| MLV | Modified-live vaccine |
| PBS | Phosphate buffered saline |
| RNA | Ribonucleic acid |
| rpm | Rotation per minute |
| RT-PCR | Reverse transcriptase polymerase chain reaction |
| SD | Standard deviation |
| $TCID_{50}$ | Median tissue culture infective dose |
| VP | Viral protein |

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# ABSTRAK

Abstrak daripada kertas projek yang dikemukakan kepada Fakulti Perubatan Veterinar untuk memenuhi sebahagian daripada keperluan kursus VPD 4999 – Projek

INFEKSI PERCUBAAN INTRAOKULAR KEPADA PUYUH JEPUN (*Coturnix coturnix japonica*) MENGGUNAKAN VIRUS PENYAKIT BERJANGKIT BURSAL (IBDV) STRAIN PERANTARAAN.

Oleh

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2016

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Penemuan pertama penyakit berjangkit bursal (IBD) ialah pada tahun 1957 di Gumboro, Delaware, USA dan pertama kali diterangkan di Malaysia pada tahun 1991. IBD merupakan penyakit bawaan virus yang membawa kerugian ekonomi yang besar kepada industri poltri kerana menyebabkan imunotindasan dan kadar kematian yang tinggi. Eksperimen ini dijalankan bertujuan mencepatkan jangkitan kepada puyuh jepun menggunakan vaksin hidup dilemahkan strain perantaraan IBD secara intraokular. Parameter yang diperolehi adalah pemerhatian tanda klinikal, lesi post-

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mortem, pengesanan antigen menggunakan transcriptase membalik reaksi berantai polimerase konvensional (RT-PCR) dan perubahan histopatologikal pada puyuh dari group A, B dan C. Spesifik primer telah direka untuk mensasarkan protein major luar kapsid iaitu gen protein virus 2 (VP2). Parameter ini diukur selepas vaksin diberikan dan bilangan puyuh tertentu dikorbankan pada hari ke 5, 9, dan 15 pos-infeksi dan 24 bursa telah dikumpulkan. Hasil eksperimen menunjukkan pengurangan limfoid minor pada kumpulan A, tanda klinikal yang jelas dan pengurangan limfoid sederhana pada kumpulan B dan tiada lesi post-mortem yang ketara pada semua kumpulan. Analisis transcriptase membalik reaksi berantai polimerase menunjukkan hasil negatif bagi semua sampel yang diuji. Kesimpulannya, ujian klinikal, patologikal dan molekular menunjukkan IBDV strain perantaraan tidak menghasilkan immun respons yang cukup pada puyuh untuk menjadikan meraka pembawa atau perumah.

Kata Kunci: IBDV, intraokular, puyuh jepun (*Coturnix coturnix japonica*), RT-PCR, histopathologi.

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ABSTRACT

An abstract of the project paper presented to the Faculty of Veterinary Medicine in partial requirement for the course VPD 4999 – Project

EXPERIMENTAL INTRAOCULAR INFECTIONS OF JAPANESE QUAILS (*Coturnix coturnix japonica*) WITH INFECTIOUS BURSAL DISEASE VIRUS (IBDV) INTERMEDIATE STRAIN.

By

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2016

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Infectious Bursal Disease (IBD) was first discovered in 1957 at Gumboro, Delaware, USA and was first described in Malaysia in 1991. IBD became an important viral disease in poultry industry due to its significant economic losses with high mortality and profound immunosuppression. This experiment was conducted to induce the Japanese quails with IBD modified-live vaccine intermediate strain intraocularly. The parameters obtained were observation of clinical signs, post-mortem lesions, antigen detection using conventional reverse transcriptase PCR and histopathological changes

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in quails from group A, B and C respectively. Specific primer was designed to target the major outer capsid protein which is viral protein 2 gene (VP2). These parameters are measured after vaccine administration and selected number of quails from each group were euthanized at day 5, 9 and 15 post-infection and the total of 24 bursas were collected. The result reveals minor lymphoid depletion in Group A, prominent clinical signs and mild lymphoid depletion for Group B and no significant post-mortem findings in all groups. RT-PCR analysis gave negative findings in all samples tested. In conclusion, clinical, pathological and molecular results indicate that IBDV intermediate strain does not produce sufficient immune respond in quails to warrant them as carrier or host.

Keywords: IBDV, intraocular, Japanese quails (*Coturnix coturnix japonica*), RT-PCR, histopathology.

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## 1.0 INTRODUCTION

Infectious Bursal Disease is often referred to as Gumboro disease, was discovered in 1957 in Gumboro, Delaware, USA (Khan *et al*., 2005). The outbreak of IBD was first described in Malaysia in 1991 by Hair-Bejo (1992). According to Washington Disease Diagnostic Laboratory (2015), the natural host of IBDV is the domestic fowl including chickens and turkeys and young chickens within the age of 3 to 6 weeks are the most susceptible to clinical diseases. The wild birds such as healthy ducks, guinea fowl, quail and pheasants, have been found to be naturally infected with IBDV (Washington Disease Diagnostic Laboratory, 2015). The most recent survey of international poultry specialists, conducted by *World Poultry,* highlighted continuing concern in the sector over the sanitary status of poultry. Gumboro diseases topped the list of the most serious poultry diseases (Tsukamoto *et al.,* 1999).

IBD is a highly contagious viral disease that affects mainly young chickens and is economically important to the poultry industry (Van den Berg, 2000) due to significant economic losses as it lead to high mortality and morbidity, impaired growth and profound immunosuppression. According to IDERIS (1999), IBDV causes severe inflammation of the bursa of Fabricius that leads to immunosuppression due to destruction of immature B-lymphocytes within the bursa of Fabricius and finally leads to lymphoid depletion and significant depression of the humoral antibody response.

The indirect economic impact of the disease is also considerable, due to virus-induced immunosuppression and potential interactions between IBDV and other

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viruses, bacteria or parasites. These indirect losses are due to secondary infections, growth retardation and condemnation of carcasses at the slaughterhouse (Van den Berg, 2000). Moreover, the increased use of antibiotics against secondary infections constitutes a growing public health concern. The birds infected with IBD became susceptible to get infection of other diseases that can result in an increase of occurrence of disease caused by opportunistic pathogens and prevents young chickens from responding optimally to vaccination (Ojeda *et al*., 1997).

The objectives of this study are:

1) To observe the clinical signs shown by challenged quails.
2) To examine the post-mortem lesions on challenged quails.
3) To examine the histopathological changes on bursa of Fabricius of challenged quails.
4) To demonstrate the presence of antigen of IBDV in challenged quails via molecular method which is RT-PCR.

Therefore, the hypotheses for this study are:

1) There are significant clinical signs shown by challenged quails.
2) There are significant post-mortem lesion on challenged quails.
3) There are significant histopathological changes on challenged quails.
4) There are presence of antigen in challenged quails upon tested with RT-PCR.

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## 2.0. LITERATURE REVIEW

### *2.1. Japanese quails (Coturnix coturnix japonica)*

Japanese quails belong to the order *Galiformes*, genus *Coturnix*, and species *japonica*. The scientific designation for Japanese quail is *Coturnix japonica*, different from the common quail (*Coturnix coturnix*). The Japanese quails are found in Japan, Korea, Eastern China, Mongolia and Sakhalin as migrating birds. Japanese quail are now farmed mainly for egg production in Japan. In Europe, they are selected for meat production due to its efficiency in body weight increase (Mizutani, 2007). These birds are the commonly raised species of quails in Philipines because of their large size and adaptability to the surrounding environment (Abao *et al.,* 2015). In Malaysia, Japanese quails are the common species raised by small holder farmers and being commercially reared for meat and egg production according to Seet and Azizah (1987).These birds require a short period of time to reach market stage and can produce eggs at the age of six s to seven weeks.

### *2.2. Infectious Bursal Disease Virus*

Infectious Bursal Disease (IBD) is caused by IBDV which belongs to genus Avibirnavirus and family Birnaviridae. The morphology of this virus is non-enveloped, icosahedral symmetry with 60nm in diameter (Dobos *et al.,* 1979). The genome of this bipartite double stranded RNA is 3.4kb for segment A and 2.8kb for segment B (Delmas *et al*., 2005). The virus has 5 viral proteins (VP) which are VP1, VP2, VP3, VP4 and VP5. The epitopes responsible for the induction of neutralising and protective antibodies are located on the VP2 protein (Bayliss *et al.,* 1990), and several groups in Europe, the USA and Australia have prepared neutralising

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monoclonal antibodies against the VP2 protein (Eterradossi *et al.,* 1997). Neutralising monoclonal antibodies are serotype-specific. The non-neutralising monoclonal antibodies are directed against either VP2 or VP3; some are group-specific, others are type-specific (Jackwood, 1990).

Two serotypes of IBDV exist, namely: Serotype I which is pathogenic to chickens and can cause serious problems in chicken flocks; however, individual strains differ markedly in their virulence (Tereza *et al.,* 2008) and Serotype 2 which is apathogenic as it cause neither mortality nor bursal lesions in SPF chickens and are thus apathogenic for chicks (Van den Berg et al., 2000). In addition to serological classification, the viral strains may be classified according to virulence (mortality and bursal lesions). Thus, strains of IBDV may be considered apathogenic, attenuated, classical virulent, variant or hypervirulent (vvIBDV).

Although the other lymphoid organs are affected (Sharma *et al.,* 1993), according to Nishizawa *et al.,* (2007), the primary target organ of IBDV is the bursa of Fabricius as this organ act as reservoir for B lymphocytes in birds.

The clinical signs of IBD can be divided into three principal clinical forms, as follows:

a) The classical form is caused by the classical virulent strains of IBDV. Specific mortality is relatively low, and the disease is most often subclinical, occurring after a decline in the level of passive antibodies (Faragher,1972).

b) The immunosuppressive form is caused by low-pathogenicity strains of IBDV, as well as by variant strains which partially resist neutralisation by antibodies against the so-called 'classical' viruses (Snyder, 1990).

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c) The acute form, first described in Europe, and then in Asia, is caused by 'hypervirulent' strains of IBDV, and is characterised by an acute progressive clinical disease, leading to high mortality rates on affected farms (Stuart, 1989).

### *2.3. Infectious Bursal Disease in quails*

Randall and Bolla (2008) found that Japanese quails can be affected by common poultry disease but fairly disease resistant. Japanese quails along with chicken can be susceptible to common disease that can reduce egg and meat production in poultry according to Abao *et al.* (2015). An experimental study done by Sonfada *et al*. (2014) revealed the bursae of infected quails with IBDV shown interfollicular edema, lymphocytolysis, haemorrhages, fibroplasia and keratinization of the bursal substances. The evaluation of bursa weight and the determination of bursa bodyweight ratio is the most frequently used method to estimate the degree of bursae atrophy (Bolis *et al.,* 2003). Akhter *et al*. (2006) stated that the measurement of length and breadth of bursa of Fabricius helps in understanding the changes in population of immunocompetent cells. Moraes *et al*. (2004) proved the use of bursometry to measure diameter of bursa of Fabricius was considered inadequate to evaluate vaccine pathogenicity thus, required for histopathological examination on bursae. In chickens, some of the IBD intermediate strain vaccines used caused severe bursal lesions as those observed in the field IBD outbreak (Hair-Bejo *et al.*, 2000). The vaccination of IBD on 14 days old chicken was effective as it is able to induce high antibody titre and level of protection (Hair-Bejo *et al.,* 2004). According to Grindstaff (2009), the maternal-derived antibody will remain detectable in quail chick's circulation for an average of 14 days.

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### *2.4. IBDV vaccine*

The IBD vaccines have been categorized into mild, intermediate and hot vaccine strains according to the bursa/body weight ratios detected following vaccine administration (Guittet *et al*., 1994). Mild vaccines did not induce bursal lesions and used in parent chickens to produce primary response prior to vaccination with inactivated vaccine (Skeeles *et al.,* 1979). Intermediate strains enlarge the bursa up to twice the normal size and used to protect broiler chicken, commercial layer replacements and young parent chicken if there is a high risk of infection with virulent IBDV (Rosales *et al*., 1989). Hot strains cause enlargement in the bursal size three times the normal size and subclinical IBD, used in young chicks in very severely affected areas (Lohren, 1994). To date, more than 46 types of imported IBD vaccines are used to control the disease in West Malaysia (Chin, 1993).

In chickens, some of the IBD intermediate strain vaccines can cause severe bursal lesions as those observed in the field IBD outbreak (Hair-Bejo *et al*., 2000). Recommended vaccination program of IBD normally starts at14 days old and it is effective as it is able to induce high antibody titre and protective level of antibody (Hair-Bejo *et al.,* 2004). In quails, maternal derived antibody remained detectable in chicks' circulation for an average of 14 days (Grindstaff, 2009) thus; vaccine challenge is required after this age.

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## 3.0. MATERIALS AND METHODS

### *3.1. Experimental chicks*

24 of one week old quail chicks were obtained from Puyumas Farm Best Sdn. Bhd, Melaka. The quail chicks were separated into three groups which were group A, B, and C respectively. The quail chicks were raised for 15 days under controlled environment in separated room in Animal House Facility, Faculty of Veterinary Medicine, Universiti Putra Malaysia. The quails were raised in two-tier cages with unlimited access to drinking water in dispenser and quails starter mash feed (Cargill, Malaysia) in feeding trough ad-libitum. Bilateral intraocular infection was induced at the seventh day of age to the all groups once. This experimental study was approved by the Institutional Animal Care and Use Committee (IACUC), with reference number: UPM/IACUC/FYP.2015/FPV.032.

### *3.2. Vaccine challenge*

Volvac® IBD MLV intermediate strain (Boehringer Ingelheim Vetmedica, Mexico) with titer of $10^{4.5}$ $TCID_{50}$ /ml was used in this study. The vaccine titre were increased to two fold which is $10^{4.8}$ $TCID_{50}$ /ml and 10 fold which is $10^{5.5}$ $TCID_{50}$ /ml and were given to Group A and B, respectively. Quails from Group A was inoculated with 0.03ml of $10^{4.8}$ $TCID_{50}$ /ml via intraocular bilaterally, Group B with 0.03ml of $10^{5.5}$ $TCID_{50}$/ml of the same vaccine via the same route and for Group C which is control group, the quails were inoculated with 0.03ml of sterile phosphate buffered saline (PBS) via the same route.

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### 3.3. *Monitoring of clinical signs*

The quails were observed for the manifestation of clinical signs by daily monitoring from day 1 post-infection to day 15 post-infection. The findings were then recorded according to the groups.

### 3.4. *Sample collection*

#### 3.4.1. *Post-mortem examination*

A total of three post-mortem examinations were carried out with a total number of 24 chicks on day 5, 9 and 15 post-infections. Two chicks from Group A, B and C were euthanized on day 5, three chicks from each group on day 9 and three chicks from each group on day 15. The transverse incision was made on ventral abdomen to examine bursa of Fabricius, spleen, breast and thigh muscles. The post-mortem examination was done on the organs such as breast muscle, thigh muscle, spleen and bursa of Fabricius to examine the gross lesions.

#### 3.4.2. *Histopathological examination*

A total of 6 bursa samples were collected during the third post mortem and subjected for histopathological examination.

##### 3.4.2.1. *Tissue fixation*

The bursa of Fabricius of two quails from each group which are Group A, B and C were collected during post-mortem. All the bursae were placed in three different bottles containing 10% formalin for fixation.

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***3.4.2.2. Tissue dehydration and clearing***

Each of the bursa was cut transversally at median region and placed in the three different cassettes according to the group. Both sections were dehydrated by passing the bursa through increasing concentrations of ethyl alcohol from 0 to 100%. Then the bursa samples were immersed into xylene and this step is called as clearing. The whole steps takes about 14 hours to be completed.

***3.4.2.3. Tissue embedding***

The bursae sample in the cassettes were then rearranged and the cassettes were placed into the well. Then, warm paraffin wax was poured into the well and was left to cool down using embedded machine (Leica EG 1150 H and Leica EG 1150 C, USA).

***3.4.2.4. Tissue sectioning***

The cassettes were detached from the well and the bursa samples on the wax were trimmed using microtome (Reichert-Jung 2045 Multicut Rotary Microtome, Canada). The thin section cut of the bursa samples were then placed in water bath $42^0$c for “fishing” onto the glass slides and then air dried.

***3.4.2.5. Tissue staining and mounting***

Harris’ Haematoxylin and Eosin staining procedure were used in this study. First, the slides were submerged in xylene for 5 minutes, followed by 100% alcohol for 5 minutes and then in 70% alcohol for 5 minutes. The slides were rinsed using tap water. Next, the slides were submerged in Haematoxylin for 5 minutes, then rinsed for 3-5 times and dipped in 1% acid alcohol for 3 seconds. The slides were rinsed under the

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running tap water for 5 minutes then submerged in Eosin for 1 minute. Next the slides were sprayed with 95% alcohol then rinse in running tap water 5-10 seconds. The slides were sprayed with 95% alcohol, cleaned using gauze and air dried. Finally, the slides were dipped in xylene for three times and mounted with DPX. The whole steps were performed in fume hood.

***3.4.2.6. Microscopic examination***

The histology slides were observed under the light microscope Olympus CX31 (Olympus, Japan) and displayed using DigiAcquis Xcam-Alpha (DigiAcquis 2.0) software.

## 3.5. Demonstration of Antigen

### *3.5.1. IBDV RNA extraction*

The RNA was extracted from bursa samples by followed RNA extraction using TRIzol® method. The bursae samples were pooled into a 15 ml centrifuge tube according to the groups. 2ml of TRIzol® reagent (Invitrogen, USA) were pipetted into to the centrifuge tube. The bursas were homogenised using tissue homogeniser (OMNI TIP™ Homogenizing Kit, USA). 1ml of homogenized product was pipetted into 1.5ml centrifuge tube. Next, 2ml of chloroform was pipetted into the tube, gently vortex and incubated at room temperature for 15 minutes. Then the tube was centrifuged at 12000rpm for 15 minutes at $4^0$c. Three layers were formed and the upper layer was pipetted out and transferred into a new 1.5ml centrifuge tube. 0.8ml of isopropyl alcohol was pipetted in and incubated at room temperature for 15 minutes. Then, the

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tubes were centrifuged at 12000 rpm for 15 minutes at $4^0$c. The supernatant were pipetted out, leaving the RNA pellet. RNA pellet was washed with 100% alcohol by pipetting it in 1 ml into the tube, gently vortex and centrifuged at 7500 rpm for 5 minutes at $4^0$c for two times. The supernatant were pipetted out and RNA pellet were dried. RNA pellet were dissolved with 15ul of nucleus free water.

### *3.5.2. RNA concentration and purification*

The extracted RNA was measured for the concentration and purification using spectrophotometry (TECAN M200 PRO, Switzerland) and interpreted using i-control 1.10 software. The RNA samples then stored at -80$^0$c before proceed with conventional reverse transcriptase (RT-PCR).

### *3.5.3. Primer selection*

A primer was designed for this study by referring to several studies. The forward and reverse primers were designed based on the virulence properties of the virus, the major outer capsid protein which is viral protein 2 (VP2) genes. Thus, VP2 gene was selected as forward primer and reverse primer. The primer designed for this study was coded for gene of VP2F and VP2R. The housekeeping gene used to test the bursa samples is 12SrDNA which target on avian mitochondrial DNA. The primer set and the journal sources were described in Table 3.1.

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Table 3.1 Primer set for detection of antigen in bursa samples.

| Primer | Sequences | References |
|---|---|---|
| **VP2 Forward** | 5'-CGC CAG GGT TTT CCC AGT CAC GAC AAC AGC CAA CAT CAA CG-3' | Moemen *et al.*, 2014 |
| **VP2 Reverse** | 5'-TCA CAC AGG AAA CAG CTA TGA CGC TCG AAG TTR CTC ACC C-3' | |
| **12SrDNA Forward** | 5'-CGA TTA GAT ACC CCA CTA TGC-3' | Bert *et al.*, 2005 |
| **12SrDNA Reverse** | 5'-AGG GTG ACG GGC GGT ATG TAC G-3' | |

### *3.5.4. Reverse Transcriptase Polymerase Chain Reaction (RT-PCR).*

An Access RT-PCR System Promega test kit was used and the manufacture protocol was followed (Promega, USA) to run the RNA samples. The assemble reaction was done in biosafety cabinet.The PCR reaction is set up as below. The master mix was prepared as shown in Table 3.2.

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Table 3.2 Master mix preparation.

| Reagent | Volume (µl) |
| --- | --- |
| **Nuclease-free water** | 30 |
| **AMV/*Tfl* 5X Reaction Buffer** | 10 |
| **dNTP Mix 10 mM** | 1 |
| **25mM $MgSO_4$** | 2 |
| **AMV Reverse Transcriptase (5µ/5µl)** | 1 |
| **Recombinant RNAsin® ribonuclease inhibitor** | 1 |
| ***Tfl* DNA polymerase (5µ/µl)** | 1 |
| **Primers (VP2F and 12SrDNA-F)** | 1 |
| **Primers (VP2R and 12SrDNA-R)** | 1 |
| **RNA samples** | 2 |

The entire reagents were kept on ice during handling. Then, the mixture was gently vortexed. The reaction was initiated by adding in 2µl of each RNA samples in tube 1 to tube 9 to the final volume of 50µl for each tube. 48µl of nuclease-free water was added into tube as negative control and 2µl of Volvac® IBD live vaccine was also added into tube 11 to the final volume of 50µl as positive control. The tubes were then placed in PCR thermocycler (SensoQuest labcycler, Germany) as set in Table 3.3 below.

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Table 3.3 Optimized cycling condition of RT-PCR assay for detection of IBDV.

| **Steps** | **Time** | **Temperature** | **Cycle** |
|---|---|---|---|
| **Reverse transcription** | 45 sec | $45^0$c | 1 |
| **Initial heat activation** | 45 min | $45^0$c | |
| **Denaturation** | 30 sec | $94^0$c | 40 |
| **Annealing** | 1 min | $52^0$c | |
| **Extension** | 2 min | $68^0$c | |
| **Final extension** | 7 min | $68^0$c | 1 |
| **Soak** | | $4^0$c | 1 |

### *3.5.5. Agarose gel preparation.*

1.5% of Agarose gel was prepared by diluting 1.5g of agarose powder into 100 ml of TAE buffer and medium and was heated in microwave (Electolux, USA) for 3 minutes to dissolve the powder. The gel was then cooled down and mixed with 2µl of SYBR ® Safe DNA gel stain (Invitrogen, USA). The gel then poured into gel cassette with a well forming comb to solidify.

### *3.5.6. Agarose gel electrophoresis.*

The agarose gel was transferred into Bio-Rad electrophoresis tank (Bio-Rad, USA). The TAE buffer was filled into the tank to immerse the gel. 1µl of 100bp DNA ladder was diluted from stock (Invitrogen, USA) into 9µl of deionized water. 2µl

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ladder solution was mix with 2µl of Blue Orange 6x Loading Dye (Promega, USA) and pipetted into well number 1. 3µl of PCR products were pipetted out from each tube and mixed with 1µl Blue Orange 6X Loading Dye (Promega, USA) then pipetted into well number 2 to 14. Agarose gel electrophoresis was ran at voltage 80 volt for 60 minutes using Bio-Rad electrophoresis set (Bio-Rad, USA.). The gel was then placed in Bio-Rad Gel Doc Imaging System (Bio-Rad, USA) under UV exposure. The image was captured and analysed with Image Lab ™ software.

### 3.6. Statistical analysis

Statistical analysis was used to analyse the possible correlation of clinical signs and vaccine dosage used Chi-square and length of bursa of Fabricius used Two-way ANOVA by GraphPad Prism 5 software. The result were interpreted at significance level of $p < 0.05$.

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# 4.0. RESULTS

## *4.1. Observation of clinical signs*

The most prominent clinical signs shown by the quails from group A and B were depression and ruffled feathers while Group C shown no clinical signs at all as the quails were bright and alert as shown in table 4.1 below.

Table 4.1 clinical signs of quails.

| Clinical signs | Ruffled feather | Depressed | No clinical signs |
|---|---|---|---|
| Group A | 8/8 | 0 | 0 |
| Group B | 8/8 | 8/8 | 0 |
| Group C | 0 | 0 | 8/8 |

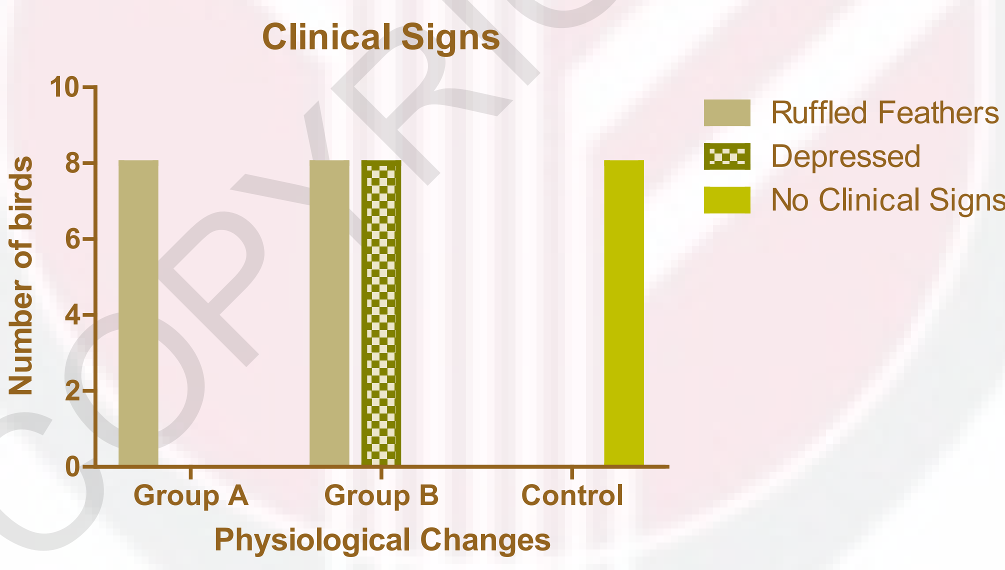


Figure 4.1 graph of clinical signs shown by quails from each group. There is significant clinical signs on quails infected with IBDV intermediate strain at $p < 0.05$.

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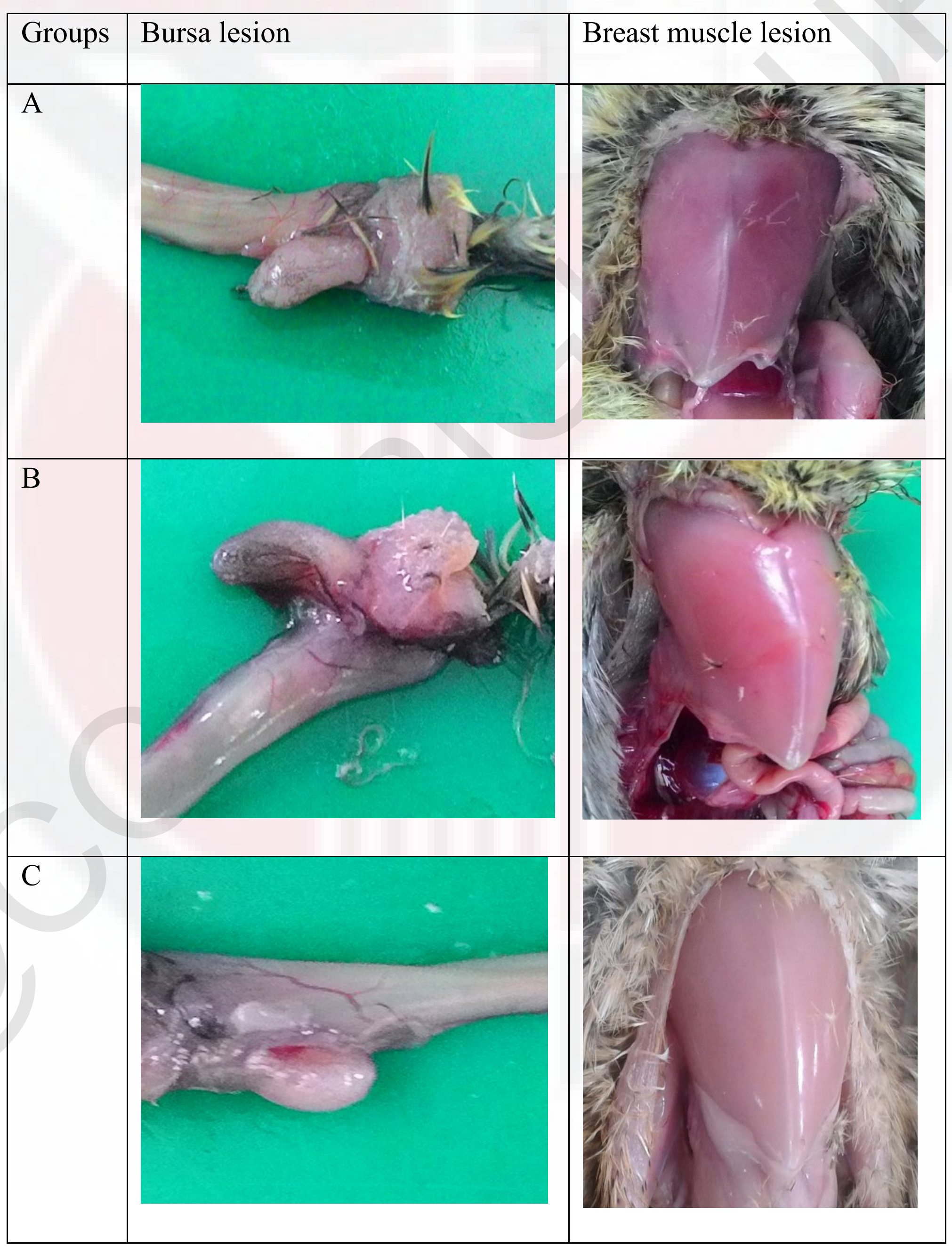
### *4.2 Post-mortem examination*

Table 4.2 below shown the post-mortem examination on breast muscle and bursa of Fabricius on quails from each groups. Among the groups there are no significant gross post-mortem lesions on the organs examined.

Table 4.2 Bursa of Fabricius and breast muscle of quails from each group.



| Groups | Bursa lesion | Breast muscle lesion |
|---|---|---|
| A | | |
| B | | |
| C | | |

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### *4.2.3. Bursal length*

Bursal length were measured to see the effect of vaccine given on the length of bursae between all groups at three different days post-infection.

Table 4.3: Length profile of bursa of Fabricius of IBD infected and non- infected quails at different days post infection (Mean ±SD)

| Age (days post-infection) | Bursa length (mm) Group A | Bursa length (mm) Group B | Bursa length (mm) Group C |
|---|---|---|---|
| 5 | 0.70 ± 0.282 | 0.80 ± 0.00 | 0.50 ± 0.00 |
| 9 | 11.0 ± 1.00 | 10.0 ± 2.00 | 10.5 ± 4.95 |
| 15 | 13.33 ± 1.15 | 13.33 ± 1.55 | 12.0 ± 2.88 |

There were not many differences of mean of bursal length among Group A, B and C on day 5, 9 and 15 post-infection.

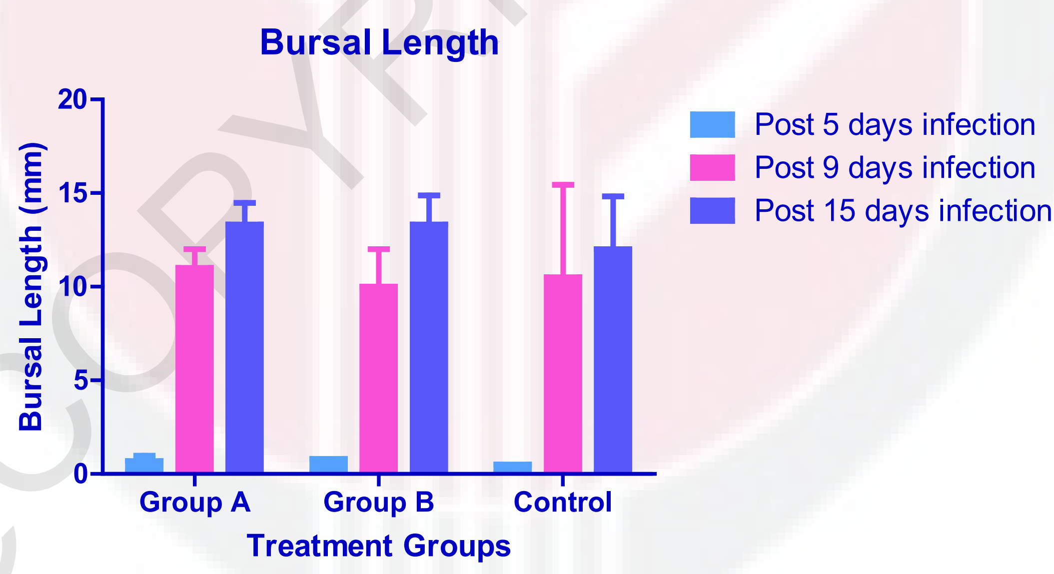


Figure 4.2 Bursal length of quails for each group at three different days post-infection.

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Based on the graph above, the result were interpreted at significant level of $p < 0.05$. The comparison of bursal length according to each group on different day post-infection can be summarized as in table 4.4 below.

| Group | A | B | C |
|---|---|---|---|
| Days 5 vs 9 | Extremely significant | Very significant | Very significant |
| Day 5 vs 15 | Extremely significant | Very significant | very significant |
| Day 9 vs 15 | No significant | No significant | No significant |

Table 4.4 The comparison of bursal length from all group at three different days post-infection.

The comparison of bursal length between vaccines challenged group vs control group shown very significant difference of bursal length. The comparison of bursal length between Day 5, Day 9 and Day15 post-infection showed no significant difference of the bursal length. The comparison of bursal length for challenged group vs days post-infection showed no significant difference of bursal length between each group and days post-infection.

### ***4.2.2. Histopathological examination***

The Muskett score were used for lymphoid depletion scoring according to Muskett et al. (1979).

Table 4.5. Microscopic examinations on histopathological changes on bursae sample.

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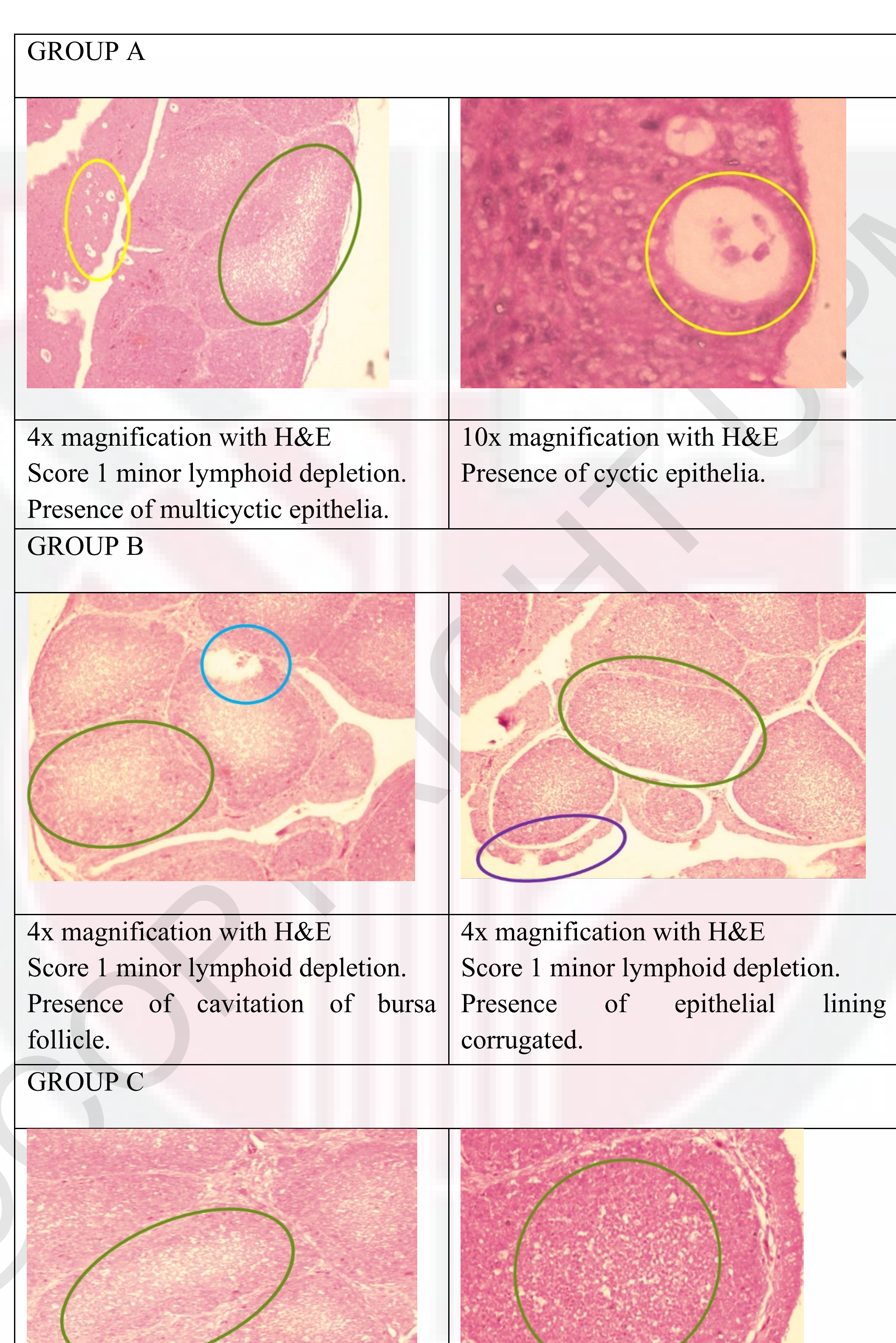

| GROUP A | |
|---|---|
| 4x magnification with H&E<br>Score 1 minor lymphoid depletion.<br>Presence of multicyctic epithelia. | 10x magnification with H&E<br>Presence of cyctic epithelia. |
| GROUP B | |
| 4x magnification with H&E<br>Score 1 minor lymphoid depletion.<br>Presence of cavitation of bursa follicle. | 4x magnification with H&E<br>Score 1 minor lymphoid depletion.<br>Presence of epithelial lining corrugated. |
| GROUP C | |

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| 4x magnification with H&E stain<br>Score 1 minor lymphoid depletion | 10x magnification with H&E stain<br>Score 1 minor lymphoid depletion |
|---|---|

### *4.3. Demonstration of antigen using RT-PCR*

All bursae sample tested shown negative result upon tested with primer VP2R and VP2F.

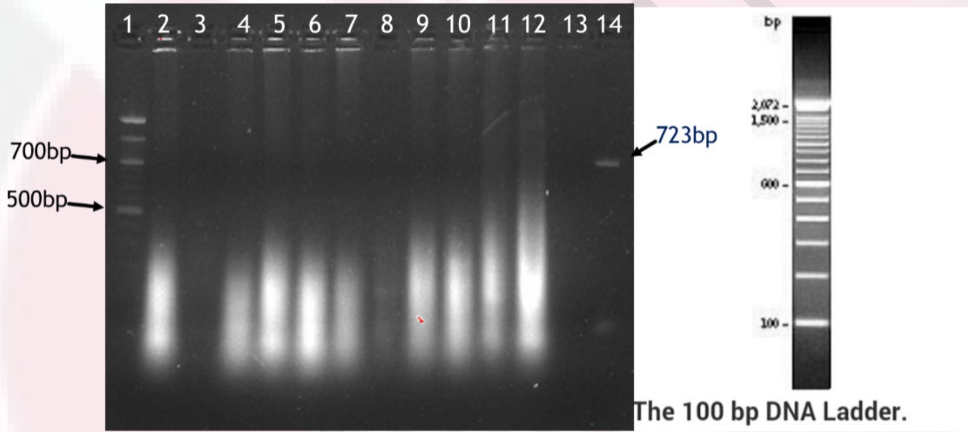


Figure 4.3 RT-PCR of bursae sample. 1)100bp ladder, 2-10) bursae sample, 11-12) 12SrDNA, 13) negative control, 14) positive control.

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## 5.0. DISCUSSION

The significant clinical signs shown by quails from each group are related to the titer of vaccine administered intraocularly once on 7 days old quail chicks on day-1 study is conducted. Group A that were administered 0.03ml of $10^{4.8}$ $TCID_{50}$/ml Volvac® IBD MLV, the quails showed ruffled feathers. Group B that were administered 0.03ml of $10^{5.5}$ $TCID_{50}$/ml Volvac® IBD MLV; the quails showed ruffled feathers and depression. Group C as control that was administered 0.03ml sterile phosphate buffered saline (PBS); the quails showed no clinical signs at all as the quails were bright and alert throughout the experiments. By comparing the clinical signs shown from each group, it can be concluded that there is significant clinical signs on quails infected with IBDV intermediate strain at P ($<0.05$).

The gross post-mortem examination on breast muscle, thigh muscle, spleen and bursa of Fabricius for quails from each group on day 5, 9 and 15 post-infection showed no lesion as the quails might have high maternal-derived antibody and the vaccine titre given may not be sufficent enough to neutralise the high maternal derived antobody in the quail chicks.

The measurement of bursal length is indicated in this study to know the effects of IBDV on bursal length other than bursal weight. Based on the mean and standard deviation, there were not much difference between bursal length of quails from each group especially on day 9 and day 15 post-infection. By using two-way ANOVA and interpreted at significant level of $p<0.05$, the bursal length on day 5 vs day 9 and day 5 vs day 15 showed an extremely significant difference for Group A, very significant difference for Group B and Group C. The bursal length comparison between groups shown an extremely significant difference. The histopathological examination

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revealed minor lymphoid depletion on all bursae sample from group A, B and C with score 1 according to Muskett *et al.* (1979). There were also presence of multicyctic epithelia, cavitation of bursa follicle and epithelial corrugated. However, the bursae lesion is considered as normal for score 1 shown by bursae sample from Group A, B and C as the quails may under stress as the room condition did not mimic the natural environment for quails.

Demonstration of antigen using RT-PCR revealed negative findings for all bursae sample from each group on all three time point of post-infection day on lane 2 to 10. The range of RNA extract following RNA concentration and purification was 1138.8 ng/µl to 3027.84 ng/µl and the ratio was 1.05 to 1.99. VP2F and VP2F primer were optimized before RT-PCR being run. However, for 12 SrDNA-F and 12 SrDNA-R primers which target the avian mitochondrial DNA; no optimisation of cycle condition being done before due to time constraint and resulted in negative expression of band on lane 11 and lane 12.

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## 6.0. CONCLUSION

There is significant clinical signs shown by the quails among Group A, B and C with no gross post-mortem lesion being examined on the breast muscle, thigh muscle, spleen and bursa of Fabricius, no significant histopathological changes found upon microscopic examination and absence of IBDV antigen for all samples when analysed with RT-PCR.

Based on these findings, it can concluded that there is no correlation between bursal length and histopathological changes and also clinical, pathological and molecular results of this experiment indicate that IBD MLV intermediate strain do not produce sufficient immune response in quails to warrant them as a carrier or host.

## 7.0. RECOMMENDATION

Optimization of cycle condition of primer 12SrDNA should be performed before RT-PCR being conducted. Bursal bodyweight ratio and bursal index should be measured instead of taking the length and breadth to determine the degree of bursae atrophy. Further study is needed to determine the susceptibility of quails towards IBD as this study used MLV vaccine instead of field strain IBD virus. Finally, the involvement of quails in epidemiology of IBD to the chickens need to be clarified as quails farms were in the same area with chicken farms.

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