UNIVERSITI PUTRA MALAYSIA

EXPERIMENTAL INTRAOCULAR INFECTION OF JAPANESE QUAILS (*CORTUNIX COTURNIX JAPONICA*) WITH GENOTYPE VII NEWCASTLE DISEASE VIRUS

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LIZMA FELISHA BINTI MAZLAN

A project paper submitted to the

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partial fulfillment of the requirement for the

DEGREE OF DOCTOR OF VETERINARY MEDICINE

Universiti Putra Malaysia,

Serdang, Selangor Darul Ehsan.

MARCH 2016

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CERTIFICATION

It is hereby certified that we have read this project paper entitled "Experimental Intraocular Infection of Japanese Quails with genotype VII Newcastle Disease Virus", by Lizma Felisha binti Mazlan and in our opinion it is satisfactory in terms of scope, quality, and presentation as partial fulfillment of the requirement for the course VPD 4999– Project

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DEDICATIONS

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

To my family,

Mazlan bin Amat

Haliza Hamim binti Munif

Lizma Alfasha binti Mazlan

To my supervisor,

Dr. Mohd Hezmee bin Mohd Noor

To my co-supervisors,

Prof. Dr. Abdul Rahman bin Omar

Dr. Lokman Hakim bin Idris

Dr. Tan Sheau Wei

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LIST OF ABBREVIATIONS

%	Percent
µl	Microliter
µM	Micromolar
˚C	Degree Celsius
APMV-1	Avian paramyxovirus serotype-1
BHQ1	Black hole quencher
Cq	Quantification cycle
Dpi	day post-infection
ELD_{50}	50 percent of embryo lethal dose
F	Fusion
FAM	Fluorescent reporter dye 5-carboxyfluorescein
HA	Haemagglutination
HI	Haemagglutination Inhibition
H&E	Hematoxylin and eosin
IACUC	Institutional Animal Care and Use Committee
ICPI	Intracerebral pathogenicity index
Ml	Milliliter
MDT	Mean death time
n	Sample size
Nm	Nanometer
N/D	Not detected
ND	Newcastle disease
NDV	Newcastle disease virus
NTC	No template control

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OD	Optical density
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
rpm	Rotation per minute
RNA	Ribonucleic acid
RT-qPCR	Reverse transcription real-time polymerase chain reaction
SPF	Specific pathogen free
TRBC	Total red blood cell
UPM	Universiti Putra Malaysia
x	Relative concentration
x g	Relative centrifugal force

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ABSTRAK

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

EKSPERIMEN JANGKITAN INTRAOKULAR PUYUH JEPUN (*COTURNIX COTURNIX JAPONICA)* DENGAN VIRUS NEWCASTLE DISEASE GENOTYPE VII

Oleh

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2016

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Kadar prevalens kajian Newcastle disease (ND) dalam industri puyuh di Malaysia tidak direkodkan dengan terperinci untuk mengenalpasti peranan sebenar puyuh Jepun dalam epidemiologi ND. Genotype VII Newcastle disease virus (NDV) ialah virus utama yang tersebar di Malaysia dan kajian ini bertujuan untuk menentukan kecenderungan

pendedahan puyuh Jepun terhadap genotype VII NDV. Tanda-tanda klinikal, perubahan kasar patologi organ, pengesanan virus yang positif di dalam organ dan calitan kloaka dan penunjukkan titer antibodi digunakan sebagai parameter untuk mengetahui kecenderungan pendedahan puyuh Jepun terhadap genotype VII NDV. Di dalam kajian ini, sebanyak 20 ekor puyuh telah dibahagikan kepada tiga kumpulan (n= 8 untuk Kumpulan A and B; n= 4 untuk Kumpulan Kawalan). Puyuh yang berada Kumpulan A dan B, masing-masing telah dijangkitkan dengan 0.03 ml $10^{3.5}$ ELD_{50} dan $10^{7.0}$ ELD_{50} NDV strain IBS 002, melalui kaedah intraokular manakala kumpulan kawalan hanya menerima 1x larutan penimbal fosfat. Kemurungan serta bulu tidak kemas, rales (berderak), kelumpuhan kaki dan tortikolis telah dilihat oleh sebahagian puyuh untuk kedua-dua kumpulan jangkitan. Berpandukan analisis statistik, tiada perbezaan signifikan *($p > 0.05$)* bagi tanda-tanda klinikal antara kedua-dua kumpulan jangkitan Calitan kloaka dilakukan pada hari ketujuh selepas jangkitan untuk kesemua puyuh. Calitan itu tertakluk kepada transkripsi-berbalik reaksi realiti masa rantai polimerase (RT-qPCR) untuk pengesanan virus dan keputusannya ialah negatif untuk kesemua kumpulan puyuh. Nekropsi puyuh dijalankan pada hari ketujuh selepas jangkitan dan tiada perubahan kasar patologi organ-organ dalaman puyuh untuk kedua-dua kumpulan jangkitan. Trakea, proventrikulus, dan seka tonsil telah diambil untuk pengesanan NDV oleh RT-qPCR. Terdapat pengesanan positif virus sebahagian daripada sampel organ untuk kedua dua kumpulan jangkitan. Ujian serologi menggunakan ujian Penghemaglutinatan-Perencatan menunjukkan peningkatan purata titer antibodi merentas masa dan kumpulan jangkitan. Analisis statistik menunjukkan tiada perubahan signifikan *($p > 0.05$)* reaksi antibodi

merentas masa tetapi terdapat perubahan signifikan *($p < 0.05$)*antara kumpulan-kumpulan jangkitan. Rumusannya, puyuh Jepun cenderung terdedah kepada genotype VII NDV berdasarkan parameter yang dikaji.

Kata kunci: Virus Newcastle disease genotype VII, RT-qPCR, Ujian Penghemaglutinatan-Perencatan, kecenderungan pendedahan

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ABSTRACT

Abstract of the project paper presented to the Faculty of Veterinary Medicine in partial requirement for the course VPD 4999 - Project

EXPERIMENTAL INTRAOCULAR INFECTION OF JAPANESE QUAILS (*CORTUNIX COTURNIX JAPONICA*) WITH GENOTYPE VII NEWCASTLE DISEASE VIRUS

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2016

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The prevalence of Newcastle disease (ND) in quail industry in Malaysia is not well documented in order to clarify the actual role played by Japanese quails in the epidemiology of ND. Genotype VII Newcastle disease virus (NDV) is the most predominant virus circulating in Malaysia, thus this study was aimed to determine the susceptibility of Japanese quails towards genotype VII NDV. Clinical signs, gross pathological lesions of organs, positive detection of virus in organs and cloacal swabs as

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well as the expression of the antibody titer were used as parameters to assess the susceptibility of Japanese quails following infection of genotype VII NDV. In this experimental study, 20 quails were divided into three groups (n= 8 for Group A and B; n= 4 for Control Group). The quails in the Group A and B were infected via intraocular route with 0.03 ml of $10^{3.5}$ ELD_{50} and $10^{7.0}$ ELD_{50} of NDV strain IBS 002, respectively, while the control group received 1x phosphate-buffered saline (PBS). Depression and ruffled feathers, trachea rales, leg paralysis and torticollis were shown in some of the quails in both infected groups. Based on statistical analysis, there was no significant difference ($p > 0.05$) in clinical signs between the infected groups. Cloacal swabs that were taken on day seven post-infection for all quails were subjected to one-step reverse transcription real time polymerase chain reaction (RT-qPCR) for detection of virus and the results were found to be negative for all groups. Necropsy was conducted on day seven post-infection and there were no gross pathological lesions of organs observed for quails in both infected groups. Trachea, proventriculus, and caecal tonsil were taken for the detection of NDV by RT- qPCR, and some of the organ samples showed positive detections of virus in both infected groups. Haemagglutination inhibition (HI) assay showed an increase in mean titers of antibody across time and between infected groups but statistical analysis revealed no significant difference ($p > 0.05$) of antibody reaction across time, but significantly difference ($p < 0.05$) between infected groups. In summary, Japanese quails are susceptible to genotype VII NDV based on parameters assessed.

Key words: Genotype VII Newcastle disease virus, RT-qPCR, HI assay, susceptibility

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1.0 INTRODUCTION

Newcastle disease virus (NDV) or avian paramyxovirus type 1 (APMV-1) is a non-segmented, single-stranded, negative-sense RNA virus which is a member of the genus Avulavirus of the family Paramyxoviridae. The virus widespread among wild and domestic birds with all bird species and some other vertebrates, including humans (transitory conjunctivitis) are susceptible to be infected (Leighton and Heckert, 2007).

Generally, strains of NDV are classified as highly (velogenic), moderately (mesogenic), and weakly pathogenic (lentogenic) pathotypes (Alexander, 1988). NDV strains also grouped into different genotypes based on the sequence and phylogenetic analysis of the F gene. NDV strains have been classified into ten genotypes (I-X), with genotypes VI and VII being further divided into seven (VIa–VIg) and five (VIIa–VIIe) sub-genotypes, respectively (Liu *et al.,* 2003; Tsai *et al.,* 2004). Recently, Shohaimi *et al*. (2015) reported the emergence of new subgenotype known as subgenotype VIIh, showing that many NDV isolates recovered during 2010-2011 were caused by subgenotype VIIh. Nowadays, genotype VII NDV is the predominant virus circulating in Asia including Malaysia and numerous geographical regions, such as Europe, China, Middle East and South Africa were also reported about the virus circulation in those regions since 1990s (Roohani *et al.*, 2015).

The prevalence of Newcastle disease (ND) in quail industry in Malaysia is not well documented in order to clarify the actual role played by the quails (*Coturnix coturnix japonica*) in the epidemiology of ND, particularly genotype VII NDV.

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This study was undertaken to determine the susceptibility of Japanese quails towards velogenic genotype VII Newcastle disease virus by observing clinical signs and gross pathological lesions of organs, presence of viruses in organs and cloacal swabs as well as expression of antibody titer.

For this research, the proposed hypothesis was Japanese quails is susceptible towards genotype VII NDV with the expectations of:

i) Clinical signs of ND will be observed in quails following the infection of genotype VII NDV
ii) Gross pathological lesions of organs will be seen following infection of genotype VII NDV
iii) NDV will be detected in organ samples of quails by one-step reverse transcription real-time polymerase chain reaction (RT-qPCR)
iv) NDV will be detected in cloacal swab specimens of quails by one-step reverse transcription real-time polymerase chain reaction (RT-qPCR)
v) Expression of antibody against NDV will be detected in quails following infection of genotype VII NDV

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2.0 LITERATURE REVIEW

2.1 Japanese quails

The Japanese quail belongs to the order *Galiformes*, genus *Coturnix*, and species *japonica*. The scientific designation for Japanese quail is *Coturnix coturnix japonica*. The Japanese quails are widely distributed in many countries around the world due to good performance in meat and egg production (Banks, 1979). It is a suitable model for poultry research due to their small size that require little cage space for rearing and a short reproductive life cycle (Ruskin, 1991). Seet and Azizah (1987) claimed that quail farming in Peninsular Malaysia, as compared with chicken farming, is rather scarce.

2.2 Newcastle disease threat in Japanese quails

Newcastle disease (ND) is complicated and unique as different isolates and strains of the virus may induce enormous variations in the severity of disease (Beard and Hanson, 1984). For years, it had been established that quails are resistant to diseases commonly affecting poultry. Fah (2005) claimed quails appear even hardy than chickens and with proper management, mortalities is not an issue. Hence, they require no preventive measure such as vaccination. For the quail raisers, this assumption is being debated as some deaths encountered in the farms are manifesting similar signs to the common diseases affecting poultry. However, this was not validated since no diagnostic confirmation or testing were performed. Higgins and Wong (1968) and Higgins (1971) stated that Japanese quails are rather resistant to Newcastle disease virus (NDV) infection with exception they may become infected under stress condition. Lima *et al*. (2004) reported all Japanese quails

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infected with viscerotropic strain of NDV do not show clinical signs or lesions indicatives of NDV, however the SPF-broilers allocated with the infected quails were dead three days post-infection and, showed clinical signs and macroscopic lesions and had positive antibodies titers, suggesting Japanese quails as potential carrier of virus in the epidemiology of ND in extensive productions. Oladele *et al.* (2008) have recorded histopathological and haemagglutination inhibition (HI) antibody titer in Japanese quails experimentally infected with NDV. Czirjak *et al.* (2007) recorded 100% morbidity and mortality in an outbreak of ND in Japanese quails with severe clinical symptoms and pathological lesions in both digestive and nervous systems.

2.3 Genotype VII Newcastle disease virus

Recently, Roohani *et al*. (2015) claimed genotype VII Newcastle disease virus (NDV) strains were frequently reported from numerous geographical regions, such as Europe, China, Middle East and South Africa since 1990s. In addition, molecular epidemiology studies have indicated that genotype VII NDV is the predominant virus currently circulating in Asia including Malaysia (Roohani *et al.,* 2015) and most frequently associated with outbreaks of Newcastle disease (ND) in Malaysia (Shohaimi *et al.,* 2010). Tan *et al*. (2010) reported outbreaks of ND in Malaysia from 2000 to 2010 were primarily caused by genotype VII NDV.

Outbreaks and sporadic cases involving genotype VII NDV occurred occasionally although intensive vaccination programs have been implemented in many countries, even in vaccinated farms in South America (Abolnik *et al*., 2004) and Asian countries such as

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China (Rui *et al.,* 2010), Korea (Jeon *et al*., 2008), and Malaysia (Berhanu *et al., 2010*). The index for reported genotype VII outbreaks recorded in commercial broiler farm by the Department Veterinary Services, Ministry of Agriculture and Malaysia (unpublished data) in year 2009 and 2010 were 5 and 75 respectively. The outbreaks become more severe with index of 153 in year 2011 (Roohani *et al.*, 2015). The clinical manifestations of genotype VII of are highly virulent causing mortality rates up to 100% (Beard and Hanson, 1981). Conclusively, genotype VII strain may have increased its virulence in poultry over the years and these viruses are spreading to other locations around the world.

2.3.1. IBS 002 strain genotype VII Newcastle disease virus

Rasoli *et al.* (2014) reported Newcastle disease virus (NDV) isolate IBS002 was isolated in 2011 from a vaccinated broiler farm in Johor, Peninsular Malaysia. A sequence analysis of the fusion (F) cleavage site of the IBS002 confirmed that the virus is virulent (GenBank Accession No. JQ809695). Characterization of IBS002 intracerebral pathogenicity index (ICPI) of 1.76 and the mean death-time (MDT) of 51.2 hour indicated that the virus is classified as velogenic NDV. According to phylogenetic study based on the F gene, IBS 002 belongs to genotype VII (Roohani *et al.*, 2015).

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3.0 MATERIALS AND METHOD

3.1 Quails and management

Twenty Japanese quails (*Coturnix coturnix japonica*) of one week of age were purchased from Puyumas Farm Best Sdn Bhd. The quails were raised according to standard procedures in an open house system before being transferred to Universiti Putra Malaysia (UPM). For the Newcastle disease virus (NDV) challenge study, the quails were transferred to Experimental Animal House at Faculty of Veterinary Medicine, UPM. The Japanese quails were housed in cages, with water and feed offered ad libitum. The diet was based on corn and soy bean meal. Handling of the quails was conducted in accordance with the laboratory animal care guidelines, and the study protocol was approved by the Institutional Animal Care and Use Committee at the Faculty of Veterinary Medicine, UPM (reference no. UPM/IACUC/FYP.2015/FPV.032).

3.2 Experimental design

The quails were divided into three groups, which were eight quails for both Group A and Group B, and four quails for Control Group. Quails in the Group A and Group B were separately placed at different levels of a double-storey cage, in a room with controlled environment whilst quails in the Control Group was located in the different room and placed in a cage with a controlled environment. All the quails were given commercialized feed based on corn and soy bean meal and water ad libitum. Blood was taken in the representatives of quails and serum was taken for screening purpose before the infection to establish that quails were clinically healthy and serologically negative for Newcastle

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disease (ND) by hemagglutination inhibition assays (HI). Inoculation of IBS 002 strain of genotype VII Newcastle disease virus (NDV) was done via intraocular route; quails in Group A were given $10^{3.5}$ ELD_{50} and quails in Group B were given $10^{7.0}$ ELD_{50}. Quails in the Control Group received 0.03 ml of 1x sterile phosphate buffered saline (PBS) via intraocular route. Clinical lesions in all quails after the infection with NDV were recorded. Cloacal swab sampling was done on 7 days post-infection (dpi) for all quails from both infected groups and the Control Group for detection of NDV by reverse transcriptase real-time polymerase chain reaction (RT-qPCR). Necropsy was conducted on the same day, 7 dpi, for halves of the quails from each group to examine the gross lesions of the organs following experimental infection with NDV. Organs such as trachea, proventriculus, and caecal tonsil were taken for the detection of NDV by RT-qPCR. In addition, the remaining quails (n = 4 for both Group A and B; n = 2 for Control Group) were kept for blood collection to obtain the serum for the measurement of antibody against NDV using haemagglutination inhibition (HI) assay. Blood was collected at basilic vein on 10 dpi, 14 dpi and 21 dpi. After third blood collection was performed on 21 dpi, all quails were euthanized through cervical dislocation method. All experimental procedures were carried out according to the UPM's Institutional Animal Care and Use Committee (IACUC) guidelines.

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3.3 Genotype VII Newcastle disease virus

The virus stock of IBS 002 strain, $10^{7.5}$ ELD_{50} /0.1ml was obtained from the Laboratory of Vaccine and Immunotherapeutics, Institute of Bioscience (IBS), UPM. Virus with a concentration of $10^{7.0}$ ELD_{50} /0.03ml was prepared and 10-fold serial dilutions with 1x phosphate buffered saline (PBS) was done to produce $10^{3.5}$ ELD_{50} /0.03 ml.

3.4 Observation of clinical signs

The clinical signs of quails were observed twice daily from 1 day post-infection (dpi) until 21 dpi to observe if there were clinical lesions manifested by Japanese quails following infection of IBS 002 strain. The clinical signs that were expected to be shown by quails were based on research studies conducted by Rasoli *et al.* (2014) and Roohani *et al*. (2015) ; clinical signs of infected chickens with IBS 002 strain include diarrhea, trachea rales, depression and ruffled feathers, loss of appetite, mortalities and neurological signs.

3.5 Gross pathological lesions of organs

Necropsy of quails was performed on 7 days post-infection (dpi) involving four birds from both group A and B and two birds from the Control Group. Observation of gross post mortem lesions in quails is important to prove that infection of Newcastle disease virus (NDV) will produce significant lesions signs towards quails. The gross pathological lesions of organs that were expected to be shown by quails infected with IBS 002 were referred to gross pathological lesions of organs of chicken that have been infected with velogenic NDV. Lesions such as congestion and haemorrhages at muscle, trachea, and

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visceral organs such as proventriculus, intestine, caecal tonsil, and spleen was expected to be seen in infected quails.

3.6 Histology examination

Histology examination was carried out to determine the tissue lesions at the microscopic level. The tissue were cryopreserved and sectioned to 10 micron thickness. The sections were stained with haematoxylin and eosin (H&E) with slight modification as per the standard procedures (Luna, 1968).

3.7 Serology testing

3.7.1. Serum collection

A total of 37 serum samples were collected including eight blood samples prior infection for screening purpose, 10 blood samples (n = 4 for both Group A and B; n = 2 for Control Group) on 10 days post-infection (dpi), 10 blood samples (n = 4 for both Group A and B; n = 2 for Control Group) on 14 dpi and nine blood samples (n = 4 for Group A, n = 3 for Group B ; n = 2 for Control Group) quails at 21 dpi. Blood was collected at the basilic vein using 29G of 1 ml syringe (TERUMO ® SYRINGE U-100 INSULIN, 29G × 1/2" (0.33 × 13mm). Prior to the procedure, collection site was cleansed and disinfected using 70% ethanol in a form of alcohol swabs (Heinz Herenz Hamburg / Germany). Collected blood was placed in a sterile plain test tube with rubber stopper and labelled accordingly. The blood collected in plain tubes were centrifuged at 5000 rpm for 15 minutes to obtain

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the serum. The hemagglutination (HA) assay and haemagglutination inhibition (HI) assay were conducted according to standard protocols (Allan and Gough, 1974).

3.7.2 Hemagglutination assay

Sterile 1x phosphate-buffered saline (PBS) with volume of 50 µl was added from second well through twelfth well. Next, 100 µl of genotype VII Newcastle disease virus (NDV) was added to the first well. A serial two-fold dilutions was made by transferring 50µl from the first well to successive eleventh and the final 50µl from eleventh was discarded. The twelfth well contains only 1x PBS as red blood cell control. Next, 50µl of 2% TRBC suspension was added to each well on the plate. The plate was gently mixed and the plate was incubated at room temperature until the cells settling out in the cell have formed a distinct button control well. Hemagglutination assay (HA) titration end point were measured by determining the highest dilution of virus that produced 100 % haemagglutination. Next, the dilution of antigen with 1x PBS was calculated by dividing the end point HA titer by eight. One part of virus with calculated parts of 1x PBS were mixed to obtain the four HA units of NDV genotype VII that was used as test antigen in completing the HI test.

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3.7.3 Haemagglutination inhibition assay

Sterile 1x phosphate-buffered saline (PBS) with volume of 25 µl was added from second well through twelfth well. Next, 50 µl of serum sample was added to the first well. A two - fold serial dilutions was made by transferring 25 µl from the first well to successive eleventh, and the final 25 µl from eleventh well was discarded. The well was added with 25 µl of four hemagglutination assay (HA) units of genotype VII Newcastle disease virus (NDV) from first well into eleventh well. The plate was gently mixed and incubated at room temperature (RT) for 30 minutes and later 25 µl of 2% TRBC suspension was added to each well on the plate. Antibody titers were measured by determining the highest dilution of serum that produced 100 % inhibition of haemagglutination.

3.8 Detection of Newcastle disease virus

3.8.1 Detection of Newcastle disease virus in cloacal swab specimens

Cloacal swab sampling was done on 7 days post-infection (dpi) from eight quails in both Group A and B, and four quails from Control Group. Sterile collection swab (Ref: CHIGW020S) was used for swabbing and the swab was immersed in 1 ml of 1x sterile phosphate-buffered saline (PBS) solution. Cloacal swab specimens were kept in -80°C for later use.

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3.8.2 Detection of Newcastle disease virus in organ samples

Trachea, proventriculus and cecal tonsil were collected on 7 days post-infection (dpi) after the necropsy was performed from four quails for both Group A and B and two quails from Control Group. In summary, each sample was minced using a pair of scissors and then transferred into individual sterile tube (CELLSTAR® Centrifuge Tube) containing 3 ml of 1x phosphate-buffered saline (PBS). The suspension was then homogenized using a homogenizer (TissueRuptor®, Qiagen, Germany) and kept in -80°C for later use.

3.8.3 Extraction of RNA

3.8.3.1 Extraction of RNA in cloacal swab specimens

RNeasy plus mini kit (Qiagen, Germany) was used to extract viral RNA. A sterile tube (CELLSTAR® Centrifuge Tube) containing a sterile swab with 1 ml of sterile 1x phosphate-buffered saline (PBS) were centrifuged at 1500 rpm for five minutes (Centrifuge 5415 D, Eppendorf, Germany). Aliquot of 400 µl was pipetted using a micropipette (Eppendorf, Germany) into a 1.5 ml collection tube (Eppendorf ® tubes) and 600 µl of Buffer RLT Plus was added. The collection tube was vortexed (Type 37600 Mixer, Thermolyne Maxi Mix® II Vortex Mixer) for 30 seconds. Aliquot of 600 µl then was transferred to gDNA Eliminator spin column placed in a 2 ml collection tube (supplied). The column was centrifuged for 30 seconds at 11,000 rpm (Centrifuge 5415 D, Eppendorf, Germany).The column was discarded and the flow through was kept for further processing. The process was continued with an addition of 600 µl of 70% ethanol to flow-through and mixed by pipetting using a micropipette (Eppendorf, Germany).

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Next, 700 µl of sample including any precipitate was transferred to an RNeasy spin column placed in a 2 ml of collection tube (supplied) and centrifuged for 15 seconds at 11,000 rpm (Centrifuge 5415 D, Eppendorf, Germany). The flow-through was discarded and 700 µl of Buffer RW1 was added to the RNeasy Mini spin column (in a 2 ml collection tube). The aliquot was centrifuged for 15 seconds at 11, 000 rpm (Centrifuge 5415 D, Eppendorf, Germany) and 500 µl Buffer RPE was added to the RNeasy spin column. The flow-through was discarded after centrifuged for 15 seconds at 11,000 rpm (Centrifuge 5415 D, Eppendorf, Germany). Next, 500 µl Buffer RPE was added to the RNeasy spin column and centrifuged for 2 minutes at 11,000 rpm (Centrifuge 5415 D, Eppendorf, Germany). The flow-through was discarded. RNeasy column was placed in a new 1.5ml collection tube (supplied) and 30 µl RNase free-water (Promega, USA) was added directly to the spin column membrane. Centrifugation for 1 minute at 11,000 rpm (Centrifuge 5415 D, Eppendorf, Germany) was carried out next to elute the RNA. The sample was stored in -20°C for future use.

3.8.3.2 Extraction of RNA in organ samples

The organ suspension was thawed and then centrifuged at 4000 rpm at 17ºC for 5 minutes (Centrifuge 5810 R, Eppendorf, Germany). Aliquot of 300 µl was pipetted into a 1.5 ml microcentrifuge tube (Eppendorf tubes®) and 750 µL TRIzol ® RNA Isolation Reagents (Invitrogen, USA) was added and mixed thoroughly by vortexing (Type 37600 Mixer, Thermolyne Maxi Mix® II Vortex Mixer) and was left still for 5 minutes at room temperature. Next, 200 µl of chloroform was added and mixed thoroughly by vortexing (

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Type 37600 Mixer, Thermolyne Maxi Mix® II Vortex Mixer) and was left still for 10 minutes and next, was centrifuged at 12000 rpm for 10 minutes (Centrifuge 5415 D, Eppendorf, Germany) . Aliquot of 500 µl was transferred into a new 1.5 ml microcentrifuge tube (Eppendorf tubes®) and 800 µl of isopropanol was added into microcentrifuge tube. The aliquot was mixed gently for the formation of white pellet (RNA) and later incubated in ice for 20 minutes. The aliquot was centrifuged at 14,000 rpm at 5ºC for 15 minutes (Centrifuge 5810 R, Eppendorf, Germany) and all the aliquot was discarded, leaving white RNA pellet to be only settled in the bottom of microcentrifuge tube. Next, 800 µl of 75% ethanol was added and centrifuged at 8000 rpm for 5 minutes (Centrifuge 5415 D, Eppendorf, Germany). Ethanol was discarded and 800 µl of 100% ethanol was added and centrifuged at 8000 rpm for 5 minutes (Centrifuge 5415 D, Eppendorf, Germany). The ethanol was discarded and the microcentrifuge tube was air-dried for 10 minutes. The RNA pellet was re-suspended in 30 µl RNase free water (Promega, USA) and kept in -20°C for later use.

3.8.4 Measurement of RNA concentration

After the RNA extraction, 2 µl of the aliquot was transferred into a µCuvette G1.0 (Eppendorf, Germany) using a micropipette (Eppendorf, Germany) and placed in a BioSpectrometer® (Eppendorf, Germany). For quantitating the amount of RNA, readings were taken at the wavelengths of 260 nm and 280 nm. An OD of 1 corresponds to 40 µg/ml for single-stranded RNA. The ratio between the readings at 260 nm and 280 nm (OD 260: OD 280) provides an estimate of the purity of the nucleic acid. Pure preparations of RNA have OD 260: OD 280 values of 1.8 and 2.0 respectively.

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3.8.5 One-step reverse transcription real-time polymerase chain reaction

Detection of Newcastle disease virus (NDV) was performed for 30 cloacal swab specimens and 20 organ samples. Extracted RNA was subjected to one-step RT-qPCR by iTaq Universal probes One-step Kit (Bio-Rad, USA). iTaq Universal Probes reaction mix and other frozen reaction components were thawed at 4°C. The solution was mixed thoroughly, centrifuged (Eppendorf, Germany), and stored and protected from light. The master mix reactions were prepared and all the components were scaled proportionally according to sample number and reaction volumes (Table 3.8.5.1). In RT-qPCR, the positive control used was velogenic NDV reference strain. Distilled water was used for no template control (NTC). Positive control and NTC were included to ensure the reaction has been set up correctly with no contamination of reagents or foreign RNA.

Table 3.8.5.1 : Reaction setup for one-step reverse transcription real-time PCR

Component	Volume	Final concentration
iTaq Universal Probes reaction mix (2X)	10.0 µl	1x
iScript reverse transcriptase	0.5 µl	1 unit
Forward primer (10µM)	1.0 µl	0.5 µM
Reverse primer (10 µM)	1.0 µl	0.5 µM
Velogenic Probe (5µM)	1.0 µl	0.25 µM
RNA (add later)	5.0 µl	100 ng/ul
dH_2O	1.5 µl	-
Total	20. 0 µl	-

All the required components were added except RNA and the reaction setup was mixed thoroughly to ensure homogenicity of aliquots in each PCR tube. Next, 5 µl RNA

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was later added to PCR tubes containing the reaction setup, tubes were tightly capped, and vortexed (Eppendorf, Germany) gently to ensured thorough mixing of the reaction components. The PCR tubes were loaded onto the real-time PCR instrument and the RT-qPCR run was started. The primers and probe used in the one-step RT-qPCR was described in Table 3.8.5.2.

Table 3.8.5.2: Primers and probe used in one-step reverse transcription real - time PCR

Primer/ Probe	Sequence	Position on F gene
Probe	5'(FAM)-AAGCGTTTCTGTCTCCTTCCTCCA-(BHQ)3'	396 to 373
Forward	5'TCCGCAAGATCCAAGGGTCT3'	342 to 361
Reverse	5' CGCTGTTGCAACCCCAAG3'	442 to 425

The amplification was performed using CFX96 ™ Real-Time system (Bio-Rad, USA). The optimized cycling conditions of the one-step RT-qPCRwere carried out as recommended by Rasoli *et al.* (2014) as described in Table 3.8.5.3. Data analysis was performed based on CFX™ Manager Software (Bio-Rad, USA).

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Table 3.8.5.3: Optimized cycling conditions of RT-qPCR assay for detection of IBS 002 strain of NDV

Step	Time	Temperature
Reverse transcription reaction	10 minutes	50 ºC
Polymerase activation and DNA denaturation	2 minutes	95 ºC
Denaturation	20 seconds	95 ºC
Annealing	30 seconds	58 ºC
Extension	15 seconds	72 ºC + data acquisition
Number of cycles	40 cycles	

3.9 Statistical analysis

The statistical analysis was conducted using the computer package GraphPad Prism, version 5.0. Test of independence, (Chi-square) was performed to measure the association of different challenge dose of virus towards the clinical signs shown by quails. Antibody titers were expressed as mean ± standard deviation of Log_2 HI titer. Next, analysis of variance (Two way ANOVA) was performed to measure the significant differences of antibody reaction across time and between groups. Differences were considered significant at alpha = 0.05.

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4.0 RESULTS

4.1 Clinical signs

Clinical signs of Newcastle disease (ND) in quails were observed started from 7 days post-infection (dpi). Clinical signs shown include depression and ruffled feathers, trachea rales, leg paralysis and torticollis. Depression and ruffled feathers were observed for all quails in infected group started from 7 dpi and markedly seen on 10 dpi. Depression and ruffled feathers were prominently expressed in quails from the Group B (Figure 4.1.3) as compared to quails in the Group A (Figure 4.1.1). Trachea rales started to be observed on 10 dpi with one quail in the Group A and two quails in the Group B. One quail from the Group A showed leg paralysis on 13 dpi (Figure 4.1.2) whereas leg paralysis was observed on 16 dpi in quail from the Group B (Figure 4.1.4). Torticollis was observed in one quail from the Group B on 14 dpi (Figure 4.1.5) and the quail was found dead on 16 dpi. All quails in the Control Group were bright and alert (Figure 4.1.6 and Figure 4.1.7). The frequency distribution of clinical signs shown by quails following infection of genotype VII Newcastle disease virus (NDV) was shown in Figure 4.1.8. Based on statistical analysis of Chi-square test (Graphpad Prism 5), there was no significant difference *($p > 0.05$)* of association of different challenged dose of virus towards the clinical signs shown by quails between Group A and B. All quails in infected group showed clinical signs of ND regardless of virus infection dosage.

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Figure 4.1.1: Mild depression and ruffled feathers in quails from the Group A

Figure 4.1.2: Leg paralysis observed on 13 dpi in quail from the Group A

Figure 4.1.3: A quail in the Group B showed ruffled feathers and depression

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Figure 4.1.4: Leg paralysis was showed in a quail from the Group B on 16 dpi

Figure 4.1.5: Torticollis was observed on 14 dpi in a quail from the Group B

Figure 4.1.6: Quails in the Control Group were bright and alerts

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Figure 4.1.7: A quail from the Control Group with bright eyes

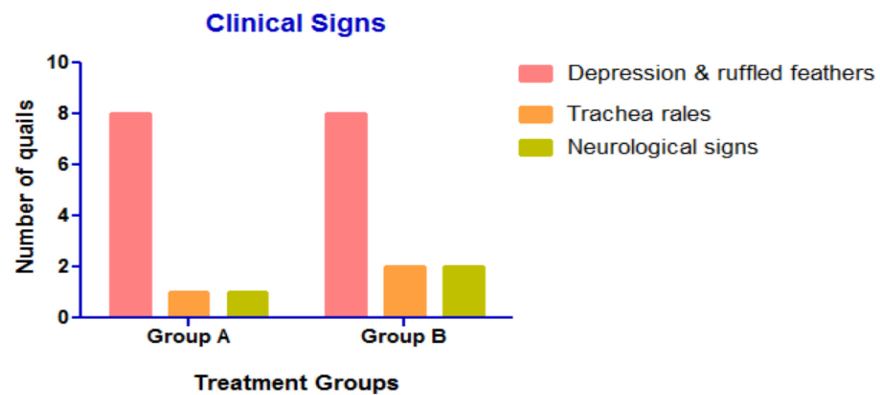

Table 4.1.8: Frequency distribution of clinical signs shown by quails following infection of genotype VII NDV

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4.2 Gross pathological lesions of organs

Quails in Control Group that were given 1x phosphate- buffered saline (PBS) did not show any gross pathological lesions (Figure 4.2.1) upon necropsy on 7 days post-infection (dpi). The result is also similar with quails in infected groups as there were no gross pathological lesions of organs were seen in quails for both Group A (Figure 4.2.2) and B (Figure 4.2.3) following infection of velogenic genotype VII Newcastle Disease virus (NDV) in quails. In addition, necropsy was done on the respected quail with torticollis that was found dead on 16 dpi, and there was no gross pathological lesions observed in brain and organs (Figure 4.2.4) following infection of genotype VII NDV.

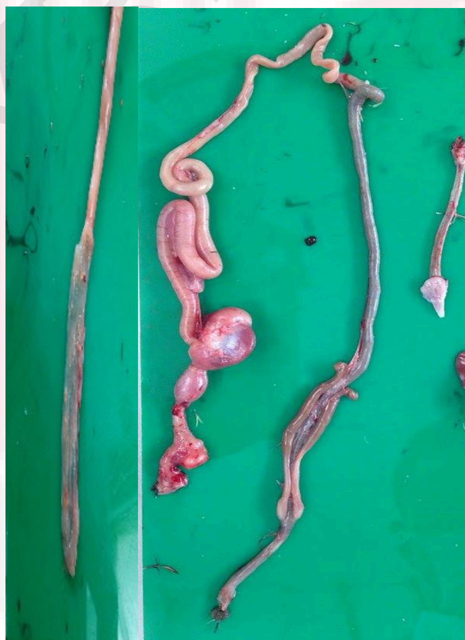

Figure 4.2.1: No gross pathological lesions of organs observed in quail (Control Group)

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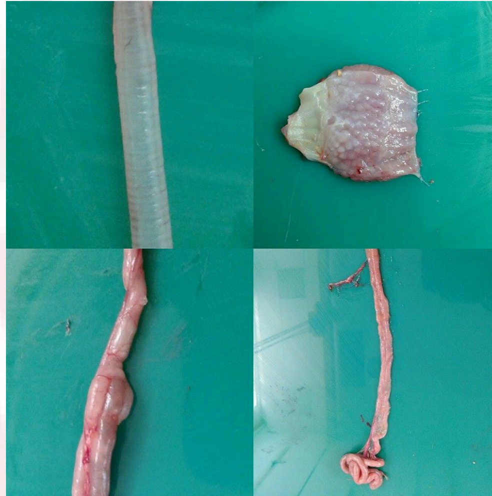

Figure 4.2.2: No gross pathological lesions observed in trachea (top left) proventriculus (top right), caecal tonsil (bottom left) and small intestine (bottom right) (Group A)

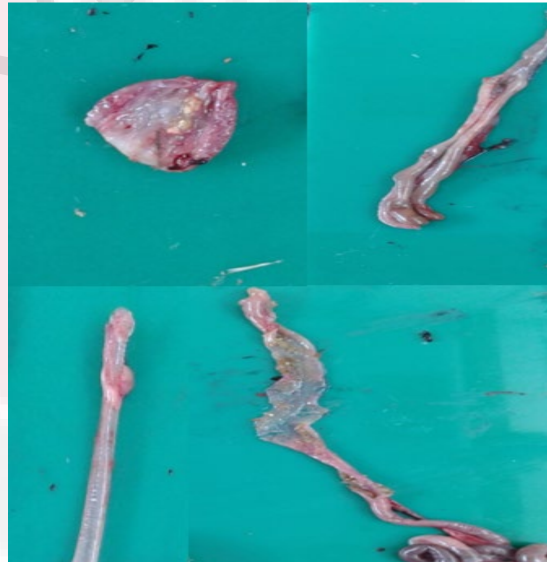

Figure 4.2.3: No gross pathological lesions observed in proventriculus (top left), caecal tonsil (top right), trachea (bottom left) and small intestine (bottom right) (Group B)

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Figure 4.2.4: No gross pathological lesions observed in brain and organs of a quail with torticollis

4.3 Histology examination

A quail with torticollis was found dead on 16 days post-infection (dpi) and the brain was processed for histology examination. Histology examination revealed the brain was filled with the accumulation of blue-stained lymphocytes, indicate encephalitis when observed under 10x magnification of compound microscope. There was also accumulation of lymphocytes within vessels of brain indicate vasculitis (Figure 4.3)

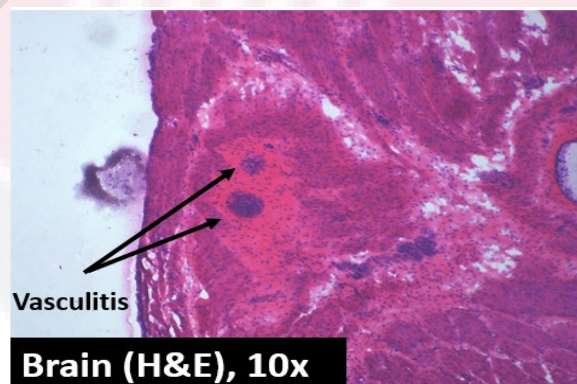

Figure 4.3: Histology examination reveals accumulation of blue-stained lymphocytes in brain (encephalitis) and vessels of brain (vasculitis)

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4.4 Serology testing

Screening was done in the representatives of quails before infection of genotype VII Newcastle disease virus (NDV) to prove that quails were clinically healthy and serologically negative for Newcastle disease (ND) by hemagglutination inhibition assays (HI). HI assay showed quails were serologically negative for ND. On 10 days post-infection (dpi) and 14 dpi, serum was collected from four quails for both Group A and Group B and two quails from Control Group. Positive expression of antibody is described by the presence of red blood cell that inhibit 100% agglutination, and confirmed by the formation of tear-drop shape of red blood cell when the titer plate is tilted. On 10 dpi, HI titer of quails from the Group A were ranging from not detectable to 16 whereas HI titer for quails from the Group B were ranging from 4 to 64. On 14 dpi, HI titer of quails from the Group A were ranging from not detectable to 128 whereas HI titer for quails from the Group B were ranging from 16 to 64. Meanwhile on 21 dpi, HI titer of quails from the Group A were ranging from not detectable to 128 whereas HI titer for quails from the Group B were ranging from 32 to 128. All the data were tabulated in Table 4.4.1. All the data were expressed as mean ± standard deviation of log_2 HI titer (Table 4.4.2). The data was analysed by Graphpad Prism 5.0, to measure the extent of antibody reaction across time and challenged dose. Based on statistical analysis of two way ANOVA, there was no significant difference $(p > 0.05)$ of antibody reaction across time, however the antibody reaction was significantly difference $(p < 0.05)$ between groups A and B. (Figure 4.4).

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Table 4.4.1: Antibody titer of quails on 10 days post-infection (dpi), 14 dpi, 21 dpi following infection of genotype VII Newcastle disease virus (NDV)

Group / Days post-infection	10 days post-infection		14 days post-infection		21 days post-infection	
* Group A ($10^{3.5}$ ELD_{50} IBS 002 strain)	HI titer	Log_2 HI titer	HI titer	Log_2 HI titer	HI titer	Log_2 HI titer
	N/D	N/D	N/D	N/D	N/D	N/D
	N/D	N/D	4	2	4	2
	2	1	8	3	16	4
	16	4	128	7	128	7
** Group B ($10^{7.0}$ ELD_{50} IBS 002 strain)	HI titer	Log_2 HI titer	HI titer	Log_2 HI titer	HI titer	Log_2 HI titer
	4	2	16	4	32	5
	16	4	32	5	64	6
	16	4	32	5	128	7
	64	6	64	6	-	-
*** Control group (1x sterile phosphate-buffered saline)	N/D	N/D	N/D	N/D	N/D	N/D
	N/D	N/D	N/D	N/D	N/D	N/D

- : mortality
N/D : not detected

* Four quails were used as sample size in Group A (n = 4) on 10, 14 and 21 days post-infection
** Four quails were used as sample size in Group B (n = 4) on 10 and 14 days post-infection. Only three quails were used as sample size on 21 days post-infection (one of the quail was found dead on 16 days post-infection due to torticollis)
*** Two quails were used as sample size in Control Group (n = 2) on 10, 14 and 21 days post-infection

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Table 4.4.2: Mean ± SD of antibody expressed in Log_2 HI titer

Group / Days post-infection	10 days post-infection	14 days post-infection	21 days post-infection
Group A ($10^{3.5}$ ELD_{50} IBS 002 strain)	1.3 ± 1.9	3.0 ± 2.9	3.3 ± 3.0
Group B ($10^{7.0}$ ELD_{50} IBS 002 strain)	4.0 ± 1.6	5.0 ± 0.8	6.0 ± 1.0
Control Group (1x sterile phosphate-buffered saline)	N/D	N/D	N/D

N/D: not detected

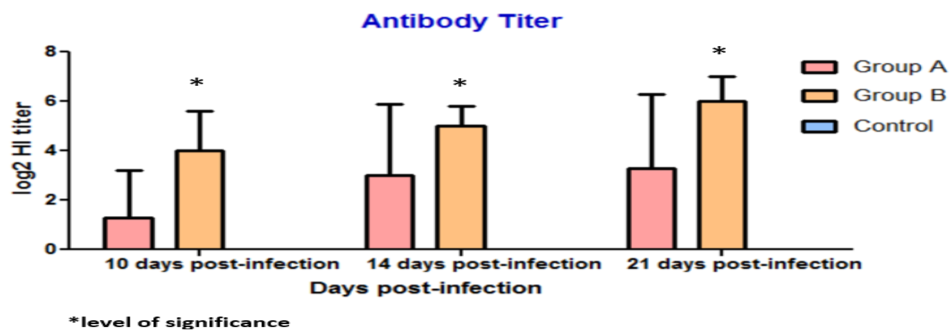

Figure 4.4: Expression of antibody across time and dose of infection based on two-way of ANOVA

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4.5 Detection of Newcastle disease virus by one-step reverse transcription real time polymerase chain reaction (RT-qPCR)

Reverse transcription real time polymerase chain reaction (RT-qPCR) was performed using specific primers targeting partial of the fusion (F) gene. The forward primer used was NDV- F: 5’ TCCGCAAGATCCAAGGGTCT 3’, and reverse primer was NDV – R: 5’ CGCTGTTGCAACCCCAAG 3’ with the velogenic probe of 5’ (FAM) - AAGCGTTTCTGTCTCCTTCCTCCA-(BHQ1) 3’. Quantification cycle (Cq) is the intersection between an amplification curve and threshold line. Cq indicates the specific amplification product of the partial fusion (F) gene that represents positive detection.

4.5.1 Detection of Newcastle disease virus in cloacal swab specimens

Cloacal swab sampling was done for all the quails from both infected groups and the control group (n = 20). Reverse Transcription Real Time Polymerase Chain Reaction (RT-qPCR**)** assay showed there was no amplification curve of cloacal swab specimens detected (Figure 4.5.1). All the generated data for RT-qPCR was tabulated (Table 4.5.1)

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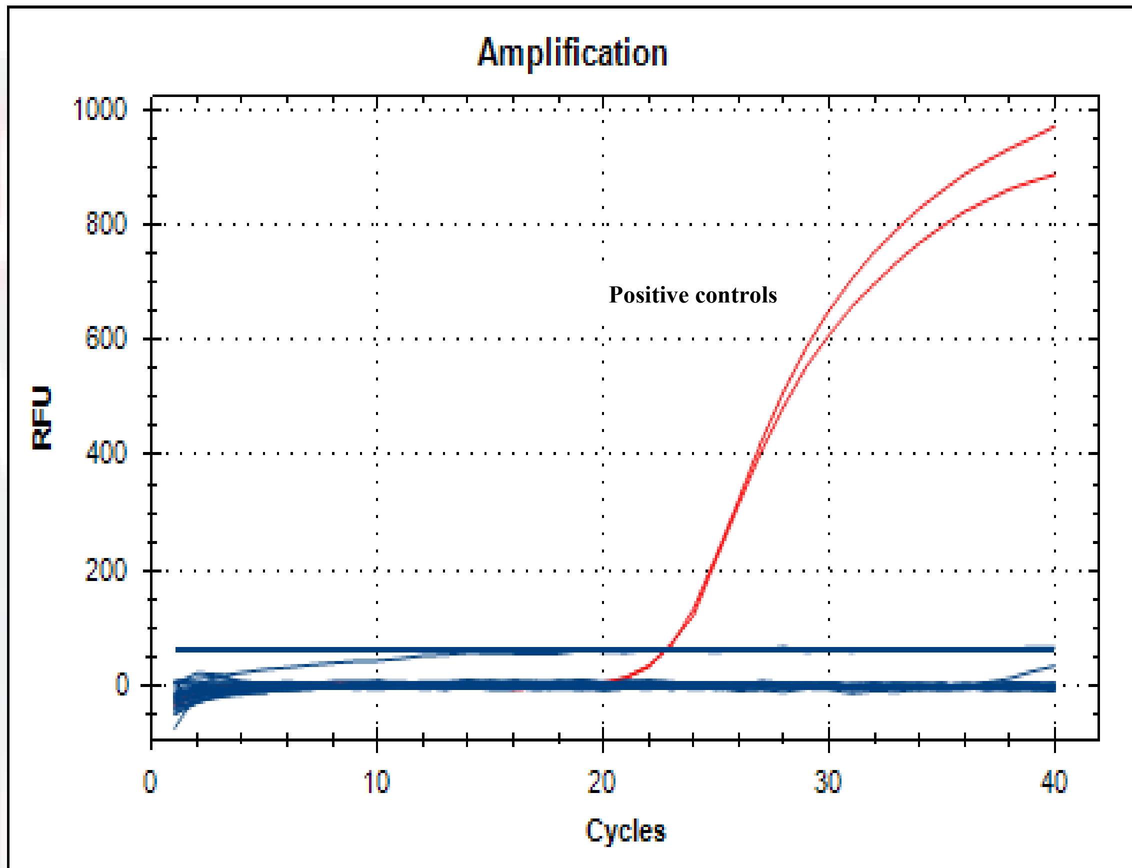

Figure 4.5.1: Assay of RT-qPCR for cloacal swabs specimens using specific primers targeting the partial of fusion (F) gene of velogenic genotype VII NDV. The amplification were observed only in positive controls (red curves)

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Table 4.5.1 : Detection of virus in cloacal swab specimens by RT-qPCR

Well	Fluorescent *	Target	Sample name**	Quantification cycle, Cq ***
A06	FAM	Velogenic	A1	N/D
B06	FAM	Velogenic	A2	N/D
C06	FAM	Velogenic	A3	N/D
D06	FAM	Velogenic	A4	N/D
E06	FAM	Velogenic	A5	N/D
F06	FAM	Velogenic	A6	N/D
G06	FAM	Velogenic	A7	N/D
H06	FAM	Velogenic	A8	N/D
A07	FAM	Velogenic	B1	N/D
B07	FAM	Velogenic	B2	N/D
C07	FAM	Velogenic	B3	N/D
D07	FAM	Velogenic	B4	N/D
E07	FAM	Velogenic	B5	N/D
F07	FAM	Velogenic	B6	N/D
G07	FAM	Velogenic	B7	N/D
H07	FAM	Velogenic	B8	N/D
A08	FAM	Velogenic	C1	N/D
B08	FAM	Velogenic	C2	N/D
C08	FAM	Velogenic	C3	N/D
D08	FAM	Velogenic	C4	N/D
A10	FAM	Velogenic	Positive Control	**22.73**
B10	FAM	Velogenic	Positive Control	**22.80**
C10	FAM	Velogenic	NTC	N/D

*FAM : fluorescent reporter dye 5-carboxyfluorescein

** A1-A8 = 8 quails from Group A, B1-B8 = 8 quails from Group B, C1-C4 = 4 quails from Control Group, NTC = no template control

**N/D : not detected

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4.5.2 Detection of Newcastle disease virus in organ samples

From the total of eight infected quails and two quails from the control group that were sacrificed for this study, 30 organ samples such as trachea, proventriculus and caecal tonsil were processed for PCR assay. Reverse transcription real time polymerase chain reaction (RT-qPCR) assay showed six organ samples were positive for NDV (Table 4.5.2). Amplification curves of organ samples that represent positive detection are shown for Quail A4, B2, B3, and B4 (Figure 4.5.2). Out of the eight infected quails that were necropsied on 7 days post-infection (dpi), four were found to be positive with detection of genotype VII Newcastle disease virus (NDV) by RT-qPCR (50%). In summary, one out of four quails (25%) from Group A showed positive virus detection. Three out of four quails (75%) from Group B showed positive virus detection.

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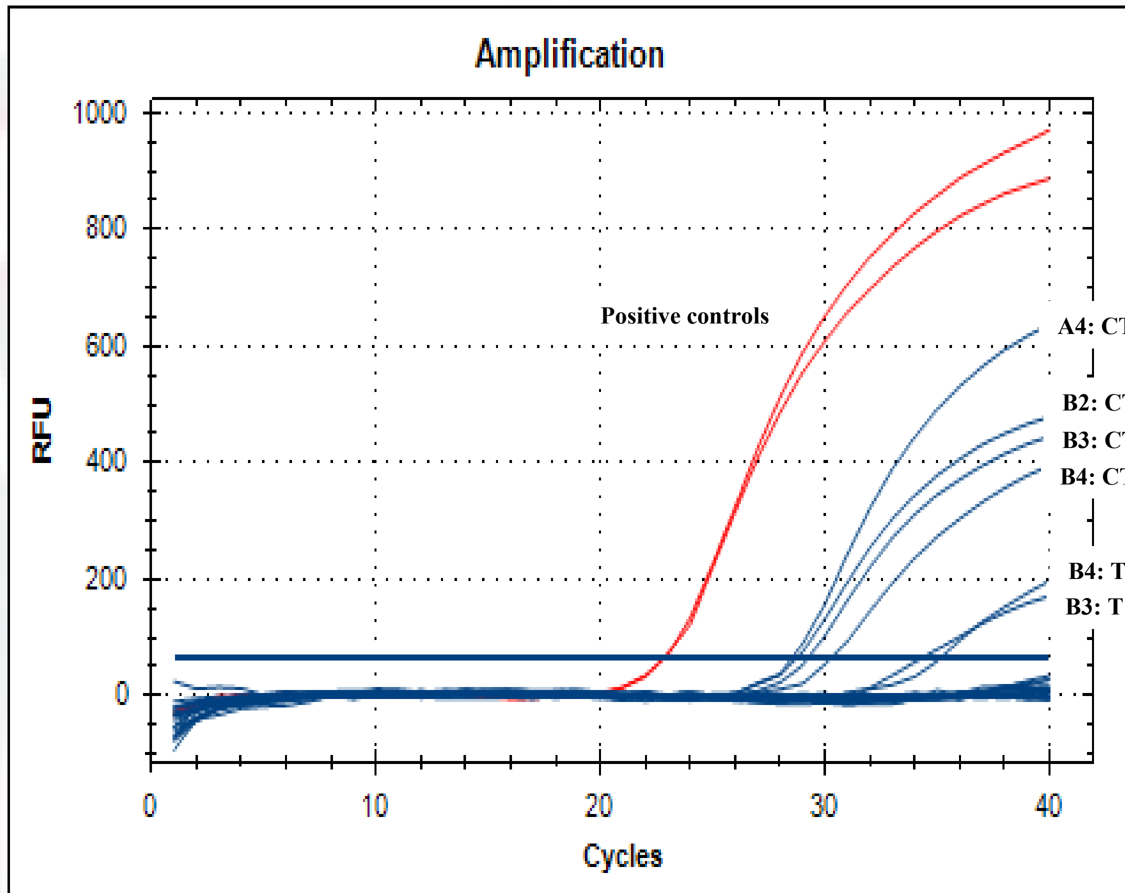

Figure 4.5.2: Assay of RT-qPCR for organ samples using specific primers targeting the partial of fusion (F) gene of velogenic genotype VII NDV. The amplification were observed in positive controls (red curves) and different organ samples (blue curves)

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Well	Fluorescent*	Target	Sample name **	Quantification cycle, Cq ***
A01	FAM	Velogenic	A1 : CT	N/D
B01	FAM	Velogenic	A1 : P	N/D
C01	FAM	Velogenic	A1 : T	N/D
D01	FAM	Velogenic	A2 : CT	N/D
E01	FAM	Velogenic	A2 : P	N/D
F01	FAM	Velogenic	A2 : T	N/D
G01	FAM	Velogenic	A3 : CT	N/D
H01	FAM	Velogenic	A3 : P	N/D
A02	FAM	Velogenic	A3 : T	N/D
B02	FAM	Velogenic	A4 : CT	**28.48**
C02	FAM	Velogenic	A4 : P	N/D
D02	FAM	Velogenic	A4 : T	N/D
A03	FAM	Velogenic	B1 : CT	N/D
B03	FAM	Velogenic	B1 : P	N/D
C03	FAM	Velogenic	B1 : T	N/D
D03	FAM	Velogenic	B2 : CT	**28.67**
E03	FAM	Velogenic	B2 : P	N/D
F03	FAM	Velogenic	B2 : T	N/D
G03	FAM	Velogenic	B3 : CT	**29.20**
H03	FAM	Velogenic	B3 : P	N/D
A04	FAM	Velogenic	B3 : T	**35.08**
B04	FAM	Velogenic	B4 : CT	**30.21**
C04	FAM	Velogenic	B4 : P	N/D
D04	FAM	Velogenic	B4 : T	**34.21**
A10	FAM	Velogenic	Positive control	22.73
B10	FAM	Velogenic	Positive control	22.80
G10	FAM	Velogenic	NTC	N/D

Table 4.5.2 : Detection of virus in organ samples by RT-qPCR

*FAM : fluorescent reporter dye 5-carboxyfluorescein

**CT: caecal tonsil, P: proventriculus, T: trachea, NTC = no template control

***N/D: not detected

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5.0 DISCUSSION

Newcastle disease virus (NDV) or avian paramyxovirus type 1 (APMV-1) is widely spread among wild and domestic birds, with all bird species are susceptible to be infected (Leighton and Heckert, 2007). The susceptibility of Japanese quails towards genotype VII NDV was determined in this study by assessing parameters such as clinical signs, gross pathological lesions of organs, virus detection in organs and cloacal swabs by one-step reverse transcription real time polymerase chain reaction (RT-qPCR) and expression of antibody titer by haemagglutination inhibition (HI) assay.

Quails infected with genotype NDV IBS 002 strain showed depression and ruffled feathers, trachea rales, and neurological signs such as leg paralysis and torticollis. Depression and ruffled feathers was started on 7 days post-infection (dpi) and markedly seen on 10 dpi, trachea rales was noticed started on 10 dpi and neurological signs (torticollis and leg paralysis) was observed started on 13 dpi. A quail with torticollis died on 16 dpi. The clinical signs vary with the pathogenicity of the isolate and the species of bird (USDA, 2016). Rasoli *et al.,* (2014) reported chickens infected with IBS 002 showed diarrhea, trachea rales, depression ruffled and loss of appetite and mortalities. The variation in manifestation of clinical signs was observed when comparing between species. Clinical signs associated with the various strains can be different in species other than chickens (USDA, 2016).

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The velogenic pathotype is divided into a neurotropic form, which has respiratory and neurologic signs, and a viscerotropic form with hemorrhagic intestinal lesions (Dortmans *et al.*, 2011). This classification is not always that clear and many strains have various manifestations in different birds (USDA, 2016). Quails in both infected group showed no gross pathological lesions of organs following infection of velogenic genotype VII NDV. The finding is similar with an experimental study conducted by Mohamed *et al.*, 2016 where Japanese quails infected with velogenic NDV did not show overt gross lesions. They hypothesized that NDV undergo limited replication and persisted for a short period of time in tissue, or it replicated at very low level in quails. In addition, histology examination was done in a quail with torticollis that was found dead on 16 dpi, and the examination revealed encephalitis where there was lymphocytes accumulation in the brain and the blood vessel of the brain (vasculitis) under 10x magnification observed under compound microscope. This finding proves the neurological problem manifested by the quail is due to NDV as lymphocytes participate in a variety of host defence mechanisms against virus infections (Denman, 1979).

For detection of virus in organs by RT- qPCR, the presence of virus can be detected in trachea and caecal tonsil. Trachea which is a part of respiratory tract, is the main tissue tropism of NDV (Levy *et al.*, 1975). Caecal tonsil which is a lymphoid organ that favours the replication of virus was also claimed to be one of the tissue tropism of NDV (Samuel *et al,* 2013). There was high occurrence of detection of virus in quails given a higher infection dose suggesting that extensive of replication of virus occurred in organs, thus

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making the detection is much possible. Negative detection in proventriculus could be due to less predilection of tissue tropism compared to trachea and caecal tonsil provided that the virus replicated only at low level in quails as mentioned before. Cloacal swab was done on 7 dpi for detection of NDV by RT-qPCR. As RNA extracted from swab showed lower yield and concentration as compared to organs, the proper time of sampling is crucial for detection of NDV in swabs. The negative detection of virus in cloacal swabs might due to the poor estimation of time of sampling. Ginocchio *et al.,* (2011), claimed that swabs should be collected within three to five days of symptom onset for optimal results. The low replication of NDV in quails as mentioned by Mohamad *et al*., 2016 could also be one of the factor for the negative detection of virus in cloacal swabs.

Antibodies against NDV can be detected approximately after 6-10 days (Miller *et.al*, 2013). From 10 dpi to 21 dpi, almost all the quails in both group A and B exhibited positive HI titers for NDV. Based on the statistical analysis (Graphpad Prism 5) of Two-Way ANOVA, there was no significant difference *(* $p > 0.05$*)* of antibody reaction across time, however the antibody reaction was significantly different *(* $p < 0.05$*)* between Groups A and B. Extent of antibody reaction was significantly different between groups, suggests that extensive virus replication occur in quails given with high virus titer, and led to significant high production of antibody as high virus and/or virus-infected cells can stimulate B lymphocytes to produce significant higher amount of antibody (Denman, 1979).

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6.0 CONCLUSION

A clear pathogenesis of Newcastle Disease (ND) was observed in Japanese quails in this experimental study. Clinical signs were clearly observed in quails following infection of Newcastle disease virus (NDV) and the replication of virus in the quails was proven by the detection of virus in organ samples through one-step reverse transcription real time polymerase chain reaction (RT-qPCR). Japanese quails showed immune response towards NDV infection by the expression of antibody against ND. Japanese quails showed positive clinical signs, positive detection of virus in organs and positive expression in the antibody titer thereby confirming the susceptibility of Japanese quails towards infection of genotype VII NDV.

7.0 RECOMMENDATIONS

This experimental study involved only a small number of samples. A larger sample size for experimental replicates is necessary to assess the underlying variability in measurement of the parameters assessed in the experimental study. On top of that, histology examination is highly recommended to be performed to have a clear idea about the lesions of organs occurred due to virus infection at the microscopic level. In addition, a transmission study could be conducted to further clarify the actual role of Japanese quail as a potential carrier for Newcastle disease virus (NDV).

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9.0 APPENDICES

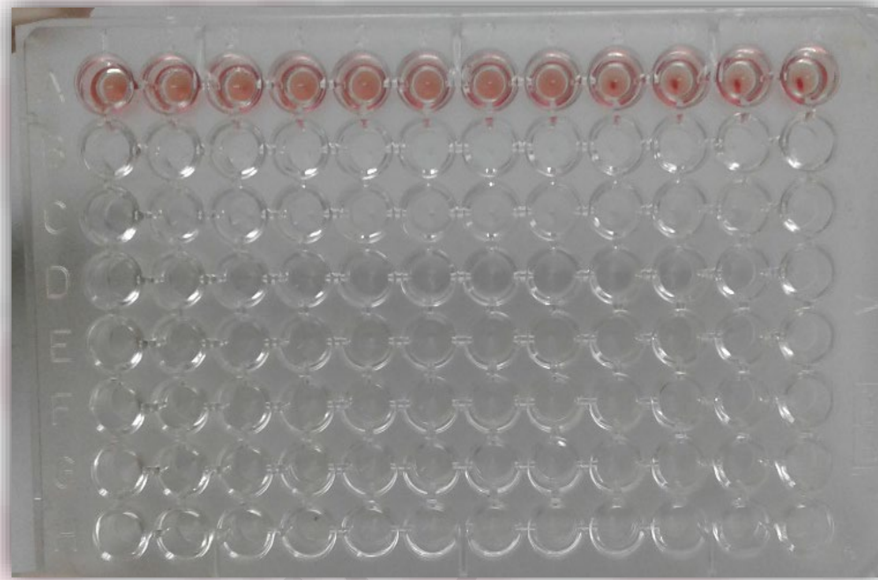

Figure 9.1: Haemagglutination (HA) assay

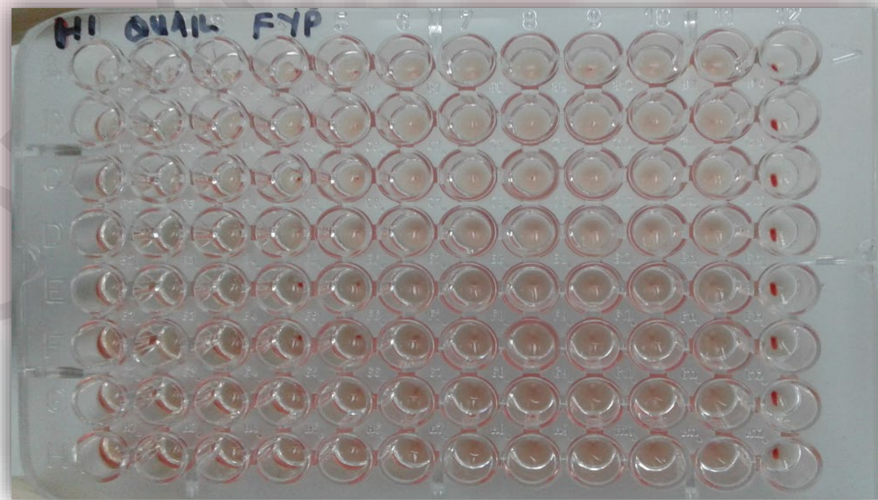

Figure 9.2: HI assay of pre-screening of quails showing 100% agglutination indicates quails were serologically negative against ND

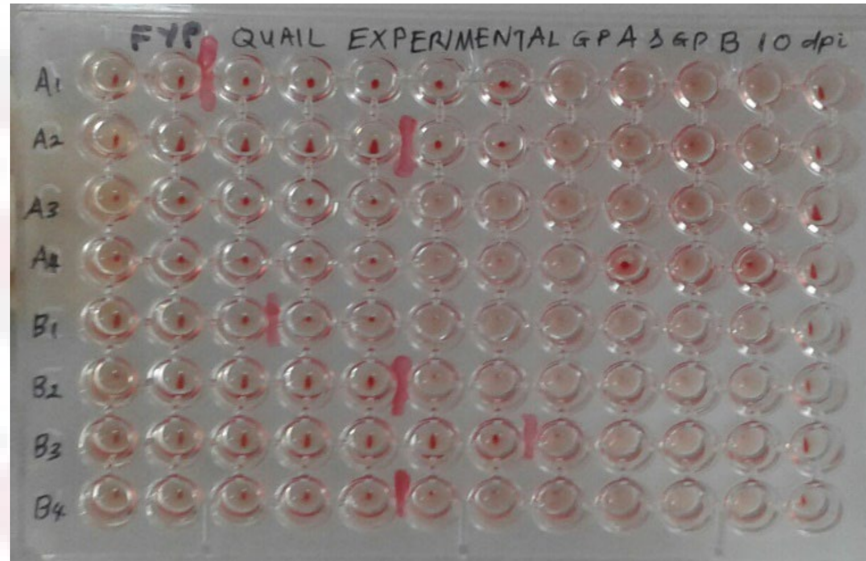

Figure 9.3: Some of the quails from Group A and Group B showed positive antibody titers on 10 dpi in HI assay

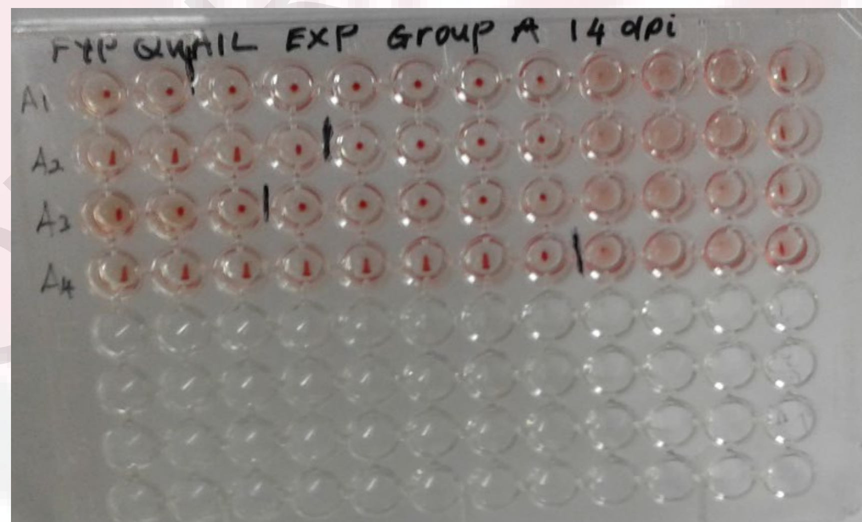

Figure 9.4: Variation of antibody titers of quails from Group A on 14 dpi in HI assay

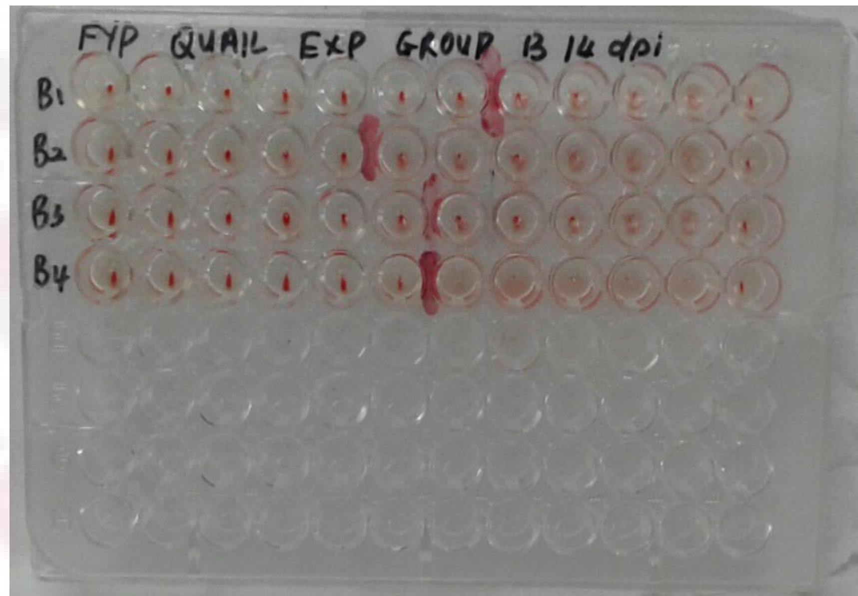

Figure 9.5: All quails from Group B showed positive antibody titers on 21 dpi in HI assay

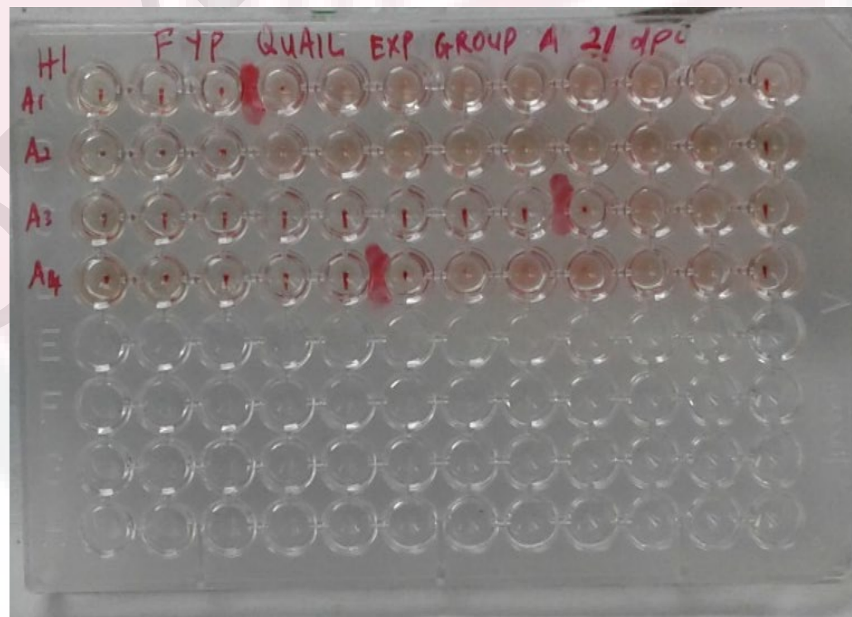

Figure 9.6: Variation of antibody titers of quails from Group A on 21 dpi in HI assay

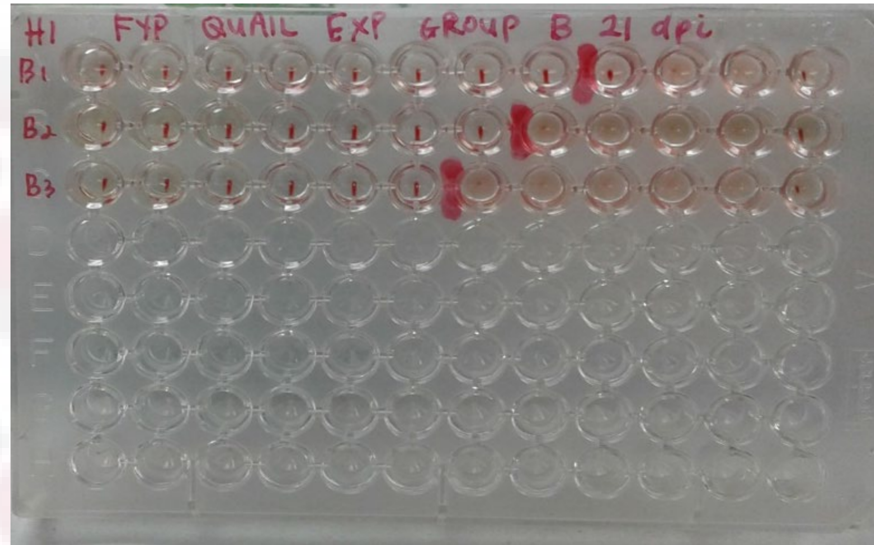

Figure 9.7: Antibody titers of quails from Group B on 21 dpi in HI assay

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