
UNIVERSITI PUTRA MALAYSIA

# SEROPREVALENCE OF NEWCASTLE DISEASE VIRUS ANTIBODIES IN PSITTACINE BIRDS IN KLANG VALLEY

SAFWAH NABIHAH BINTI NOOR MOHD EMRAN

# SEROPREVALENCE OF NEWCASTLE DISEASE VIRUS ANTIBODIES IN PSITTACINE BIRDS IN KLANG VALLEY

**SAFWAH NABIHAH BINTI NOOR MOHD EMRAN**

A project paper submitted to the

Faculty of Veterinary Medicine, Universiti Putra Malaysia

In partial fulfillment of the requirement for the

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## CERTIFICATION

It is hereby certified that we have read this project paper entitled "Seroprevalence of Newcastle Disease Virus Antibodies in Psittacine Birds in Klang Valley", by Safwah Nabihah 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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**ASSOC. PROF. DR. JALILA ABU**

**DVM (UPM), M.Sc. (UPM), Ph.D. (Minnesota)**

Head of Department, Department of Veterinary Clinical Studies

Faculty of Veterinary Medicine

Universiti Putra Malaysia

(Supervisor)

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**ASSOC. PROF. DR SITI SURI ARSHAD**

**DVM (UPM), MSc (UPM), PhD (London)**

Associate Professor

Faculty of Veterinary Medicine

Universiti Putra Malaysia

(Co-Supervisor)

## DEDICATION

This project paper is dedicated in the name of Allah S.W.T., The Most Gracious, The Most Merciful and had make everything possible,

To my father, Noor Mohd Emran Amir, my mother, Aini Abdul Rahim, my brothers, for believing in me and for the endless support and motivation,

to my family members,

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

And of course to all avian enthusiasts.

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| | |
|---|---|
| % | Percent |
| µL | Microliter |
| µM | Micromolar |
| mm | Millimeter |
| no. | Number |
| °C | Degree Celsius |
| ND | Newcastle Disease |
| NDV | Newcastle Disease virus |
| AVMA | American Veterinary Medicine Association |
| RNA | Ribonucleic acid |
| VRI | Veterinary Research Institute |
| g | Gram |
| min | Minutes |
| mL | Milliliter |
| RT-PCR | Reverse Transcriptase Polymerase Chain Reaction |
| NGS | Next Generation Sequencing |
| HI | Hemaggutination Inhibition |
| HA | Hemagglutination |
| x g | Relative centrifugal force |
| ELISA | Enzyme-linked immunosorbant assay |
| IACUC | International Animal Care Unit Committee |

## ABSTRAK

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

**SEROPREVALEN UNTUK ANTIBODI BAGI VIRUS PENYAKIT NEWCASTLE DALAM BURUNG PSITTACINE DI LEMBAH KLANG, MALAYSIA**

**Oleh**

**SAFWAH NABIHAH NOOR MOHD EMRAN**

**2017**

**Penyelia: Prof. Madya Dr. Jalila Abu**

**Penyelia bersama: Prof. Madya Dr. Siti Suri Arshad**

Penyakit Newcastle adalah salah satu penyakit pemusnah yang penting dalam industry ternakan ayam dan spesis avian yang lain yang disebabkan oleh virus ND dari famili Paramyxoviridae. Ianya juga berpotensi sebagai penyakit zoonotik yang menyebabkan konjuntivitis dan simptom flu pada manusia. Informasi berkenaan prevalens terhadap virus ND adalah penting sebagai panduan klinikal kerana kumpulan psittaformes berpotensi sebagai reservoir atau pembawa penyakit kepada kumpulan galliformes lain di Malaysia. Satu ujian telah dijalankan untuk mengetahui kadar antibodi terhadap virus ND dalam burung peliharaan dari spesis psittacine di Lembah

Klang, Selangor. Empat puluh dua ekor spesis psittacine terdiri daripada kumpulan parrot, cockatoo dan macaw telah diperoleh secara hidup dan sampel darah diperoleh dari burung klien, zoo dan kedai haiwan peliharaan dengan metod sampel konvenien. Sample darah diproses dan serum digunakan untuk dicerakinkan. Dari empat puluh dua sampel, 54.76% (23/42) adalah positif dengan antibodi terhadap virus ND. Berdasarkan lokasi burung diambil sampel seperti klien, zoo dan kedai haiwan peliharaan, seroprevalens adalah 21.43% (9/42), 4.76% (2/42) and 28.57% (12/42. Kumpulan parrot menunjukkan seroprevalens tertingi dengan 33.33% (14/42) diikuti oleh kumpulan cockatoo dengan 21.43% (5/42) dan kumpulan macaw iaitu 9.52% (4/42). Analisa chi-square menunjukkan terdapat assosiasi antara kumpulan spesis psittacine dengan seroprevalens terhadap virus ND. Secara konklusi, spesis psittacine terdedah kepada virus ND. Ujian lebih lanjut perlu untuk mengetahui adakah spesis ini adalah reservoir atau pembawa penyakit melalui virus ND yang dikeluarkan ke alam sekitar dengan deteksi virus dari sampel tinja dan discaj.

Kata kunci: Cockatoo, ujian HI, macaw, virus Newcastle Disease, parrot, burung Psittacine birds, seroprevalen

## ABSTRACT

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

**DETECTION OF AVIAN POLYOMAVIRUS FROM PSITTACINE BIRDS IN KLANG VALLEY, MALAYSIA**

**By**

**SAFWH NABIHAH NOOR MOHD EMRAN**

**2017**

**Supervisor: Assoc. Prof. Dr. Jalila Abu**

**Co-supervisor: Assoc. Prof. Dr. Siti Suri Arshad**

**SEROPREVALENCE OF NEWCASTLE DISEASE VIRUS ANTIBODIES IN PSITTACINE BIRDS IN KLANG VALLEY, MALAYSIA**

Newcastle Disease is an important devastating disease in poultry industry and other avian species caused by Newcastle Disease virus from family *Paramyxoviridae*. It is also a potential zoonotic disease that can cause conjunctivitis and flu-like symptoms in human. The information on seroprevalence against NDV in psittacine birds is important

for clinical measures as they may serve as reservoirs of NDV or carriers to other galliformes group in Malaysia. A study was carried out to determine the ND antibodies in pet birds from psittacine species in Klang Valley, Selangor. Forty-two psittacine species consisted of macaw group, parrot group and cockatoo group were captured alive and blood were obtained from clients owned, zoo and pet shop using convenient sampling method. Blood samples were processed and serum subjected for HI assay. Out of forty-two samples, 54.76% (23/42) were positive for ND antibodies. Based on location and purposes which were clients owned, zoo and pet shop, the seroprevalence were 21.43% (9/42), 4.76% (2/42) and 28.57% (12/42), respectively. Parrot group showed the highest seroprevalence 33.33% (14/42) followed by cockatoo group with 21.43% (5/42) and lastly, macaw group with 9.52% (4/42). Chi-square analysis revealed that there was association between the risk factor of psittacine group and the seroprevalence towards NDV. In conclusion, psittacine species are susceptible to NDV. Further work should determine whether this species is a reservoir and excreting virus to the environment by virus detection from feces and discharges.

Keywords: Cockatoo, hemagglutination inhibition test, macaw, Newcastle Disease virus, parrot, Psittacine birds, seroprevalence

## 1.0 INTRODUCTION

The virus family Paramyxoviridae is a single-stranded, enveloped RNA virus contains at least six structural proteins and additional non-structural proteins which are hemagglutinin–neuraminidase (HN), nucleoprotein (NP), fusion (F), phosphoprotein (P), matrix (M), RNA-dependent RNA polymerase (L). According to OIE 2013, Avian Paramyxovirus (APMV) is classified as type 1 to 10 categorised as lentogenic, mesogenic and velogenic strain. The first case of Newcastle Disease was reported in Newcastle Upon Tyne and Indonesia and after 30 years of the first initial isolation in 1956 obtained in chickens from Yucaipa, California, Avian Paramyxovirus type-1 was serologically distinctively known as Newcastle Disease virus (Alexander, 1988) which can commonly hosted in avian species (Leighton and Heckert, 2007). According to OIE, Newcastle disease (ND) is one of the notifiable disease listed when it meets certain criteria of virulence. ND is important in the avian industry as it is one of economics contributors and food resources for human (Ashraf and Shah, 2014) and and potential zoonotic disease which can cause flu-like symptoms, conjunctivitis and laryngitis in human (Evans, 2011). Based on previous study, (Kang *et al*., 2016) NDV is known to infect at least 250 bird species through either experimental or natural routes in wide range of hosts.

Although ND is pathogenic to the domestic chickens, turkeys and guinea fowls, other avian species might be developed the antibody to this virus without showing clinical symptoms (Ameji *et al.,* 2015). When there was a sudden emergence of virulent

NDV in a region, the researches came up with the cause of emergence virus that was due to the movement of captive caged birds especially psittacine species (Madadgar *et al.,* 2013).

Based on the prevalence study in Kogi State, Nigeria in the apparently healthy psittacine birds, they found that among wild birds in research of ND prevalence through HI test, psittacine birds showed negative antibody titer probably due to small sampling size (Ameji *et al.*, 2015). Based on reported cases of chronic ND infection in wild birds, Newcastle Disease virus is believed to be spread by the wild birds and psittacine birds to local and commercial chickens (Afayoa, 2010). An outbreak in 1970 in Netherland had traced infected psittacine birds with actively trading activity of psittacine and other birds to various countries and believed that psittacine particularly is a reservoir for the domestic avian species (Clavijo *et al.,* 2000). Newcastle Disease is also a potential zoonotic disease with highly risk spreading through laboratory works and people who are working and in contact directly with the diseased birds, hence, it is a subject to an official control measures in any endemic country (OIE, 2012).

Thus, the objective for this study was to detect the presence of Newcastle Disease virus antibodies in psittacine birds by conventional method of hemagglutination inhibition (HI) assay. As there is no study done for Newcastle Disease virus seroprevalence in psittacine birds compared to commercial chickens, there is a need for preliminary research on the seroprevalence of NDV antibodies in psittacine birds. This information is important for control measure as they may serve as reservoirs of ND virus or carrier to

other galliformes groups in Malaysia. The hypothesis of this study is psittacine birds in Klang Valley have antibody titer against the Newcastle disease virus.

## 2.0 LITERATURE REVIEW

### 2.1 Newcastle Disease

Paramyxovirus isolated form avian species have been classified by serological testing and phylogenetic analysis into ten subtypes known as Avian Paramyxovirus-1 (APMV-1) to Avian Paramyxovirus-10 (APMV-10), (OIE, 2013). It is a highly contagious viral disease which can cause a bad economic impact in avian industry (CAB International, 2013). The susceptibility of ND varies where we can see the different pathogenicity in different species with the severity depending on the strain affected the bird (CAB International, 2013).

According to OIE 2000, ND is an endemic infection in many countries with some of the European country had reached the free status after few years of endemic condition. It is believed that the ND outbreak started in Java, Indonesia in 1926 and in Newcastle Upon-Tyne, United Kingdom (Alexander, 1977).

In Malaysia, there are few studies to determine the status of ND. Based on cases and samples received in Veterinary Research Institute (VRI), ND status study was conducted to observe the prevalence of ND in between 2004 to 2009. The high incidence of ND during this period may be due to increased avian cases caused by Highly Pathogenic Avian Influenza (HPAI) outbreak in Malaysia where they found high chances of samples to have ND during surveillance of HPAI outbreak (Leow *et al*., 2011). Based on the study, the cases received by Veterinary Research Institute was

consistent throughout the year and increasing as initiated by climatic stress (Leow *et al.*, 2011). In Malaysia, Newcastle Disease is an important disease of poultry that required rapid detection and identification of virus to efficiently control the disease. The disease is being controlled by culling method, quarantine, and prevented by vaccination program and application of strict biosecurity level in the farm (Leow *et al.*, 2011).

**2.2 Psittacine Birds**

According to Brown (2013), Psittaciformes which are commonly referred to psittacine birds or parrots, well-known as caged pet birds or aviary birds. The order Psittaciformes contains two families which include 6 genera of Cacatuidae or cockatoo with 21 species and 74 genera of Psittacidae knowns as parrots, macaws, conures, lories and parakeet with roughly 350 species (Homberger, 2006).

Psittacine birds are popular among pet owners to be kept as pets (Seibert, 2006). Due to the popularity of keeping psittacine birds as pets, the owners must understand the social behavior in natural and captive environment, and they need to be provided with suitable physical needs (Seibert, 2006). It is being attracted by people to be kept as pets because of their playful antics and vocal mimicry, most parrots species seen relatively quiet and rest occupy over 24 hours (Bergman and Reinisch, 2006).

According to Harcourt-Brown (2009), there was evidence in history of earliest captive pet birds from parrot species, ring-necked parakeets that have been recorded brought from India to Europe by Alexander the Great. Since then, over the next 40

years, thousands birds were brought in and imported from Australia. Parrots are well-known as companion animals as they have potential for taming and training, attractive features and popular with its intelligence (Harcourt-Brown, 2009). Trading of live birds started in the fifteenth or sixteenth century when the European brought back parrot species from their voyages to Asia, America and Australia (Yvonne *et al*., 2016).

Conservation of wild species of psittacine birds started when habitat destruction occurred and increase the percentage of threatened animals (Munn, 2006). However, captive parrots also being on threat to welfare categories of bad husbandary, unsuitable environment and human interactions in case of improper training and sexual bonding between parrots (Meehan and Mench, 2006).

A statistical study made by the American Veterinary Medicine Association (AVMA) recorded 11 to 16 million companion and exotics birds in the United States in 2007.

**2.3 Pathology of Newcastle Disease**

The signs in infected animals are generalized and not pathognomonic which can be seen in reproductive, respiratory, enteric and nervous system. This can be influence by virulence and tropism of the virus (McFerren, 1988). In Grey Parrots, the symptoms that are usually seen are dilation of pupil, twisting head, trembling, spasmodic breathing, convulsion, lameness and CNS disturbances with eyes and nasal discharge, diarrhea and crouching of floor (Mundt, 1987). According to Mundt (1987), generally Newcastle

Disease in birds will develop clinical signs of anomalies in movement and carriage with occasional S-shaped twisting on neck. In quarantine station there was also high mortality in parrots.

In Psittacformes, the signs are almost confined to nervous system with abnormal behavior, movement, ataxia, torticollis and paralysis (McFerren, 1988). Cattoli (2011) in his paper stated that the clinical signs in infected psittacine birds varies from inappetance to exhibition of neurological syndrome like tremors, respiratory distress, lateral recumbency, ataxia, wing dropping and leg paralysis with incubation period of NDV from 2 to 3 days and can be up to 14 days.

Upon gross pathology, vasculitis of the brain can be suggestive of ND and histological study should be done to see the neuronal degeneration, gliosis, hypertrophy and proliferation of endothelial cells and perivascular lymphocytic infiltration (McFerren, 1988). In addition, Clavijo (2000) documented the gross and histological lesion in psittacine birds upon exposure of VVNDV which were Neuronal necrosis, diffuse spongiosis of the gray and white matter, varying degrees of endothelial cell hypertrophy, and occasional mild perivascular infiltration of mononuclear cells.

**2. 4 Newcastle Disease in Psittacine Birds**

In large quarantine stations, Amazons and Grey Parrots were seen to frequently get infected. This indicates that virus is species and adapts only slowly to another group of birds (Mundt, 1987). A shipment of pet birds consisted of 543 birds of Cacatuidae

(cockatoo) and Psittacidae (parrots) from Netherland had 23 birds death cases upon quarantine session and 13 cases in the following week, with all the infected birds exhibited symptoms of Newcastle Disease (Clavijo *et al.*, 2000). The two dead cockatiels and three parrots were submitted to National Centre for Foreign Animal Disease for necropsy and virus isolation revealed the typical paramyxivirus morphology and based on the monoclonal activity in an HI assay, the result indicated that the isolate virus was velogenic NDV (Clavijo *et al.,* 2000).

Wild birds and semi-domestic birds are believed to be susceptible and can develop antibody hence, it can be the source of infection to other domestic birds especially in poor poultry husbandary (Ameji *et al.*, 2015). Based on research done by Ameji *et al.,* (2015), the seroprevalence against NDV antibodies in psitacine birds captured by using HA, HI assay and ELISA showed low antibody titer. These detection methods either HI assay or ELISA, do not represent the true status of susceptibility of the species especially in the absence of ND antibodies in small sample size (Ameji *et al*., 2015).

Among the avian paramyxovirus serotypes, there can be cross-reactivity between APMV-1 and APMV-3 particularly with psittacine variant of APMV-3 commonly isolated form pets and exotic birds (OIE, 2013). A declining of wild bird species was observed in a conservation study and they obtained vaccine-derived Newcastle Disease virus from India in an endemic psittacine (Ayala *et al*., 2016). In the study conducted by

the team, they found Psittacine birds had developed antibodies and it was isolated in 1997 (Ayala *et al*., 2016).

**2.5 Diagnostic Techniques of Newcastle Disease**

When there are cases of Newcastle Disease in a poultry flock which involve high mortality in the flock, virus isolation will be done to the dead birds or apparently sicked birds (OIE, 2013). In virus isolation techniques, samples taken from trachea or orpharyngeal and cloacal swab in live birds, while in dead birds, samples are collected from lung, kidney, intestine and brain (OIE, 2013).

Virus identification is useful for proceeding with HI assay as the antigen is produced from the bird itself by inoculation of virus in an embryonated egg, however we cannot distinguish the serotype of avian paramyxovirus that infect the birds, thus, RT-PCR is used to identify the serotype (OIE, 2013).

The demonstration of virus with presentation of multiple basic amino acid and F protein cleavage can confirm the virulence of virus using molecular technique, however, the failure to demonstrate of multiple basic amino acid and F protein cleavage cannot confirm the absence of virulent of virus (OIE, 2013). There are several studies done by using molecular techniques to determine the F protein in the isolated virus of infected birds from tissue and feces, which established the in vitro test for virulence (Miller *et al*., 2010). In another study of velogenic NDV among imported caged birds, the virulency of isolated virus is obtained by using intracerebral pathogenicity index (ICPI)

in day-old-chicks and intravenous pathogenicity index (IVPI) in 6-week-old chickens (Clavijo *et al*., 2000).

In researches and studies to determine the ND status in wild birds, HI test and Enzyme-link Immunosorbent assay are used for detection of antibodies against NDV (Ameji *et al.*, 2015). Previous study to determine the prevalence of ND conducted by Veterinary Research Institute comprising of cloacal swab, tracheal swabs and pooled organs for virus isolation and antibody titer where HA and HI assay were conducted to confirm ND virus besides molecular detection of ND virus RNA extracted form allantoic fluid using TRI LA reagent (Leow *et al*., 2011).

In a nutshell, to report an outbreak of ND, the subjects must meet the criteria of virulency which are the virus intracerebral pathogenicity index is 0.7 or greater and the characteristic of pattern of amino acid residues obtain by the isolated virus through ICPI test (OIE, 2013).

**2.6 Antibody Detection Method in Newcastle Disease**

According to OIE 2013, hemagglutination inhibition test is the most widely used for antibody detection. The procedures each laboratory practiced might be different, however, OIE recommended the usage of V-bottomed microtiter plate with had final volume of 0.075mL in each well (OIE, 2013). The antibody titer will be expressed as reciprocals of the highest dilution of virus that caused erythrocyte agglutination inhibition using a microtiter plate as recommended (Madadgar *et al*., 2013).

In a research done in Kogi State, Nigeria, the absence of antibodies in few wild birds species was due to the non susceptibility of the species against the this virus however, the psittacine species are susceptible to NDV based on the antigen detection method with absence of antibody titer might be relative to the few sampling number that is not representing the true status of this species (Ameji *et al*., 2015).

Serological testing can demonstrate infection with virus or to monitor vaccination and conventionally, HI test is used commonly but in recent years, they had developed Enzyme Immunosorbent assay (ELISA) (Alexander, 2000).

**2.7 Newcastle Disease as Zoonotic Potential Disease**

As psittacine birds are popular among pet owners as pet birds, birds get contact with human, thus increase the risk to the human to get infected from diseased birds. There is lack of papers on reported cases of ND in human, with no human to human transmission (Alexander, 2000).

The virulence of NDV isolates does not appear to differ for humans in contrast to the wide difference in virulence for chickens (Swayne, 2003). Similar signs were resulted from the experimentation with a virulent field isolate and low virulence NDV strains, such as B1, which are commonly used as ND vaccines (Dardiri *et al*., 1962; Alexander, 2000).

The documented cases of ND in human were caused by the direct contact with the virus especially the laboratory workers and diagnostic veterinarians, while the farm workers

and pet owners are potentially to get infected from diseased birds and carcass of infected birds (Alexander, 2000). According to Evans (2011), Newcastle disease is a potential zoonotic disease where it can cause conjunctivitis, flu-like symptoms and laryngitis in human.

Rarely, NDV infection has resulted in symptoms of fever, chills, headache, pharyngitis, depressed appetite, photophobia, and general apathy (Chang, 1964). Generalized infection occurred when there was aerosol exposure of NDV in human as stated in a study (Hanson, 1958).

# 3.0 MATERIALS AND METHODS

## 3.1 Animals

The study area is in Klang Valley,Selangor including Sg. Buloh, Klang and Kuala Lumpur. Data on population size of psittacine birds in this state is unknown, and it may be a major challenge for designing the prevalence study in birds. Hence, the sample size was not predetermined but it was sampled using convenient sampling method where it is reasonable to limit the sample size to available resources. The captured and released of birds were done by workers and bird owners.

The birds that kept in household for pets, show birds in zoo and birds for sale were sampled. Based on history, none of the places visited encountered with Newcastle Disease cases. A total of 50 birds from Psittacidae and Cacatuidae family were restrained by using pisttacine technique with the usage of net and towel. They were divided into 3 groups which were macaw, parrot and cockatoo. Ages and sexes of some of the species were not determined.

The birds were selected through convenient sampling method. All the birds are healthy and never had history of Newcastle Disease. Sampling of animals was approved by IACUC dated 30$^{th}$ December 2016 (AUP. No: FYP2016/FPV.34)

### 3.2 Risk Factors

The samples of psittacine birds were categorized in groups known as parrots, cockatoos and macaws groups. There is no study done on the different groups and breed based on antibody titers of NDV antibodies. As these birds are kept for breeding, pets and exhibition, they were on various ages and sexes. Some of them are sexually unknown as the gender could only be determined by DNA sexing.

According to origin and location of birds, the birds were from different places and kept with different management. For client owned pets, they may be originated from other country, same goes to the breeder farm, zoo and pet shops. Based on the records, there is no outbreak of ND or any similar cases reported in the area or in the radius.

### 3.3 Sample collection

The birds were physically restrained with psittacine technique by using net and towels. Blood was collected from few species of apparently healthy after properly restrained. The samples are collected by blood venipuncture through brachial vein at 0.5 ml per birds by using 25G needles (B.Braun®, Germany) and a 3mL syringe (TERUMO® syringe, Philippines). After the blood was withdrawn, the blood was allowed to stand in room temperature for few hours for clotting to occur and serum decanted in the tube. They are then transport to the laboratory and the serum is extracted

in a 1.5 mL Eppendorf tube (Eppendorf Tubes ®, Germany) and kept in temperature of -20°C until assayed.

### 3.4 Serum Extraction and Chicken Red Blood Cell Preparation

After being transport from the field, it was then being centrifuged at 1500 x *g* for 5 minutes. By using 100µL pipette, the serum were withdrawn and stored in a 1.5mL Eppendorf Tube (Eppendorf Tubes ®, Germany). The samples are then kept in temperature of -20 $^{0}$C before assayed. Red blood cell was obtained from Specific Pathogenic-free (SPF) chicken through blood venipuncture via brachial vein at 3mL by using 25G needle and 3ML syringe. The blood is kept in a 3 mL plain vacutainer tube (BD Vacutainer ®, USA) then transport to the laboratory for processing. It is being centrifuge at 1500 x*g*, 4°C in 5 minutes and the blood plasma or Phosphate-Buffer saline (PBS) and buffy coat were removed. Then, the PBS was added into the tube. The steps of centrifuging, removing and adding PBS were repeated twice. The calculation for RBC was done by using formula of $M_1V_1=M_2V_2$ and the RBC is diluted with PBS. The dilution will produce 0.25% RBC and it was stored at temperature of 4°C before used.

### 3.5 Serology Test

All sera collected were put into a 1.5 mL Eppendorf tube (Eppendorf Tubes ®, Germany) and kept in -20°C before assayed. To assess the antibody level in the serum of birds, hemagglutination and hemagglutination inhibition test were done. As this virus is

capable to agglutinate red blood cells in avian species due to the presence of F protein, the antigen is prepared to provide 8 HA unit to proceed for HI assay.

### 3.5.1. Antigen Titration

The antigen is reconstituted or thawed and the appropriate dilution is determined by titration. A microtiter plate was used and marked as Well 1 to Well 12 in one row. 0.05mL PBS was added into Well 2 to Well 12 of all rows and the titration was performed in duplicate. 01mL of antigen extracted from egg inoculation and being put in Well 1. By using 0.05mL diluting loops was carried out in serial two-fold dilution starting from well 1 to Well 11. The last well was left without antigen or control. The 0.25% RBC suspension was added in each well. The plates were covered and kept in room temperature until the cells settling out in the cell control well. The end-point of the titration is the highest dilution of antigen with 100% hemagglutination. The HA unti in 0.05mL of the undiluted antigen is equal to the dilution factor of the endpoint. The virus stock was prepared as 8units HA per 0.05mL.

### 3.5.2. Serum Titration

HI assay was done by using 4HA unit antigen. A serial two-fold dilution of each serum was prepared so that serum was diluted out through the wells. Well 1 will have no antigen and it will be non-specific agglutination control. 0.025mL of PBS was added in Well 2 to Well 12. 0.05mL serum was added into Well 1, and by using 0.025 diluter, transferred and mixed 0.025mL of serum from Well 1 to the second well and continued

through Well 11 and 0.025mL was discarded from the last well. Virus dilution containing 4HA unit was added to each serum dilution starting from Well 2 to Well 11. The 4HAU dilution was obtained by taking out 0.025mL virus dilution from 8HAU virus stock. For 10 seconds, the microtiter plate was shaked and incubated at room temperature for 20 minutes. Then, 0.05mL of 0.25% RBC suspension in each well and it was maintained in room temperature. As the RBC was settled out in approximately 30 minutes, the HI assay was read. The serum titers were expressed as the reciprocal of the highest dilution having complete inhibition of the hemagglutination of the antigen.

### 3.6 Statistical Analysis

The data were assessed as percentage calculation to measure the seroprevalence by number of seropositive birds over the total samples taken. Chi-square method was used to study the association of the different bird's groups and presence of antibodies against NDV. If the calculated P value is less than 0.05, it is considered statistically significant.

Figure 1: Different bird's group categorized in this study, Cockatoo; Macaw and Parrot

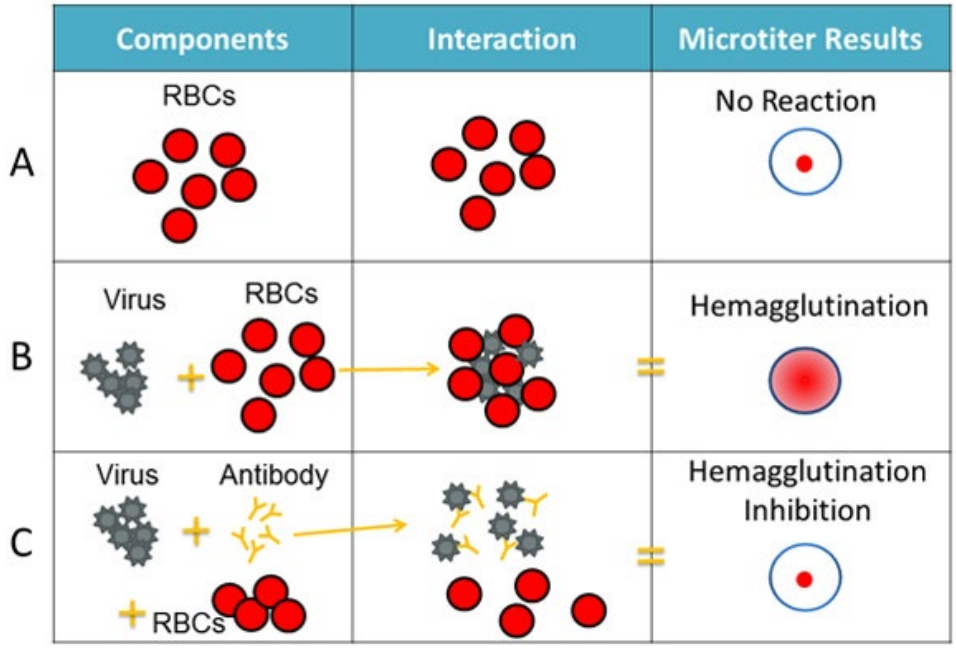


Figure 2: Hemagglutination inhibition (HI) assay concept

## 4.0 RESULTS

A total of 42 sera samples of birds were collected from different location in Klang Valley area and analyzed for presence of antibodies against NDV using hemagglutination inhibition assay. In general, 54.76% (23/42) of bird's sera sample detected to be seropositive and having antibodies against NDV.

An overview of the distribution of all 45 sera samples based on bird group had as shown in Figure 1. The highest seroprevalence was in parrot group which was 33.33% (14/42) followed by cockatoo group which was 11.90% (5/42) and lastly macaw group which was 9.52% (4/42). Statistically, there was a low association between the bird's groups and seroprevalence of NDV since the P value was less than 0.05 (p-value = 0.01)

The samples also being divided into different location which were clients' owned birds around Klang Valley, zoo confined birds and pet shop birds as birds are in contact with human, including owners, zookeepers, pet shop owners and customers, this grouping can give a limelight to the people to get aware that these birds having antibodies and may or may not carrying disease. Based on the data obtained, highest seroprevalence was in pet shop birds with 28.57% (12/42), followed by clients owned birds with 21.43% (9/42) and lastly from the zoo confined birds with 4.76% (2/42).

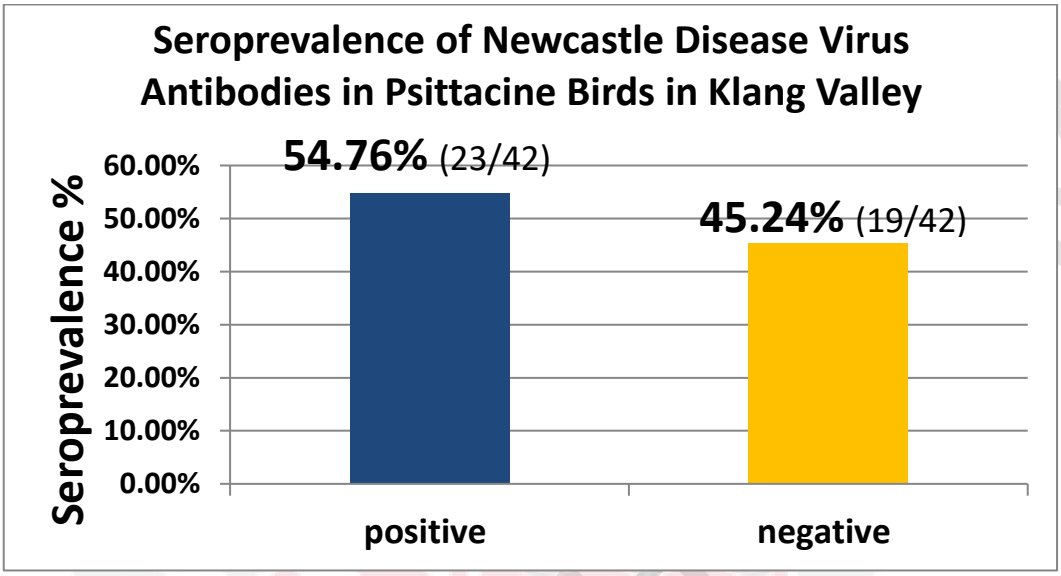


Figure 3: Seroprevalence of NDV antibodies in psittacine birds in Klang Valley

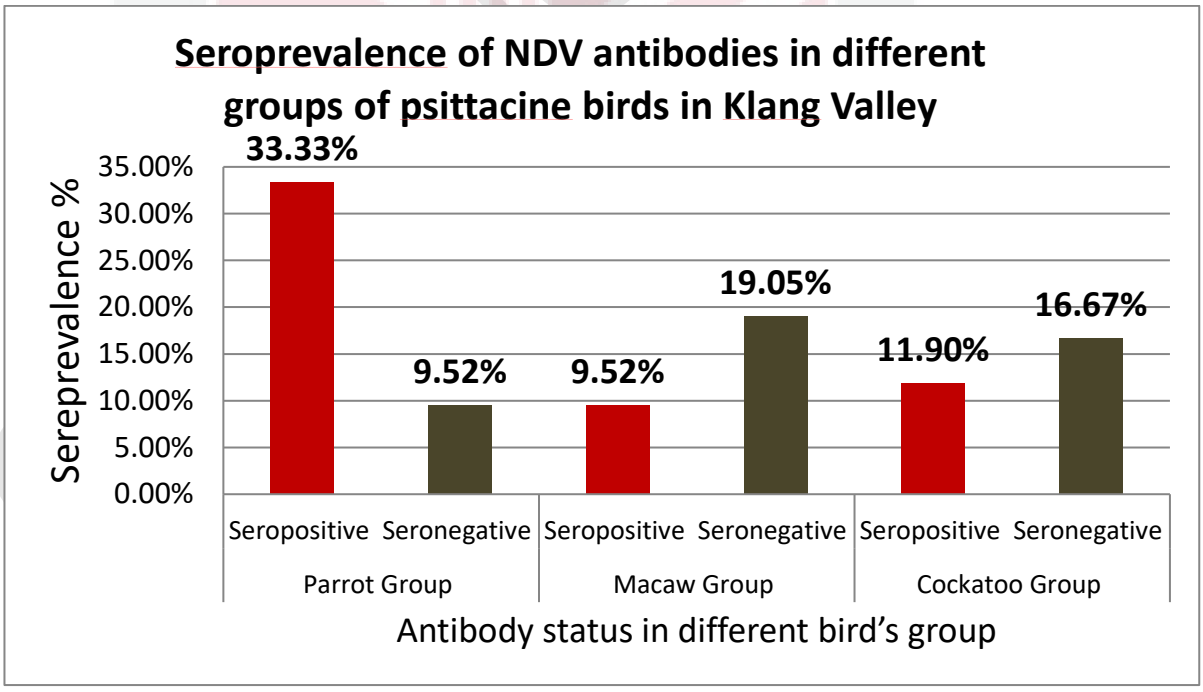


Figure 4: Seroprevalence of NDV antibodies in different groups of psittacine birds in Klang Valley

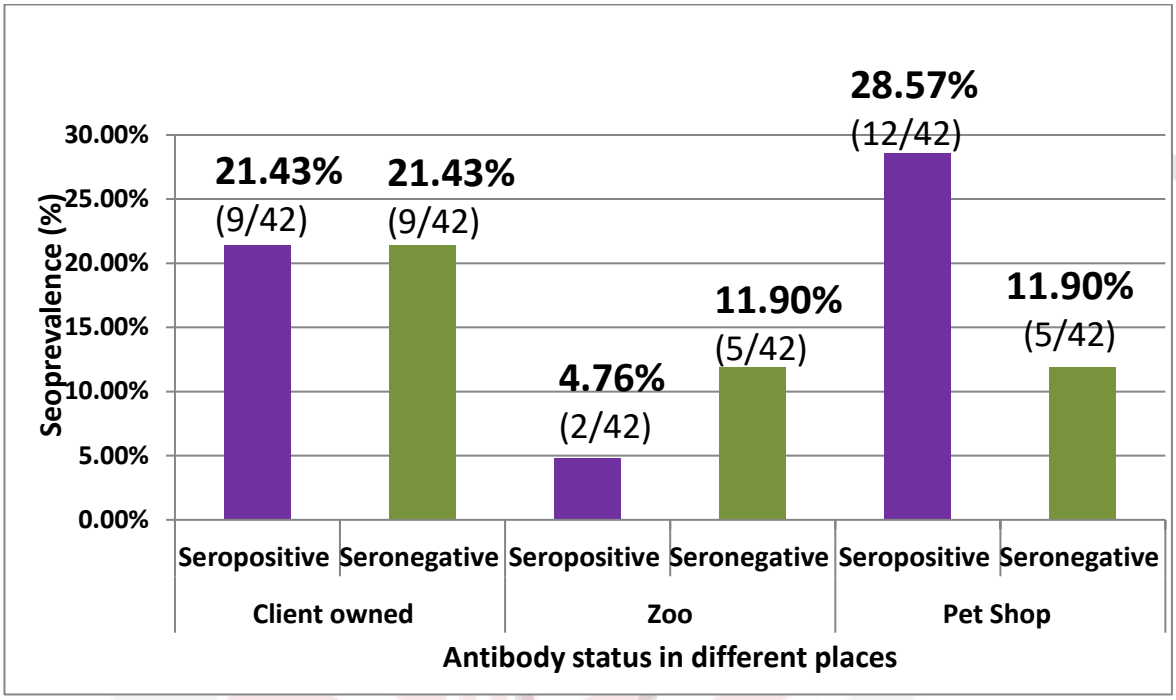


Figure 5: Seroprevalence of NDV antibodies according to different places in Klang Valley

## 5.0 DISCUSSION

In this study, the seroprevalence of NDV of psittacine birds in Klang Valley area was found to be 54.76%. This showed that among 42 birds sampled, 23 of them are positive with antibody against NDV and it is relatable to study done for seroprevalence towards NDV antibodies in psittacine birds which stated that psittacine birds can produce antibodies against the ND virus, and to represent true status of this prevalence study more serum samples should be obtained (Ameji *et al.*, 2015). However, in a study conducted in Tehran on ND in exotic caged birds, through HI assay, psittacine birds marked negative for antibodies probably due to low concentration of virus to cause the antibody development in the birds (Madadgar *et al.,* 2012). According to Murphy *et al.,* (1999), HA assay needs $10^6$ particles of virus to be detected, hence, in low concentration of virus, it may cause negative antibody titer.

As these samples were categorized into three groups which were parrots, cockatoos and macaw as in they were mostly kept in Malaysia and based on owners' consent to be sampled. In this study, parrots showed the highest seroprevalence followed by cockatoos and macaws. Statistically, the association between the birds groups and seroprevalence of NDV antibodies is low. Based on this findings, we can say these groups may or may not represent the true population however, this should be a good information to the owners in choosing a pet bird because there is a possibility for particular bird group to get infected and developed antibodies.

Further study should be done to study on the relation of birds groups and their ability to develop antibodies. In few studies, there were particular birds said to be susceptible to NDV. Amazon parrots, budgerigars and cockatoos are highly susceptible to Newcastle Disease virus while other species like macaws, lories and African Grey parrots may not showing sign but exhibit as carriers (Highfil, 2003). In my study, we found cockatoos showed higher seroprevalence than macaws.

According to Cattoli *et al.,* (2011) in a study, three species of psittacine birds including macaw, cockatiel and red breasted parakeet were shedding VVNDV without showing clinical sign. As in my study, though the macaws group showed antibody titer against NDV, they were apparently healthy.

Budgerigars, Amazon parrots and conures showed neurological signs after being exposed with isolated VVNDV after 2 weeks post-infection (Cattoli *et al.,* 2011). This is relatable to the possibility of parrots group to be seropositive in my study as they are able to get infected with presentation of clinical signs after exposure.

This study also had divided the samples into three categories of places of birds sampled. The divisions are from clients owned, zoo and pet shop. This is to consider the people for instance the pet owners, zookeepers, the zoo vets, pet shop owner and staffs and also the customers that come into contact with the birds whether seropositive birds can infect human.

We found pet shop birds showed the highest seroprevalence followed by clients owned birds and zoo-confined birds. None of the people showed clinical signs of ND. Absence of recent reports suggested that whenever there was an infection occurred in human, it was self-limiting with mild clinical signs (Swayne, 2003).

According to Babjee A. *et al.,* (1976) there was a case of laboratories technician to get infected with NDV after doing a laboratory work which was confirmed through virus isolation from eye swab and paired serum sample. The possibility of human to get infected with NDV transmitted from diseased birds is only with presence of high concentration of virus from faeces and discharges (Swayne, 2003).

Based on the data provided by OIE (2009), Malaysia has been reported to be endemic with positive disease status. Peninsular Malaysia (OIE, 2009), Sabah and Sarawak (OIE, 2005) have recorded the present cases of ND in chicken. While, in another study, it stated that ND occurs throughout the year in Malaysia and the pattern of ND cases were consistent based on reported cases to Veterinary Research Institute (Leow *et al*., 2011).

According to these studies, this country have consistent circulating virus and Malaysia is endemic with vaccination done to effectively control NDV in poultry industry. These findings might be related to the presence of antibody titer in psittacine birds in study area due to past exposure in its some stage of life or from the consistent circulating virus in this country. This results can be reliable for birds' status as HI assay

is a gold standard (OIE, 2012), rapid test with good specificity and quantitatively measuring the antibodies in serum (Senne, n.d).

## 6.0 CONCLUSION AND RECOMMENDATION

This study revealed that psittacine birds in Klang Valley have antibody titer against NDV with seroprevalence of NDV antibodies is 54.76%. The highest seroprevalence of NDV antibodies found in parrot's group followed by cockatoo and macaw's group. Statistically, the association in between birds groups and seroprevalence of NDV antibodies is low. Although there was presence of antibodies in sera samples, none of the birds nor human that get contact with birds are exhibiting the clinical signs of ND. As Malaysia is ND endemic, to determine the true status of birds is important.

This study provides the first database on antibodies status against NDV in psittacine birds in Klang Valley, Selangor. Thus, further study should be done to determine the true status of psittacine birds as they may serve as reservoirs of NDV or the carriers by antigen detection methods such as virus isolation. Virus isolation procedure can be conducted by using virus inoculation in egg or cell culture and molecular study in Reverse Transcription Polymerase Chain Reaction (RT-PCR) together with virus sequencing by Next Generation Sequencing. Various samples such as feces, cloacal and tracheal swabs should be collected for the above test. In addition to the present status in Klang Valley, increasing sample size is recommended to determine the status in this country. Hence, justification for vaccination against NDV in psittacine birds can be applied as this species are very valuable.

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## APPENDICES

| Group | Common name | Scientific name |
|---|---|---|
| Macaw (n=12) | Blue and Gold Macaw | *Ara ararauna* |
| | Red-bellied Macaw | *Orthopsittaca manilatus* |
| | Chesnut-fronted Macaw | *Ara severus* |
| | Green Coloured Macaw | *Primolius auricollis* |
| | Green-winged Macaw | *Ara chloropterus* |
| Parrot (n=18) | Eclectus Parrot | *Eclectus roratus* |
| | African Grey Parrot | *Psittacus erithacus* |
| | Amazon Parrot | *Amazona sp* |
| | Blue-cheeked Amazon | *Amazona dufresniana* |
| | Patagonian Conure | *Cyanoliseus patagonus* |
| Cockatoo (n=12) | Galah Cockatoo | *Eolophus roseicapilla* |
| | Greater Sulphur crested Cockatoo | *Cacatua galerita* |
| | Mollucan Cockatoo | *Cacatua moluccensis* |
| | Medium Sulphur Crested Cockatoo | *Cacatua galerita eleonora* |

Appendix 1: List of the psittacine birds involved in this study

Appendix 2: Psittacine technique in an *Ara ararauna* and blood venipuncture

| **Antibody titer** | **$2^4$** | **$2^5$** | **$2^6$** | **$2^7$** |
|---|---|---|---|---|
| **No. of samples(n=42)** | 15 | 7 | 1 | 0 |
| **Percentage** | 35.71% | 16.67% | 2.38% | 0.00% |

Appendix 3: No. of samples tested positive in HI assay and the antibody titer