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RESEARCH NOTE

SEROLOGICAL AND MOLECULAR DETECTION OF NEWCASTLE DISEASE VIRUS IN CAPTIVE PSITTACINES IN A WILDLIFE RESCUE CENTER IN LUZON, PHILIPPINES

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ABSTRACT

Newcastle disease (ND) is a viral infectious disease affecting wild and domestic poultry and is one of the major causes of the world's economic losses due to very high mortality. Limited information is known on the presence and distribution of Newcastle disease virus (NDV) in captive wild birds in the Philippines. The samples in this study were from captive psittacines in a wildlife rescue center in Luzon, Philippines. The birds were unvaccinated and did not show clinical signs of ND during the time of study. Thirty-four (91.89%) samples were seropositive for NDV using hemagglutination inhibition test with geometric mean titers ranging from 2^2 to $2^{10.5}$. Reverse Transcription Polymerase Chain Reaction (RT-PCR) showed 7 (18.34%) of the pooled oropharyngeal and/or cloacal swab samples of birds belonging to *Agapornis* spp., *Tanygnathus lucionensis*, *Cacatua galerita*, *Psittacus erithacus*, and *Melopsittacus undulatus* were NDV positive. Presence of antibody without detectable virus suggests that the birds have already recovered from infection and may not have been shedding the virus at the time of sample collection. However, RT-PCR positive results indicates active infection during sampling. Furthermore, seropositive results in RT-PCR positive birds could mean that there is prolonged viral shedding in these species.

Key words: *captive psittacines, hemagglutination inhibition, Newcastle disease, RT-PCR, wild birds*

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INTRODUCTION

Newcastle disease (ND) is a viral infectious disease affecting wild and domestic poultry and is one of the major causes of the world's economic losses due to very high mortality, reaching 100% in susceptible flocks (Alexander *et al.*, 2012). Previous studies determined that wild birds serve as reservoir hosts for Newcastle disease virus (NDV) (Alexander *et al.*, 2012) and that ND may be found in apparently healthy birds where non-virulent ND may be maintained and cause outbreaks (Thomazelli *et al.*, 2012). According to Ganar *et al.* (2014), NDV strains circulating among avian species have varying degrees of virulence. Moreover, Shengquing *et al.* (2002) have demonstrated and have proven that even several lentogenic NDV strains isolated from wild birds are pathogenic when passage to chickens. When it comes to Newcastle disease in captive wild birds, limited information are available. It has been discovered that NDs of lentogenic and even velogenic pathotype can be found in captive wild birds in spite of being apparently healthy (Roy *et al.*, 1998; Garcia *et al.*, 2013). Vaccine-derived Newcastle disease viruses have also been isolated form different wild bird species across different continents, implicating reverse spillover from domestic animals to wildlife (Ayala *et al.*, 2016).

Psittacines have been implicated in the maintenance of Newcastle disease virus infection and transmission of the disease to poultry species (Cattoli *et al.*, 2011). The most important point about ND in psittacines is the duration of viral shedding. The length of shedding varies in certain species and may last for over one year, stressing

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the potential of psittacines as a source of outbreaks (Falcon, 2004).

ND remains endemic in Asia despite of control and preventive measures applied including intensive vaccination programs which had been implemented for decades (Alexander *et al.*, 2012). Recently, an outbreak of Newcastle disease occurred in various commercial poultry farms and provinces in the Philippines which started late December 2015 (Umali *et al.*, 2017). However, despite the awareness about the disease in the country, there is very scarce information about the distribution and genetic characteristics of NDVs in the wild and in particular, in wildlife facilities. Since wild birds are considered reservoirs and vectors of ND, molecular detection of this pathogen and research on its genetic characterization will give further important insights to the current level of information about the disease in the Philippines. Due to its pathogenic potential, disease surveillance on captive wild birds is likewise important as with the free-living wild birds. Furthermore, these information will be useful for future prevention and control of ND outbreaks. Generated data on this investigation will also provide important information on the possible relationship of NDVs from wild birds to NDVs affecting commercial poultry.

MATERIALS AND METHODS

Captive Psittacines

A total of 76 out of 206 captive psittacines belonging to 18 different species, namely: Chattering Lory (*Lorius garrulus*) = 2, Black-capped Lory (*Lorius lory*) = 1, White Cockatoo (*Cacatua alba*) = 5, Sulfur-crested cockatoo (*Cacatua galerita*) = 1, Black Palm Cockatoo (*Probosciger aterrimus*) = 1, Eclectus Parrot (*Eclectus roratus*) = 1, Blue-naped Parrot (*Tanygnathus lucionensis*) = 3, Red-vented Cockatoo (*Cacatua haematuropygia*) = 3, Ring-necked Parakeet (*Psittacula krameri*) = 2, Rainbow Lory (*Trichoglossus moluccanus*) = 1, Sun Conure (*Aratinga solstitialis*) = 1, Green-cheecked Conure (*Pyrrhura molinae*) = 1, Cockatiel (*Nymphicus hollandicus*) = 14, Budgerigar (*Melopsittacus undulatus*) = 9, African Lovebird (*Agapornis* spp.) = 21, African Grey Parrot (*Psittacus erithacus*) = 6, Green-winged Macaw (*Ara chloropterus*) = 2, Blue-and-gold Macaw (*Ara ararauna*) = 2, were included in this study using convenience sampling in determining the samples selected. All samples were housed at the Biodiversity Management Bureau National Wildlife Rescue and Research Center (BMB-NWRRC), Quezon City, Philippines. The use of animals in this experiment was authorized by the Department of Environment and Natural Resources (DENR) and the Institutional Animal Care and Use Committee (IACUC) of the College of Veterinary Medicine, University of the Philippines Los Baños (UPLB) with assigned protocol number: 2017 – 0058. Handling of the animals and collection of samples were performed under the supervision and assistance of the BMB-NWRRC staff.

Sample Collection

Oropharyngeal and cloacal swabs were taken from 74 captive psittacines in the facility. Rubber cap glass test tubes with isotonic phosphate buffered saline (PBS) was used as storage and transport media. The swabs were pooled based on their enclosure assignments with maximum of five (5) swabs per pool for a total of 38 pools. The blood samples successfully collected from the wing vein of 37 birds, wherein 35 were part of the 74 birds from which swabs were taken, were centrifuged and the harvested sera were transferred to sterile 1.5 ml microcentrifuge tubes.

Hemagglutination Inhibition (HI) Test

Commercial LaSota vaccine (Hipra, Spain) was reconstituted with five (5) mililiters phosphate buffered saline (PBS) solution and hemagglutinating titer was determined by Hemagglutination Test. First, 0.025 ml PBS was added into each well, and then 0.025 ml of the reconstituted vaccine was added in the first well. A two-fold dilution was made across the well and the last 0.025 ml was discarded. Another 0.025 ml PBS was added to each well. Lastly, 0.025 ml 1% chicken red blood cells (RBCs) was added to each well. The plate was tapped gently to mix the contents of the wells and was allowed to settle for 30 minutes under room temperature. The HA titer of the vaccine was determined and 4-hemagglutinating units (HAU) of the antigen was prepared, as the working antigen for the HI test. Back titration was performed. Antibodies against NDV from serum samples were identified using HI test. Indication for hemagglutination is the formation of thin film of RBCs while inhibition reaction forms a sharp button of RBCs (Young *et al.*, 2002). HI test was performed following the OIE protocol (2012). Initially, 0.025 ml of PBS was dispensed in each well of a 96-well U-bottomed microtiter plate. Then, 0.025 ml of the collected serum was dispensed on the first well of the plate and a two-fold dilution was made across the plate. 4-HAU antigen in 0.025 ml was added to each well and was allowed to incubate for 30 minutes at room temperature. Afterwards, 0.025 ml 1%

chicken RBC was dispensed in each well. The solution was mixed by tapping the plate gently and the RBCs were allowed to settle for 40 minutes at room temperature.

RT–PCR

Using the pooled swabs, molecular detection was performed through RT-PCR. Viral RNA extraction was performed using QIAamp® Viral RNA Mini kit (Qiagen, West Sussex, UK). Following the extraction, complementary DNA (cDNA) strand synthesis was done using random hexamers and Primescript® Reverse Transcriptase (Takara Bio-Inc., Shiga, Japan). A two-step nested PCR was performed to amplify the region comprising the 3' end of the matrix protein (M) gene and the 5' end of the fusion protein (F) gene using SapphireAmp® Fast PCR Master Mix, 5 μM of external and internal primers as described previously (Mase *et al.,* 2002; Umali *et al.,* 2014). The initial denaturation was performed at 95˚C for 2 min followed by 35 cycles of denaturation at 95˚C for 10 s, annealing at 55˚C for 10 s and extension at 72˚C for 10 s. Final extension was done at 72˚C for 2 min. In the first step, a 921-bp primary product was obtained by using the external primers and a 766-bp product was obtained in the second step using the internal primers. The PCR product was analyzed in 1.1% agarose in Tris-borate-EDTA (TBE) buffer gel containing 1% GelRed (Biotium Inc., California, USA).

RESULTS AND DISCUSSION

This study aimed to detect the presence of NDV in different captive psittacines in a wildlife rescue center in Luzon, Philippines with the use of serological and molecular diagnostic techniques, HI Test and RT-PCR, respectively. Records of the psittacine birds used in the study were reviewed and it was found out that the birds were either confiscated or donated to the rescue center around 2009 to 2017 however, the origin of the birds were not indicated in the records. All birds tested in this study were unvaccinated nevertheless, the birds did not present clinical signs of ND.

ND antibody titers were detected using HI test and the results were evaluated in comparison with the negative and positive control sera. Thirty-seven (37) sera were tested and the geometric mean HI titer of the samples ranged from 2^2 to $2^{10.5}$. According to OIE (2012), the HI titers are regarded positive when inhibition at a serum dilution of 1/16 (2^4) or more is observed using 4-Hemagglutinating units (4HAU) as a working antigen. Out of 37 sera, 34 (91.89%) of the samples tested were seropositive for NDV.

For molecular detection of NDV, a total of 38 pooled swab samples were tested for NDV using RT-PCR. Analysis of all samples revealed positive reactions for pool number 5, 8, 14, 15, 21, 30 and 31 in RT-PCR. The positive pools include

Table 1. HI titers of sampled captive psittacines at the wildlife rescue center.

Psittacine	Number of Samples (n)	HI Titer (log2)
White Cockatoo	5	6.2
Sulfur-crested Cockatoo	1	7
Black Palm Cockatoo	1	7
Eclectus Parrot	1	8
Blue-naped Parrot	3	7.8
Red-vented Cockatoo	3	9.0
Ring-necked Parakeet	2	8.8
Rainbow Lory	1	7
Green-cheecked Conure	1	7
Cockatiel	7	7.5
African Lovebird	1	6
African Grey Parrot	6	7.8
Green-winged Macaw	2	3.5
Blue and Gold Macaw	2	4.5
Geometric Mean Titer		6.8

Table 2. Identification of pooled samples of captive psittacines that tested positive for NDV in RT-PCR.

Species (n)	Endemicity to the Philippines	Pool #	Type of Sample	Swab Samples/Pool	HI Titer (log2)
African Lovebird (5)	Non-endemic	5	Oropharyngeal swab	5	7
African Lovebird (4)	Non-endemic	8	Cloacal swab	4	N/A
Blue-naped Parrot (2)	Endemic	14	Oropharyngeal swab Cloacal swab	2	8
Sulfur-crested Cockatoo (1)	Non-endemic	15	Oropharyngeal swab Cloacal swab	2	7
African Grey Parrot (5)	Non-endemic	21	Oropharyngeal swab	5	7.8
Budgerigar (2)	Non-endemic	30	Oropharyngeal swab Cloacal swab	2	N/A
African Lovebird (5)	Non-endemic	31	Oropharyngeal swab	5	6
Total Positive Rate				7/38 pools	(18.42%)

oropharyngeal and/or cloacal swabs collected from African Lovebirds (*Agapornis* spp.), Blue-naped Parrot (*Tanygnathus lucionensis*), Sulfur-crested Cockatoo (*Cacatua galerita*), African Grey Parrot (*Psittacus erithacus*) and Budgerigars (*Melopsittacus undulatus*).

Seropositive results indicate exposure of birds to NDV. One hypothesis that can be assumed is that, these birds are asymptomatic carriers of the virus. As mentioned by Jibril *et al.* (2014), recovery form clinical disease may also explain seroprevalence in healthy birds. There were three (3) birds that showed seronegative results whilst having seropositive birds on the same and adjacent enclosures. Other birds might have been infected with low virulent strain of NDV and have recovered without shedding the virus thus, hindering the transmission of the disease. Most of the seropositive birds in this study had a high HI titer. This could be attributed to previous infection with virulent strains of NDV.

Awan *et al.* (1994) cited that a positive serological result provides a clear evidence of exposure to NDV however, it does not indicate the infecting strain and often a poor indicator of the presence of highly pathogenic strains in unvaccinated populations. Conversely, more recent studies have claimed otherwise. Abraham-Oyiguh *et al.* (2014) mentioned that high antibody titers may be caused by infection with virulent strains of NDV. Different pathotypes of NDV can stimulate different antibody production response, allowing potential for different NDV pathotypes to stimulate different antibody production response. Thus, more virulent strains could produce a higher antibody titer (Rezaeianzadeh *et al.,* 2011).

Clinical signs of ND in psittacine birds are highly variable, ranging from inapparent to severe neurologic disease or even death without premonitory signs with shedding shown to last for more than a year in certain species (Cattoli *et al.*, 2011; Falcon, 2004). It has also been suggested that imported caged birds contribute to the distribution of NDV which raises concerns about the worldwide distribution of velogenic ND pathotypes through trading and migration across regional and international borders (Shabbir *et al.*, 2013). Inadequate biosecurity measures and uncontrolled circulation of wild birds in the vicinity of poultry farms serve as a major factor in the transmission of the avian diseases as it also creates opportunity for wild and domestic birds to interact (Khulape *et al.*, 2014). Spill over of ND infection from commercial poultry market to wild birds may also be a cause of transmission of the disease (Garcia *et al.*, 2013). Furthermore, interaction with wild birds results to wide range

dissemination of the disease (Snoeck *et al.*, 2013). Surveillance studies have been conducted to determine the prevalence of the disease in wild birds. Even with the low prevalence of ND in wild bird populations, its pathogenic potential cannot be ignored (Umali *et al.*, 2014).

Due to insufficient amount of sera extracted in some birds, only 5 out of 7 RT-PCR positive pools had representative samples for HI Test. These are the samples from African Lovebirds (*Agapornis* spp.), Blue-naped Parrot (*Tanygnathus lucionensis*), Sulfur-crested Cockatoo (*Cacatua galerita*), and African Grey Parrot (*Psittacus erithacus*). Antibody titers of the RT-PCR positive birds mentioned ranged from 2^6 to 2^9, which are considered seropositive. In this case, despite yielding high antibody titers, the RT-PCR results indicate that there was an ongoing or active infection at the time of sample collection which means these birds were still actively shedding the virus. Furthermore, a possibility of prolonged viral shedding in these species can also be speculated based on these findings.

Additionally, enclosures of the RT-PCR positive birds were located separately from each other hence, infection may be restricted to the respective enclosures. This may also indicate that there could be different sources of NDV infections in the facility. Birds in the adjacent enclosures negative for NDV in RT-PCR as well as other avian species in the facility are still at risk of contracting, incubating, and shedding the virus. Furthermore, birds that had negative HI titer are at higher risk because these birds are more susceptible due to the lack of antibodies against NDV. Seropositive results that tested negative for RT-PCR may suggest that the birds are recovering from infection and were no longer shedding the virus. Moreover, Camenisch *et al.* (2008) mentioned that the viral shedding period is much shorter than the duration of persistence of antibodies in the blood, leaving a shorter diagnostic window for RT-PCR.

There is limited literature about serological or molecular detection of NDV in wild birds, especially psittacines. In the Philippines, there was an attempt by Johnson *et al.* (1986) to demonstrate viscerotropic velogenic Newcastle disease in native and exotic psittacines however, the birds tested negative for velogenic ND. Camenisch *et al.* (2008) also screened wild birds for NDV in Switzerland and all samples tested were negative in RT-PCR which is in contrast with the previously reported serologic screenings by Schelling *et al.* (1999), having NDV antibody prevalence of 10%. Likewise, Rezaeianzadeh *et al.* (2011) were not able to detect NDV by RT-PCR in seropositive village chickens in Fars province, Iran. In the Philippines, serological and molecular detection of NDV in live bird markets (LBMs) by Resplandor and Umali (2017) and in captive raptors by Lastica-Ternura *et al.* (2016) also had a negative RT-PCR outcome despite the seropositive results.

In summary, this study showed that most of the sampled captive psittacines in the wildlife rescue center were seropositive for NDV as demonstrated by the HI Test. The presence of antibody titers without detectable virus suggests that the birds have already recovered from the infection and may not have been shedding the virus at the time of sample collection. However, there were birds that tested positive for NDV in RT -PCR in this study. This indicates active infection during sampling. Since there were seropositive birds with RT-PCR positive result, it is possible that there is prolonged viral shedding in different psittacine species. The birds did not show any clinical sign of ND during sample collection suggesting that the birds are asymptomatic carriers.

It is important to keep track of the origin of the animals to provide additional information which may give useful insights on the local epidemiology of NDV. Most of the birds used in the study were confiscated although tracking the exact origin was not possible. This denotes a risk of introduction and spread of NDV due to illegal transportation of these birds especially since psittacines are known reservoirs of the virus. Screening for NDV and other disease in the facility is recommended upon arrival and release of the animals for the prevention and control of the spread of disease. Disinfection and improvement of enclosures and strict implementation of biosecurity measures are also recommended to prevent further spread of the disease. Lastly, phylogenetic analysis of RT-PCR positive samples in this study may provide us more insights with regards to NDV in the Philippines.

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STATEMENT ON COMPETING INTEREST

The authors declare no competing interests.

AUTHOR'S CONTRIBUTION

DV Umali and EA Lastica-Ternura conceived and designed the study. DV Umali, EA Lastica-Ternura and JA Baydo performed the experiments and analyzed the data. JA Baydo, EA Lastica and DV Umali wrote the manuscript. All authors read and approved the final version.

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