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Serologic Status of Newcastle Disease in Native Chickens by Hemagglutination Inhibition Test

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ABSTRACT

Newcastle disease (NCD) is a poultry disease caused by avian Paramyxovirus type 1, characterized by gastrointestinal, respiratory and neurological symptoms. The study established the prevalence of NCD in native chickens and evaluated the protection levels of vaccinated chickens. Blood serum samples were subjected to hemagglutination inhibition test. A total of 75 blood samples were collected from five sites in Davao City: 60 samples from four unvaccinated native chicken farms, and 15 from a vaccinated broiler farm. Results showed seven (7) unvaccinated native chickens with positive titer levels ranging from 2 to 32, of

which two(2) were considered significant, indicating protection even without an elicited immune response. This cannot be simply attributed to environmental factors considering uniform exposure of other individuals to similar conditions but exhibited no positive titers. The significant titer count of vaccinated samples ranging from 16 to 128 is attributed to their vaccination history. Differences in titer levels despite similar vaccine administration indicate a disparity in levels of protection due to different individual antibody immune responses, and efficacy of vaccines. Analysis by Chi-square goodness of fit test showed no difference in the titer levels of native chickens, which was expected as they did not have previous exposure to NCD and most had no titers. The two significant titer levels were considered outliers and provided a possible genetic perspective with pre-immune antibodies and natural resistance of native chickens as the focus. Gene analysis and isolation, as well as the prevalence of NCD in other localities, are recommended for future studies.

Keywords **—** Newcastle Disease, Hemagglutination Inhibition test, native chickens, Davao City

INTRODUCTION

Newcastle Disease (NCD) is a poultry disease caused by avian paramyxovirus serotype-1 which is characterized by gastrointestinal, respiratory and neurological symptoms (Tapdasan et al., 2016). It is one of the most important viral diseases in industrial aviculture that affect domestic poultry resulting in significant economic consequences that reduce production in the industry (Orsi et al., 2010). In chickens, it can reach up to a mortality rate of 100%, along with a decline in the production and quality of eggs. Despite the availability and global employment of NCD vaccination since 1950, it remains a constant threat to poultry producers worldwide (Kapczynski, Alfonso & Miller, 2013). No treatment has been identified for NCD; only preventive measures can be taken to avoid outbreaks. Recently, however, incidents of vaccine failure have been reported (Alfonso et al., 2013).Hemagglutination Inhibition Test is the most widely used serological method for measuring antibody levels and is considered the standard laboratory test for Newcastle Disease (Carrasco, Neto, Lages, Sousa, Junior, & Pinto, 2008). Behind it is the principle of hemagglutination, which occurs when hemagglutinins found on the virus envelope interact with receptors on the surface of red blood cells. On the other hand, inhibition of hemagglutination happens

when subtype-specific antibodies are present in the serum. The result of the HI test is the HI titer, which is taken as the highest dilution of serum to prevent hemagglutination. A positive result is seen as the formation of a button of RBC's, while a negative result is the clumping or agglutination of RBC's. The higher the titer of a serum sample, the higher the resistance of the organism.

Chickens are considered the most popular poultry though the native breed of this bird is widely used for rural backyard poultry production, their susceptibility to Newcastle Disease has not been thoroughly established (Padhi, 2016). Significantly less attention has been given to native chickens leading to the lack of studies being conducted, and absence of surveillance data in many regions despite their ability to adapt, survive, and reproduce under adverse conditions with marginal care and low production inputs (Lopez, Lambio, Vega & De Guia, 2015).

NCD outbreak in native chickens in Bohol was investigated by Tapdasan et al., (2016) wherein the distribution of infected chickens was the primary source of the virus, which therefore highlights the need for surveillance and working vaccination programs. A study on the genetic resistance of native chicken breeds in Egypt was conducted by Hassan, Afify & Ali, (2004) wherein various breeds were challenged with a virulent strain of NCD. Mandarah chickens emerged as a resistant breed with only 20% mortality. Aside from natural disease resistance, their survival was also attributed to the possibility of non-encounter of pathogens and immunity after recovery from infection. The outcome of this particular study is indicative of the possibility of NCD resistance in native chickens.

The study surveyed the prevalence of Newcastle Disease among native chickens in selected farms in Davao City. Specifically, it aimed to: measure NCD titers of serum samples collected from four (4) native chicken farms by hemagglutination inhibition (HI) test: Chua Game Fowl, DAVOC, Kahayag Farms and Range Chicken Farm and; evaluate the protection levels of vaccinated chickens from NCD virus by measuring titers of broiler chickens in Grande farm.

By considering the susceptibility of unvaccinated native chickens unexposed to NCD virus and their possible resistance, genetically-related dynamics may surface which could pave the way for studies that could enhance innate defense mechanisms for the poultry organisms without having to conduct frequent and costly vaccination programs. Since no other studies have been conducted on local chickens in the region, the results will also serve as baseline data for future studies.

METHODOLOGY

Research Design

Sixty-two (62) blood samples from available and randomly chosen native chickens were collected from four unvaccinated sites in Davao City, of which only sixty (60) samples were considered viable for analysis given the amount of serum that separated from the blood samples. For comparative purposes, fifteen (15) blood samples were collected from the vaccinated site: Grande Farm, wherein all samples were considered viable for analysis.

2 mL of blood from the brachial vein of one wing of each chicken were collected using 3 mL syringes and placed a tilting position to allow serum separation. Centrifugation was employed to take out the clot and the supernatant left is the serum which was carefully separated. Phosphate Buffered Saline (PBS) and Washed RBC were already prepared in the laboratory.

Hemagglutination test was used to quantify the amount of Newcastle disease virus in a suspension. This was done by carrying out two-fold serial dilutions of the viral suspension in a microwell plate and then testing to determine an endpoint. This result was then used to determine the amount of hemagglutinin in the suspension and was expressed as a Hemagglutination (HA) titer. Twenty-five (25) µL of PBS was dispensed in all wells starting from columns 1 to 12 of two rows. Twenty-five (25) µL of the antigen/virus suspension was then added in the wells of column 1. Serial two-fold dilutions were carried out by transferring 25 µL from column 1 to column 2 and every previous column to the next, until column 11. The 25 µL from column 11 was discarded. Twenty-five (25) µL of 1% RBC suspension was then added to all wells beginning from column 12. Column 12 serves as the RBC control. The plate was well-covered and mixing was done by gently tapping the sides of the microplate. Incubation lasted for 45 minutes at 4°C.

A positive HA result was observed as hemagglutination or clumping of RBCs. "Button" formation or settling of the RBCs into a compact mass which streaks when the plate is tilted towards the operator is indicative of the absence of hemagglutinating activity. The endpoint was taken as the highest dilution to produce 100% RBC agglutination (no streaking) and the reciprocal of this well gives the HA titer. The result was then recorded under Antigen Titration. The working dilution of the antigen for the Hemagglutination Inhibition test was prepared by dividing the hemagglutination titer obtained with the required working dilution (4HAU or 4 Hemagglutinating Unit) to get the dilution factor.

Back titration method was employed to validate the concentration of antigen. The back-titration of antigen was performed twice since the antigen concentration did not match with the standard 4HAU. The adjustment of antigen concentration is done by adding more antigen if the concentration is below the required HA units, while PBS is added if the antigen concentration is above the required HA units.

HI test was conducted by dispensing 25 µL of PBS to wells of columns 1 to 12. Twenty-five (25) µL of test serum sample was then dispensed to columns 1 and 2. Column 1 served as the serum control. Serial two-fold dilutions of 25 µL volumes were carried out from columns 2 to 11. Twenty-five (25) µL from column 11 was discarded. Twenty-five (25) µL of the working dilution of antigen was then added to wells of columns 11 to 2 working backward. The plate was sealed and incubated for 1 hour at room temperature. Twenty-five (25) µL of 1% RBC suspension was dispensed to all wells beginning from column 12. The plate was incubated for 45 minutes at 4°C and was read immediately.

The procedure adopted herein was sourced from the Manual of Diagnostic Tests and Vaccines (OIE, 2012).

Research Site

The study was conducted on four (4) different sites located in Davao City. These were the Chua Game Fowl in Matina Crossing; Davao Organic Chicken (DAVOC) in Eden, Toril; Kahayag Farms in Sitio Acacia, Brgy. Biao Escuela; and Range Chicken Farm in Purok 2, Tacunan. Processing and analysis of all samples were conducted in the Department of Agriculture Regional Animal Disease Diagnostic Laboratory.

Participants

The conduct of the study was limited to the participation of native chickens from farms within Davao City.

Instrumentation

For the preparation and analysis of samples, the following materials and equipment were used: eight (8) 96-well microplates with U-bottom wells, four (4) HTL pipettes, centrifuge, autoclave, Newcastle Disease antigen source, prepared antigen concentration, Alsever's solution, prepared Phosphate-buffered solution, and RBC suspension as provided by the Department of Agriculture Animal Disease Diagnostic Laboratory.

Research Ethics Protocol

The study involved the use of vertebrate animals, particularly, *Gallus gallus domesticus* (Philippine native chickens). Blood serum samples were obtained with prior consent and understanding of the study by the farm owners. The animals and the collection of blood samples were handled by a licensed veterinarian. Fresh blood samples were then immediately transported to the Department of Agriculture Regional Animal Disease Diagnostic Laboratory for HI testing. Standard microbiological practices were employed in the laboratory. No other procedures in the study directly involved the said animals after sample collection.

The research was generally considered a BSL-1 study as it only involved the use of domestic animal blood and therefore posed a low risk to personnel and the environment. Serum samples were decontaminated by autoclaving. To inactivate infectious agents from the serum, the samples were placed in a water bath (56^{o}C) for 30 minutes. Laboratory work was supervised by trained personnel.

Data Collection

The presence or absence of a "button" formation after HI test served as the data recorded and interpreted in the study. Complete inhibition, exhibited by "button" formation and the presence of tear-shaped formation upon tilting of the microwells, was considered positive and wells are showing agglutination as negative. The endpoint for HI titer for each test row is the dilution of the last well showing complete inhibition of hemagglutination. Newcastle disease antibody-positive samples were those showing inhibition at or above 1:16 serum dilution or 2^4 using 4HAU antigen.

Statistical Techniques

Chi-square goodness of fit test was used for data analysis.

RESULTS AND DISCUSSION

NCD Titer Evaluation in Native Chickens

Among the blood samples taken from 60 native chickens from all of the identified farms, seven (7) samples were found to exhibit Newcastle Disease titers, of which two (2) were considered significant and five (5) were insignificant. These two native chickens with significant NCD titers were from Chua Game Fowl and DAVOC farm with titer counts of 32 and 16, respectively. Three of the

five native chickens with insignificant NCD titers were from DAVOC with titer counts of 2, 4 and 8, while the other two were from Range Chicken Farm with titer counts of 2 and 8.

Immune Status

HI titers are expressed as a value of 2^N, where N is the serum dilution concentration of antibodies that inhibit the antigen. A HI titer of 16 or greater following vaccination has been considered significant against virulent Newcastle Disease virus, and a titer of 64 or greater with no vaccination history suggests a recent infection by the virus. HI titers below 16 have been associated with lower levels of protection (Abdi et al., 2016).

The occurrence of titers can usually be explained based on the type of immunity elicited from the organism; either passive, from the use of attenuated vaccines or active, from its direct exposure to the pathogen. This study, however, considers the possibility of pre-immune antibodies in native chickens – antibody molecules produced even in the absence of antigen stimulation, because of their perceived resistance to diseases that allows them to thrive in a naturally selective environment even without vaccination (Alberts, Johnson, Lewis, Raff, Roberts & Walter, 2002).

NCD Titer Evaluation in Vaccinated Commercial Broiler Chickens

Broiler chickens in Grande Farm located at Kilometer 12, Sitio Ubat, Catalunan Grande, Davao City served as baseline comparison on the nature of NCD titers in vaccinated chickens. Purposive sampling was employed on the basis of the commercial production characteristic of the farm associated with the regular administration of vaccines, and the availability for access. Titer values ranged from 16 to 128, with all the samples having significant titer levels.

The positive titer level count for NCD antibodies exhibited by all the vaccinated chickens was expected given their vaccination history. Vaccines invoke a defense mechanism which allows the organism to develop immunity to the virus. The increase in the concentration of antibodies due to the elicited immune response thereby explains the significant higher titer counts. However, even with the use of a similar vaccine for each of these organisms, there is an apparent difference in their NCD titer counts, consequently indicating a difference in their levels of protection. This difference is attributed to variability in the physiological processes of organisms particularly their individual antibody immune responses, and the efficacy of administered vaccines (Van Boven, Bouma, Fabri, Katsma, Hartog & Koch, 2008).

Genetic Resistance Perspective

The two native chickens which exhibited positive or significant titer count for NCD antibodies came from unvaccinated farms, which mean that their immunity against the disease was not previously elicited with the use of vaccines. Meanwhile, the focus on environment-induced factors lies on the assumption that the organisms were exposed to certain agents under similar conditions, in this case, the NCD virus. It has already been established that chickens that survive infection with virulent NCD virus develop a long-lasting immunity to further infection with the said virus, but the possibility of having previous exposure to the disease in the case of the native chickens in the study, is not entirely plausible as the birds would have been carriers of the virus themselves and would have caused either an infection or an immune response to their flock. This is due to the contagious viral characteristic of Newcastle Disease transmitted by the introduction of new birds, selling or giving away sick birds, exposure to fecal and other excretions from infected birds and contact with contaminated feed, water, equipment and clothing (Hossain, Ali, & Yamato, 2010).

The infection or exposure of a single bird to the disease can immediately affect a whole population in a given area thereby causing the appearance of symptoms or in milder cases, the building up of resistance by the birds through their titer levels which was not observed for most of the native chickens (Centers for Disease Control and Prevention, National Center for Immunization and Respiratory Diseases, 2017)

Preventive Measures in the Philippine Context

Newcastle Disease outbreaks cause huge economic losses in the poultry industry of the Philippines, which therefore warrant traceback investigations to determine the cause and source of the outbreak and recommend effective control measures. In Bohol, the study of Tapdasan, Wongsathapornchai, Chanachai, Benigno, Gundran, Daguro & Lapiz (2013) attributed the NCD infection to uncontrolled ports of entry in the region which allowed infected poultry to be easily introduced. With regards to vaccination and quarantine methods, Cadeliña, Capuno and Batoy (2008), tested for the efficacy of DA-RFU 7, the locally produced ND vaccination, which showed significant production of antibodies against ND on free-range native chickens with various management systems.

Statistical Analysis

Subjecting the data values obtained to Chi-square goodness of fit test revealed no significant difference in terms of titer levels of the farms which were expected

as most of the native chickens show no titers, thereby indicating susceptibility to the virus for the majority. However, the two (2) native chickens out of sixty (60) which were identified to exhibit significant antibodies for the disease are considered outliers and cannot be simply attributed to environmental factors since fellow species were similarly exposed to the same conditions but exhibited no significant positive titers. The presence of titer counts, though insignificant, from five (5) other native chickens further support the possible genetic prospect of the study which considers the idea of pre-immune antibodies.

The study of Sun et al., (2012) on Immunoglobulin genes from domestic animals provided insights on antibody diversity of chickens. In support, the relative genetic resistance of birds to some viruses has already been recently established in a study conducted by Ruiz-Hernandez et al. (2016) wherein host-genetic control of infection was given focus and resistant birds, though infected, were found to have been unable to transmit the virus to contact birds. Accordingly, this resistance to infection was independent of adaptive immune responses.

As the concerns for the loss of genetic resistance to diseases in commercially bred birds arise due to one-sided selection for production traits, there is an apparent need to enhance genetic variation, specifically the alleles resistant to diseases. Considering that the process of adaptation of the native chickens to often harsh and extreme environmental conditions demanded selection of survival traits, it is likely that these birds are carrying alleles that determine their immuno-competence. However, this link, which is the baseline of the study, remains to be clarified through gene analysis as a support to the outlier phenomenon.

CONCLUSIONS

After analysis by hemagglutination inhibition test, two (2) samples collected from the unvaccinated native chickens were significantly positive, one (1) each from DAVOC and Chua Game Fowl, with titer levels of 1:16 and 1:32 respectively.

Titer levels of vaccinated broiler chickens from Grande Farm ranged from 1:16 to 1:128 which were all significantly positive as expected, given their vaccination history. The apparent difference in their NCD titer counts despite similar administration of vaccines, however, indicate a difference in their levels of protection attributed to variability in individual antibody immune responses, and the efficacy of vaccines which do not provide perfect immunity in organisms.

Statistical analysis shows that there was no significant difference in the titer levels of the unvaccinated farms as compared to Grande Farm, which was expected considering that most of the unvaccinated native chickens show no titers. The two (2) serum samples collected from unvaccinated chickens which tested significantly positive for NCD titers were considered outliers.

TRANSLATIONAL RESEARCH

Since the current research is a baseline study, the findings may be translated into various media of communication that are best accessible to students and researchers who can compound on the study. Magazines, newspapers and social media may be used in the information dissemination.

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