ANTIBODY TITER AND VIRUS SHEDDING IN THE CHICKEN VACCINATED AGAINST INACTIVATED LOCAL STRAIN OF NEWCASTLE DISEASE VIRUS (NDV) GENOTYPE VII

BY

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ABSTRACT

Newcastle disease is a very contagious disease in chickens and one of the most important diseases of poultry in the world. This study aimed to compare the effect on immune response and virus shedding in vaccinated chickens of the new inactivated vaccine of Newcastle disease virus (NDV) and commercial vaccine LaSota. The local subtype of NDV Genotype VIId (isolate 12234/10) was chosen as inactivated vaccine candidate and three different adjuvants were used which includes Oil-emulsion Complete Freund's, Oil-emulsion Squalene-based, and aluminium hydroxide gel (Alum). Ten different groups of chicken consists of ten each, were vaccinated with and without adjuvant of the inactivated vaccine via intramuscular and subcutaneous, respectively. As a comparison, a group of chicken was vaccinated with commercial vaccine and control group was not vaccinated. Then, all of the chickens were challenged with NDV Genotype VIIh (isolate 7979/01) two weeks after vaccination. Blood sera of the chickens were taken weekly for haemaglutination-inhibition (HI) test starting from prior to vaccination. Virus shedding of the chickens were calculated based on the virus isolation done within two weeks after challenge. With 100% survival rate and highest mean HI titre of $\log_2$ 7.8, the inactivated vaccine with adjuvant Oil-emulsion Complete Freund's surpassed the LaSota and the virus shedding also reduced significantly. Alum-adjuvanted inactivated vaccine gave most impressive results as LaSota with all chickens survived at $\log_2$ 6 of antibody titer and zero virus shedding. Whereas, without adjuvant, the chicken HI antibody titre is below $\log_2$ 4 after vaccination and only 20-30% were survived. Based on the post-mortem findings on the survived chicken from each vaccinated group, their internal organs appeared normal and no sign of haemorrhage or pathognomonic signs of Newcastle disease (ND). Conclusively, vaccinated chicken are effectively protected from morbidity and mortality against virulent genotype VII challenge with the addition of adjuvant into inactivated local strain of NDV genotype VII vaccine. Thus, the development of inactivated local NDV genotype VII vaccine is a promising candidate to control the current ND endemic in Malaysia.

خلاصة البحث

إن مرض نيوكاستل مرض معدي، وهو من خطر أمراض الدواجن في العالم. تهدف هذه الدراسة الى مقارنة الاستجابة المناعية اثناء استخدام اللقاح الجديد بالفيروس الخامل (ndv) واللقاح التجاري Losata. النوع المحلي للقاح الخامل (عزل ١٢٢٣٤) VII إختير مع ثلاث مكملات تحتوي على مستحلب دهن-شب-، مستحلب دهن سكوالين، هيدروكسيد الالمنيوم. تم اختيار عشر مجموعات من الدواجن تحتوى كل مجموعة على عشرة أفراد، حقنت باللقاح الخامل وبدون المكملات عن طريق الحقن داخل العضلة وتحت الجلد. تمت مقارنة هذه المجموعة من الدواجن بأخرى حقنت باللقاح التجاري Losata ومجموعة أخرى لم تحقن. عرضت جميع الدواجن للنوع Ndv، VII (عزل ٧٩٧٩٠١) لمدة اسبوعين بعد اللقاح وتم أخذ عينات من الدم لاختبار HI. بعد اسبوعين تم نقيس بواسطة العزل الفيروسي كمية الفيروس المثبط : ١٠٠% دجاج حي أعلى معدل في log2.78 الذي حقن باللقاح الخامل مع المكمل-الشب- متجاوز اللقاح التجاري Losata في حين الدجاج المحقون باللقاح دون مكمل يوجد فقط ٢٠الى٣٠% على قيد الحياة. بناء على نتائج فحص للدجاج الباقي على قيد الحياة من كل المجموعات ظهر أن الاعضاء الداخلية ما زالت طبيعية ولا يوجد أثر لأعراض نزيف أو أعراض مرض نيوكاستل. نستنتج من ذلك ان الدجاج الملقح استفاد من حماية من الاصابة و الموت ضد النوع VII المعرض له. ولذلك فمن المتوقع أن اللقاح المطور لمرض نيوكاستل نوع VII يكون وسيلة ناجحة للسيطر على مرض نيوكاستل DN المنتشر في ماليزيا.

APPROVAL PAGE

I certify that I have supervised and read this study and that in my opinion, it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a thesis for the degree of Master of Health Sciences

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In the name of God, Most Gracious, Most Merciful. Be praised to Allah, prayers and greetings to our beloved prophet Muhammad S.A.W. and his family and companions. I am grateful to Allah because by His permission and blessing so I could successfully complete this thesis writing.

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TABLE OF CONTENT

LIST OF TABLES

LIST OF FIGURES

CHAPTER ONE

INTRODUCTION

1.1 Background of Study

Newcastle disease (ND) is one of the most important diseases of poultry worldwide. Economically, ND severely affects the domestic poultry industry with mortality approaching 100% in fully susceptible flock (Alexander & Senne, 2008). It is highly contagious and known to infect over 241 species of birds (Kaleta & Baldauf, 1988). The disease is susceptible in all types of commercially reared poultry with chicken the most highly at risk (Alexander, 2000). Likewise, some avian species are resistant to Newcastle disease virus (NDV) and usually act as carrier and appear asymptomatic such as Japanese quails (Czirják et al., 2007), white Pekin duck (Nishizawa et al., 2007), turkeys, guinea fowls and exotic birds (Snoeck et al., 2013).

ND was first described in 1926 in Newcastle-on-Tyne, England and Java, Indonesia (Ashraf & Shah, 2014). NDV genotypes II, III, and IV were responsible for the first penzootic which began from Southeast Asia (Berhanu et al., 2010). The ND outbreak which appeared from Middle East in the 1960s had taken only 4 years to spread across the world becoming the second penzootic (Alexander et al., 2012). The emerging of commercial poultry industry form the Middle East extended to Southern Africa subsequently lead to the third penzootic in late 1970s (Shane, 2006) which primarily involved pigeons and doves (Wildlife Health Australia, 2011). The latest pandemic involving virulent NDV genotypes VII and VIII had emerged in the Far East, Europe, and South Africa since late 1980s (Czeglédi et al., 2006).

1.2 Statement of Research Problem

Major epidemic of ND had occurred in Peninsular Malaysia from 2000–2001 with peaking 84 outbreaks in 2001 (Berhanu et al., 2010). Additionally, Roosevien and Azri (2011) reported that there were 533 ND outbreaks occurred during 2000–2010 where the highest came from Perak with 129 outbreaks. Previously, NDV genotype VII was isolated from different states in Malaysia for causing ND outbreaks in chicken flocks from year 2000–2001, 2004–2005, and 2010 (Maizan et al., 2011; Tan et al., 2010). Until recently, more than 80% of NDV genotype VII had been identified and isolated from reported positive cases all over Malaysia (Veterinary Research Institute, 2014).

Although routine vaccination had been implemented since 1950s, ND outbreaks still manage to occur occasionally worldwide. The disease has causing endemic in 57% of the countries that raise poultry while further 23% subject to one or several introduction of the virus (Czeglédi et al., 2006). According to Alexander (2001), most countries that rear poultry commercially and where the disease is endemic are depend on vaccination to control the disease. However, several studies indicated that current available NDV vaccines are not sufficiently immunogenic against virulent NDV. The vaccines are able to induce protection against morbidity and mortality but they do not prevent the spreading of infection and virus shedding (Kapczynski & King, 2005; Miller et al., 2007; Jeon et al., 2008; Hu et al., 2009).

Most common commercial vaccines that being used worldwide such as LaSota and HitchnerB1 were isolated from low virulence NDV strains of genotypes I and II. The vaccine strains were sequestered more than 60 years ago which provide optimal protection against NDV during at the time compare to currently circulating virulent virus strains (Miller et al., 2007; Bwala et al., 2011).Thus, numerous studies had

suggested to vaccinate using homologous vaccines which will induced high haemaglutination-inhibition antibody (HI) titres and significantly reduced virus shedding (Hu et al., 2009; Miller et al., 2007; Jeon et al., 2008; Samuel et al., 2013; Miller et al., 2013).

HI antibody titre is used to indicate immune status of the host as it will ensure the protective efficacy of the vaccines (Reynolds & Maraqa, 2000). In addition, vaccination strategies such as the chick's age, vaccine doses, routes of administration and protocols will also greatly influence the immune response of chickens (Maragon & Busani, 2006; Kapcyzinski & King, 2013). More importantly, existence of the chick's maternal antibodies during vaccination rendering the vaccine futile because the vaccine antigen will be neutralized (Al-Zubeedy, 2009). Hence, this study was conducted for development of improved vaccines and vaccination regime that is directed toward maintaining high antibody levels and preventing the spread of infection.

1.3 Importance of Study

Implementation of intensive vaccination programs still does not managed to eradicate sporadic cases and ND outbreaks. According to previous study, the current vaccines are no longer effective to render complete immunity protection against NDV infection and outbreaks (Alexander, 2001). The potency of the vaccines are reduced since the isolates of vaccines are from low virulence NDV genotype I and II, whereas NDV genotype VII is the predominant causing agent of outbreaks (Perozo et al., 2012). Compared to the heterologous vaccine, the vaccine that have same genotype with infectious virus will induced high level antibody production and eliminate or reduce the virus shedding from chicken's secretions (Miller et al., 2007). Some studies

suggest that by increasing the capability of immune response, it will elevate the probability of flock's survival and reduced the spreading of infection (Kapczynski & King, 2005). Moreover, immune status of the host has been found to be greatly influenced the severity of the NDV (Reynolds & Maraqa, 2005).

Thus, by developing local vaccine from NDV genotype VII that was conducted in the present study, it will provide effective protection against virulence NDV strain to our local commercial and backyard chicken. The success of developing this vaccine in this study will provide a promising candidate of homologous NDV vaccine that may lead to control and eradication of NDV in Malaysia and bringing Malaysia towards ND free status.

1.4 Objectives of Study

The main objective of this study is to analyse the inactivated vaccine from local NDV genotype VII and challenged the vaccinated chicken against virulent NDV. Specific objectives of the study are further elaborate as:

1. To compare the effect on immune response between chickens administered with non-adjuvant inactivated vaccines, adjuvant inactivated vaccine, and commercial vaccine LaSota.
2. To determine the most successful route to administer the inactivated vaccine which is either through intramuscular or subcutaneous.
3. To observed the capability of virus shedding after-challenged in chicken between vaccination with homologous-matched NDV genotype VII inactivated vaccine and heterologous-matched commercial vaccine.

1.5 Scope of Research Work

The study involved the development of inactivated vaccine from NDV genotype VIId and conducting the experimental study of the developed vaccine on Specific-Pathogen-Free (SPF) and broiler chickens. The vaccine strain isolate 12234/10 and challenge virus strain isolate 7979/01 were provided by Veterinary Research Institute, Malaysia. Both strains are chosen based on their ability to circulate causing the occurrence of sporadic recent outbreaks.

Firstly, the NDV isolates were propagated by inoculation in the allontoic fluid of 9-10 day old embryonated SPF chicken eggs. Then, the concentration of virus suspension were measured in 50 percent Embryo Infectious Dose (EID_{50}) using the mathematical technique devised by Reed and Muench (1983). The propagated virus was inactivated using chemical binary ethylanimine (BEI), following the standard protocol for vaccine preparation.

In experimental study, one hundred of two-week old SPF chickens were obtained from the Veterinary Research Institute and were maintained in isolation units. The chickens were randomly divided into ten groups of ten each. Each group was assigned with different vaccination regime based on the mixture between the vaccine and types of adjuvant. This study used three types of commercial adjuvants which are Complete Freund's Adjuvant (Sigma Aldrich), Squalene-based oil-in-water Adjuvant (AddaVax), and aluminium hydroxide gel (AddaVax), and one type commercial vaccine LaSota. With virus concentration at $10^{8.8}$ EID_{50}, four groups of the chicken received 0.2 – 0.4 ml of the vaccine via intramuscular while the other four groups by subcutaneous. A group of chicken vaccinated by LaSota through eye drop as prescribed and one control group was unvaccinated. Two weeks after vaccination,

all groups were challenged with virulent NDV genotype VIIh at concentration $10^{4.3}$ EID_{50} via intramuscular.

The chickens were bleed weekly via intravenous prior and after vaccination, and their sera were subjected to haemaglutination-inhibition (HI) assay for antibody titre evaluation. Within two weeks after challenged, virus shedding study was completed with virus isolation test by using the swab samples taken from tracheal and cloacal. The swabs were transport using Tryptose Phosphate Buffer with PSK (penicillin, streptomycin, and kanamycin) antibiotics. The samples were filtered before inoculated into SPF eggs to determine the positive samples and evaluate the virus shedding. Finally, all of the surviving chickens were culled and subjected to post-mortem by certified veterinarian for further investigation.

CHAPTER TWO

LITERATURE REVIEW

2.1 Newcastle Disease Virus

Pathogenic newcastle disease virus is an enveloped avian paramyxovirus serotype 1 (APMV-1) that has an intracerebral pathogenicity index (ICPI) greater than 0.7 and have multiple basic amino acids (at least three arginine or lysine residues) at the C-terminus of the F2 protein and phenylalanine at residue 117 of N-terminus F1 protein (Office International des Epizooties [OIE], 2012).

2.1.1 Aetiology

Taxonomically, NDV belongs to the genus Avulavirus, within the family *Paramyxoviridae* from the order Mononegavirales. It consists of non-segmented, negative-sense, single-stranded RNA with genomic size of approximately 15kb. So far, its genome codes for at least six structural proteins including nucleoprotein (N), phosphoprotein (P), matrix (M) protein, fusion (F) protein, hemagglutinin-neuraminidase (HN) protein and RNA polymerase (L). The F and the HN proteins are the two interactive glycoproteins which form the spike structures on the virion surface and involved in cell surface attachment and cell membrane fusion (Figure 2.1). The F protein is synthesized as a precursor F_0 is proteolytically cleaved into 2 disulfide-link subunits, F_1 and F_2, which is necessary for the infectivity of paramyxovirus (Nagai et al., 1976; Glickman et al., 1988; Alexander & Senne, 2008).

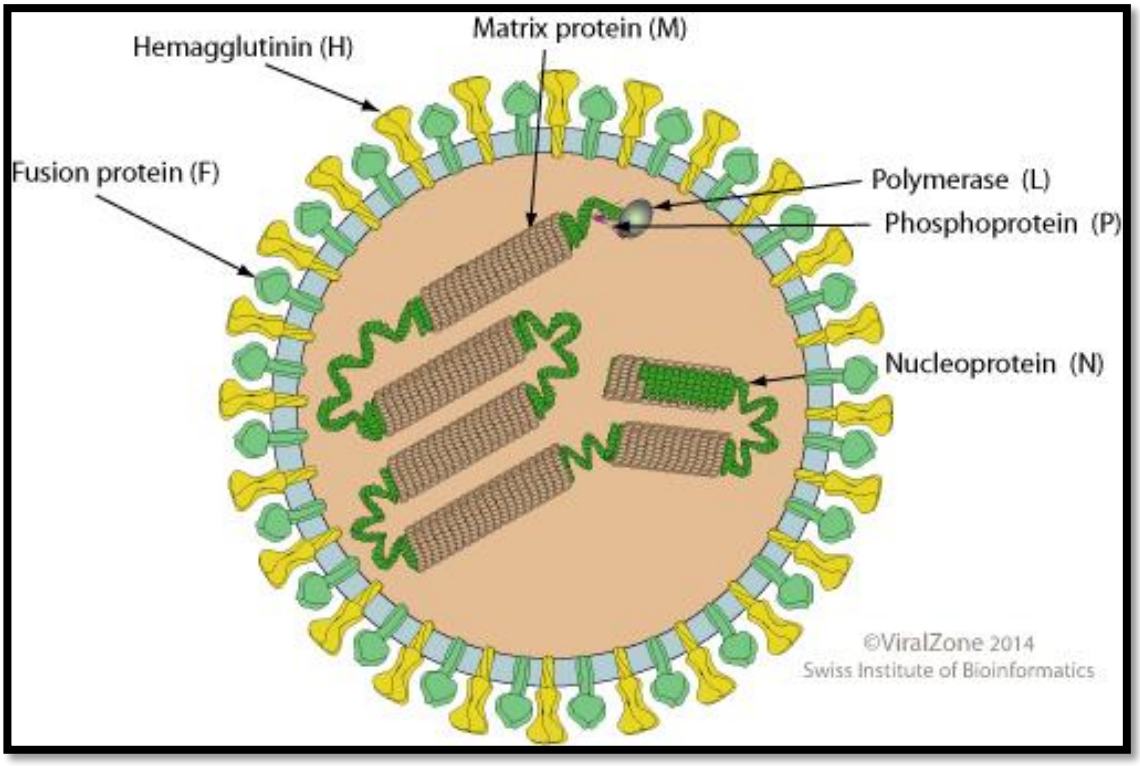

Figure 2.1: Diagram of NDV

Based on analysis of the F and HN gene sequences, nine genotypes of Class I and 10 of Class II NDV (Genotypes I to X) strains have been identified. Most NDV sequences of Class I are low virulence and isolated from waterfowl. Meanwhile NDV isolates from Class II viruses were commonly from poultry, pet, and wild birds. Genotypes I-IV of Class II are primarily isolated during early years before 1960. Genotypes V-X from Class II are all virulent viruses, and of these, genotype VII is particularly important given it has associated with many of the most recent outbreaks in Asia, Africa, and the Middle East (Gould et al., 2003; Czeglédi et al., 2006; Kim et al., 2007; Diel et al., 2012). Moreover, the genotype VII of NDV is still predominant in the domestic poultry of Asia, and these viruses are not evolutionary widely different from the 1990s (Ebrahimi et al., 2012).

In recent decades, Malaysia had experienced several outbreaks in backyard (village chicken) and also commercial poultry which affected the economic's states specialized in poultry production cause by NDV genotype VII. Shamsiah *et al.* (2015) stated that 129 out of 151 isolates from positive ND cases reported around Malaysia during 1999-2012 were originated from genotype VII. Based on further analysis of phylogenetic tree, eight subgenotypes (a-h) of NDV genotype VII had been identified. NDV genotype VIIb isolate had been reported as early as 1990 which then reisolated in 1999 of the ND positive case (Tan et al., 2010; Berhanu et al., 2010; Maizan et al., 2011). However, according to Shamsiah *et al.* (2015), subgenotype VIId and VIIh are predominantly isolated strain which associated with ND outbreaks in Malaysia.

2.1.2 Pathogenicity

Formerly, studies had defined that the difference at the amino acid sequences surrounding the F protein cleavage site is a major determinant for NDV virulence. Virulent isolates have the multibasic amino acid sequence ^{112}R/K-R-Q-K/R-R^{116} at the C terminus of the F_2 protein and a phenylalanine at residue 117 located at the N terminus of the F_1 protein which are cleaved intracellularly by ubiquitous furin-like proteases. Whereas, low virulent NDV have a monobasic amino acid of ^{112}G/E-K/R-Q-G/E-R^{116} at the C terminus of the F_2 protein and leucine located at residue 117 of the N terminus of the F_1 protein which are sliced extracellularly by trypsin-like proteases (Panda et al., 2004; de Leeuw et al., 2005; Dortmans et al., 2011). This suggested that low virulence viruses are restricted to replicate at site with trypsin related enzymes which mainly found at the respiratory and intestinal tracts. Meanwhile, virulent viruses can cause fatal systemic infections resulting from damages of tissues and organs (Rott, 1979; Alexander et al., 2012).

By pathotyping, NDV strains were classified into the highly pathogenic (velogenic), moderately pathogenic (mesogenic), and lowly pathogenic (lentogenic) categories. Lentogenic strains of NDV may cause minimal or no clinical sign in adult chicken, but often cause mortality in young and fully susceptible. In adult chicken, mesogenic strains usually cause respiratory problems and drop in egg production, while mortality only affects the young. With mortality reach up to 100%, the velogenic forms can be further classified into viscerotropic (VVND) and neurotropic (NVND) types based on clinical manifestations and lesions (Alexander & Senne, 2008).

2.1.3 Diagnostic Tests

The divergent in virulence of NDV isolates and massive usage of live vaccine means that detection of ND from birds showing clinical signs is inadequate for diagnosis. As emphasized by previous study, some isolates submitted to an International Reference Laboratory were avirulent even though the birds were showing similar clinical signs as virulent NDV. Thus, further assessments of the isolates are required by pathogenicity testing or nucleotide sequencing (Alexander et al., 1987; Bennejean, 1988).

ND is diagnosed with isolation of virus mostly obtained from tracheal or cloacal swabs. Then, it is either cultured into cells or specific pathogen-free eggs. The chicken eggs must be free from at least NDV antibodies and at 9 to 11 days old. The NDV isolation will further verify with serological tests as it may indicates the virus infection. The haemaglutination inhibition (HI) test had been used regularly for antibodies detection against APMV-1 in birds. Standard method of HI test for avian haemagglutinating viruses includes application of V-bottomed microwell plastic plates

in normal room temperature which is between 20-25 °C. However, if the surrounding temperature is high, it is recommended for all the solutions and plate that are used during the test to be in 4°C condition. Usually, it is unnecessary to pre-treat poultry sera prior to the test except in regard to serum samples from avian other than chicken which sometimes tend to give false positive results. Serum samples from unvaccinated or uninfected birds will constantly give HI titres less than 1/8 (Brugh, 1978; Alexander, 1988, 2000). Alternatively, the enzyme linked immunosorbent assay (ELISA) tests also able to measure the concentration of antigens in blood serum. There are three common types of ELISA tests depending on its binding structure between antibody and antigen which are Indirect, Sandwich, and Competitive ELISA. The ELISA test is preferable in flock screening for multiple pathogens with its rapid and easily semi-automated procedures. However, complex measurement of enzyme activity and expensive commercially available test kits might be the downsides of this test (Kfir & Genthe, 1993; Pokhrel, 2015).

Virulence characterisation of NDV can be distinguished using several in- vivo tests specifically the mean death time (MDT) in embryonated chicken eggs, the intravenous pathogenicity index (IVPI) in six-week-old chickens, and the intracerebral pathogenicity index (ICPI) in one-day-old chickens. MDT and IVPI may use as reference but it tend to be inaccurate in some occasion particularly involving hosts other than chicken. Meanwhile, ICPI were recognized for its precision and sensitivity and had been the general method to evaluate the pathogenicity of NDV (Alexander, 1988; Dortmans et al., 2011; OIE, 2012).

The conventional diagnostic method is regard as slow, laborious, require significant amount of animal's usage, and limited information on epidemiology. Thus, molecular techniques were developed to further understand the origin of the virus and

its spread of infection. Currently, real-time polymerase chain reaction (rT-PCR) is widely used method for the detection, identification and characterization of NDV. This method is rapid on-set since the virus can be amplified directly from infected tissue (Jestin, Cherbonnel, & Arnauld, 1993; Aldous & Alexander, 2001; Creelan, Graham, & McCullough, 2002). However, the presence of PCR inhibitors specifically blood and faeces may cause hindrance. Hence, it is crucial to ensure that the inhibitors had been deactivated by RNA extraction method (Wilde et al., 1990; Gohm et al., 2000). Overall, rapid diagnosis of ND is one of the important factors in controlling outbreaks.

2.2 Avian Immune System

Successful vaccination strategies can be achieved by having a firm understanding in the basic of the avian immune system and its interaction with the disease. Besides, study found that the severity of NDV is influenced by immunity level of the host against the disease (Reynolds & Maraqa, 2000).

2.2.1 Innate versus Adaptive immunity

The layers of avian defence mechanism mainly consist of innate and adaptive immunity (Figure 2.2). The basis of their action is as barrier against pathogen entry and spread of infection. As a first line defence, innate immune response are capable of instant response towards microbes, thus, prevent their entrance. The system includes physical and chemical barriers (e.g.: skin, mucosal epithelium, gastric secretions), serum proteins, and phagocytic cells (e.g.: macrophages, heterophils, thrombocytes, natural killer cells). However, this mechanism unable to provide specific protection against various types of pathogens (Davison, 2008).

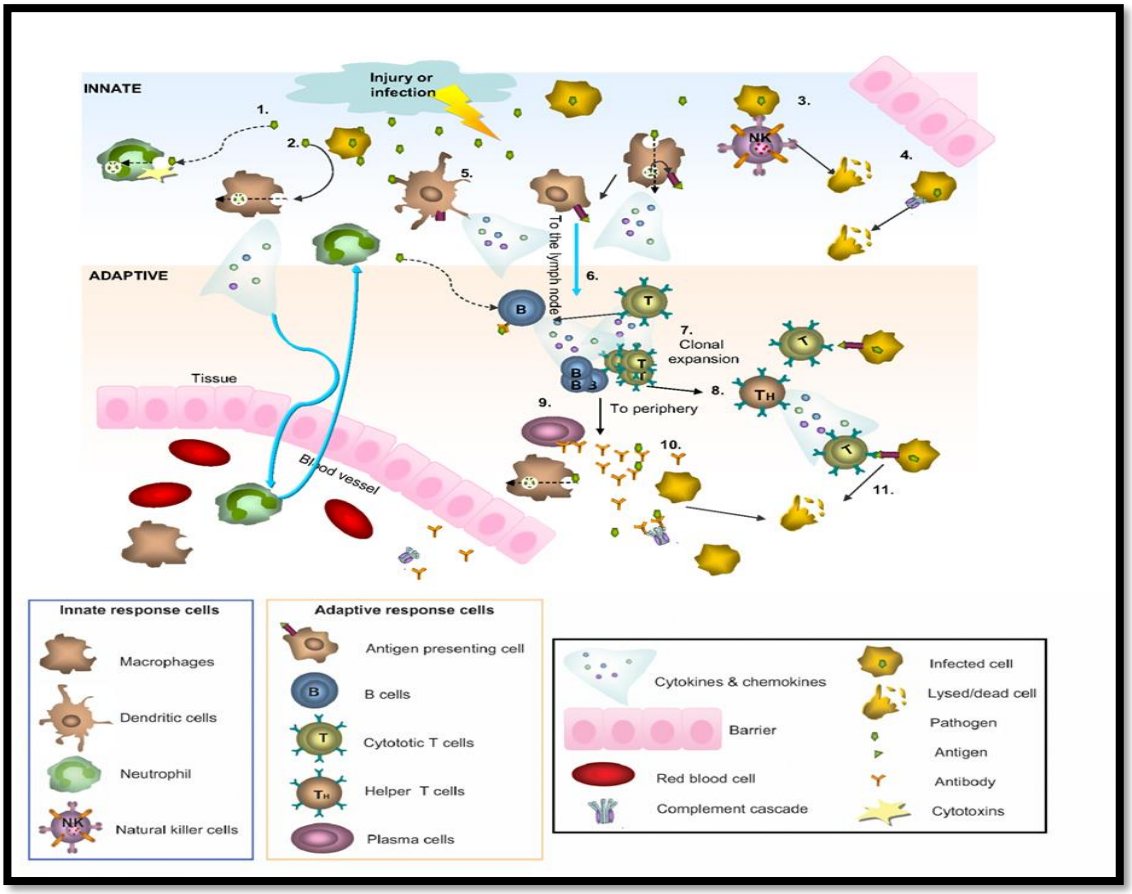

Figure 2.2: Innate and Adaptive immunity (Source: Frontiers Synaptic Neuroscience)

The immediate reaction of innate immune response had enabled the adaptive immune response to develop specific protection and inhibit virus growth. This specific protection can be delivered by either passive immunity or active immunity. The passive immunity or also known as Maternal Derived Antibodies (MDA) are naturally present and provide protection to chicks within 1-2 weeks after hatch. Whereas, active immunity develops through exposure to pathogens and can be further classified to humoral and cell-mediated immunity. B and T lymphocytes are the main cells responsible for their immune response immunity (Jeurissen et al., 2000; Grogan et al., 2007).