KnE Life Sciences
VMIC 2017
The Veterinary Medicine International Conference 2017
Volume 2017

Conference Paper

Detection of Newcastle Disease Virus by Immunohistochemistry on the Brains of Laying Birds with Clinical Signs Torticollis and Curled Toe Paralysis

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Received: 03 October 2017
Accepted: 10 October 2017
Published: 29 November 2017

Publishing services provided by Knowledge E

Selection and Peer-review under the responsibility of the VMIC Conference Committee.

Abstract

Poultry is one of animal protein sources that have been consumed much in Indonesia. Various of health disorders are caused by environmental changes, woof and infectious agents have caused decline in production and economic loss. One of diseases that have a big impact on poultry husbandry is Newcastle disease (ND). Newcastle disease virus (NDV) is an important disease in Indonesia, because this has spread around Indonesia and caused a big loss for poultry industry moreover, this has high morbidity and mortality and very rapid spread of the virus. The purpose of the research is to find out and determine a fast, exact and accurate, and efficient and effective application immunohistochemistry streptavidin biotin (IHK SB) test on NDV diagnosis affirmation and for NDV fast detection as a cause of disease on commercial laying hens with clinical torticollis and curled toe paralysis symptoms. The samples used in the form of tissues from 20 commercial laying hens with an indication of clinical torticollis and curled toe paralysis symptoms. Brains would be tested using streptavidin biotin. Inspection result data obtained by immunohistochemistry streptavidin biotin (IHK SB) method is analyzed in a descriptive and qualitative way. The result of the research proves that IHK SB coloration, NDV antigen could be detected on brains. Based on the result of the research, it could be concluded that IHK SB could be applied for NDV diagnosis affirmation and it proves that commercial laying hens with clinical torticollis and curled toe paralysis symptoms.

Keywords: Newcastle disease virus, immunohistochemistry streptavidin biotin, torticollis, curled toe paralysis.

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1. Introduction

Newcastle disease (ND) is a deadly endemic disease caused by Newcastle disease virus (NDV), avian paramyxovirus type -1 (APMV-1). NDV is considered as top ranking viral diseases of poultry by the Office International des Epizooties. These viral disease is responsible for serious economic losses to the poultry industry all over the world every year [1, 2]. ND is another highly contagious viral disease of many domestic and wild species of birds throughout the world [3] which is caused by an enveloped, single stranded, negative sense and nonsegmented genomic ribonucleic acid (ssRNA) virus. NDV belonging to the genus Rubulavirus and family Paramyxoviridiae [4]. Based on the pathogenic and virulence properties, NDV is categorized into three major pathotypic strains e.g. lentogenic, mesogenic and velogenic strains. Velogenic Newcastle disease (VND) is the most severe form of the disease and is probably the most serious viral disease of poultry throughout the world. Newcastle disease was first reported in 1926 in the East Indies and then in 1927 at Newcastle-on-Tyne, England, after which it is named.

NDV is spread primarily through direct contact between healthy birds and the bodily discharges of infected birds. The disease is transmitted through infected birds droppings and secretions from the nose, mouth, and eyes. High concentrations of the NDV are found in body discharges, therefore, the disease can be spread easily by mechanical means. NDV can survive for several weeks in a warm and humid environment on Birds feathers, manure, and other materials. It can survive indefinitely in frozen material. NDV affects the respiratory, nervous, and digestive systems. Symptoms are very variable depending on the strain of NDV, species of bird, concurrent disease and preexisting immunity. The incubation period for the disease ranges from 2 to 15 days. An infected bird may exhibit the signs of respiratory problems including sneezing, gasping for air, nasal discharge, coughing, greenish, watery diarrhea, nervousness, depression, muscular tremors, drooping wings, twisting of head and neck, circling, complete paralysis and swelling of the tissues around the eyes and in the neck. Both NDV and AIV often display almost similar clinical signs, post-mortem lesions and the pattern of outbreak that need to be differentiated [5].

Non-purulent encephalomyelitis with neuronal degeneration, foci of glial cells, perivascular cuffing of lymphocytes, and hypertrophy of endothelial cells of the brain are seen in ND in chickens. These brain lesions are invariably observed in the chickens infected with the neurotropic velogenic pathotype and commonly with viscerotropic and mesogenic pathotypes. Generally, histologic lesions of the central nervous system are found in the cerebellum, medulla, midbrain, and spinal cord but rarely found in the

cerebrum. However, the lesions were most frequently observed in the cerebrum in the broilers naturally infected with ND. The lesions of the present case were seen in the medulla, cerebrum, and cerebellum in decreasing order of frequency [6].

Immunohistochemistry has emerged as a powerful investigative tool that can provide supplemental information to the routine morphological assessment of tissues. The use of immunohistochemistry to study cellular markers that define specific phenotypes has provided important diagnostic, prognostic, and predictive information relative to disease status and biology. The history of immunohistochemistry has been a constant effort to improve sensitivity for detection of rare surviving antigenic targets with the ultimate goal of integrating tissue-based analysis.

The advantages of using immunohistochemical streptavidin-biotin is its strong binding between streptavidin and biotin. Streptavidin biotin method is better when compared to the avidin biotin complex method for streptavidin biotin binding capability which has more and more powerful than avidin. The use of streptavidin biotin method could improve the sensitivity test because in one molecule, there are four binding sites of streptavidin to biotin, which is expected sensitivity four times higher than with other methods of immunohistochemistry [7].

2. Materials and Methods

The sample is conducted using approximately 20 laying hen tissues which have the clinical symptoms torticollis and curled toe paralysis. The samples collected were brains. The samples were fixed with 10% of neutral buffer formalin. It is made preparation of histopathologic process and the color is applied using immunopathology immunohistochemistry streptavidin biotin. The first step is deparaffinization and rehydration, and the second step is immunostaining. The first step of deparaffinization and preparation, tissue was put into xylene for 3 times and each took for two minutes. Next, rehydration was conducted by putting continuously the tissue preparation into absolute ethanol solution for two times, each one for two minutes each, 95% ethanol 1 time for 2 minutes each, 50% ethanol 1 time for 2 minutes, Aquadest 2 times for 2 minutes each. After deparaffinization and rehydration, the tissue preparation was rinsed with phosphate buffered saline (PBS) 0.01 MpH 7.1 for 5 minutes and then the tissue preparation is ready for the second step, i.e that procedure of immunopathology immunohistochemistry streptavidin biotin.

For second step of Immunostaining, at first, the tissue preparation was incubated with 3% of H_2O_2 in absolute methanol solution for 10 minutes. Then, it was rinsed

again with PBS solution for 10 minutes. The tissue preparation was incubated with blocking serum solution for 10 minutes. After the incubation, the leaving remnant of blocking serum solution in the tissue preparation was rinsed with tissue paper. Next, the tissue preparation was incubated with polyclonal antibody of anti NDV or anti AIV for 45 minutes at room temperature. The tissue preparation was rinsed with PBS solution for 10 minutes. The tissue preparation was incubated with solution of secondary antibody labeled with biotin for 10 minutes. Then, the tissue preparation was rinsed with PBS solution for 10 minutes. The tissue preparation was incubated with streptavidin peroxidase conjugated for 5 minutes. Then, the tissue preparation was rinsed with PBS solution for 10 minutes. The tissue preparation was incubated with mixed-substrate (H_2O_2) chromogen (3.3^1-diaminobenzidine) solution for 15 minutes at room temperature and then the tissue preparation was rinsed with aquadest for 10 minutes. Next, the tissue preparation was dropped with hematoxylin solution as a basic dye and it was incubated for 3 minutes. It was rinsed, with aquadest, dehydrated and was given a glycerol adhesive medium, then closed with the cover glass. The tissue preparation which has been applied the immunohistochemistry streptavidin biotin staining was then observed under light microscope.

3. Results and Discussion

Results of the present study showed that on the uninfected NDV tissues, the IHC SB staining was negative (Fig.1). Meanwhile, the NDV antigens can be detected in the brains (Fig.2-3), respectively. NDV are seen in the form of a group of spread reddish brown discoloration or spreading throughout of the tissue. The existence of horseradish peroxidase (HRP) as an enzyme on the bond of complex antigen-antibody has caused the changing color during substrate-chromogen application. The substrate which is conducted in this method is hydrogen peroxide (H_2O_2) and the chromogen is diaminobenzidine (DAB) which enable to see the changing color into reddish brown on the infected tissue, meanwhile there cannot be found any changing color (reddish brown) on the uninfected tissue [8]. In the method of IHC SB color application, it will only need approximately 3-5 hours to detect NDV antigen on chickens tissue. Hydrogen peroxide (H_2O_2) solution is given to block endogenous enzyme (hydrogen peroxide) which is commonly located in all kinds of tissues and cells, especially in erythrocyte and leukocyte. If peroxidase is not blocked first by H_2O_2, the given substrate-chromogen will give a reaction toward peroxidase inside the cell or tissue, then it will create a false positive. Blocking endogenous peroxidase is conducted by giving H_2O_2as a peroxide substrate in the tissue preparation. H_2O_2 substrate will take peroxidase

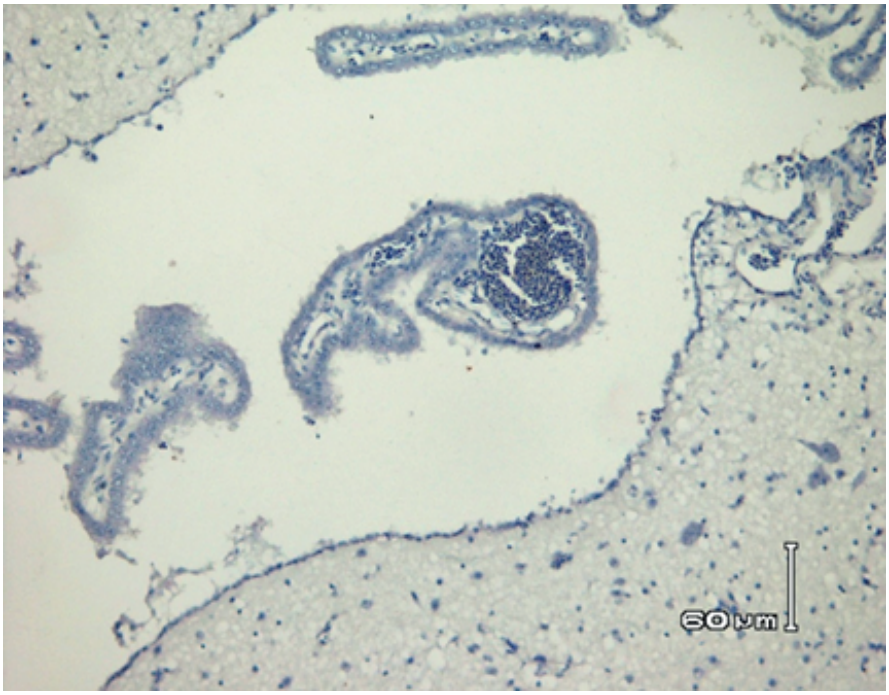

Figure 1: The brains of the laying hen with clinical symptoms of torticollis and curled toe paralysis (negative control). Immunohistochemistry streptavidin biotin negative, no brown discoloration (Streptavidin biotin, 250x.).

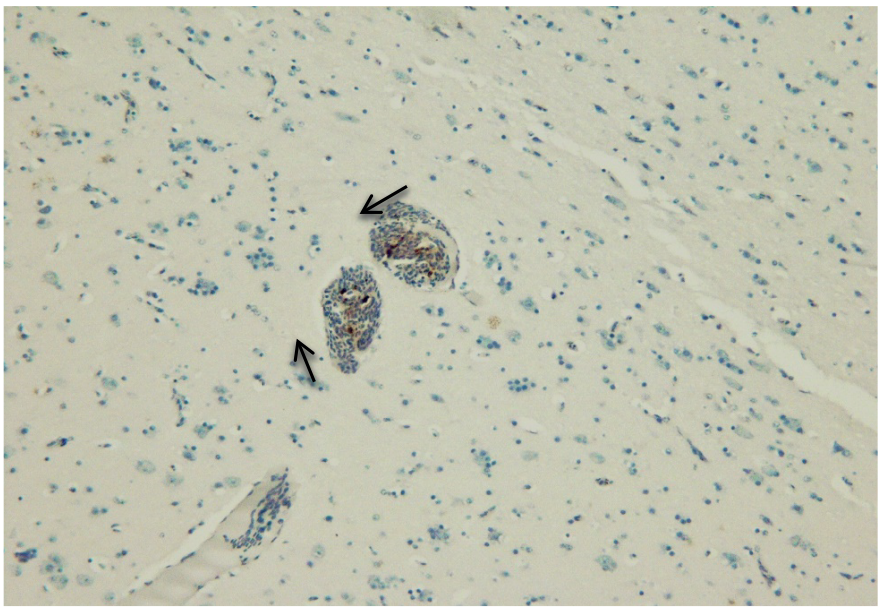

Figure 2: The brain of the laying hen with clinical symptoms of torticollis and curled toe paralysis. Immunohistochemistry streptavidin biotin positive NDV seen as reddish brown discoloration (↑) (Streptavidin biotin, 250x.).

from inside of cell, therefore peroxidase which is attached with antibody will give reaction to substrate and chromogen [9]. Chromogen conducted in IHC SB method is diaminobenzidine (DAB) that will give a result for the located antigen to change its color into reddish brown in the tissue preparation which is positively infected by NDV.

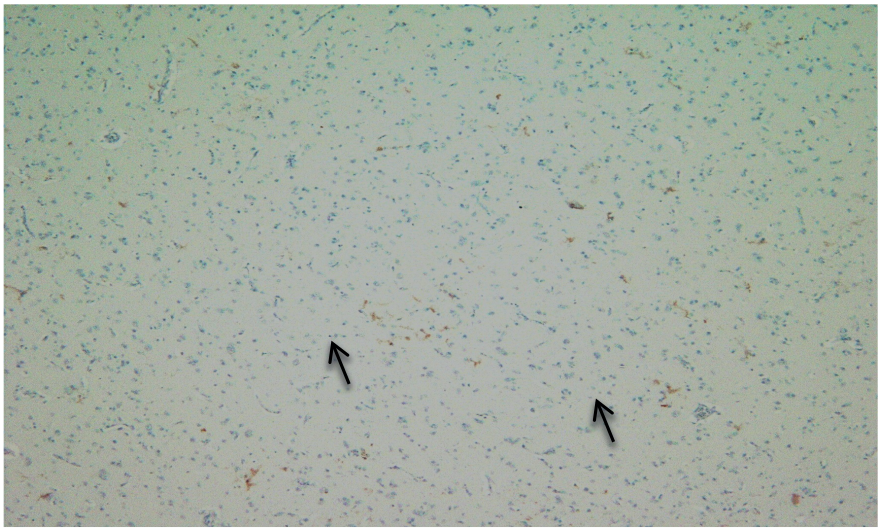

Figure 3: The brain of the laying hen with clinical symptoms of torticollis and curled toe paralysis. Immunohistochemistry streptavidin biotin positive NDV seen as reddish brown discoloration (↑) (Streptavidin biotin, 250x.).

The blocking procedure is to close or minimize the non-specific bond between molecules in the tissue preparation with antibody. The non-specific antibody can prevent the emergence of non-specific color (false positive), otherwise the false positive result will be received from the reaction of the non-specific bond of antigen and primary antibody [9].

In the present study, the given primary antibody specifically functions to detect the emergence of the virus, which causes the ND in the tissue preparation of the chickens. The tissue preparation needs to be wet during the application of color in IHC SB. The given secondary antibody was labeled with biotin, when it was given HRP enzyme conjugated with streptavidin, it created a complex streptavidin-biotin. Then, it was added H_2O_2 substrate which reacted to HRP and the result was that it released ion hydrogen. Ion hydrogen was attached with chromogen by creating precipitate and if the tested tissue consists of the targeted antigen, it will create a final product in the form of reddish brown discoloration [10]. Chromogen is functional groups of chemical compounds that creates colored compound if it is reacted with certain compound. The interaction between antigen and antibody is an invisible reaction, then it is needed a visualization on both interactions with the used antibody molecule with enzyme. Enzyme is reacted with chromogen substrate in order to create colored final product and it is insoluble that can be observed using light microscope [9].

The special quality of using IHC method is to trace the virus distribution on every organ. It will be used to acknowledge and decide the pathogenesis virus infection in chicken. The IHC is considered as a safe way because it is conducted on the fixed

tissue with formalin. Therefore, the tracked virus is an inactive virus, then the viral transmission on the sensitive host can be avoided [19]. Moreover, the use of IHC SB method can increase the test sensitivity because one molecule streptavidin consists of four attachment sites toward biotin then it will be assumed that the sensitivity will increase four times higher than using common IHC method [7]. SB method will be conducted better if it is compared with complex avidin biotin method because streptavidin has an ability to attach biotin greater and stronger than avidin [10].

In the histopathology examination, there is a significant change on the brain, such as hiperemia, lymphocytic perivascular cuffings and endothelia cells hypertrophy [11]. The virus transmission from the blood flows until bone marrow and brain and will cause nerves symptoms resembles with torticollis and tremor [12]. Torticollis can appear because of virus replication inside of the brain cell which can cause necrosis brain cell and it is followed the development of mild lymphocyte perivascular cuffing to the heavy one. This symptom is commonly found with other clinical symptoms, such as decreasing of appetite, emaciation, decreasing of eggs production, breathing symptom, such as coughing, sneezing, sticking the neck out, hyper-lacrimation, dull feather, edema on face and feet, cyanosis on featherless skin and diarrhea [13].

ND can be induced by some factors, such as the not optimal vaccination program, the level of immunosuppressive disease and the management aspect that makes ND still becomes fowl disease which can damage the financial problem of the poultry breeder [14]. ND virus is able to infect more than 200 fowl species, however the worst level of the disease caused by NDV infection is various and it depends on host (Fowls species) and NDV furrow. NDV furrow which has less pathogenic can cause the worst disease in fowl if it is followed with secondary infection by other microorganism and the bad environment condition [15]. The existence of NDV in the tissue of laying hen might be caused that the hens have not been given NDV vaccine or have already given NDV vaccine, but it is not optimal then the immune body cannot develop perfectly toward NDV virus. This causes the chicken are potentially infected with NDV virus. The result shows that the importance of perfect vaccination toward commercial laying hens in the frame of NDV epidemic eradication is crucial. The success of vaccination can be influenced by how the vaccine is given in the right dose [14]. Moreover, the exact neutralization epitope also influences toward the development of immune response in neutralizing virus that infected the chicken. The success of vaccination can happen because the developed antibody is expected to be able to neutralize the field virus which infects the chicken and it doesn't cause the appearance of clinical symptom and death [16].

NDV antigen can be detected with IHC method in lymphocyte, macrophage, lymph, respiratory and digestive tracts, bone marrow, kidney, liver, heart and particularly inside eyelid, lymphoid organ and lymphoid aggregate which is associated with mucous membrane in chickens infected by NDV [17]. NDV genome is successfully detected with RT-PCR method on lymph, lungs, brain, and the broiler's colon and the laying pullet's infected by NDV [18]. There are a lot of factors that can give various results, such as virus differentiation, virus infection dose, the transmission route, hospes sensitivity, variability between animal individuality, the step of method and reagent that is used in that method [19].

The results of the present study indicated that NDV infection on the commercial laying chickens showing torticollis and curled toe paralysis. The NDV are identified in the brains by using immunohistochemistry streptavidin biotin approach.

4. Conclusions

Immunohistochemistry staining of streptavidin-biotin can be applied to the detect Newcastle disease virus (NDV) in the brains. IHC SB can be applied for confirmation of the diagnosis of NDV and proven infection of the virus can be differentiated by this technique.

Acknowledgements

This study was supported by Direktorat Riset dan Pengabdian Masyarakat, Direktorat Jenderal Penguatan Riset dan Pengembangan, Kementerian Riset, Teknologi, dan Pendidikan Tinggi and Gadjah Mada University.

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