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Conference Paper

Immunopathological Approach for Avian Influenza Virus Detection in Brain of Laying Bird with Clinical Signs of Torticollis and Curled Toe Paralysis

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Abstract

Infection with avian influenza virus (AIV) in laying birds field cases often lead to clinical signs of torticollis and curled toe paralysis. Clinical signs of torticollis and curled toe paralysis is similar to the clinical signs of Newcastle disease virus infection in poultry, making it difficult for the confirmation of diagnosis of infection with both viruses. Immunopathological immunohistochemistry streptavidin-biotin (IHC SB) is an antibody-based test to detect the presence of pathogens, especially AIV in poultry with the principle of antigen detection in tissue specimens. This research aims to detect AIV infection in laying birds field case with clinical signs of torticollis and curled toe paralysis. The samples (brains from 20 layer chicken) were taken from the cases of the disease in poultry in several commercial poultry farms. The chickens showed clinical signs of torticollis and curled toe paralysis, and oedema in the brain suspected of being infected AIV. After being necropsied, then the brains were tested IHC SB and observed with a digital microscope camera system and analyzed descriptively qualitative. The results showed that the avian influenza virus antigens was found in the parenchyma, neural and endothelial cells of brain blood vessels. Based on these results, we can conclude that Immunopathologic immunohistochemical staining of streptavidin-biotin can be applied for confirmation of the diagnosis of avian influenza virus (AIV) on the brain of commercial laying chickens showing torticollis and curled toe paralysis.

Keywords: AIV; IHC SB; brain; torticollis; curled toe paralysis.

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1. Introduction

Avian influenza (AI) is a disease of poultry with high morbidity and mortality rate and can cause huge economic losses. AI is zoonotic viral diseases in birds. Influenza viruses that infect birds called avian influenza virus (AIV). AI is caused by Influenza A virus, which belongs to the family Orthomyxoviridae [1]. AI is an infectious disease of birds, ranging from a mild to severe form of illness which is caused by AIV, a single stranded negative-sense enveloped RNA virus belonging to the family Orthomyxoviridae [2]. AIV infecting poultry can be divided into two distinct groups on the basis of their ability to cause disease named highly pathogenic avian influenza (HPAI) and low pathogenic avian influenza (LPAI) viruses [3]. In avian species, most influenza viruses cause mild localized infections of the respiratory and intestinal tract, but highly pathogenic strains such as H5N1 cause infections in which mortality approaches virtually 100% [4]. AI is a "notifiable" highly infectious disease affecting many species of birds, including chickens, duck, turkeys and geese. Since first report of outbreak in 2003, the H5N1 in poultry reached epidemic proportion with reports of serious outbreaks in several Asian countries including Indonesia, Vietnam, Thailand, South Korea, Laos, Cambodia, Japan and Malaysia [5]. In 2013, the incidence of AI in poultry in Indonesia is low pathogenic clinical signs of emaciation, dirty fur, torticollis, and curled toe paralysis. Pathologic anatomic lesions are edema and petechial hemorrhages of the lung. In serology and molecular reverse transcriptase-polymerase chain reaction (RT-PCR) of serum and lungs of these birds had tested positive for H5N1 AIV [6].

Clinical signs of torticollis and curled toe paralysis in AIV infection frequent in the poultry field cases, especially in laying birds. Infection of AIV in poultry can cause clinical signs, including neurological symptoms, such as tremors of the head and neck, torticollis, curled toe paralysis, paresis, inability to stand, abnormal head position, and behavioral abnormalities [23] as well as bleeding found in the proventriculus and the ventriculus [26]. Meanwhile, according to [6], torticollis and curled toe paralysis is also often found in infectious Newcastle disease virus (NDV) and hemorrhages at the border portion of the esophagus and proventriculus, proventriculus and ventriculus [18]. Clinical signs and pathologic anatomic lesions similar due to infection of AIV and NDV, especially neurological symptoms such as torticollis and paralysis as well as pathologic anatomic lesions form of hemorhhages in the gastrointestinal tract of poultry seen in field cases, making it difficult for the confirmation of diagnosis of infection with both viruses. Both AIV and often display almost similar clinical signs, post-mortem lesions and the pattern of outbreak that need to be differentiated [2].

Diagnosis of AIV in poultry can be made by virus isolation, antigen detection, real-time reverse transcriptase-polymerase chain reaction (real-time RT-PCR), in order to agar gel immunodiffusion (AGID), hemaglutination inhibition (HI), and enzyme-linked immunosorbent assay (ELISA) [21]. Poultry farm that implements routine AIV vaccination program still be exposed to the avian influenza virus. This is due to commercial vaccines are used in accordance with the antigenic virus circulating in the field, then the diagnosis by serological tests such as HI tests, AGID or ELISA antibody improper use, because it is not able to distinguish seropositive due to vaccination or natural infection [27]. Virus isolation takes a long time for test. The polymerase chain reaction (PCR) requires a substantial cost if samples are tested a lot. Immunohistochemistry techniques has been applied as a qualitative test that is effective, accurate, easy to implement and affordable to detect the presence of viral antigens [7, 12, 15, 24]. 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 detecton of rare surviving antigenic targets with the ultimate goal of integrating tissue-based analysis.

Streptavidin-biotin immunohistochemistry test (IHC SB) is immunohistochemical methods are widely used because it has advantages over other methods. 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 [28]. 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 [14].

The aims of this study was to detect AIV brain laying birds with clinical symptoms of torticollis and curled toe paralysis with the technique immunohistochemistry test approach imunopatologis streptavidin biotin.

2. Materials and Methods

The sample is conducted using approximately 20 laying hen tissues which have torticollis and curled toe paralysis clinical signs, and pathologic anatomic lesion of foci

necrotic and petechial hemorrhages on the digestive tract and the lungs. The samples collected were brains. The samples were fixed with 10% of bufferd formalin. It is made preparation of histopathology and the color is applied using immunopathology immunohistochemistry streptavidin biotin. The first step is deparaffinization, rehydration, and the second step is immunostaining. The first step for deparaffinization, preparation tissue was put into xylene for 3 times and each took for two minutes. Next, rehydration was conducted by the tissue preparation was put continuously into absolute ethanol solution for two times, each one for two minutes, 95% ethanol for 1 time and 2 minutes, 50% ethanol for 1 time and 2 minutes, aquadest for 2 times, each one for 2 minutes. After deparaffinization and rehydration, the tissue preparation was cleansed with phosphate buffered saline (PBS) 0.01 MpH 7.1 for 5 minutes and then the tissue preparation is ready to detect immunopathology immunohistochemistry streptavidin biotin.

The second step is Immunostaining. At the beginning, the tissue preparation was cleansed with 3% of H_2O_2 in absolute methanol solution for 10 minutes. Then, it was cleansed again with PBS solution for 10 minutes. The tissue preparation was incubated inside blocking serum solution for 10 minutes. After the incubation, the leaving remnant of blocking serum solution in the tissue preparation was cleaned with tissue paper. Next, the tissue preparation was incubated with antibody of anti NDV or anti AIV (primary antibody) for 45 minutes at room temperature. The tissue preparation was cleaned with PBS solution for 10 minutes. The tissue preparation was incubated with secondary antibody solution labeled with biotin for 10 minutes. Then, the tissue preparation was cleansed with PBS solution for 10 minutes. The tissue preparation was incubated with streptavidin peroxide conjugation for 5 minutes. Then, the tissue preparation was cleansed 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 cleansed with aquadest for 10 minutes. Next, it was dropped with hematoxylin solution as a basic dye and it was incubated for 3 minutes. It is cleansed, with aquadest and given a glycerol adhesive medium then closed with the cover glass. The tissues preparations which has been applied the immunohistochemistry streptavidin biotin staining was observed with light microscope.

3. Results and Discussion

Results of the present study showed that on the uninfected AIV tissues, the IHC SB staining was negative (Fig.1). Meanwhile, the AIV antigens can be detected in

parenchyma and neural cells (Fig.2), endothelial cells of blood vessel (Fig.3) of brain, respectively. AIV 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 [1]. In the method of IHC SB color application, it will only need approximately 3-5 hours to detect NDV or AIV 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 from inside of cell, therefore peroxidase which is attached with antibody will give reaction to substrate and chromogen [10]. 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 AIV. 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 [10].

In the present study, the given primary antibody specifically functions to detect the emergence of the virus, which causes the AIV 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 [19]. 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.

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

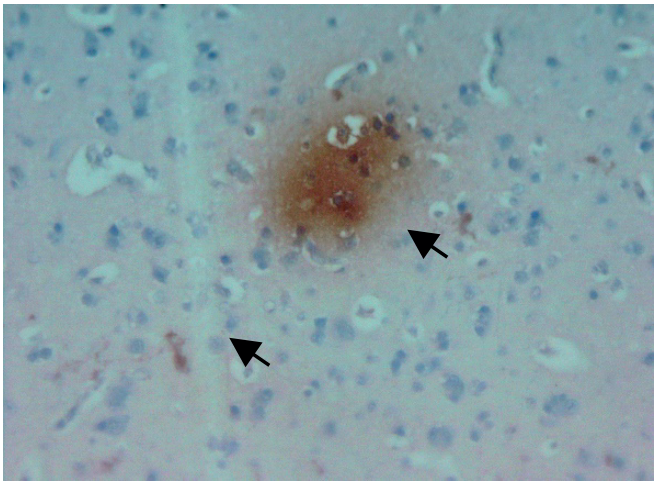

Figure 2: The brain of the laying hen with clinical signs of torticollis and curled toe paralysis. Immunohistochemistry streptavidin biotin positive AIV seen as reddish brown discoloration in parenchyma and neural cells (↑) (Streptavidin biotin, 1000x.).

Enzyme is reacted with chromogen substrate in order to create colored final product and it is insoluble that can be observed using light microscope [10].

The special quality of using IHC method is to trace the virus distribution on every organs. 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 hospes can be avoided [16]. 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 [14]. SB method will

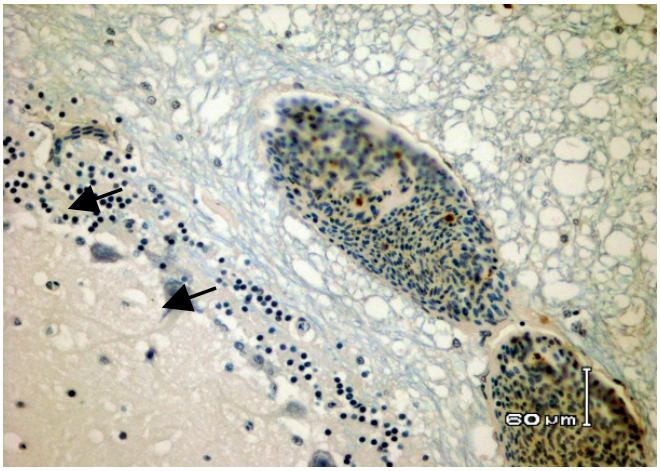

Figure 3: The brain of the laying hen with clinical signs of torticollis and curled toe paralysis. Immunohistochemistry streptavidin biotin positive AIV seen as reddish brown discoloration in lumen and endothelial cells of brain blood vessel (↑) (Streptavidin biotin, 1000x.).

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 [28, 29].

In the histopathology examination, there is a significant change on the brain, such as hiperemia, lymphocytic perivascular cuffings and endothelia cells hypertrophy [4]. The virus transmission from the blood flows until bone marrow and brain and will cause nerves symptoms resembles with torticollis and tremor [11]. 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, hyperlacrimation, dull feather, edema on face and feet, cyanosis on featherless skin and diarrhea [9]. The virus that replicated with the blood will carry over to the target cell or organ, so the AI virus is not only found in the brain, but are also found in other organs, such as respiratory and digestive organs. AIV mainly occupies an area that is in the vascular endothelium of blood vessels [5, 13, 17, 23]. The AIV damage looks very prominent in vascular areas in all organs. Antigens are located in the area of vascular ischemia created the clinical vascular infarction that can be found in chickens with cyanosis of the comb and wattles, and other circulatory disorders [25].

The results of the present study indicated that AIV infection on the commercial laying chickens showing torticollis and curled toe paralysis can be proven. The AIV are identified in the parenchyma, neural cells and endothelial cells of brains by using the immunohistochemistry streptavidin biotin approach.

4. Conclusions

Immunohistochemistry staining of streptavidin-biotin can be applied for confirmation of the diagnosis of avian influenza virus (AIV) on the laying birds showing torticollis and curled toe paralysis. The AIV antigens can be detected clearly in the parenchyma, neural cells and endothelial cells of brains.

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