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RESEARCH ARTICLE

Detection of Mixed Infection of Avian Influenza and Newcastle Disease Viruses in Chickens in Indonesia by Immunopathologic Immunohistochemistry Double Staining

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ABSTRACT

Avian influenza virus (AIV) and Newcastle disease virus (NDV) are systemic disease that is highly infectious with high morbidity and mortality rates, and highly detrimental to economic value in the domestic and commercial poultry industries. Generally, in the field there is a mixed infection of AIV and NDV, each of which is highly pathogenic. Clinical signs are difficult to distinguish, so animuno-pathological immunohistochemistry (IHC) double staining was developed with the ability to confirm the diagnosis of mixed infection. In the present study, 20 chickens infected naturally with clinical signs of torticollis and curled toe paralysis that had a mixture of AIV and NDV infections and there are variations of tropism distribution of AIV and NDV in tissues were used. The AIV and NDV were visualized by performing the double staining technique DAB & Fast-Red with monoclonal antibody anti-hemagglutinin AIV-mouse (AIVHA) and polyclonal antibody anti-hemagglutinin-neuraminidase NDV-rabbit (NDVHN) proteins. AIV infected-lungs or brains had red coloration (AIVHA) and NDV infected lungs or brains had brown coloration (NDVHN). The IHC double staining technique is sensitive and accurate in the confirmation of the diagnosis of a mixed infection to determine the difference between AIV and NDV.

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INTRODUCTION

Avian influenza virus (AIV) and Newcastle disease virus (NDV) are pathogenic and causing very fatal disease in chickens. AIV and NDV have been endemic in Indonesia, and infectious in humans (zoonotic) as well. In humans, NDV infection has long been known to cause conjunctivitis. According to Office of International Epizootes, only AIV and NDV are diseases included in List A that are very contagious and infectious (Anonymous, 2008).

AIV has been prevalent in chickens in almost all parts of Indonesia (31 provinces among 33 provinces) (Sedyaningsih *et al.*, 2006)). In Indonesia, AIV in addition to infecting chickens, also infect and cause deaths in various other types of birds and ornamental birds, such as Bangkok chickens, ducks, entok, quail, pigeons and parrots (Wuryastuti *et al*., 2005). In the field, the clinical signs of AIV are difficult to distinguish from other viral infections such as Newcastle disease (NDV). Clinical symptoms of NDV are similar to AIV, among others: the head is seen irregularly moving left, right and downward (torticollis), curled toe paralysis, respiratory distress and acute diarrhea.

Until now routine laboratory diagnostics for the identification of AIV and NDV are still based on isolation and virus identification. Various molecular tests, including reverse transcriptase-polymerase chain reaction (RT-PCR) and multiplex reverse transcriptase-polymerase chain reaction (mRT PCR) have been successfully developed (Wasito *et al*., 2014). However, RT-PCR and mRT-PCR assays allow for the contamination of laboratory and environmental space, relatively expensive devices (biosafety cabinet Level 2, refrigerated microcentrifugation and PCR machines) and PCR results obtained cannot distinguish between natural and contaminated infections, cannot be used in the determination of primary pathological lesions in tissues or organs.

Basically, for the diagnosis of AIV and NDV laboratories, strict biosafety (and biosurveillance) procedures are required, and a relatively long time. The complicated diagnosis of AIV and NDV is the consequence of the low pathogenic forms of AIV and the NDV, each mutating into a highly pathogenic AIV and velogenic viscerotrophic NDV which is highly infectious and lethal to infected poultry. A late confirmation of AIV and NDV diagnosis may inhibit early detection and elimination efforts so as to enable an outbreak of disease to spread naturally in the field. Rapid, accurate and economical diagnosis is a very critical way of monitoring and preventing the disease outbreaks. Identification of both viruses is particularly important in the surveillance program (Koopman *et al*., 2004).

This double staining immunopathologic immunohistochemistry technique is expected to be developed and applied to address the above-mentioned problems, so that the identification and differentiation of mixed AIV and NDV infections on the tissues can be performed simultaneously, early, rapidly and accurately. The immunopathologic immunohistochemistry approach can also avoid laboratory contamination (and field) (Anonymous, 2014). In the present study, the development and application of immunopathologic techniques based on immunologic streptavidin biotin with monoclonal and polyclonal anti nucleoprotein antibodies respectively for the detection of NDV and AIV in poultry and was then applied the double staining DAB and Fast Redmouse-HRP + rabbit-AP with anti-hemagglutinin monoclonal and polyclonal antibodies anti-hemagglutinin-neuraminidase for the simultaneous detection of AIV and NDV in poultry were performed.

MATERIALS AND METHODS

Immunopathological immunohistochemistry streptavidin biotin of avian influenza and Newcastle disease viruses: In the present study, 20 chickens infected with virus showing the clinical symptoms of torticollis and curled toe paralysis were necropsied and then, brains and lungs, which are the target organs of AIV and NDV (Wasito *et al*., 2017) were observed for the presence or absence of anatomical pathological lesions in the form of petechial and/or linear hemorrhages. The brains and lungs were collected and were processed for histopathologic preparation. The 3-5 µm preparation of tissue (lung and brain) were attached to glass objects and then immunopathologically tested immunohistochemical streptavidin biotin with polyclonal antibody anti nucleotrotein avian influenza virus (AIV) and Newcastle disease virus (NDV). Initially, the histopathologic preparations of the lungs were deparafinized with xylene, rehydrated with ethanol solution of multilevel concentration, washed with aquades and finally cleared with PBS 0.05M, pH 7.1. The tissue preparation was then immersed in a 3% H 2 O 2 solution in absolute methanol to inactivate endogenous peroxidase activity, washed 3x PBS and then immersed in normal serum of rats for 20 min. Furthermore, the tissue preparation was incubated with AIV polyclonal anti-nucleoprotein antibodies or NDV for 45 min at room temperature (Santa Cruz Biotechnology). The tissue preparation is then washed with sterile aquades and directly incubated with a secondary antibody (pig antibody (Yunita *et al*., 2017) labeled biotin anti rabbit IgG) (Dako) for 10 minutes. Furthermore, given the conjugate streptavidin-horseradish peroxidase (BD PharMingen, San diego, CA) for 10 minutes at room temperature. And, hereafter was given a solution of 3,3'-diaminobenzidine (Zymed Corp., San Francisco, CA) as a dye-anti nucleoprotein AIV or NDV bound to lung or brain tissues. Furthermore, the tissue preparation was given a counterstain of hematoxylin-eosin, dehydrated, cleaned by washing with aquades flows, subjected to glycerol adhesive and covered with a glass cover to be observed under a microscope.

Immunopathological immunohistochemistry double staining DAB and Fast Red of avian influenza and Newcastle disease viruses: IHC paraffin block of brains and lungs- positive AIV and NDV streptavidin biotin single staining were recut with microtom with a thickness of 3-5 µm, then tissues preparation were made on the object glasses and subjected to an immunopathologic double stainining as follow: The histopathologic preparation were deparaffinized with xylene 3x, 5 min each, rehydrated with ethanol solution of reduced concentrations (absolute, 95%, 75% and 50%, respectively 5 min), washed aquades and finally cleaned by immersion into PBS 0.05M, pH 7.1 for 2 min. The tissue preparation was then immersed into a 3% H2O2 solution in absolute methanol to inactivate endogenous peroxidase activity, washed 3 times of PBS 2 minutes each. To inactivate the possibility of non-specific endogenous antibodies, the tissue preparation then immersed in normal serum of rats for 20 min. Furthermore, the tissue preparation was incubated with the monoclonal antibody anti-hemagglutinin AIV-mice and polyclonal antibody anti-hemagglutinin-neuraminidase-NDV-rabbit and incubated for 45 minutes at room temperature. The tissue preparation will then be washed with 2x sterile aquadest 3 minutes each, and immediately incubated simultaneously with two different secondary antibodies, which are labeled AP (alkaline phosphatase) -mice and HRP (horseradish peroxidase-rabbit (Abcam), 20 minutes each and washed with 2x PBS for 3 minutes each. The tissue preparation was dyed with 2 drops of hematoxylin, washed with running water, then immersed in 2 min aquades. Next, it was given a gliserol adhesive and covered with a deck glass to be observed under a microscope and were analyzed descriptively.

RESULTS

In the present study, the IHC double staining was developed by using monoclonal antibodies anti-hemagglutinin avian influenza virus (AIV) and polyclonal antibodies anti-hemagglutinin-neuraminidase Newcastle disease virus (NDV). Based on the results of this study, it is evident that immunopathologic-immunohistochemical double staining can be applied in the confirmation of infection diagnosis mixture between AIV and NDV in cases of avian disease showing clinical signs of torticollis and curled toe paralysis in the field. The sensitivity in diagnosis to determine the difference between AIV and NDV is brains and lungs, respectively 100%. The infected cells or tissues of AIV are red coloration (AIVHA**)** (Fig. 1) and of NDV (NDVHN) are brown coloration (Fig. 2).

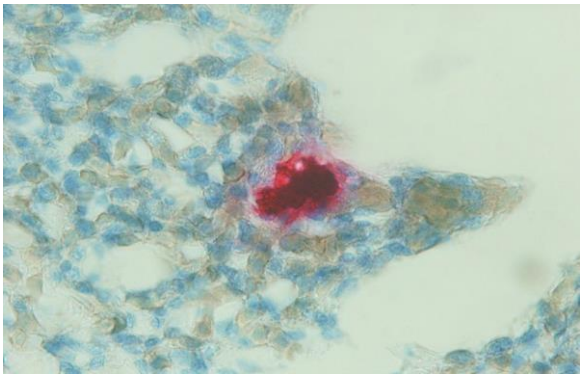

Fig. 1: Immunopathological immunohistochemistry double staining of the lung positively infected with avian influenza hemagglutinin virus (AIVHA) (red) and Newcastle disease hemagglutinin-neuraminidase virus (NDVHN) (brown) (DAB &FastRed, 1000x.).

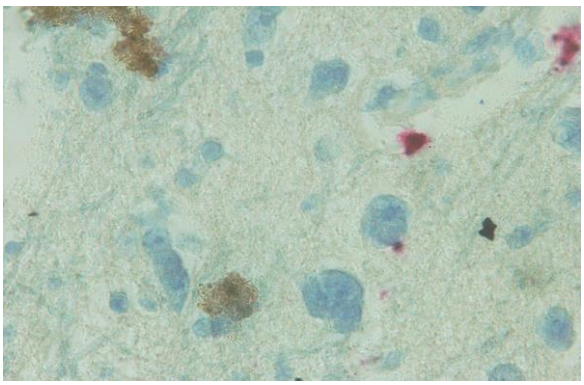

Fig. 2: Immunopathological immunohistochemistry double staining of the brain positively infected with avian influenza hemagglutinin virus (AIVHA) (red) and Newcastle disease hemagglutinin-neuraminidase virus (NDVHN) (brown) (DAB &FastRed, 1000x.).

DISCUSSION

Immunopathologic-immunohistochemical (IHC) double staining is a biotechnology approach through modern immunopathologic test which is very useful to be applied in Research Laboratory and Clinical Diagnostic Laboratory. The IHC double staining can be applied to determine the existence of specific antigen in tissues with labeled antibodies based on reaction interaction between antibodies-antigen. Immunoreactive products can be observed and determined by enzymatic markers (Nielsen *et al*., 2014).

The most interesting and important findings of this study were detectable AIV and NDV present in the same cells in pneumocytes of the lungs and brainsparen-chymeswith double infection by both viruses (AIV and NDV). It clearly shows that AIV and NDV are located in the same cells in the lungs and the brain. It proves that AIV and NDV have the same cell of taget to infected cells, tissues or infected host (poultry). Avian influenza virus and NDV have the same pathological lesions, among others: focal necrosis of the central nervous system (brain) accompanied noduli of glial cells (Nakamura *et al*., 2008). Poultry, especially chicken, is a harmonious and potential replication site for AIV and NDV, since most AIV and NDV are infective and replicate in poultry (Wasito *et al*., 2017). Like AIV in poultry, that pig is also a potential site for AIV replication, which is different from AIV in poultry, AIV in pigs is thought to be infectious to humans.

In the case of poultry disease in the field, AIV and NDV-infected chickens show similar neurologic and respiratory clinical symptoms. In neurologic symptoms, the head appears irregularly tilted to the left, right and downward (torticollis) and curled toeparalysis. Although neuroinvasiveandneurovirulence in infected chickens of both AIV and NDV is one of the main factors that can lead to relatively high morbidity and mortality (± 100%) with torticollis and curled toe paralysis, and petechial hemorrhages in the gastrointestinal tract, there have been only a few published reports describing viral neuropathogenesis in poultry. It has been reported that in poultry, the virus enters the central nervous system through three ways: hematogenus, olfactory and through nerve fibers (neurons). Infection of blood vessel endothelial cells and viremia are the main factors of pathogenesis during viral infection in poultry (Toffan *et al*., 2008). Viremia determines the spread of the virus to various organs and eventually to the central nervous system, especially the brain (Swayne, 2007).

In humans infected with AIV subtypes H5N1 shows a disturbance regulation of cytokines and chemokines are often referred to as cytokine storm. The cytokine storm is considered one of the major mechanisms in the pathogenegesis of AIV subtype H5N1. Based on the results of *in vitro* studies with primary cell of bronchial and alveolar epithelial cells, and macrophages, showed that AIV subtype H5N1 induces cytokines and chemokines more potentially and significantly than cytokines and chemokines induced by seasonal AIV infections (Lam *et al*., 2010). Research on experimental animals *in vivo* also proves that the induction of proinflammatory cytokines and chemokines is due to H5N1 infection (Maines *et al*., 2012). With the reverse transriptase-polymerase chain reaction (RT-PCR) molecular approach, it is evident that in humans infected with AIV subtype H5N1, local expression of cytokines and chemokines in the lungs become irregular.

The results of double immunohistostaining studies of AIVHA and NDVHN antigen detection in the brains and lungs are unique and unreleased findings. Infection with AIV and NDV, in addition to show similar clinical signs (torticollis and curled toe paralysis), AIV and NDV also have similar pathologic lesions. Based on the anamnesa, the chickens used in this study had been vaccinated against NDV and AIV. The findings based on the results of the present study are very interesting, and should be studied again in detail, especially the association of AIV subtype H5 (Guan and Smith, 2013) and H7 (Lai *et al*., 2013) infections reported to be infectious in humans which are known to have the ability of cross-reaction hospes, ie genetic shift or genetic reassortment so as to infect humans (Poovorawan *et al*., 2013).Cases in humans infected with AIV subtype H5N1 were first reported in Hong Kong in 1997. And the fatal case of AIV H7N9 infection in humans reported by the Department of Health, Hongkong Special Administrative Region, also occurred in Hong Kong on January 5, 2017. Out of a total of 106 human infected with H7N9 in Hong Kong, 35 people died, 80 of whom contracted AIV H7N9 from live poultry in poultry and poultry farms found in the commercial market (WHO, 2017).

Conclusions: Immunopathologic immunohistochemistry double staining for AIV and NDV can be accurately, rapidly, simultaneously and securely applied in the confirmation of poultry infection showing clinical symptoms of torticollis and curled toe.

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Authors contribution: BS plays a role in field sampling and histopathologic preparation of the lungs for testing. HW with RW plays a role in performing immunopathologic immunohistochemistry double staining and reading the results. All the researchers discussed together before declaring their consent, that the manuscript is ready for publication.

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