Mini Review Article

# Production of Antibody for Direct Fluorescence Antibody Assay against Avian Influenza H9N2

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**Abstract** | The H9N2 influenza A viruses are endemic in some countries for many years and had caused economic losses in poultry industries. Infection with this virus decreases egg production and increases chicken mortality. In human health, cases of this zoonotic agent are being reported and some cases showed fatal infection. The H9N2 is highly contagious, and transmission can occur from chicken to chicken, chicken to humans through the air, or sometimes from humans to humans. H9N2 belongs to alpha influenza virus genus of the family *Orthomyxoviridae*. It has a minus sense RNA genome of eight segments. This fact makes the virus to easily re-assort, which might yield a new virus strain, and causes an outbreak in unimmunized humans or chickens. In reducing losses due to H9N2 infection, rapid diagnosis is an option to prevent further infections. Office International des Epizooties (OIE) suggested some methods for the detection of H9N2 infection including the immunofluorescence method that is commonly known as the direct fluorescent antibody (DFA) assay. This method is a combination of immunology and bio-imaging. This DFA assay is rapid and sensitive, but specificity is influenced by antibody production methods. Therefore, the aim of this review was to describe various antibody production methods, which might be used in developing DFA assay to diagnose H9N2 infection in humans or chickens, and we discussed avian influenza H9N2 virus, immunogens, various H9N2 antibody production, and principle of DFA assay.

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## Introduction

Avian influenza is one of various contagious diseases and is caused by influenza A virus of the *Orthomyxoviridae* family (Guan *et al*., 1999; Mehta *et al*., 2005; Peacock *et al*., 2019). These viruses are zoonotic viruses, and transmission of these viruses is effective from chicken to chicken, chicken to humans through the air, or sometimes from humans to humans. Some infection of avian influenza virus in human is related to a history of contact with poultry, farms or contaminated objects (Harder *et al*., 2016).

Avian influenza viruses are divided into several subtypes based on two surface glycoproteins, Hemagglutinin and Neuraminidase. A total of eighteen HA (H1-H18) and 11 NA (N1-N11) subtypes have been identified to date (Henry and Murphy, 2018). One subtype of the avian influenza virus that can threaten human and animal health is H9N2. The first outbreak of this subtype was reported in 1992 that infected poultry in China (Sun and Liu, 2015). Subsequent outbreaks in poultry were reported in Iran and Pakistan in 1998 (Ali *et al*., 2019). Subtype H9N2 infects the reproductive organs in poultry so that economic losses occur due to a decrease in egg production up to 70%. Large numbers of deaths from these subtype infections can be due to bacterial or other viral secondary infections. Generally, H9N2 is classified as low pathogenic avian influenza (LPAI), and cases of H9N2 subtype infection in humans have been reported in several countries such as China, Bangladesh, Egypt, Oman, and Pakistan. Several cases mentioned the occurrence of gene insertion from other subtypes that caused changes in the pathogenicity of H9N2 (Pusch and Suarez, 2018).

In the last decades, some methods have been developed to diagnose H9N2 infection. Office International des Epizooties (OIE) recommended reverse transcriptase polymerase chain reaction (RT-PCR) and virus isolation in an embryonated egg as gold standards in H9N2 diagnosis (Chaharaein *et al*., 2007). In humans, H9N2 virus infections are generally examined on clinical specimens in the form of throat swabs and nasal fluid. To confirm an H9N2 virus infection, an examination must be carried out to isolate the virus, to detect either the H9N2 genome by polymerase chain reaction (PCR) method using a pair of specific primers, or an increase in antibody titre against H9N2, and examination by western blotting to detect H9N2 specific proteins. For a definitive diagnosis, one or more of the above confirmatory tests must be positive (Hosseini *et al*., 2018).

Another method that might be used to diagnose H9N2 infection is direct fluorescent antibody (DFA) assay. The DFA assay can detect H9N2 virus from both human and animal cases. Throat swabs and nasal fluid can be tested for humans with H9N2 clinical symptoms (She *et al*., 2010). Besides, this method can detect the virus in tracheal or cloacal swabs and infected organs of dead animals. The DFA assay is rapid and sensitive but the specificity of this method is influenced by antibody production methods (Prabhu *et al*., 2018).

Therefore, this article aimed to review various antibody production methods that might be used in developing DFA assay to diagnose H9N2 infection in humans or chickens, and we discussed avian influenza H9N2 virus, immunogens, H9N2 polyclonal and monoclonal antibody production, direct fluorescent antibody (DFA) imaging approach, and principles of DFA assay.

### *Avian influenza H9N2 Virus*

Avian influenza virus has a pleomorphic shape, which spherical shape with a size of about 50–120 nm is predominant, while filamentous form of more than 250 nm may also occur (Dadonaite *et al*., 2016). This virus also has an envelope and is able to survive in the air for four days at 22 °C and more than 30 days at 0 °C (Dadonaite *et al*., 2016). The Avian Influenza has a minus sense RNA genome of eight segments. This fact makes the virus to easily re-assort, which might yield a new virus strain, and causes an outbreak in unimmunized humans or chickens. The eight segments encode about ten different viral proteins. The viral proteins are divided into surface proteins, which include hemagglutinin (HA), neuraminidase (NA) and membrane ion protein (M2), and internal proteins that consist of nucleoprotein (NP), matrix proteins (M1), and polymerase complexes. Polymerase complexes consist of basic protein polymerase 1 (PB1), basic protein polymerase 2 (PB2), and acid protein polymerase (PA). There are two additional proteins, which are non-structural, i.e. non-structural protein 1 (NS1) and 2 (NS2) that is also known as nuclear export proteins (NEP) (Bouvier and Palese, 2008). Among these proteins, only two are known to play a role in causing antibody responses, i. e. HA and NA (Shao *et al*., 2017).

Hemagglutinin (HA) is an avian influenza virus surface protein that functions as a receptor binding site. HA protein attaches to sialic acid containing receptors, which are expressed in host cells, after proteolytic activation. Proteolytic activation of HA precursor molecules yields HA1 and HA2. The degree of HA cleavage determines the virulence of avian influenza virus in poultry (Sriwilaijaroen and Suzuki, 2012). Non-virulent viruses usually have HA with a single arginine residue at the cleavage site, which can only be cleaved by proteases such as

extracellular trypsin in the upper respiratory tract and digestive tract and cause local infection only. In contrast, virulent viruses have HA with several basic residues at the cleavage site, which can be activated by intracellular proteases everywhere, and therefore can cause systemic infections (Lucso *et al.*, 2015).

At the beginning of infection, HA will bind to cell receptors and release ribonucleoproteins in the cytoplasm. Hemagglutinin as the main surface influenza virus glycoprotein is translated as a single protein HA0, where HA0 must be cleaved into HA1 and HA2 in order for the virus to be activated. The activation of hemagglutinin, which is an important factor for infectivity and spread of the virus, is carried out by host serine endo-protease proteolytic enzyme at a specific place that normally contains a single basic amino acid (arginine). The difference in sensitivity of HA protein to host protease is related to the level of virulence (Dou *et al.*, 2018; Zhang *et al.*, 2012).

HA1 protein is the main target of the immune response, whereas HA2 protein with a fusigenic portion at its end facilitates fusion between viral envelope and host endosomal membrane (Dou *et al.*, 2018). Neuraminidase is an enzyme, which is located on the surface of the virus, that helps the release of viruses that are reproduced from infected cells (McAuley *et al.*, 2019). Neuraminidase must play a balanced role with HA so that NA enzymatic activity, which releases sialic acid from infected cells, does not reduce the efficiency of subsequent cell infections. If two or more strains of avian influenza virus infect cells together, it is very possible that randomization of viral genome segments including NA and HA encoding genes can lead to new strains with new genome combinations and have different host specificities from the original virus (Taubenberger and Kash, 2010).

*Immunogens*

An immunogen is a substance that is capable of eliciting an immune response. Immunogen can be defined as a complete antigen, which is composed of a macromolecule, that has epitopes (determinants), which can induce an immune response. An immunogen produces a humoral or cell-mediated immune response. All immunogens are antigens but not all antigens are immunogen. General immunogenic properties are foreignness, physical properties, complexity, forms (conformation), charge and the ability to enter a cell (Mahanty *et al.*, 2015). Several proteins of avian influenza virus are immunogenic and can stimulate the formation of antibodies. In many studies, HA and NA are more widely known as the immunogenic parts of avian influenza virus.

There are several conditions for a substance to be an immunogenic substance. The first and foremost requirement for a substance to qualify as an immunogen is that the substance is genetically foreign to the host (Chaplin, 2010). Moreover, there are other immunogenic substances, such as pathogen-associated molecular pattern molecules (PAMPs), which are derived from the virus, and damage-associated molecular pattern molecules (DAMPs), which are derived from host cell and tissue damage due to the virus. Both PAMPs and DAMPs are immunogenic (Tang *et al.*, 2012).

In general, the immune response will occur against components that are usually not present in the body or are usually not exposed to the host's lymphoreticular system. A substance can be immunogenic, if it has a certain minimum size. The smaller the size of an immunogen, the antibody response produced will also be smaller. Factors that influence the complexity of immunogens include both physical and chemical properties of molecules. The state of molecular aggregation for example can affect immunogenicity. Monometric proteins may stimulate a refractory or tolerant state, when they are in a monometric form, but are highly immunogenic, when they are in a polymeric or aggregate state (Chaplin, 2010).

The existence of certain shapes in certain molecules is a condition of a substance to be immunogenic. Linear or branched polypeptides and carbohydrates, and globular proteins are all able to stimulate an immune response. However, antibodies that are formed from various types of structural combinations are very specific and can quickly recognize differences between them. When the shape of antigens changes, antibodies that are stimulated against their original form will not recognize them (Nicholson, 2016). Immunogenicity is not limited to certain molecules; substances that are positively charged, negative and neutral can be immunogenic. However, immunogens without a charge will produce antibodies without strength. The ability to enter a determinant group in the recognition system will determine the outcome of an immune response. Recent developments have

enabled research to prepare synthetic immunogenic polypeptides that contain certain amino acids, and whose chemical makeup can be determined (Burton, 2017).

### *H9N2 antibody production*

H9N2 antibody can be monoclonal or polyclonal. Serum is a polyclonal antibody, because it is produced by derivatives of several B cells stimulated by different epitopes of a same antigen. Monoclonal antibodies (MAb) are homogeneous antibodies with the same specificity that are produced from clones of cells that produce antibodies (Ertekin *et al.*, 2018).

### *H9N2 polyclonal antibody production*

Producing antibodies naturally can be done by immunization of animals, i.e. by injecting an antigen (immunogenic substance) that we want. The immune system will oppose the antigen/immunogen. Activated lymphocytes will then multiply and develop into plasma cells that produce antibodies. In this case the antibodies are polyclonal antibodies with various compositions and could be purified from serum (Chames *et al.*, 2009). These antibodies address most epitopes so that they are less specific than monoclonal antibodies (Ertekin *et al.*, 2018).

Animals that can be used for polyclonal antibody production include chickens, sheep, guinea pigs, hamsters, horses, mice and goats. Animal selection must be based on three criteria, i.e. the number of antibodies needed; the relationship between antigen donors and antibody-producing recipients, which should be far from phylogenetic view, where generally the farther is the phylogenetic relationship, the better is the potential for an antibody response to mount high titers, and important characteristics of antibodies will be created (Thompson *et al.*, 2016).

Avian influenza subtype H9N2 polyclonal antibody production begins with making a solution of immunogenic substances. In general, two separate solutions are needed to get maximum antibody results. The first solution is made by mixing inactivated H9N2 virus antigens with incomplete Freund's adjuvant. The solution is used to prime antibody production by injecting experimental animals such as guinea pigs or goats. The second solution is a combination of inactive viral antigens with complete Freund's adjuvant. The second solution is used as a booster that can be repeated two or three times, in a period of two to three weeks. The amount of solution injected is affected by the type and size of experimental animal used (Figure 1) (Peacock, 2018).

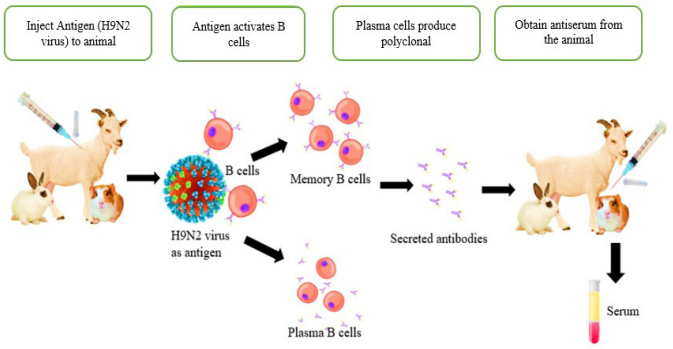


**Figure 1:** *Diagram of the process for harvesting polyclonal antibodies in response to an antigen.*

The success in immunization of experimental animals can be known by testing H9N2 antibody titres in these animals. Antibody titre can be tested by hemagglutination inhibition (HI) method (Heidari *et al.*, 2016). In addition, qualitative tests such as agar gel precipitation test (AGPT) can also be used to determine the success of the hyperimmune process to produce good H9N2 antibodies. Further, polyclonal antibody or serum is separated from whole blood by centrifugation (Hassan *et al.*, 2017).

When experimental animals are immunized with HA or NA recombinant protein, the resulting serum will contain specific antibodies to one type of antigen, HA or NA. The antibody is mono-specific polyclonal antibody and can be used for developing a specific DFA assay.

### *H9N2 monoclonal antibody production*

To produce monoclonal antibodies a pure antigenic protein, need to be produced. In the avian influenza H9N2 subtype, viral antigenic parts that are often used in monoclonal antibody production are HA and NA proteins. Both are produced as materials for developing diagnostic kits to detect the presence of avian influenza subtype H9N2 (Lukosaityte *et al.*, 2020). Monoclonal antibody production can be done by several methods, one of which is hybridoma technique (Figure 2). In this technique, mice are intra-peritoneally immunized with the H9N2 subtype influenza virus that has been inactivated. Just like making polyclonal antibodies, complete and incomplete Freund's adjuvants can be combined to produce good antibodies. Spleen of immunized mice was taken; then splenocytes were fused with myeloma cells. Ten days after fusion,

hybridoma supernatants were screened for specific antibodies (Holzlöhner and Hanack, 2017). The indirect *enzyme linked immunosorbent assay* or ELISA can be used as a screening method until specific antibodies against HA or NA are selected. Antibody secreting hybridoma clones are selected, followed by monoclonal antibody harvesting.

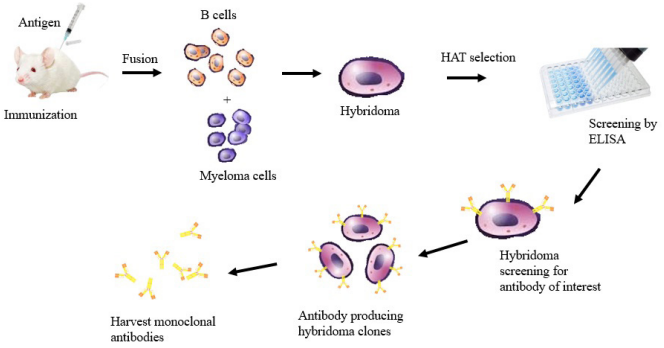


**Figure 2:** *Monoclonal antibody production by hybridoma technique.*

In two decades, the production of monoclonal antibodies against HA and NA was facilitated by recombinant processes (Figure 3) (Kunert and Reinhart, 2016). In summary, partial or whole of the HA and NA genes can be selected in GenBank and then a synthetic gene is made as the main ingredient in a recombinant method. The HA or NA specific gene is cloned in a vector in the form of a certain plasmid (selected by bioinformatics) followed by transformation of *E. coli* to harbour the plasmid. The success of cloning can be tested through PCR and sequencing. The next step is the expression of HA or NA gene, in general the expression stage is carried out by inducing *E. coli* with *Isopropyl-1-thio-β-Dgalactopyranoside* (IPTG), whose gene is inserted in the plasmid. IPTG is a molecular mimic of allolactose, which is a lactose metabolite that triggers transcription of a lac operon, and it is therefore used to induce *E. coli* protein expression where the HA or NA gene is under the control of the lac operon. After expression, the protein must be purified to be used in antibody production. Ion exchange chromatography method can be used for protein purification. The success of the process can be is tested by SDS PAGE using the Laemmli method (Brunelle and Green, 2014).

### *Direct fluorescent antibody imaging approach*

The latest advances in imaging in the diagnosis and treatment of diseases have created tremendous benefits for biological and medical sciences (Patra *et al.*, 2018). One of the main reasons is because it is simple, fast, sensitive, and has a relatively low cost (Yang *et al.*, 2019).

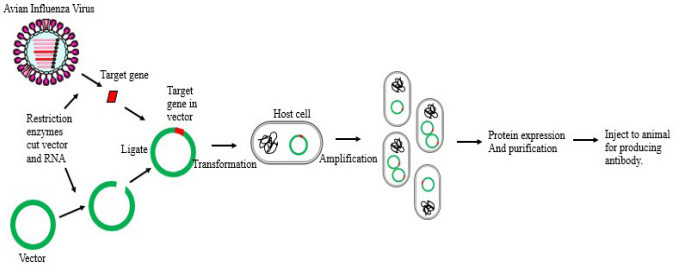


**Figure 3:** *Specific antigen production by a recombinant DNA technology to develop a monoclonal antibody.*

Direct fluorescent antibody or DFA assay is one of various methods that is based on imaging approach (Vemula *et al.*, 2016). This method can be used to diagnose a disease that is caused by virus, bacteria, fungi, or other pathogens (Steensels *et al.*, 2017). Direct fluorescent antibody assay uses a fluorescent labeled antibody to bind and illuminate a target antigen. A fluorescence microscope is used to image the interaction between the labelled antibody and antigen (Cummings *et al.*, 2008).

### *Principles of direct fluorescent antibody (DFA) assay*

The direct fluorescent antibody (DFA) assay is a microscopic procedure for detecting the presence of causative antigen (Donaldson, 2015). Some virus infection like rabies, influenza, corona and others have been reported can be tested by this test. The specific protein on the surface of virus will be recognized by the antibody in this test, where the monoclonal or polyclonal antibody is labelled by a fluorescent dye. DFA assay is a reliable tool for visualizing certain bacteria and viruses that are difficult to isolate or culture from samples (Bakerman *et al.*, 2011).

A fluorescent dye is conjugated to the constant region (FC) of an antibody. The presence of an antigen will be bound by the antibody when the labelled antibody is incubated with the sample (Figure 4). Unbound antibody can be washed away, and areas where the antigen is present can be visualized as fluorescent areas using a fluorescence microscope. If there is no specific antigen, there will be no staining in this test (Burry, 2011). Direct fluorescent antibody (DFA) assay belongs to rapid test that is started by fixing a prepared sample to a glass slide. The fixed smear is covered by specific fluorescent dye-labelled antibody and incubated at 36°C for 30 minutes in a humidified chamber. Excess antibody from the smear is washed by phosphate-buffered saline or deionized water.

Positive samples will show specific fluorescence in stained smear when observed at 200x magnification in a darkened room with the use of a fluorescence microscope (Lembo *et al*., 2006).

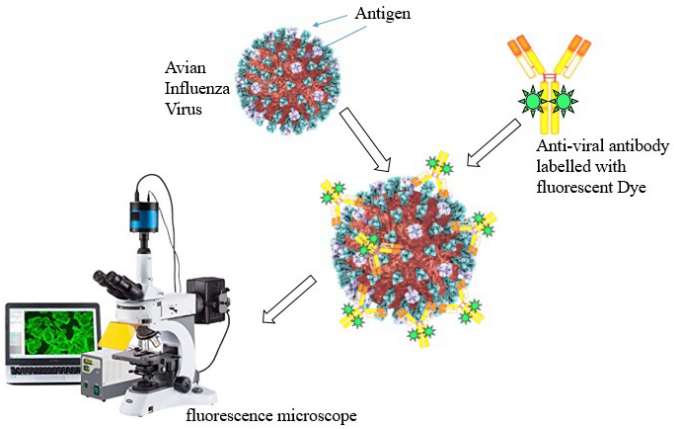


**Figure 4:** *Principle of Direct Fluorescent Antibody (DFA) Assay.*

The used of DFA assay has some advantages, such as this test allows visual assessment of a specimen that contains microbes that can't be easily cultured. In addition, it can be both sensitive and specific in labelling single cells, and therefore can visualize the presence of microbe in certain cells. Further, DFA assay can view cells in their natural environment, and can use different types of fluorescent-labelled antibodies, each with different dye, to see multiple cell types and microbes in one sample. The disadvantage of this test is related to cross reactivity. It is often difficult to develop a monoclonal antibody that works well. Besides, the operators must carefully run controls to assure that there are no false positives or negatives (Kim and Poudel, 2013).

## Conclusions and Recommendations

Direct fluorescent antibody (DFA) assay is one of the diagnostic methods that can be used to detect H9N2 avian influenza infection by imaging approach. Antibody production is an important step to get the specificity of this test. In developing DFA assay for H9N2 virus, polyclonal antibody is produced by injection whole H9N2 virus in laboratory animals. Otherwise, hybridoma technique and combination of recombinant methods are used to produce a more specific monoclonal antibody.

## Author's Contribution

CMHN conducted the literature search, chose the included studies, and drafted the figures and article. JAP added included studies, revised the figures and article, and added the discussion. The final version was approved by all authors.

### *Conflict of interest*

The authors have declared no conflict of interest.

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