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Pathogenesis of infectious bronchitis virus (IBV) and laboratory test methods available to detect IBV in chickens

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ABSTRACT

Infectious bronchitis, caused by gammacoronavirus, is a high contagious disease of chickens. The disease was characterized by respiratory, renal, urogenital, and alimentary infections. Different strains of IBV may show variable tissue tropisms. For example, the classical strains of Massachussetts serotype (M41) targeted in respiratory and reproductive system while variant serotypes including QX, 793/B, D388 have been detected in respiratory, reproductive, alimentary tract and kidney. Because the evolution and mutation of IBV have contributed to the emergence of new genotypes of IBV, the outbreak of IB still occurred in the vaccinated flocks. Therefore, diagnosis of IB plays an important role for limiting the effects caused by IBV infection. Available laboratory tests for diagnosis of IBV include virus isolation, electron microscope, immunohistochemistry, serological tests (virus neutralization, haemagglutination inhibition, enzyme-linked immunosorbent assay, agar gel immunodiffusion), and genotype identification (Reverse transcription – polymerase chain reaction (RT-PCR), genome sequencing [using next generation sequencing]).

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1 INTRODUCTION

Infectious bronchitis virus (IBV) was classified into genus *Gammacoronavirus*, subfamily *Coronavirinae*, family *Coronaviridae* and order *Nidovirales*. The viral genome of IBV is a single-stranded positive sense ribonucleic acid (RNA) that is approximately 27.6 Kb in length. The gene organization is 5' untranslated region (UTR)-leader-1a/1ab-S-3a-3b-E-M-5a-5b-N-3' UTR. Among these genes, Spike glycoprotein (S), which made up of 2 subunits S1 and S2, plays an important role in virus classification and virus neutralization. S1 protein can induce serotype-specific antibody and neutralizing antibody. Variation in S1 protein enables one strain of virus to avoid immune response induced by another strain of the same species (Jackwood and Wit, 2013).

Infectious bronchitis (IB) is an acute and contagious disease of the chicken caused by IBV. IB is the most economically important viral disease of chicken. Initially, IBV caused the disease in respiratory system. Then, IBV has been detected in other systems including reproductive tract, alimentary tract, and kidney. In layer and breeder chickens, the disease caused loss of production and poor egg quality. In broiler, the IB affected weight gain and reduced feed efficiency. Some strains of IBV have

targeted in kidney of young chickens, resulting in nephritis which caused high mortality due to kidney failure.

The control of IB is attempted using live attenuated and inactivated vaccines, however the IB outbreaks still occur with variable types including respiratory disease, nephritis, and poor egg production and quality. Other infectious diseases including Newcastle disease (ND), infectious Laryngotracheitis (ILT), infectious Coryza and Avian influenza (AI) also have these types of infection. Therefore, diagnostic methods are needed to identify IBV infections in relation to a clinical problem. Generally, IBV can be detected by traditional methods [virus isolation, electron microscope, serotype tests (Haemagglutination inhibition-HI), virus neutralization (VN), enzyme-linked immunosorbent assay (ELISA)]; molecular methods [reverse transcription - polymerase chain reaction (RT-PCR), real time quantitative RT-PCR (qPCR) and sequencing methods]; and other methods [immunofluorescence assay (IFA), immuneperoxidase assay (IPA)].

Therefore, this paper is to discuss and explain the pathogenesis of IBV and available laboratory methods for IB diagnosis.

2 PATHOGENESIS OF IBV

IB is predominant disease of chicken (*Gallus gallus*). The pathogenesis of IBV can differ widely between serotypes. The clinical outcome of an IBV infection depends on many variables such as the virus strain and type; sex and age of the chicken; immune status (vaccination, immune suppression, and maternally derived antibodies); secondary infection (*Escherichia coli*, *Mycoplasma* spp., and *Haemophilus paragallinarum*); and environmental circumstances such as climate, dust, ammonia, and cold stress (Jackwood and Wit, 2013).

In general, IBV infects initially the upper-respiratory tract regardless of the tissue tropism of IBV strain including respiratory (Massachusetts strains (M41)), kidney (LX4, SXL, NMC, HBC), reproductive (D388, M41), and alimentary target (G, LX4, 793/B). IBV replicates and causes lesions in nasal turbinates, Harderian gland, trachea, lungs, and air sacs of respiratory tract. After a brief viraemia, IBV continue to attack other epithelial surfaces including kidney, oviduct, and testes depending on strain of IBVs. The virus also grows in esophagus, proventriculus, duodenum, jejunum, cloacal, bursa, cecal tonsils, rectum, and cloaca of the alimentary tract.

2.1 Respiratory infection

The replication of IBV in the respiratory tissues causes clinical signs of gasping, coughing, tracheal rales and nasal discharge. Occasionally, signs of puffy, inflamed eyes and swollen sinuses may be observed during infection. Trachea is the key site of IBV replication, after entering and damaging the trachea, a viraemia occurs, and the virus disseminates broadly to other organs such as kidney, reproductive tract, and gut system. The virus is primarily epitheliotropic and enters the epithelial cells by viropexis. Many researches revealed that using immunofluorescence, immunoperoxidase and electron microscopy methods have identified the presence of IBVs in ciliated epithelial and mucus-secreting cells. Virus titers in the trachea reach peak from 5 to 10 days post inoculation and gradually decrease and maintain at low level up to 28 days after infection (Raj and Jones, 1997). The presence of IBVs also detected in the epithelial cells of air sacs and lungs (Janse *et al.*, 1994). High level of virus is detected in these tissues from 4 to 11 days post infection. Cloudy air sacs and pneumonia may be observed after post-mortem (Raj and Jones, 1997).

The progression of IBV infection in the trachea includes three stages: degenerative, hyperplastic and recovery. In the first 1 or 2 days of infection, deciliation and desquamation of epithelial and mucous-secreting cells with a mild infiltration of heterophils and lymphocytes in the lamina propria can be detected. Heterophils are usually seen infiltrating among ciliated epithelial cells and in the lumen of the trachea. In hyperplastic stage, newly-formed epithelial cells, which usually have no cilia, are detected. Complete recovery of trachea is performed within 10 to 20 days (Chen *et al.*, 1996) and infected air sacs, epithelial cell desquamation, oedema and some fibrinous exudate may be seen during this stage (Jackwood and Wit, 2013).

2.2 Nephritis

IBV nephropathogenic strains (B1648, QX, AZ23.74, and B1648) caused extensive and reproducible kidney disease in broiler or in meat-type chickens, many strains of IBV may be associated with nephritis to some degree in the field. Firstly, a virulent nephropathogenic IBV is published in Australia, then, in USA, certain areas of Europe, and Asian countries (Lambrechts *et al.*, 1993; Zhou *et al.*, 2004; Chacón *et al.*, 2014;).

Initially, nephropathogenic IBV strains infect respiratory tract followed by damaging kidney combining with signs of increased water intake and watery dropping. The first two-week chicks are very

sensitive with nephropathogenic IBVs that normally show more severe nephritis and higher mortality than older birds. However, mortality rates depend on several factors such as gender, nutrition, environment and management factors. Nutrition also affects nephropathogenetic infection. Chickens fed with high levels of calcium may reduce urolithiasis and kidney lesions when infected with nephropathogenic Gray IBV strain. The virulence of nephropathogenic strains also depends on the age of infection. Greater mortality was seen in males, with cold stress, in certain breeds (Raj and Jones, 1997).

The replication of IBV in the kidneys has been revealed in the lower nephron down to the collecting duct epithelial cells (Chen *et al.*, 1996). IBV also replicated in many parts of tubules and ducts, however the presence of virus in the epithelial cells of the collecting ducts, tubules, Henle's loops and distal convoluted tubules is very common. Chickens infected with nephropathogenic IBV showed increasing in urinary water losses (Raj and Jones, 1997). Infected chickens caused by nephropathogenic IBV strains show gross lesions in kidney including swollen and pale with deposition of urate in tubules and ureters. In addition, the haematocrit values are reduced and plasma uric acid levels are increased (Afanador and Roberts, 1994). In microscopic lesions, degeneration and desquamation of the tubular epithelium with heterophils infiltration in the interstitium can be detected in acute IB (Raj and Jones, 1997).

Variant nephropathogenic IBV strains cause similar lesions in kidney but their severity is different. The effect of IBV on the trachea is apparently independent of the effect on kidney.

2.3 Reproductive tract infection

In laying hens, the presence of virus in the epithelium of the oviducts is detected from 6 to 9 days after IBV infection (M41)(Raj and Jones, 1997). Infected chickens showed mild respiratory signs with severe loss of egg production and quality including soft-shells, misshapen or rough-shelled, watery albumen (Jackwood and Wit, 2013). Microscopic lesions were observed in the oviduct including shorten height of the epithelial cells, absence or disappearance of cilia, dilation of glands, infiltration of lymphocytic foci and cellular in the lamina propria and inter tubular stroma (Jackwood and Wit, 2013).

In young chickens, day-old chicks inoculated with IBV strain M41 revealed that IBV is detected from 5 to 11 days post inoculation and found in the epithelium of the oviducts. Less than 2 week-old female chicks infected with IBV may affect their fertility when they become mature (Raj & Jones, 1997). The middle third of the oviduct is the most severely affected with areas of localized hypoplasia seen between normal patent oviducts. Similar to laying hens, microscopic lesions in the oviduct include shorten height of the epithelial cells, absence or disappearance of cilia from epithelial cells, dilation of the tubular glands, infiltration of heterophils, lymphocytes and plasma cells.

2.4 Alimentary tract infection

The replication of IBV has been revealed in cells resembling histiocytes and lymphoid cells in the caecal tonsils and epithelial cells of the villi in rectum and ileum (Ambali and Jones, 1990; Owen *et al.*, 1991). In addition, IBVs have been isolated in other parts of alimentary system such as oesophagus, proventriculus, duodenum and jejunum; however, the replication of IBVs in these tissues has not been confirmed. A variant IBV strains including strain G and 793/B-like were classified as enterotropic type instead of pneumotropic type because they located in the gut longer compared to in the respiratory tract (Raj and Jones, 1996). IBVs have a wide tropism for alimentary tract but no gross or microscopic lesions have been reported.

3 LABORATORY TESTS AVAILABLE TO DETECT IBV

Laboratory tests for diagnosis of IB include virus isolation, electron microscope, immunohistochemistry (IHC), IFA and IPA, serological tests (VN, HI, ELISA, agar gel immunodiffusion), genotype identification (RT-PCR, NGS). Among them genetic based tests (RT-PCR or RT-PCR) are used commonly to identify an isolate as IBV.

3.1 Virus isolation

Virus isolation is a standard method for diagnosis of IB. Virus isolation from fresh tracheal tissue, kidney, proventriculus, tonsil, and oviduct is the preferred samples. Titers of IBV are very high in the trachea at 3-5 days after inoculation, then rapidly decline. IBV initially grows in the upper respiratory tract and then spreads to nonrespiratory tissues such as kidney, cecal tonsils, and oviduct which should be considered depending on the clinical history of the flock (De Wit, 2000).

IBV is most commonly grown in embryonated eggs, tracheal organ culture (TOC), and chicken kidney cell culture. The embryonated egg is preferred for primary isolation attempts.

3.1.1 Embryonated chicken eggs

Most IBV strains grow well when inoculated into the allantoic cavity of a 9–11-day-old SPF chicken embryo. Clinical samples from tracheal swab, broth, or tissue homogenate are inoculated into the allantoic cavity of specific pathogen-free eggs and incubated at 37^0C after inoculation. Eggs are candled daily tomonitorembryo viability; death within 24 hours is considered nonspecific. After 48-72 hours, allantoic fluid is harvested and tested for the presence of IBV using serological tests or RT-PCR. The allantoic fluid needs to be subjected to several passages to allow the virus to adapt and replicate to high titer. Inoculated eggs are opened and observed for characteristic IB lesions such as curling and dwarfism of the infected embryo at 5-7 days post inoculation.

3.1.2 Organ cultures

Chicken TOC can be used to propagate IBV. TOC is prepared from tracheal rings of 20-day-old chicken embryo. The tracheal rings are maintained in a roller bottle and infected with IBV-suspected samples. The culture is observed under light microscope for evidence of ciliostasis. A positive culture shows complete impairment of ciliary activity. Successful growth of IBV has been demonstrated in organ cultures derived from kidney, intestine, proventriculus, and oviduct. However, susceptibility of these organs to IBV can be influenced by the strain of the virus and the amount of virus presence in the sample (Bande *et al.*, 2016).

3.1.3 Cell cultures

The isolation of IBV has been used in various primary and secondary cells, such as chicken embryo kidney fibroblast and Vero cells. Infected cultures are characterized by rounding, development of syncytia, and subsequent detachment from the surface of the plate. However, this technique showed a major limitation in virus adaptation in cell culture and requirement of primary isolation in embryonated eggs and several passages (Bande *et al.*, 2016).

3.2 Electron microscope

Electron microscopy is a useful tool that has been used for IB diagnosis based on morphological characteristics of *coronavirus*. Positive cultures are confirmed based on the presence of *coronavirus*-like pleomorphic structures with spike projections, following negative staining with phosphotungstic acid. Using this technique, characteristics of IBV including shape and diameter (120 nm) should be carefully considered. Apart from the negative staining method, transmission electron microscopy is also a useful tool which enables the visualization of virus-like particles in infected cells. This method is often applied to understand viral attachment and entry into the cell (Hodgson *et al.*, 2006).

3.3 Immunohistochemistry (IHC)

IHC includes immunoperoxidase and immunofluorescence that are two main histochemistry methods used for detection and confirmation of IBV antigen from infected tissues and cells. These methods perform based on antigen-antibody reactions. Both immunoperoxidase and indirect immunofluorescent assays have been used successfully to localize IBV antigen in tissue samples (Bande *et al.*, 2016).

3.4 Serotype specific tests

A number of tests have been used for diagnosis of IB disease. Those considered here including VN, agar gel immunodiffusion, HI and ELISA. Each test has advantages and disadvantages in terms of practicality, specificity, sensitivity and cost.

3.4.1 Virus neutralization (VN)

VN is a standard test for the detection of IBV serotype specific antibodies. This test also used to investigate the flock protection following vaccination. Classification of new IBV isolates is mostly by VN tests *in ovo* or in organ and cell cultures. These methods are rather time-consuming and are not always unambiguous. Furthermore, they require virus growth in eggs or in organ or cell culture, and monospecific antisera to the new isolate (Bande *et al.*, 2016).

3.4.2 Haemagglutination inhibition (HI)

Similar to VN, HI can be used to detect, serotype IBV strains and measure the antibody responses after vaccination. However, this test is less reliable compared to VN and ELISA assays. IBV can cause agglutination of red blood cells of chickens. HI antibodies are induced primarily by the S1 protein. A previous study reported the results of the HI test for the Massachusetts serotype after vaccination with Holland strain of the Massachusetts serotype. After the primary inoculation, the HI test failed to detect the presence of the Holland strain when using M41 as antigen, but the strain was well detected by HI test when using Holland antigen (Bande *et al.*, 2016).

3.4.3 Enzyme-linked immunosorbent assay (ELISA)

Currently the ELISA test is the most widely used serologic test for antibodies against IBV because it is inexpensive and can be used to test a large number of samples in a short time. The ELISA technique is a sensitive serological method and gives earlier re-

actions and higher antibody titers than other serological tests. Commercial tests are available and typically detect antibodies (IgY) after one week post infection. The ELISA is widely used to identify IBV infected flocks (broilers) based on high antibody titers (Bande *et al.*, 2016).

3.4.4 Agar gel precipitin test (AGPT)

AGPT has a high specificity although the precipitation lines for IBV are more difficult to read comparing to AGPT for infectious bursal disease virus or Newcastle disease virus. However, variable and transient results of AGPT make it not suitable test for characterization of strain.

3.5 Molecular identification

3.5.1 Reverse transcriptase polymerase chain reaction assay (RT-PCR)

This assay applies viral RNA, amplified either directly (one-step RT-PCR) or following cDNA synthesis (two-step RT-PCR). RT-PCR assays have been popularly used to detect and classify genotype or serotype of IBVs based on nucleotide and amino acid sequences of S1 protein. The UTR and N gene in many IBV serotypes are the most conserve regions, so RT-PCR based on these regions are used for universal detection. A serotype specific PCR assay has been designed to enable differentiation of Massachusetts, Connecticut, Arkansas, and Delaware field isolates (Bande *et al.*, 2016).

3.5.2 Restriction Fragment Length Polymorphism (RFLP)

RFLP assay is used to identify and genotype IBV strains based on unique electrophoresis banding patterns defined by restriction enzyme digestion. A sequence of IBV S1 glycoprotein could be targeted for amplification and enzymes analysis. RFLP allows differentiation of various known IBV strains.

3.5.3 Real-Time RT-PCR Assays

Real-time RT-PCR assays are the most sensitivity and specificity test have been utilized for IBV detection. Beside of detection, Real time RT-PCR is possible to quantify IBV viral load and gene expression from host's clinical samples. This technique allows for direct detection of PCR product during the exponential phase of the reaction, combining amplification and detection in one single step.

3.5.4 Nested PCR

Routine diagnosis of IB using nested RT-PCRs is seldom applied because of their extreme sensitivity and the high risk of contamination during both sampling and performing the assay, resulting in false-positive results. However, this technique is more sensitive than conventional RT-PCR. Optimal test-conditions are even more critical for performing nested RT-PCRs than conventional RT-PCR.

3.5.5 Sequencing methods

RT-PCR products are purified and sequenced based on traditional sequencing method as Sanger sequencing or advanced methods including next-generation sequence [pyrosequencing, sequencing by synthesis (Illumina), sequencing by ligation (SOLID sequencing)], and nanopore DNA sequencing. Currently, next-generation sequence have been applied to sequence whole genomes within limited periods of time, though this approach has been used only in the laboratory (Bande *et al.*, 2016).

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