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Molecular interventions and pathogenic clues for identification of infectious bursal disease virus in poultry

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Abstract

Infectious bursal disease virus (IBDV), a non-enveloped double stranded virion is the etiological agent causing clinical infection in young poultry. IBDV has two serotypes and serotype 1 affects chicken, by destroying the IgM bearing lymphocytes in bursa of fabricus. The poultry sector encounters severe economic loss when the birds get infected, as the entire flock is lost. Appropriate diagnosis and proper vaccination schedules need to be followed to combat the odds of the disease. The pathogenic pathways of IBDV virus and the molecular interventions in diagnosis is discussed in this review.

Keywords: IBDV pathogenesis, ELISA, VNT, RT-PCR, Reverse genetics

Introduction

Infectious bursal disease is an acute contagious immunosuppressive disease with severe mortality rates in poultry. IBDV belongs to the genus *Avibirnavirus* and family *Birnaviridae*. (Wahome *et al.*, 2017; Mwenda *et al.*, 2018) [40, 32]. This virus has bisegmented genome, segment A and segment B. Segment A has two partial overlapping open reading fragments as ORF1 and ORF2. These overlapping open reading frames have five different viral proteins, and are designated as VP1, VP2, VP3, VP4, and VP5. (Etteradossi and Saif, 2020) [11]. Large segment A consists of four viral proteins. The two capsid protein, Viral protease and nonstructural proteins are 3261 nucleotide long. The two capsid proteins are VP2 and VP3 (Kasanga *et al.* 2007, Muller *et al.* 2003) [22, 31]. VP2 protein size is 48kDa from the first aminoacid to 512 aminoacids, VP3 with 32kDa size extends from 792 to 1012 aminoacids, the viral protease protein is VP4 with 24 kDa size and in the position of 513 to 791 aminoacids. The nonstructural protein is VP5 which is 17kDa in size. The smaller segment B which encodes the VP1 protein is 97kDa in size with an RNA polymerase and exists as a free polypeptide and as a genome linked protein. (Dey *et al.* 2019. Liu and Vakharai., 2004) [8, 27]. VP2 protein, the major host protective capsid antigen is responsible for eliciting neutralizing epitopes (Hamoud *et al.*, 2007) [11]. VP2 protein has three different major domains namely the shell, base and projection domains. VP3 is the other major structural protein which elicits non-neutralizing antibodies. VP3 supports viral polymerase activity and interactions involving the correct assembly of VP2 protein (Boot *et al.* 2002) [6]. VP4 protein is a viral protease that plays a major role in the maturation of VP2 capsid protein, by trimming several peptides progressively. VP5 is a nonstructural protein, which has a regulatory function in virus release and dissemination in the early stages of infection (Lombardo *et al.* 2000) [28].

Serotypes of IBDV

In IBDV there are two different serotypes which are designated as serotype I and II. Serotype II does not cause disease in poultry and is considered as nonpathogenic. However serotype I consists of different pathogenic strains of IBDV causing infection in poultry (OIE, 2016) [33]. According to their pathogenicity, serotype I viruses are classified as avirulent, classical virulent strains (cIBDV), antigenic variant strains (vaIBDV), and very virulent strains (vvIBDV) (Maqbool *et al.*, 2020) [29]. The Emergence of the acute phase of IBDV has drastically changed the epidemiology, although the origins of vvIBDVs have spread all over the world in a very explosive but in a conserved manner. Fingerprints of VP2 on vvIBDVs are considered as more evolutionary markers than as virulence markers. (Berg, 2000) [4]

Pathogenesis

The oral route is the most common mode of infection in chicken. The virus is initially transported from the gut to other tissues. The viral antigen has been detected in kidney within few hours of infection, later in the liver. The viral replication starts primarily in the bursa of Fabricius. Exposure of IgM-bearing B lymphocyte within hours after the virus replication results in the appearance of virus-containing cells in the bursa. Subsequently, the virus spreads rapidly through the whole bursal follicles and start targeting all IgM-bearing B lymphocytes. Targeting all B-lymphocyte results in massive destruction of lymphoid cell in the medullary and the cortical regions of the bursal follicles followed by cellular destructive process, by apoptosis of virus-free bystander cells. The acute phase of infection is associated with the reduction in circulating IgM cells, with no detectable circulating immunoglobulins. T cells are resistant to viral infection of IBDV. Thymus undergoes atrophy with extensive apoptosis of thymocytes during the acute phase of virus infection. The acute phase of infection targets the IgM bearing B-lymphocytes particularly the actively dividing and differentiating B lymphocytes. Clinical signs associated with acute disease include anorexia, depression, diarrhea, prostration, ruffled feathers and death.

The acute phase of the IBD virus lasts for about 7 to 10 days. In this phase, there is total depletion of B cells in bursal follicles. After deploying B cells in the bursal follicles, the viral antigen starts targeting the peripheral lymphoid organs such as the cecal tonsils and spleen. Accumulation of CD4+ and CD8+ T cells starts at the site of virus replication near the deployed B-cell organs with activated T cells. The virus induced T-cells exhibit upregulation of cytokine genes and proliferation of T-cells in response to the *in vitro* stimulation. In this condition death is encountered among poultry due to the virulence of the strain. If survived, the virus is cleared and the bird starts to recover from its pathologic effects, by repopulation of IgM B cells in the bursal follicles.

The clinical and subclinical infection of IBD virus may lead to immunosuppression. Both humoral and cellular immune responses are compromised, resulting in the inhibition of humoral immunity which is attributed to the destruction of immunoglobulin producing cells. Orakpoghenor *et al.*, in 2020 remarked the association of altered antigen-presenting and helper T cell. The infection with IBDV leads to a transient inhibition of the proliferative responses of T cells to mitogens under *in vitro* condition. The inhibition is therefore mediated by macrophages which are activated in virus-exposed chickens and exhibits expression of a number of cytokine genes. Sharma *et al.*, in 2000 [39] speculated that T cell cytokines such as interferon (IFN)- γ may stimulate macrophages to produce nitric oxide (NO) and other cytokines with anti-proliferative activity.

Diagnosis

Isolation and identification provide the most certain diagnosis of IBD. In laboratory practice, diagnosis of IBD depends on detection of specific antibodies to the virus, or on detection of the virus antigen and nucleic acid in tissues, using immunological or molecular methods. Confirmatory diagnosis of IBDV is most commonly performed by serology using Enzyme linked immunosorbent assay (ELISA), Agar gel precipitin test (AGPT) and Virus neutralization test (VNT) of bursal sections (Sharma *et al.*, 2000) [39]. Zafar *et al.*, 2020 [43] used gold nanoparticle-based lateral flow assay for the detection of IBDV using anti-IBDV antibodies.

Agar Gel Precipitation Test (AGPT)

It is the most commonly recommended test for IBD diagnosis by OIE, but lacks sensitivity and identifies primarily group-specific soluble antigens. Antigen is prepared from a suspension of infected bursa of fabricus. Briefly, a 50% suspension is homogenized and then processed by centrifugation. Subsequently the antigen is checked for the sensitivity and specificity against known positive and negative sera. Test sera were placed in adjacent wells to positive control sera to enhance sensitivity and to establish specificity of precipitin lines. (Ley *et al.*, 1983) [25]. The results of the tested sample are interpreted when a clear precipitin line is formed when the positive antigen and antibody interacted with each other, popularly known as a "line of identity" AGID is the simplest diagnostic test but is least sensitive. (Salik, 2019) [37].

Virus Neutralization Test (VNT)

VNT is useful for differentiating the strains and evaluating the vaccine responses. This test is not required for routine diagnostics but has proven to be more sensitive for detecting the IBDV antibody against the antigen. Virus neutralization tests (VNT) are carried out in animal cell culture system. Serum and virus are made to react with each other in equal volumes and inoculated into a susceptible cell line. If the antibodies to the virus are present cytopathic effect (CPE) will not be observed in the cell line, as it results in the inhibition of virus replication followed by neutralization of virus. (Lindal 2004) [26].

Reverse Transcriptase Polymerase Chain reaction (RT-PCR)

RT-PCR is the most sensitive diagnostic assay, as it has the ability to detect the presence of even a single gene copy by multiplying it to millions of copies by thermal cycling. Nucleic acid of the virus has to be extracted. In IBDV as RNA is the nucleic acid, it is extracted by triturating the bursal tissues in a mortar and pestle with sterile sand and PBS. The triturated sample along with 100 μ l of triton-x 114 is vortexed for 1 minute followed by 3 times of freezing and thawing. The supernatant was collected and processed for extraction of nucleic acid using TRI Reagent.

In this method 0.25 ml of tissue supernatant was taken to which 1ml of TRI reagent was added and incubated at room temperature for 5 minutes for complete dissociation of nucleoprotein complexes. Subsequently 200 μ l of chloroform was added and mixed vigorously for 10 -20 seconds and incubated at room temperature for 5 minutes. After incubation the samples were centrifuged at 4°C for 10 minutes at 14,000rpm. The RNA containing aqueous layer was carefully transferred into a fresh tubes and equal volume of isopropanol was added and kept for 10 minutes at room temperature. The samples were centrifuged at 12,500 rpm for 10 minutes. The RNA pellet was washed with 70 % ethanol, air dried and resuspended in 15 μ l of RNase free water and quantitated. (Adamu *et al.*, 2013) [1]. The extracted total RNA was converted to complementary DNA (cDNA). Primers for very virulent IBDV (vvIBDV) was used for detecting the presence of IBDV by RT-PCR. (Meenambigai *et al.*, 2017) [30].

Restriction fragment length polymorphism (RFLP)

RFLP detects the presence of minor genetic changes known as polymorphisms among strains of IBDV. To perform this technique, RT-PCR products were digested with suitable

restriction enzymes known as molecular scissors (Eg) MboI (Ozbey *et al.* 2003) [35]. Specific enzymes were used to generate RFLP patterns that distinguish the viruses into molecular groups.

To perform RFLP, ten microliter aliquots of RT-PCR reaction products were digested with 10 units of enzyme and incubated for 2 hour at 37°C. The MboI digested products were separated on a 2.5% agarose gel and visualized under a UV transilluminator. (Hernandez *et al* 2011) [15].

Loop mediated isothermal amplification (LAMP)

LAMP is a simple, specific and less laborious technique for diagnosing IBDV. Reverse Transcriptase PCR products with several primers specific for any viral protein of IBDV can be used in the test. For example the VP5 gene including two outer primers (F3 and B3), two inner primers (FIP and BIP) and two loop primers-F loop and B loop which were reported by Wang *et al.*, in 2011 [41] were used. The RT-LAMP reaction was carried out using RNA amplification kit as reported by Khan *et al.*, in 2018 [23] which resulted in prodigious specificity without cross reactions with other pathogens.

Enzyme Linked Immunosorbent Assay (ELISA)

One of the best methods in serological diagnosis is ELISA. The principle of ELISA is that antibodies are precoated in a 96 well plate and attached to their specific antigen with an enzyme linked antibody followed by the addition of the substrate. ELISA enables the quantification of antibodies towards IBDV and is therefore used for screening the immune status of the chicken flocks with or without vaccination and also for monitoring the natural field exposure and weaning of maternal antibody titers. ELISA is a sensitive diagnostic assay in which a large number of samples can be tested at the same time. (Howie and Thorsen 1981) [15]. The antigenic variation of infectious bursal disease virus was demonstrated from different host systems (bursa of Fabricius, embryos, cell cultures) by ELISA (indirect and antigen capture). In this study 27 non-neutralizing anti-VP2 monoclonal antibodies were used with a reference panel of nine neutralizing monoclonal antibodies, and 13 neutralizing anti-IBDV chicken polyclonal antibodies. (Rodriguez-Chavez *et al.*, 2002, Ching wu *et al.*, 2007) [36, 7].

Peptide ELISA

Peptide ELISA is the most rapid diagnostic assay with increased specificity and sensitivity. Peptides are safe, chemically defined and noninfectious alternative antigens which can be used instead of whole virus antigen in serodiagnosis of IBDV. Peptide ELISA was done by Saravana *et al.*, in 2004 [38] utilizing Multiple antigenic peptides (MAPs) to predict the antigenic determinants on the VP2 protein of infectious bursal disease virus (IBDV) and were used as antigens in enzyme-linked immunosorbent assay (ELISA) as an alternative to the whole viral antigen to detect anti-IBDV antibodies in chicken sera.

Dot blot hybridization assay

The dot blot hybridization assay was conducted using two cDNA clones. cDNA clones were used to prepare probes which were identified from a library of clones prepared using the STC viral genome. The cDNA clone STC-1 represents a portion of STC genome segment A. The Clone STC-119 represents the 3' end of STC genome segment B. (Jackwood

et al. 1989) [18]. Radiolabeled probes were prepared from the STC-1 and STC-119 clones using 32P-dCTP and a nick-translation kit. These probes were combined before use in the hybridization assays (Jackwood *et al.*, 1990) [17]. Dot blot results in greater sensitivity of the hybridization assay due to the ability of the cDNA probes to detect the viral genomic RNA and the viral mRNA, which are synthesized during early infection state. (Henderson and Jackwood 1990) [13].

Immuno-electrophoresis

Counter immuno electrophoresis (CIE) test is a standard diagnostic method used to detect unknown antigen and antibodies with known positive reference controls. 1% agar was prepared in 0.025 M bicarbonate acetate buffer which was layered onto each slide. Wells were punched on polymerized gels using standard CIE template. Each pair of wells in the slide were connected and placed in position of cathode and anode. Cathodal well was filled with the antigen while the anodal well was filled with the antiserum. 0.05M barbitone acetate buffer was poured into the electrophoresis tank and the slide was connected with the buffer in the tank with a strip of Whatman filter paper on each end of the slide. The test was run for 30 to 45 min under a relatively constant current (12mA per slide). The anode and cathode wells were filled with the unknown sera and the known positive antigen respectively to form a precipitin line in between them. The slides were read in presence of illuminated background. If there is appearance of a precipitin line between an antigen and serum it indicates positive reaction. CIE a rapid and simple technique for the detection of IBD viral antigen and antibody. (Durojaiye *et al.*, 1985) [10].

Real-Time RT-PCR

This is a very rapid and sensitive diagnostic method. It employs the fluorescence resonance energy transfer (FRET) technique to identify the RT-PCR products. Moody *et al.*, in 2000 used two different probes one labeled with fluorescein isothiocyanate and other labeled with the red 640 fluorophore. By using this taq man system sequence identity or mutations can be assessed by comparing the melting temperature (Jackwood 2004) [20].

Reverse genetics

In a reverse genetics system over 300 IBDV isolates were analysed and investigated using a panel of monoclonal antibodies and by cross-neutralization assays in embryonated eggs and vaccinated chicken. In addition to this the sequences of a large portion of the gene were determined using DNA STAR laser gene software online tool (Durairaj *et al.* 2011) [9].

Conclusion

Infectious bursal disease or gumboro disease is one of the viral diseases that mainly affect poultry all over the world with severe economic losses. It mainly affects young chickens between 3-6 weeks old with severe immunosuppression. The bursa of Fabricius is the major organ affected during an infection with the virus. Diagnosis of IBD is dependant on clinical signs, differential diagnosis, gross lesions, histopathological lesions, virus isolation, serological and molecular diagnosis. Although confirmatory diagnosis of IBD is traditionally performed with serological methods such as ELISA or virus neutralization, a number of molecular diagnostic methods based on RT-PCR are now available for the detection of IBDV and differentiation of IBDV subtypes.

Through these different identification methods more appropriate control measures could be developed in future to afford a higher degree of protection to young birds. Hence, early diagnosis of IBDV must be targeted to adopt effective control strategies.

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