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Efficacy of two diagnostic tests for the detection of infectious bursal disease viruses in chicken from different types of bursal samples

Fahmida Afrin[1,2], M. M. R. Chowdhury[2,3], Chang-Yeon Cho[2], Seungchang Kim[2], Hee-Jong Roh[2], Dong-Kyo Kim[2], Md. Alimul Islam[1] and Sukumar Saha[1*]

[1]Department of Microbiology and Hygiene, Bangladesh Agricultural University, Mymensingh-2202, Bangladesh.
[2]Animal Genetic Resources Research Center, National Institute of Animal Science, Rural Development Administration (RDA), Hwang San-ro 1214-13, Unbong-up, Namwon, 55717, Jeonbuk, Republic of Korea.
[3]Department of Physiology and Pharmacology, Faculty of Animal Science and Veterinary Medicine, Patuakhali Science and Technology University, Bangladesh.

Infectious Bursal Disease (IBD) is an economically important irreversible immunosuppressive disease of young birds. The present study was designed to confirm the efficacy of two common diagnostic tests for the detection of Infectious Bursal Disease Virus (IBDV) from the three types of bursal samples collected from a recent outbreak in layer and broiler chickens of Gazipur district, Bangladesh. This study compared the degree of sensitivity between Ouchterlony Double Immunodiffusion Test (ODIT) and Reverse Transcription Polymerase Chain Reaction (RT-PCR) for the detection of IBD viral antigen from the bursal samples. A total of 180 field bursal samples (80 broiler and 100 from layer chicken) from suspected IBDV infected dead chickens were collected from 50 different poultry farms. Bursal homogenates were used to detect IBDV using ODIT and RT-PCR. Three types of bursal samples, hemorrhagic bursa (90), edematous bursa (78) and atrophied bursa (12) were selected for the detection of viral antigen. A panel of anti-sera and IBDV specific primer for VP2 gene was used for RT-PCR. The data demonstrated that, out of 180 field samples, 164 (91%) were positive by RT-PCR and 120 (67%) were positive by ODIT. Haemorrhagic bursas were more sensitive compared to oedematous bursas while no virus was detected from the samples of atrophied bursa. This study demonstrated that, RT-PCR was more sensitive and effective diagnostic tool compared to that of ODIT.

Key words: Infectious bursal disease virus (IBDV), ouchterlony double immunodiffusion test (ODIT), reverse transcription polymerase chain reaction (RT-PCR), bursa, broiler, layer.

INTRODUCTION

Infectious bursal disease or Gumboro is a vastly transmissible immunosuppressant disease which is triggered with a virus belonging to the genus *Avibirnavirus* of family Birnaviridae. IBD is associated with high

*Corresponding author. E-mail: sukumar.saha@bau.edu.bd. Tel: 008801740847339.

mortality affecting mainly 3 to 6 weeks old age group but subclinical and less acute form is also mutual in 0 to 3 week old chickens (Teshome and Admassu, 2015). IBD can cause an acute viral disease with 80 to 100% morbidity and mortality that varies from 20 to 30% in broilers whereas it is 40 to 80% in layers, respectively, depending on the strain virulence (Van den Berg et al., 2000; Chowdhury et al., 1996). There are three different pathotypes of IBD virus namely, very virulent (vvIBDV), classical virulent (cIBDV) and variant (Michel and Jackwood, 2017), the later one being responsible for prolonged immunosuppression. The clinical form of IBD is associated with significant economic losses with irreversible immune suppression, reduced effectiveness of vaccination, retarded growth, poor feed consumption, decreased egg quality and decreased egg production. In addition, the excessive hemorrhages within the muscles consequences in increased mortality of the chickens. The characteristic clinical sign of the IBD infected chicken includes whitish watery droppings followed by depression, anorexia, severe prostration, trembling and finally death (Sali, 2019). Virus strain, age and also the breeds of chickens affect the severity of the disease (Teshome and Admassu, 2015). Infection with less virulent strains may not show remarkable clinical signs besides the chickens may have fibrotic or cystic bursa of Fabricius that might turn into atrophied prematurely (before six months of age). During postmortem diagnosis, the chickens showed hemorrhages in the pectoral or thigh muscles, dehydration, urate deposits within the kidneys, enlarged, edematous, hyperemic and or atrophic bursa of Fabricius (Aliyu et al., 2016; Khan et al., 2009). In chronic cases, presence of hemorrhage within the connection between gizzard and proventriculus is observed (Khan et al., 2017; Aliyu et al., 2016). The virus generally infected the lymphoid tissue especially the bursa (Dey et al., 2019; Wang et al., 2011; Van den Berg et al., 2000) and thus others immune organs like as spleen, bone marrow, thymus are also involved. Several diagnostic methods are used for the identification of IBDV through various levels of specificity and sensitivity (Daodu et al., 2018; Oluwayelu et al., 2014; Okwor et al., 2011; Hussain et al., 2003; Nachimuthu et al., 1995; Aliev et al., 1990; Allan et al., 1984). Common serological tests such as agar gel immunodiffusion test (AGIDT), enzyme-linked immunosorbent assay (ELISA), immunofluorescent test and indirect hemagglutination (IHA) test are being used for the detection of IBDV (Daodu et al., 2018). Although, the confirmatory diagnosis of IBDV is very important for formulating an effective strategy intended for control of the disease. ODIT is also familiar as passive double immunodiffusion or AGID test. ODIT is a simple method to detect specific antibody and measure antigenicity (Hornbeck, 2017). It is simplest but least sensitive serological method (Sali, 2019). It measures primarily group specific soluble antigens and cannot distinguish serotypic variances. It can be used to produce

a precipitin line by estimating antibody size through dilutions of serum in the test well and taking the titer as the highest dilution (Sali, 2019). The molecular methods like RT-PCR is used to amplify the reverse transcription of the DNA code (Saiki et al., 1985). RT-PCR is the method which helps for precise and early diagnosis of viruses from field samples (Hasan et al., 2010). Among these diagnostic methods, in this study we chose ODIT and RT-PCR as ODIT is simplest of all, inexpensive and has the ability for detecting specific antibodies (Dey et al., 2019; Hornbeck, 2017; Ouchterlony, 1948; Ouchterlony and Nilsson, 1986) and RT-PCR is specific, sensitive, speedy, reliable and accurate techniques for detection of IBD viruses (Hasan et al., 2010).

IBD is an economically significant disease in poultry sector. The irreversible immune suppression caused by IBD virus in young chicks increases their susceptibility to an assembly of opportunistic avian pathogens that are normally non-pathogenic in healthy groups (Michel and Jackwood, 2017). Usually the farmers are worried about the present monetary mortality value from the lost flock and never see beyond if the chickens were to get relief from the disease. These collectively consequences remarkable economic losses for the poultry farmers which are often high and alarming if not properly diagnosed. Fresh samples from the affected chickens are usually more suitable to detect the virus but in rural areas of Bangladesh, there is very limited opportunity for the identification of poultry disease at the field level. The diagnosis is done mainly based on the clinical history, sign-symptoms and necropsy findings where there is a high possibility of misinterpretation and wrong diagnosis, because many poultry diseases produce similar clinical signs, symptoms and postmortem findings which are sometimes very difficult to differentiate (Hasan et al., 2010; Sali, 2019). Therefore, this study was designed to compare the sensitivity of the molecular technique (RT-PCR) and serological technique (ODIT) for the detection of IBDV from three types of bursal samples.

MATERIALS AND METHODS

Ethical approval

The research was carried out following the guidelines set forth by the Animal Welfare and Experimentation Ethics Committee of Bangladesh Agricultural University, Mymensingh, Bangladesh (ref. no. AWEEC/BAU/2019(38)).

Sample collection

A total of 180 (broiler 80 and layer 100) bursa of Fabricius (BF) (hemorrhagic, edematous and atrophied bursa) were collected from a recent outbreak in broiler and layer chickens of Sadar Upazila in Gazipur district, Bangladesh.

Sample preparation

Three types of bursal samples were macerated separately with

sterilized mortar and pastel to prepare 10 to 20% (w/v) suspension in sterile PBS. The suspension was centrifuged at 4000 rpm for 30 min to separate tissue debris from the supernatant. The supernatant thus obtained was treated by a broad-spectrum antibiotic (Gentamycin) at 50 µg/ml and the prepared inoculates were tested for bacterial contamination by culturing 0.1 ml of each on nutrient agar and blood agar plates for 24 h at 37°C.

Hyper immune serum preparation for ODIT

Four non vaccinated chickens were immunized with the vaccine (Nobilis® Gumboro 228E, Intervet International, The Netherlands) orally at 7, 14, 21, 28 and 35 days of age. After 10 days of last vaccination blood were collected to obtain serum which was used as hyper-immune serum in ODIT.

ODIT

Immunodiffusion plates were prepared by melting 8 g of sodium chloride in 100 ml of distilled water followed by the addition of 1.25 g agar noble. The mixture was gently mixed and boiled in a water bath until the agar was absolutely dissolved. The agar was given left to cool at 50°C before it was poured in 6 of 9 cm immunodiffusion plates and allowed to solidify. The plates were then kept overnight at 4°C until used. Applying a template and well cutter (4 mm), seven wells of 4 mm (a group of six wells surrounding a centre well) were made. The central well of the glass slide filled with melted agarose gel was loaded by known hyper-immune sera against IBDV and peripheral wells by bursal suspensions. Slides were kept in moist chamber for 24 to 48 h at 4°C and observed for antigen antibody reaction in the form of appearance of precipitation lines in between the peripheral and central well.

Extraction of viral RNA

Viral RNA of IBD virus was extracted from three types of bursal suspensions using the QIAamp Viral RNA Mini Kit (QIAGEN GmbH, Hilden, Germany) according to the manufacturer's informations. Then the extracted RNA was treated with RNase-free DNAse I (Fermentas, Canada) and after that using the first strand cDNA synthesis kit, HyperScript (GeneAll), cDNA was synthesized from the treated RNA.

RT-PCR

RNAs from bursal samples were reverse transcribed to cDNA and amplified by a one-step RT-PCR. RT-PCR performed on a MJ Mini Thermocycler, Bio-Rad, USA. Primers Vv-fp775 (forward primer, 5′-AATTCTCATCACAGTACCAAG-3′) and Vv-rp1028 (reverse primer, 5′-GCTGGTTGGAATCACAAT-3′) were used to amplify a 253 bp fragment of the VP2 gene (Hasan et al., 2010). RT-PCR was performed at 42°C for 1 h followed by activation of Taq polymerase at 94°C for 2 min, then 35 cycles of denaturation at 94°C for 30 s, annealing at 45°C for 1 min and elongation at 60°C for 1.5 min. A final extension step at 60°C for 10 min was performed. After amplification, RT-PCR product was subjected to electrophoresis on 2% agarose gel containing ethidium bromide. The band was examined under UV-trans-illuminator.

Statistical analysis

Statistical analyses were accomplished using InStat® software (GraphPad, USA). Correlations between the proposed techniques were analyzed followed by Chi-squared test. Differences were considered statistically significant at *$P < 0.05$ or ***$P < 0.001$.

RESULTS AND DISCUSSION

IBDV endures to be a main concern for the commercial and subsistent poultry farmers including an utmost essential threat regarding poultry production systems in Bangladesh. So, in this study, we compared two to identify the effective diagnostic approaches for the detection of IBDV through ODIT and RT-PCR from the three types of bursal samples.

Apparently, no previous reports were found documenting the sensitivity of the tests using three types of bursal samples for virus isolation. ODIT is one of the substitute techniques suggested for IBDV diagnosis by Organization for Animal Health (OIE) for international trade (Butt et al., 2015). The ODIT is the most useful serological technique for the detection of specific antibodies in sera, or for identifying viral antigen or antibodies in bursa. The ODIT is used to detect prominent white line of precipitation between bursal homogenates of the peripheral wells and known positive anti-IBDV hyper-immune serum of the central well due to antigen and antibody reaction (Hornbeck, 2017). Our data demonstrated that, in case of ODIT, hemorrhagic and edematous bursal homogenates were found positive for IBDV. Prominent white line of precipitation was observed between known positive anti-IBDV hyper-immune serum of the central well and bursal homogenates of the peripheral wells due to antigen and antibody reaction within 24 to 48 h which agrees with Abraham-Oyiguh et al. (2015), Okwor et al. (2011) and Roy et al. (2008) findings. By ODIT, out of 180 samples, 120 samples (H.B.S. 34; E.B.S. 17 from broiler and H.B.S. 44; E.B.S. 25 from layer) were positive for IBDV (Table 1 and Figure 1). No line of precipitation was found in the atrophied bursal homogenates that were considered as negative for IBDV antigen. These results suggested that preparation of hyper-immune serum was appropriate and properly functioned to identify the IBDV antigen in ODIT test, while hyper-immune serum is not invariably available and commercially highly expensive. Additionally, hemorrhagic bursa was more effective compared to edematous bursa whereas no virus was detected in atrophied bursa. Previous report suggested that for viral replication, bursa of Fabricious is the vital target organ. In acute case, bursa of Fabricious is haemorrhagic, oedematous, turgid and within 7 to 10 days turns in atrophic (Dey et al., 2019). These differentiations in clinical signs depend on the subsistence of maternal immunity, virulence of causative agent and bird's age (El-Samadony et al., 2019; Rauw et al., 2007; Hassan, 2004). In the bursa of Fabricious, the stage of B cell differentiation keeps a vital role for viral replication as the stem cell (Dey et al., 2019). Our study

Table 1. Rate of identification of IBDV from the three types of bursal samples of broiler and layer chickens.

Sample type	Broiler identification		Sample type	Layer Identification		Comparative sensitivity of identification of IBDV in broiler and layer chickens	
	ODIT (%)	RT-PCR (%)		ODIT (%)	RT-PCR (%)	ODIT	RT-PCR
H.B.S. (40)	34 (85)	40 (100)	H.B.S. (50)	44 (88)	50 (100)		
E.B.S. (34)	17 (50)	32 (94)	E.B.S. (44)	25 (57)	42 (95)	120 (67)	164 (91)
A.B.S. (6)	0 (0)	0 (0)	A.B.S. (6)	0 (0)	0 (0)		

H.B.S. = Haemorrhagic bursal suspension; E.B.S. = Oedematous bursal suspension; A.B.S. = atrophied bursal suspension.
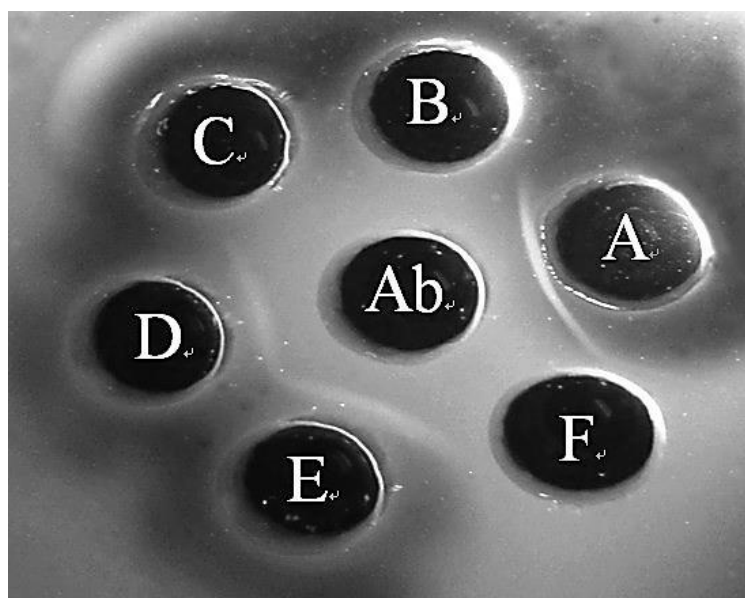

Figure 1. ODIT indicating the occurrence of IBD virus in bursal samples using hyperimmune serum. Ab = Hyperimmune serum against IBDV, A = haemorrhagic bursal suspension, B = atrophied bursal suspension 1, C = atrophied bursal suspension 2, D = oedematous bursal suspension 1, E = oedematous bursal suspension 2, F = mock solution.

observes that, there might be the possible difference among the bursal samples for pathogenesis of IBD were irreversible bursal follicle damage and IgM-bearing B lymphocytes and others cell damage (Rodriguez-Chavez et al., 2002), those were more severe in haemorrhagic bursa compared to oedematous and atrophied bursa which increased the sensitivity in haemorrhagic bursa compared to oedematous and atrophied bursa for IBDV.

RT-PCR is used to detect viral RNA in homogenates of infected organs or embryos without considering the viability of the virus present (Hasan et al., 2010). RT-PCR works on hypervariable region (VP2). The VP2 contains a hypervariable region which displays the greatest amount of amino acid sequence variation between strains. This area is responsible for antigenic variation, tissue-culture adaptation and it is slightly responsible for viral virulence (Escaffre et al., 2013). Finally, in terms of the three types of bursal samples for virus detection through RT-PCR, this study highlighted that out of 180 field samples, 164 samples (H.B.S. 40; E.B.S. 32 from broiler and H.B.S. 50; E.B.S. 42 from layer) were positive for IBDV whereas no virus was detected in atrophied bursa (Table 1 and Figure 2). Moreover, we observed that the association between ODIT and RT-PCR in IBDV affected broiler and layer chickens bursal samples was considered statistically significant (Tables 2 to 4). Amplification of VP2 gene (Zahoor et al., 2010) by RT-PCR is obvious by the presence of 253 bp band (Figure 2). The size and location of the bands for each type of samples were identical. These results were in good coordination with the findings of Zohair et al. (2017), Mawgod et al. (2014), Jackwood and Stoute (2012), Kusk et al. (2005). Generally, RT-PCR plays a vital role in the identification of viral antigens through enzymatic amplification of DNA
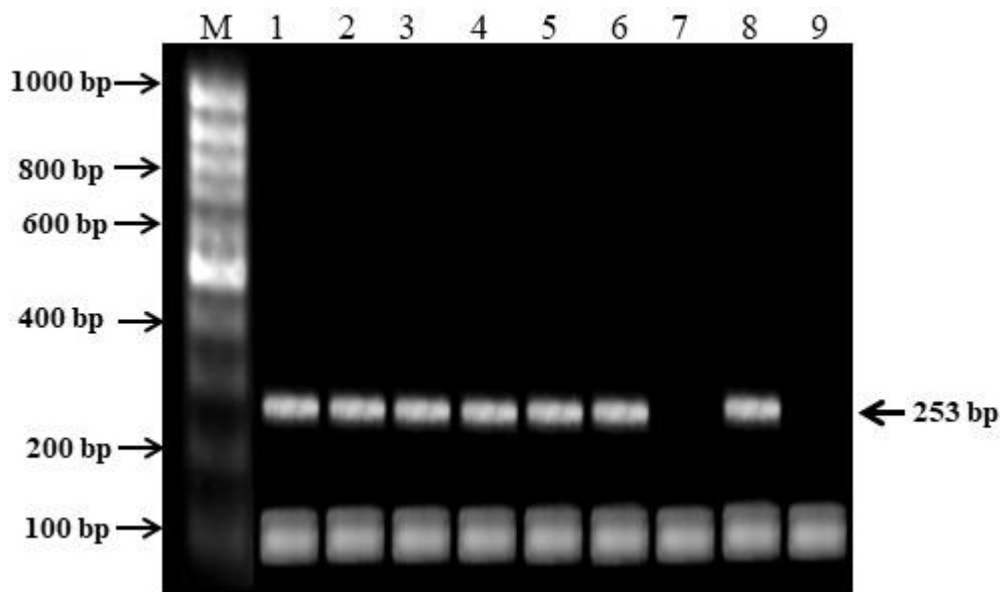
Figure 2. Results of RT-PCR products (253 bp) of IBD virus from bursal samples of chickens analyzed using 2% agarose gel electrophoresis. M = DNA Marker (100 bp), Lane-1 = haemorrhagic bursal suspension 1, Lane-2 = haemorrhagic bursal suspension 2, Lane 3 = haemorrhagic bursal suspension 3, Lane-4 = oedematous bursal suspension 1, Lane-5 = oedematous bursal suspension 2, Lane-6 =oedematous bursal suspension 3, Lane-7 = atrophied bursal suspension, Lane-8 = positive control, and Lane-9 = negative control.

Table 2. Correlation between ODIT and RT-PCR in IBDV affected broiler and layer chickens bursal samples.

Sample types (B + L)	ODIT		RT-PCR		Chi-squared ($\chi 2$) value between ODIT and RT-PCR	Degree of freedom (DF)	P-value
	Pos. (B + L)	Neg. (B + L)	Pos. (B + L)	Neg. (B + L)			
H.B.S. (90)	78	12	90	0	10.8035	1	0.0005***
E.B.S. (78)	42	36	74	4	32.3094	1	0.0001***
A.B.S. (12)	0	12	0	12	-	-	-

From each type of bursal samples and for individual diagnostic test, the number of positive samples of broiler and layer chickens were combined together and similarly all number of negative samples of broiler and layer chickens were added together and analyzed using Chi-squared (χ^2) test. ***P < 0.001 indicated the association was considered statistically significant. B = Broiler; L = layer; H.B.S. = haemorrhagic bursal suspension; E.B.S. = oedematous bursal suspension; A.B.S. = atrophied bursal suspension; Pos. = positive; Neg. = negative.

Table 3. Correlation between ODIT and RT-PCR in IBDV affected broiler chickens bursal samples.

Sample type	ODIT		RT-PCR		Chi-squared (χ^2) value between ODIT and RT-PCR	Degree of freedom (DF)	P-value
	Pos.	Neg.	Pos.	Neg.			
H.B.S. (40)	34	6	40	0	4.5045	1	0.0169*
E.B.S. (34)	17	17	32	2	14.3157	1	0.0001***
A.B.S. (6)	0	6	0	6	-	-	-

*P < 0.05 or ***P < 0.001 indicated the association were considered statistically significant. H.B.S. = Haemorrhagic bursal suspension; E.B.S. = oedematous bursal suspension; A.B.S. = atrophied bursal suspension; Pos. = Positive; Neg. = Negative.

code (Kralik and Ricchi, 2017). It is due to identification of important veterinary and clinical viruses through serological approaches are time-consuming or impossible, whereas ODIT is not universally available and sustain from comparatively low specificity and sensitivity. However, this study demonstrated that RT-PCR was more sensitive than ODIT (Mahmood and Siddique, 2006). Previous study reported that RT-PCR give   more

Table 4. Correlation between ODIT and RT-PCR in IBDV affected layer chickens bursal samples.

Sample type	ODIT		RT-PCR		Chi-squared (χ^2) value between ODIT and RT-PCR	Degree of freedom (DF)	P-value
	Pos.	Neg.	Pos.	Neg.			
H.B.S. (50)	44	6	50	0	4.4326	1	0.0176*
E.B.S. (44)	25	19	42	2	16.0113	1	0.0001***
A.B.S. (6)	0	6	0	6	-	-	-

*P < 0.05 or ***P < 0.001 indicated the association were considered statistically significant. H.B.S. = Haemorrhagic bursal suspension; E.B.S. = oedematous bursal suspension; A.B.S. = atrophied bursal suspension; Pos. = Positive; Neg. = Negative.

specific and sensitive data than serological methods for amplification of VP2 gene of IBD virus (Van den Berg et al., 2000). RT-PCR again confirmed that, haemorrhagic bursal sample reveals highest effectiveness than the oedematous bursal samples for the detection of IBD antigen. Accordingly, these results suggested that out of the two available methods for IBDV detection, RT-PCR was more efficient than ODIT and the possible reasons might be RT-PCR is highly sensitive diagnostic technique (Vogel et al., 2012) that generates rapid and precise results with amplification of a specific component of DNA (Garibyan and Avashia, 2013).

Conclusions

Confirmatory diagnosis of IBD in both clinical and subclinical cases is very important for the control and prevention of infection in the poultry farms with confirmation of effective strategy as there is a substantial economic loss to the farmers due to IBD. This study will be very helpful for the subsistent poultry farmers in this regard. The study recommended hemorrhagic bursal samples remained highly sensitive for detection of IBD viral antigen compared to that of edematous bursal samples whereas no virus was detected by atrophied bursa. This study suggested that between the two different methods (ODIT and RT-PCR), RT-PCR was highly efficient compared to that of ODIT and hemorrhagic bursal samples were more suitable compared to edematous bursal samples for the detection of IBDV antigen.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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