Diagnosis and Molecular Identification of Virulent Infectious Bursal Disease in Naturally Infected Broiler Chickens.

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ABSTRACT

Infectious bursal disease (IBD) is a serious problem in young chickens and losses occurs in face of restricted biosecurity, frequently vaccination of breeder to maintain uniform high maternal antibody titers and trials to apply more effective vaccines in broiler. The disease was diagnosed based on clinical signs, pathology, and AGPT as well as virus isolation and detection of viral antigen in tissue by RT-PCR. Therefore, bursae were collected from 5 IBD suspected flocks aged 4 to 5 weeks having clinical signs, mortality (35-70%) and lesions suggestive to infected with virulent IBDV. AGP test results showed positive precipitation lines in 5/5tested flocks (100%) between the prepared bursal homogenate or CAM of inoculated ECE with high embryo mortality of virulent IBDV. The 5 bursal homogenate extract were subjected to RT/PCR products of VP2 full gene and electrophoretic pattern of amplified samples revealed the presence of specific PCR products (642 bp). BY restriction end nuclease enzymes BstNI and SspI the samples are negative at 210 bp, 171 bp, 151 bp and 110 bp bands suggested either to be very veriolant IBDV (vvIBDV), while Sspi restriction enzyme analyzed on 2 % agarose gel electrophoresis showed that Results clarify the isolates to be very virulent subtype. The recorded histopathological changes in the examined bursal sections were similar to those previously reported due to infection with veriolant and vvIBDV.

Conclusions:We can concluded that virulent IBDV still circulating and cause losses in vaccinated broiler and RT-PCR is useful, rapid and accurate in diagnosis IBDV outbreaks.. It is important to look for a more suitable vaccine *and vaccination programs for both breeder and broiler chicken to face and controlling very virulent IBDV mutation.*

Key Words: Broiler chicken, IBDV, isolation and identification AGP-test, RT-PCR , histopathology

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INTRODUCTION

The name of Infectious bursal disease (IBD) was designated by Hitchner [1] for an acute contagious disease of young chicks reported for the first time 1957 in the Gumboro area of Southern Delmarva, USA by Cosgrove [2]. The disease was characterized by ruffled feather, watery diarrhea, trembling and severe prostration. Morbidity was 10-25 % and mortality averaging 5% where the characteristic enlargement of

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infected bursa was described. IBDV belongs to the Avibirnavirus genus. A major antigenic difference between IBDV strains of serotype-1 isolated in different poultry producing areas in USA [3,4,5] as well as major antigenic shift of serotype-I IBDV strains [6,7] . Mortalities of up to 60 % due to acute IBD outbreaks of highly pathogenic virus in broilers [8,9,10] **,** while El-Batrawi [11] indicated that the majority of the highly virulent pathotype, producing acute disease with severe clinical picture and high mortalities up to 70%. The vvIBDV strain was differed from standard strain (CU-1) in the viral protein 2. It was reported that AGP test is useful for detection of IBDV antigen in bursa [12, 13]. RT/PCR-RE assay is a rapid method for differentiation and identification for detection of IBD viral RNA in bursa of infected chicken with unknown field isolates [14, 15]**.**

RT-PCR was used to identify the point mutation for amplification of 743 bp fragment of VP2 gene followed by RE analysis using BSTN1, Sty1 and MboI that cut at bp 759, 852 and 1063 respectively. BstNI enzyme cut at 759 bp, StyI enzyme cut at 852 bp and MboI enzyme that cut at 1063 bp [16]. IBDV isolates can be differentiated into several groups using restriction enzymes (SSPI, BstNI, MboI, StyI) as most of the classic strains were BstNI and StyI double positive while most of the variant strains were BstNI and StyI double negative [14,17,18] . The reverse transcriptase / polymerase chain reaction using restriction enzyme analysis assay (RT-PCR-RE) could be used to diagnose IBDV in chickens [19]**.**

The pathological changes induced by a highly virulent IBDV causing sever clinical signs and high mortality was recorded [20, 21,22].

This study was carried out for diagnosis of IBDV infection in natural affected 3-4weeks old broiler chickens flock with sudden high mortality based on clinical as well as isolation and molecular detection of virus with histopathological examination of collected bursae.

MATERIAL AND METHODS

Field history:

Fife flocks with stock number of 6,000- 40,000 broiler chickens from different broiler breeds derived from vaccinated breeder flocks. these flocks were vaccinated with intermediate IBD vaccine at 8-12 days of age via drinking water and feed on commercial pelted ration. Chicken Flocks were aged 4 to 5 weeks showing clinical signs, high mortalities (35- 72% within 3-4 days) and lesions suggestive to naturally infected with IBDV [23].

Samples:

Ten Bursae per flock were aseptically collected from sacrificed infected diseased chickens with recording of signs and postmortem lesions. Each bursa was divided into 2 pieces one half was homogenized and the homogenates were subjected to agar gel precipitation (AGP) test, viral isolation , passage in ECE and RT-PCR, while the 2nd half was fixed in 10% neutral formol saline for histopathological examination for diagnosis of this outbreak.

Embryonated chicken eggs (ECE):

Specific pathogen free (SPF) ECE were obtained from Koum Osheim, El-Fayoum and used for virus isolation from suspected field bursal sample , virus passage and preparation of positive antigen through inoculation on the chorioallantoic membrane (CAM) at 10-11 days of age [24,25].

Agar gel precipitation test (AGPT):

It was carried out according to Wood et al**.** [26] the test was done to demonstrate the presence of IBDV antigen against positive immune serum and positive antigen prepared from CAM homogenate of inoculated ECE with E228 vaccine [24]. Also naturally infected bursa was prepared to be antigen [24, 27].

Preparation of bursal samples for virus isolation:

The half of collected field bursae were prepared for ECE inoculation according to Hitchner [1,28].

Conventional RT-PCR:

RT-PCR was carried out according to Cardoso et al. [29]**.** RNA extraction from bursa and CAM of 2nd passage in ECE was extracted according to Cardoso et al. [30]. Primers synthesized by Metabion Company, Germany as described according to the published sequence of CU-1 and STC strains by **Bayliss et al.** [31] in the following tables:

Primer	Primer Design	Position
VP2 upstream	5` GCGATGACAAACCTGCAAGAT 3`	93-114 bp of CU-1 strain
VP2 downstream	5` AGGTGGGAACATGTGGAGAC 3`	1470-1490 bp of CU-1 strain
HVR upstream	5` TCACCGTCCTCAGCTTAC 3`	587-604 bp of STC strain
HVR downstream	5` TCAGGATTTGGGATCAGC 3`	1212-1229 bp of STC strain

Histopathological examination:

The fixed bursae halves with the 10% neutral formol saline, dehydrated in grade alcohol, cleared with xyline and embedded in paraffin. Sections of an average thickness of 5 micros were stained with hematoxyline and eosin according to Sharma et al. [32] and Bancroft et al. [33].

RESULTS AND DISCUSSION

IBD is a serious problem in young chickens and losses occurs in face of restricted biosecurity, frequently vaccination of breeder to maintain uniform high maternal antibody titers and trials to apply more effective vaccines in broiler [23]. The disease was diagnosed based on clinical signs, pathology, and AGPT. The field cases showed the following signs, lower feed intake, ruffled feather, watery diarrhea and sudden increased morbidity (range 35- 72% within 3-4 days) out of flock number 6,000-40,000 chickens from different broiler breeds derived from vaccinated breeder flocks. Similar signs and mortifies were reported [9,10,11,20,22,34,35]. Appearance of very virulent IBDV 1987 which cause mortalities up to 100 % in vaccinated birds had been reported to increase the economic importance of such disease [8, 11, 36, 37]. This result can be supported by virulent strains of IBDV of same serotype have been reported to overcome high MDAs in commercial flocks vaccinated with vaccines developed from different variants, causing up to 60% to 70% mortality [38].

Died and sacrificed chickens showed post mortem lesions including patches of hemorrhage in brest and thigh muscles (Figure 1 a), hemorrhagic atrophied bursa (Figure 1 b), enlarged edematous bursa with hemorrhages (Figure 1c), full hemorrhagic bursa (Figure 1d), nephritis, petichial hemorrhages in proventriculus mucosa [20,23]

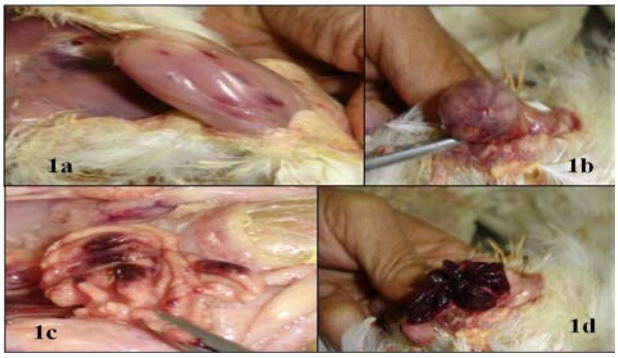

Figure 1. Lesion in naturally infected broiler chickens.

1 a: Patches of hemorrhage in brest and thigh muscles. 1 b: hemorrhagic atrophied bursa. 1c: enlarged edematous bursa with hemorrhages.
1d: full hemorrhagic bursa.

AGP test results showed positive precipitation lines in 5/5tested flocks (100%) between the prepared bursal homogenate or CAM of inoculated eggs used for viral passage and the positive serum [24, 26, 27].

Table 1. Results of AGP positive bursa (n=10) and ECE in passages of isolate IBDV(n= 5).

Sample No	Bursal samples		Passage 1		Passage 2	
	Positive	%	Positive ECE	%	Positive ECE	%
1	4/10	40	3/5	60	5/5	100
2	8/10	80	4/5	80	5/5	100
3	5/10	50	3/5	60	4/5	80
4	5/10	50	2/5	40	3/5	60
5	7/10	70	3/5	60	5/5	100

Infected embryos with AGP positive homogenates in 5 flocks showed increased both embryo lesions and mortalities with passage as hemorrhages on head and toes with sever congested liver, edematous body, greenish liver, homogenates on skin feather tract, dwarfing embryo (Table 1) [39,40]. Similar embryo mortality was reported due to veriolent IBDV [41].

The bursal homogenate and CAM of 2nd passage in ECE extract were subjected to RT/PCR products of VP2 full gene was used using internal specific primers covering the hyper variable region within the VP2 gene for confirmation of the VP2 full gene RT/PCR product and for amplification of the hyper variable region for genotyping of the IBDV local isolates. The electrophoretic pattern of amplified samples revealed the presence of specific PCR products (642 bp) at the correct expected size of the VP2 gene [29,35,42].

RFLP was carried out using 2 different restriction of endonuclease enzymes BstNI and SspI for genotyping of the IBDV isolated strains. 2 µg DNA of each sample was digested with BstNI restriction enzyme. The resulted fragments were analyzed by 2% agarose gel electrophoresis. The samples are negative at 210 bp, 171 bp, 151 bp and 110 bp bands suggested either to be vvIBDV [16, 36].

Samples were purified from low melting agarose gel and digested with Sspi restriction enzyme analyzed on 2 % agarose gel electrophoresis. Results clarify the isolates to be very virulent subtype as the enzyme digested gave 2 bands at 390 bp and 253 bp indicating very virulent subtypes [14, 17, 18, 22].

The recorded histopathological changes in the examined bursal sections of sacrificed field cases showed vacullation of the lymphoid follicles with mononuclear leukocytes infiltration (Figure 2a). Atrophy of bursal lymphoid follicles withinterfollicular edema (Figure 2 b), necrosis of medullar lymphocytes interfollicular with congestion (Figure 2 c), complete follicular necrosis with cyst like space containing eosinophilic with homogenous mass and cell debris (Figure 2 d) . The recorded bursal cells changes were similar to those previously reported due to infection with veriolant and very veriolant IBDV [22,32,43,44,45].

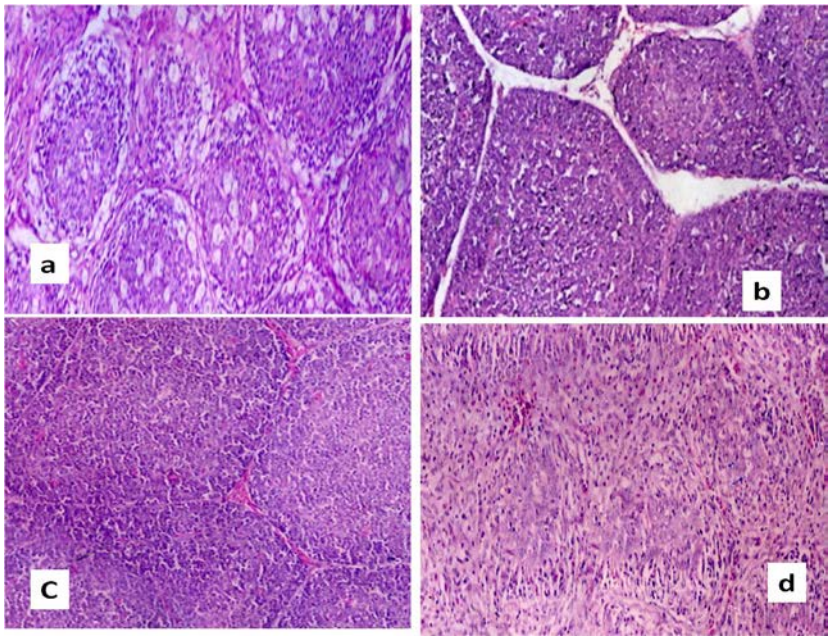

Figure 2. Bursal stained sections of naturally infected chicken with IBDV. (H&E X 100).
2a). Vacullation of the lymphoid follicles with mononuclear leukocytes infiltration.
2b). Atrophy of bursal lymphoid follicles with interfollicular edema.
2c).Necrosis of medullar lymphocytes interfollicular with congestion.
2d). Complete follicular necrosis with cyst like space containing eosinophilic.

Clinical signs, gross lesions, histopathological findings, ECE inoculation and results of AGP test were characteristic of virulent IBDV. This result was also confirmed by results of RT-PCR.

CONCLUSION

It can be concluded that virulent IBDV still circulating and cause losses in vaccinated broiler and RT-PCR is useful, rapid and accurate in diagnosis IBDV outbreaks.. it is important to look for a more suitable vaccine and vaccination programs for both breeder and broiler chicken to face and controlling very virulent IBDV mutation.

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