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molecular diagnosis of new reassortant infctious bursal disease virus in broiler chicken

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Abstract

Infectious bursal disease, causing immunosuppression in chickens, poses significant financial challenges to the poultry sector. The onset of clinical symptoms and viral shedding is influenced by factors such as age, immunological condition, route of infection, and virus type. In this study, one hundred suspected infectious bursal disease (IBD) cases from nineteen chicken farms were examined, resulted in an overall positive rate of approximately 80%. Clinical signs in affected birds included ruffled feathers, decreased appetite, diarrhea, and a dirty vent, with hemorrhage seen on thigh muscles.

Identification was done based on molecular methods, polymerase chain reaction (PCR), and gross pathological observations. Ten samples were randomly selected and tested using specific IBD primers. All those samples were positive, despite the absence of a known cure for IBD, preventive measures, including immunization and rigorous biosecurity protocols, can help mitigate its impact. The study underscores the importance of molecular techniques by using special Primer Design for diagnosing reassortant very virulent IBDv sttain in natural infected broiler in Iraq(data don't mention). offering insights into its prevalence and the effectiveness of preventive measures.

Keywords: Infectious bursal disease, polymerase chain reaction (PCR), gross pathological lesions

I. INTRODUCTION

Infectious bursal disease (IBD) is as an acute and highly contagious disease of primarily of young chickens causing mortality and immunosuppression and it's caused by infectious bursal disease virus (IBDV) (1). IBD is commonly known as Gumboro disease, the causative agent of this disease has emerged as a significant threat for the global poultry industry and affecting chickens worldwide (2).IBD was first recognized in broiler flocks near Gumboro, Delaware USA (3).IBDV has two separate serotypes with different pathogenicity: serotype 1, which is harmful to chickens, and serotype 2, which is a nonpathogenic strain (4). Serotype 1 can be further categorized into classical, variant, highly virulent, and attenuated strains(5, 6). The virus's genome consists of two segments of double-stranded RNA, with Segment A being roughly 3,200 base pairs long and including two open reading frames (ORFs). The smaller open reading frame (ORF) codes for the non-structural protein VP5(7), whereas the larger ORF codes for a polyprotein (pVP2-VP4-VP3). VP4 enzymatically splits this polyprotein into the precursor pVP2 and VP3(8). VP2, generated through further processing of pVP2, interacts with VP3 as a scaffold protein to assemble the capsid of IBDV (9). VP2 acts as the primary host protective antigen, responsible for stimulating neutralizing antibody production (10). A hypervariable region (HVR, aa 206–350) of VP2, showing frequent amino acid mutations, determines the virulence, antigenic variation, and cell tropism of IBDV (11, 12, 13, 14). Consequently, for phylogenetic analysis VP2 is the most used viral protein.

The RNA-dependent RNA polymerase VP1, which is crucial for transcribing and translating the IBDV genome, is encoded by Segment B, which is approximately 2,800 base pairs long(15). Segment B and its encoded VP1 play a crucial role in the virulence and development of IBDV(16). A phylogenetic marker for VP1, known as the B-marker (amino acids 110–252), has been discovered (17). VP1 nucleotide sequence phylogenetic analysis separates two major genetic groups: the very virulent strains

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are in one group, while IBDVs from subclinical to classical virulent pathotypes (referred to as the "non-vv group") are in the other (18). In addition to mutations, genome reassortment plays a role in the birnavirus's evolution; recombination has only occasionally been documented. When two different IBDV strains co-infect cells, there is a greater chance of reassortment due to the segmented form of the IBDV genome. It has previously been shown that intraserotypic reassortants are produced by reassortment between serotype 1 strains (19.20). When compared to their most virulent parental strains, recombinant viruses frequently exhibit reduced pathogenicity (19,21). There has also been new evidence of occasional reassortment increasing virulence (22), and chrono-phylogenetic analyses indicate that reassortment events may have contributed to the formation of vvIBDV by allowing vvIBDV segment A and vvIBDV segment B to be subsequently incorporated into IBDV strains that were circulated in chicken flocks (23). Data showing that both segments contribute to vvIBDV pathogenicity are consistent with the idea that reassortment may alter the pathogenesis of the reassorted IBDV strains (24). Interestingly, reassortment between serotype 1 and 2 viruses (interserotypic reassortment) was shown to be feasible using reverse genetics in laboratory settings, with segment A of the serotype 1 parent strongly defining the tropism for bursal lymphocyte in the resulting recombinant viruses (25) In recent years, naturally occurring reassortant IBDV strains have been reported frequently from different parts of the world, which included reassortment between serotype 1 and serotype 2 (26, 27) and reassortment between different pathotypes/genotypes of serotype 1 IBDVs (28, 29). revealed IBDV might make the hens more vulnerable to septicemic infections caused by high- and low-virulence E. coli strains (30). Besides implementing biosecurity measures, vaccination emerges as the foremost strategy to control IBDV in the field. The significance of detecting and recognizing IBDV strains is in the antigenic subtypes present within serotypes, which requires customized vaccination programmers based on the prevailing antigenic type in the bird's surroundings. Despite following sanitary conditions in chicken breeding halls and using different types of vaccines produced locally or imported, the disease appears constantly due to the repeated phenomenon of failure of vaccination programs (31). clinical symptoms on days 2, 4, and 6 following vaccinations. Serum surface area was covered in yellowish transudate, indicating the presence of edema. The bursa swelled as a result, appearing congested. The longitudinal striation on the surface also became more pronounced and congested, and little gray foci developed on the splenic surface (32). Therefore, future studies are needed to compare these histological changes with serum immunological tests(33) Conventional methods of isolating and characterizing viruses are not viable for the routine diagnosis and identification of IBDV strains (34). PCR-based molecular diagnostic methods are more efficient in diagnosing viruses and identifying strains (35).

Focusing the endemic nature of IBD in Iraq, the disease was reported in 1978 for the first time (36), and since then, IBD has extensively spread among poultry farms in Iraq. The need for advanced molecular diagnostic approaches becomes essential, under lining their significance role in understanding the prevalence and diversity of IBDV strains.

II. MATERIALS AND METHODS

Filed sample collection

A total of seventy-two samples were collected from suspected infected birds, encompassing various tissues including Bursa of Fabricius, spleen. These samples were gathered from twenty poultry farms located in different provinces, namely Baghdad, Al Kut, Al Anbar, Al Najaf, Dialya, Kirkuk T**able (1).** The collection focused on chickens exhibiting symptoms such as vent picking, depression, trembling, white watery diarrhea, ruffled feathers. The collected samples were carefully dissected under sterile conditions, and each was appropriately labeled. To preserve their integrity, the samples were promptly transported on ice to the laboratory and then stored at -20°C until further processing. Notably, a subset of these samples was preserved in 10% formalin solution for subsequent histopathological examination.

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Table 1:

No.	Number of farms	Number of birds	Number of samples	The result positive	The result negative	region
1.	4	12	12	12	-	Dialya
2.	3	20	20	12	8	Al-Anbar
3.	6	20	20	20	-	Bagdad
4.	1	3	3	3	-	Al Najaf
5.	2	8	8	8	-	Kirkuk
6.	3	9	9	6	3	Al-Kut
Total of positive				61		
Total of negative				11		

First-Strand cDNA synthesis protocol

The retro-transcription step, conversion of RNA into complementary DNA (cDNA), was performed using EasyScriptTM kit (Abm, Canada) according to the instructions of the manufacturer and the reaction mixture was shown as in Table 2.

Table 2: The composition of the reaction mixture used for the synthesis of the first strand of cDNA.

Component	**Volume**	**final concentration**
Total RNA or poly(A) + mRNA	Variable	1.0 ng – 2.0 µg/rxn 1.0 pg – 2.0 ng/rxn
Oligo (dT) (10 µM) or Random Primers (10 µM) or Gene-Specific Primer	1.0 µL 1.0 µL Variable	
dNTPs (10 mM each	1.0 µL	500 µM
5X RT Buffer	4.0 µL	1X
RNasin (40 U/µl)	0.5 µL	20 U/rxn
EasyScriptTM	1.0 µL	200U/rxn
Nuclease-free H_2O	Up to 20 µL	-------

Primer Design

Three primer sets were developed for the amplification of VP2 segment A, and segment B (37). All designed primers were synthesized by DNA Integrated Biotechnology (IDT Co., Canada). The conditions for segment A&B are shown in table 3&4 respectively.

Extraction of total RNA from Tissue

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Using the AccuZolTM kit from BIONEER, total RNA was extracted from tissue samples of Bursa of Fabricius. Each tissue sample was treated with AccuZol, which sped up the process of lysing cells. Chloroform was then added, and the mixture was shaken ferociously before centrifuging. After that, the resultant RNA was dried, centrifuged, and precipitated with 80% ethanol. Subsequently, the RNA pellet was dissolved in either 0.5% SDS solution or RNase-free water, and it was incubated for ten minutes at 55–60°C. For long-term stability, the extracted RNA samples were kept in storage at -80oC.

Nano-drop estimations for DNA and RNA samples

total RNA from all samples under study were analyzed using Nano-drop spectrophotometers. RNA were measured at 260 nm. The concentrations of RNA samples were expressed in terms of ng/µL

Agarose gel electrophoresis

Agarose gel electrophoresis involves adding 1.0g agarose to 100mL of TAE buffer, heating it to melt, and adding ethidium bromide at a final concentration of 0.5 µg/mL. Subsequently, the liquefied agarose is transferred into a gel electrophoresis apparatus designed for submarine applications, where it solidifies and is subsequently submerged in a tank specifically designed for submarine electrophoresis. The gel is fully immersed by adding TAE buffer. Before gel loading, RNA samples are combined with 1X gel loading dye. The electrophoresis procedure is performed at 5-8 volts per centimeter for 45 minutes. Using a UV-Transilluminator, the agarose gel is observed under ultraviolet (UV) light

Table 3: Primer design and PCR condition for segment A

Primer sequence	PCR recipe [a]	PCR conditions [b]	PCR product (bp)
VP Fw- CTTCCAAGGGAGCCTGAGTG Rv- ACCACCGGTACAGCTATCCT	cDNA : 3.0 µL Fw-primer: 2.0 µL Rv-primer: 2.0 µL 2XPCR master mix: 12.5 µL NFW[c]: 5.5 µL Tot volume: 25 µL	1 cycle: Initial denaturation: 95 °C, 5 min 30 cycles: each cycle Denaturation: 94 °C, 1 min Annealing : 60,55,58°C, 1 min Extension : 72 °C, 25 seconds 1cycle: Final extension: 72 °C, 10 min	993bp
Fw- GAGCCTAGCAGTGACGATCC Rv- GCTGTTCAGTGCTTTGGGTG			739bp
Fw- CACCCAAAGCACTGAACAGC Rv- AGCTACCCATTCCGGTGTTG			917 bp

Table 4: primer design and PCR condition for fragment B

Primer sequence	PCR recipe [a]	PCR conditions [b]	PCR product (bp)
VP Fw- AAGCAAGATCTCAGCAGCGT Rv- AAGGCTTGTCATCCTCACCG	cDNA : 3.0 µL Fw-primer: 2.0 µL Rv-primer: 2.0 µL 2XPCR master mix: 12.5 µL NFW[c]: 5.5 µL Tot volume: 25 µL	1 cycle: Initial denaturation: 95 °C, 5 min 30 cycles: each cycle Denaturation: 94 °C, 1 min Annealing : 59,55,52,59,60°C, 1 min Extension : 72 °C, 25 seconds 1cycle: Final extension: 72 °C, 10 min	632 bp
Fw- TTGTGGCCATGAAGGAGGTC Rv- ATTGTCTCTCCCTTGGTGCG			286bp
Fw- CGCACCAAGGGAGAGACAAT Rv- ATGTAGCTGACCACCCAAGC			937bp
Fw- GTACCTGAGTGGGGGTGTTG Rw- CCACTCAGTCCGGCTTCATT			546 bp
Fw- AATGAAGCCGGACTGAGTGG Rw- CCATTGGTCTGCTCGTTCCT			382 bp

The clinical signs and gross lesion

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The chicken exhibited symptoms of sadness, such as white watery diarrhea, ruffled feathers, vent plucking, anorexia, dehydration, and increased water consumption. Additionally, visible hemorrhages were found on the thigh muscle. **(figure1)**. Bursa of Fabricius which showed doubling in size with a yellowish gelatinous material that may surround it and sometimes hemorrhages may be seen on the surface of it **(figure 1).**

Figure 1: A - Chicken infected with IBD shows feather ruffling. B - Chicken infected with IBD exhibits enlargement and hemorrhage of the Bursa Fabricius. C - Hemorrhages are evident in the thigh muscle, D- Enlarged, edematous and hemorrhagic bursa.

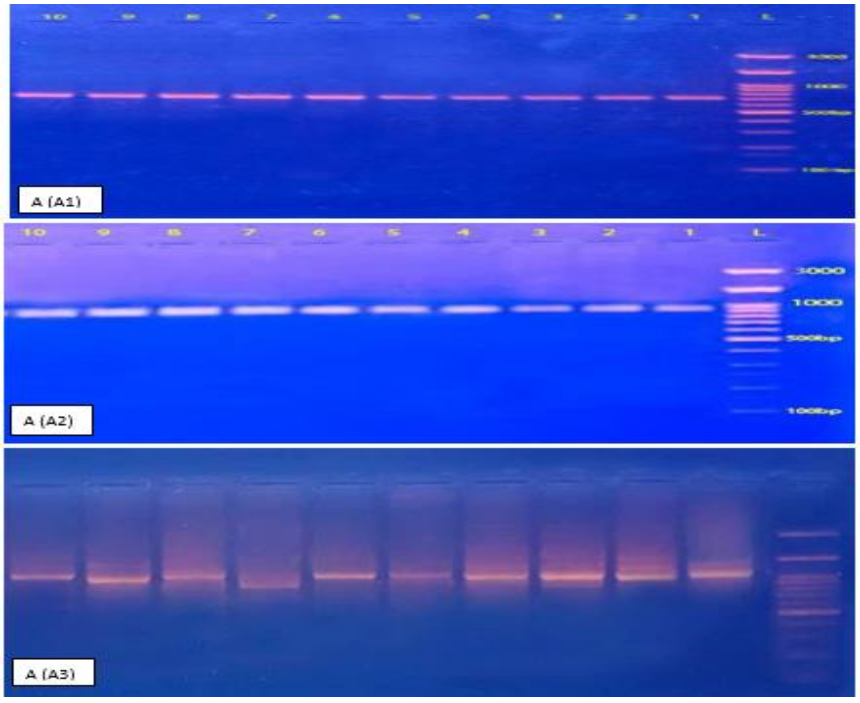

Figure 2: Molecular detection of infectious bursal disease virus (segment A) Amplification results stained with ethidium bromide. Agar gel electrophoresis of PCR products. M= 100bp DNA ladder, Lanes 1 to 10 positive sample with amplicon size of 739bp of segment (A) (A1), Lanes 1 to 10 positive sample with amplicon size of 917bp of segment (A) (A2), Lanes 1 to 10 positive sample with amplicon size of 993b of segment (A) (A3).

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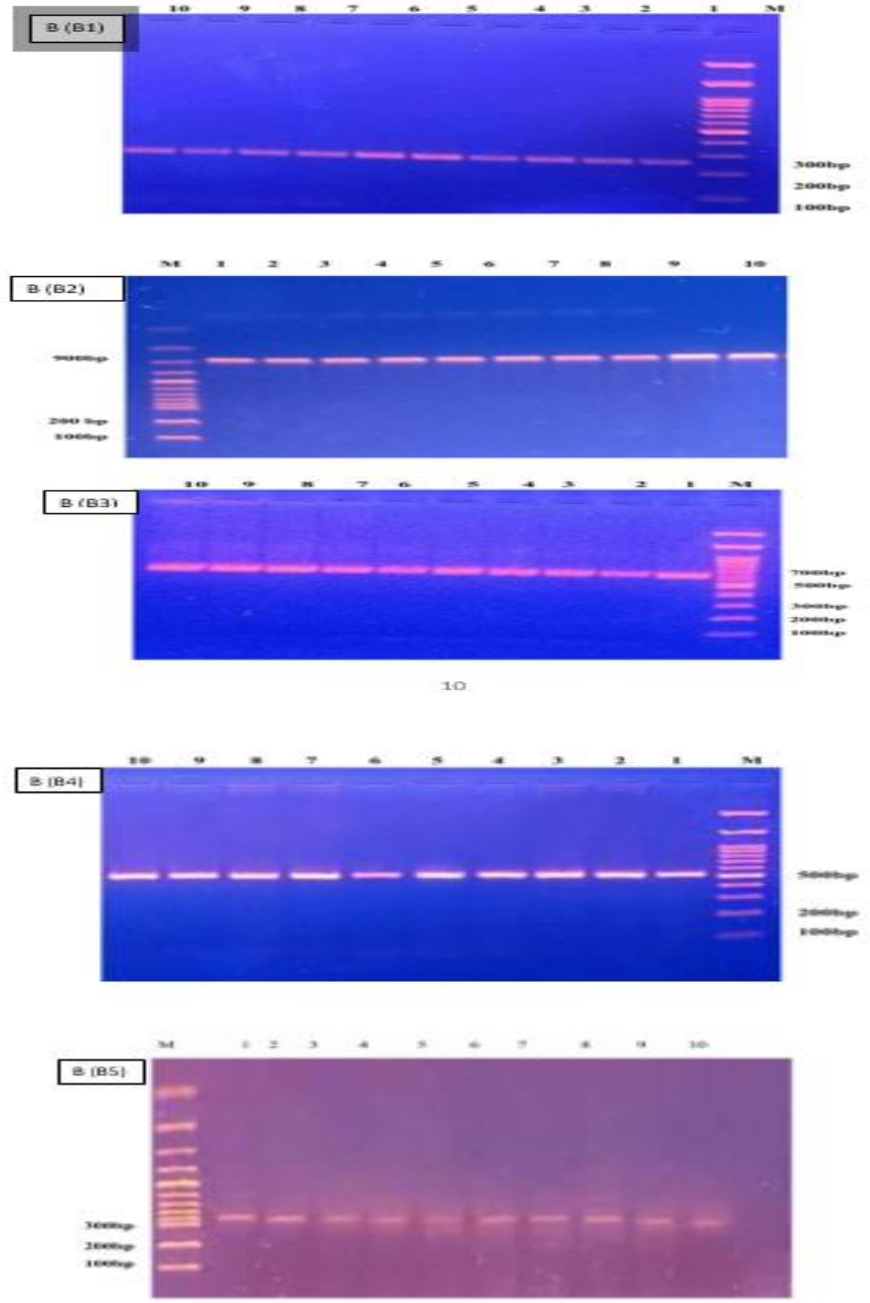

Figure 3: Molecular detection of infectious bursal disease virus (segment B)

Amplification results stained with ethidium bromide. Agar gel electrophoresis of PCR products. M= 100bp DNA ladder, Lanes 1 t0 10 positive sample with amplicon size of 286bp of segment (B) (B1), Lanes 1 t0 10 positive sample with amplicon size of 973bp of segment (B) (B2), Lanes 1 t0 10 positive sample with amplicon size of 632bp of segment (B) (B3), Lanes 1 t0 10 positive sample with amplicon size of 564bp of segment (B) (B4), Lanes 1 t0 10 positive sample with amplicon size of 382bp of segment (B) (B5).

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III. Discussion

Chickens under the age of 3 weeks typically show no clinical signs of disease, whereas those older than 3 weeks often exhibit clinical disease 38). The Infectious Bursal Disease Virus (IBDV) is characterized by a brief incubation period of 2-3 days, and the infection typically persists for 5-7 days. An early indication of IBDV infection is vent picking behavior (39). The clinical manifestations reported include a sudden onset of depression, trembling, diarrhea that is white and runny, loss of appetite, and feathers that appear ruffled 40. Severe cases may progress to dehydration, subnormal temperature, and ultimately death. The severity of IBD lesions and the distribution of the virus within tissues depend on the strain's pathogenicity (41). Post-mortem examinations revealed dehydrated carcasses, with hemorrhages evident on the thigh muscle, bursa, and spleen(39). Notably, major lesions in the Bursa of Fabricius were included edematous swelling, hemorrhagic bursa, and a gelatinous lesion covering the serosal surface (42). Molecular technique namely the PCR results of this study revealed that bursa's samples were from IBD infected broiler. This was shown in figure 2&3 were all the tested samples were positive. The result in our study consist with previous descriptions(43) .

IV. Conclusion

The clinical, gross pathology, and molecular findings collectively support the diagnosis of Infectious Bursal Disease in suspected chickens. Despite advancements in virology, molecular biology, and the availability of new vaccinations, this study showed the ongoing prevalence of IBD in numerous poultry farms. It emphasizes the need for continued a wariness and updated strategies in disease management within the poultry industry.

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Conflicting Interest

There are no conflicts of interest.

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