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RESEARCH ARTICLE

Sequence Analysis and Comparison of Infectious Bursal Disease Virus Affecting Indigenous Kurdish Breed and Broiler Chickens in Sulaymaniyah, Kurdistan Region of Iraq

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A B S T R A C T

Infectious bursal disease (IBD) is a contagious infection of chicken that causes complicated immunosuppressive disease, leading to significant economic loss. Genetic characterization of the circulating field virus plays a significant role in controlling the disease in any particular region. Therefore, this study investigated IBD cases in indigenous Kurdish breed chicken and broiler farms and to investigate the accessible vaccine strain that homologs with the field strain in Sulaymaniyah province, Kurdistan Region of Iraq. The viral protein 2 (VP2) nucleotide and amino acid sequences of two field strains were analyzed in conjunction with 74 IBD virus (IBDV) sequence data in the world from GenBank. Also, the field strains were genetically compared with 30 vaccine strains from GenBank. Phylogenetic analysis indicated that the very virulent IBDV strain that is circulating in indigenous Kurdish poultry was different from that of broiler farms. Genetic analysis indicated that the available vaccine strain, D78, that is used in the vaccination program probably does not provide adequate protection from current field strain. Therefore, this study suggests field assessment of a higher homology vaccine, MB vaccine strain in term to evaluate safety and protective immunity.

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INTRODUCTION

Infectious bursal disease (IBD), traditionally named by Gumboro disease, is an acute and highly contagious viral disease characterized by the destruction of B lymphoid cells in the Fabricius gland leading to severe immunosuppression (Banda *et al.*, 2003). Compromisation in the immune response leads to the failure of the host to defend against subsequent other infectious agents and vaccination (Müller *et al.*, 2003). The causative agent, IBDV, is non-enveloped, icosahedral in shape with double-stranded RNA, and belongs to the genus Avibirnavirus, family Birnaviridae (Ye *et al.*, 2018). The first discovery of IBD was in Gumboro in the USA in 1962 (Le *et al.*, 2019). The genome of IBDV is bi-segmented, and the smaller segment B encodes viral protein 1 (VP1), which has RNA-dependent RNA polymerase activity. The larger segment A encodes VP2, VP3, VP4, and VP5 (Wang *et al.*, 2004). The VP2 protein has vital neutralizing antigenic domains and produces a defensive immune response. Nearly most of the amino acid substitutions are in the hypervariable region (HVR) of VP2. Therefore, the HVR of VP2 is selected as a target for molecular characterization and identification of IBDV and strain evolution studies (Bayliss *et al.*, 1990). In the HVR of VP2, the most amino acid variations are in four hydrophilic loops. These are two primary sets of hydrophilic A and hydrophilic B, from amino acid residue 210 to 225 and 312 to 324, respectively, with minor hydrophilic peaks present between them (Schnitzler *et al.*, 1993). IBDV has two serotypes, but only viruses that belong to serotype I cause natural infection in chickens, while serotype II only infects turkeys and ducks (Yamazaki *et al.*, 2016). The IBDV is classically classified based on virulence and antigenicity into the variants attenuated, classical, and very virulent IBDV (vvIBDV) (Jackwood *et al.*, 2008). In 2017, a better classification was done for IBDV based on genetic diversity and phylogenetic characteristics. Accordingly, IBDV is classified into seven genogroups (Michel and Jackwood, 2017).

In Iraq, the first diagnosis of IBDV was in 1978. Currently, IBDV is endemic in Iraq and some of the neighboring countries (Amin and Jackwood, 2014;

Norouzian *et al.*, 2017; Yilmaz *et al.*, 2019). One option for controlling IBD is vaccination. However, the classical strains' vaccines did not produce protective immunity for variant strains, leading to infection and complicated immunosuppression (Besseboua *et al.*, 2015). This study was conducted to explore the IBD cases in indigenous Kurdish breed chicken and broiler farms and to investigate the current vaccine strain that homologizes with the circulatory field strain in Sulaymaniyah province, Kurdistan Region of Iraq.

MATERIALS AND METHODS

Sample collection: In 2019, two farms of 45-day-old broilers and 60-day-old indigenous Kurdish chickens in Sulaymaniyah Province showed mortality rates of 8 and 10%, respectively. The broiler farm was vaccinated by the D78 vaccine at the age of 12 days, while the local Kurdish chickens were not vaccinated. Necropsy revealed gelatinous exudates and hemorrhages in the Fabricius gland.

RNA extraction: Pool samples were taken from hemorrhagic bursae of indigenous Kurdish chickens and broiler chickens. The samples were subjected directly to total RNA extraction according to the manufacturer's instructions of GeNet Bio (Korea).

RT-PCR amplification: The extracted RNA was subjected to one-step RT-PCR to detect and amplify a 643 bp hyper-variable region of the VP2 gene of IBDV. The reaction was carried out in 0.2 ml tubes using the SuPrimeScript RT-PCR premix (GeNet Bio, Korea). The tube contained 10 µl of the master mix, 4 µl of the extracted RNA, 1 µl of the forward primer (10 pmol) TCACCGTCCTCAGCTTAC, 1 µl of the reverse primer (10 pmol) TCAGGATTTGGGATCAGC (Patel *et al.*, 2016). The volume was then completed to 20 µl by adding 4 µl of diethylpyrocarbonate (DEPC) water. The thermocycler was programmed for 50°C for 30 minutes. An initial denaturation was conducted at 95°C for 10 minutes, followed by 40 cycles of denaturation at 95°C for 30 seconds, annealing at 54°C for 30 seconds, and extension at 72°C for 40 seconds. A final extension at 72°C for 5 minutes was also included.

PCR products were analyzed by loading 7 µL on 1% agarose gel in 1× Tris//EDTA (TE) buffer. The gel was stained with 5 µL safe dye. Electrophoresis was done on 120 volts for 50 minutes using the Safe-Blue Illuminator/ Electrophoresis System. The 643 bp amplicons of PCR products were analyzed according to the migration pattern of a 100 bp DNA ladder.

Direct Sequencing: Thirty microliters of the PCR product were sequenced by Macrogen sequencing service in South Korea. The coding sequences were submitted to the GenBank database with accession numbers.

Sequence comparison and phylogenetic analysis: The VP2 sequence identity of IBDV isolates was determined by the blast method in National Center for Biotechnology Information (NCBI) Homepage. The nucleotide sequences were analyzed in conjunction with 74 IBDV sequence data from GenBank. For simplicity, one reference IBDV sequence was selected for each group to set NCBI multiple sequence alignment and identity percentage. Two phylogenic trees were constructed, one with VP2 hypervariable region of 74 IBDV isolates and the other with identified Slemani strains and 30 vaccine strains. The Phylogenic tree was based on the neighbor-joining method using the Kimura 2-parameter model in Mega 6 (Tamura *et al.*, 2013). The bootstrap values were settled from 1000 replicates of the original data (Felsenstein, 1985).

RESULTS

IBDV identification by RT-PCR: The suspected samples were positive for IBDV, based on the agarose gel electrophoresis, which demonstrated the expected 634 bp amplicon size. Sequencing of the PCR products affirmed the results, and the sequences were submitted to NCBI GenBank. The isolate Slemani/Kurdi/2019 (accession # MN782240) was isolated from local chicken, and Slemani/3/2019 (accession # MN782241) was isolated from broilers.

Phylogenetic analysis: A phylogenetic tree was built based on the VP2 hypervariable region (HVR) of IBDV nucleotide sequence alignment of the 74 genomes (Fig. 1). The isolates were distinctly distributed into seven genotypes from G1 to G7. The groups were also named according to the classical categorization of serotype and pathogenicity, as reported by Michel and Jack-wood (Michel and Jackwood, 2017). The very virulent strains of group 3 were subdivided into five subgroups (vvIBDV 1-vvIBDV 5). The topology of the tree showed that the field isolates of Slemani/Kurdi/2019 belonged to the very virulent strain vvIBDV4 that shares a common ancestor with previous isolates in Slemani province and Iraq. The Slemani/3/2019 was clustered in vvIBDV1 that contains seven isolates from Iraq, a reference HN strain, and other isolates from Kuwait, India, and Turkey.

The Phylogenic tree based on amino acid sequences of VP2 of both Slemani/Kurdi/2019 and Slemani/3/2019 with 30 vaccine strains from GenBank indicated that the Sulaymaniyah field isolates have a common ancestor with the MB vaccine, but the D78 vaccine strain was in a different cluster (Fig. 2).

Sequence analysis: Sequence analysis was carried out for 74 IBDV isolates. The analysis included 432 nucleotides, which express 144 amino acids (position: 211–355) in the VP2 hypervariable region. Alignment of the amino acid sequence revealed that both Slemani strains have the amino acid residue hallmarks of vvIBDV strains, which are A222, I242, I256, I294, and S299. Furthermore, they had Q253 and A284 epitopes (Fig. 3). There was no mutation at the minor hydrophilic peak 1, major hydrophilic peak B, minor hydrophilic peak 2, and the major hydrophilic peak B, compared to the reference strain of HN. However, the isolate Slemani/Kurdi/2019 was characterized by N112D variation in the major hydrophilic peak A as compared to the HN reference strain and Slemani/3/2019 isolate.

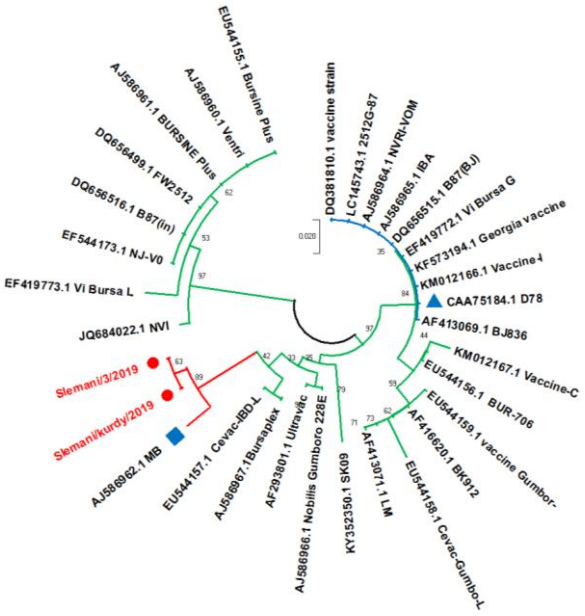

Fig. 1: Phylogenetic tree based on the VP2 hypervariable region (HVR) of IBDV nucleotide sequence alignment of 74 genomes from GenBank. Isolates from the current study are labeled by red circles, while previously isolated viruses from Slemani are labeled by red triangles.

Table 1: VP2 amino acid homology percentage of Slemani Field strain with different vaccine strains

Accession No./Vaccine strain	Kurdi/2019 (isolated from local chicken)	Slemani/3/2019 (isolated from broilers)
AJ586962.1 MB	97.931	98.621
EU544157.1 Cevac-IBD-L	95.139	95.833
AJ586967.1Bursaplex	95.105	95.804
AJ586966.1 Nobilis Gumboro 228E	94.483	95.172
AF293801.1 Ultravac	93.836	94.521
DQ656515.1 B87(BJ)	93.007	93.706
EF419772.1 Vi Bursa G	92.701	92.701
AJ586964.1 NVRI-VOM	92.414	93.103
AJ586965.1 IBA	92.414	93.103
CAA75184.1 D78	92.414	93.103
AJ586960.1 Ventri	92.414	93.103
AF413071.1 LM viral	92.361	93.056
EU544159.1 vaccine Gumbor-L	92.361	93.056
AF413069.1 BJ836	92.361	93.056
LC145743.1 vaccine 2512G-87	92.361	93.056
KF573194.1 Georgia vaccine	92.308	93.007
AJ586961.1 BURSINE Plus	91.724	92.414
AF416620.1 BK912	91.667	92.361
KY352350.1 SK09	91.667	92.361
JQ684022.1 NVI Vaccine strain	91.667	91.667
EU544155.1 Bursine Plus	91.667	92.361
EF419773.1 Vi Bursa L	91.608	90.972
DQ656499.1 FW2512	91.608	92.308
DQ656516.1 B87(in)	91.608	92.308
EU544156.1 BUR-706	91.429	92.143
EU544158.1 Cevac-Gumbo-L	90.278	92.308
DQ381810.1 vaccine strain	90.244	90.244
EF544173.1 NJ-V0	90.071	90.78
KM012166.1 Vaccine-I	89.362	90.426
KM012167.1 Vaccine-C	87.755	88.776

The nucleotide sequence identity percentage of the isolate Slemani/Kurdi/2019 with reference sequences of the groups was as follows: G1 classical (92.69%), G2-Antigenic (92.01%), G3-variant very virulent (97.03%), G4-dIBDV (88.89%), G5-variant/classical-recombinant (86.92%), G6-ITA (89.02%) and G7-Australian (86.76%). The nucleotide and amino acid identities of Slemani/Kurdi/2019 with Slemani/3/2019 were 96.12% and 99.32%, respectively.

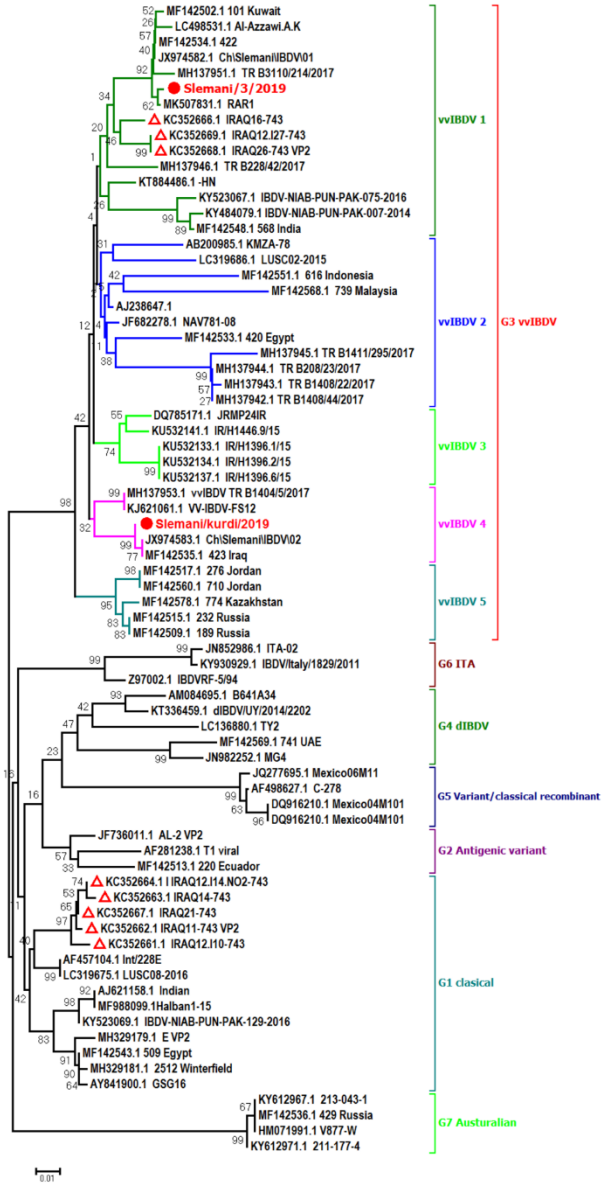

Fig. 2: Phylogenic tree based on amino acid sequences of VP2 of Slemani/Kurdi/2019 and Slemani/3/2019, with 30 vaccine strains from GenBank. Current study's isolates are labeled by red circles. The MB vaccine is indicated by the blue square and the used D78 vaccine is shown by a blue triangle.

Amino acid analysis based on the hypervariable VP2 region was carried out for both Slemani isolates with 30 vaccine strains, the result indicated that the best amino acid homology was with MB vaccine accession # AJ586962, which showed 97.93% with Slemani/Kurdi/2019 and 98.621% with Slemani/3/2019 strains. However, the most popularly used D78 vaccine strain in Slemani showed 92.414% with Slemani/Kurdi/2019 and 93. 1% with Slemani/3/2019 strain (Table 1).

DISCUSSION

The IBDV continues to be a severe problem for the Iraqi poultry industry since it was reported first in 1978 (Amin and Jackwood, 2014). However, the IBD cases in the indigenous breed were infrequent and not documented in Sulaymaniyah province before this study. There is no molecular study on IBDV strains circulating in the

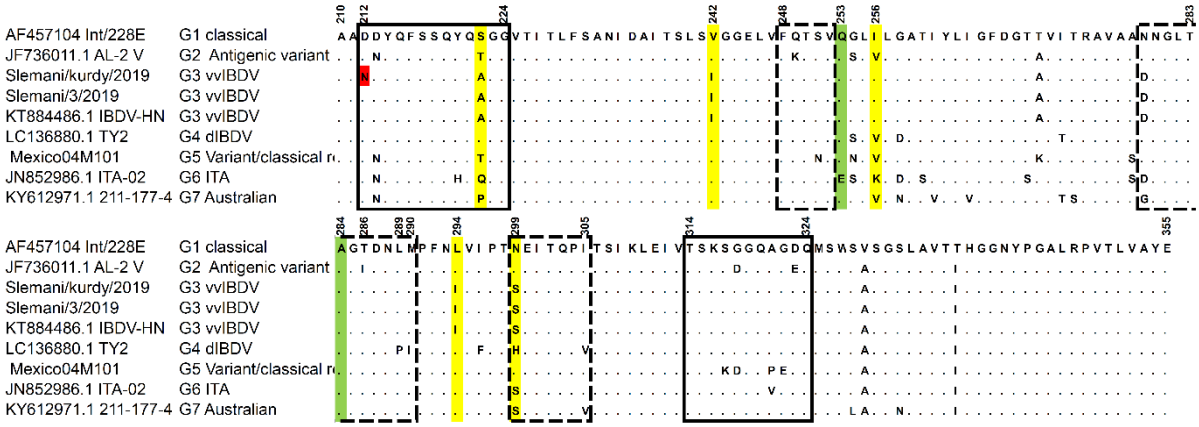

Fig. 3: Alignment of amino acids corresponding to 210–355 of the VP2 hypervariable region of Slemani field strains with the reference strains in each genogroup. Major hydrophilic domains are boxed with solid lines and minor hydrophilic domains are boxed with dashed lines. Residues exclusive to vvIBDV strains are highlighted in yellow color. Residue 212 in Slemani/Kurdi/2019 is highlighted with red. Residues responsible for the replication of IBDV in cell culture are colored with green.

indigenous breed in Iraq. Therefore, this research was undertaken to investigate the relationship of field strains circulating in the local breed and broiler chicken and compare them to the vaccine strain.

This study concentrated on the analysis of the hypervariable VP2 (206-350 amino acids) region of IBVD because it contains the key amino acids at positions of 222, 242, 253, 256, 279, 284, 294, 299 and 329 that are responsible for viral pathogenicity and antigenicity (Jackwood and Sommer-Wagner, 2007). The molecular analysis revealed that both Slemani IBVD isolates belonged to a vvIBDV because they had amino acid residue hallmarks of vvIBDV strains, which are A222, I242, I256, I294, and S299 (Van den Berg *et al.*, 2004). Furthermore, both Slemani strains also had the highest amino acid homology (97.03%) with G3-very virulent IBVD.

Amino acid substitutions Q253H and A284T were responsible for pathogenicity, and are distinctive to cell tropism and cell culture adaptation (Brandt *et al.*, 2001). It was found that none of the identified strains in this study were from the vaccine source due to the absence of H253 and T284 sight mutations, which are markers for vaccine strains (Van Loon *et al.*, 2002; Jackwood *et al.*, 2008).

Both Slemani strains were found to share only 96.12 and 99.32% identity at the nucleotide and amino acid levels, respectively. Furthermore, amino acid sequence alignment indicated that Slemani/Kurdi/2019 was characterized by N212D substitution in major hydrophilic peak A as compared to Slemani/3/2019 and other previous IBVD isolates that were reported in Slemani province in 2012. This finding suggested that there may be two different isolates that infected broilers and the local breed in Slemani province. Other Asian strains have the same sight substitution, N212D, including 616-Indonesia, 739-Malaysia, and 75-Pakistan strains (Khan *et al.*, 2019). There was no published data about the effect of N212D substitution that was present in Slemani/ Kurdi/2019 in IBVD. However, it was reported that the major hydrophilic region A (210-225) is significant for the neutralization of monoclonal antibodies. Therefore, any variation in this amino acid sequence more probably induces significant antigenic variation (Eterradossi *et al.*, 1998; Domanska *et al.*, 2004).

The phylogenetic analysis of the VP2 region led to the grouping of the IBDV isolates into different pathogenic groups, from G1 to G7 (Khan *et al.*, 2019). The G3 vvIBDV was distributed to five clusters. Slemani/ Kurdi/2019 belonged to the vvIBDV4 strain, clustering with previously identified IBDV from Slemani Province and Iraq (Fig. 1). Slemani/3/2019, on the other hand, was located within the vvIBDV1 cluster. The former cluster included most of the vvIBDV from Slemani and other provinces of the Kurdistan region, Iraq, Kuwait, and Turkey, which means the circulation of the same vvIBDV across neighboring countries. This is probably due to inadequate restrictions on the movement of poultry products from neighboring countries to the Kurdistan region (Amin and Jackwood, 2014; Yilmaz *et al.*, 2019).

The vvIBDV strains identified in the present study were found to belong to two independent clusters, which proposes the existence of multiple introduction sources of the vvIBDV.

This study also investigated the most homologous vaccine related to the field strain of Slemani province because the second most economical method after biosecurity to protect against IBD is vaccination (Müller *et al.*, 2012). Most of the poultry farms in Iraq, especially in Sulaymaniyah, were vaccinated with the classical strain D78 (Amin and Jackwood, 2014). However, significant problems in the poultry field are due to the recurrent outbreaks of Gumboro despite the massive vaccination with the available IBD vaccines. This failure in vaccination may be due to alteration in VP2 (the major protective antigen of IBDV), which may result from genetic reassortment or immunological pressure (Hon *et al.*, 2006). Therefore, to apply optimal preventive and control strategies, both field and vaccine strains must be monitored (Müller *et al.*, 2012). Genetic analysis by the alignment of hypervariable region amino acids of Slemani strains with most vaccine strains indicated that the highest vaccine strain homology was with the MB vaccine. The most commonly used D78 vaccine strain in Slemani showed a much lower homology percentage as compared to the MB vaccine (Table 1).

The bootstrap values in Fig. 2 indicated significant differences between the D78 vaccine strain and the

Slemani field virus. Moreover, MB vaccine strain appears to be more closely related to the field strain based on the topology of the phylogenic tree. This finding explains why Slemani/3/2019 crosses the antibody protection level of the D78 vaccine and the poorly efficient D78 vaccine to protect the field strain in most of Iraq. According to the finding, it is recommended to use the MB vaccine stain instead. However, some of intermediate vaccines like MB vaccine strains may have adverse effect on bursa, which might result in immunosuppression. Hence, practical administration of the vaccines to poultry farms should be performed to investigate the efficiency of protection with the present circulating virus (Müller *et al*., 2012).

Conclusions: This study disclosed that the circulating genotypes in Slemani Province, the Kurdistan Region of Iraq, were very virulent strains. Phylogenetic analyses indicated that the IBDV strain circulating in indigenous Kurdish poultry was different from that of broiler farms. Genetic analysis indicated that the available vaccine strain probably did not provide reasonable protection from the current field strain. Therefore, this study suggests vaccination with the higher homology MB vaccine and recommends *in vivo* and field assessments of the degree of protection by the new proposed vaccine.

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