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MOLECULAR CHARACTERIZATION OF INFECTIOUS BURSAL DISEASE VIRUS ISOLATED FROM BROILER AND PULLET FLOCKS IN ALGERIA

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ABSTRACT

Infectious bursal disease (IBD) or Gumboro disease is an acute, highly contagious viral disease of young chickens characterised by haemorrhagic syndrome, severe damage in the cloacal bursa, immunosuppression, and high mortality, generally at 3–6 weeks of age. The present study was performed to investigate the molecular characteristics and histopathological effects of infectious bursal disease virus (IBDV) isolated from broiler and pullet flocks in eastern and central Algeria. Fifty-five chickens collected from eleven broiler and pullet farms were investigated for IBD outbreaks over the period of 2019–2020. Only the birds with clinical signs and macroscopic lesions indicating IBD were selected for histopathological examination and molecular investigations using reverse transcription-polymerase chain reaction (RT-PCR) followed by sequencing. Except for Flock N°4, all the birds in the study showed microscopic lesions of IBD. RT-PCR confirmed IBDV infection in samples from flocks N°1, 2, 3, 9, 10, and 11. Using sequencing, a very virulent IBDV (vvIBDV) strain was detected in samples N°10 and 11. The studied strains exhibited four conserved amino acids (222A, 256I, 294I, and 299S), characteristic of vvIBDV. According to the phylogenetic tree, the two strains in the study were closely related to previously isolated vvIBDVs and clustered together. This result may explain the frequent vaccine failure against IBD observed in broiler and pullet flocks in Algeria.

Keywords: Molecular characterization, sequencing, vvIBDV, chickens, Algeria

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INTRODUCTION

In Algeria, poultry industry plays a significant role in the agricultural sector. As per FAOSTAT (2023), the country produced 257,000 tons of poultry meat and 305,000 tons of eggs in 2021. This activity is vital for the population as it serves as a crucial source of protein through meat and eggs, while simultaneously providing a reliable source of income for farmers. Despite these facts, the contribution of poultry production to human nutrition and the country's economy is still limited by various factors such as high production costs, a lack of appropriate breeding practices, and infectious diseases. Infectious bursal disease (IBD) is an immunosuppressive disease of young chickens of worldwide prevalence (Eterradossi and Saif, 2020). The disease, also named "Gumboro disease" according to the location of the first outbreaks in Gumboro, Delaware, USA, was later designated infectious bursal disease (IBD) according to varying morphologic and histological changes observed in the bursa of Fabricius (Hitchner, 1970). IBD causes severe direct and indirect economic losses to the poultry industry and farmers worldwide (Spackman *et al*., 2017; Brown Jordan *et al*., 2018).

The causal agent of IBD is infectious bursal disease virus (IBDV), a non-enveloped double- stranded RNA (dsRNA) virus that is, a member of the family *Birnaviridae* and genus *Avibirnavirus* (Dobos *et al*., 1979; Müller *et al*., 2003). The genome of IBDV is composed of two segments known as A and B. Segment A comprises a larger open reading frame 1 (ORF1), which encodes a polyprotein of 110 kDa that splices into three viral proteins (VP2, VP3, and VP4) through auto-catalytic mechanisms, according to Mató *et al*. (2020). VP2 and VP3 are structural proteins, while VP4 acts as a protease, as reported by Morgan *et al*. (1988) and Biđin *et al*. (2001). Additionally, a small ORF1 gene present in segment A encodes VP5, which is a non-structural protein as Mundt *et al.* (1995) pointed out. On the other hand, segment B contains only one ORF gene that encodes VP1, which is the viral RNA polymerase (Hudson *et al*., 1986; Berg, 2000; Qin and Zheng 2017).

Two serotypes of IBDV (Serotype 1 and serotype 2) and several antigenic subtypes of serotype 1 viruses have been identified using cross-virus neutralization (VN) assays (Jackwood and Jackwood, 1997). The serotype 1 strains are pathogenic to chickens and vary in their virulence, whereas serotype 2 strains, isolated from turkeys, are apathogenic for both turkeys and chickens (McFerran *et al*., 1980). Serotype 1 has been classified into different groups based on antigenic variation, virulence, and molecular characteristics of the hypervariable region (HVR) of VP2. These groups include attenuated IBDV, classical IBDV, variant IBDV, and very virulent IBDV (vvIBDV) (Sharma *et al*., 1989; van den Berg *et al*., 2004; Jackwood *et al*., 2018).

The four strains of IBDV have a global distribution and are present in most countries in poultry industry. The classical strains of IBDV can cause damage to the bursa and lymphoid necrosis, leading to a mortality rate of 1-30% (Müller *et al*., 2003; Mawgod *et al*., 2014). On the other hand, variant IBDVs are characterised by antigenic drift, which is caused by point mutations affecting the neutralising epitopes of VP2 (Vakharia *et al*., 1994; Rajkhowa *et al*., 2018). These strains were first identified in North America, where they caused B lymphocyte depletion without inducing an inflammatory response or clinical signs of disease (Sharma *et al*., 1989). In the mid-1980s, vvIBDV strains emerged in Europe and caused devastating outbreaks, resulting in 50-100% mortality in young chickens (Berg, 2000; Eterradossi and Saif, 2013). These strains then spread to the Middle East, Asia, South America and Africa (Abdel-Alim *et al*., 2003; Gómez *et al*., 2018). The main approach to prevent IBD is a combination of immunisation and biosecurity measures (Müller *et al*., 2012; Thomrongsuwannakij *et al*., 2021). However, despite vaccination, farmers still encounter difficulties with this disease (Ali Khan *et al*., 2019), as vaccination failures have been documented in numerous locations worldwide (Müller *et al*., 2012). These failures have been attributed to various factors (Islam *et al*., 2008; Becheur and Oumouna, 2019), including inadequacy between the vaccine strain and the field strain of IBDV circulating in farms.

In Algeria, IBD was first observed in poultry production in the late 1980s (Allamigeon and Comte, 2001; Abed *et al*., 2018). In 2000, the partial sequencing of the VP2 gene from seven IBDV isolates led to the initial suspicion of vvIBDV in Algeria (Bouadoud and Alloui, 2008). After a few years, the clinical picture of IBD in various regions of Algeria shifted, with significantly higher mortality rates, compared to the classical form. This led to the hypothesis that hypervirulent strains may have spread as a result of genetic mutations within the IBDV genome. In this context, Boudaoud (2015) reported the presence of a highly virulent IBDV strain in Eastern Algeria. Three years later, another study (Abed *et al*., 2018) revealed the circulation of IBDVs related to reassortments and the European vvIBDV.

Although a wide range of vaccines against IBD are used in Algeria, losses due to this disease have not decreased likely due to the circulation of vvIBDV, which has been implicated in most vaccination failures (Lukert and Saif, 2003; Thomrongsuwannakij *et al*., 2021). The present study aimed to check the persistance of vvIBDV in eastern and central Algeria by conducting molecular characterization of circulating strains. Additionally, it sought to establish the relationship between the identified viruses and IBDV reference strains from Algeria and other regions worldwide. The study also included a histopathological examination of the investigated birds, in order to explore the effect of these field strains on the bursa of Fabricius.

MATERIALS AND METHODS

Ethical approval: All experimental procedures were approved by the Institutional Animal Care Committee of the National Administration of the Algerian Higher Education and Scientific Research (Ethical approval number: 98–11, Law of 22 August, 1998) and were conducted according to the recommendations of the "Guide for the Care and Use of Laboratory Animals".

Flock history: To investigate IBD outbeaks, fifty-five birds collected from eleven chicken flocks were examined in eastern and central Algeria between 2019 and 2020. The suspected outbreaks of IBD were identified in broiler chickens (flocks N°1, 2, 3, 4, 5, 6, 7, and 8) and pullet chickens (flocks N°9, 10, and 11) based on clinical signs and gross lesions.

The mortality rate in affected broilers ranged from 2% to 9%, while in affected pullets, it was 71% and 47.8% in flocks N°10 and 11, respectively. In both broiler and pullet affected flocks, the clinical signs presented were reduced appetite and activity, whitish watery diarrhoea, prostration ruffled feathers, huddling, somnolence, and death. At necropsy, various gross changes were seen in bursa of Fabricius in bird flocks detected IBDV. There were edema, hemorrhage, and enlargement in some bursae of Fabricius. In addition, petechial and hemorrhages were seen on the breast and thigh muscles of IBDV infected birds.

All of the flocks had been immunized against IBD, with the exception of flock N°10's pullets. The live vaccines that were predominantly used were of the "intermediate" strain, with the exception of flock N°3, which was given an attenuated vaccine. Information regarding the sampling region, type of breeding, the IBD primary vaccination date, IBD vaccine strains, age of birds at clinical outbreak, mortality rate and age at sampling time is summarised in Table 1.

Table 1. Flock history.

Flock number	Region in Algeria	Breeding	Age at first IBD vaccination	IBD vaccines used	Age at the outbreak	Mortality rate (%)	Age at sampling
1	Center	Broilers	16 d	Intermediate	32 d	6.2	33 d
2	Center	Broilers	15 d	Intermediate	24 d	8%	26 d
3	Center	Broilers	15 d	Low vaccine strain	27 d	4.5%	29 d
4	East	Broilers	15 d	Intermediate	27 d	2.2%	31 d
5	East	Broilers	15 d	Intermediate	30 d	3%	33 d
6	East	Broilers	11 d	Intermediate	21 d	9%	24 d
7	East	Broilers	13 d	Intermediate	37 d	4.6%	38 d
8	East	Broilers	14 d	Intermediate	28 d	3.9%	29 d
9	Center	Broilers	14 d	Intermediate	20 d	1.8%	28 d
10	Center	Pullets	**Not vaccinated**	**Not vaccinated**	24-26 d	**71%**	27_30 d
11	Center	Pullets	20 d	Intermediate	24-27 d	**47.8%**	24_27 d

IBD: Infectious bursal disease

Histopathology: Fifty-five bursae from freshly dead or sacrificed birds were collected from 11 flocks and stored in 10% buffered formalin solution for histopathological analysis. Only the bursa of Fabricius showing necropsi findings (edema, heamorrhages and enlargement) were examined histopathologically.

The tissues were cut into sections, identified and dehydrated using a series of graded alcohols (70%, 80%, 90%, 95% and 100%). The blocks were cleared in xylene and infiltrated with molten paraffin wax. Sections (5 µm) were cut from the embedded tissues using a microtome. The tissues were mounted on clean grease-free glass slides, and kept at 25 °C, and then stained with hematoxylin and eosin (H&E) (Luna, 1968). The prepared slides were examined using a light microscope at 20x, 100x, and 200x magnification. Photomicrographs of the tissues were captured using a digital microscopic objective camera.

Molecular characterization

Sample collection: Fifty-five fresh bursas were collected from 11 broiler and pullet flocks suspected of IBD. Bursas from each outbreak were cut open, and imprinted directly onto a Flinders Technology Associates® card (FTA® card (Whatman)). These cards are suitable for capturing and preserving viral RNA, as well as facilitating transportation (Moscoso *et al*., 2006).

Viral RNA extraction from FTA® card: Three discs (2 mm in diameter) were cut from the spotted area of each FTA® card (Whatman, GE Healthcare, UK) using a sterile puncher (Harris Micro-Punch) and placed in 1.5 mL Eppendorf tubes. For each tube containing FTA® paper discs, 250 µL of TE buffer was added, vortexed, and incubated for 10 min at 25 °C (Moscoso *et al*., 2006; Amin and Jackwood, 2014). The QIAamp® Viral RNA Mini Kit (QIAGEN GmbH, Germany) was used to extract RNA from the FTA® paper according to the manufacturer's instructions. The RNA product was stored at -20 °C until analysis.

Reverse transcription-polymerase chain reaction (RT-PCR): RNA purity and concentration were determined using NanoDrop spectrophotometre (NanoDrop 1000c, Thermo Scientific, Waltham, USA). Conventional RT-PCR was performed using an automatic DNA thermal cycler (Chromo-4; Bio-Rad)). A one-step RT-PCR Kit (Qiagen, Germany) was used according to the manufacturer's instructions. Primers VP2 F5'-GCCGATGATTACCAATTC-3' and VP2 R5'-GTGACGGGACGGAGGGCC-3' were used to amplify a 422-bp sequence of the hypervariable region of the VP2 gene of IBDV from bp 762-1184 region. (Regarding the reference of the primers, please refer to our reply in the cover letter)

A VP2 region was amplified as follows: 20 min at 50 °C (RT reaction); 15 min at 95 °C (initial PCR activation); 35 cycles at 94 °C for 30 s (denaturation), 59 °C for 40s (annealing) and 72 °C for 1 min, and 72 °C for 10 min (final extension). Positive (IBD vaccine strain) and negative (nuclease-free water) controls were included in each PCR run.

After amplification, RT-PCR products were subjected to electrophoresis on 2% agarose gel containing ethidium bromide. The band was examined using a UV-light trans-illuminator. The PCR products were purified using a QIA-quick Gel Extraction Kit (Qiagen) following the manufacturer's instructions.

Sequencing of the hypervariable region of VP2 gene: The purified PCR products were sequenced using a BigDye Terminator V3.1 Cycle Sequencing Kit (Perkin-Elmer, Foster City, CA, USA). The sequencing reactions were then purified, followed by loading onto a sequencer plate with capillary electrophoresis using an ABI PRISM 3730xl Genetic Analyser developed by Applied Biosystems (ABI), USA. Nucleotide sequences were assembled and edited using BioEdit software, version

7.2.1. DNA sequences for the present study were submitted to GenBank of NCBI (National Centre for Biotechnology Information, USA) and deposited in this database under accession numbers: MT104575 and MT104576.

Sequence and phylogenetic analysis: The nucleotide and deduced amino acid (aa) sequences were aligned to those of IBDV reference strains from GenBank (listed in Table 2). The Clustal W method was used to perform multiple alignments using the molecular evolutionary genetics analysis (MEGA) version 7. The phylogenetic tree was constructed using the neighbour-joining method with 1000 bootstrap replicates in MEGA 7.

The aim of the phylogenetic analysis was to determine the relationship between the two Algerian strains isolated during the current study and reference strains of IBDV (including classical, attenuated/vaccinated, vv, and variant reference strains of IBDV) from Algeria and various other parts of the world (as enumerated in Table 2).

Table 2. Infectious bursal disease virus (IBDV) reference strains used in the present study.

N°	IBDV Strains	Virulence	Origin and year	GenBank accession number	IBDV segment
1	**Faragher 52-70**	Classic	England	Y14958	VP2 (segment A)
2	**STC**	Classic	USA	D00499	Segment A of IBVD
3	**Cu-1 M**	Attenuated/vaccine	Germany	AF362771	
4	**Bursa Vac**	Attenuated/vaccine		AF498633	VP2 (Segment A)
5	**Cloned D78 strain**	Attenuated/vaccine	NOBILIS (1997)	Y14962	VP2 (Segment A)
6	**Cevac IBDL**	Attenuated/vaccine	CEVA (2008)	EU544157	VP2 (Segment A)
7	**150124**	vvIBDV/reassortant	Algeria (2018)	KY555572	VP2 (Segment A)
8	**150128**	vvIBDV	Algeria (2018)	KY555578.1	VP2 (Segment A)
9	**150133**	vvIBDV	Algeria (2018)	KY555586.1	VP2 (Segment A)
10	**150142**	vvIBDV/reassortant	Algeria (2018)	KY555594	VP2 (Segment A)
11	**150144**	vvIBDV/reassortant	Algeria (2018)	KY555598	VP2 (Segment A)
12	**03ALG**	vvIBDV	Algeria (2015)	KP729478	VP2 (Segment A)
13	**09ALG**	vvIBDV	Algeria (2015)	KP729479	VP2 (Segment A)
14	**20ALG**	vvIBDV	Algeria (2015)	KP729480	VP2 (Segment A)
15	**38ALG**	vvIBDV	Algeria (2015)	KP729481	VP2 (Segment A)
16	**50ALG**	vvIBDV	Algeria (2015)	KP729482	VP2 (Segment A)
17	**399_Algeria**	vvIBDV	Algeria (2017)	MF142528	VP2 (Segment A)
18	**P07**	vvIBDV	Tunisia	AY665672	Segment A
19	**UK661**	vvIBDV	England (1989)	X92760	VP2 (segment A)
20	**D6948**	vvIBDV	Netherland (1989)	AF240686	Segment A
21	**849VB**	vvIBDV	Belgium (1989)	AY321949	Segment A
22	**89163**	vvIBDV	France	HG974563	Segment A
23	**OKYM**	vvIBDV	Japan (1991)	D49706	Segment A
24	**HK46**	vvIBDV	Hong Kong (1994	AF092943	Segment A
25	**E/Delaware**	Variant	USA (1985)	AF133904	Segment A
26	**GLS**	Variant	USA (1987)	M97346	Segment A
27	**OH**	Serotype 2	USA (1982)	M66722	**Non-pathogenic** (Segment A)
28	**MBechIBDVdz1**	To be determined	Algeria (present study)	MT104575	VP2 (Segment A)
29	**MBechIBDVdz2**	To be determined	Algeria (present study)	MT104576	VP2 (Segment A)

RESULTS

Clinical signs: The disease appeared suddenly with death, and spread rapidly in the infected flocks.The age at the outbreak was 21 to 30 day for all investigated flocks. Mortality rates in clinically affected broiler flocks ranged from 2% to 9%. Very high mortality was reported in pullet flocks N°10 and 11 (71% and 47.8%, respectively). The clinical signs were anorexia, whitish watery diarrhea, depression, ruffled feathers, huddling together, somnolence, and death.

Clinical signs observed in broilers were similar to those observed in pullets, except that the disease was accompanied with very high mortality in pullets.

Necropsy and histopathological findings: The gross lesions of the muscles and bursa of Fabricius (BF) of the affected birds are presented in Table 3 and illustrated in Figure 1 (necropsy).

The main histopathological bursal findings consisted of lymphocyte depletion and interstitial oedema in the medullary area of the bursal follicles. The lymphocytes were soon replaced by heterophils, pyknotic debris, and hyperplastic reticuloendothelial cells.

The details of these histopathological lesions are presented in Table 3 and illustrated in Figures 3, 4, 5, and 6. The normal appearance of the bursa is illustrated in Figure 2.

Table 3. The gross and histopathological lesions of infected flocks.

Flock number	Mortality rate (%)	Gross lesions	Histopathological lesions of the bursa of Fabricius
1; 2; 3.	6.2; 8 and 4.5 respectively	Swollen oedematous bursa (Figure 1-b) Muscular haemorrhages (Figure 1-a)	Mild to extensive depletion of lymphocytes. Interstitial oedema and influx of heterophils and macrophages (Figure 3).
4	2.2	0	Normal Bursa (Figure 2).
5; 6; 7; 8; 9.	3; 9; 4.6; 3.9 and 1.8 respectively	Swollen oedematous bursa. Muscular haemorrhages.	Follicular lymphocytic depletion. Extensive heterophil infiltration of interstitium. Apoptotic and degenerate lymphocytes. Macrophage and heterophil infiltration. Interstitial oedema (Figure 4).
10	71	Swollen oedematous bursa. Severe haemorrhages in breast and thigh muscles.	Extensive depletion of lymphocytes with densely staining necrotic foci. Marked interstitial oedema and hyperplasia. Epithelium reactive and hyperplastic. Necrotic focus consisting of macrophages and cellular debris. Interstitial inflammatory cells (Figure 5).
11	47.8		Marked pallor, lymphocyte depletion and oedema, reactive and extensively hyperplastic epithelium (Figure 6).

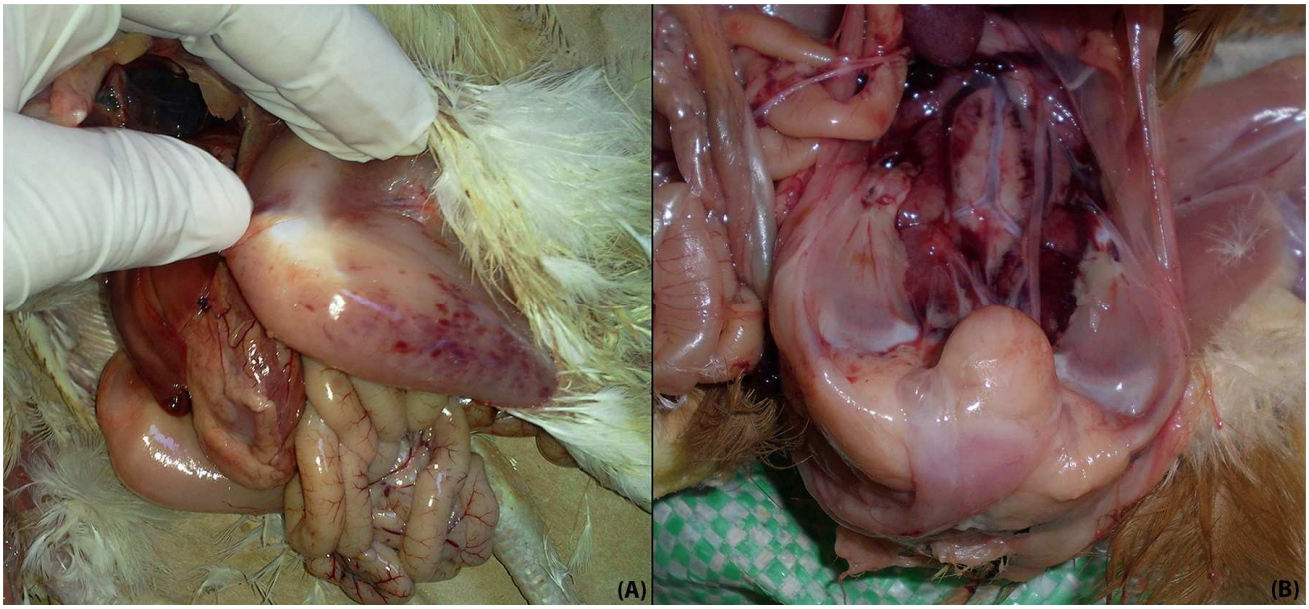

Figure 1. Gross lesions in IBD-suspected birds. (A): Muscular (thigh) haemorrhages, (B): Swollen oedematous bursa.

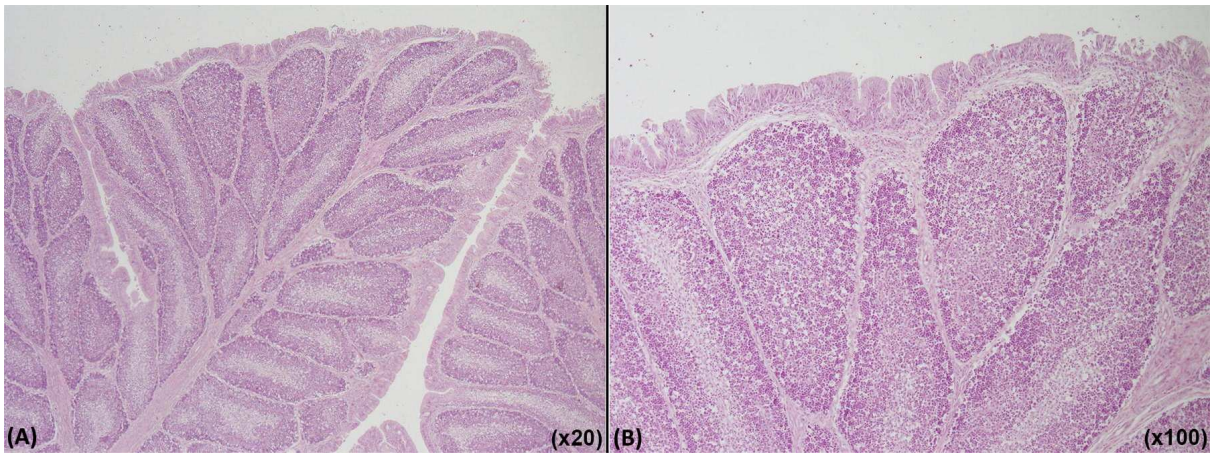

Figure 2. (A): Normal bursa with densely populated outer cortices of lymphocytes and sparsely populated medullae. Lobules subdivided by connective tissue. Luminal surface covered with pseudo stratified columnar epithelium (H&E, 20×). (B): Normal bursa, as left (H&E, 100×).

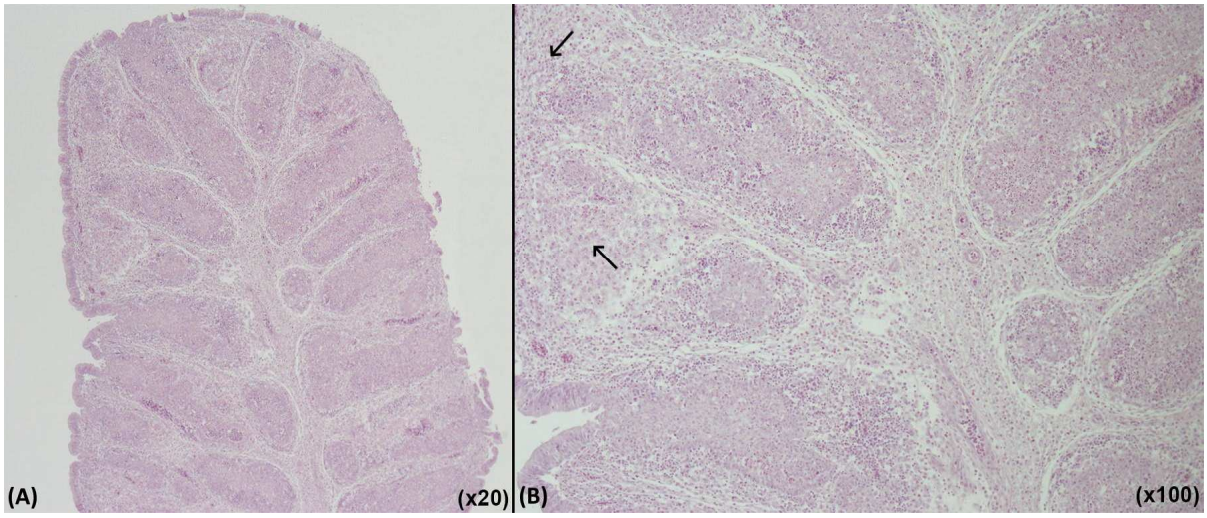

Figure 3. (A): Marked pallor due to extensive depletion of lymphocytes. Structure, architecture, and epithelium normal (H&E, 20×). (B): Extensive depletion of lymphocytes of the BF (arrows). Interstitial oedema and influx of heterophils and macrophages (H&E, 100×).

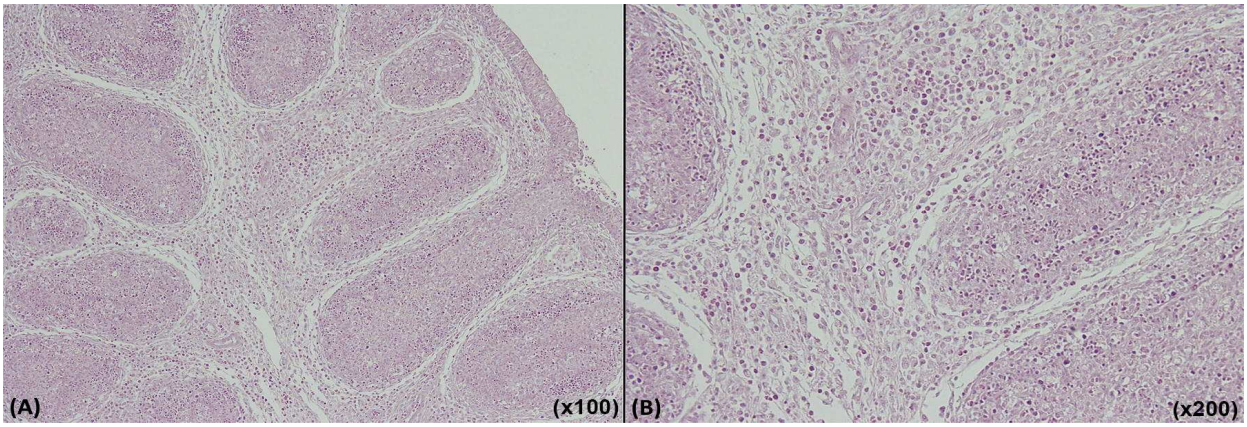

Figure 4. (A): Follicular lymphocytic depletion in the BF. Extensive heterophil infiltration of interstitium (H&E, 100×). (B): Apoptotic and degenerate lymphocytes. Macrophage and heterophil infiltration. Interstitial oedema (H&E, 200×).

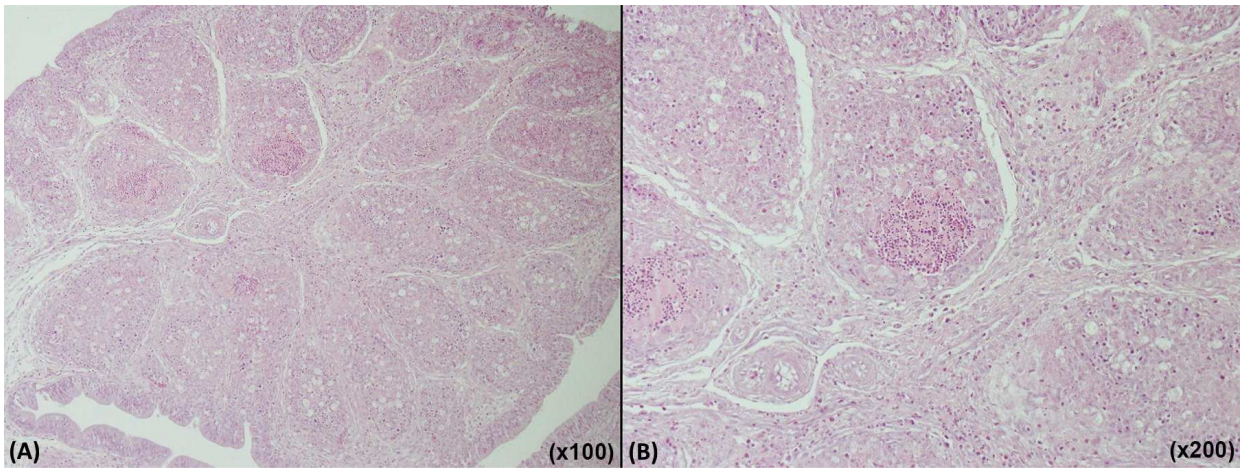

Figure 5. (A): Extensive depletion of lymphocytes with densely staining necrotic foci in the BF. Marked interstitial oedema and hyperplasia. Epithelium is reactive and hyperplastic (H&E, 100×). (B): Necrotic focus consisting of macrophages and cellular debris. Interstitial inflammatory cells (H&E, 200×).

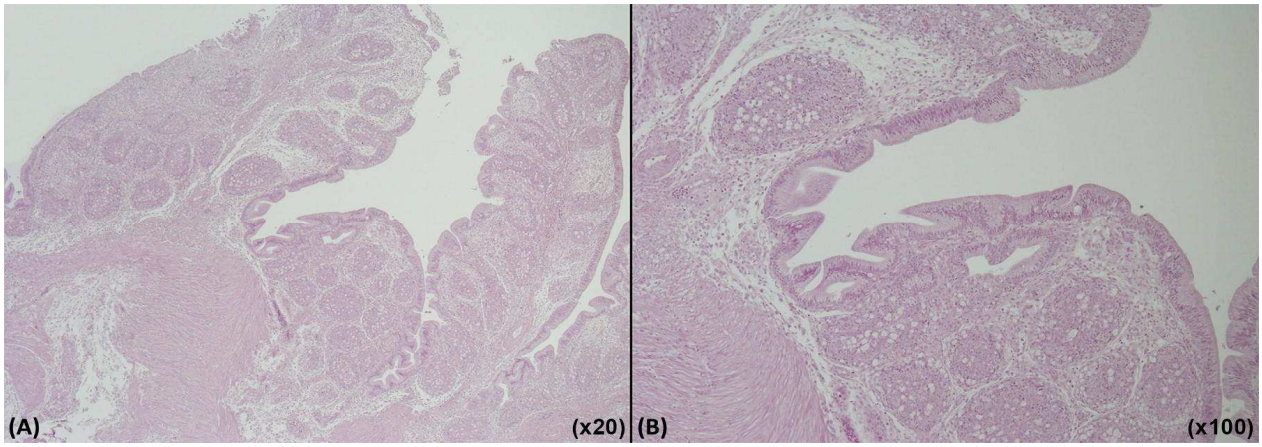

Figure 6. Marked pallor, lymphocyte depletion, and oedema in the BF. Reactive and extensively hyperplastic epithelium. (A): H&E, 20×; (B): H&E, 100×.

Molecular characterization

RT-PCR: Using RT-PCR, the IBDV was detected in 6 out of the 11 investigated chicken flocks (54.5%) namely, flocks N°1, 2, 3, 9, 10, and 11. The viral RNA extracted from the FTA® card showed specific amplification of the VP2 gene with a size of 422 bp. The specificity of the RT-PCR was further confirmed by the absence of amplification in the negative control.

Amino-peptide analysis: Two out of the six RT-PCR positive samples were selected for sequencing. In the current study, the two nucleotide sequences (MBechIBDVdz1 and MBechIBDVdz2) were aligned and their deduced amino-acid sequences at positions 211-350 were compared to the VP2 hypervariable region of 27 global isolates of both virulent and attenuated IBDVs, which included 11 Algerian IBDVs and serotype 2 of IBDV available in the NCBI database. Figure 7 illustrates this comparison.

The studied strains were found to possess four conserved amino acids (222A, 256I, 294I, and 299S), which are characteristic of vvIBDV according to previous studies (Brown and Skinner, 1996; Yilmaz *et al*., 2019), as shown in Figure 7.

In addition, the virulent phenotype of our Algerian strains was determined by the co-existence of amino acids 279D and 284A, as reported in previous studies (Yamaguchi *et al*., 1996; Brandt *et al*., 2001; Mahgoub *et al*., 2012).

Figure 7 shows that the two strains analyzed in this study had the serine-rich heptapeptide sequence '326SWSASGS332' adjacent to the second major hydrophilic region, which is a known marker of virulent IBD strains, as previousely reported by Brown and Skinner (1996), Hernández *et al*. (2006) and Alkhalefa *et al*. (2019).

The different substitutions are summarised in Table 4.

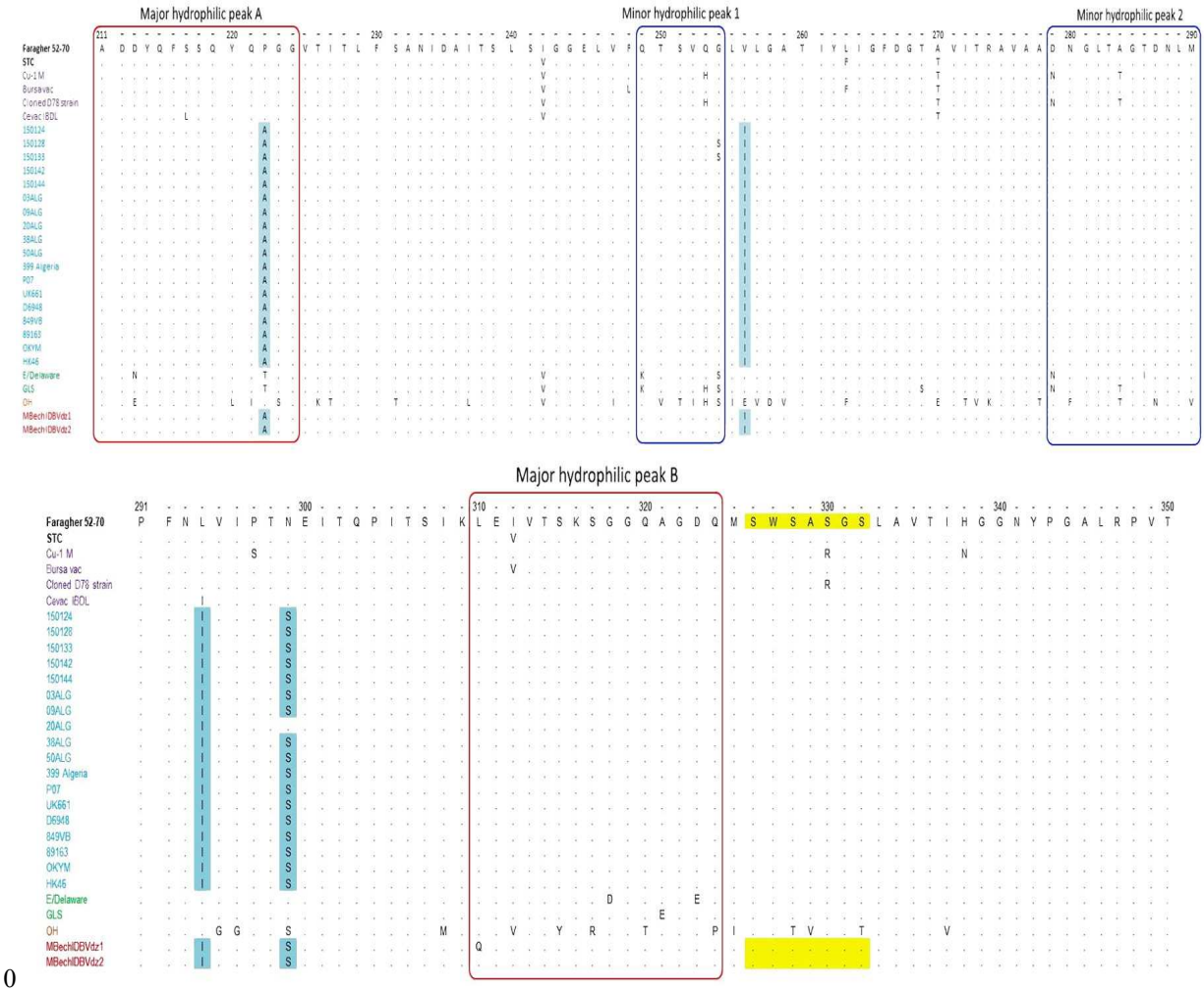

Figure 7. Alignment of deduced amino acid sequences of the VP2 hypervariable region from aa positions 211-350 of IBDV strains. Dots indicate positions with aa identical to the reference Faragher 52-70 strain. Major hydrophilic peaks are boxed with red lines and minor hydrophilic peaks are boxed with blue lines. Amino acid positions are as per Bayliss et al. (1990). The 222 (A), 256 (I), 294 (I), and 299 (S) aa positions are characteristic of vvIBDV. Heptapeptide 'SWSASGS', a serine rich area (enclosed in yellow box), is indicative of virulent IBD strains.

Table 4. Comparison of the characteristic amino acid residues between the strains of the present study with infectious bursal disease virus (IBDV) reference strains.

	Amino acid position (as per Bayliss et al., 1990)									
	222	249	253	254	256	279	284	294	299	330
Classical strains (Faragher 52-70).	P	Q	Q	G	V	D	A	L	N	S
Attenuated/vaccine strains (Cu-1 M, Bursa Vac, D 78, IBDL).	P	Q	Q[1] H[2]	G	V	N[2] D[1]	T[2] A[1]	L	N	R[2] S[1]
vvIBDV strains (Algerian vvIBDV strains (Boudaoud, 2015; Abed et al, 2018) and other vvIDV (UK661, 849VB, 89163, OKYM…)).	A	Q	Q	G	I	D	A	I	**S**	S
Variant strains (E Delaware, GLS).	T	K	Q[3] H[4]	S	V	N	A[3] T[4]	L	N	S
Strains of present study (MBechIBDVdz1, MBechIBDVdz2).	A	Q	Q	G	I	D	A	I	**S**	S

[1] Bursa Vac; IBDL [2] Cu-1 M; D78 [3] E Delaware [4] GLS

Nucleotide identity percentage: The sequence analysis involved a comparison of the nucleotide (genetic) identity of the studied strains with that of reference IBDV strains. To calculate pairwise sequence identity and similarity from multiple sequence alignments, the SIAS tool was utilised. The detailed results are presented in Table 5.

Table 5. Nucleotide identity percentage between strains of the present study and selected infectious bursal disease virus (IBDV) strains.

				Identity percentage between strains of the present study and selected IBDV strains	
Nº	**IBDV Strains**	**Virulence**	**Origin and year**	**MBechIBDVd1**	**MBechIBDVd2**
1	**Faragher 52-70**	Classic	England	93.79	94.03
2	**STC**	Classic	USA	93.07	93.31
3	**Cu-1 M**	Attenuated/vaccine	Allemagne	91.40	91.64
4	**Bursa Vac**	Attenuated/vaccine		93.07	93.31
5	**Cloned D78 strain**	Attenuated/vaccine	NOBILIS(1997)	91.88	92.12
6	**Cevac IBDL**	Attenuated/vaccine	CEVA (2008)	93.07	93.31
7	**150124**	vvIBDV	Algeria (2018)	99.28	99.52
8	**150128**	vvIBDV	Algeria (2018)	98.32	98.56
9	**150133**	vvIBDV	Algeria (2018)	98.56	98.8
10	**150142**	vvIBDV/Reassortant	Algeria (2018)	99.76	100
11	**150144**	vvIBDV/Reassortant	Algeria (2018)	99.76	100
12	**03ALG**	vvIBDV	Algeria (2015)	98.8	99.04
13	**09ALG**	vvIBDV	Algeria (2015)	98.8	99.04
14	**20ALG**	vvIBDV	Algeria (2015)	99.04	99.28
15	**38ALG**	vvIBDV	Algeria (2015)	98.32	98.56
16	**50ALG**	vvIBDV	Algeria (2015)	98.8	99.04
17	**399_Algeria**	vvIBDV	Algeria (2017)	98.8	99.04
18	**P07**	vvIBDV	Tunisia	96.89	97.13
19	**UK661**	vvIBDV	England (1989)	96.42	96.65
20	**D6948**	vvIBDV	Netherland (1989)	97.13	97.37
21	**849VB**	vvIBDV	Belgium (1989)	97.13	97.37
22	**89163**	vvIBDV	France	96.89	97.13
23	**OKYM**	vvIBDV	Japan (1991)	96.65	96.89
24	**HK46**	vvIBDV	Hong Kong (1994	96.89	97.13
25	**E/Delaware**	Variant	USA (1985)	91.64	91.88
26	**GLS**	Variant	USA (1987)	90.93	91.16
27	**OH**	Serotype 2	USA (1982)	72.31	72.55

N.B: nucleic acid identity percentage between MBechIBDVdz1 and MBechIBDVdz2 is 99.76%

In the current study, the nucleotide identity between the two studied strains and the serotype 1 viruses, including classical, attenuated, variants, and hypervirulent, varied. It was comparatively lower in classical, attenuated, and variant viruses (ranging from 90.93% to 94.03%), and higher in hypervirulent viruses (ranging from 97% to 100%), as presented il Table 5.

The lowest percentage of nucleotide identity was recorded for the OH strain, serotype 2 (apathogenic), which showed values of 72.31% and 72.55% (Table 5).

The strains analysed in this study shared a high degree of similarity (ranging from 98.32% to 99.28%) with Algerian hypervirulent strains identified by Boudaoud in 2015 (03Alg, 09Alg, 20Alg, 38Alg, and 50Alg) and strain 399_Alger (Michel and Jackwood, 2017). They also exhibited a very high level of similarity (ranging from 99.28% to 100%) with hypervirulent and reassortant strains studied in Algeria in 2018 by Abed *et al*. (150124, 150128, 150133, 150142, and 150144) (refer to Table 5).

Phylogenetic analysis: A phylogenetic tree of VP2 gene-based sequences was constructed using the neighbor-joining method with 1000 bootstrap replicates, as shown in Figure 8.

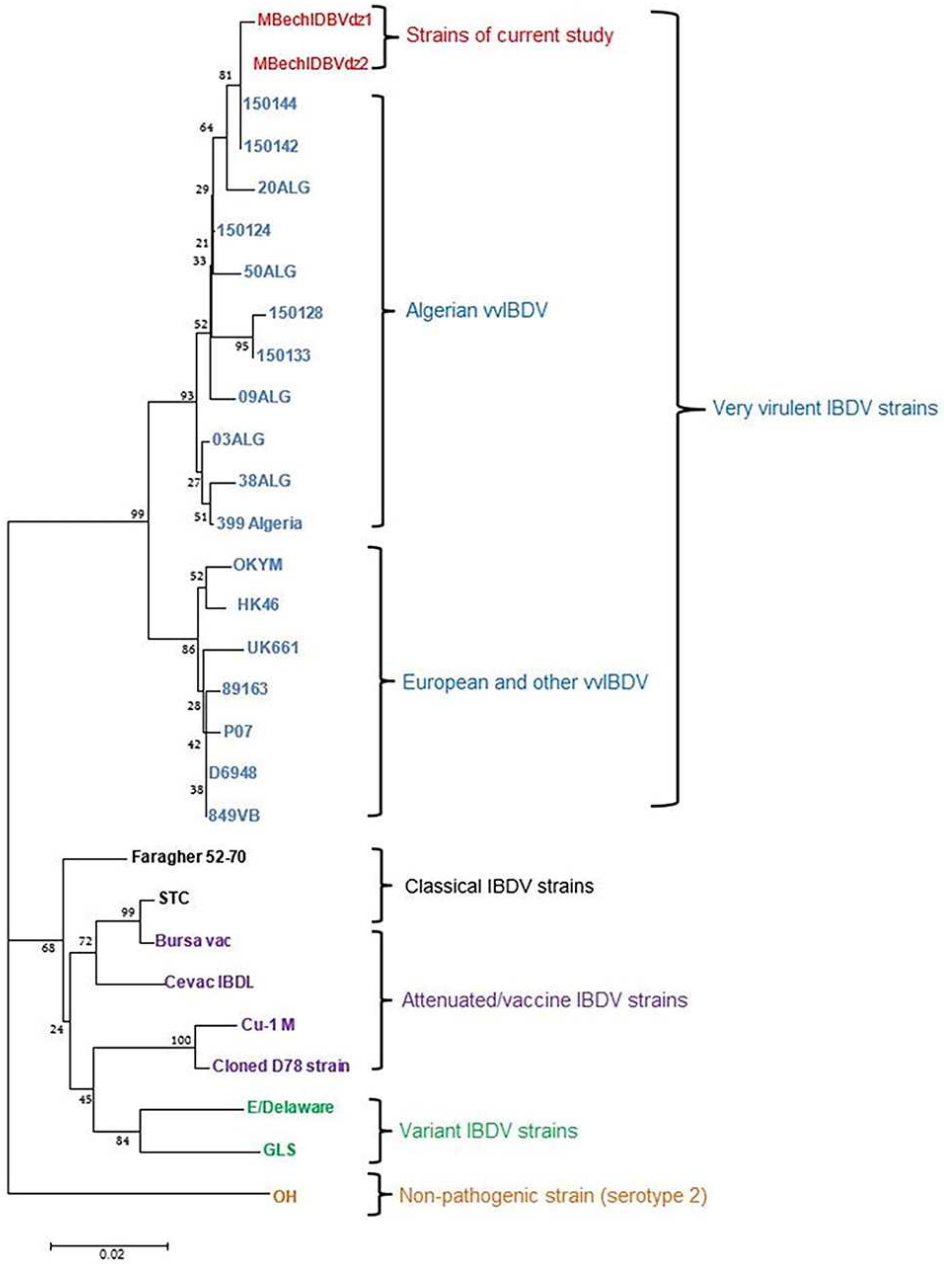

Figure 8. Phylogenetic tree based on partial sequencing of the VP2 gene, showing the relationship between the two Algerian strains isolated in the present study and other Algerian and IBDV reference strains (classical, attenuated/vaccinated, vv and variant reference strains of IBDV).

Phylogenetic investigation of nucleotide sequences indicates clustering of IBDV based on virulence and geographical locations. Classical, attenuated/vaccinated and variant IBDV formed a separate clade from vvIBDVs.

According to the phylogenetic tree (Figure 8), the two strains examined in the present study (MBechIBDVdz1 and MBechIBDVdz2) are closely related to previously isolated Algerian vvIBDVs, as they are clustered together.

DISCUSSION

IBD, known for its immunosuppressive effect on young chickens, has caused significant financial losses in the poultry industry worldwide, particularly in the last decade (Wagari, 2021).

To control the IBDV infection, broiler and pullet chicks are immunized with intermediate or intermediate-plus strains, while breeders receive live attenuated and inactivated vaccines (Thai *et al*., 2021).

Despite strict biosecurity measures and intensive immunization programs, IBD outbreaks still occur worldwide (Spackman *et al*., 2017). This may be due to frequent genetic changes, genomic recombination, and reassortment of the viral genome, which can result in vaccine failure (Mwenda *et al*., 2018).

The sudden shift in the clinical picture of IBD across various regions in Algeria, characterized by significantly higher mortality rates compared to the classical form, has prompted the hypothesis that hypervirulent strains may have spread as a result of genetic mutations within the IBDV genome.

The aim of this research was to check the persistance of vvIBDV by conducting molecular analysis of strains present in eastern and central Algeria. Additionally, it aimed to establish the relationship between the viruses identified in this study and IBDV reference strains from Algeria and other regions worldwide.

The mortality rates observed in pullet flocks affected by the vvIBDV strain align with previously documented rates in literature on this strain, as reported by Berg (2000), Eterradossi and Saif (2013) and Shekhar and Dalai (2020). Research has shown that layer-type chickens, or pullets, are more vulnerable to vvIBDV compared to broiler-type chickens, as noted by Silva *et al*. (2016) and Shekhar and Dalai (2020). Additionally, it was found that the birds from one of the investigated pullet flocks were not vaccinated against IBD. This could also clarify the reason for the exceptionally elevated mortality rate of IBD in pullets especially in Flock N°10.

Even though flock N°11 received vaccine against IBD, it still contracted the virus. This can be attributed to vaccine failure due to a inadequacy between the circulating viral strain, which in this case was the vv strain, and the vaccine strain that was administered, which was an intermediate strain. As a result, mild vaccines are not very effective against vvIBDV, as stated by Thomrongsuwannakij *et al*. (2021). Indeed, the characteristic of vvIBDVs is their ability to cause disease in the presence of high levels of maternal antibodies (MDAs) (Berg, 2000; Tolba *et al*., 2019).

The moderate recorded mortalities, in affected broiler flocks, could suggest that they were infected by classical IBDV strain. In the same context, it has been reported that mortality associated with infection due to classic strains may range from 1-30% (Müller *et al*., 2003; Mawgod *et al*., 2014). In contrast, the variant strain cannot be involved in the outbreaks of the present study because it does not produce overt clinical signs, but causes immunosuppression and may cause mortality due to secondary opportunistic infections (Kegne and Chanie, 2014).

On the other hand, the age at the time of the outbreaks was similar to the information reported in the literature. Clinical disease resulting from vvIBDV infection is typically seen in chicks between 3-6 weeks old, which coincides with the period of maximal development of the bursa of Fabricius (BF), as observed with classical pathogenic strains, according to Mahgoub *et al*. (2012).

The main necropsy findings are in line with previous field studies, as reported by Khenenou *et al*. (2017) and Umm-i-Habiba *et al*. (2020). In post-mortem examination, bursa of Fabricious is the principal diagnostic organ for birds that died during the acute phase of vvIBD. At the macroscopic level, BF appears swollen, edematous, and sometimes hemorrhagic. Within 7-10 days, the BF becomes atrophic, as noted by Ingrao *et al*. (2013).

No bursal atrophy was found in the present study, indicating that the disease had started recently because bursal atrophy is detected only in advanced cases of IBD (Wani *et al*., 2021). According to Bejo *et al*. (2004), the BF undergoes atrophy as a result of the progression of lymphoid cell degeneration and necrosis during the later stage of the infection.

Post-mortem findings did not differ markedly between infections with vvIBDV and classical virulent strains, such as F52/70, except that hemorrhaging in the bursae and other tissues may be more extensive and frequent in vvIBDV infections. Additionally, the disease may be more pronounced, acute, and associated with high mortality (Ignjatovic, 2004; Ingrao *et al*., 2013).

The histopathological changes observed in the bursa of Fabricius, including lymphoid follicle depletion, were consistent with previous reports of vvIBDV infection (Omer and Khalafalla, 2022; Tanimura, 2022). The depletion of lymphoid B cells in the BF following IBDV infection is attributed to both necrosis and apoptosis, as noted by Ingrao *et al*. (2013).

The bursa is the preferred tissue for assessing microscopic changes. In addition, the literature does not indicate a significant difference between the microscopic lesions of the BF caused by hypervirulent and classical IBDVs (Ignjatovic, 2004).

According to Lukert and Saif (2003), and Mahgoub *et al*. (2012), degeneration and necrosis of lymphocytes starts in the medullary area of the bursal follicles as early as 1 dpi. Then, lymphocytes are replaced by heterophils of pyknotic debris and hyperplastic reticuloendothelial cells. According to the same authors, by days 3 or 4 post-infection, all lymphoid follicles are affected. Severe oedema, hyperaemia, and marked accumulation of heterophils are evident, which causes an increased bursal weight.

According to Ignjatovic's in 2004, chicks infected with classical IBDV strains that have low pathogenicity show regeneration of the bursa, characterized by the repopulation of lymphocytes, within 8-21 days after infection. In contrast, chicks infected with vvIBDV do not exhibit a recovery phase, and instead,

chronic lesions develop in their bursae three weeks after infection.

Using RT-PCR, the IBDV was detected in 54.5 % of the investigated chicken flocks. This rate is consistent with the findings reported by Boudaoud in 2015 (46.15%) and Abed *et al*. in 2018 (63%).

The VP2 gene is frequently used to detect IBDV in field samples using RT-PCR because of its high specificity to the virus, and as a result, it is commonly used to differentiate and classify IBDV isolates by researchers (Jackwood and Sommer-Wagner, 2007; Adamu *et al*., 2013; Awandkar *et al.,* 2018). Additionally, the VP2 protein is known to play a critical role in the pathogenicity, virulence, and tropism of the virus, as noted by Cheggag *et al*. (2021).

The hypervariable region of VP2, spanning amino acids 206-350, contains two major hydrophilic domains known as major hydrophilic peaks A (amino acids 212-224) and B (amino acids 314-325), which form hairpin loops PBC (amino acids 219-224) and PHI (amino acids 316-324), respectively (Azad *et al*., 1987, Bayliss *et al.,* 1990*;* Garriga *et al*., 2006; Mahgoub *et al*., 2012). The minor hydrophilic peaks 1 (aa 248-254) and peak 2 (aa 279-290) of hVP2 form loops PDE (aa 249-254) and PFG (aa 279-284), respectively (Coulibaly *et al*., 2010).

The studied strains were found to contain four conserved amino acids (222A, 256I, 294I, and 299S) that are distinctive of vvIBDV, as reported in previous research (Brown and Skinner, 1996; Yilmaz *et al*., 2019), as shown in Figure 7.

As reported by other authors (Ndashe *et al*., 2016; Drissi Touzani *et al*., 2019; Yilmaz *et al*., 2019), certain conserved residues (A222, I242, Q253, I256, D279, A284, I294, and S299) are considered characteristic of vvIBDV. Our isolate's amino acid sequence analysis, as presented in Table 4, corroborates these findings.

In addition, our isolates exhibited the amino acid Q249, which has been detected in numerous vvIBDV strains (Lachheb *et al*., 2021). The residues I272, M290, Q324, and S330 found in our study's isolates are also frequently observed and distinctive of vvIBDV strains, as stated in prior research (Hernández *et al*., 2006; Abed *et al*., 2018).

According to previous studies (Yamaguchi *et al*., 1996; Brandt *et al*., 2001; Mahgoub *et al*., 2012), the presence of both amino acids 279D and 284A in our Algerian strains determines their virulent phenotype.

In addition, glutamine (Q) at aa position 253, aspartic acid (D) at aa position 279, and alanine (A) at aa position 284 have been identified as the putative amino acids responsible for virulence and cellular tropism in several studies (Mundt, 1999; Brandt *et al*., 2001; Mwenda *et al*., 2018).

The two strains analyzed in this study were found to contain the heptapeptide sequence 'SWSASGS' rich in serine, which is adjacent to the second major hydrophilic region. This sequence is commonly associated with virulent strains of IBD, as noted by previous studies conducted by Brown *et al*. (1994), Hernández *et al*. (2006), Felice *et al*. (2017), and Alkhalefa *et al*. (2019). Additionally, Lachheb *et al.* (2021) reported that the most virulent strains were those with the region with the highest number of serine residue. Moreover, the absence of aa 249R in the studied isolates, characteristic of attenuated IBDV strains (Gómez *et al*., 2018), confirmed their virulent phenotype.

Unlike variant viruses which typically lack aa 222T and 254S as noted in a previous study by Heine *et al*. (1991), these amino acid residues were present in the strains analysed in this study. This indicates that there was no antigenic drift observed among these strains. Additionally, variant strains of IBDV are known to cause immunosuppression (due to early and severe atrophy of the BF) and a reduction in production performance, even though they don't cause obvious clinical signs (Kegne and Chanie, 2014; Zachar *et al*., 2016; Xu *et al*., 2020).

The strains analysed in this study were found to be very similar to the Algerian hypervirulent strains that were previously identified by Boudaoud in 2015, namely 03Alg, 09Alg, 20Alg, 38Alg, and 50Alg, as well as the strain 399_Algeria reported by Michel and Jackwood in 2017. Additionally, they were almost indistinguishable (99.28-100%) from the hypervirulent and reassortant strains reported in Algeria in 2018 by Abed *et al*. (2018), specifically strains 150124, 150128, 150133, 150142, and 150144.

Based on the phylogenetic tree, it was observed that our two studied strains (MBechIBDVdz1 and MBechIBDVdz2) were closely related to previously isolated Algerian vvIBDVs, and they were grouped together in a cluster.

The initial appearance of IBD in poultry production in Algeria occurred after the virus surfaced in Europe during the late 1980s, as noted by Allamigeon and Comte (2001). In (2015), Boudaoud identified a vvIBDV strain in the eastern region of Algeria. Recently, Abed *et al*. (2018) conducted a study that confirmed the circulation of IBDVs within the country. Based on genetic analyses of partial sequences for segments A and B, the research identified four groups of Algerian IBDV isolates: i) viruses related to vvIBDV for segments A and B; ii) viruses, whose segment A is related to vvIBDV and whose segment B is more distantly related to vvIBDV; iii) viruses related to vaccine strains; and iv) potential reassortant viruses, with segment A genetically related to vvIBDV and segment B of undetermined origin.

vvIBDVs have also been reported in countries neighbouring Algeria. As per Cheggag et al. (2021), nearly 40% of the IBDVs that were in circulation in

Morocco from 2013 to 2016 were highly virulent strains. According to Lachheb and colleagues (2021), their research on Tunisian IBDV strains showed that the isolates from this country are closely related to Algerian vvIBDV strains based on phylogenetic analyses of the five gene sequences. In contrast, Moroccan strains, despite being in close geographical proximity, were found to be less closely related and were not on the same tree branch.

According to Drissi Touzani *et al*. (2020), segment A of Moroccan IBDV isolates was phylogenetically related to Algerian and Tunisian vvIBDV isolates, which is likely due to the close geographical proximity and transboundary between Morocco and its neighboring countries. However, in the phylogeny of segment B, Moroccan IBDV isolates were found to be more closely related to French and Malaysian isolates, as reported by the same authors.

Conclusion: IBDV was detected in 6 out of the 11 chicken flocks investigated using RT-PCR. Additionally, sequencing revealed the presence of vvIBDV in two out of the six IBDV-positive flocks.

Because of the vaccination failures they generate, special attention should be paid to these highly virulent strains, which continue to spread in Algeria. This necessitates considering a more effective vaccination strategy, selecting the most appropriate vaccine strains against the circulating pathotypes of IBD, and implementing continuous epidemiological surveillance.

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