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Pathotypic, Molecular, and Serological Response of Specific-pathogen-free Chickens Inoculated by Three Very Virulent Infectious Bursal Disease Virus Strains

Maryame Cheggag[1,2]*, Ghizlane Sebbar[3], Khalil Zro[4], Loutfi Chafiqa[3], Mohamed Mouahid[5], Mohammed El Houadfi[2], and Faouzi Kichou[2]

[1]*Division of Pharmacy and Veterinary Inputs, ONSSA, Rabat, Morocco*
[2]*Department of Veterinary Pathology and Public Health, Agronomy and Veterinary Institute Hassan II, Rabat, Morocco*
[3]*Society of Veterinary Pharmaceutical and Biological Productions (Biopharma), B.P. 4569, Rabat, Morocco*
[4]*Ministry of Agriculture, Directorate of Development of Production Sectors, Dbagh Station, Av. Hassan II, Rabat, Morocco*
[5]*Mouahid's Veterinary Clinic, Temara, Morocco*
* Corresponding author's Email: cheggagmaryame@gmail.com; ORCID: 0000-0002-8093-0149

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ABSTRACT

Three field strains of infectious bursal disease virus (IBDV) were isolated in 2015 from the bursa of Fabricius of affected broiler chickens originating from Moroccan broiler farms and were characterized as very virulent IBDV. Thus, the aim of this study was to investigate the pathogenicity of the above-identified IBD strains (accession number), MN241434.1, MN241436.1, and MN241439.1. In this trial, 75 three-week-old specific-pathogen-free chickens were randomly divided into four groups. Three groups of 1, 2, and 3 (n = 20 chicks/group) were inoculated via intra oculonasal route with $10^{3.5}$ EID_{50} of MN241434.1, MN241436.1, and MN241439.1, respectively. Group 4 (n = 15) was kept as control. Chickens from all groups were monitored during 14 days post-challenge (dpc). Dead and euthanized birds were necropsied and their lymphoid organs (bursa, thymus, and spleen) were subjected to histopathological examination and lesion scoring. Serological response and presence of IBDV in the bursa of Fabricius were investigated using ELISA and rRT-PCR, respectively. Chickens in all challenged groups showed characteristic signs of IBD, including prostration, dehydration, aqueous diarrhea, and ruffled feathers, starting mostly at 2 dpc. The total mortality rate was 100% at 4 dpc in groups 1 and 2 and 76.47% in group 3. Bursa of Fabricius from affected birds revealed severe lymphoid depletion and necrosis with mean bursal lesion score between 3 and 5 (4 for MN241434.1 and MN241436.1, and 4.125 for MN241439.1). Similar severe lesions were observed in the spleen of all infected broiler chickens. Histological lesions in the thymus were detected from 2 dpc with individual cell necrosis. Serological results revealed that survived chickens in group 3 developed Ab anti-IBDV at 7, 9, and 14 dpc. RT-PCR confirmed the presence of the IBDV in BF of all infected chickens. These results indicated that the investigated local field IBDV strains genetically characterized as vvIBDV were phenotypically hypervirulent pathotypes of IBDV in SPF chickens hence reflecting the epidemiological Moroccan and regional IBD. This constitutes a potential candidate reference strains to be used in the development of strategies for the prevention and control of IBD in Morocco.

Keywords: ELISA, Histopathology, Infectious bursal disease, Pathogenicity, RT-PCR, SPF chickens

INTRODUCTION

Infectious bursal disease virus (IBDV) of chickens, member of the family *Birnaviridae*, genus *Avibirnavirus*, is a non-enveloped, icosahedral, and double-stranded (dsRNA) virus with segments A and B (Azad et al., 1985). The virus is composed of two genome segments, A (3.17 kb) and B (2.8 kb) (Müller et al., 2010). Segment B encodes the RNA polymerase VP1 (879 amino acids) while segment A encodes a precursor polyprotein that is autocatalytically cleaved into proteins pVP2 (which subsequently matures into VP2), VP4, and VP3. There are two serotypes of IBDV, serotype 1 and serotype 2, although only serotype 1 viruses are pathogenic to chickens (Ismail et al., 1988; Wang et al., 2007). Infectious bursal disease (IBD) induced by serotype 1 IBDV strains infect B lymphocytes in the bursae of Fabricius (BF) of young chickens. Very virulent IBDV (vvIBDV) strains have emerged and spread worldwide

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since 1987, causing high morbidity and mortality in susceptible chickens (Lasher and Shane, 1994).

In Morocco, IBD was identified in poultry flocks in 1977. Since that time, this infection has become endemic and the introduction of very virulent strains of IBDV was reported in 1991 (Bouzoubaa et al., 1992; Kichou et al., 1999; Tahiri et al., 2011). Three Moroccan vvIBDV isolates (accession number: MN241434.1, MN241436.1, and MN241439.1) isolated in 2015 from broiler chicken farms located in three different peri-urban areas of Morocco (Temara, Tit Milil, and El Jadida) were clustered phylogenetically with vvIBDV from Africa (Nigeria and Ethiopia, Cheggag et al., 2020). These strains were isolated from field cases of IBD in broilers chickens, however, their pathogenic effects were still to be confirmed. Thus, the aim of the present study was to investigate and compare the pathogenicity of these three Moroccan of IBDV isolates in specific-pathogen-free (SPF) chickens.

MATERIALS AND METHODS

Ethical approval

The experimental protocol for the current study was conducted after the approval of the Board of Studies by the local ethical committee of Biopharma (Biopharma Ethics and Scientific Committee) directed by its veterinarian in charge; with the requirement of the welfare guidelines of the Manual of Diagnostic Tests and Vaccines for Terrestrial Animals/OIE (chapter 7.8. Use of Animals in Research and Teaching), and the Guide of good manufacturing practices (GMP) for veterinary use (Specific guidelines: LPD 4 Section 28, 29 and 30).

Viral strains

Three IBDV field strains (accession number, MN241434.1, MN241436.1, and MN241439.1), were previously isolated from the bursa of Fabricius of affected broiler chicken farms in Morocco in 2015 and stored at -80°C. Based on the comparison of the nucleotide sequence spanning, the hypervariable region (HVR) of these three IBDV strains with the genome sequences of IBDV strains isolated in different countries and available in PubMed, were identified to belong to vvIBDV strains (Cheggag et al., 2020). Phylogenetically, these three IBDV strains were grouped in the same cluster with Malaysian and European vvIBDV isolates (Cheggag et al., 2020).

The IBDV isolates were propagated by inoculation in 9 to 11day-old SPF embryonated eggs as described by Cheggag et al. (2020). For virus titration, 6-fold dilutions were inoculated into the allantoic cavity of six SPF chicken embryos, and incubated for 6 days at 37°C and relative humidity of 75 % +/- 10 %. The titer–was calculated according to the Reed and Muench method (Reed and Muench, 1938).

Specific-pathogen-free chickens

A total of 75 SPF chicks aged three weeks were used in the present experimental infection. Chickens were from the SPF chickens flock unit of Biopharma Laboratory, Rabat, Morocco. Chickens of different groups were housed in separate isolators with negative pressure, lightning program and temperature were the same for all groups. Water and food were provided *ad libitum* throughout the study. The adaptation period lasted for four days.

Pathogenicity assays

Chickens were randomly divided into four groups. The first group (n = 20) was inoculated with vvIBDV MN241434.1 strain, the second group (n = 20) was inoculated with vvIBDV MN241436.1 strain while the third group (n = 20) was inoculated with vvIBDV MN241439.1 strain. The last group inoculated with Phosphate Buffered Saline (PBS) was considered a negative control (n = 15). Chickens were labeled via leg rings for individual identification. Chickens of challenged groups were inoculated with 200 µl ($10^{3.5}$ EID_{50}) of vvIBDV strains suspension via oculo-nasal route using a micropipette. Chickens in the control group were administered the same volume of PBS.

Clinical signs and mortality were recorded daily by the same examiner during 14 days post-challenge (dpc). Clinical signs were scored as 0 (no signs), 1 (mild), 2 (moderate), 3 (severe). On days 1, 2, 3, 4, 7, 9, and 14 pc, one chicken from each experimental group was weighed and then euthanized humanely by exsanguination under ether anesthesia. Chickens that survived the challenge were euthanized at 14 dpc (two birds from group 3). Dead and euthanized chickens were necropsied and BF, spleen, and thymus were collected, weighed, and each organ was divided into two portions, one fixed in 10 % neutral-buffered formalin (NBF) for histopathological examination, and the other portions of BF were stored at -80°C until used for RNA extraction and real-time PCR (rRT-PCR) detection of the IBDV.

Blood was collected from the wing vein from chickens who were then euthanized at 0, 1, 2, 3, 4, 7, 9, and 14 dpc. Blood samples were then centrifuged at 1500 × g for 10 minutes at 4°C after clotting, the serum was

separated and stored at -20°C for the detection of antibodies against IBDV by enzyme-linked immunosorbent assay (ELISA).

Organs to body weight ratios

Organs/body weight ratios of the bursa and spleen were calculated from challenged and control chickens for comparison purposes using the following formula: Organ/body weight ratio = Organ weight (gm)/ Body weight (gm) × 1000 (Sharma et al., 1989).

Histopathological examination

Ten % NBF-fixed tissues were processed for histopathological examination according to standard methods. They were dehydrated and embedded in paraffin wax. Five µm thick sections were marked with hematoxylin and eosin and examined under a light microscope (ZEISS SIGMA, Germany).

Histopathological changes in the BF were identified and their severity assessed by the bursal lesion score (BLS) according to the method of Muskett et al. (1979), with slight modifications, and the obtained results of groups were compared. The scoring system was based on score 0 for no lesions, score 1 for 1-25% of follicles indicating lymphoid depletion (less than 50% depletion per follicle) and accumulation of heterophils, score 2 for 26-50% of follicles revealing almost complete lymphoid cell depletion (more than 75% depletion per follicle), necrosis, and accumulation of heterophils, score 3 for 51-75% of follicles show almost complete depletion of bursa follicles with necrosis and heterophils; score 4 for 76-100% of follicles showing almost complete depletion of bursa follicles with necrosis and heterophils, hyperplasia, and cysts in some cases, and score 5 for 100 % of follicles demonstrating almost complete depletion of bursa follicles with loss of bursa architecture, and fibrosis. In the spleen, microscopic lesion scores (SLS) were attributed from 0 to 5 as described by Scanavini Neto et al. (2004), 0 refers to the normal spleen (periarteriolar sheath (PAS) and periellipsoidal white pulp (PWP) without reticuloendothelial cells hyperplasy), 1 signifies hyperplastic PAS, an increased number of mononuclear like cells in the PWP, 2 shows hyperplastic PAS, increased number of mononuclear cells around the central vein, PWP and reactive PALS and heterophils in the sinusoids; 3 indicates ellipsoid cells (EC) and PAS reticuloendothelial cells are degenerated and also heterophils and mononuclear cells increase in the PWP, 4 is score 3 plus infiltration of heterophils in the PAS and PWP and also erythrocytes and hemosiderin deposition in the sinusoid, and 5 includes PAS necrosis associated with massive heterophil infiltration.

In the Thymus, microscopic lesions were assessed and thymic lesion score (TLS) was attributed according to Sharma's scale (Sharma et al., 1993). These scores were includes 0 as no lesions, 1 as mild, multifocal cortical necrosis, or mild cortical atrophy (i.e., cortex 30-40% of follicular cross-section), 2 as moderate multifocal cortical lymphocyte necrosis, 3 as moderate diffuse cortical atrophy (that is cortex 20-30% of follicular cross-section), and 4 as severe diffuse cortical atrophy (i.e., cortex < 20% of follicular cross-section).

rRT-PCR assay

Viral RNA was extracted from 150 µl of suspension of bursa using the Nucleospin RNA Virus Extraction kit (Machery-Nagel, Germany) following the manufacturer's instructions. The real-time PCR (rRT-PCR) targeted VP5/VP2 overlapping region of segment A using the specific primers and probes designed by Bioneer (Korea) as described by Tomás et al. (2012). The rRT-PCR protocol performed was as previously described by Cheggag et al. (2018).

Detection of antibodies against infectious bursal disease virus by ELISA

Serum samples were tested for antibodies directed against IBDV using commercial indirect classical ELISA kits (ID Vet, France). According to the manufacturer's instructions, samples were tested at a dilution of 1:500, and IBD immune status was considered negative if ELISA titer is less than 875.

RESULTS

Clinical signs and mortality

The three vvIBDV strains used in pathogenicity assay (MN241434.1, MN241436.1, and MN241439.1) caused 100% morbidity in the three-week-old SPF chickens at 2 dpc. Challenged chickens became depressed and showed typical signs of IBD, including ruffled feathers, watery diarrhea, depression, and prostration. In groups 1 and 2 inoculated respectively with MN241434.1 and MN241436.1, most of the chickens died at 3 dpc and the mortality rate reached 100% at 4 dpc. In MN241439.1 inoculated group, 76.47% of chickens died during the experimental period with a peak of mortality at 4 dpc (9/20). All chickens of the control group remained healthy throughout the experiment and showed no clinical signs neither mortality.

At necropsy of dead chickens at 2-4 dpc, typical pathological changes were observed, such as thigh and breast muscle petechial hemorrhages, kidney and bursa swelling, and hemorrhages. From 9 and 14 dpc, the bursa of the remaining chicks in group 3 exhibited severe atrophy (Figure 1).

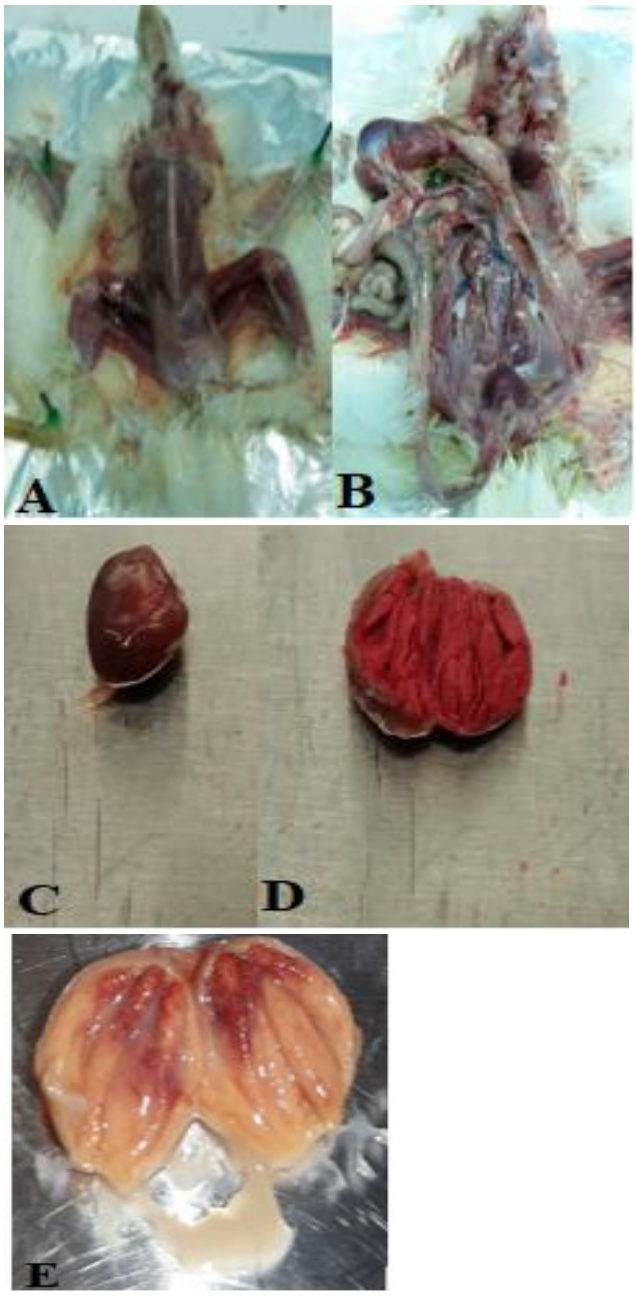

Figure 1. Pathological lesions observed in infected chickens with vvIBDV strains (accession number, MN241434.1, MN241436.1, and MN241439.1) **A:** Hemorrhages on thigh and breast muscles. **B:** Swollen and edematous kidneys with multifocal pinpoint petechial hemorrhages and edematous and hemorrhagic bursa. **C, D,** and **E:** Swollen, edematous, and hemorrhagic cloacal bursa.

Organs to body weight ratios

Means of the bursa to body weight ratio are presented in Figure 2. The bursa to body weight ratio increased in all challenged groups; however, the increase was more severe in challenged chickens of group 3 at 3 dpc. The hypertrophy of bursa observed in group 3 was followed by a severe decrease in weight, with the means of the bursa to body weight ratio going from 10.81 at 3 dpc to 0.81 at 14 dpc. In control chickens, there was no decrease in means of the bursa to body weight ratio. The means of the spleen to body weight ratio increased in all challenged groups and control chickens (Figure 3). However, the increase was more severe in challenged chickens of group 3 with a peak at 6 dpc

Infectious bursal disease virus-specific one-step rRT-PCR assay

The bursal homogenates from chickens inoculated with MN241439.1 (Group 3) at 1, 2, 3, 4, 5, 6, and 7 dpc (the length of the experimental period) revealed the presence of RNA of IBDV by rRT-PCR. In groups 1 and 2, the bursal homogenates from chickens inoculated with MN241434.1 and MN241436.1 revealed the presence of IBDV's RNA respectively at 1, 2, 3, and 4 dpc. However, IBDV's RNA was detected in bursal homogenates from chickens of the control group (Figure 4).

Antibodies against infectious bursal disease virus

Infectious bursal disease virus antibodies were detected on the 7, 9, and 14 dpc in serum samples from chickens inoculated with MN241439.1 (Group 3) with the highest antibodies concentration at 9 dpc. No virus antibodies were detected in serum samples collected from chickens inoculated with MN241434.1 (Group 1) and MN241436.1 (Group 2) and the control group (Group 4).

Microscopic lesions and scores

Bursal microscopical changes were detected in the IBDVs inoculated chickens starting at day 2 PI. From days 2 to 6 PC these changes included depletion, necrosis, and/or infiltration by heterophils of the bursal lymphoid follicles, and hyperemia and edema of inter-follicular stroma along with its infiltration by heterophils, macrophages, and/or lymph-plasmocytic inflammatory cells. The lining epithelium was disrupted in some areas and in a few plicae cystic cavities of variable diameters were observed in lymphoid follicles. The mean BLS at the 2 dpc was 4 in birds from groups 1, 2, and 3 in chickens of group 3. At 4 dpc, the mean BLS was 4 for the three inoculated groups (Table 1). From days 7 to 14 PC, bursal changes as detected in birds inoculated with MN241439.1

(Group 3) were marked lymphoid depletion and necrosis of the lymphoid follicles involving both cortex and medulla with fibrosis of the interstitial spaces and a complete loss of the bursal architecture. There was no intact lymphoid follicle and the remaining areas were replaced by fibrous tissue and the lining epithelium was highly corrugated (Figure 5).

At 2 dpc, lesion score (SLS) in the spleen was 2 for groups 1 and 3 corresponding to the hyperplastic periarteriolar sheath, increased number of mononuclear cells in periellipsoidal white pulp, and heterophils in the sinusoids (Table 1). For group 2, mean SLS at 2 dpc was 4 as indicated by the degeneration of ellipsoid cells and periarteriolar sheath reticuloendotelial cells (Figure 6). At 4 dpc, the mean SLS was 4 in all inoculated groups. At 9 and 14 dpc in group 3, the mean SLS was 5 as indicated by severe periarteriolar sheath necrosis associated with massive heterophil infiltration.

Microscopic changes in the thymus were mainly individual cell necrosis of thymocytes evident as vacuolated spaces in the cortex and medulla. The mean TLS was 1 at 2 dpc in groups 1 and 2 inoculated by MN241434.1 and MN241436.1 respectively, and 1 at 3 dpc in group 3 inoculated with MN241439.1 (Figure 7, and Table 1). At 4 dpc, mean TLS reached 2.33 in group 1 indicating more severe thymocytes necrosis in chickens of this group as shown by more abundant large vacuolated spaces of cortex and medulla. No histopathological changes were detected after 4 dpc in the thymus of survival chickens of group 3.

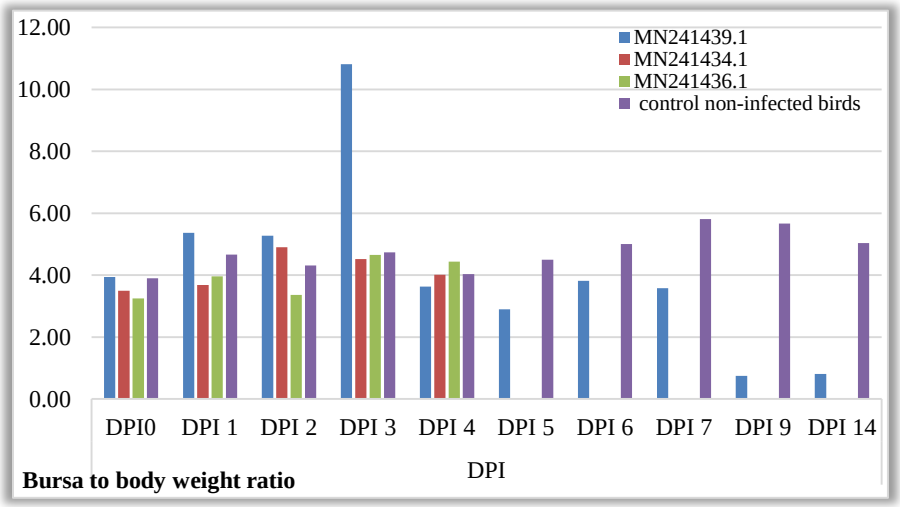

Figure 2. Means of bursa to body weight ratio in infected birds at specified time intervals and control non-infected birds

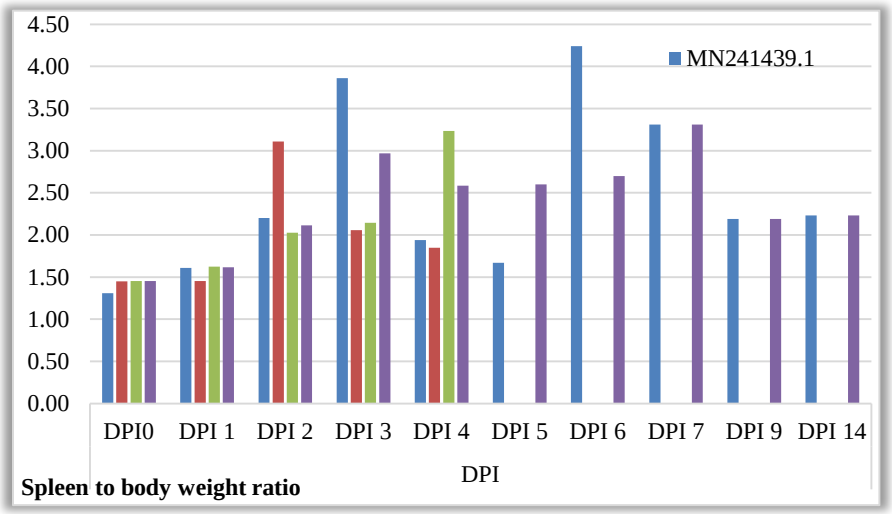

Figure 3. Means of spleen to body weight ratio in infected birds and non-infected birds

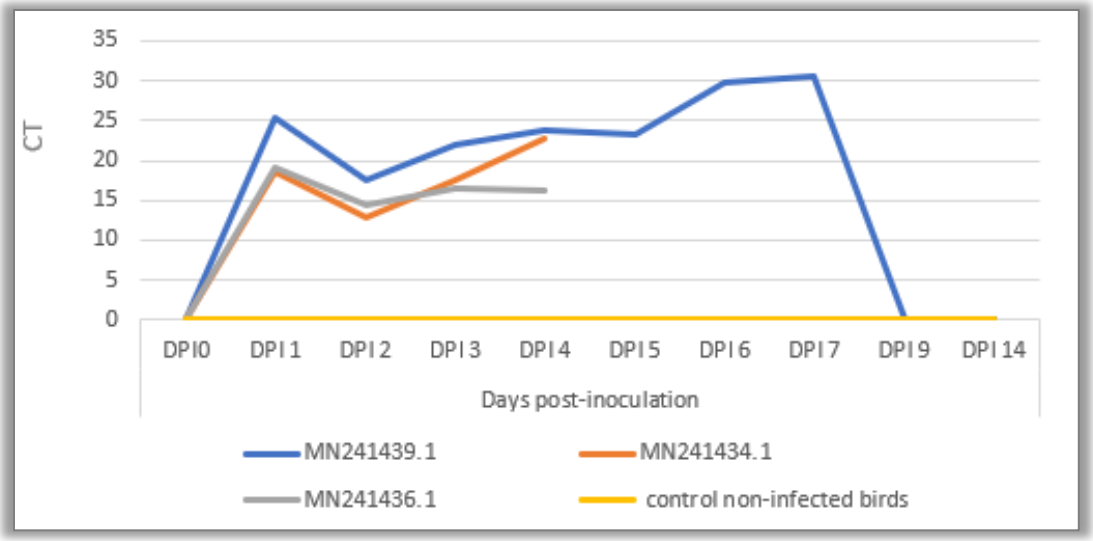

Figure 4. Cycle Threshold (CT) values at RT-PCR of bursal homogenates from birds inoculated with MN241434.1, MN241436.1, and MN241439.1 and control group

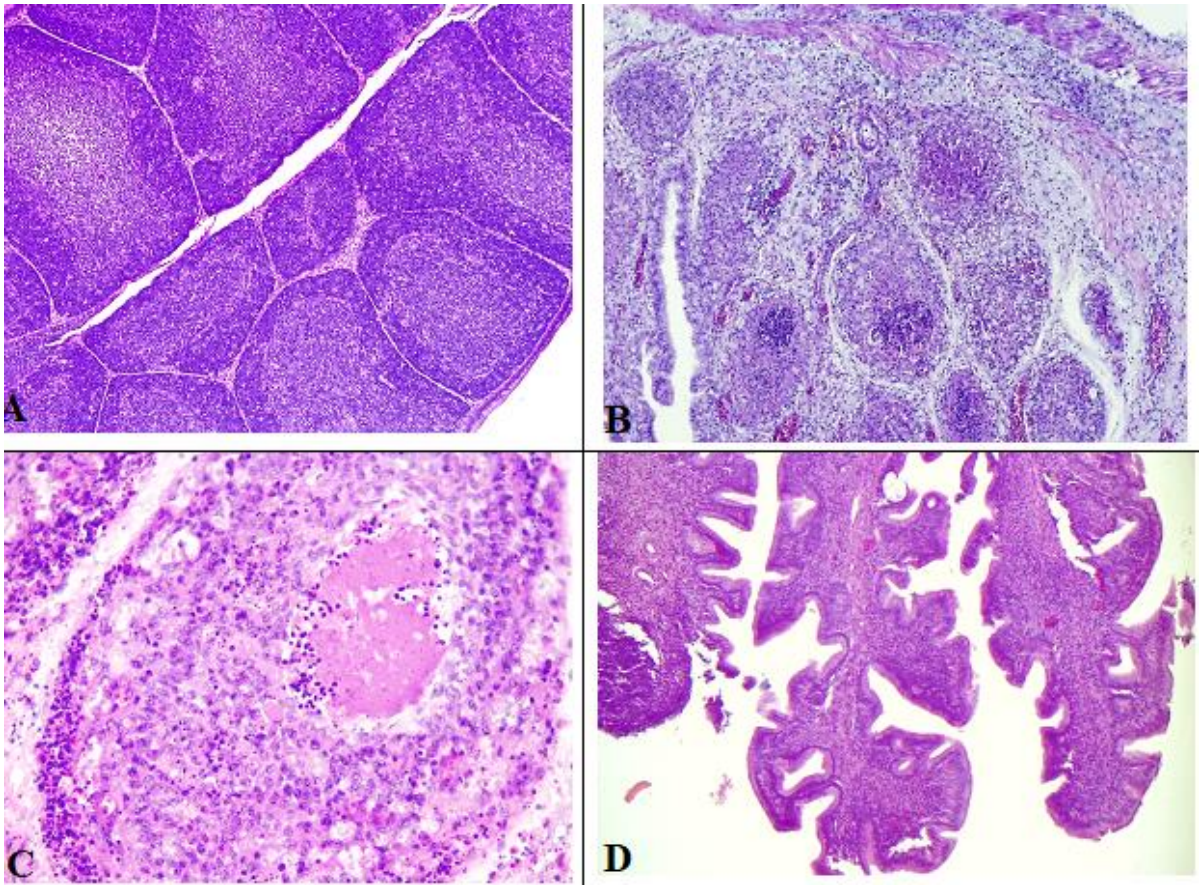

Figure 5. Histopathology of bursa of Fabricius of inoculated and uninoculated SPF chickens. **A:** Normal bursa of Fabricius from the control group. **B:** Severe depletion and necrosis of lymphoid follicles, and edema and inflammatory cell infiltrate of inter-follicular stroma in the bursa of Fabricius of a bird inoculated with MN241434.1 at 3 dpc. Original magnification x100 (H&E). **C:** A lymphoid follicle showing severe lymphoid depletion and necrosis of both cortex and medulla, infiltration by heterophils and presence of caseous material within the center of the bursa of Fabricius of a bird inoculated with MN241434.1 at 3 dpc. Original magnification x400 (H&E). **D:** Severe bursal atrophy with complete loss of the bursal architecture in a survived bird from group 3 inoculated with MN241439.1 at 14 dpc. Original magnification x40 (H&E).

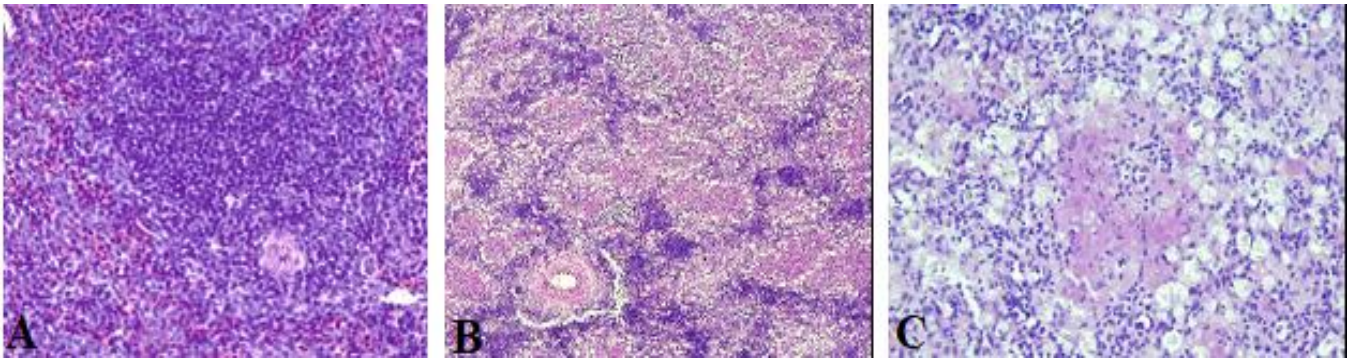

Figure 6. Histopathology of spleen of inoculated SPF chickens and uninoculated SPF chickens. **A:** Normal spleen from a bird of control group. **B:** Spleen showing extensive necrosis of periarteriolar sheaths from infected chickens with MN241439.1 at 9 dpc. Original magnification x100 (H&E). **C:** Higher magnification from B- Severe necrosis of periarteriolar sheaths and infiltration of mononuclear cells in red pulp. Original magnification x400 (H&E).

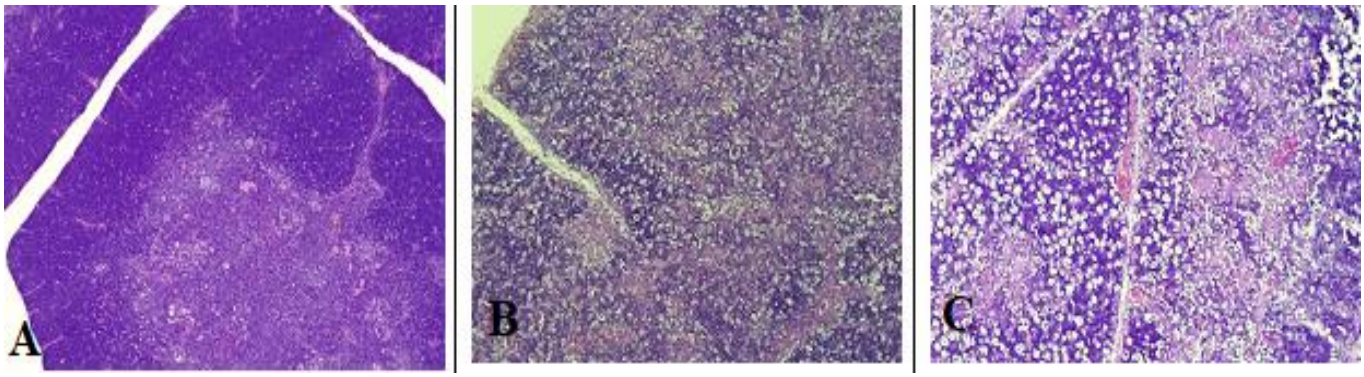

Figure 7. Histopathology of thymus of IBDV inoculated and uninoculated SPF chickens. **A**: normal thymus from bird of control group. Original magnification x100 (H&E). **B** and **C:** Severe individual cell necrosis of thymocytes as shown by abundant large vacuolated spaces in the cortex and medulla of thymus from infected chickens with MN241434.1 (B; original magnification x40) and MN241439.1 (C; original magnification x100) IBDV strains at 4 dpc and 14 dpc, respectively (H&E).

Table 1 Mean microscopic lesion scores of the Bursa of Fabricius, spleen, and thymus at days 1 to 14 post challenge in MN241434.1-group 1, MN241436.1-group 2, and MN241439.1-group 3

	Mean BLS (Number of birds)			**Mean SLS (Number of birds)**			**Mean TLS (Number of birds)**		
	G1	**G2**	**G3**	**G1**	**G2**	**G3**	**G1**	**G2**	**G3**
Day 0 PC	0 (1)	0 (1)	0 (1)	0	0	0	0	0	0
Day 1 PC	0 (1)	0 (1)	0 (1)	0	0	0	0	0	0
Day 2 PC	4 (1)	4 (1)	3 (1)	2	4	2	1	1	0
Day 3 PC	4 (14)	4 (15)	4 (2)	3.2	4.71	4	1.66	1.5	1
Day 4 PC	4 (3)	4 (2)	4 (9)	4.66	4	4.75	2.33	1	-
Day 5 PC	-	-	4 (1)	-	-	5	-	-	-
Day 6 PC	-	-	4 (1)	-	-	5	-	-	-
Day 7 PC	-	-	4 (1)	-	-	5	-	-	-
Day 9 PC	-	-	5 (1)	-	-	5	-	-	0
Day 14 PC	-	-	5 (2)	-	-	5	-	-	0

BLS: bursal lesion scores, SLS: Splenic lesion score, TLS: Thymic lesion scores, PC: post challenge

DISCUSSION

In this study, we have investigated the pathogenesis of 3 vvIBDV strains which were isolated in 2015 (Cheggag et al., 2020). An experimental infection in three weeks old SPF chickens was carried out to evaluate the pathogenicity of these three Moroccan vvIBDVs (accession number, MN241434.1, MN241436.1, and MN241439.1). The experimental IBDV infection with appropriate controls was successfully implemented in the four groups. Previous characterization of the 3 vvIBDV strains used in this study was based on molecular characterization and pathogenicity in embryonated chicken eggs and cell cultures (Cheggag et al., 2020). Consequently, there was an apparent need to ascertain their pathogenicity in vivo on SPF chickens to demonstrate their hypervirulent phenotype.

Results showed that all the three tested strains of IBDV were capable of inducing clinical signs of IBD starting at 48 hours post-challenge. Inoculated chickens were exhausted, prostrated, dehydrated, and showed aqueous diarrhea and ruffled feathers. The mortality was commenced on the third day of infection in the three inoculated groups and reached a peak at 3 dpc in groups 1 and 2 and at 4 dpc in group 3. Then, it dropped rapidly at 5 dpc in group 3, and the surviving chickens recovered a state of apparent health at 7 dpc. The mortality caused by MN241434.1 and MN241436.1 vvIBDV strains was much higher (100%) than that induced by MN241439.1 vvIBDV strains (76.47%). A mortality rate of 90–100% was also reported in SPF flocks inoculated with vvIBDV European strains (Chettle et al., 1989). The mutations revealed within the HVR of the vvIBDV strain MN241439.1. can explain the decrease of the total mortality rate observed in challenged chickens of group 3 (Cheggag et al., 2020). Mutations in the major hydrophilic peak region A ((G) at position 225 (V→G)), in the minor hydrophilic peak region ((H) at position 249 (Q→H)) and outside of the hydrophilic regions of VP2 ((P) at position 238 (T→P) and (T) at position 353 (A→T)) which were detected in MN241439 were considered to influence IBDV antigenicity (Durairaj et al., 2011; Jackwood and Sommer-Wagner, 2011; Cheggag et al., 2020). Gross lesions shown on infected chicks included petechial hemorrhages in skeletal muscles, kidneys, and bursal swelling. In conclusion, clinical signs, gross and histopathological lesions observed in this experimental infection are in accordance with the findings described for vvIBDV infection investigated elsewhere (Chettle et al., 1989; Jackwood et al., 2011; Ingrao et al., 2013).

The severe decrease in the means of bursa to body weight ratio observed in challenged chickens of group 3 with survival chickens was compatible with bursal atrophy previously described by other researchers as shown by a decrease in means of bursa to body weight ratio in chickens inoculated with vvIBDV strains (Tanimura et al., 1995; Hoque et al., 2001) reported that the means of the spleen to body weight ratio increased in the group challenged with vvIBDV, this increase was also observed in the present study in all challenged groups. BF increase in size and weight during 3-4 dpc could be due to the presence of edema and hyperemia (Lukert and Saif, 1997) and spleen showed reduced size due to germinal centers and perivascular sheaths necrosis (McFerran, 1993). Reduced size of BF at 9 and 14 dpc, compared to 3 dpc can be related to loss of lymphoid tissue due MN241439.1 strain virus infection. The enlargement of the spleen at 2 dpc in group 1, 3 dpc in group 3, and 4 dpc in group 2 can be related to the reactive white pulp due to antigenic stimulus (Cheville, 1967).

In the current study, ELISA test was used to evaluate levels of antibody (Ab) against IBDV in three infected groups. An anti-IBDV was detected at 7, 9, and 14 dpc in serum samples from birds inoculated with MN241439.1 (group 3) with the highest Ab anti-IBDV concentration at 9 dpc. This result confirms that infected chickens, from group 3, seroconverted against IBD, after the manifestation of specific clinical signs. Indeed, Ashraf et al. (2006) showed that ELISA could be sensitive and specific for detecting IBD antibodies which titers increase from 7 dpc (McFerran, 1993; Ashraf et al., 2006).

Ab anti-IBDV were detected only in the sera of challenged chickens from day 7 pc (Okoye and Uzoukwu, 1990) for this no virus antibodies were detected in serum samples collected from birds inoculated with MN241434.1 (group 1) and MN241436.1 (group 2) and control group (group 4) because the mortality rate was 100% at 4 dpc in groups 1 and 2.

Bursal microscopical changes detected in the IBDVs inoculated birds were very severe as shown by high mean microscopic BLS thus indicating pronounced damage to the BF in all birds from the 3 IBDVs infected groups. The mean BLS was 4 in birds from groups 1 and 2 and the reported value in group 3 ranged 3-4. During the first phase of the infection from days 2 to 6 dpc, these microscopic changes were mainly depletion, necrosis of lymphoid follicles, and hyperemia, and edema of inter-follicular spaces along with its infiltration by heterophils and mononuclear inflammatory cells. During this first

phase (from days 2 to 6 PC), mortality was very high in groups 1 and 2 with no survivals after 4 dpc. In the second phase of the infection from days 7 and 14 PC in the remaining birds of group 3 inoculated with MN241439, bursal changes evolved to more chronic changes with a progressive fibroplasia and fibrosis of the interstitial spaces and a complete loss of the bursal architecture. This pathological trend in the bursa of Fabricius agrees with the one that has been previously described for vvIBDV by other authors (Tanimura et al., 1995; Sharma, 2000; Hoque et al., 2001; Nouën et al., 2006). In the thymus, microscopic changes induced by the MN241434.1 and MN241436.1 strains were found to have a higher degree of pathogenicity than MN241439.1 strain.

These two 2015-vvIBDV field strains 1 and 2 (MN241434.1 and MN241436.1) seem to be slightly more virulent than another Moroccan laboratory vvIBDV strain isolated in 2011 from SPF birds maintained in conventional non protected areas. Indeed, mortality at 4 dpc induced by strains 1 and 2 was higher (100% mortality) than that obtained by 2011-vvIBDV (90% mortality) after experimental infection in SPF chickens (Tahiri et al., 2011). This finding indicates that field strains of IBDV may continue to gain in virulence despite vaccination programs implemented by the industry which call for more efforts to alleviate the burden of this very contagious infectious disease of broilers.

Based on the comparison of the high mutating VP2 variable domain (vVP2) sequence together with epidemiological observations and mortality studies, it has been suggested that European vvIBDVs, and Asiatic very virulent strains belong to the same and common very virulent genetic lineage (van den Berg et al., 1996; Eterradossi et al., 1997; Yamaguchi et al., 1997). However, there is a significant difference between these strains and the African highly pathogenic strains of IBDV isolated in the late 1980s, indicating independent evolution (van den Berg, 2000). Furthermore, there was strong evidence that vvIBDV-related strains differ genetically and/or phenotypically from the vvIBDV reference strains isolated in the late 1980s (Hoque et al., 2001; Eterradossi et al., 2004; van den Berg et al., 2004) and seemed to have evolved from this same initial vvIBDV clone and resulted in the emergence of new and diversified vvIBDV-related virus strains (Nouën et al., 2006).

Genetic characterization by sequencing and analysis of the HVR of the VP2 gene of the 3 vvIBDV isolates used in the present study and other recently identified Moroccan strains of IBDV (Drissi Touzani et al., 2019) led to the classification of strains into genogroup 3 (predominantly vvIBDV) with VP2 nucleotide sequence similarity of 96.2-100% (Cheggag et al., 2020). Furthermore, the phylogenetic analysis showed that all the Moroccan vvIBDV field strains were grouped in the same cluster with the African vvIBDV isolated in Nigeria and Ethiopia, which suggested their ancestral relationships. Moreover, in a comprehensive phylogenetic analysis of the hvVP2 of IBDV strains worldwide, a Moroccan isolate of IBDV was classified as a member of the genogroup 1 (predominantly classical), which included most US strains and other strains from Central and Latin America, Europe (France, UK, Russia), Algeria, and Egypt but without focusing on the pathotypic profile of tested viruses (Michel and Jackwood, 2017). This indicates that the diversity of the IBDVs circulating in Morocco is still questionable and under-evaluated, which needs further investigations.

CONCLUSION

The obtained results of the current study show clearly that the present local field IBDV strains (accession number, MN241434.1, MN241436.1, and MN241439.1), which have been genetically characterized as vvIBDV, induced very high mortality and severe pathological changes in SPF chickens. Therefore, these strains reflect the epidemiological Moroccan IBD situation. This constitutes a potential candidate reference strains to be used in the development of strategies for the prevention and control of IBD in the Moroccan poultry field.

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Competing interests

All authors declare no competing interest.

Consent to publish

Not applicable.

Availability of data and materials

The manuscript contains all datasets generated and/or analyzed in the current study.

Authors' contributions

The conceptualization of this paper was carried out by Maryame Cheggag, Ghizlane Sebbar, Khalil Zro, Loutfi Chafiqa, Mohamed Mouahid, Mohammed El Houadfi, and Faouzi Kichou. The pathogenicity assays and formal

analysis was performed by Maryame CHEGGAG, Ghizlane SEBBAR, Khalil ZRO, Loutfi Chafiqa, Mohamed MOUAHID, Mohammed EL HOUADFI, and Faouzi KICHOU. Maryame CHEGGAG wrote the first draft of the manuscript. Faouzi KICHOU, Ghizlane SEBBAR, Mohamed MOUAHID, Mohammed EL HOUADFI edited the manuscript prior to the submission. All authors read and approved the final manuscript.

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Ethical considerations

Ethical issues (including plagiarism, consent to publish, misconduct, data fabrication and/or falsification, double publication and/or submission, and redundancy) have been checked by the authors.

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