Bursa atrophy at 28 days old caused by variant infectious bursal disease virus has a negative economic impact on broiler farms in Japan

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ABSTRACT
Infectious bursal disease (IBD), caused by IBD virus (IBDV), is highly contagious, immunosuppressive and causes a negative economic impact on poultry industry. IBDV-vaccinated broiler farms at south Kyushu, Japan had a bursa-to-bodyweight ratio (BB ratio) reduction at 28 days (d) old, followed by high mortality 30 d later. We analysed the influence of the IBDV on atrophy of the bursa of Fabricius (BF) and the subsequent mortality after 30 d. Ten broilers were sampled at each timepoint from the farm with high mortality at 21, 25, 28 and 35 d. A second flock from the same farm was sampled at 14, 21, 25, 28, 35 and 42 d. IBDV was detected in BF samples at 25, 28 and 35 d and at 21, 25, 28 and 35 d in the first and second flocks, respectively, using immunohistochemical staining and RT-PCR. IBDV isolates from both flocks were closely related to the China KM523643 strain. Histopathology and TUNEL assay indicated apoptosis, severe lymphoid depletion, vacuoles within follicles, lymphoid follicle atrophy and fibrosis in the BF. We observed 75% of the polyserositis and 10% of the airsacculitis at 30 D in dead broilers. The antigenic variant IBDV infection was appeared to be the main influencing factor on BF atrophy and BB ratio reduction in the broilers. High mortality in the broilers after 30 d could be due to secondary infection. The disease caused by IBDV had a negative economic impact in the farm.

RESEARCH HIGHLIGHTS
- New variant IBDV caused bursa atrophy and reduced BB ratio in 28-day-old broilers.
- After vIBDV had infected broilers, at 21 days old they became immunosuppressed.
- High mortality at 30 days old in broilers was due to secondary infection.
- New vIBDV has a negative economic impact on broiler farms in Japan.

Introduction
Infectious bursal disease (IBD), known as Gumboro disease, is a highly contagious infectious disease that usually occurs in young chickens at 3–6 weeks of age after maternal antibody has waned (Kumar et al., 2000; Eterradossi & Saif, 2008; Badij et al., 2016). IBD virus (IBDV) belongs to the family Birnaviridae and genus Avibirnavirus. The primary target cells for IBDV are lymphocytes in the bursa of Fabricius (BF), resulting in lymphoid depletion and destruction in the BF. Consequently, the chickens become immunosuppressed and more susceptible to other viral and bacterial infections. IBDV infections can cause high mortality in chicken flocks and a negative economic impact on the poultry industry (Müller et al., 2003; Eterradossi & Saif, 2008).

IBDV strains have been classified into serotype, pathotype and genotype based on the detection methods used. There are two distinct serotypes: serotype 1 viruses are pathogenic in chickens and serotype 2 viruses are avirulent for chickens (Müller et al., 2003; Eterradossi & Saif, 2008). The pathotypes can be classified as classic virulent (cIBDV), subclinical (sIBDV) & very virulent (vIBDV) (van den Berg, 2000). Michel & Jackwood (2017) described the classification of seven major genogroups of IBDV. There are six genogroups: 1 (classical viruses), 2 (antigenic variant viruses), 3 (very virulent IBDV), 4 (distinct IBDV), 5 (recombination between classical and variant viruses), 6 (93% identity to the ITA genotype observed in Italy), and 7 (IBDV from Australia as well as from Russia). The degree of pathogenicity of IBDV for chickens depends on the virus strain (Tsukamoto et al., 1992).

In Japan, the first IBD outbreak occurred in 1967, and vIBDV outbreaks with high mortality occurred in many broiler and layer flocks from 1990 to 1991.
(Tsukamoto et al., 1992). Yamaguchi et al. (2007) showed that IBDV from the samples of BF collected during 1996–2004 was of cvIBDV and vvIBDV types. IBDV infection in commercial poultry farms throughout Japan is a major economic problem.

In Japan, some IBDV-vaccinated broiler farms at south Kyushu had atrophy of the BF lymphoid follicles and remarkable reduction of bursa-to-bodyweight ratios (BB ratios) (less than 0.03) at 28 days (d) old. The subsequent mortality on these farms became high after 30 d of age. Consequently, these farms became low performance broiler (LPB) farms. The BB ratios of broilers from good performance broiler (GBP) farms were 0.2 or higher at around 28 d of age and the mortality rate was low in the high BB ratio farms.

There are several causes for BB atrophy and BB ratio reduction on poultry farms. In addition to IBDV, chicken infectious anaemia virus, Marek’s disease virus, Reovirus, Newcastle disease virus, Escherichia coli (E.coli) and Mycotoxicosis infections were also associated with BB atrophy and lymphoid depletion in the BF (Hoerr et al., 1982; Nakamura et al., 1986; Wang et al., 2007; Ett eradossi & Saif, 2008; Jones, 2008; Schat & Santen, 2008; Adi et al., 2010; Cazaban et al., 2015; Berthault et al., 2018).

A new antigenic variant IBDV (vIBDV) strain was considered as one of the main causes for the BB ratio reduction and BF atrophy because the vIBDV was detected at 28 d of age during a preliminary survey of broiler farms. We investigated the involvement of IBDV infection in the LPB farms. The aim of this study was to investigate the negative impact of IBDV infection-induced BF atrophy, BB ratio reduction and the subsequent high mortality in broilers after 30 d of age on a LPB farm using histopathological and molecular techniques. Genetic sequence analysis was also conducted to determine the genotype of the IBDV strain. This study has demonstrated through detailed pathological and genetic analyses that the new vIBDV invasion causes BF atrophy and affects mortality on the LPB farm. Information on this virus will hopefully help with the control and prevention of the infection.

Materials and methods

Farm information for surveillance of BB ratio

The 2012–2017 surveillance of BB ratios and mortality was carried out on IBDV-vaccinated broiler farms, which are located at south Kyushu, Japan. Based on the BB ratios and mortality, six broiler farms, which included three GBP farms (Farm D, E, F) and three LPB farms (Farm A, B, C), were chosen for this study. IBDV detection from BF samples of the six broiler farms was done by RT-PCR and sequence analysis. Based on the results of BB ratio reduction at 27–29 d and the subsequent high mortality at 35 d of age, one of new LPB farms was chosen for further analysis. A chronological investigation was conducted on two flocks: the first flock in November 2018 and the second flock in January 2019. Taishu vaccine (Kyotobiken, Uji, Japan) was used at 14 and 21 d for IBDP prevention in both flocks on this farm.

Sample collection

Samples of 10 vaccinated broiler chickens (Ross 308 breed) were collected at 21, 25, 28 and 35 d from the first flock and at 14, 21, 25, 28, 35 and 42 d from the second flock on the LPB farm. The 10 vaccinated broiler chickens collected at each sampling included five normal growth (NG) and five poor growth (PG) broiler chickens. These chickens were necropsied after euthanasia and examined for gross lesions. BF samples from the chickens were collected for both histopathological and molecular tests. Thus, 40 broilers from the first flock and 60 broilers from the second flock were used in this study. In addition, 100 dead broiler chickens were also necropsied for gross lesions between 32 and 46 d of age, at the time high mortality was observed in the second flock. The Chicken Pathologic Autopsy Ethics Committee of the broiler company, Japan (2019-1) approved this experiment procedure.

Histopathological examination

The BF samples were fixed with 10% buffered formalin and then processed for paraffin embedding. After making 4 µm paraffin sections, these tissues were stained with haematoxylin and eosin (H&E). The histopathological lesion scoring system for the BF lesions was determined based on the degree of lymphoid depletion: 0 = no significant lesions; 1 = less than 24%; 2 = between 25% and 49%; 3 = between 50% and 74%; 4 = between 75% and 100% lymphoid depletion in affected follicles; 5 = atrophy of follicles, BF plicae and vacuoles within follicles with lymphoid depletion.

Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling (TUNEL) assay

For the detection of apoptotic lesions in the BF, the TUNEL assay was conducted by using a commercial kit (Apophtosis in situ detection kit, Wako, Japan) according to the manufacturer’s instructions. After the deparaffinizing and rehydrating steps, these slides were immersed in the protein digestion enzyme solution at 37°C for 10 min. After washing with phosphate buffered saline (PBS), the slides were labelled with Terminal deoxynucleotidyl transferase solution at 37°C for 10 min. After washing with phosphate buffered saline, they were inactivated with 3%
hydrogen peroxide solution for 5 min, washed with PBS again and labelled with horseradish peroxidase-conjugated antibodies at 37°C for 10 min. Colour development for apoptotic cells was done by using 3,3′-diaminobenzidine substrate solution at room temperature for 5 min. Haematoxylin was then used for counter-staining. The scoring system for TUNEL assay was as follows: 0 = no stained cells; 1 = less than 50%; 2 = greater than 51% stained cells within the cortex or medulla of follicles; 3 = less than 30%; 4 = 31–50%; 5 = above 51% stained cells within both the cortex and medulla of follicles.

**Immunohistochemistry (IHC) test procedure**

The BF tissue slides were deparaffinized and dehydrated. Antigen retrieval was done in citrate buffer solution (pH 6) for 12 min at 110°C using a microwave. After blocking endogenous peroxidase activity with 3% hydrogen peroxide solution, these slides were incubated with Blocking One solution (Nacalai Tesque, Kyoto, Japan) to avoid non-specific reactions. The monoclonal IBDV antibody (IBDV9, HyTest Ltd, Turku, Finland) was used as a primary antibody and the Histofine Simple Stain MAX PO (Multi) (Nichirei Biosciences, Tokyo, Japan) was used as a secondary antibody. Diaminobenzidine solution was applied as a substrate and haematoxylin as a counter-stain. The scoring system for IHC was as follows: negative: no lymphocytes stained; 1+: one to two lymphocytes stained; 2+: less than 30% lymphocytes stained within lymphoid follicles; 3+: above 31% lymphocytes stained within lymphoid follicles.

**Reverse transcription-polymerase chain reaction (RT-PCR) and nucleotide sequence analysis**

Total RNA extraction was done from 250 µl of sample homogenate by using ReliaPrep™ RNA Cell Miniprep kits (Promega Corporation, WI, Madison, USA) according to the manufacturer’s instructions. IBDV was detected by AccessQuick™ RT-PCR kit (Promega Corporation). The IBDV forward primer (5′ACCTTCCAAGGAA GCCGTGAGTG-3′) and reverse primer (−5′ATCACGTCC AAGTTG CTCACC-3′) were used and the amplicon size was 720 base pairs (Pikula et al., 2017). The RT-PCR procedure was modified from Diep et al. (2015). The reverse transcription step for IBDV detection was conducted at 45°C for 45 min followed by incubation at 94°C for 2 min. For the PCR, 35 cycles of 94°C for 30 s, 61°C for 1 min and 72°C for 1 min were conducted. The terminal extension of PCR was 72°C for 10 min (Pikula et al., 2017). The RT-PCR products were visualized on a 1.2% agarose gel. The sequencing procedure was performed, as previously described by Diep et al. (2015).

**Data analysis**

The formula of BB ratio = [BF weight in gram/body weight in gram] × 100 was described by Cazaban et al. (2015). The difference in mortality between the low and high BB ratio farms was analysed by Student’s T-test. The difference in number of broilers with airsacculitis between 21, 25, 28 and 35 d groups was analysed by Chi-square test. All analyses were performed using computer programming language R (version 3.4.3; R development core team, Vienna, Austria).

**Results**

**Low BB ratio and high mortality in IBDV-vaccinated broiler farms from 2012 to 2017**

The BB ratio was examined in broilers raised on farms where IBD vaccines were used at south Kyushu, Japan. Some broiler farms used intermediate virulence (IV) vaccines at 14 or 18 d of age and some broiler farms used mild virulence (MV) vaccines at 14 and 21 d for IBD prevention. The BB ratio on some MV-vaccinated broiler farms was found to be significantly reduced compared to that of IV-vaccinated broiler farms, as shown in Figure 1. The average BB ratios at 27–29 d in IBDV-vaccinated broiler farms are shown in Figure 1. In 2017, BB ratio of MV-vaccinated broiler farms dropped severely from at least 0.2 in 2012 to 0.03 with BF atrophy. Figure 2 shows the broiler farm with low BB ratio did not show high mortality at 28 d demonstrating BF atrophy, but the mortality was significantly increased at 30 d.

**Different BB ratios and mortality on broiler farms**

Six different broiler farms (Farms A, B, C, D, E and F) were chosen for the analysis of mortality and BB ratios. We also determined the genotype of the IBDV strains detected on these farms. Farm A had BB ratios of 0.03, severe BF atrophy and high mortality. Farms B and C were located near Farm A where the TY2 variant IBDV strain was isolated in 2002. These farms had low BB ratios and high mortality. Both Farms D and E showed good performance. Farm F had high BB ratios and low mortality and was GPB farm. The broiler populations and distances between these six broiler farms are shown in Table 1.

The new antigenic variant IBDV (vIBDV) strains like China KM 523643 were identified from Farms A, B, C in 2017 and Farm D in 2018. One classical IBDV strain, the same as Canada EF138968, was identified from Farms E and F in 2017 and 2018,
respectively. One representative IBDV strain from each of Farms A, B, C, D, E and F was selected for phylogenetic analysis of IBDV.

When three GPB farms with high BB ratios and three LPB farms with low BB ratios at 27–30 days of age were analysed, the mortality of the low BB ratio broiler farms was significantly higher than that of the high BB ratio broiler farms at 31–49 d (P < 0.05) (Table 2). One of the new LPB farms with low BB ratios and high mortality was located near Farm A and was chosen for further investigation in detailed chronological analysis as the LPB farm. This new selected LPB farm was another separated farm and not involved in Farms A, B, C, D, E and F.

Chronological comparison for mortality and BB ratios between GPB farm and the LPB farm

Two flocks on the LPB farm were examined in comparison with a GPB farm, Farm F. The BB ratios of the first and second broiler flocks at 28 d of age were 0.11 and 0.06, respectively, in comparison with 0.27 BB ratio in the GPB farm. The mortalities at 30–50 d of the first and second flocks were 5.7% and 10.4%, respectively, in comparison with 1.06% low mortality in the GPB farm. The first and second flocks of the LPB farm with low BB ratios encountered a higher mortality than the GPB farm at the 35–50 d. The differences in the mortality and BB ratios between the GPB farm and the LPB farm were compared in Figure 3.

Table 1. Six different broiler farms for comparison of BB ratio and mortality by the impact of IBDV infection.

| Broiler farm | Different or same house/farm | Number of broilers in the house | Number of broilers in the farm | Distance | GPB/LPB farm |
|--------------|------------------------------|---------------------------------|--------------------------------|----------|--------------|
| A            | Different house in the same farm which showed 0.03% BB ratio and high mortality after 30 d | 26,000                           | 269,000                        | -        | LPB           |
| B            | House near the farm where TY2 was isolated in 2002 | 18,000                           | 72,000                         | 15 km    | LPB           |
| C            | Another house near the farm where TY2 was isolated in 2002 | 20,000                           | 80,000                         | 14 km    | LPB           |
| D            | New farm (first chick placement) | 27,000                           | 188,000                        | 6 km     | GPB           |
| E            | House in small-scale farm | 10,000                           | 50,000                         | -        | GPB           |
| F            | House in high rank farm (small-scale farm) | 11,000                           | 33,000                         | 12 km    | GPB           |

Note: LPB farm - Low performance broiler farm, GPB farm - Good performance broiler farm.

Figure 1. The BB ratio on IBDV-vaccinated broiler farms at 14, 18 and/or 21 d old from 2012 to 2017. The oval shape around the 2017 data points indicates that mild virulence IBDV-vaccinated broiler farms showed severe BB ratio reduction to a value of 0.03.

Figure 2. Low BB ratio broiler farm showing high mortality at 35–49 d old in the farm vaccinated with mild virulence IBDV in 2017. Each bar represents the mortality in each day of this farm. The solid dot is the BB ratio which reduced at 28 d old broilers. The figure shows the mortality on this farm increased 35 d later after BB ratio had reduced significantly at 28 d old. The standard minimum BB ratio of broilers is 0.11 (Cazaban et al., 2015).
Clinical signs and gross lesions on the LPB farm

The broilers showed slight sneezing at 21 d of age in the LPB farm. Only BF atrophy, and atrophy with some congestion were grossly found in the first and second flock, respectively.

Airsacculitis, which indicates inflammation caused by the secondary bacterial infection, usually *E. coli*, was seen at 25, 28 and 35 d in the first flock and at 14, 21, 25, 28, 35 and 42 d in the second flock. In the first flock, the number of broilers with airsacculitis at 28 d was significantly higher (*P* < 0.05) than at 21 and 25 d. In the second flock, there was no significant difference in the number of broilers with airsacculitis between the age groups (*P* > 0.05). The number of broilers with airsacculitis from the two flocks is shown in Supplementary Table 1.

Histopathological findings and evaluation of the BF

The histopathological evaluation of the BF showed that there were no obvious lesions in 21-d-old broilers of the first flock. Lymphoid depletion, vacuoles within follicles and follicular atrophy were not seen in 14 D old broilers of the second flock. Severe apoptosis of lymphocytes in the BF occurred at 25 and 21 d of the first and second flock, respectively. Apoptotic lesions were gradually reduced in older age birds of both flocks. Severe lymphoid depletion in the BF started at 25 d and was most severe at 35 d of the first flock. In the second flock, lymphoid depletion in the BF was found at 21–42 d, where lymphoid depletion was most severe at 28 d.

In both flocks, reticular cells and macrophages replaced lymphocytes in the lymphoid follicles during lymphocyte depletion. Then, vacuoles and cystic cavities appeared within the lymphoid follicles. Follicular atrophy and loss of lymphoid follicles were seen from 28 to 35 d in the first flock and from 21 to 42 d in the second flock. Infolding epithelium into damaged follicles of the BF was found from 28 to 35 d in the first flock and from 25 to 42 d in the second flock. Fibrous tissue infiltrations between follicles of the BF were seen from 25 to 35 d in the first flock and between 21 and 42 d in the second flock. The data and scores for histopathological evaluation of the BF from both flocks are shown in Table 3. The histopathology of the BF from different age groups for the second flock is shown in Figure 4.

Comparing the severity of the lesions in each group of NG and PG broiler chickens, PG chickens had more severe damage to lymphoid follicles than NG chickens of both flocks.

TUNEL assay results

The lymphocyte apoptotic lesions of the BF were examined by the TUNEL assay in both flocks. Two tissue sections from each group were selected for

### Table 2. Broiler farms with low BB ratio showed high mortality.

| Broiler farm | BB ratio at 27–30 d | Mortality at 31–49 d (%) |
|--------------|---------------------|-------------------------|
| A            | 0.04                | 23.6                    |
| B            | 0.06                | 12.5                    |
| C            | 0.04                | 18.3                    |
| Average      | 0.05                | **18.1**                |
| D            | 0.15                | 3.0                     |
| E            | 0.17                | 2.9                     |
| F            | 0.27                | 1.6                     |
| Average      | 0.20                | **2.5**                 |

*The data within the same column with the different superscripts are significantly different at (*P* < 0.05).
The TUNEL assay appeared clearer than with H&E staining. The TUNEL assay-stained apoptotic cells were found in all broiler groups of both flocks. The highest lymphocyte apoptotic lesion scores were found at 25 d in the first flock and at 21 d in the second flock. The TUNEL results for apoptotic cells in the BF from the second flock are shown in Figure 5.

**IBDV detection by IHC**

In the first flock, there were no IHC-labelled cells in the BF at 21 d. At 25, 28 and 35 d, IBDV-positive cells were observed in lymphocytes and macrophages of the BF. The IHC-positive score for IBDV in the BF was highest at 28 and 35 d.

In the second flock, IBDV antigen was detected in lymphocytes and macrophages of the BF at 21, 25, 28 and 35 d. The IHC-positive scores for IBDV in the BF were highest at 21 d. However, there were no immunolabelled cells in the BF at 14 and 42 d. The IHC staining of the BF for the different age groups is shown in Figure 6.

**Detection of IBDV in BF by RT-PCR**

In the first flock, IBDV was detected in the BF samples by RT-PCR at 25 and 28 d of age, and at a low level at 35 d, but was absent at 21 d. In the second flock, IBDV RT-PCR detected positive samples at 21, 25 and 28 d,

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**Table 3. Histopathological evaluation of BF.**

| Histopathological lesions                                      | Flocks                        |
|---------------------------------------------------------------|-------------------------------|
|                                                               | First flock                  | Second flock                |
|                                                               | 21*  25*  28*  35*           | 14*  21*  25*  28*  35*    |
| Apoptosis                                                     | 0/10  7/10  10/10  10/10     | 5/10  10/10  9/10  9/10    |
| Average apoptosis score                                       | 3.2  2.3  1.8  1.5           | 3.1  2.2  2.3  1.6         |
| Lymphoid depletion                                            | 0/10  8/10  10/10  10/10     | 0/10  9/10  10/10  10/10   |
| Average lymphoid depletion score                              | 2.7  4.6  4.9  3.5           | 3.9  4.5  3.8  3.4         |
| Infolding epithelium into damaged lymphoid follicles          | 0/10  1/10  5/10  8/10       | 2/10  1/10  6/10  9/10     |
| Vacuoles within follicles                                     | 0/10  0/10  7/10  9/10       | 0/10  3/10  6/10  7/10     |
| Cystic cavity within follicles                                | 0/10  4/10  1/10  5/10       | 1/10  1/10  6/10  6/10     |
| Lymphoid follicle atrophy                                     | 0/10  1/10  9/10  10/10      | 0/10  5/10  4/10  6/10     |
| Note: No. of IBD histopathological lesions present in broilers/no. of detected broilers. |
| *days of age.                                                 |                               |                               |

Figure 4. Histopathological appearance (H&E) of the BF in different age groups at 14, 21, 25, 28, 35, and 42 d old in the second flock broilers. (a) 14 d; no obvious change; inset: no obvious change in lymphoid follicles, (b) 21 d; lymphoid depletion and fibrosis; inset: apoptosis of lymphocytes, lymphocyte necrosis with pyknotic nuclei within follicles, (c) 25 d; lymphoid depletion, lymphoid follicle atrophy, vacuoles within follicles and fibrosis; inset: apoptosis of lymphocytes, lymphocyte necrosis, macrophages and vacuoles within follicles, (d) 28 d; lymphoid depletion, lymphoid follicle atrophy, vacuoles within follicles and infolding surface epithelium into damaged follicles and fibrosis; inset: vacuoles, necrotic lymphocytes, macrophage and reticular cells within the follicles, (e) 35 d; lymphoid depletion, loss of some lymphoid follicles, vacuoles within follicles, infolding surface epithelium into damaged follicles and fibrosis; inset: vacuoles, necrotic lymphocytes, macrophage and reticular cells occupied the follicles, (f) 42 d; cystic cavities, lymphoid depletion in some follicles, some regenerative lymphoid follicles and some fibrosis; inset: regenerative lymphocytes (right), necrotic lymphocytes (left) and reticular cells were seen.
and faint positive at 35 d. However, samples from 14 and 42 d were negative (Supplementary Figure 3). The number of IBDV-positive samples by RT-PCR and IHC in each group from both flocks is shown in Table 4.

**Phylogenetic analysis of IBDV**

Phylogenetic analysis of IBDV isolates from the BF samples was carried out by following the genogroup classification of Michel & Jackwood (2017). The seven IBDV isolates from both flocks, 16 IBDV isolates from Farms A, B, C, D, E, F, and the Taishu IBDV vaccine strain were registered into GenBank. The genotype, sequence accession numbers and isolate or strains of reference for the IBDV, Taishu IBDV vaccine strain, six representative IBDV isolates from the preliminary survey LPB Farms A, B, C, D, E, F and seven IBDV isolates from this study are shown in Table 5.

Phylogenetic analysis demonstrated that the IBDV strain from the preliminary survey of LPB Farms A, B, C and D (Accession numbers from MT 215072 to

![Figure 5. TUNEL assay results for the second flock.](image)

![Figure 6. The immunohistochemical staining of BF in different age groups in second flock broilers.](image)
Table 4. IBDV detection in BF by IHC and RT-PCR tests.

|                     | No. of IBDV-positive broilers |
|---------------------|------------------------------|
|                     | 14 * | 21 * | 25 * | 28 * | 35 * | 42 * |
| First flock IHC     | 0/10 | 9/10 | 10/10| 10/10|      |      |
| RT-PCR              | 0/10 | 9/10 | 9/10 | 6/10 |      |      |
| Second flock        | 0/10 | 10/10| 10/10| 10/10| 10/10| 10/10|
|                      | 0/10 | 10/10| 10/10| 4/10 | 0/10 |

Note: No. of IBDV IHC and RT-PCR test positive broilers/no. of tested broilers.
*days of age.

Based on the partial viral protein (VP2) gene sequences of the isolates from the LPB farm, these isolates were highly homologous to China isolates, KMS23643, JX134483, MH879092 and MN485882 strain, sharing 98.5–99.1%, 96.4–97.1%, 96.8–97.5% and 97.1–97.7% nucleotide identity, respectively. The IBDV isolates from the LPB farm were not similar to the vaccine strain (MN700903), which was used on this farm. This vaccine strain was clustered into the classical IBDV genogroup. The phylogenetic analysis, based on the partial VP2 gene for IBDV, is shown in Figure 7.

Pathological findings of 100 dead broilers collected at the time of high mortality in the second flock

In gross findings, pericarditis and pericarditis were seen in 75% and ascites was found in 10% of the 100 dead broilers from the second flock at 32–46 d of age.

Discussion

During 2017, some broiler farms vaccinated with a mild-virulent IBDV from south Kyushu, Japan, had severely reduced BB ratios around 28 d of age and then high mortality followed at 30 d of age. When disease diagnosis after 35 d of age was carried out by RT-PCR during the time of high mortality, the involvement of IBDV disappeared in these broilers. However, antigenic vIBDV-like KMS23643 strain was isolated from a preliminary survey of LPB broiler farms (Farms A, B and C) before a high mortality was observed. The BB ratio of LPB farms A, B and C was severely reduced at 27–30 d. Although vIBDV infection was also found in Farm D, which was a GPB farm, a severe decrease of BB ratio with BF atrophy at 28 d was not observed because IBDV introduction in this farm occurred after 28 d.

When the mortality, based on BB ratio of the six preliminary surveys in farms A, B, C, D, E and F, was analysed, the mortality for low BB ratio farms was significantly higher than that for high BB ratio farms between 31 and 49 d. Therefore, the factors causing BF atrophy, reduced BB ratios and high mortality after 30 d of age in broilers were investigated in two flocks from one of the LPB farms.

This study indicated that the new antigenic vIBDV in Japan was the main influencing factor for BF atrophy, and BB ratio reduction, and may be followed by immunosuppression resulting in high mortality after 30 d of age in broilers. Our results showed that vIBDV caused not only severe bursa damage resulting in reduced BB ratio but also immunosuppression resulting in high mortality in broilers (Kurukulsuriya et al., 2016).

McMullin (2004) described that the normal weight of the BF in broilers was about 0.3% of the body
weight. Cazaban et al. (2015) showed that the minimum BB ratio standard of broilers at 7–42 d of age was 0.11. The BB ratio from the first and second broiler flocks of this study was lower than the new minimum standard BB ratio. In this study, the mortality of the second flock was also higher than the first flock and this is correlated with a lower BB ratio observed in the second flock.

The clinical signs of IBDV-infected broilers are soiled vent feathers, diarrhoea, anorexia, depression, ruffled feathers, trembling, severe prostration, dehydration and mortality (Eterradossi & Saif, 2008; Mahgoub, 2012). However, diarrhoea, dehydration and soiled vent feathers were not seen in our study. Eterradossi and Saif (2008) described that the severity of the lesions depends on the pathogenicity of the IBDV strains. The vvIBDV causes the most severe lesions. The variant IBDV causes no obvious clinical signs or death, although BF atrophy, thymus atrophy and muscular haemorrhage were occurred in chickens (Fan et al., 2019; Xu et al., 2020) The clinical signs and gross findings for broilers were not severe in our study, presumably because the IBDV strain from this study may be a less pathogenic vIBDV strain.

Although many studies on IBDV infection with histopathological findings have been conducted experimentally, there is limited information on the histopathological lesions from week-to-week in broilers naturally infected with IBDV. The lymphocyte apoptotic lesions in the BF were most pronounced at 25 and 21 d of age in the first and second flocks in this study. The apoptotic cells were frequently present near the IBDV antigen-presenting cells because apoptosis-inducing factor, like interferon (IFN), would induce inhibition of protein synthesis and cause apoptosis in double-stranded RNA virus infections (Lee & Esteban, 1994; Jungmann et al., 2001). Therefore, if IBDV replication increases in the B-lymphocytes of the BF, the apoptotic cell proportion would also be expected to increase. The highest apoptotic cells due to IBDV infection were demonstrated at 48 hr post-infection in chicken embryo cells (Jungmann et al., 2001) and after 2 d post-infection in the BF (Nieper et al., 1999). The apoptotic lesions indicated that IBDV was introduced before 25 and 21 d in the first and second flocks, respectively. The TUNEL assay gave a clearer indication of apoptosis lesions compared to the H&E staining in this study.

IBDV destroyed the lymphoid follicles, and lymphoid depletion occurred from 25 to 35 d of age in the broilers of the first flock, and from 21 to 42 d of the second flock. After nearly 100% lymphoid depletion had occurred, atrophy of lymphoid follicles followed. Many vacuoles and cystic lesions were observed in the follicles and infolding epithelium into the damaged lymphoid follicles was a distinct indicator of a severe BF damage. The most severe lymphoid depletion was found at 35 and 28 d in the first and second flock, respectively. During this stage, we observed the most severe lymphoid depletion and it

Figure 7. Phylogenetic tree based on the alignment of partial VP2 gene sequence for IBDV infection. It represents the IBDV isolates from the LPB farm, and the IBDV isolates from the preliminary survey of Farms A, B, C, D, E and F. The phylogenetic tree was constructed by the maximum likelihood method.
would be the peak period of BF damage. This suggests that the broilers were immune suppressed. IBDV histopathological lesions of the BF, such as numerous vacuoles and cysts, infolding epithelium within follicles and atrophy of lymphoid follicles, were more severe in PG broilers than NG broilers. PG broilers may be more susceptible to IBDV because BB ratios of PG broilers were slightly lower than those of NG broilers. Apoptosis occurs in BF due to pathological and physiological stimuli, chemical and physical agents (Arai et al., 2000). Some apoptosis, cystic lesions and infolding epithelium into lymphoid follicles were also seen at 14 d in PG broilers of the second flock before IBDV introduction. It may be due to the physiological stimuli and other pathological agents.

Rauf et al., (2011) observed that the BF damage and inflammatory response to classical IBDV were more pronounced than vIBDV. However, the inflammatory response of heterophils and plasma cells was not seen in the study. Severe lesions due to vIBDV infection were lymphoid follicle atrophy, connective tissue proliferation, infiltration of macrophages and reduced lymphocyte population in the BF (Fan et al., 2019; Xu et al., 2020). Our findings were similar to those of Sharma et al., (1989), Fan et al. (2019), and Xu et al. (2020), but histopathological findings from week-to-week were more briefly described in this study. The vIBDV caused extensive lesions in the BF but did not cause an inflammatory response.

IBDV antigens were detected in the lymphocytes and macrophages by the IHC test in this study. Previously, it was reported that IBDV antigen was detected in the cytoplasm of lymphocytes, epithelial cells and inflammatory cells, mostly macrophages, of the BF in IBDV-infected chickens (Nunoya et al., 1992; Tanimura et al., 1995; Oladele et al., 2009). IHC and RT-PCR test results for IBDV antigen detection in the BF samples were similar. According to these results, IBDV entered around 25 d in the first flock and 21 d in the second flock. After that, IBDV gradually disappeared at 35 d in both flocks. Therefore, IBDV was not detected after 35 d at high mortality on the preliminary survey farms. The introduction of IBDV in the second broiler flock was earlier than that of the first flock. It is probably due to environmental factors and insufficient IBDV maternal immunity in the second flock of broilers. Müller et al. (2003) reported that chickens were highly susceptible to IBDV at 3–6 weeks of age because the BF developed to a maximum size at this age.

The IBDV isolates from both the flocks and the A, B, C and D farms were clustered into the antigenic vIBDV genogroup and were closely related to Chinese strains of KM523643. This suggests that this antigenic vIBDV strain had been circulating in this LPB farm and is not related to the vaccine strain being used. This strain may have originated by transmission from China. This vIBDV may be circulating not only in this LPB farm but also neighbouring farms. These IBDV isolates from both flocks were new antigenic vIBDV in Japan.

Although the clinical signs, gross findings and inflammatory cell infiltration in histopathological lesions were not severe, the vIBDV strain was a likely cause of the immunosuppression and high susceptibility to secondary infections. Therefore, airsacculitis in both types of flocks, 75% pericarditis, pericarditis and 10% ascites in the 100 dead birds of the second flock were caused by secondary infections in this study and were most likely due to immunosuppression in the broilers. The number of broilers with airsacculitis in the first flock at 28 d was significantly higher than that of the others in the first flock, and 25 d from the second flock was numerically higher in the incidence of airsacculitis than 21 d. It is probable that the higher susceptibility to secondary bacterial infections started after 1 week of IBDV infection (Supplementary Table 1).

Many studies reported that airsacculitis and fibrous polyserositis in broilers were found as the main gross lesions of colibacillosis (El-Sukhon et al., 2008). Our study clearly describes the role that a new vIBDV infection played as a main influence factor on BF atrophy and reduced BB ratios in the commercial broiler farms in Japan. After vIBDV infection at about 21 d of age, the broilers most likely became immune suppressed. Consequently, high mortality was observed at 30 d and later, and was most likely due to secondary infections. The new vIBDV infection in Japan should be considered as a primary factor in the case of lower BB ratios at 27–29 d and high mortality at 30 d and later in commercial broiler farms.

**Disclosure statement**

No potential conflict of interest was reported by the authors.

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