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TITOLO DELLA TESI

PATHOGENICITY OF A NEW ITALIAN GENOTYPE OF INFECTIOUS BURSAL DISEASE VIRUS IN SPF CHICKENS

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Abstract

Infectious Bursal Disease (IBD) is a highly contagious immunosuppressive disease of chickens caused by a *Birnaviridae* (IBDV). The ITA genotype was first detected in 2011 in Italy in IBD-live vaccinated chickens. Full genome characterization confirmed ITA to be a genetically distinctive IBDV. The aim of the study was to determine the pathogenicity of the ITA genotype in SPF chickens. Birds were housed in poultry isolators and inoculated at 35 days of life with ITA or STC IBDV strains. A control group was housed in analogous conditions and inoculated with sterile water. All groups were daily observed for clinical signs up to 28 days post-inoculation (dpi). At 0, 7, 14, 21 and 28 dpi birds were bled for IBDV antibody detection by an ELISA Kit. At 2, 4, 7, 14, 21 e 28 dpi 5 birds from each of the inoculated groups, and 3 from the control group, were euthanized and subjected to necropsy. Bursal and Thymus indexes were calculated; histological sections were examined and scored; viral tissue distribution determined by qRT-PCR in the bursa of Fabricious (BF), thymus, kidney, cecal tonsils, spleen, proventriculus, harder gland and bone marrow. No clinical signs, nor mortality were recorded in any group during the study. BF of both inoculated groups showed enlargement and oedema in the acute phase of the infection (2 dpi), followed by atrophy, which lasted until the end of the trial. Microscopic lesions of the BF of ITA IBDV inoculated birds consisted in lymphocyte depletion, cystic cavities and poor regeneration process. Viral RNA was persistently detected until the end of the trial in lymphoid tissues. The study showed that ITA genotype, though it has a subclinical course, causes a severe and persistent damage of BF, therefore, its circulation in broilers might be a threat for the poultry industry.

Table of content

Introduction 6

Chapter I: Infectious Bursal Disease

1. **Etiology**
    - a. Taxonomy 8
    - b. Morphology 8
        - i. Genome 9
        - ii. Viral proteins 9
    - c. Virus Replication 10
    - d. Susceptibility to physical and chemical agents 11
    - e. Strain classification 11
        - i. Antigenicity 11
        - ii. Pathogenicity 12
        - iii. Genetic types and genogroups 12
2. **Epidemiology**
    - a. Geographical distribution 14
    - b. Natural and experimental hosts 15
    - c. Transmission 15
    - d. Morbidity and mortality 15
3. **Pathogenesis**
    - a. Pathogenesis 16
    - b. Immunosuppression 17
    - c. Clinical signs 18
    - d. Gross lesions 19
    - e. Histopathological lesions 22
        - i. Bursal histopathological lesions 22
        - ii. Thymus histopathological lesions 25

4. Diagnosis

a. Gross lesions 25

b. Virus detection/isolation 26

c. Serology 26

5. Disease control 26

Chapter II: Infectious bursal disease virus of ITA genotype (G6) reveals in SPF chickens its aggressivity for lymphoid tissues though (despite) a subclinical course 28

Chapter III: Comparative dynamic tissue distribution and shedding of infectious bursal disease virus of ITA (G6) or classical (G1) genotypes by qRT-PCR after experimental infection of SPF chickens 53

Conclusions 66

References 67

Introduction

Infectious bursal disease (IBD) is a highly contagious immunosuppressive disease of chickens caused by infectious bursal disease virus (IBDV). IBDV belongs to the family *Birnaviridae*, genus *Avibirnavirus*, and has a bi-segmented dsRNA genome (Delmas et al., 2004). There are two recognized serotypes of IBDV, designated serotype 1 and 2; only serotype 1 IBDVs have been known to cause naturally occurring disease in chickens. The primary target organ of the virus is the Bursa of Fabricious (BF) where the virus infects and destroys dividing IgM–bearing B cells. IBDV includes different antigenic and pathogenic strains. Classical (cIBDV) isolates, firstly reported in USA in the 60's (Cosgrove, 1962), cause, in receptive birds, acute disease characterized by ruffled feather, dehydration, watery diarrhea and prostration. Infection of sensitive birds with classical strains can be also characterized by absence of clinical signs and mortality in the presence of bursal damage (Abdul et al., 2013; Sreedevi et al., 2007). In the early 1980s, antigenic variants (vaIBDV) of the virus were identified in the United States (Rosenberger and Cloud, 1985; Saif, 1984); vaccine strains available at that time did not elicit full protection against the variant, which were antigenically different from the classical isolates (Heine et al., 1991). vaIBDV isolates typically do not cause clinical signs but can cause discernible immunosuppression (Jayasundara et al., 2016; Sharma et al., 1989). In the mid-1990s, very virulent (vvIBDV) strains emerged in several European and Asian countries, causing >70% mortality in sensitive chickens (Chettle et al., 1989; Eterradossi et al., 1992); these strains were shown to be mostly antigenically similar to the classical isolates (Abdel-Alim and Saif, 2001).

The high mutation rate of IBDV RNA genome and the high selection pressure generated by application of intensive vaccination programs in birds can lead to the emergence of IBDV strains with new properties allowing them to persist in immune populations (Ingrao et al., 2013).

Independently from the pathogenicity of the strain and the severity of clinical signs, IBDV infection is always associated with damage to the bursa of Fabricious and immunosuppression, often associated in field with impaired response to vaccinations and secondary infections (Sreedevi et al., 2007).

IBDV genotype ITA was first detected in 2011 in Italy in IBD-live vaccinated broilers (Lupini et al., 2016). Full genome characterization confirmed ITA to be a genetically distinctive IBDV genotype (Felice et al., 2017) and a recently proposed classification of IBDV into genogroups, placed ITA genotype into genogroup 6 together with few other strains from Saudi

Arabia and Russia (Michel and Jackwood, 2017). At the present, genetics characteristics of IBDV have not been demonstrated to be factors in pathogenicity, and formal demonstration of the pathogenicity of an IBDV isolate requires it to be experimentally inoculated in susceptible chickens in comparison with a strain of known pathogenicity (Abdul et al., 2013). Currently available epidemiological and clinical data do not allow to define the pathogenicity of ITA genotype.

To assess this point, an *in vivo* experimental study was conducted in specific pathogen-free (SPF) chickens inoculated with ITA or a classical IBDV strain. Clinical signs, mortality, gross and microscopic lesions, bursal and thymus indexes, antibody response and IBDV loads on lymphoid and non-lymphoid tissues were evaluated.

Chapter 1: Infectious Bursal Disease

1. Etiology

a. Taxonomy

Infectious bursal disease virus (IBDV) belongs to *Birnaviridae* family, genus *Avibirnavirus* (Delmas et al., 2004). Viruses of this family has genomes consisting in a bisegmented double-stranded RNA (dsRNA) (Macdonald, 1980; Mueller et al., 1979). The *Birnaviridae* family is composed of 4 genera: *Aquabirnavirus*, dsRNA viral agents of fish, mollusks and crustaceans; *Blosnavirus*, whose type species is blotch snakehead virus (BSNV); *Avibirnavirus*, of which IBDV is the only species; and *Entomobirnavirus*, which includes viruses that infects insects (Delmas et al., 2004).

b. Morphology

The IBDV virion is non-enveloped, single-shelled, with an icosahedral symmetry capsid of about 70 nm in diameter, composed of 260 trimmers of VP2 that form spikes projecting radially from the capsid (Figure 1). The peptides derived from pre-VP2 C-terminal cleavages remain associated within the virion. VP3 forms a ribonucleoprotein complex with the genomic RNA. Minor amounts of VP1 are also incorporated in the virion (SIB, 2018).

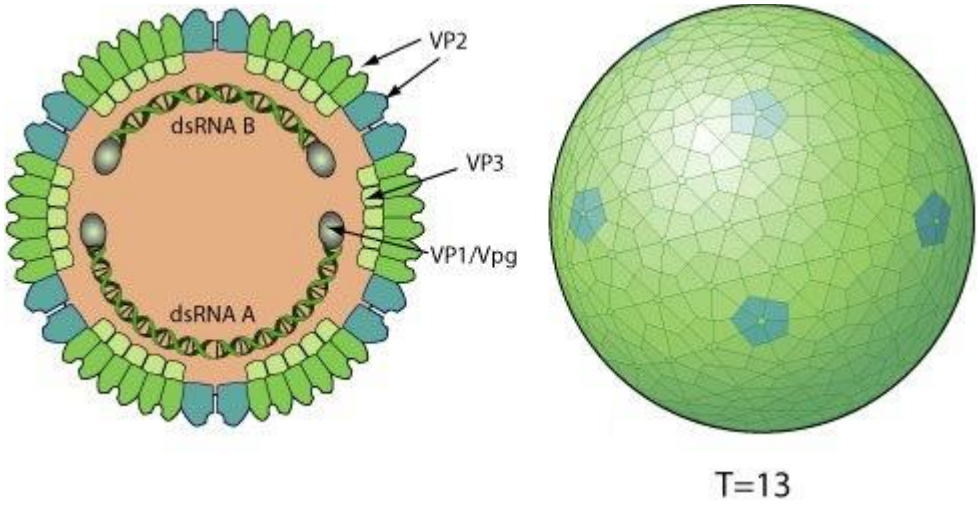

Figure 1. Morphology of an *Avibirnavirus* (SIB, 2018).

i. Genome

The genome is composed by two segments (Figure 2), A and B, of double-stranded RNA, coding for 5 viral proteins (VP): VP1, VP2, VP3, VP4 and VP5 (Eterradossi and Saif, 2013). Segment A is the larger one and consists of 3,254 base pairs (bp) that contains two open reading frames (ORF). The larger ORF encodes a polyprotein precursor (N-pVP2-VP4-VP3-C) that is self-processed from its protease in three viral premature proteins known as VP2, VP4 and VP3 (Lejal et al., 2000). A small ORF partially overlaps the previous one and encodes the VP5 protein (Ganguly and Rastogi, 2018). Segment B consists of 2,817 bp that encodes for the polymerase VP1, an RNA-dependent RNA polymerase (RdRp) (von Einem et al., 2004).

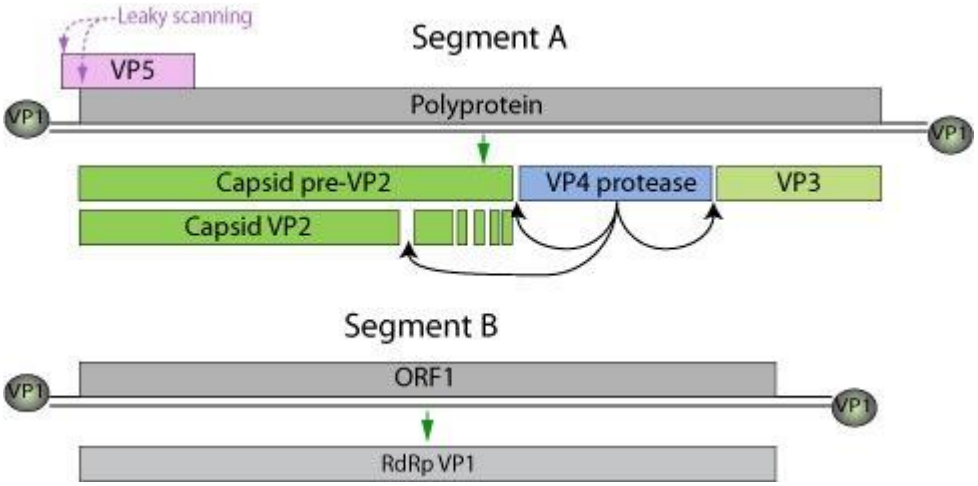

Figure 2. Organization of the IBDV genome. Segment A and Segment B and proteins encoded by them. ORF = open reading frames; VP = viral protein. Green: structural proteins; Rose: non-structural protein VP5 and Blue: protease VP4 (SIB, 2018).

ii. Viral proteins

Five viral proteins are currently recognized in IBDV: VP1, VP2, VP3, VP4 and VP5 with approximate molecular weights of 97 KD, 41 KD, 32 KD, 28 KD, and 21 KD, respectively (Qin and Zheng, 2017). VP2, VP3, and VP1 are the structural proteins of IBDV. VP4 is a non-structural protein, together with VP5 (Dobos, 1979).

VP1 is presumed to be the viral RNA-dependent RNA polymerase (RdRp), involved in replication and transcription of the virus, and exhibits an original organization as compared with other viral RdRps (von Einem et al., 2004).

VP2 is the main capsid protein and it is, until now, the most important determinant for immunogenicity by eliciting the neutralizing antibody response of the host; it represents the molecular basis for antigenic variation (Vakharia et al., 1994). It forms trimmers which are the basic units of the virus shell (Coulibaly et al., 2005).

VP3 is the internal capsid protein and the second major structural protein, it induces group-specific antibodies in the host, and interacts with all other components of the virus particles. It plays a critical role in virion morphogenesis, encapsidation, and replications (Chevalier et al., 2004; Garriga et al., 2007; Lombardo et al., 1999; Mertens et al., 2015; Tacken et al., 2000).

VP4 is a viral protease that performs cleavages to control IBDV reproduction at several levels (Birghan et al., 2000). VP4 plays a major role in the maturation of capsid protein VP2, by progressively trimming several peptides at the VP2 carboxy-terminal extremity during virus assembly (Lejal et al., 2000).

VP5 has no yet an established function but is predicted to be a structural analog of leucin rich repeat (LRR) family of proteins and to have functional implications arising from its structural similarity to host Toll-like receptor (Tlr) 3; this function relates to the dual role of the protein in first abolishing and later inducing host cell apoptosis (Ganguly and Rastogi, 2018).

c. Virus Replication

The cellular viral cycle of IBDV consists of an adsorption phase followed by viral penetration and replication in the cell cytoplasm (Yip et al., 2012). The first step of the infection occurs by the binding of the virus to specific receptors present on the surface of the host cells. The virus was shown to be able to attach to chicken embryo kidney cells 75 minutes after inoculation (Lukert and Davis, 1974). The replication cycle in chicken embryo cells last 10-36 hours and the latent period is 4-6 hours (Becht, 1980; Jackwood and Saif, 1983; Lukert and Davis, 1974; Nick et al., 1976). In VERO and BGM-70 cells, a longer (48-hour) replication cycle was described (Jackwood and Saif, 1987; Kibenge et al., 1988; Lukert et al., 1975). The internalization of the bound IBDV particles occurs by a clathrin-independent endocytosis mechanism (Yip et al., 2012) and the virus replicates its nucleic acid by a strand displacement mechanism (Spies et al., 1987).

The current proposed model for the assembly of IBDV particles involves most viral proteins, VP3 interacts with itself, pVP2 and VP1 with the viral genome, playing an important chaperone role in morphogenesis and in encapsidation (Tacken et al., 2000). The assembly process ends with the final maturation of pVP2 which cleavages the final peptide at the N-terminal level (Chevalier et al., 2004). Finally, the viral particles accumulate in the cytoplasm of the infected cells. This phenomenon is certainly promoted by the ability of VP5 to prevent apoptosis in the initial stages of infection by interfering with caspase and NF-KB (Liu and Vakharia, 2006).

d. Susceptibility to physical and chemical agents and environment persistence

IBDV is chemically and physically very stable virus and the main cause of its worldwide spread is its ability to persist in the environment, remaining infectious for 54-122 days after the removal of infected birds, cleaning and disinfection of sheds. IBDV is ultraviolet rays (UV) resistant, withstands several physical and chemical agents as ether, chloroform and acidic pH (2.0), while it is rapidly inactivated at pH 12. Chloroderivatives and glutaraldehyde inactivate IBDV quicker than formol and iodine; the phenols and the quaternary ammonium salts are scarcely effective. Studies carried out by evaluating a high number of disinfectants have shown that a 2% chloramine solution, formalin at adequate temperature, glutaraldehyde and complex disinfectants containing formaldehyde, glutaraldehyde and alcohol dimethylbezolamino chlorohydrate can be used as efficient disinfectants (Eterradossi and Saif, 2013).

e. Strain Classification

IBDV strains are classified according to antigenicity, immunogenicity, molecular characteristics and pathogenicity.

i. Antigenicity

Serotypes 1 and 2 of IBVD have been recognized and they share only 30% antigenic relatedness (McFerran et al., 1980). The 2 serotypes are differentiated by virus neutralizing (VN) tests, but not by fluorescent antibody tests or enzyme-linked immunosorbent assay (ELISA) and viruses pathogen for poultry belong to the serotype 1, while serotype 2 viruses are considered avirulent (Kibenge, 1988). Antigenic variant IBDV strains, which belongs to serotype 1, are antigenically different from classical IBDV strains (Ismail and Saif, 1991; Saif, 1984). Several amino acid changes in VP2 hydrophilic peaks are correlated with the antigenic changes observed in

the vaIBDV viruses (Heine et al., 1991; Lana et al., 1992; Vakharia et al., 1994). vvIBVD strains, first described in Europe (Chettle et al., 1989), are mostly antigenically similar to the cIBVD (Abdel-Alim and Saif, 2001; Eterradossi et al., 1992; Van Den Berg and Meulemans, 1991; Van der Marel et al., 1990).

ii. Pathogenicity

Virulent IBDV strains belong all to serotype 1, while serotype 2 strains include only avirulent strains. The terms "classical", "variant", and "very virulent" have been used to define IBDV strains that exhibit differences in pathogenicity. Based on the signs and lesions observed in two lines of White Leghorn SPF chickens, during experimental IBD following viral challenge, North American vaIBDV induced little if any clinical signs and no mortality but marked bursal lesions, cIBDV induced approximately 10–50% mortality and IBD typical signs and lesions, whereas vvIBDV induced approximately 50–100% mortality and typical IBDV signs and lesions (OIE, 2016).

vaIBDV strains do not show certain VN epitopes that are typically present on classical strains. Therefore, they can break through high levels of maternal derived antibody elicited after cIBDV exposition and cause an early IBDV infection characterized by severe bursal atrophy and consequent immunosuppression (Sharma et al., 2000).

iii. Genetic types and genogroups

Genetic characterization of IBDV strain is generally based on the sequence of the VP2 gene.

A protocol of Reverse transcriptase- Polymerase chain reaction - Restriction enzyme fragment length polymorphisms (RT-PCR-RFLP) has been developed by Jackwood and Sommer (1997) and applied for classification of IBDV strains (Jackwood et al., 2001); six genetic types were identified.

This method of classification has been recently superseded by a new classification in genogroups proposed by Michel and Jackwood (2017) based on phylogenetic analysis of the hypervariable region of the capsid protein VP2 (hvVP2).

In Table 2 classification in genogroups of reference IBDV strains is reported. Seven groups are currently recognized. cIBDV strains follow in genogroup 1, vaIBDV strains in-genogroup 2 and vvIBDV strains in genogroup 3.

ITA genotype cluster in genogroup n. 6 separately from other IBDV strains, having distinctive molecular characteristics as previously reported by Felice et al. (2017),

Table 2. Classification of IBDV reference strains in genogroup (Michel and Jackwood, 2017).

Genogroup	Previous classification	Reference strains (*accession number)
1	Classical	228E (AF457104)
		D78 (AF499929)
		F52-70 (AY321953)
		Lukert (AY918948)
		STC (D00499)
2	Variant	AL-2 (JF736011)
		DelE (AF133904)
		T1 (AF281238)
3	Very virulent	Henan (KT884486)
		HK46 (AF092943)
		OKYM (AF092943)
		UK661 (NC_004178)
4	dIBDV	dIBDV/UY/2014/2202 (KT336459)
		MG4 (JN982252)
		TY2 (LC136880)
5	Variant/classical recombinant	Mexico04M101 (DQ916210)
6	ITA genotype	ITA-02 (JN852986)
7	Australian	V877-W (HM071991)

**GenBank*

2. Epidemiology

a. Geographical distribution

IBD is reported worldwide, occurring in all major poultry producing areas of the world. Essentially, all flocks are exposed to the virus during the early stages of life, either by natural exposure or vaccination. Due to vaccination programs carried out by most poultry producers, all chickens eventually become seropositive to IBDV (Sharma, 2000). vaIBDV strains seem to be predominant in the United States (Jackwood and Sommer-Wagner, 2005) but scattered outbreaks due to vvIBDV were recorded in California (Jackwood et al., 2009). In Canada recently vvIBDV were detected (Zachar et al., 2016). In Australia cIBDV viruses (Sapats and Ignjatovic, 2000) and a local type of vaIBDV (G7) have been reported (Jayasundara et al., 2016).

In contrast, in Europe, vvIBDV seem to be the prevalent pathotype; the vvIBDV first detections date back to the 90's (Chettle et al., 1989; Eterradossi et al., 1992; Nunoya et al., 1992; Tsukamoto et al., 1992; Van Den Berg and Meulemans, 1991). However, de Wit et al. (2018) highlight the issue that surveys to attempt the detection of subclinical IBDV infections are quite uncommon in Europe. Therefore, this may lead to underdetection of IBDV strains that cause less or hardly any mortality but might cause serious immunosuppression such as the vaIBDV strains or other subclinical IBDV strains like the ITA genotype (Lupini et al., 2016; Felice et al., 2017) that could be emerging in Europe. Several reports suggest that they might be an underestimation of IBDV in Europe since mid-2000 (Jackwood and Sommer-Wagner, 2005; Letzel et al., 2007). In Brazil vvIBDV and vaIBDV seem to be circulating even in vaccinated flocks with live or recombinant vaccines (Muniz et al., 2018); while in other South American countries dIBDV strain, belonging to genogroup 4 has been frequently detected (Hernandez, 2016).

vvIBDV are prevalent also in Africa (Abed et al., 2018; Mwenda et al., 2018; Sedeik et al., 2018).

IBDV in Italy has been isolated for the first time in 1965, associated with classical forms of IBD accompanied by high mortality (Asdrubali and Franciosini, 1993). IBDV circulating strains in Italy belonged mainly to vvIBDV, followed by cIBDV and vaccinal strains (Moreno et al., 2010). A significant emergence of the genotype ITA has been reported (Lupini et al., 2016). Full genome characterization confirmed ITA to be a genetically distinctive IBDV genotype (Felice et al., 2017) recently classifies by Jackwood et al. (2017) into genogroups 6, together with few other strains from Saudi Arabia and Russia

b. Natural and experimental hosts

Only chickens develop IBD after infection by serotype 1 viruses. Turkeys may be asymptomatic carrier (Abdul et al., 2013). A serotype 1 virus was isolated from two 8-week-old ostrich chicks that had lymphocyte depletion in the bursa of Fabricius, spleen, and/or thymus (Eterradossi and Saif, 2013). Another serotype 1 isolate was obtained from healthy ducks (McFerran et al., 1980). Van den Berg et al. (2001) inoculated pheasants, partridges, and guinea fowl with a vvIBDV strain and did not report the occurrence of clinical signs or lesions referable to IBV in these species., Consistently Weisman and Hitchner (1978), were not able to produce disease in coturnix quail with a chicken-origin IBDV. Similarly, an earlier report showed that IBDV-inoculated Guinea fowl did not develop lesions or antibody (Okoye and Okpe, 1989). However, in quails the virus can replicate in BF and be shed for five days after infection (Van den Berg et al., 2001).

Several species of free-living and captive birds of prey were examined by serology for antibodies to IBDV, and positive results were obtained from accipitrid birds (Ursula et al., 2001), from Rooks and wild pheasants (Campbell, 2001), from Antarctic penguins (Gardner et al., 1997), from ducks, gulls and shearwaters (Wilcox and Flower, 1983) and from crows, gulls and falcons (Ogawa et al., 1998).

c. Transmission

IBDV is transmitted horizontally and no evidence suggests that IBDV is transmitted through eggs or that a true carrier state exists in recovered birds. The resistance of the virus to heat and disinfectants is enough to account for virus survival in the environment between out-breaks (Eterradossi and Saif, 2013). Snedeker et al. (1967) demonstrated that lesser mealworm (*Alphitobius diaperinus*), taken from a poultry house 8 weeks after an outbreak of IBDV, was able to carrier IBDV to susceptible chickens when fed as a ground suspension. In another study performed by McAllister et al. (1995) found that the virus was isolated from several tissues of surface-sterilized lesser mealworm adults and larvae that were fed the virus earlier.

d. Morbidity and mortality

IBD appears suddenly, in fully susceptible flocks, showing a high morbidity rate, that can reaches 100%. Mortality may be nil but can be as high as 20%, higher after vvIBDV infection,

usually beginning on day 3 post-infection and peaking and receding in a period of 5–7 days (Eteradossi and Saif, 2013). Strains of vvIBDV can cause mortality rates up to 90% (Chettle et al., 1989) or 100% (Van Den Berg et al., 1991) in 4-week-old susceptible white leghorn chickens. IBDV infections can be subclinical depending on the age of birds, in birds less than 3 weeks old, depending on the pathotype of the strain and on the presence of maternal antibody (Eteradossi and Saif, 2013).

3. Pathogenesis

a. Pathogenesis

The most common way of IBDV infection is the oral route. Chickens are highly susceptible to IBDV infection from three to six weeks after hatching. Experiments in which bursectomized chickens survived IBDV infection demonstrated that the bursa of Fabricious is the main target organ for the virus (Aricibasi et al., 2010). The acute phase of IBD usually lasts a week, and peak clinical signs and mortality are recorded 3- 4 days post-infection (Jackwood and Sommer-Wagner, 2011). After the entry via the gastrointestinal tract (GIT) IBDV is then transported to other tissues by phagocytic cells, thought to be resident macrophages (Aricibasi et al., 2010). IBDV has been shown to infect and destroy IgM bearing B cells in active mitosis within the BF (Rodenberg et al., 1994). In addition to targeting B cells, IBDV has been shown to be capable of infect and replicate in the macrophages. After infection IBDV has been reported to stimulate the production of pro-inflammatory mediators and cytokines, which peak during the early phase of viral replication (Aricibasi et al., 2010). Within the spleen cells, IBDV induces expression of IL-1β, IL-6, IL-18 and inducible nitric oxide synthase (iNOS) (Aricibasi et al., 2010; Eldaghayes et al., 2006). T cells, although not actively infected by the virus have been suggested to modulate the pathogenesis of the infection by limiting the viral replication in the BF during the early phase of the infection, 5 days post-infection. This occurs via promoting tissue damage in the BF, via releasing cytokines and cytotoxic effects, and delaying tissue recovery (Rautenschlein et al., 2002). Pathogenesis and immune responses of IBDV infection may vary depending on the age of the affected chickens and the maturity of the bird's immune system (Rautenschlein et al., 2007). IBDV infection of birds of more than 2 weeks induce T cell accumulation in the BF coinciding with the replication of the virus (Aricibasi et al., 2010). These intrabursal T cells are activated and play a significant part in viral clearance but also in bursal recovery (Rautenschlein et al., 2007).

b. Immunosuppression

Both clinical or subclinical IBDV infection cause immunosuppression in birds, compromising humoral and cellular immune responses (Sharma et al., 2000). The destruction of immature B lymphocytes in the BF creates an immunosuppression, which will be more severe in younger birds (Faragher et al., 1974). In fact, the most severe and long-lasting immunosuppression occurs when day-old chicks are infected by IBDV. In field conditions, this rarely occurs since chickens tend to become infected at approximately two to three weeks, when maternal antibodies decline. Evidence suggests that the immunosuppressive effect of the virus least up to six weeks of age (Van Den Berg et al., 2000). In commercial chicken flocks, immunosuppression may be clinically manifested in several ways. In general, the flock performance is reduced. Specifically, immunosuppressed flocks tend to experience an increased incidence of secondary infections, poor feed conversion, reduced protective response to commonly used vaccines, and an increased rate of carcass condemnation at the processing plant (Sharma et al., 2000).

IBDV causes a lytic infection of IgM^+ B lymphocytes. Although B cell destruction is most pronounced in BF, evidence of viral replication and associated cellular destruction can also be found in several secondary lymphoid organs including cecal tonsils and spleen (Ivanyi & Morris, 1976; Okoye et al., 1990; Darteil et al., 1995). The cytolytic effect of IBDV on B cells leads to a dramatic reduction in circulating IgM^+ B cells (Giambrone et al., 1977; Rodenberger et al., 1994). Only the primary antibody responses are impaired; the secondary responses remain intact (Sharma et al., 2000). Although the data on the effect of IBDV on antigen-specific T cell functions are controversial, there is convincing evidence that in vitro mitogenic proliferation of T cells of IBDV-exposed birds is severely compromised. T cells in the spleen as well as in the peripheral circulation were affected (Confer et al., 1981; Kim et al., 1998). The mitogenic inhibition occurred early, during the first 3±5 days of virus exposure. Subsequently, the mitogenic response of T cells returned to normal levels. IBDV modulates macrophage functions. There is indirect evidence that the in vitro phagocytic activity of these cells may be compromised (Lam, 1998).

A recent study conducted by Li, et al. (2018) demonstrates that IBDV infection leads to changes in the gut associated-lymphoid tissue (GALT) and the microbiota composition of chickens. An increase in the number of mast cells was observed in the BF and cecal tonsils during the

acute phase of the disease what confirms previous studies showing IBDV infection may affect the number and morphology of mast cells (Wang et al., 2009). vvIBDV-infected birds had a lower abundance of *Clostridium XIVa and a*n increase in the abundance of *Faecalibacterium* was observed *in the acute phase of IBD. The trend was reversed after this phase.* Therefore, *it may* suggest that vvIBDV interferes with the delicate balance of gut mucosal immunity and may support harmful intestinal inflammation.

c. Clinical signs

Severity of signs and lesions in infected birds depend on the virulence of the IBDV strain, race or genetic lineage, age and immune status, co-infection with other viruses, the dose and route of inoculation of the virus in experimentally infected birds. In studies based on the experimental reproduction of acute IBD in SPF white leghorn chickens, vaIBDV induce little if any clinical signs and mortality but marked bursal lesion. cIBDV induced approximately 10–50% mortality with typical signs and lesions, and vvIBDV induced approximately 50–100% mortality with typical signs and lesions (Skeeles et al., 1979).

The incubation period is short and clinical signs can appear in 2-3 days from virus exposure (Helmboldt and Garner, 1964). Birds younger than 14 day-old do not appear susceptible to the disease if serologically positive to maternal immunity; birds of 3 to 6 week of age can demonstrate severe clinical signs (Mahgoub, 2012).

In the acute clinical form Birds are depressed, with ruffled feathers and droopy appearance, they may be seen pecking at their vent. Whitish or watery diarrhea and anorexia is also described (Figure 3) (Cosgrove, 1962).

Figure 3. Clinical signs of IBD: ruffled feathers, depression and watery diarrhea (https://albeitar.portalveterinaria.com/noticia/13000/articulos-aves/La-enfermedad-de-Gumboro-I.html).

Subclinical IBD occurs when chickens are exposed to IBDV during the first two weeks post hatch and have enough maternal antibody at time of infection to prevent clinical disease but not to prevent viral replication in the bursa. Immunosuppression and resultant increased susceptibility to secondary infections are the major problem of subclinical IBD (Sharma, 2000).

d. Gross lesions

Birds that are affected by IBD, at post mortem examination are dehydrated, with dark discoloration of pectoral muscles. Pectoral, thigh and leg hemorrhages can be present (Figure 5) (Rinaldi et al., 1965). In advanced stages of the disease, kidneys can be pale due to severe urate diathesis caused by dehydration (Figure 6) (Cosgrove, 1962). Occasionally hemorrhages can be observed at the juncture of the proventriculus and gizzard (Figure 7) (Pikuła et al., 2018).

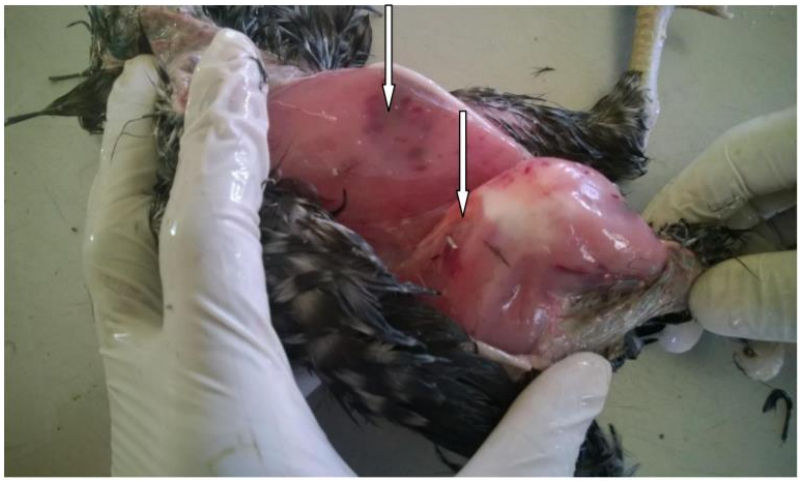

Figure 4. Petechial and ecchymosis on breast and thigh muscles (white arrows) in a chicken experimentally infected with vvIBDV (Raji et al., 2017).

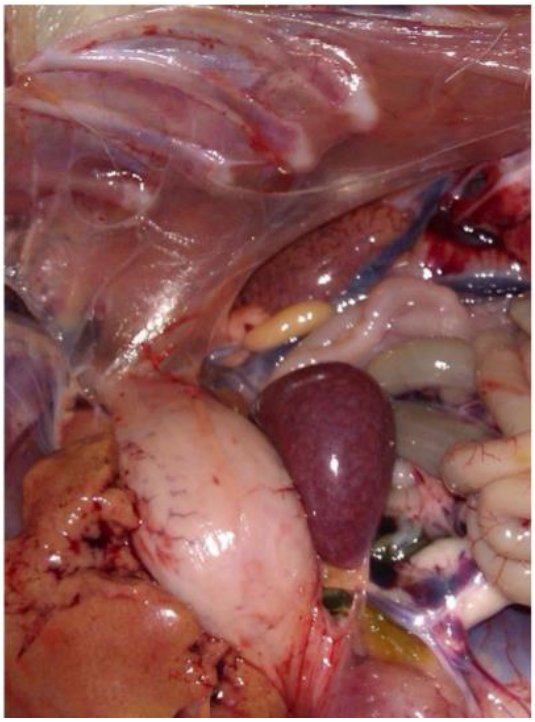

Figure 5. Chicken experimentally inoculated with the IBDV strain Bpop/03: spleen enlarged showing small gray foci uniformly dispersed (Pikuła et al., 2018).

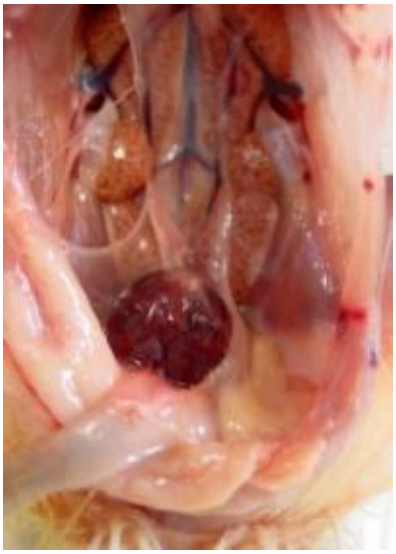

Figure 6. Palled kidneys observed at post-mortem examination during IBD. (https://albeitar.portalveterinaria.com/noticia/13000/articulos-aves/La-enfermedad-de-Gumboro-I.html).

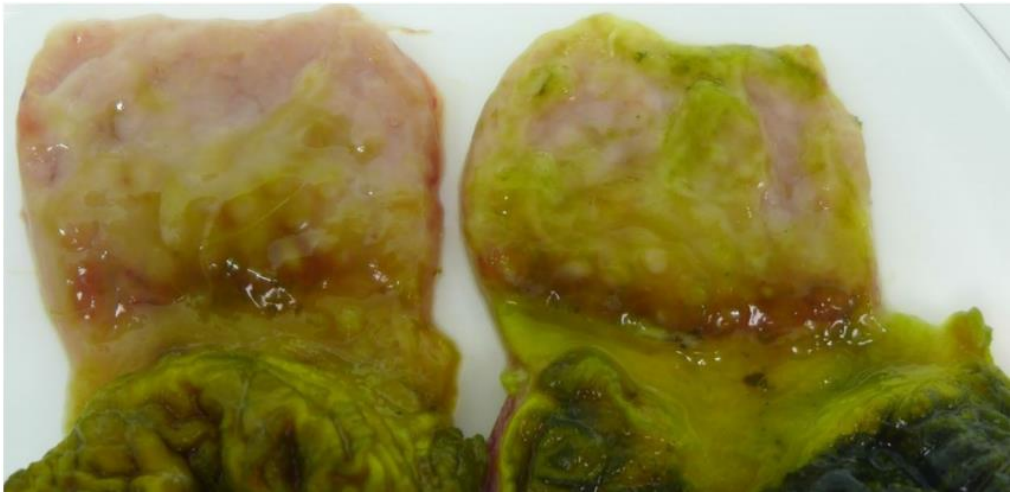

Figure 7. Hemorrhages at the juncture of the proventriculus and gizzard in chickens experimentally inoculated with the IBDV strain Bpop/03 (Pikuła et al., 2018).

The most severe macroscopic lesions are seen in bursa of Fabricious. In the early stages of the infection BF begins to increase in size because of edema and hyperemia. A gelatinous yellowish transudate is covering the serosal surface of the organ (Figure 8). Longitudinal striations on the surface become prominent, and the normal white color turns to cream color. After 4-5 dpi, BF returns to normal weight, the transudate disappears, but it continues to atrophy, and the organ

may become gray. From 8 dpi forward, it is approximately one-third its original weight, or even less (Cheville, 1967). The BF often shows necrotic foci and, at times petechial or ecchymotic hemorrhages on the mucosal surface. Occasionally, extensive hemorrhage throughout the entire bursa has been observed; in these cases, birds may void blood in their droppings (Eterradossi and Saif, 2013).

vvIBDV, however, cause similar lesions as cIBDV described above, although, bursal atrophy appears faster (Van Den Berg et al., 2000).

vaIBDV were reported not to induce an inflammatory response (Rosenberger and Cloud, 1985; Sharma et al., 1989), although 1 variant strain (IN) did so (Hassan, 1996) and cause a quicker and more severe bursal atrophy (Sharma et al., 1989).

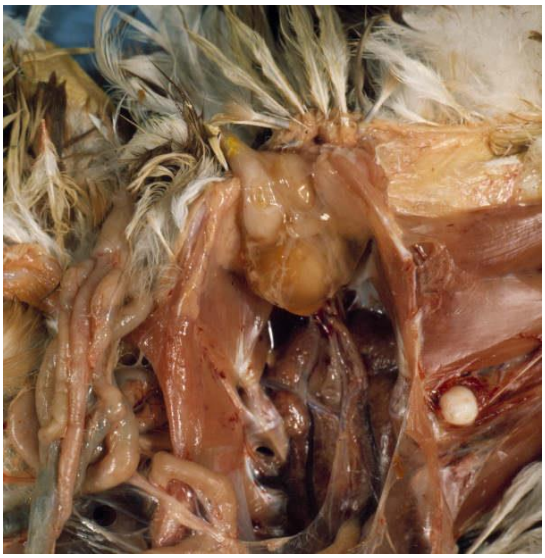

Figure 8. Chicken affected by IBD: bursa of Fabricious enlarged and oedematous.

e. Histopathological lesions

Microscopic lesions of IBD occur primarily in the lymphoid tissues like BF, spleen, thymus, Harderian gland, and cecal tonsil and are notable more severe in BF (Eterradossi and Saif, 2013).

i. Bursal histopathological lesions

Histopathological lesions due to cIBDV infection have been vastly described by Helmboldt and Garner (1964), Cheville (1967), Mandelli et al. (1967) and Peters (1967).

Degeneration and necrosis of lymphocytes in the medullary area of bursal follicles can be recognized in the early days post infection, Lymphocytes were soon replaced by heterophils, and hyperplastic reticuloendothelial cells. Hemorrhages often appear but are not consistently present. All lymphoid follicles can be affected by 3- 4 dpi (Figure 9) and severity depends of the pathogenicity of the strain. The enlargement of the bursa seen at this time is caused by edema, hyperemia, and marked accumulation of heterophils. As the inflammatory reaction declines, cystic cavities developed in medullary areas of follicles; and there is a fibroplasia in the interfollicular connective tissue.

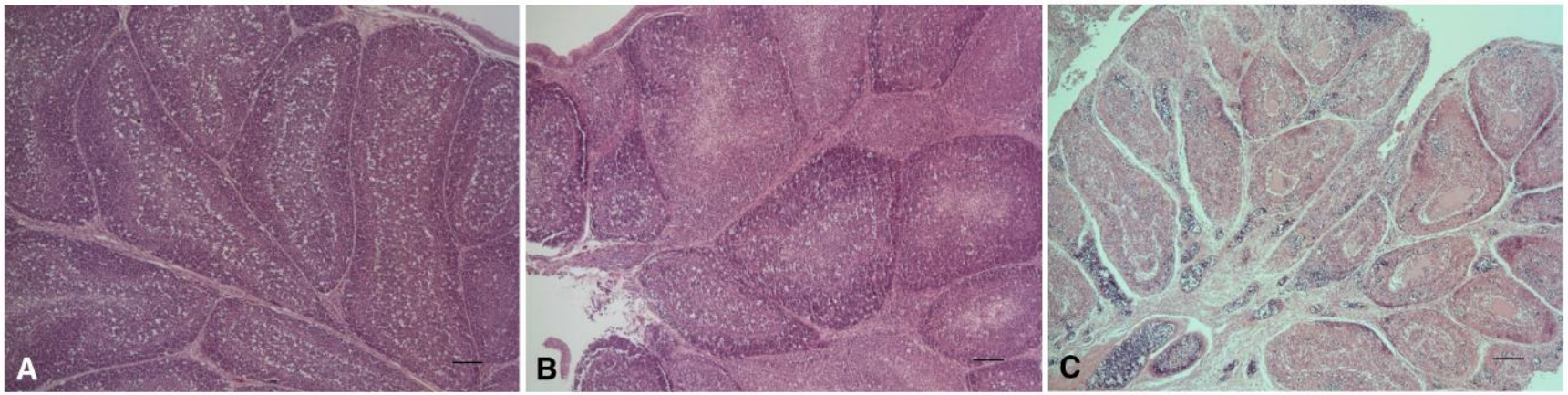

Figure 9. Differences in the severity of histopathologic lesions in sections of bursa of Fabricius 3 days post experimental inoculation of SPF chickens with cIBDV and vvIBDV . A) Control group; B) D78 cIBDV strain; C) 75/11 vvIBDV strain (Pikula1et al., 2018).

ii. Thymus histopathological lesions

In the early stages of infection thymus can exhibit cellular reaction in the lymphoid tissues; classical strains are reported to cause more severe lesions in this organ than variant strains (Sharma et al., 1989; Tanimura and Sharma, 1998). Cortical atrophy (Figure 10) (Sharma et al., 1989) and pyknotic foci (Tanimura and Sharma, 1998) in the initial phase of the disease can be observed.

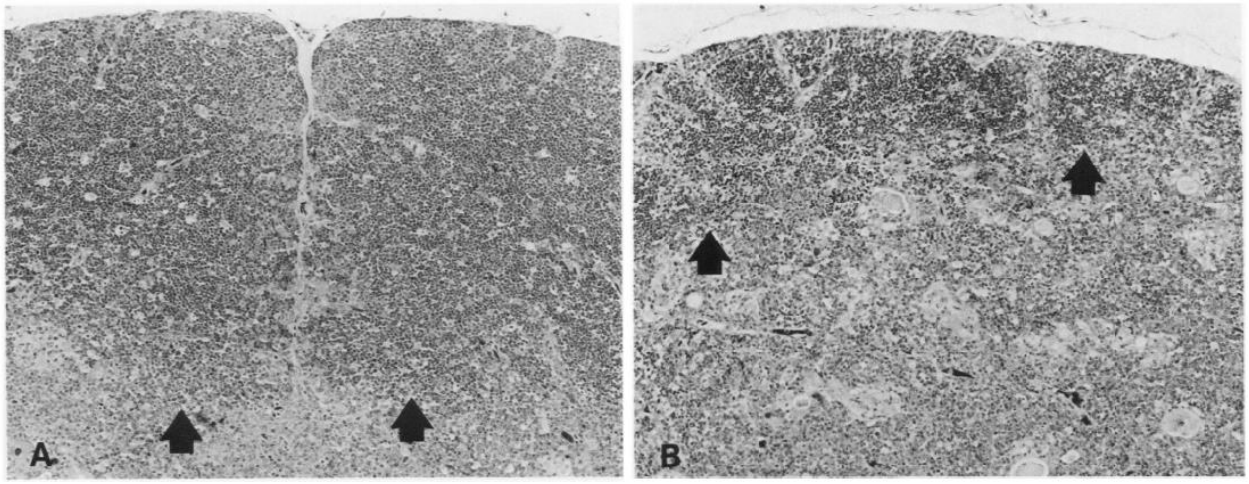

Figure 10. Histological sections of thymus of birds experimentally inoculated (5 dpi) with a vaIBDVstrain (A) or with a cIBDV strain (B). Corticomedullary junction is denoted by arrows cIBDV causes cortical atrophy (Sharma et al., 1989).

4. Diagnosis

a. Gross lesions

When the virus reaches its main target organ (BF) it produces lesions and their extensions are compatible with its virulence and pathogenicity, viral load and the host's immunity. In addition, other organs and tissues may present lesions, as demonstrated previously, due to these same factors. The evaluation of the appearance, consistency and size of the bursae are widely used to follow the dynamics of the disease. This is a useful tool for tracking the disease but should not be used as a single diagnostic method, since other diseases also cause considerable changes in BF (Eteradossi and Saif, 2013).

Birds affected by IBD show injuries at BF as enlargement and turgid with a pale-yellow discoloration. Thigh and leg hemorrhages, enlargement of the liver and spleen and hemorrhages at the juncture of the proventriculus and gizzard can also be detected in the acute phase of the disease. In advanced stages of the disease, kidneys can be pale due to severe urate diathesis caused by dehydration (Eteradossi and Saif, 2013). Differences between acute and clinical forms are related with the severity and velocity of appearance of the of clinical signs and lesions (van Den Berg et al., 2000). Subclinical form commonly present only bursal atrophy (Sharma, 2000).

b. Virus detection/isolation

The viral antigen can be detected in smears BF or bursal sections by an immune-agar gel diffusion test (AGID), by immunofluorescence or immunostaining.

Several antigen-capture enzyme-linked immunosorbent assays (AC-ELISA) have been developed for antigenic typing of IBDV strains. Virus isolation can be attempted by inoculation of the samples in SPF chicken embryonated eggs, or chicken embryo fibroblasts. Molecular tests are currently use in avian laboratories

RT-PCR (Lin et al., 1994; Long Huw Lee et al., 1992; Vakharia et al., 1994) or real-time RT-PCR protocols (Moody et al., 2000; Peters et al., 2005) have been developed targeting the VP2 gene; some can differentiate classical, variant or very virulent strains of IBDV.

c. Serology

Serological tests as AGID, virus neutralization or ELISA are currently available to detect antibodies against IBDV in serum samples, but the most used routinely is the ELISA test. This test aim to measure titers of passive antibodies to determinate the date of vaccination in broiler flocks or in laying hens to verify proper vaccine intake (Sharma et al., 2000).

5. Disease control

The prophylaxis of Gumboro disease employs tools for the prevention and control of the occurrence or spread of this disease. It must necessarily be linked to its epidemiological chain that is applied to the sources of infection, transmission of the disease and susceptible birds. Measures of direct prophylaxis include cleaning and disinfection, with physical or chemical agents, of all fomites that get contact with the birds, as well as all the environment where they are housed. In addition, efforts should be made to avoid the contact of birds with possible disseminators of IBDV, such as wild birds and insects, and to maintain optimal hygiene of farm workers.

Disinfectants such as formalin, chloramine and iodophor compounds have been shown to be effective against IBDV (Eteradossi & Saif, 2013).

Control of IBD is achieved by vaccination. Live attenuated vaccines are commercially available and, according to the virulence of the strain, are classified in “mild”, “mild intermediate”, “intermediate”, “intermediate plus” or “hot”.

However, effective control using conventional live vaccines requires proper timing of vaccination based on level of maternal antibody. While the live vaccines are susceptible to neutralization by maternal antibodies, there is the potential for immunosuppression if birds are vaccinated when their antibody levels are too low (Rautenschlein et al., 2005).

Inactivated vaccines are available and used in breeders at 16-20 weeks of age after the priming with an attenuated live IBDV vaccine at 8 weeks of age (Müller et al., 2012). These vaccines are used to produce a passive immunity in the progeny (Van Den Berg et al., 2000).

Immune complex and recombinant vaccine administered safely and effectively at hatchery have been recently developed and can be used in many countries. Immune complex vaccines are produced by mixing a well-defined proportion of attenuated IBDV, produced in embryonated eggs, with IBDV specific antibodies produced in SPF chickens inoculated with IBDV (Jeurissen et al., 1998). The recombinant vaccines use the Herpes virus of turkeys (HVT) as vector (Bublot et al., 2007) that is currently used to prevent Marek disease also with considerable efficacy (Gimeno et al., 2016). The VP2 gene from a donor IBDV is inserted into the genome of the HVT vaccine, which expresses the protein of IBDV as it replicates, thus inducing IBDV protection in vaccinated birds. Studies demonstrated high safety and efficacy of recombinant HVT-IBD vaccines, although they do not spread, or spread poorly, from bird to bird (Cho, 1976; Zhou et al., 2010).

Chapter II

Infectious bursal disease virus of ITA genotype (G6) reveals in SPF chickens aggressiveness for lymphoid tissues despite a subclinical course

Running title: *in vivo* pathogenicity study of an IBDV strain of ITA genotype (G6)

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Summary

Recently, a new genotype of Infectious Bursal Disease Virus (IBDV), named ITA, was detected in IBD vaccinated Italian broilers. Genome characterization revealed ITA to be a genetically different IBDV placed into genogroup 6 of a recent IBDV classification. The currently available clinical data do not allow definition of the degree of pathogenicity ITA-IBDV genotype. In the present study a pathogenicity trial has been conducted by inoculation of SPF chickens. Birds were housed in poultry isolators and inoculated at 35 days of life with a strain of the ITA IBDV genotype (35 birds) or a classical reference strain (35 birds). Control birds (25 birds) were contextually mock inoculated with sterile water. Birds were daily observed for clinical signs and at 0, 7, 14, 21- and 28-days post-inoculation (dpi) bled for IBDV antibody detection. At 2, 4, 7, 14, 21 e 28 dpi, 5 birds from each of the inoculated groups, and 3 from the control group, were euthanized and subjected to post-mortem examination bursa and thymus-body weight ratios were

calculated. Microscopic lesions of bursa and thymus were scored on the basis of lymphoid necrosis and/or depletion or cortex atrophy, respectively. Both viruses induced a subclinical course of disease, as neither clinical signs nor mortality were recorded during the study, in presence of IBDV typical post-mortem lesions. Bursal damage, measured by the bursa-body weight ratio, was more noticeable, precocious and persistent after ITA-IBDV inoculation, significantly at 4 and 28 dpi. Histopathology of the bursa and thymus confirmed the higher aggressiveness for lymphoid tissues of the ITA-IBDV strain, indicating the scores a more severe lymphoid depletion. The study showed that ITA genotype, though it has a subclinical course, causes a severe and persistent damage of lymphoid tissues. Therefore, its circulation in birds might be a threat for the poultry industry.

Keywords: Avibirnavirus, Chicken, Gumboro Disease, Infectious Bursal Disease, Pathogenicity trial

INTRODUCTION

Infectious bursal disease (IBD) is a highly contagious immunosuppressive disease of chickens caused by a bi-segmented dsRNA virus (IBDV), which belongs to the family *Birnaviridae,* genus an *Avibirnavirus* (Dalmas et al., 2019). There are two recognized serotypes of IBDV, designated serotypes I and II; only serotype I viruses have been known to cause naturally occurring disease in chickens. The primary target organ is the Bursa of Fabricius where the virus infects and destroys dividing IgM–bearing B cells (Hirai and Calnek, 1979). IBDV of serotype I includes strains with different antigenicity and pathogenicity. Isolates known as “classical”, firstly reported by Cosgrove (1962), can cause, in receptive birds, acute clinical disease characterized by ruffled feathers, dehydration, watery diarrhea, prostration and mortality. Infection with classical

strains can be also characterized by absence of clinical signs and mortality, in the presence of bursal damage (Abdul et al., 2013; Sreedevi et al., 2007). In the early 1980s, antigenic “variants” of the virus were identified in USA (Rosemberg and Cloud, 1985; Saif, 1984) in respect of which vaccine strains available at that time were not able to elicit full protection (Heine et al., 1991). “Variant” isolates typically do not cause clinical signs of disease but always cause discernible immunosuppression (Jayasundara et al., 2017; Sharma et al., 1989). In the mid-1990s, “very virulent" strains of IBDV appeared in European and Asian countries, these strains were shown to be mostly antigenically similar to the classical isolates (Abdel-Alim and Saif, 2001) and were able to cause outbreaks of disease characterized by an exacerbated acute phase and more than 70% mortality in sensitive chickens (Chettle et al, 1989; Eterradossi et al., 1992);

Independent of the pathogenicity of the strain and the severity of clinical signs, IBDV infection is always associated with damage to the bursa of Fabricius and immunosuppression, often followed, in the field, by infections with other pathogens and impaired immune response to other vaccinations (Sreedevi et al., 2007).

The high mutation rate of IBDV genome can lead to the emergence of strains with new antigenic and pathogenic properties, which can persist and circulate in immunized commercial chickens (Ingrao et al., 2013).

In 2011, a new genotype of IBDV, named ITA, was detected in IBD-live vaccinated Italian broilers (Lupini et al., 2016). Full genome characterization confirmed ITA to be a genetically different IBDV (Felice et al., 2017) and a recently proposed classification for IBDV into genogroups, placed ITA genotype into genogroup 6, together with few other strains detected in Saudi Arabia and Russia (Michel and Jackwood, 2017). The currently available epidemiological and clinical data regarding IBDV of ITA genotype, do not allow definition of its degree of path-

ogenicity since the viruses have been detected in IBDV vaccinated broilers, sometimes with poor performances, hence provided with some kind of immune protection which could have masked the clinical course of the disease.

The ITA-IBDV genotype shows peculiar molecular characteristics, as it has most of the mutations that affect charged or potentially glycosylated amino acids in key positions of the hypervariable region of the VP2 protein. These mutations may be virtually associated with major changes in virus properties, with VP2 being the primary antigenic and virulence determinant of IBDV (Nagarajan and Kibenge,1997).

The aim of this study was to determine the virulence of IBDV of ITA genotype. A pathogenicity study has been conducted by inoculation of Specific-pathogen-free (SPF) chickens in secure isolation conditions with an IBDV strain recognized to belong to the ITA genotype and observation of clinical signs, macro/microscopic lesions, antibody response and damage of bursa of Fabricius and thymus.

MATERIAL AND METHODS

Ethics statement

Experimental trial was performed in agreement with national regulations on animal experiments and animal welfare (UE Directive 2010/63/EU), according to authorization N°635/2015-PR provided by the Italian Ministry of Health.

Chickens

Ninety-five SPF chickens were used. Birds were housed in secure isolation facilities for the duration of the study and food and water were given *ad libitum*.

Viruses

Two field isolates of IBDV were used in the study, named, according with the new nomenclature proposed by Jackwood et al. (2018), IBDV 1/chicken/Italy/1829/11/(G6) (ITA genotype, genogroup 6) and IBDV 1/chicken/Italy/24II/12/(G1a) (STC genotype, genogroup 1). The virus of the STC genotype was used as reference control in the pathogenicity test as suggested by OIE (2016).

Virus isolation was obtained from bursal homogenates that tested positive for IBDV ITA or STC genotypes by RT-PCR and sequencing (Jackwood et al., 2008). Inocula were prepared as a 20% (weight/volume) suspension of positive bursae in Minimal Essential Medium (MEM) containing antibiotics and antimycotics (penicillin, streptomycin and amphotericin B) (Applied Biosystems, CA, USA). The supernatant (0.2 ml/egg) was used to inoculate ten 12-day-old specific pathogen free (SPF) chicken embryonated eggs via the chorioallantoic membrane (CAM) route (Senne, 2008). The eggs were incubated at 37.7°C until embryo death was recorded or up to 7 days post-inoculation. From the eggs suspected to be infected, due to embryo death or presence of embryo lesions, the CAMs were aseptically harvested, homogenized, pooled and prepared as 20% (w/v) suspensions. IBDV isolation was confirmed by RT-PCR (Jackwood et al., 2008). Viruses were afterwards titrated in SPF eggs (Villegas, 2008) and titers were calculated by the method of Reed and Muench (1938). Viral stocks were examined by PCR assays for avian adenovirus (Raue and Hess,1998) and chicken anemia virus (Imai et al.,1998), to exclude contamination.

Experimental design

Birds were tagged and divided into three groups, housed in separate isolators, and named as follows: ITA-IBDV (35 birds), STC-IBDV (35 birds) and a CONTROL group (25 birds). At 35

days of age ITA-IBDV and STC-IBDV groups were orally inoculated with a dose of $10^{4.5}$ EID_{50}/per bird of IBDV1/chicken/Italy/1829/11/(G6) or IBDV1/chicken/Italy/24II/12/(G1a) viruses, respectively. Chickens of the control group were kept as negative control and mock inoculated with sterile water. After inoculation birds were monitored and scored daily for clinical signs as previously reported (Le Neuen et al., 2012).

Before inoculation, and at 7, 14, 21- and 28-days post-infection (dpi), ten birds per group were bled and sera were tested for circulating anti-IBDV antibody by ELISA assay. At 2, 4, 7, 14, 21 and 28 dpi, five birds from ITA-IBDV group, five from STC-IBDV group and three from the control group were euthanatized, weighted and post-mortem examined for macroscopic lesions. Bursae and thymuses were collected, weighted for the subsequent calculation of the bursa-body weight (B:BW) or thymus-body weight (T:BW) ratios, and fixed in 10% neutral buffered formalin for histological score of lesions. The experiment was terminated at 28 dpi.

Serology

Anti-IBDV antibody titers were determined using the commercial Infectious Bursal Disease Virus Antibody test kit (BioCheck, Reeuwijk, The Netherlands) following the manufacture's instructions. Sera samples with an antibody titer >391 were considered positive.

Geometric mean of ELISA antibody titers were compared through t-student test, for differences among groups. A p-value <0.05 was considered statistically significant.

Bursa-body weight and thymus-body weight ratios

Body weight and bursa or thymus weights were used to calculate the bursa-body (B:BW) or thymus-body (T:BW) ratios, respectively, according to the following formula (Sharma et al., 1989):

B:BW or T:BW ratio = [organ weight (g)/body weight (g)]×1000

The means of the B:BW or T:BW ratios per group, at each day-point sampling, were compared through Wilcoxon-Mann-Whitney non-parametric test for differences among groups. A p-value <0.05 was considered statistically significant.

Histological Scores of bursa and thymus lesions

Fixed tissues were dehydrated in grades alcohols, cleared with xylene, embedded in paraffin wax, sectioned, and stained with haematoxylin and eosin.

Bursa lesions were scored from 0 to 4, on the basis of lymphoid necrosis and/or depletion according to Sharma et al. (1989) as follows: 0=less than 5% of the lymphoid follicles affected; 1= 5-25% of lymphoid follicles affected; 2= 25-50% of lymphoid follicles affected; 3=50-75% of lymphoid follicles affected and 4= more than 75% of lymphoid follicles affected.

Thymus lesions were evaluated on the bases of cortex atrophy and expressed as percentage of cortex area/lobule area. Photomicrographs of two thymic lobules from each thymus sample were acquired with a digital camera connected to an optical microscope; digital image analysis was performed using ImageJ software (Schneider et al., 2012). Thymic medulla and lobule images were manually delineated in order to calculate their areas. Scores were expressed as percent of cortex area/lobule area.

Mean histological scores were compared through t-student test, for differences among groups. A p-value <0.05 was considered statistically significant.

RESULTS

Clinical signs and gross lesions

Neither clinical signs, nor mortality were observed during the study in all experimental groups. At post-mortem examination, from 2 dpi, bursae of Fabricius of birds of both virus-inoculated groups were enlarged, and a gelatinous yellowish transudate was covering the serosal surface. Atrophy of the bursae takes over from 4 dpi in birds of the ITA-IBDV group (Figure 1) and from 7 dpi in birds of STC-IBDV group. The atrophy lasted until the end of the trial and, at 28 dpi was more severe in birds of the IBDV ITA group (Figure 2). In both inoculated groups, from 2 to 7 dpi, hemorrhages in the thigh muscles (Figure 3) and/or a slight enlargement of spleen, showing grey foci uniformly dispersed on the surface, were observed. No post-mortem lesions were observed in the control birds at any time.

Bursa-body weight and thymus-body weight ratios

Bursa-body weight and thymus-body weight ratios means are reported in table 1. At 4 dpi, the mean of the B:BW ratios of the ITA-IBDV group was significantly lower than the means of the B:BW ratios of the STC-IBDV group and of the control group (p values<0.05), confirming what was observed at the post-mortem examination. At 7, 14 and 21 dpi, the means of the B:BW ratios of both virus-inoculated groups were significantly lower than the mean of the B:BW ratios of the control group (p value<0.05) but did not differ each other (p values>0.05). At 28 dpi the mean of the B:BW ratios of the ITA-IBDV group was significantly lower that the one of the STC-IBDV group (p values<0.05).

At 4 dpi the mean of T:BW ratios of the STC-IBDV group was significantly lower ($p<0.005$) than the one of the ITA-IBDV group. Both differed significantly, at this day-point, from the

mean of the T:BW ratios of the controls ($p<0.005$). Both virus-inoculated groups, at the end of the trial, showed a higher mean of the T:BW ratios than the control group ($p<0.005$) (Table 1).

Serology

No ELISA anti-IBDV antibodies were detected in the control group at any time during the trial and in the ITA-IBDV and STC-IBDV groups before inoculation. From 7dpi, anti-IBDV antibody have been detected, and titers have increased up to the end of the trial in both inoculated birds. At 7 dpi, birds of ITA-IBDV group showed a significantly higher mean antibody titer (9650 ± 1300) than the birds of the STC-IBDV group (7329 ± 1285), while were not statistically different in the following days of sampling (Figure 4).

Bursa and thymic histologic lesion scores

Mean histologic lesion scores of bursa and thymus are reported in table 2. Extensive microscopic lesions were observed in the bursa of virus-exposed birds from 2 dpi, which persisted through the observation period of 28 days (Figure 5, 6 and 7). A statistically significant difference between mean of bursa scores of virus-inoculated groups was recorded at 2dpi ($p<0.05$), scoring higher the ITA-IBDV group. At the following sampling days, the means of bursa scores of both virus-inoculated groups were significantly higher than the mean of the control group (p value<0.05) but did not differ each other (p values>0.05).

Thymus cortical atrophy was observed in birds of virus-inoculated groups from 2dpi up to the end of the trail (Figure 8). A statistically significant difference between mean of thymus scores of virus-inoculated groups was recorded at 2dpi ($p<0.05$), scoring lower the ITA-IBDV group. At the subsequent sampling days, the means of thymus scores of virus-inoculated groups were significantly lower than the mean of the control group (p value<0.05) but did not differ each other (p values>0.05).

DISCUSSION

In the present study a pathogenicity trial has been conducted by inoculation of 5 weeks-old SPF chickens with an IBDV strain recognized to belong to the ITA genotype, in order to assess its virulence in comparison to a "classical" STC strains. Both viruses induced a subclinical course of disease, as neither clinical signs nor mortality were recorded during the study, in presence of IBDV typical post-mortem lesions. Bursal damage, measured by the bursa-body weight ratio, was more noticeable, precocious and persistent after ITA IBDV inoculation, significantly at 4 and 28 dpi. This feature has been previously described for "variant" IBDV, which have been reported to cause earlier and more severe bursal atrophy than "classical" strains (Hussan et al., 1996, Sharma et al., 1989; Jayasundara et al., 2017). Histopathology scores of the bursa and thymus confirmed the higher aggressiveness for lymphoid tissues of the ITA-IBDV strain, indicating the scores a more severe lymphoid depletion, as previously reported by Sharma et al., (1989) for American "variant" strain.

It is universally recognized that IBDV-induced damage of the bursa of Fabricius is related to immunosuppression; moreover, IBDV strains that have been shown to involve other lymphoid organs, such as thymus, can cause even more severe immune disorders (Sharma et al., 1989, 1992). A damage in thymus lymphoid tissues is reported to be indicative of a highly aggressive nature of the examined IBDV strain (Timura and Sharma, 1998; Rauf et al., 2013), and may represent a generalized inflammatory response to a acute virus infection (Sharma et al., 1989). In the present study at microscopic level, a significant reduction of the thymus cortex was observed in the ITA-IBDV inoculated birds, not associated to atrophy of the organ as previously described (Sharma et al., 1989).

A significantly earlier antibody response was detected in ITA-IBDV group, when compared to STC-IBDV group. This characteristic was previously addressed as high virulence indicator, in the acute phase of IBDV infection, for an Australian variant strain (Jayasundara et al., 2017). The authors also observed that the early antibody response was correlated to a faster virus clearance from host tissues; this point need to be further investigated for the IBDV of the ITA genotype by performing a dynamic virus distribution *in vivo* study. It is known that during IBDV infection, the immune response against the virus itself is not affected; this seems to be a paradox as there is immunosuppression in respect of other antigens. Different mechanisms could be involved that selectively stimulate the proliferation of the B cells committed to anti-IBDV antibody production (Withers et al., 2005; Jakka et al., 2014).

The molecular markers of virulence of IBDV have not yet firmly established, therefore the assessment of the pathogenicity of a new viral genotype of epidemiological relevance, trough experimental infection of sensitive birds remains an essential point. It has been suggested that more than one molecular determinant, either located in segment A or B of the genome, contribute to the virulence of IBDV (Nagarajan & Kibenge, 1997). Complete sequence analysis of a strain of the ITA IBDV genotype revealed the coexistence in the aminoacidic sequence of some residues, including virulence markers, in common with very virulent strains, and others typical of IBDV strains at low degree of virulence (Felice et al., 2017).

Our present study shows that a strain belonging to the ITA genotype, inoculated in SPF chickens, is able, though it has a subclinical course, to cause a severe and persistent damage of bursal tissues and, in addition, to involve the thymus. For its unique molecular characteristics ITA genotype was shown to cluster phylogenetically apart from classical, variant and very virulent IBDV reference strains (Michel and Jackwood, 2017), together with few Saudi Arabia and Russian iso-

lates, into Genogroup 6. Michel and Jackwood (2017) report that Saudi Arabia strains were detected in flocks with suspected non-very virulent IBDV infection; this could suggest that these strains have a similar subclinical course as experimentally demonstrated in the present study for an IBDV of the ITA genotype. Moreover, the protection offered by common vaccination schedules to ITA IBDV is still unknown and need to be further investigated by *in vivo* cross-protection studies using existing IBDV vaccines.

Strains of ITA genotype are still circulating in Italy (Lupini et al., 2018) often under detected during routine diagnostic activity; and due the absence of overt clinical signs and mortality can exercise uncontrolled their deleterious and underhand immunosuppressive potential.

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Table 1. Mean Bursa: BW and thymus:BW ratios (± standard deviations) of experimental groups by day post-inoculation (dpi). Different superscripts indicate that the difference between groups is statistically significant ($p<0.05$).

	Mean Bursa:BW ratio			Mean Thymus:BW ratio		
dpi	STC-IBDV	ITA-IBDV	Control	STC-IBDV	ITA-IBDV	Control
2	6.1^{*} (±2.33)	6.3^{*} (±1.44)	$4,8^{*}$ (±1.27)	10.7^{*} (±1.07)	10.0^{*} (±2.96)	9.6^{*} (±0.80)
4	4.7^{*} (±1.11)	2.3^{**} (±0.29)	4.2^{*} (±0.18)	5.0^{*} (±1.17)	7.0^{**} (±0.87)	$9,8^{***}$ (±0.63)
7	1.0^{*} (±0.35)	1.3^{*} (±0.14)	4.3^{**} (±1.02)	7.4^{*} (±1.16)	6.3^{*} (±0.35)	7.6^{*} (±1.27)
14	0.7^{*} (±0.50)	0.3^{*} (±0.15)	4.1^{**} (±1.58)	10.0^{*} (±2.86)	7.4^{*} (±1.69)	9.8^{*} (±4.92)
21	1.0^{*} (±0.38)	0.7^{*} (±0.43)	3.0^{**} (±0.42)	10.0^{*} (±2.00)	9.3^{*} (±2.02)	8.1^{*} (±1.85)
28	0.6^{*} (±0.12)	0.3^{**} (±0.15)	3.9^{***} (±0.76)	8.3^{*} (±1.39)	9.2^{*} (±2.53)	6.0^{**} (±1.03)

Table 2. Mean histological lesion scores of bursa and thymus of experimental groups by day post-inoculation (dpi). Different superscripts indicate that the difference between groups is statistically significant ($p<0.05$).

	Bursa lesion score			Thymus lesion score		
dpi	STC-IBDV	ITA-IBDV	Control	STC-IBDV	ITA-IBDV	Control
2	2.2^{*}	4^{**}	0^{***}	$65,2^{*}$	$58,9^{**}$	$71,2^{***}$
4	3.6^{*}	4^{*}	0^{**}	$46,4^{*}$	$49,5^{*}$	$65,1^{**}$
7	3.6^{*}	4^{*}	0^{**}	$65,7^{*}$	$62,2^{*}$	$72,9^{**}$
14	4^{*}	4^{*}	0^{**}	$64,6^{*}$	$67,9^{*}$	$65,7^{**}$
21	4^{*}	4^{*}	0^{**}	$65,1^{*}$	$67,2^{*}$	$66,9^{**}$
28	4^{*}	4^{*}	0^{**}	$59,9^{*}$	$56,7^{*}$	$57,8^{**}$

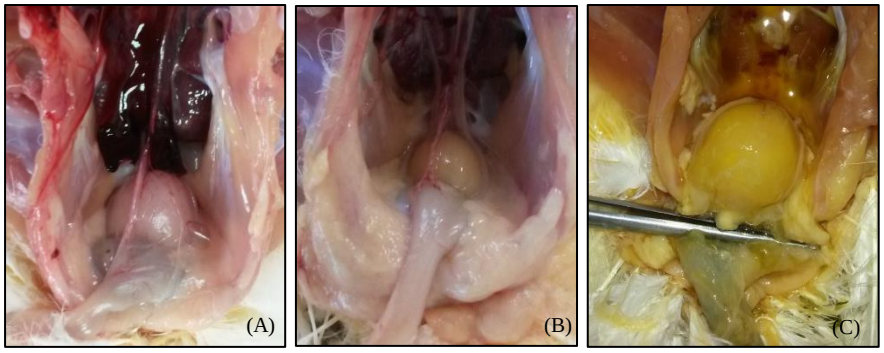


Figure 1. Post mortem lesions in SPF chickens at 4 dpi: (A) control group (B) ITA-IBDV group and (C) STC-IBDV group. A gelatinous yellowish transudate is covering the serosal surface of bursae of birds of both virus-inoculated groups. Furthermore, atrophy of the bursa begins to be present in the ITA-IBDV group.

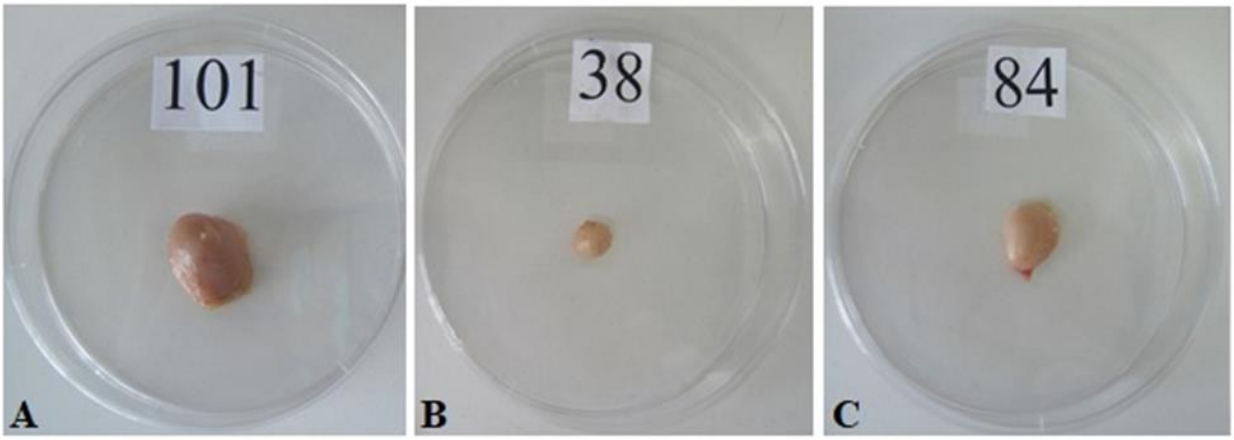


Figure 2. Bursae of Fabricius of SPF chickens at 28 dpi: (A) control group (B) ITA-IBDV group and (C) STC-IBDV group. The bursae of viruses-inoculated birds showed atrophy, which appeared more severe in birds of the ITA-IBDV group.

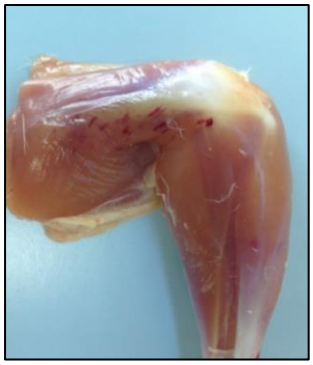


Figure 3. Hemorrhages in the thigh muscle of a SPF chicken inoculated with the IBDV strain of the ITA genotype (2 dpi).

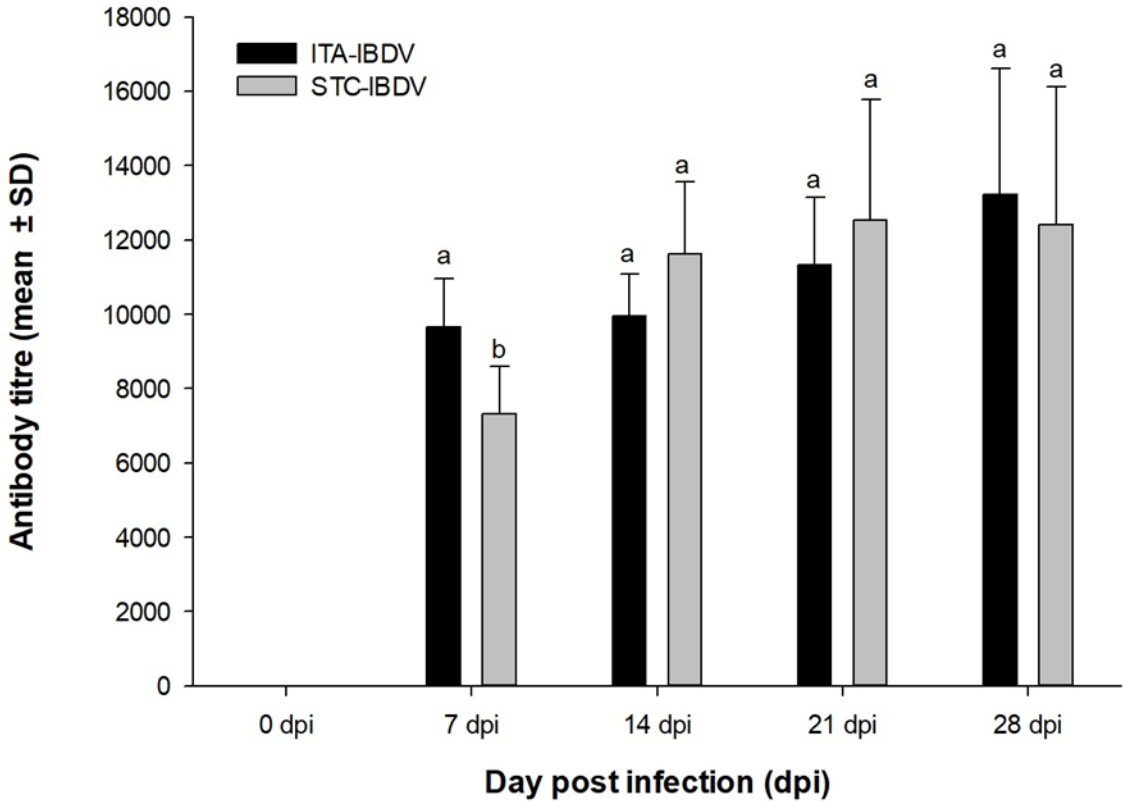
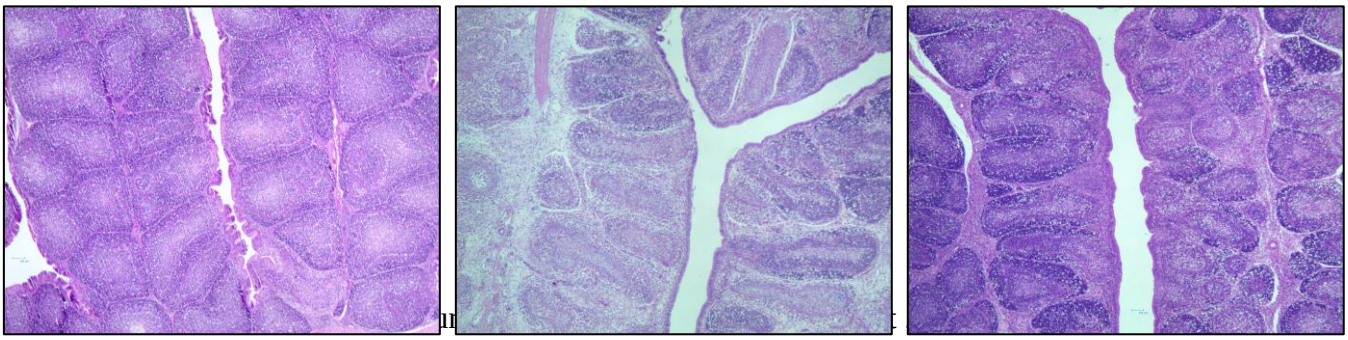
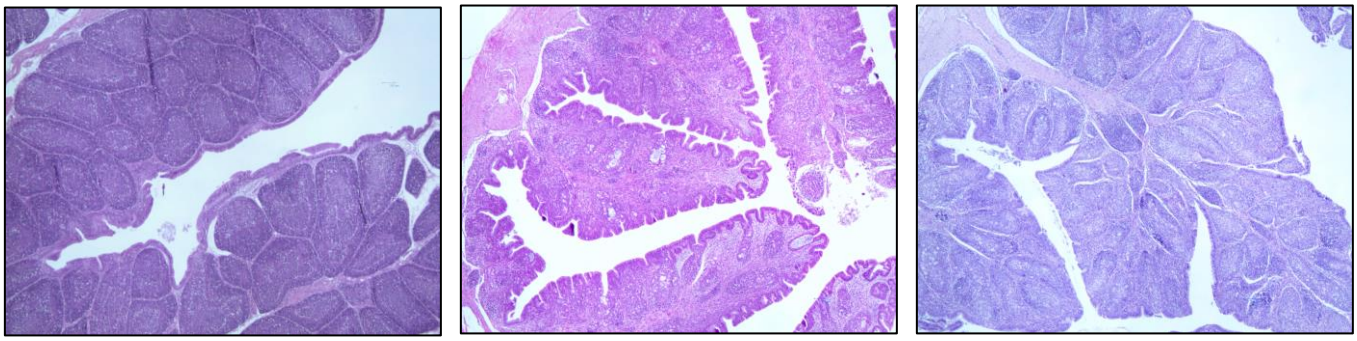


Figure 4. Mean IBDV antibody titres in ITA-IBDV and STC-IBDV groups by days post-inoculation. Different letters indicate that the difference between groups is statistically significant ($p<0.05$).



(A) (B) (C)

Figure 5. Normal lymphoid follicles in control birds; (B) severe follicular lymphocytes depletion in birds of ITA-IBDV group; (C) mild follicular lymphocytes depletion in birds of STC-IBDV group.



(A) (B) (C)

Figure 6. Histopathology of bursa of Fabricius of SPF chickens at 14 dpi (X40 magnification): (A) Normal bursal plicae in control birds; fold atrophy and follicular lymphocytes depletion in birds of ITA-IBDV (B) and STC-IBDV (C) groups.

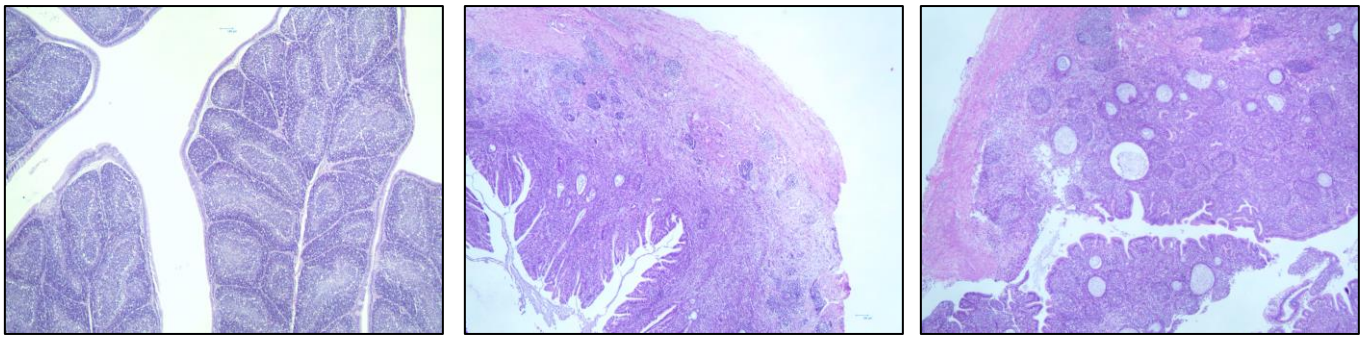
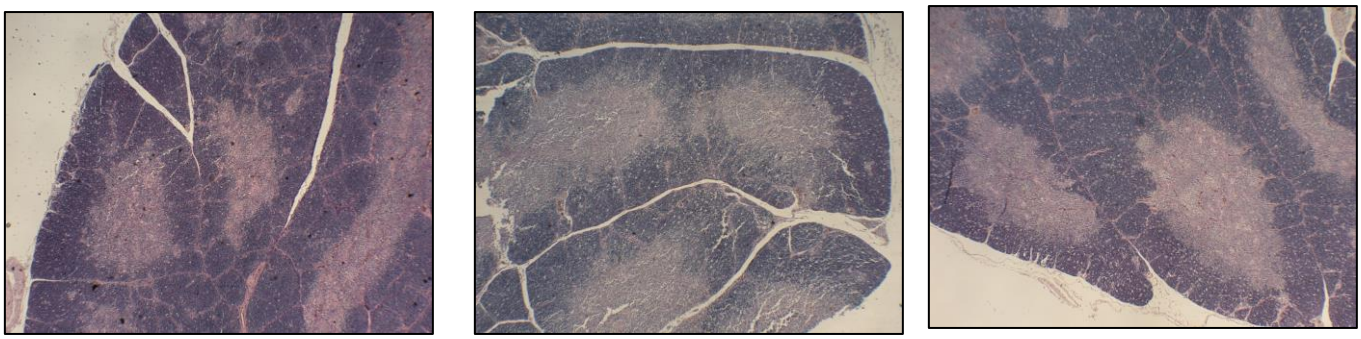


Figure 7. Histopathology of bursa of Fabricius of SPF chickens at 28 dpi (X40 magnification): (A) Normal pattern of bursa in control birds; fold atrophy, cistic degeneration and early lymphoid repopulation in bursae of birds of the ITA-IBDV (B) and STC-IBDV (C) groups.



(A) (B) (C)

Figure 8. Histopathology of thymus of SPF chickens at 2 dpi (X40 magnification): (A) virus-free control; cortical atrophy in ITA-IBDV (B) and STC-IBDV (C) groups.

Chapter III

Comparative dynamic tissue distribution and shedding of infectious bursal disease virus (IBDV) of ITA (G6) or classical (G1) genotypes by qRT-PCR after experimental infection of SPF chickens

Aim of the study

The present study was aimed to evaluate by quantitative reverse transcription PCR (qRT-PCR), the tissue distribution, persistence and fecal shedding of IBDV following experimental infection of specific pathogen free (SPF) chickens with a strain recognized to belong to the ITA genotype (G6) or a classical (G1) reference strain.

Material and methods

Ethical statements

Experimental trial was performed in agreement with national regulations on animal experiments and animal welfare, according to authorization N°635/2015-PR by the Italian Ministry of Health.

Chickens

Seventy-eight SPF chickens were used. Birds were housed in secure isolation facilities for the duration of the study and food and water were given *ad libitum*.

Viruses

Two field isolates of IBDV were used in the study, named, according with the new nomenclature proposed by Jackwood et al. (2018), strain IBDV 1/Italy/1829/11/(G6) (ITA genotype, genogroup 6) and strain IBDV 1/Italy/23II/12/(G1) (STC genotype, genogroup 1).
Viruses were isolated and inocula were prepared and titrated as described in chapter 2.

Experimental design

Birds were tagged and divided into three groups named as follows: ITA-IBDV group (30 birds), STC-IBDV group (30 birds) and a control group (18 birds). At 35 days of age, birds of ITA-

IBDV and STC-IBDV groups were orally inoculated with $10^{4.5}$ ELD_{50} of ITA or STC viruses, respectively. Chickens of the control group were kept as negative control and mock inoculated with sterile PBS. The groups were housed in separate isolators and the experiment was terminated 28 days post infection (dpi).

At 2, 4, 7, 14, 21 and 28 dpi, samples were collected for virus detection and quantification by qRT-PCR. From five birds of ITA-IBDV group, five from STC-IBDV group and three from the control group, cloacal swabs were collected. Birds were subsequently euthanized and from each of them tissue samples from bursa of Fabricius (BF), spleen, thymus, bone marrow, cecal tonsils and harderian gland, kidney, liver and proventriculus were collected.

Cloacal swabs processing

After sampling, the cloacal swabs were let to air dry under laminar flow cabinet. Once dried, the swabs were kept at room temperature until processing.

For elution each swab was dip in 250 µl of PBS in a 2 mL tube and vortexed for 1 min. Afterwards, the tubes were centrifuged at 4,500 rpm for 5 minutes at +4 °C. The swab was then removed from the tube and the eluted was stored at -80°C until RNA extraction.

Tissue collection and processing

Tissues were collected aseptically using separate sterile scissors and forceps and stored at -80 °C until processing. Tissue samples were weighed in a sterile 2 mL tube, and, to obtain a 10% suspension weight/volume (w/v), sterile PBS was added in accordance with the following formula:

PBS (ml) = weight of the organ or tissue (g) x 9

After homogenization for 10 seconds, samples were centrifuged at 2,000 rpm for 15 minutes at + 4 °C, the supernatant was collected and placed into a new 2 ml tube for storage in at -80 °C until viral RNA extraction.

RNA extraction and qRT-PCR for IBDV

The viral RNA extraction from eluted samples was carried out using Solution D containing Guanidine Thiocyanate according to Jing et al. (1993).

qRT- PCRs were developed and validated for this study to specifically detect and quantify genomes of IBDV of ITA or STC genotypes. Primers and probes were designed (Table 1) on the variable regions of VP2. The assay was performed using SuperScript® III Platinum® One-Step qRT-PCR Kit (Invitrogen^{TM}, Carlsbad, CA, USA) on LightCycler® Nano Instrument (Roche, Basel, Switzerland) following the manufacturer's instructions. The reaction profile used was: 50 °C for 15 min for RT step, 95 °C for 2 min, and 45 cycles of 95 °C for 15 sec, and 60 °C for 30 sec for the PCR step. The assay was validated using titrated virus suspensions (5.4 Log_{10} EID^{50}/mL for the STC genotype strain, 5.2 Log_{10} EID^{50}/mL for the ITA genotype strain), accounting for the qRT-PCR detecting both viable and non-viable viral particles in tissues or swab samples. The qRT-PCR limit of detection (LoD_{50}) was evaluated using serial 10-fold RNA dilutions in negative matrices, performed in quadruplicate, ranging from the undiluted virus to a final 10^{-7} dilution. LoD_{50} was defined as the lowest viral amount detectable in at least 50% of the replicates. LoD_{50} was $10^{0.49}EID^{50}$/mL for the STC strain and $10^{0.49}EID^{50}$/mL for the ITA strain. Standard curves generated for each titrated IBDV genotype strain were obtained and used for quantification by fitting a linear regression relating Cp and Viral titer. Additionally, the curves allowed to evaluate reaction efficiency and coefficient of determination, which were proven to be 92%-103% and higher than 0.97, respectively. All samples were tested in duplicates.

Statistical analysis

Data were analyzed using Fisher exact test and significant statistical differences between groups were reported when $p<0,05$. The viral genome quantity means were transformed to Log_{10}, and then the non-parametric Wilcoxon test was performed (SPSS, IBM) to assay statistical differences in RNA loads between groups, with 95% confidence interval.

Results

Results of IBDV detection in samples collected from birds of ITA-IBDV or STC-IBDV group, are reported in Table 2. No IBDV RNA was found in any birds of the control group.

Detection and quantification of ITA-IBDV and STC-IBDV genomes in lymphoid tissues

Bursa of Fabricius

No statistically significant differences between virus-inoculated groups were found in the number of bursa IBDV positive birds per days of sampling (Table 2). Quantification of viral RNA in bursal tissues showed that, at 4 and 7 dpi, the mean of RNA load in ITA-IBDV group was significantly higher ($p<0.05$) than the RNA load in STC-IBDV group (Figure 1).

Spleen

At 2 dpi spleens of virus-inoculated birds were all positive for IBDV (Table 2), although the quantification showed that ITA-IBDV group had a higher RNA load ($p<0.05$) compared to STC-IBDV group (Figure 2). At 14 dpi, higher number of positive birds and higher viral load ($p<0.05$) were also observed in ITA-IBDV group compared to STC-IBDV group. At 28 dpi, STC-IBDV group showed higher number of positive birds ($p<0,05$) compared to ITA-IBDV group (Table 2), but the viral load was not statistically different between groups (Figure 2).

Thymus

A significant higher number of IBDV positive birds ($p<0.05$) were observed in ITA-IBDV group at 14 dpi compared to STC-IBDV group. Furthermore, at 2, 4, 7 and 14 dpi ITA-IBDV group had a significant higher RNA load ($p<0.05$) compared to STC-IBDV group (Figure 2).

Bone marrow

IBDV RNA was more frequently detected in ITA-IBDV group than in STC group in bone marrow, as is deductible by total number of detections and RNA quantification at 2, 4, 7, 14 and 28 dpi ($p<0.05$) (Figure 2).

Cecal tonsils

No statistically significant differences between IBDV-inoculated groups were found in the number of cecal tonsil positive samples per day of sampling. Quantification of viral RNA showed that, at 4, 14, 21 and 28 dpi, ITA-IBDV group had a higher mean RNA load in cecal tonsils ($p<0.05$) compared to STC-IBDV group (Figure 4).

Harderian glands

Only at 2 dpi, ITA-IBDV group showed a higher RNA load ($p<0.05$) compared to STC-IBDV group (Figure 2).

Detection and quantification of ITA-IBDV and STC-IBDV genomes in non-lymphoid tissues

Kidney

A statistically higher number of positive birds ($p<0.05$) were observed at 14 dpi in ITA-IBDV group compared to STC-IBDV group. Furthermore, at 2, 4, 7 and 14 dpi kidneys of ITA-IBDV group had a higher RNA load ($p<0.05$) compared to kidneys of STC-IBDV group (Figure 3).

Liver

Only at 4 dpi, ITA-IBDV group showed a statistically higher RNA load in liver ($p<0.05$) compared to STC-IBDV group (Figure 3).

Proventriculus

At 2, 4 and 28 dpi STC-IBDV group shower higher number of birds IBDV positive in proventriculus ($p<0,05$) compared to ITA-IBDV group ($p<0.05$). The viral load was not different between IBDV-inoculated groups ($p>0.05$) (Figure 3).

Viral shedding

A higher number of positive swabs were found at 4 dpi, in birds of STC-IBDV group ($p<0,05$) compared to the birds of ITA-IBDV group ($p<0.05$) (table 2); however the viral load was not statistically different between virus-inoculated groups ($p>0.05$) (Figure 4).

Discussion

This study showed that a strain of the ITA genotype could be detected in SPF birds experimentally infected, for up to 28 dpi in lymphoid or non-lymphoid tissues, with higher load in bursa of Fabricius, cecal tonsils and bone marrow. As expected, the bursa of Fabricius has been confirmed to be the most important site for IBDV replication. Both viruses were detected in bursa until 28 dpi and at notable load. Seen the higher viral load detected, most lymphoid organs are likely to play an important role in viral persistence in chickens.

Moreover, in the present study is confirmed, for both tested viruses, that non-lymphoid tissues, play a marginal role in IBDV persistence, as previously reported by Abdul et al. (2013) for a variant and a classical IBDV strains. In particular within non-lymphoid organs, viral load is negligible in proventriculus.

Significant higher viral loads (p <0.05) were found in ITA strain- inoculated birds, in all organs tested except for proventriculus. Most part of those differences is found in the first two-week post-infection, especially in bursa, thymus, bone marrow and kidney. Moreover, bone marrow presented the same tendency until the endpoint of the experiment. Cecal tonsils of ITA-IBDV inoculated birds showed a notable viral load, higher than the one detected in the bursa, at the end point of the trial.

For this reason, cecal tonsils and bone marrow may serve as non-bursal lymphoid tissues supporting virus replication at later time points post-infection. Nevertheless, the detection of vRNA is not indicative of the presence of the infectious virus, and virus isolation has to be performed to prove the presence of infectious particles.

IBDV fecal shedding is known to be short lasting after infection (Takase et al., 1982; Zhao et al., 2013), this is confirmed by our results for both tested viruses, although the shedding of the ITA IBDV strain is even shorter, being the virus excreted only for the first 48 hours post-infection. The faster antibody response observed in ITA IBDV strain inoculated birds (see chapter 2) can be one reason explaining this behaviour. A similar hypothesis was previously advanced to explain a faster clearance for lymphoid organs of birds experimentally infected with Australian or US variants (Abdul et al., 2013; Jayasundara et al., 2017).

The dynamic distribution and persistence of an IBDV strains of the ITA genotype after experimental inoculation of SPF chickens, reported in the present study, show an overall high replica-

tion rate of the tested virus in lymphoid tissues. This can be taken as support to the notion that this new genotype possesses a high aggressiveness, as demonstrated in the pathogenicity study reported in chapter 2.

Table 1. Primers and probes designed and used for detection and quantification by qRT-PCR, of STC and ITA genotypes of IBDV in tissues and swabs.

Primer	Classical	Position in the genome
STC-IBDV		
Forward	TGGAGACTATGGGCATCTAC	2715-2734[a]
Reverse	CGGTATTTCTCGTGTGTTCT	2805-2824[a]
Probe	TAGCACTCAATGGGCACCGA	2754-2773[a]
ITA-IBDV		
Forward	CTCAGCCTGCCCACATCATA	389-408[b]
Reverse	CGTTACCCCACCTTGTTGGT	549-568[b]
Probe	AGGCTTGGWGACCCCATTCC	425-444[b]

[a] Based on the sequence of IBDV, strain 150127/0.2 (GenBank accession no.: MF969107).

[b] Based on the sequence of IBDV, strain IBDV/Italy/1829/2011(GenBank accession no.: KY930929.1).

Table 2. IBDV RNA detection in lymphoid and non-lymphoid tissues, and in swabs.

Sample	2 dpi[a]		4 dpi		7 dpi		14 dpi		21 dpi		28 dpi		Total	
	STC	ITA	STC	ITA	STC	ITA	STC	ITA	STC	ITA	STC	ITA	STC	ITA
Lymphoid tissues														
Bursa	5	5	5	5	5	5	4	5	4	5	4	4	27	29
Spleen	5	5	5	3	5	5	1*	4*	2	3	5*	1*	23	21
Thymus	4	5	5	5	4	5	1*	5*	0	0	2	2	16	22
Bone marrow	4	4	5	5	3	5	3	5	0	3	0*	5*	15*	27*
Cecal tonsils	5	4	5	5	5	3	2	4	4	5	3	5	24	26
Harderian gland	3	3	5	3	1	2	2	3	0	1	2	3	13	15
Non-lymphoid tissues														
Kidney	5	2	4	4	4	5	0*	5*	0	1	1	0	15	17
Liver	5	5	5	4	3	3	3	1	0	0	0	0	16	13
Proventriculus	4*	0*	5*	1*	1	1	1	0	0	1	4*	0*	15*	3*
Shedding														
Swabs	5	4	4*	0*	3	1	0	0	0	0	0	1	12	6

[a] Day post-inoculation.
[b] Number of birds out of 5 from which viral RNA was detected.
* Statistically significant difference ($p<0.05$)

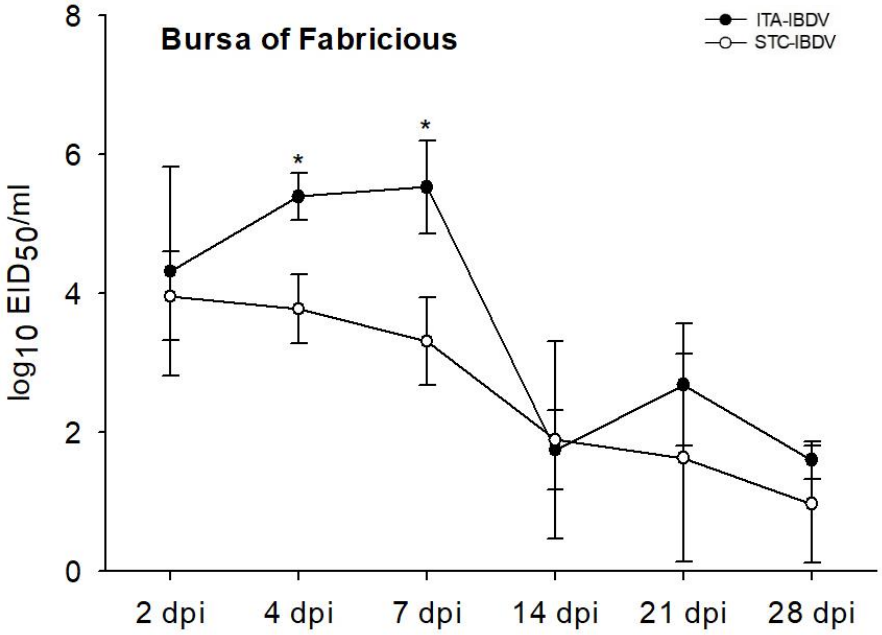


Figure 1. IBDV RNA load in bursa of Fabricius of birds of virus-inoculated experimental groups. The asterisk (*) indicates a statistically significant difference ($p<0.05$).

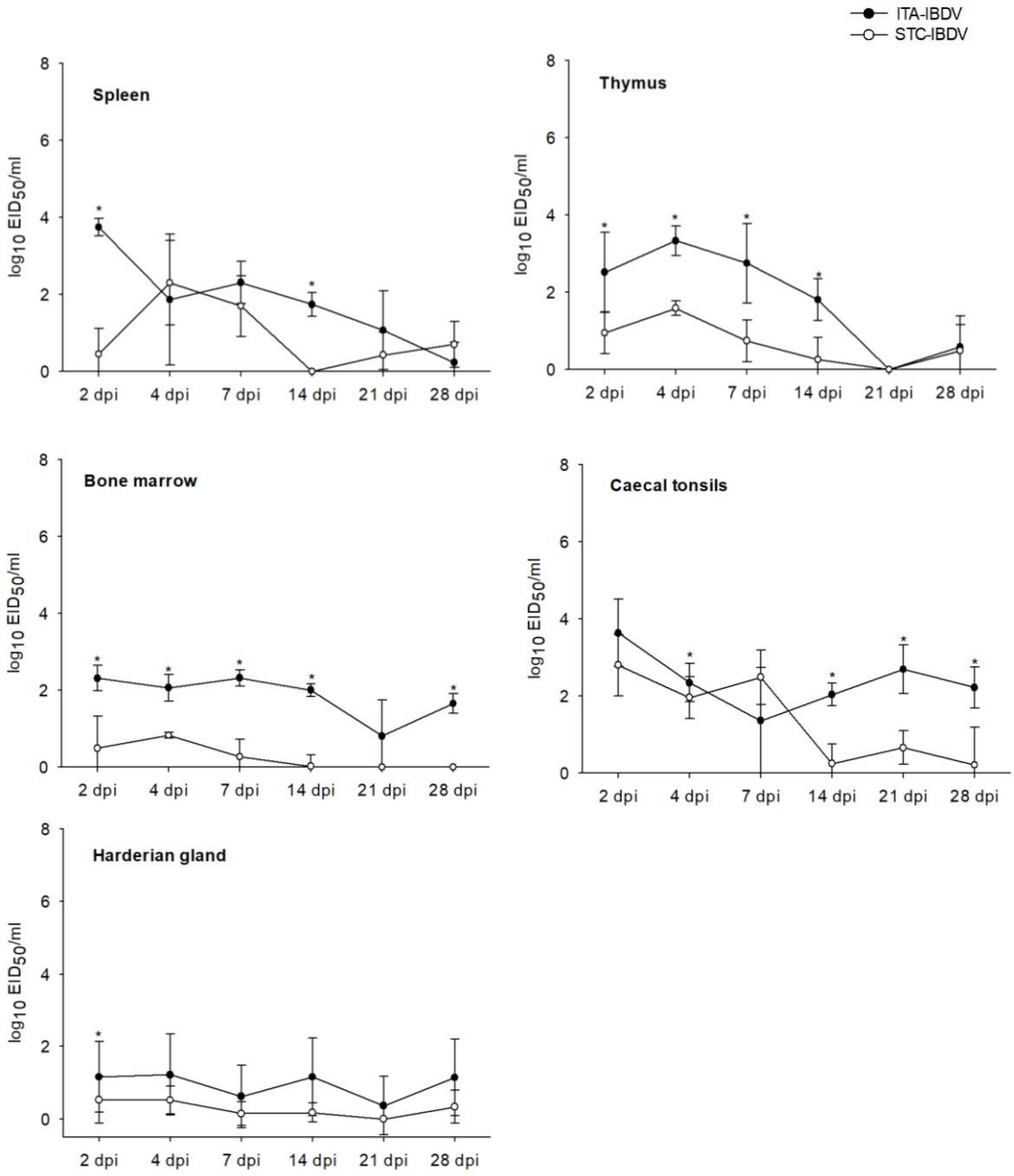


Figure 2. IBDV RNA load in lymphoid tissues of birds of virus-inoculated experimental groups. The asterisk (*) indicates a statistically significant difference ($p<0.05$).

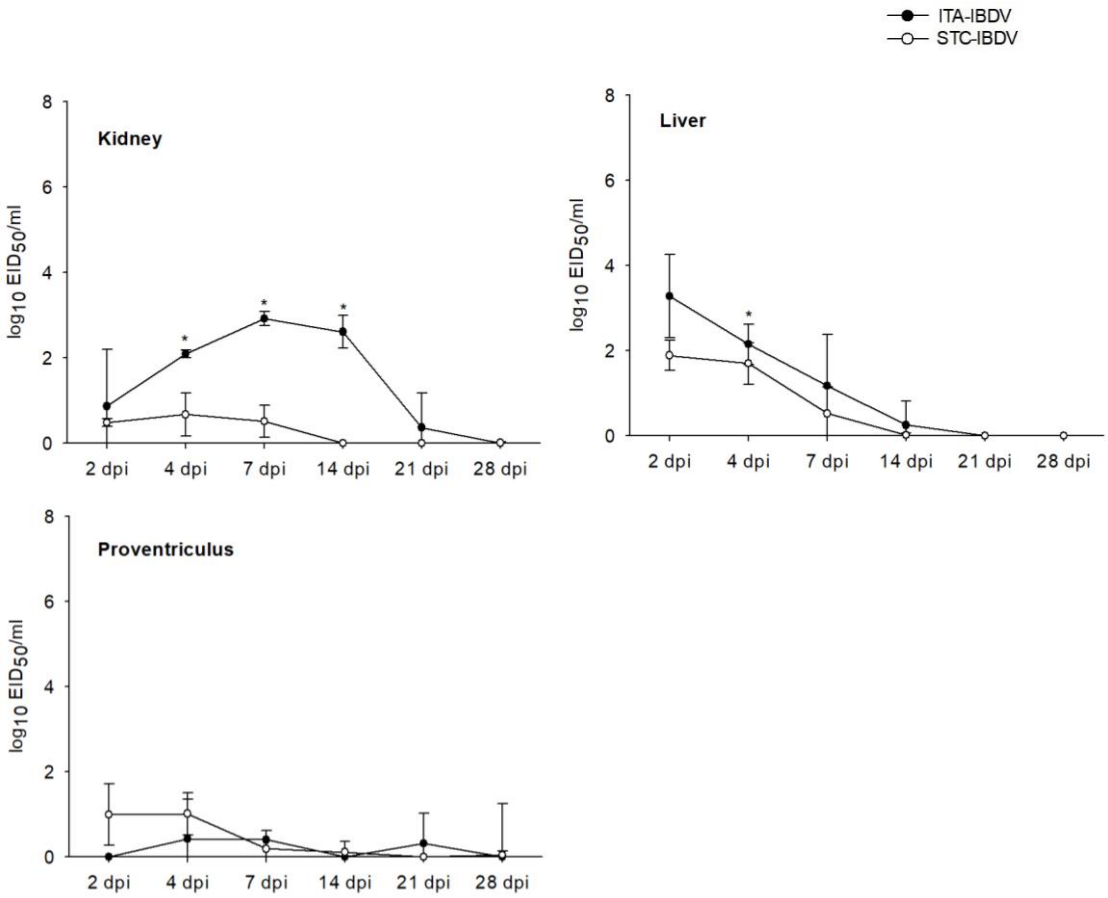


Figure 3. IBDV RNA load in non-lymphoid tissues of birds of virus-inoculated experimental groups. The asterisk (*) indicates a statistically significant difference ($p<0.05$).

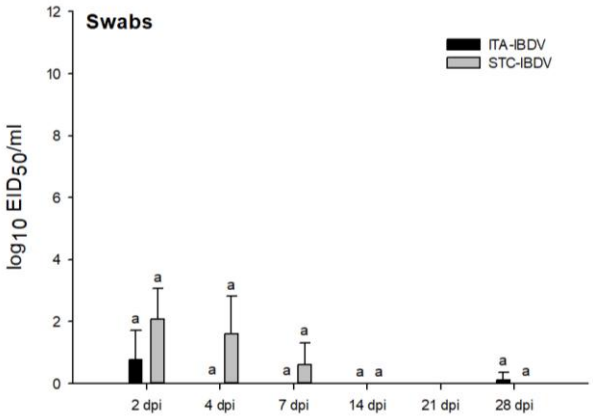


Figure 4. Mean RNA shedding in ITA-IBDV or STC-IBDV inoculated groups. The different letters (*) indicate a statistically significant difference ($p<0.05$)

Conclusions

The pathogenicity characterization of a strain of a distinctive genotype of IBDV as ITA genotype is extremely important to understand how IBDV has evolved in order to circumvent the control strategies that have been developed over time. The in-depth pathological study of these new strains is fundamental to improve strategies to control and prevent IBD.

Recently, the emergence of distinctive strains of IBDV has occurred worldwide and strains causing subclinical form of IBDV have been shown to be a big challenge to poultry health, since they can lead to infections with secondary pathological agents, such as Escherichia coli, through immunosuppression and a decreased response to vaccines for other important poultry pathogens.

In the present thesis a strain belonging to the IBDV ITA genotype, strain IBDV 1/Italy/1829/11/(G6) was, for the first time, inoculated in SPF chickens to determine virulence and tissue distribution in comparison to an IBDV strain belonging to the classical STC genotype.

Results showed that ITA genotype is able, thought it as a subclinical course, to cause a severe a persistent damage of bursal tissues and, in addition to involve the thymus. Moreover viral load in lymphoid tissues of ITA-inoculated birds resulted to be significantly higher than in birds inoculated with the classical strain. Taken together these results indicate aggressiveness of the new genotype for the lymphoid tissues, which may be indicative of a significant immunosuppressive potential.

Protection conferred by the existing commercial vaccines to ITA IBDV genotype infection is still unknown and need to be further investigated trough in vivo cross-protection studies in order to implement efficacious vaccination plans in commercial poultry farms.

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