

Original Article

Isolation, Characterization and Standardization of New Infectious Bursal Disease Virus for Development of a Live Vaccine

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Abstract

Background and Aims: Infectious bursal disease (IBD) is an acute contagious viral disease of birds worldwide. The causative virus induces a persistent immune suppression following destroy B lymphocytes precursors in bursal lymphoid follicles. Vaccination is the main strategy for prevention of the disease in commercial poultry industry.

Materials and Methods: To produce a live vaccine against the disease, a new virus strain was isolated from the affected bursa of Fabricius. Diagnostic serological tests, histopathology and molecular examinations were done for pathotyping the isolate.

Results: The results indicated that the virus is a new strain and named IBD07IR. In case of developing live IBD vaccine, the bacteriology, purity, titration, reversion to virulence, bursa-body index, immunosuppression and efficacy tests were performed on the candidate vaccine virus seed. The live vaccine was produced following propagation of the IBDV seed in SPF embryonated eggs. The proper dose of the vaccine was found by administration of SPF chicken groups. Afterward, the laboratory trial was scaled and the clinical trials were done three times with different administration routes.

Conclusion: According to serological assays and challenge, the developed live IBD vaccine induces an adequate immunity in both SPF and commercial chickens and had no adverse effects.

Keywords: Infectious Bursal Disease; Intermediate; Poultry; Vaccine; Production

Introduction

Infectious Bursal Disease (IBD) is an acute and contagious progressive disease of chickens that induces high morbidity and mortality in young chickens about 3-6 weeks of age. The causative agent is a virus belonging to the family *Birnaviridae* of the genus *Avibirnavirus* characterized by a nonenveloped icosahedral capsid containing a double-stranded RNA genome consisting of A and B

segments (1). Two serotypes of IBDV have been recognized, of which serotype 1 may cause disease in chickens, whereas serotype 2 is avirulent (1, 2). Infection with the pathogenic strains of serotype 1 leads to severe necrosis and destruction of B-lymphocytes in the bursa of Fabricius (3, 4). The pathogenicity of IBDV strains might be involved in the induction of apoptosis in host cells (5). The IBDV classical strain causes bursal inflammation and severe lymphoid necrosis in infected chicken, resulting in immunodeficiency and moderate mortality from 20–30% in specific pathogen free (SPF) chicken (6). In 1980's the very virulent IBDV

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(vvIBDV) first appeared in the Netherlands rapidly spread all over the world including Europe, Africa, the Middle East, South America and Asia (7, 8). The strains have been characterized by severe clinical signs and high mortality ranging from 60-100%. The disease in younger chickens is usually sub-clinical and results in immunosuppression with subsequent poor immune response to other infections and negative interference with effective vaccination. Therefore, the disease has a significant economic impact on all areas of intensive poultry farms (2, 8).

From the early 1970s, numerous IBD vaccines and vaccination programs have been studied for prevention and control of IBD (9). It has been suggested that, vaccine should be prepared only from strains of virus which are of attenuated virulence or from naturally occurring strains of low virulence (10). A live attenuated vaccine based on a classical virulent strain was developed to provide maternal immunity to the off-springs. The use of live IBDV vaccine has been very successful in controlling the disease, with the aim of maintaining the immune response induced by the parent virus (11-14). The live vaccine mimics infection in poultry and can induce both cellular and humoral immunity. Hyper-immunization of parent breeder chickens with live IBDV vaccine followed by booster vaccination to maintain the protective antibody levels protects the young chicken against clinical and/or subclinical disease.

This study was carried out based on a) characterization a local IBDV isolate, b) standardization the vaccine seed, and c) developing a live IBD vaccine and evaluating the induced protective immunity in farm.

Methods

Virus isolation and characterization

Specimen from broiler farms suffered from mild hemorrhagic and oedema of bursa were collected. The bursa was removed aseptically, chopped and homogenized in peptone broth containing antibiotics. Freez-thaw processing was done in three repeated steps after 15 minutes interval. Homogenate was centrifuged

at 4000 RPM for 20 min. The supernatant was collected carefully and filtered through 0.22 μ m filter and stored at -70°C until use. 0.1 ml of the homogenate was inoculated in to the allantoic cavity of five 9-11-day-old embryonated SPF eggs. Up to five days post-inoculation (pi) the embryos were examined for lesions including subcutaneous oedema, congestion and intracranial haemorrhages, and abnormalities of internal organs. The presence of IBDV was also tested in embryo by agar gel immunodiffusion (AGID) and virus neutralization (VN) assays.

Agar gel Immunodiffusion (AGID) test. The gel was made with 1.5% Nobel agar containing 8% sodium chloride and 0.1% Merthiolate-aspreservative in distilled water. When the agar was set, a pattern of wells punched in agar dish. -The serum sample, central wells and the virus samples, surrounding wells. Dishes were incubated in a humid atmosphere and examined 48 h later for lines of precipitation.

Virus Neutralization (VN) test. The α -procedure (constant-serum, diluted-virus) was used for serological identification of the isolated virus. 10-fold dilution of virus was added to undiluted sera against IBDV. The virus/serum mixtures were incubated onto the dropped CAMs of 9-11 day-old fertile SPF chicken eggs. Eggs were incubated at 37°C up to 7 days and the neutralising index (NI) was calculated.

Molecular identification of IBDV isolates. Viral RNA was extracted from the bursal homogenates using High Pure RNA Extraction Kit (Roche, Germany). A 743 bp fragment of VP2 gene was amplified using a primer set: forward 5'-GCCAGAGTCTACACCAT-3' and reverse 5'-CCCGGATTATGTCTTTGA-3' and one-step RT-PCR kit (iNtRON Biotechnology, Korea). The RT-PCR products were digested with BstNI and MboI restriction enzymes (15) to identify the pathogenic characterization of IBDV isolates. The nucleotide compositions sequenced and their phylogenetic relationships with 48 VP2 gene sequences of IBDV isolates previously deposited in GenBank were determined using the minimum evolution analysis with 1000 bootstrap replication in MEGA4.

Histopathology. The bursa of treated chickens was placed in 10% formalin and embedded in paraffin wax. The sections were stained with H&E and examined microscopically.

Vaccine seed preparation and standardization

According to serological, molecular, and pathological analysis, one IBDV isolate was candidate for vaccine seed preparation. At first step, the master seed virus was prepared by inoculating of the selected isolate in 9-11-day-old embryonated SPF eggs. The infected embryos were homogenated and centrifuged. Then the sterility, purity, reversion to virulence, and immunosuppression assays were managed on the supernatant according to the standard protocol (10). Reversion to virulence test was carried out by inoculation of the virus to ten 7-day old SPF chicks. Bursa was removed aseptically four days pi then homogenated and inoculated to other ten chickens. At least five consecutive chicken-to-chicken passages were applied at four day intervals. Induction of immunosuppression was performed by eye-drop administration of the virus to each 20 SPF chickens at 1-day old. Two weeks pi, each bird was received the Newcastle disease vaccine (B1 strain, Razi Institute, Iran) and the haemagglutinin inhibition (HI) response was measured after two weeks.

IBD vaccine development and validation

Vaccine production. IBV vaccine was developed according to OIE (16) and FAO (10) protocols.

Dose finding. Vaccine dose was determined by inoculation of 2, 2.5, 3, 3.5, and 4 EID₅₀ log₁₀ of the seed virus into five groups of 7-day-old SPF chickens (n=10) via eye-drop method. Serum samples were taken prior and three weeks pi and IBDV antibody titers evaluated using VN and ELISA commercial kit (IBD-Idexx®).

Experimental procedure in SPF chickens.

Eighty SPF chickens were divided into two treatment groups; A and B comprising 30 birds were vaccinated by eye-drop and drinking water on day 7. The remaining 20 birds were kept as non-vaccinated control. At 2, 3, 4, 6, and 8 weeks post-vaccination sera

were collected and assayed for antibody levels against IBDV by ELISA. At three weeks pi the VN test was done. Twenty chickens per groups were challenged by eye-drop with >10² CID₅₀ of a vvIBDV strain at 3 weeks pi. At the end of experiment, 10 birds were individually necropsied and the bursa collected and tested. For safety test, ten field doses of vaccine were administered by eye-drop to each of 15 SPF chickens of 1-wk-old age. The chickens were observed up to 21 days for any clinical symptom or mortality.

Experimental procedure in commercial chickens. A total number of 1000 Ross broiler chicks were divided into two treatment groups of 500 each. Group A was received the IBD vaccine via intraocular route and group B vaccinated via drinking water. One hundred chickens were considered as non-vaccinated control group. Challenge and serological assessment were performed in the same manner as detailed above.

Statistical analysis. Serological data were analyzed by using t test. The data for challenge assays were compared using Pearson Chi-square test. P<0.05 was considered as meaningful differences.

Results

The isolated viruses were checked for their antigenicity and pathogenicity. In AGID test, precipitated line appeared with IBDV hyperimmune serum and the NI estimated 3.17 followed virus neutralization. Hence diagnosis of IBDV was confirmed through the serological assays. Pathotype of the isolates was identified using RFLP patterns. The PCR products were digested with restriction enzymes and the results revealed that the examined strains assigned to be intermediate and vvIBDVs (figure 1). An intermediate isolate named IBD07IR was subjected to vaccine production procedure.

Data ensuring that virus master seed used in the production of IBD vaccine were sterile and free of extraneous agents. The virulence of IBD isolates was determined by calculation of B:B index, reversion to virulence, and immunosuppression tests. The B:B index was

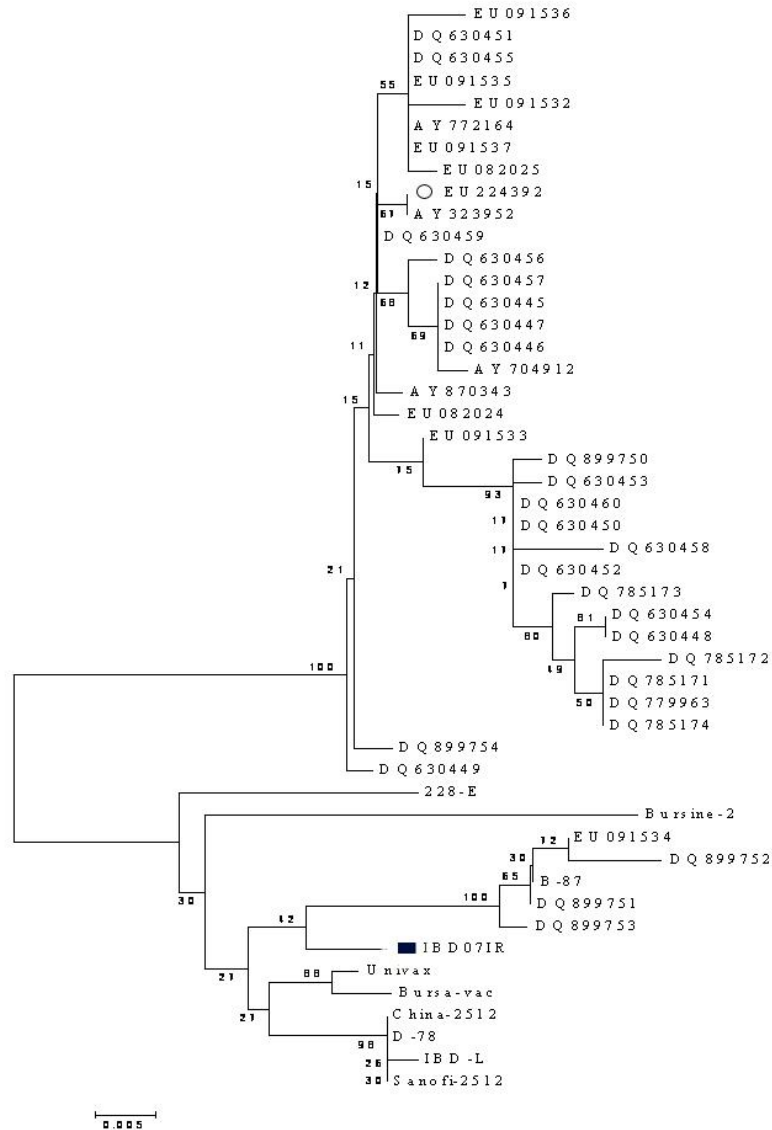


Fig. 1. Phylogenetic relationships of IBDV VP2 gene sequences deposited in GeneBank is shown by construction of a tree with 1000 bootstrapping.

examined 1.14 in chickens inoculated with IBD07IR virus and 0.21 for groups exposed with vvIBDV. None of chickens showed disease signs in all six serial passages indicated that the virus was not reverse to virulence. In Immunosuppression test, no significant difference in GMT HI titer was found between the chickens received IBD07IR alone (5.14) and others received both B1+IBD07IR (5.07). In vaccine production procedure, the best virus titer per dose was estimated ≥ 3 EID₅₀ log₁₀ (Figure 2). In SPF and commercial chicken experimental procedures, the ELISA antibody titers against IBDV were raised in both treated groups which

vaccinated by different routes. The results were summarised in figures 3 and 4.

The VN results indicated that the vaccine neutralized the virus. By the end of 21 days post vaccination the chickens were healthy and did not exhibit any clinical signs of IBD or dies from causes attributable to the vaccine virus.

In efficacy test, no seeing of IBD disease was seen in chickens of group 1 until the end of experiment. All of them showed complete protection against challenge test. Similar results were obtained for groups 2. In group 2 one chicken (5%) was shown a moderate sign of disease and 90% protection estimated. Seventeen chickens (85%) of control groups

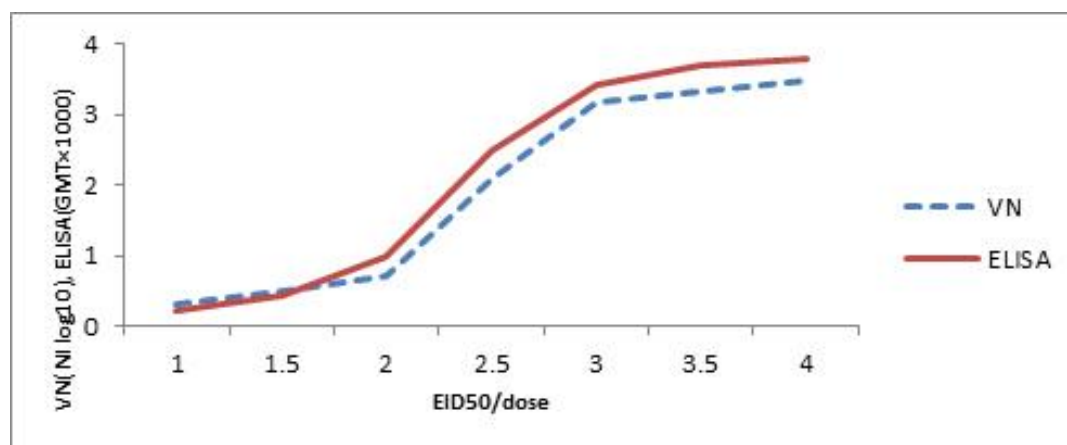


Fig. 2. Dose finding of the live IBD07IR vaccine with VN and ELISA assays.

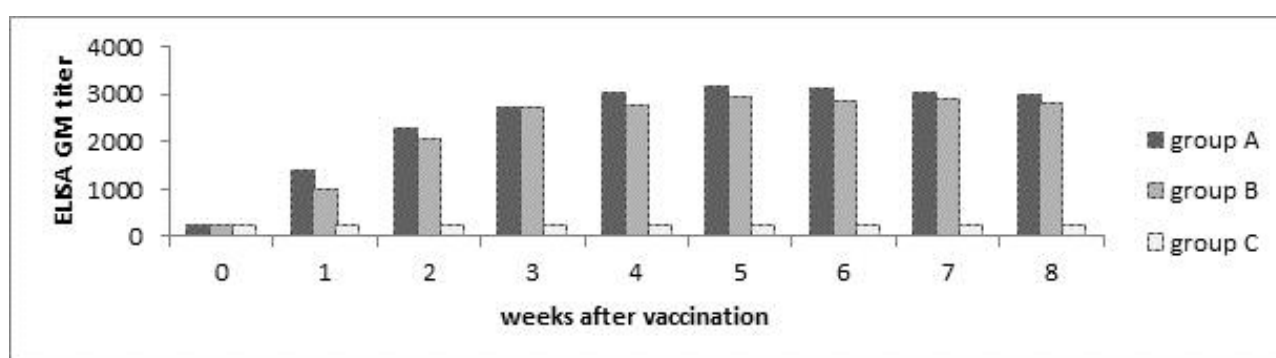


Fig. 3. The ELISA antibody titers against IBDV after vaccination of SPF chickens with IBD07IR vaccine. A: eye drop, B: drinking water, C: control.

died and the remained were shown the marked signs of IBD.

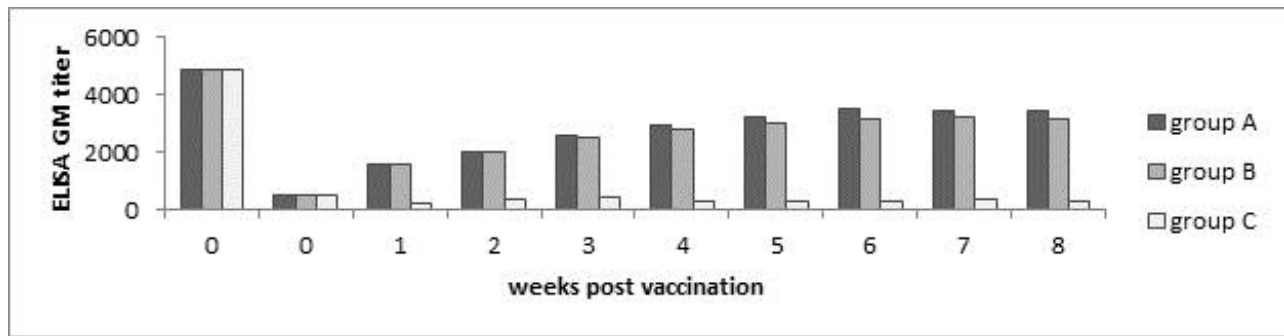


Fig. 4. The ELISA antibody titers against IBDV after vaccination of commercial chickens (Ross 308) with IBD07IR vaccine. A: eye drop, B: drinking water, C: control.

In challenge, the group A of SPF chicken trial showed 100% protection, while group B showed 95% protection in compared to the control group which had 85% dead and 15% clinical disease. On commercial chickens, group A showed 19/20 (95%) protection done 1/20(5%) mild clinical disease. Group B showed 18/20 (90%) protection and 2/20(10%) mild clinical disease while 11/20 (55%) dead and 9/20 (45%) clinical disease were observed in control group.

Discussion

Immunosuppression continues to be a major concern for the poultry industry worldwide. This condition is characterized by dysfunction of the immune system, with a suboptimal response either by the humoral or the cellular compartments. IBDV primarily impairs the humoral immune response which is followed by severe immunosuppression due to down regulation of T cells and macrophages which leads to an increased susceptibility to other pathogens (2, 4, 8). IBD vaccination program including priming with a live attenuated followed by boosting with an inactivated vaccine is used for vaccinating parent stocks (13, 17). A careful selection of virus strains with a sensitive method of determining virulence is important when establishing a live virus vaccine (10). In this study we develop a live IBD vaccine from naturally occurring strains of low virulence which we characterized as intermediate strain to produce an active immunity in young chickens. Clinical disease due to infection with the IBDV was diagnosed by a combination of characteristic signs and post-mortem lesions in an affected

farm. Laboratory diagnosis of IBDV was performed by detecting the presence of viral antigen and viral genome in bursa using VN and AGID serological tests, pathological, and molecular examinations. The serological results detected viral antigen in the bursa at the early stages of the infection. The RT-PCR/RFLP pattern was used for differential pathotyping of the isolates (15, 18). The nucleotide sequences examined for candidate virus was 98.1% homology and matched with intermediate IBDV strains. In the step, an IBDV isolate named IR/emm1/07 was characterized as an intermediate vaccine seed. Second, an IBDV isolate was candidate for vaccine seed virus preparation. The virus further standardize by testing its pathogenicity in SPF eggs and SPF chickens. According to FAO, some parameters were used for evaluation the potential of IBDV isolate including immunosuppression effect based on mean of HI titer for NDV with protection, reversion to virulence test, histopathological change for bursa of fabricius, calculation of B:B index, and antibody level against IBD which monitoring by ELISA and VN. In immunosuppression test GMT HI titers of the vaccinated and the control groups were calculated 5.07 vs 5.14 and no significant difference was found between them. The B:B index was estimated 1.14 following vaccination with the developed IBD live vaccine. There was no significant difference in the index between vaccinated and control groups after six serial passages. The calculate index higher than 0.70 is revealed normal bursa without atrophy (10). The results of sterility, safety, potency and immunogenicity were compared according to the international standards (10, 16).

For studying the efficacy of the IBD vaccine in field; the immune responses were determined by measuring ELISA and NV titer post vaccination and also by challenge (19). All birds were observed for two weeks post challenge and signs, lesions and mortalities were recorded; no clinical signs or lesions were recorded in the vaccinated groups. The bursa of control chickens (un-vaccinated) challenged with very virulent IBDV appear yellowish; hemorrhagic and turgid with prominent striations, oedema and caseous material were found in some birds.

Chickens infected with IBDV experience suppression in both humoral and cellular immunity. Humoral immunosuppression appears to be associated with IBDV-induced B-cell destruction, while the mechanism of cellular immunosuppression is largely elusive (20). Along with strict hygiene management of poultry farms, vaccination programs with inactivated and live attenuated viruses have been used to prevent IBD. The right strategy for IBD control and its success rate under field conditions depends on hygiene management, IBD field pressure, level and variation in maternally derived IBD antibodies, and the IBD vaccine strains to be used (21). As chickens are most susceptible to IBDV in their first weeks of life, active immunity to the virus has to be induced early after hatching. However, maternally derived IBDV-specific antibodies may interfere with early vaccination with live vaccines (22). Early passive protection of the broiler against infection can be conferred through maternally derived antibody. Intermediate vaccines are more capable of overcoming low levels of maternally derived antibody (11). In broilers, current strategies for prevention and control of IBD include passive immunological protection, hygiene and vaccination on the broiler farm with live vaccines. The vaccines are used to produce an active immunity in young chickens usually administered by eye dropping or in drinking water. The results of the present study showed that protection percentages were ranged between 95%-100% in vaccinated group with the intermediate live IBD vaccine depends on vaccination routes. The severity of

microscopic lesions was correlated with bursal atrophy as measured by B:B ratios. The immunosuppressive potential of the live IBD vaccine was estimated by evaluating the chicks' serological response to a Newcastle disease vaccine administered by eye-drop two weeks following vaccination against IBD disease. The vaccine also provided adequate protection against the challenge. Taken together the newly developed IBD vaccine considered satisfactory and potent. With pride CVL certification confirms our results on development of the live IBD vaccine. The mass production license was obtained from Iranian veterinary organization.

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References

1. Murphy FA, Gibbs EPJ, Horzinek MC, Studdert MJ. Birnaviridae. In: *Veterinary Virology*. 3th edn. 2000;405-9.
2. Lukert PD, Saif Y M. Infectious Bursal disease. In: YM Saif et al (Eds.) *Diseases of Poultry* 11th edn. 2008;161-79.
3. McFerran JB. Infectious Bursal Disease. In: JB McFerran et al (Eds.) *Virus Infection of Birds*, B.V. 1993;213-30.
4. Palmquist JM, Khatri M, Cha RM, Goddeeris BM, Walcheck B, Sharma JM. In vivo activation of chicken macrophages by infectious bursal disease virus. *Viral Immunology*. 2006;19:305-15.
5. Shahsavandi S, Ebrahimi MM, Sadeghi K, Mahravani H. Apoptotic response of chicken embryonic fibroblast cells to infectious bursal disease virus infections reflects viral pathogenicity. *In Vitro Cellular & Developmental Biology-Animal*. 2014;50:858-64.
6. Rautenschlein S, Samson-Himmelstjerna G, Haase C. A comparison of immune responses to infection with virulent infectious bursal disease virus (IBDV) between specific-pathogen-free chickens infected at 12 and 28 days of age.

Veterinary Immunology and Immunopathology. 2007;115:251-60.

7. Muller H, Islam MR, Raue R. Research on infectious bursal disease-the past, the present and the future. *Veterinary Microbiology*. 2003; 97:153-165.

8. Sharma JM, Kim IJ, Rautenschlein S, Yeh, HY. Infectious bursal disease virus of chickens: pathogenesis and immunosuppression. *Development Comparative Immunology*. 2000;24:223-35.

9. Kulkov L, Lekov U, Jurajda V, Juranov R. Effects of Infectious Bursal Disease Vaccination Strains on the Immune System of Leghorn Chickens. *Acta Veterinaria*. Brno. 2004;73,205-9.

10. Payla V. Preparation and control of inactivated Newcastle disease vaccine. In: Manual for the production of Marek,s disease, Gumboro disease and inactivated Newcastle disease vaccines. 1991;29-43.

11. Rautenschlein S. Yeh HY, Sharma JM. Comparative immunopathogenesis of mild, intermediate, and virulent strains of classic infectious bursal disease virus. *Avian Diseases*. 2003;47:66-78.

12. Smith J, Sadeyen JR, Butter C, Kaiser P, Burt DW. Analysis of the early immune response to infection by infectious bursal disease virus in chickens differing in their resistance to the disease. *Journal of Virology*. 2015;89:2469-82.

13. Zorman RO, Krapez U, Slavec B, Jursic-Cizerl R, Poljanec T. Field efficacy of different vaccines against infectious bursal disease in broiler flocks. *Acta Veterinaria Hungaria*. 2011;59:385-98.

14. Mazariegos LA, Lukert PD, Brown J. Pathogenicity and immunosuppressive properties of infectious bursal disease "intermediate" strains. *Avian Diseases*. 1990;34:203-8.

15. Jackwood DJ, Sommer-Wagner SE. Detection and characterization of infectious bursal disease viruses in broilers at processing. *Prevention Veterinary Medicine*. 2010;97:45-50.

16. Etteradossi N. Infectious bursal disease (Gumboro disease). In: Office International des Epizooties, World Organization for Animal Health. 4th edn. 2008;549-65.

17. Jakka P, Reddy YK, Kirubaharan JJ, Chandran ND. Evaluation of immune responses by live infectious bursal disease vaccines to avoid vaccination failures. *European Journal of Microbiology and Immunology*. 2014;4:123-7.

18. Juneja SS, Ramneek DD, Oberoi MS, Singh A. Molecular characterization of field isolates and vaccine strains of infectious bursal disease virus. *Comparative Immunology Microbiology and Infectious Diseases*. 2008;31:11-23.

19. Gallardo RA, Carrasco-Medanic R, Zhou H, Lyu S, Wang Y, Woolcock PR, Hoerr FJ. Effects of challenge with very virulent infectious bursal disease virus reassortants in commercial chickens. *Avian Diseases*. 2014;58:579-86.

20. Kim IJ, You SK, Kim H, Yeh HY, Sharma JM. Characteristics of Bursal T Lymphocytes Induced by Infectious Bursal Disease Virus. *Journal of Virology*. 2000;74:8884-92.

21. Block H, Meyer-Block K, Rebeski DE, Scharr H, de Wit S, Rohn K, Rautenschlein S.. A field study on the significance of vaccination against infectious bursal disease virus (IBDV) at the optimal time point in broiler flocks with maternally derived IBDV antibodies. *Avian Pathology*. 2007;36:401-9.

22. Muller H, Mundt E, Etteradossi N, Islam MR, Raue R. Current status of vaccines against infectious bursal disease. *Avian Pathology*. 2012;41:133-9.