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Effect of Co-Administration of 3',5'-Cyclic Diguanylic Acid with Infectious Bursal Disease Virus Vaccine on Serum Antibody Levels in Broilers

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Abstract: Cyclic diguanylic acid (c-di-GMP) is an intra-cellular bacterial signalling molecule previously shown to possess immune-stimulatory activities. However, the effects of this molecule on chicken immune responses have not been investigated. This study evaluated the humoral immune response following oral administration or Intra-Muscular injection (IM) of saline, 10 or 100 nmol c-di-GMP in conjunction with the Infectious Bursal Disease Virus (IBDV) vaccine S-706 in 96 broiler chickens. The oral vaccine and the test compounds were administrated at age 14 days and the chickens were then monitored until day 35. Blood samples were collected weekly from 8 birds per treatment to determine the antibody titers. Results showed that oral or IM administration of c-di-GMP did not induce any adverse effect in broiler chickens. Significant increases ($p<0.05$) in the total Immunoglobulin (Ig) A concentrations were observed regardless of the treatments as the birds aged. No significant effect was noted for total IgG and IgM concentrations after any of the sampling days immediately following administration of the compounds. However, on day 35, serum of birds orally administered with 10 and 100 nmol of c-di-GMP showed higher serum IgA antibody concentrations when compared to the serum of birds from the saline control ($p<0.05$) indicating that c-di-GMP stimulated IgA production in serum and confirming the potential of this molecule as a mucosal adjuvant. No significant effect on serum antivirus antibodies (IBDV infectious bronchitis virus, Newcastle disease virus and avian Reovirus) was observed for the 10 or 100 nmol c-di-GMP treatments. The results indicate that studies are warranted on the potential beneficial effects of c-di-GMP on broiler immunity.

Key words: 3',5'-Cyclic diguanylic acid, immunoglobulin quantification, IBDV, broiler, Canada

INTRODUCTION

Viral immunosuppressive infections have severely affected the economics of broiler production, often as a result of increased chick susceptibility to secondary infections and sub-optimal response to vaccination programs for Newcastle disease, Marek's disease and infectious bronchitis (Kibenge *et al*., 1988). Infectious Bursal Disease Virus (IBDV, Gumboro disease) is one of the major immunosuppressive viruses affecting broilers. This virus, belonging to the Birnaviridae family induces a highly contagious disease in 3-6 weeks old chickens and continues to be one of the major causes of economic losses in poultry farming worldwide (Bumstead *et al*., 1993). The general mode of infection is oral, the virus enters the gut and subsequently, spreads to different organs. In addition to poor vaccination response, secondary bacterial, viral and protozoan infections, the causes of economic devastations associated with IBDV also include poor growth performance of infected birds. The targets of the virus are the immature B cells from the bursa of Fabricius, the primary organ involved in the development of the chicken's immune system (Hirai and Calnek, 1976; Sharma *et al*., 2000). The consequence of IBDV infection is the depletion of immunoglobulin production by the B-lymphocytes in response to a variety

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of vaccines or pathogenic microorganisms (Van den Berg, 2008). The inability of current IBDV vaccinations to effectively prevent and limit the spread of IBDV necessitate the development of more effective, alternate interventions. Correspondingly, studies have been conducted to enhance the immunity of chickens to fight against IBDV and other infections. Recent studies have found that β-Glucan treatment can significantly stimulate immunity and improve bird growth (Rajapakse *et al*., 2010; Tang *et al*., 2011). Also, results from nutritional interventions suggested that tryptophan and arginine modulate systemic immune responses against IBDV (Emadi *et al*., 2011).

The compound 3',5'-Cyclic diguanylic acid (c-di-GMP) is an intracellular signalling molecule that is present in various bacterial species but no evidence has been shown for its presence in eukaryotes. The cellular levels of c-di-GMP are determined by the opposing activities of diguanylate cyclases and phosphodiesterases which can control virulence through modulation of motility, cell adhesion and biofilm formation (Jenal and Malone, 2006; Romling and Amikam, 2006). As such, exogenous c-di-GMP was reported to inhibit intercellular adhesive interactions between *S. aureus* cells and to reduce biofilm formation (Karaolis *et al*., 2005; Brouillette *et al*., 2005). Moreover, prophylactic treatment of mice with exogenous c-di-GMP prior to experimental *S. aureus* infection provided a protective effect and a 10,000 fold reduction of bacterial counts in tissues. Also intramuscular vaccination of mice with c-di-GMP as an adjuvant for a purified *S. aureus* ClfA antigen produced significantly higher anti-ClfA IgG antibody titers in serum compared with injections of ClfA alone (Karaolis *et al*., 2007). These immunomodulatory activities and vaccine adjuvant effects of c-di GMP have also been reported by Ogunniyi *et al*. (2008). These researchers showed that intranasal pre-treatment with c-di-GMP or intraperitoneal co-administration of c-di-GMP with the pneumolysin toxoid prior to pneumococcal challenge resulted in significant reduction of the bacterial number in the lungs and blood as well as a significant increase in antigen-specific antibody titers and the increased survival of mice when compared to control groups (Ogunniyi *et al*., 2008). Increasing the efficiency of mucosal immunity has been shown to play an important role in the resistance to mucosal pathogens. In mice, c-di-GMP as a mucosal adjuvant, produced a significant increase (512 fold) in anti-β-GAL IgG titers compared to controls and significantly stimulated release of β-GAL-specific IgA in the lungs (Ebensen *et al*., 2007). Results from Zhao *et al*. (2011) reported that the administration of 50 μg (72 nmol) of c-di-GMP 18 h prior to infection provided protection against *Acinetobacter baumannii* in a mouse model of intranasal infection. These observations suggest that c-di-GMP needs to be investigated for its remarkable properties as an immunomodulator. The objective of the present study was to investigate the capacity of c-di-GMP to increase humoral responses of broiler chickens in an IBDV vaccination model. Total immunoglobulin IgA, IgG and IgM as well as anti-virus antibodies titers to a number of relevant viruses was determined.

MATERIALS AND METHODS

Chicken housing and treatments: One hundred ninety two male day old broiler chicks (Western Hatchery Abbotsford, B.C) were randomly placed in 24 cages (8 chicks/cage). Before placement, all chicks were visually examined for health and inferior chicks were excluded from the trial. Each cage was equipped with a drinker and a feeder providing free access (*ad libitum*) to feed and water. Heat was provided through gas-fired brooders and airflow was provided by negative pressure. The temperature was initially set at 32°C and then was progressively reduced by 1.7°C each week to reach 23°C at 35 days of age. Chicks were exposed to light for 24 h for the 1st day, 23 h for the 2nd and 3rd days and then 18 h thereafter as previously described (Leusink *et al*., 2010). The composition of the diets used in this study is shown in Table 1. The starter, grower and finisher diets were formulated in accordance with the broiler diet used

Table 1: Composition of the diet that was used in this study

	Percentage inclusion in diet		
Ingredient	Starter	Grower	Finisher
Wheat	34.96	35.03	40.79
Soya	23.00	0.00	1.54
Barley	10.00	0.00	0.00
Canola	11.00	24.00	18.00
Canola oil	5.60	5.00	5.00
Corn	8.00	25.00	26.00
Corn gluten	2.30	6.00	4.00
Limestone	1.60	1.30	1.20
Dicalcium phosphate[1]	1.60	1.50	1.40
Vitamin-mineral mix[2]	1.00	1.00	1.00
Lysine	0.40	0.71	0.60
Iodized salt	0.31	0.32	0.32
Methionine	0.18	0.09	0.10
Avizyme[3]	0.05	0.05	0.05
Total	100.00	100.00	100.00

[1]A mixture of mono and dicalcium phosphate containing 18% calcium and 21% phosphate. [2]Supplied per kilogram of diet: vitamin A, 9,000 IU; cholecalciferol, 1,500 IU; vitamin E, 10 IU; vitamin K, 0.5 mg; vitamin B12, 0.007 mg; thiamine, 0.4 mg; riboflavin, 6 mg; folic acid, 1 mg; biotin, 0.15 mg; niacin, 135 mg; pyridoxine, 4 mg; choline chloride, 1,000 mg; dl-methionine, 1,184 mg; ethoxyquin, 125 mg; NaCl, 2 g; manganese sulfate, 60 mg; copper sulfate, 5 mg; selenium (sodium selenium), 0.1 mg; iodine, 0.35 mg and zinc sulfate, 50 mg. [3]Multi-enzyme system for wheat-based poultry feed (Halchemix Canada Inc., Toronto, ON, Canada) containing 5,000 U g^{-1} of xylanase and 1,600 U g^{-1} of protease

in Western Canada using wheat, barley and corn as the principal cereals and soybean and canola meals as protein concentrates to meet the National Research Council (NRC) nutrient requirements for broiler chickens (NRC, 1994). On day 7 and 14, two birds were removed from each cage and the remaining 96 birds (4/cage) were vaccinated by oral administration with 1 mL of Bursal Disease Vaccine (S-706) as recommended by the manufacturer (Canadian Poultry Consultants, Ltd. Abbotsford, BC, Canada). On the same day, the vaccinated birds were assigned to six treatment groups: Group I and group II were administrated with saline orally (gavage) or by Intramuscular (IM) injection, respectively. These two groups did not receive c-di GMP; Group III and IV received 10 nmol c-di-GMP by gavage or IM, respectively Group V and VI received 100 nmol of c-di-GMP by gavage or IM respectively. All experimental procedures performed in this study were approved by the local Institutional Animal Care Committee (Agassiz, BC, Canada) according to guidelines described by the Canadian Council on Animal Care (CCAC, 1993).

Blood collection and measurement of antibodies: From day 7-35, two birds/cage (2 birds/treatment group) were randomly selected for blood sample collection. Prior to centrifugation, blood samples were allowed to clot at room temperature. The serums were then transferred to sterile eppendorf tubes and stored at -20°C. Total serum Ig concentrations were determined using a commercial chicken IgA, IgG and IgM ELISA quantification kits (Bethyl Laboratories Inc, Montgomery, TX, USA) according to the manufacturer's instructions. Antibodies against IBDV infectious bronchitis disease virus, Newcastle disease virus and Reovirus in serum were also determined at the Animal Health Center of BC Ministry of Agriculture and Lands (Abbotsford, BC).

Statistical analysis: Statistical analyses were conducted according to a randomized complete block design using the GLM procedure of SAS (2000) with treatment groups as sources of variation and the individual cages as experimental units (four cages per treatment group). Data from serum samples were log transformed before analysis. Mode of administration and bird ages (days) were included as sources of variation. The least significance difference was used to separate treatment means whenever the F-value was significant. The 0.05 p-value was used to declare significance.

RESULTS AND DISCUSSION

The various reported immunomodulatory activities and vaccine adjuvant effects of c-di GMP (Karaolis *et al*., 2007; Ogunniyi *et al*., 2008; Hu *et al*., 2009) suggest that c-di-GMP may represent a new pathogen-associated molecular pattern recognized by the immune system of animals. Based on these observations, we evaluated in the present study the potential of c-di-GMP as an immunostimulant and a novel adjuvant for commonly used vaccines in broiler chickens.

General immunoglobulin: The effect of 10 nmol (6.9 μg) and 100 nmol (69 μg) c-di-GMP administrated orally or IM on the humoral immunity of broilers from day 14-35 was evaluated. The data showed that oral or IM administration of 10 or 100 nmol c-di-GMP did not induce any adverse effect in broiler chickens. Regardless of treatments, significant increase ($p<0.05$) in the immunoglobulins IgA and IgM concentrations were observed over time (i.e., day 7-35) whereas the serum immunoglobulin IgG titers remained relatively stable during this period (Fig. 1a-c). No significant treatment effect was noted for IgG and IgM titers on any of the sampling days after administration of 10 or 100 nmol of the test compound. On day 35, a significant treatment effect ($p<0.05$) on IgA titers was observed in birds receiving 10 and 100 nmol of c-di-GMP by gavage. These birds showed the highest IgA antibody titers when compared to birds of the control group. Thus, we can conclude that regardless of treatment, IgA and IgM titers significantly increased with age but IgA titers can be significantly increased by treatment with 10 and 100 nmol of c-di-GMP using gavage.

As stated before, stimulation of secretory IgA production in the lung by c-di-GMP had been reported (Ebensen *et al*., 2007). Mucosal IgA plays an important role in mucosal immunity which is part of the first line of defence against bacteria and viruses (Fagarasan and Honjo, 2003; Suzuki *et al*., 2007). In serum, IgA may function as a second-line of defence by eliminating pathogens that have breached the mucosal surface (Snoeck *et al*., 2006). In the study intestinal Ig concentrations were not determined however, if a significant increase of serum IgA concentration translated into an increase of the intestinal IgA concentration after oral administration of c-di-GMP this would confirm the potential of c-di-GMP as mucosal adjuvant for broilers.

Antivirus antibodies: A well-developed immune system and optimal immune response are important for the welfare and growth performance of chickens. The ability of high productive meat-type chickens to build sufficient immune responses to infections during the rearing period is of concern (Koenen *et al*., 2002). Prophylactic measures such as vaccination and antimicrobial feed-supplementation have been used to reduce infectious diseases. Despite these good management practices including vaccination against major diseases such as IBD infectious bronchitis disease, Newcastle disease and

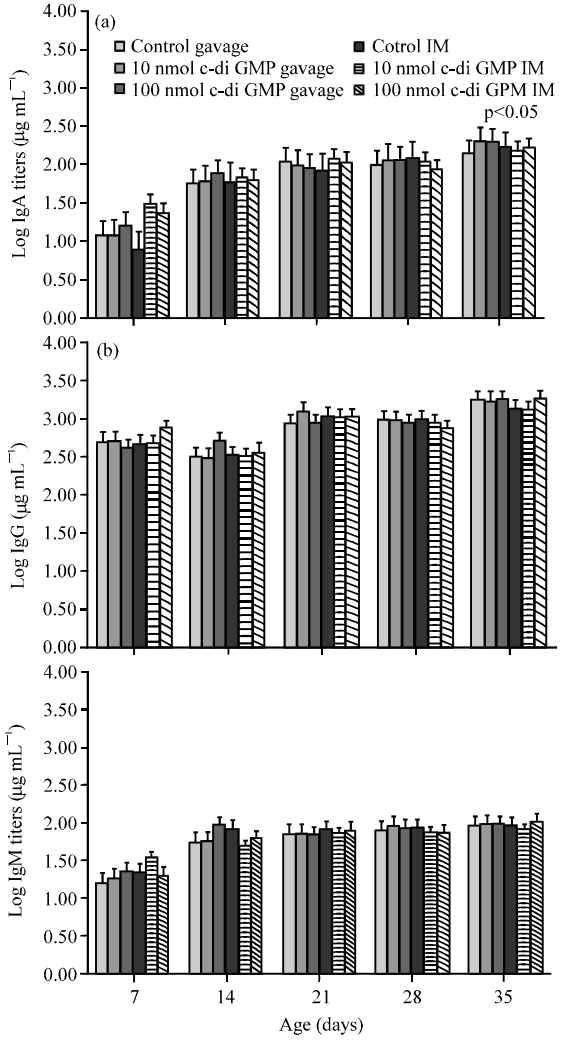

Fig. 1: General antibody profile in a broiler immunisation model with IBDV vaccine on day 14 using 100 or 10 nmol c-di GMP administrated orally (gavage) or Intramuscularly (IM). Total IgA (a), IgG (b) and IgM (c) titers in chicken serum. *Significant differences between treatments were observed in the IgA titers at the day 35 ($p<0.05$). Data represent means±SEM of 4 replicates/treatment (n = 4 pens; 2 chickens/pen at sampling day) arranged in a completely randomized block design

avian Reovirus infections, these viruses continue to be important health issues for broilers and a major constraint for the poultry industry worldwide (Hoerr, 2010). The increase in virulence and/or emerging new field virus variants is contributing to the failure of the live-attenuated viral vaccines that are presently being used. Thus, the development of new vaccination strategies using immune stimulatory adjuvants for the poultry industry would help to develop better control of major pathogenic poultry viruses. The recent interest in c-di-GMP has been stimulated by its reported immune-stimulatory effects. Therefore, in the present study, we also evaluated the effects of a co-administration of c-di-GMP with the IBDV vaccine on the humoral response of broiler chickens.

After vaccination on day 14, the antibody levels against IBDV decreased from day 7-35 and thus no significant response of birds to this vaccine was observed. It is possible that maternal IBDV antibodies which were high on day 14, may have played a role in the neutralizing antigen, thereby limiting antibody titers (Van den Berg, 2008). However, on day 35, birds receiving c-di-GMP (10 or 100 nmol) orally (gavage) or IM apparently had higher IBDV antibodies titers (Fig. 2a) than the control groups although, this was not statistically significant. Thus, we plan to evaluate the effect of a higher dose than 100 nmol of c-di-GMP since in mice, significant increases in titers of IgG1, IgG2a, IgG2b and IgG3 were observed compared to the control group in immunization studies using a dose of 200 nmol of c-di-GMP (Hu *et al*., 2009; Karaolis *et al*., 2007). It would also be interesting to evaluate the effect of c-di-GMP on the cellular immunity of broilers since some cell-mediated immune-related cytokine could play crucial roles in driving cellular immune responses during IBDV infection (Liu *et al*., 2010). Before placement, the birds were initially vaccinated on day 0 against bronchitis at the hatchery. However, we observed low antibody titers on day 21 after administration of c-di-GMP in all treatment groups (Fig. 2b). On day 28 (except for birds treated with 100 nmol c-di-GMP) and day 35, apparent increased antibody titers (not statistically significant) were observed in comparison to control groups. Birds receiving 100 nmol c-di-GMP orally showed the highest antibody titers on day 35. The antibody titers against Newcastle virus also decreased when the birds aged ($p<0.05$). On day 35, birds receiving c-di-GMP both by oral and IM administrations showed higher antibody titers than birds receiving saline (Fig. 2c). On day 35, birds receiving 100 nmol c-di-GMP orally showed higher serum titers against Reovirus (Fig. 2d). Although, this study did not find significant humoral response effects associated with two different doses of c-di-GMP on antivirus antibody levels in broilers, future research on the beneficial effects of this molecule in broilers using larger bird numbers in field and evaluations at higher dosages such as 200 nmol could lead to the development of new vaccination strategies against major poultry pathogenic viruses such IBDV.

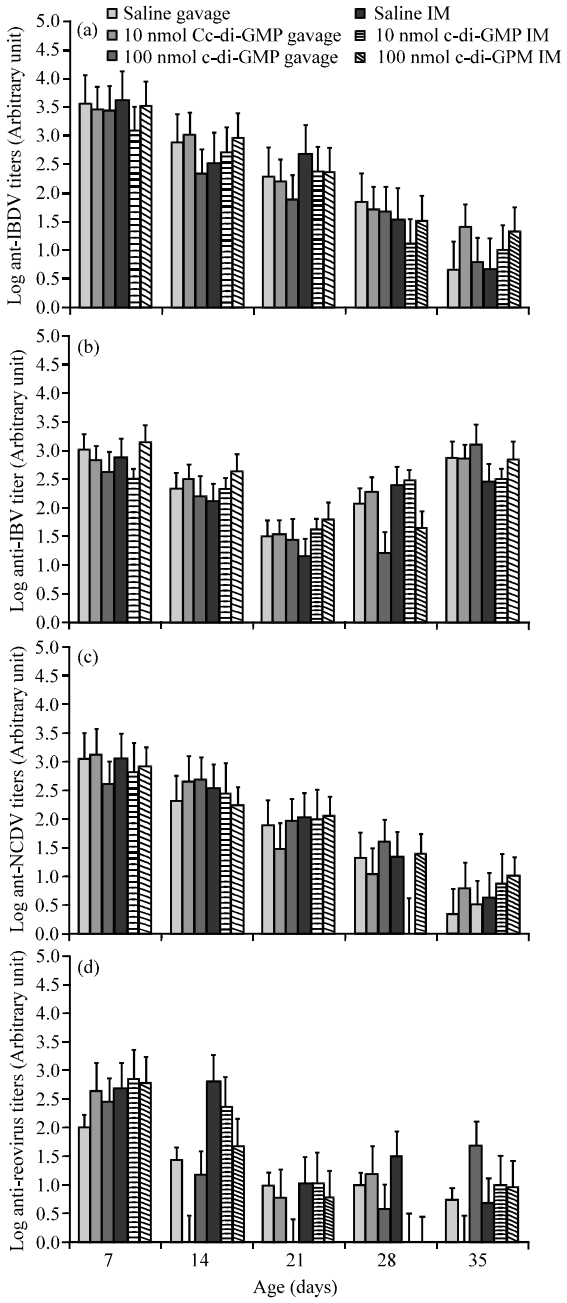

Fig. 2: Antivirus antibody titer after broiler immunisation with IBDV vaccine on day 14 using 100 or 10 nmol c-di GMP administrated orally (gavage) or Intramuscularly (IM). Titer of anti-IBDV (a), anti-infectious bronchitis virus (b), Newcastle disease virus (c) and avian Reovirus (D) in chicken serum. Data represent means±SEM of 4 replicates/ treatment (n = 4 pens; 2 chickens/pen at sampling day) arranged in a completely randomized block design

CONCLUSION

In this study the results showed no clear linear (dose dependence increase or decrease) effects on the humoral response in broilers. One of the explanations for this lack of clear effects could be due to the use of low doses of c-di-GMP. However, the observed increases in serum IgA at day 35 in birds orally administered with c-di-GMP suggest potential mucosal immune-stimulatory effects. In general the immunostimulatory effects of c-di-GMP were shortcoming in some aspects and optimal utilization of this molecule remains to be defined. Future research evaluating increased concentrations of c-di-GMP administration or ovo inoculation at the embryonic stage may promote improved humoral antibody responses, potentially reducing the morbidity and mortality in chickens.

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REFERENCES

Brouillette, E ., M. Hyodo, Y. Hayakawa, D.K. Karaolis and F. Malouin, 2005. 3',5'-cyclic diguanylic acid reduces the virulence of *Staphylococcus aureus* biofilm-forming strains in a mouse model of mastitis infection. Antimicrob. Agents Chemother., 49: 3109-3113.

Bumstead, N., R.L. Reece and J.K.A. Cook, 1993. Genetic differences in susceptibility of chicken lines to infection with infectious bursal disease virus. Poult. Sci., 72: 403-410.

CCAC, 1993. Guide to the Care and Use of Experimental Animals. Vol. 2, Canadian Council on Animal Care, Ottawa, Ontario, Canada.

Ebensen, T., K. Shulze, P. Riese, M. Morr and C.A. Guzman, 2007. The bacterial second messenger c-di-GMP exhibits promising activity as a mucosal adjuvant. Clin. Vaccine Immunol., 14: 952-958.

Emadi, M., F. Jahanshiri, K. Kaveh, M. Hair-Bejo, A. Ideris and A.R. Alimon, 2011. Nutrition and immunity: The effects of the combination of arginine and tryptophan on growth performance, serum parameters and immune response in broiler chickens challenged with infectious bursal disease vaccine. Avian Pathol., 40: 63-72.

Fagarasan, S, and T. Honjo, 2003. Intestinal iga synthesis: Regulation of front-line body defenses. Nat. Rev. Immunol., 3: 63-72.

Hirai, K. and B.W. Calnek, 1976. *In vitro* replication of infectious bursal disease virus in established lymphoid cell lines and chicken B lymphocytes. Infect. Immun., 25: 964-970.

Hoerr, F.J., 2010. Clinical aspects of immunosuppression in poultry. Avian Dis., 54: 2-15.

Hu, D.L., K. Narita, M. Hyodo, Y. Hayakawa, A. Nakane and D.K.R. Karaolis, 2009. C-di-GMP as a vaccine adjuvant enhances protection against systemic methicillin-resistant *Staphylococcus aureus* (MRSA) infection. Vaccine, 27: 4867-4873.

Jenal, U. and J. Malone, 2006. Mechanisms of cyclic-di-GMP signaling in bacteria. Annu. Rev. Genet., 40: 385-407.

Karaolis, D.K.R., M.H. Rashid, R. Chythanya, W. Luo, M. Hyodo and Y. Hayakawa, 2005. c-di-GMP (3'-5'-cyclic diguanylic acid) inhibits *Staphylococcus aureus* cell-cell interactions and biofilm formation. Antimicrob. Agents Chemother., 49: 1029-1038.

Karaolis, D.K.R., T.K. Means, D. Yang, M. Takahashi and T. Yoshimura *et al*., 2007. Bacterial c-di-GMP is an immunostimulatory molecule. J. Immunol., 178: 2171-2181.

Kibenge, F.S., A.S. Dhillon and R.G. Russell, 1988. Biochemistry and immunology of infectious bursal disease virus. J. Gen. Virol., 69: 1757-1775.

Koenen, M.E., A.G. Boonstra-Blom and S.H.M. Jeurissen, 2002. Immunological differences between layer and broiler-type chickens. Vet. Immunol. Immunopathol., 89: 47-56.

Leusink, G., H. Rempel, B. Skura, M. Berkyto and W. White *et al*., 2010. Growth performance, meat quality and gut microflora of broiler chickens fed with cranberry extract. Poult. Sci., 89: 1514-1523.

Liu, H., M. Zhang, H. Han, J. Yuan and Z. Li, 2010. Comparison of the expression of cytokine genes in the bursal tissues of the chickens following challenge with infectious bursal disease viruses of varying virulence. Virol. J., 7: 364-364.

NRC, 1994. Nutrient Requirements of Poultry. 9th Edn., National Academy Press, Washington, DC. USA., ISBN-13: 978-0-309-04892-7.

Ogunniyi, A.D., J.C. Paton, A.C. Kirby, J.A. McCullers and J. Cook *et al*., 2008. C-di-GMP is an effective immunomodulator and vaccine adjuvant against pneumococcal infection. Vaccine, 26: 4676-4685.

Rajapakse, J.R., M.D. Buddhika, M. Nagataki, H. Nomura, Y. Watanabe, Y. Ikeue and T. Agtsuma, 2010. Effect of sophy β-glucan on immunity and growth performance in broiler chicken. J. Vet. Med. Sci., 72: 1629-1632.

Romling, U. and D. Amikam, 2006. Cyclic di-GMP as a second messenger. Curr. Opin. Microbiol., 9: 218-228.

SAS, 2000. Statistical Analysis System. 1st Edn., SAS Institute Inc., Jackknife and Bootstrap, Springer-Velag, New York..

Sharma, J.M., I.J. Kim, S. Rautenschlein and H.Y. Yeh, 2000. Infectious bursal disease virus of chickens: Pathogenesis and immunosuppression. Dev. Comp. Immunol., 24: 223-235.

Snoeck, V., I.R. Peters and E. Cox, 2006. The IgA system: A comparison of structure and function in different species. Vet. Res., 37: 455-467.

Suzuki, K., S.A. Ha, M. Tsuji and S. Fagarasan, 2007. Intestinal IgA synthesis: A primitive form of adaptive immunity that regulates microbial communities in the gut. Sem. Immunol., 19: 127-135.

Tang, X.Y., J.S. Gao, F. Yuan, W.X. Zhang, Y.J. Shao, F. Sakurai and Z.D. Li, 2011. Effects of Sophy β-glucan on growth performance, carcass traits, meat composition and immunological responses of Peking ducks. Poult. Sci., 90: 737-745.

Van den Berg, T., 2008. *Birnaviridae*. In: Poultry Diseases. 6th Edn., Pattison, M., P. McMullin, J.M. Bradbury and D.J. Alexander (Eds.). Saunders Elsevier, Toronto, Canada, pp: 359-366.

Zhao, L., R. KuoLee, G. Harris, K. Tram, H. Yan and W. Chen, 2011. C-di-GMP protects against intranasal *Acinetobacter baumannii* infection in mice by chemokine induction and enhanced neutrophil recruitment. Inter. Immunopharmacol., 10.1016/j. physletb.2003.10.071.