Contents lists available at ScienceDirect

Asian Pacific Journal of Tropical Biomedicine

Comparison of total immunoglobulin A levels in different samples in Leghorn and broiler chickens

Rubén Merino-Guzmán[1], Juan David Latorre[2], Ramiro Delgado[3], Xochitl Hernandez-Velasco[1], Amanda Desha Wolfenden[2], Kyle Dean Teague[2], Lucas Elzie Graham[2], Brittany Danielle Mahaffey[2], Mikayla Francis Ann Baxter[2], Billy Marshall Hargis[2], Guillermo Tellez[2*]

[1]College of Veterinary Medicine, National Autonomous University of Mexico, Mexico City, 04510, Mexico

[2]Department of Poultry Science, University of Arkansas, Fayetteville, 72701, AR, USA

[3]Nutriavicola, Cali, 760046, Colombia

ARTICLE INFO

ABSTRACT

Objective: To standardize an ELISA protocol to quantify total immunoglobulin A (IgA) from different biological samples.

Methods: Two independent experiments were conducted. In Experiment 1, total IgA levels were quantified from the lachrymal fluid, tracheal swab, and cloacal swab at various time points from Days 30 to 89 in white Leghorn chickens. Experiment 2 was conducted to evaluate the effect of 50 or 500 ppb of aflatoxin B_1 (AFB_1) on total IgA quantified in samples from the lachrymal fluid, tracheal swab, gut content and cloacal swab in broiler chickens at 21 days of age.

Results: Lachrymal fluid contained the highest level of IgA; however, the sampling procedure was time consuming and stressful to the bird, and the sample volume depends largely on the size of the chicken. Cloacal swabs also contained a high concentration of IgA; this sampling procedure was faster than lachrymal fluid sampling and was not affected by the age of the bird. Tracheal sampling was more difficult than cloacal sampling; the age of the bird limited the sampling, and the IgA concentration was the lowest detected at all sampling ages. 500 ppb of AFB_1 significantly reduced total IgA concentration in the gut content compared with control or 50 ppb of AFB_1 treated groups. Interestingly, a significant reduction in total IgA was also observed in those chickens that received 50 ppb of AFB_1 in gut rinse when compared with cloacal swabs.

Conclusions: The results of this study suggest that cloacal swab is an easy and reliable way to evaluate mucosal IgA concentration in both Leghorn and broiler chickens.

1. Introduction

Next to the skin, the mucosa of metazoans is the largest surface area constantly exposed to external antigens [1]. However, mucosal surfaces in the gastrointestinal tract, respiratory tract, urogenital tract, eye conjunctiva, ear ducts and exocrine glands are equipped with sophisticated innate and acquired mechanisms that neutralize or reject most foreign antigens [2]. The ineffectiveness of this important line of defense will create an environment that favors pathogens. In these mucosal immune areas, 80% of lymphocytes are present, hence known as mucosa-associated lymphoid tissues (MALT) [3]. As part of the adaptive humoral immune system, plasma B cells produce large quantities of secretory immunoglobulin A (SIgA) which is the most important immunoglobulin in external secretions [3]. The intestinal mucosa, also known as the gut associated lymphoid tissue, is the largest immunological organ in metazoans, holding 70%–80% of all immunoglobulin-producing cells [4,5]. In humans, gut associated lymphoid tissue produces more SIgA (50–100 mg/kg body weight/day) than the total production of immunoglobulin G (IgG) in the body

(30 mg/kg body weight/day) [5,6]. In contrast with other immunoglobulins, SIgA is abundant in mucosal secretions and is resistant to host proteases [7]. The major functions of SIgA are to inhibit macromolecule absorption or binding of allergens to mucosal target cells, inhibit inflammatory effects of other immunoglobulins, neutralize bacterial toxins, and enhance nonspecific defense mechanisms (*e.g.*, lactoperoxidase and lactoferrin) [8,9]. It is evident that mucosal immunity is important for controlling pathogenic infections; however, a reliable and effective method to measure mucosal IgA has yet to be developed in poultry, which are necessary for evaluation of vaccination efficacy. The objective of the present study was to standardize an ELISA protocol to quantify total IgA in different biological samples taken from Leghorn chickens and then to apply that protocol in broiler chickens consuming a diet containing aflatoxin B_1 (AFB_1).

2. Materials and methods

2.1. Animal source and housing

In the present study, two independent experiments were conducted. In Experiment 1, 30 one-day-old white male Leghorn chickens (Hy-Line W36, College Station, TX, USA) were raised in a floor pen for a period of 89 days. In Experiment 2, 240 one-day-old male broiler chicks (Cobb-Vantress, Siloam Springs, AR, USA) were also raised in floor pens for a period of 21 days. In both experiments, birds were given diets and water *ad libitum*. All animal handling procedures were in compliance with the Institutional Animal Care and Use Committee at the University of Arkansas and approved by Institutional Animal Care and Use Committee of the University of Arkansas (approval number 15006).

2.2. Experiment 1, total IgA determination from different samples in white Leghorn chickens

This experiment was conducted during the months of September to November 2015. All birds were sampled for total IgA quantitation in lachrymal fluid and cloacal swab at 30, 33, 51, 55, 71 and 89 days of age. Because of the trachea diameter, tracheal swab sampling started on Day 51. Since the tracheal swab sampling was more difficult and stressful for the birds, it was decided to include saliva swab samples at 71 and 89 days of age. In order to induce lachrymation, fine sodium chloride crystals were sprinkled onto one eye while keeping the eyelids held open [10], and within 1 min lachrymation occurred. Fluid accumulated on the eye before being carefully collected by a micropipette. The fluid was immediately placed in 96-microwell plate and stored at −20 °C until tested. Cotton swabs were used to collect tracheal, cloacal and saliva swabs; after sampling, swabs were dispensed in 2 mL vials containing 0.5 mL of 0.9% saline. Vials were then vortexed thoroughly for 10 s and centrifuged at 3 000 r/min at 4 °C for 10 min without removing the swab, and supernatant was poured into a 96-microwell plate and stored at −20 °C until tested.

2.3. Experiment 2, total IgA levels in different samples from broiler chicks

This experiment was conducted to evaluate the effect of concentrations of AFB_1 (50 and 500 ppb) on total IgA in samples from lachrymal fluid, tracheal swab, gut content and cloacal swab in broiler chicks. The experiment was conducted during August 2015. One-day-old male broiler chicks were allocated randomly to three groups, namely, Group I (control feed), Group II (feed + 50 ppb AFB_1) and Group III (feed + 500 ppb AFB_1). Each group had 8 replicates of 10 chicks (n = 80/group). The experimental diets (Table 1) were formulated to approximate the nutritional requirements of broiler chicks as recommended by the National Research Council [11] and adjusted according to breeder's recommendations [12].

AFB_1 was provided by Dr. George E. Rottinghaus, Veterinary Medical Diagnostic Laboratory, University of Missouri, Columbia, MO, USA. AFB_1 was produced through the fermentation of rice and the aflatoxin content was measured by spectrophotometric analysis. The aflatoxin within the rice powder consisted of 74.62% AFB_1, 22.38% aflatoxin G_1, 2.48% aflatoxin B_2, and 0.49% aflatoxin G_2, based on total aflatoxin in the rice powder. Diets containing AFB_1 were analyzed, and the presence of aflatoxins was confirmed by high-performance liquid chromatography with fluorescence detection method by using a Romer Derivatization Unit (Romer Labs, Inc., MO,

Table 1

Ingredient composition and nutrient content of broiler chicks corn-soybean based diets used in all experiments on as-is basis with or without different concentrations of AFB_1.

Item		Starter diet	Grower diet
Ingredients (%)	Corn	59.72	64.40
	Soybean meal	33.87	29.04
	Poultry fat	2.29	2.67
	Dicalcium phosphate	1.85	1.73
	Calcium carbonate[1]	0.92	0.88
	Salt	0.37	0.37
	DL-Methionine	0.30	0.26
	Vitamin premix[2]	0.10	0.10
	L-Lysine HCl	0.15	0.14
	60% choline chloride	0.20	0.20
	Mineral premix[3]	0.10	0.10
	Threonine	0.11	0.09
	Antioxidant[4]	0.02	0.02
	Total	100.00	100.00
Calculated analysis	Metabolizable energy (kcal/kg)	3 035	3 108
	Crude protein (%)	21.00	19.00
	Digestible lysine (%)	1.18	1.05
	Digestible methionine (%)	0.45	0.42
	Digestible methionine plus cysteine (%)	0.88	0.80
	Digestible threonine (%)	0.77	0.69
	Digestible tryptophan (%)	0.18	0.17
	Total calcium (%)	0.90	0.84
	Available phosphorus (%)	0.45	0.42
	Sodium (%)	0.16	0.16

Values are expressed as amount per kg of diets. [1]: Inclusion of 10^6 spores/g of feed mixed with calcium carbonate; [2]: Vitamin premix included vitamin A (20 000 000 IU/1 000 kg), vitamin D_3 (6 000 000 IU/1 000 kg), vitamin E (75 000 IU/1 000 kg), vitamin K_3 (9 g/1 000 kg), thiamine (3 g/1 000 kg), riboflavin (8 g/1 000 kg), pantothenic acid (18 g/1 000 kg), niacin (60 g/1 000 kg), pyridoxine (5 g/1 000 kg), folic acid (2 g/1 000 kg), biotin (0.2 g/1 000 kg), cyanocobalamin (16 mg/1 000 kg) and ascorbic acid (200 g/1 000 kg) (Nutra Blend LLC, Neosho, MO 64850); [3]: Mineral premix included manganese (120 g/1 000 kg), zinc (100 g/1 000 kg), iron (120 g/1 000 kg), copper (10–15 g/1 000 kg), iodine (0.7 g/1 000 kg), selenium (0.4 g/1 000 kg), and cobalt (0.2 g/1 000 kg) (Nutra Blend LLC, MO 64850); [4]: Ethoxyquin.

USA). AFB$_1$ was added to the diets and mixed thoroughly in a graded sequence to specified concentrations. The birds were given diets with or without supplemental AFB$_1$ and water *ad libitum*. Chickens were fed with starter (Day 1–Day 7) or grower diets (Day 8–Day 21). Replicates in each experiment were used as experimental units for growth performance parameters. All broiler chicks were individually weighed, and body weight (BW), BW gain, feed intake and feed conversion ratio (FCR) were obtained every week. Lachrymal fluid, tracheal and cloacal swabs were obtained in the same way as in Experiment 1. Then, chickens were humanely killed by CO$_2$ asphyxiation. Blood was collected from the femoral vein of 10 birds per group to determine chemistry profiles. For total IgA quantitation from gut rinse samples, a section of 5 cm from Meckel's diverticulum was rinsed with 5 mL 0.9% saline, then the rinse was collected in a tube and centrifuged at 3 000 r/min at 4 °C for 10 min. Supernatant was poured into a 96-microwell plate and stored at −20 °C until tested.

2.4. ELISA

An indirect ELISA was performed to quantify IgA. The commercial chicken IgA ELISA quantitation set (Catalog No. E30-103, Bethyl Laboratories Inc., Montgomery, TX 77356) was used according to the manufacturer's instructions. In brief, 96-well plates (Catalog No. 439454, Nunc MaxiSorp, Thermo Fisher Scientific, Rochester, NY) were coated with 1 μg/100 μL of goat polyclonal anti-chicken IgA diluted in 0.05 mol/L carbonate-bicarbonate, pH 9.6. The plates were covered with a lid and allowed to incubate overnight at 4 °C. Then the contents of the plates were emptied, and the plates were tapped on a dry paper towel, and rinsed 5 times with washing solution (50 mmol/L Tris, 0.14 mol/L NaCl, 0.05% Tween 20, pH 8.0) of 350 μL/well. Individual wells were then blocked (125 μL/well) with 20% SuperBlock (Pierce Inc., Rockford, IL) in phosphate buffered saline for 60 min at room temperature. The plates were again emptied, tapped to dry and stored desiccated without further washing step. Samples were thawed to room temperature and diluted in sample/conjugate diluent (50 mmol/L Tris, 0.14 mol/L NaCl, 1% bovine serum albumin, 0.05% Tween 20) and 100 μL was added to the respective wells. A standard curve was used in order to quantify the total IgA in the samples, and chicken reference serum IgA from the quantitation kit was serially diluted in sample/conjugate diluent to get concentrations of 1 000, 500, 250, 125, 62.5, 31.25 and 15.625 ng/mL; sample/conjugate diluent alone was used as the zero standard (blank). Standard dilutions were added to the respective wells (100 μL/well). Plates were then incubated for 1 h at room temperature and rinsed 5 times with washing solution. Goat anti-chicken IgA-horseradish peroxidase (HRP) conjugated detection antibody from the IgA quantitation set was diluted (1:40 000) in sample/conjugate diluent and 100 μL were transferred to each well. The plate was incubated for 60 min at room temperature. After incubation, HRP detection antibody was removed and the plate washed again 5 times as previously described. After washing, 100 μL of tetramethylbenzidine substrate (Catalog No. TMBS-1000-01, TMB Super Sensitive One Component HRP Microwell Substrate, SurModics IVD, Eden Prairie, USA) was added to each well and incubated for 15 min at room temperature, protected from light. The reaction was stopped with 100 μL of 3%–7% maleic acid solution (Catalog No. LSTP-1000-01, BioFx® 450 nm liquid stop solution for TMB Microwell

Substrates, SurModics IVD, Eden Prairie, USA), and absorbance was measured at 450 nm using an ELISA plate reader (Synergy HT, multi-mode microplate reader, BioTek Instruments, Inc., Winooski, VT, USA). The value of absorbance at 450 nm minus the blank value for each standard concentration was plotted on the vertical (Y) axis versus the corresponding chicken IgA concentration on the horizontal (X) axis using the Gen5™ software (BioTek Instruments, Inc., Winooski, VT, USA). Chicken IgA concentration obtained was multiplied by the dilution factor to determine the amount of chicken IgA in the undiluted samples. Optimum dilution for total IgA quantitation in different samples was 1:2 000 for lachrymal fluid, 1:10 for tracheal swab, 1:20 for saliva, 1:10 for cloacal swab and 1:100 for gut rinse.

2.5. Clinical chemistry changes

In Experiment 2, blood was collected from the femoral vein of 10 birds per group prior to necropsy. The chemistry profiles of selected serum which included albumin, alkaline phosphatase, alanine transaminase, aspartate aminotransferase, and total protein were determined using a Corning clinical chemistry analyzer (Chiron Corporation, San Jose, CA).

2.6. Statistical analysis

Data were subjected to ANOVA as a completely randomized design by using the General Linear Model procedures of the Statistical Analysis Software [13]. Data are expressed as mean ± SE. Significant differences among means were determined by using Duncan's multiple-range test at $P < 0.05$.

3. Results

Experiment 1 quantified the total IgA from different biological samples in white Leghorn chickens and results are summarized in Table 2. The highest concentration of IgA was obtained from the lachrymal fluid during the six weeks of evaluation when compared with cloacal swab, tracheal swab or saliva swab ($P < 0.05$). These levels also remained reasonably constant at all times of evaluation (Table 2). Cloacal swabs had the second highest concentration of IgA which remained constant over the collection period. However, at 89 days of age there was a significant increase in cloacal IgA when compared to the same previous sample. Tracheal swabs showed the lowest concentration of IgA from all sample procedures, having the lowest levels of IgA during the last sampling at 89 days of age. Saliva swabs were only collected at the last two sampling dates (at 71 and 89 days of age). The concentration of IgA at 71 days of age in saliva swab was significantly higher than that of the tracheal samples. At Day 89, there was no significant difference in IgA concentration between saliva and tracheal swabs (Table 2).

In Experiment 2, both doses of AFB$_1$ significantly reduced BW of broiler chickens when compared with control chickens at 14 days of evaluation. However, on Day 21, chickens that received 500 ppb of AFB$_1$ showed a significant reduction on FCR when compared with controls or 50 ppb of AFB$_1$ fed chickens (data not shown).

Table 3 summarizes total IgA levels in different biological samples from broiler chickens receiving 50 or 500 ppb AFB$_1$. Similar to Experiment 1, levels of IgA were significantly higher

Table 2

Total IgA determination from different samples in white Leghorn chickens (ng/mL).

Sample	Age (days)					
	30	33	51	55	71	89
Lachrymal fluid	78 445 ± 9 658[aw]	76 522 ± 9 773[aw]	64 278 ± 12 660[aw]	64 477 ± 7 696[aw]	50 506 ± 8 721[aw]	55 842 ± 6 000[aw]
Cloacal swab	1 013 ± 125[by]	2 569 ± 273[bx]	2 012 ± 251[bx]	2 311 ± 316[bx]	2 219 ± 248[bx]	3 616 ± 406[bw]
Tracheal swab			499 ± 63[cw]	466 ± 70[cw]	501 ± 87[dw]	259 ± 22[cx]
Saliva swab					1 391 ± 180[cw]	383 ± 50[cx]

abcd: Different superscripts within columns indicate significant difference among sample source ($P < 0.05$). wxy: Different superscripts within rows indicate significant difference among sampling dates ($P < 0.05$).

Table 3

Total IgA levels in different samples from broiler chickens receiving 50 or 500 ppb AFB_1 on Day 21 (ng/mL).

Group	Lachrymal fluid	Tracheal swab	Gut rinse	Cloacal swab
Control	16 118.0 ± 1 601.4[aw]	129.0 ± 10.4[ay]	2 149.0 ± 238.7[ax]	2 567.0 ± 366.0[ax]
50 ppb AFB_1	18 579.0 ± 2 215.4[aw]	145.0 ± 11.0[az]	1 556.0 ± 297.6[ay]	2 112.0 ± 229.3[ax]
500 ppb AFB_1	18 803.0 ± 2 353.9[aw]	144.0 ± 10.3[az]	641.0 ± 100.5[by]	1 835.0 ± 172.9[ax]

ab: Different superscripts within columns indicate significant difference between treatments ($P < 0.05$). wxyz: Different superscripts within rows indicate significant difference among sampling sources ($P < 0.05$).

in the lachrymal fluid samples followed by cloacal swabs, gut rinse and tracheal swabs in all three groups evaluated (Table 3). The levels of IgA in lachrymal fluid, tracheal or cloacal swabs were similar between control, 50 or 500 ppb of AFB_1 treated chickens. However, chickens that received 500 ppb of AFB_1 showed a significant reduction of IgA in gut rinse samples when compared with control or 50 ppb AFB_1 fed chickens. Interestingly, IgA concentration in cloacal swabs was higher in groups receiving AFB_1 when compared with gut rinse (Table 3).

Table 4 shows the effect of dietary administration of 50 or 500 ppb AFB_1 on blood chemistry at 21 days of age in Experiment 2. No significant differences were observed in the levels of albumin, alkaline phosphatase, alanine transaminase or aspartate aminotransferase between control and AFB_1 treated chickens. The only significant reduction was observed for total protein in chickens fed with 500 ppb AFB_1 when compared with control chickens (Table 4).

importance of IgA in vaccinology to neutralize pathogens in their natural route of infection, serum IgG is still considered the most widely used immunoglobulin to monitor infections or immune status in humans and domestic animals [17]. Particularly in poultry, most commercial kits only monitor IgG levels. Therefore, the main objective of the present study was to standardize an ELISA protocol to quantify total IgA in different samples taken in Leghorn or broiler chickens. In the present study, the ELISA test was performed using maximum binding plates and a commercial chicken IgA quantitation kit. Total chicken IgA concentrations (ng/mL) in all samples were calculated by comparing their optical density against a standard curve used in the assay. From the results obtained in both experiments, it was clear that lachrymal fluid contained the highest concentration of IgA. However, the sampling procedure is time consuming and the sample volume depends largely on the size of the chicken.

Table 4

Effect of dietary administration of 50 or 500 ppb AFB_1 on blood chemistry in 21-day-old broiler chickens.

Group	Albumin (g/dL)	Alkaline phosphatase (IU/L)	Alanine transaminase (IU/L)	Aspartate aminotransferase (IU/L)	Total protein (g/dL)
Control	1.01 ± 0.02[a]	388.00 ± 98.03[a]	0.91 ± 0.23[a]	235.74 ± 7.03[a]	2.19 ± 0.04[a]
50 ppb AFB_1	1.01 ± 0.03[a]	475.20 ± 58.91[a]	1.00 ± 0.17[a]	248.73 ± 12.11[a]	2.16 ± 0.05[ab]
500 ppb AFB_1	0.98 ± 0.04[a]	463.20 ± 71.83[a]	0.76 ± 0.19[a]	235.01 ± 11.69[a]	2.10 ± 0.09[b]

ab: Different superscripts within columns indicate significant difference between treatments ($P < 0.05$).

4. Discussion

Most pathogens infect metazoans through mucosal surfaces (gastrointestinal, respiratory and genito-urinary mucosal surfaces, conjunctiva, ear duct or secretory glands) [3]. Regardless of their phylogenetic distance, vertebrate species use IgA produced by plasma B cells as the primary acquired defense neutralizing toxins and pathogenic microbes [14,15]. The versatility of the MALT allows activated plasma B cells to circulate through lymphatic or blood vessels to seed other MALT where they will secret specific IgA, blocking the invasion of more toxins or pathogens [16]. In spite of the

When using lachrymal sample, it is important to use the proper sample dilution and ensure the ELISA is reliable. Cloacal swab sampling was easy; IgA concentration was constant as birds aged and this sample was shown to hold an important amount of IgA (Tables 2 and 3). Tracheal swabs were not only the most difficult to collect and the ability to sample was dependent on the age of birds, but the total IgA concentration was the lowest detected (Tables 2 and 3). The results of this study suggest that cloacal swabs are an easy and reliable way to evaluate mucosal IgA concentration in Leghorn or broiler chickens. AFB_1 at 500 ppb in the feed had a negative impact on performance and caused liver damage which was

reflected by a reduction in total serum protein. In the present study, 500 ppb of AFB_1 also reduced the total IgA concentration in gut rinse compared with lachrymal fluid, tracheal or cloacal swabs when compared with control or 50 ppb of AFB_1 treatment. Interestingly, a significant reduction in total IgA was also observed in those chickens that received 50 ppb of AFB_1 in gut rinse when compared with cloacal swabs (Table 4). These results suggest that when considering sampling for IgA determination, it is also important to consider what pathogen or toxin is involved in the evaluation, in order to determine the best sampling method. For determining the concentrations of specific IgA against Newcastle disease virus and infectious bronchitis in broiler chickens, using different sampling methods is currently being evaluated.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgments

References

[1] Pastorelli L, De Salvo C, Mercado JR, Vecchi M, Pizarro TT. Central role of the gut epithelial barrier in the pathogenesis of chronic intestinal inflammation: lessons learned from animal models and human genetics. *Front Immunol* 2013; **4**: 280.

[2] Levin M, King JJ, Glanville J, Jackson KJ, Looney TJ, Hoh RA, et al. Persistence and evolution of allergen-specific IgE repertoires during subcutaneous specific immunotherapy. *J Allergy Clin Immunol* 2016; **137**(5): 1535-44.

[3] Gutjahr A, Tiraby G, Perouzel E, Verrier B, Paul S. Triggering intracellular receptors for vaccine adjuvantation. *Trends Immunol* 2016; **37**(9): 573-87.

[4] Howarth GS, Wang H. Role of endogenous microbiota, probiotics and their biological products in human health. *Nutrients* 2013; **5**(1): 58-81.

[5] Liu YY, Rhoads JM. Communication between B-cells and microbiota for the maintenance of intestinal homeostasis. *Antibodies* 2013; **2**: 535-53.

[6] Thaiss CA, Zmora N, Levy M, Elinav E. The microbiome and innate immunity. *Nature* 2016; **535**(7610): 65-74.

[7] Konkel JE, Chen W. Balancing acts: the role of TGF-β in the mucosal immune system. *Trends Mol Med* 2011; **17**: 668-76.

[8] Zhao K, Rong G, Hao Y, Yu L, Kang H, Wang X, et al. IgA response and protection following nasal vaccination of chickens with Newcastle disease virus DNA vaccine nanoencapsulated with Ag@SiO2 hollow nanoparticles. *Sci Rep* 2016; **6**: 25720.

[9] Kamada N, Núñez G. Regulation of the immune system by the resident intestinal bacteria. *Gastroenterology* 2014; **146**(6): 1477-88.

[10] Toro H, Lavaud P, Vallejos P, Ferreira A. Transfer of IgG from serum to lachrymal fluid in chickens. *Avian Dis* 1993; **37**(1): 60-6.

[11] National Research Council. *Nutrient requirements of poultry*. 9th rev. ed. Washington, DC: National Academy Press; 1994, p. 19-32.

[12] Cobb-Vantress Inc. Broiler performance and nutrition supplement. Siloam Springs: Cobb-Vantress Inc.; 2015. [Online] Available from: http://www.cobb-vantress.com/docs/default-source/cobb-500-guides/Cobb500_Broiler_Performance_And_Nutrition_Supplement.pdf [Accessed on 12th September, 2016]

[13] Cody R. *An introduction to SAS® university edition*. Cary: SAS Institute Inc.; 2015, p. 92-106.

[14] Chou WK, Chen CH, Vuong CN, Abi-Ghanem D, Waghela SD, Mwangi W, et al. Significant mucosal sIgA production after a single oral or parenteral administration using *in vivo* CD40 targeting in the chicken. *Res Vet Sci* 2016; **108**: 112-5.

[15] Rochereau N, Pavot V, Verrier B, Jospin F, Ensinas A, Genin C, et al. Delivery of antigen to nasal-associated lymphoid tissue microfold cells through secretory IgA targeting local dendritic cells confers protective immunity. *J Allergy Clin Immunol* 2016; **137**(1). 214–22.e2.

[16] Fujkuyama Y, Tokuhara D, Kataoka K, Gilbert RS, McGhee JR, Yuki Y, et al. Novel vaccine development strategies for inducing mucosal immunity. *Expert Rev Vaccines* 2012; **11**(3): 367-79.

[17] Kuttappan VA, Bielke LR, Wolfenden AD, Berghman LR, Tellez G, Hargis BM, et al. Comparison of different ELISA protocols for the detection of IgA against influenza nucleoproteins in trachea of vaccinated chickens. *Poult Sci* 2015; **94**(2): 181-4.