Mucosal, cellular and humoral immune responses induced by different live infectious bronchitis virus vaccination regimes and the protection conferred against infectious bronchitis virus Q1 strain

Rajesh Chhabra[1,2], Anne Forrester[1], Stephane Lemiere[3], Faez Awad[1], Julian Chantrey[1] & Kannan Ganapathy[1#]

[1]University of Liverpool, Leahurst Campus, Neston, South Wirral, UK

[2]College Central Laboratory, Lala Lajpat Rai University of Veterinary & Animal Sciences, (LUVAS) Hisar, India

[3] Merial S.A.S., 29 avenue Tony Garnier, 69348 Lyon cedex 07, France

#Corresponding author Tel.: +44 151 7946019; fax: +44 151 7946005.

E-mail address: gana@liv.ac.uk

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ABSTRACT

The objectives of present study were to assess the mucosal, cellular and humoral immune responses induced by two different infectious bronchitis virus (IBV) vaccination regimes and their efficacy against challenge by a variant IBV Q1. Day-old broiler chicks were vaccinated with live H120 alone (Group I) or in combination with CR88 (Group II). Both groups were again vaccinated with CR88 at 14 days of age (doa). One group was kept as the control (Group III). A significant increase in lachrymal IgA levels was observed at 4 doa, which then peaked at 14 doa in the vaccinated groups. The IgA levels in group II were significantly higher than group I from 14 doa. Using immunohistochemistry to examine changes in the number of CD4+ and CD8+ cells in the trachea, it was found that overall patterns of CD8+ were dominant compared to CD4+ cells in both vaccinated groups. CD8+ were significantly higher in group II compared to group I at 21 and 28 doa. All groups were challenged oculo-nasally with a virulent Q1 strain at 28 doa, and their protection was assessed. Both vaccinated groups gave excellent ciliary protection against Q1, though group II's histopathology lesion scores and viral RNA loads in the trachea and kidney showed greater levels of protection compared to group I. These results suggest that greater protection is achieved from the combined vaccination of H120 and CR88 of day-old chicks, followed by CR88 at 14 doa.

Keywords: Infectious bronchitis virus, Chicken, Vaccination, Mucosal-humoral-cell mediated immune responses, Protection, Q1-challenge

INTRODUCTION

The prevention of infectious bronchitis (IB) in chickens is achieved through the use of live and inactivated vaccines, which provide protection against virulent field IB viruses in the event of an exposure. Despite these preventative measures, outbreaks of IB frequently occur in many poultry producing countries (1-3). This is probably due to the emergence of new variants of infectious bronchitis virus (IBV) (1-5). For the successful protection of chickens against infection, it is essential to identify the prevalent genotypes in the region and to determine the cross-protective potential of available vaccines and optimise strategic vaccination programmes.

IB was first described in the USA during the 1930s and was identified in the UK in 1948. Thereafter, many IBV variants were isolated from Europe, significantly a variant called 793B that emerged in the 1990s (6). Later, IBV QX was first identified in China (7) before spreading to Europe (8). Another IBV genotype, Q1, genetically and serologically distinct from the classical IBVs, was also reported in China (9), the Middle East (10) and Europe (11). To contain this strain, an effective vaccination programme is needed. However, very little is known about the cross protection induced by the commercially available vaccines or vaccination regimes against this variant Q1.

An effective and long-lasting protection against IBV infection requires the activation of effector, memory cell-mediated and humoral immune responses against the virus (12). A number of studies have reported the systemic and local humoral immune response (HIR) to IBV vaccination (12-14). In chickens~~,~~ experimentally challenged with IBV, the development of a cell mediated immune response (CMI) has been correlated with effective virus clearance, reduction of clinical signs and resolution of lesions (15, 16). The presence of cytotoxic CD8+ T lymphocytes (CTL) represents a good correlation for decreasing infection and corresponds with a reduction in clinical signs, as CTL activity is major histocompatibility complex

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restricted and these T cells mediate cytolysis (17). It has additionally been shown that the transfer of CTLs obtained from spleen of IBV-infected chickens, was protective to naïve chicks against a subsequent IBV challenge (15, 18). During the course of experimental viral infection, Kotani *et al* (2000) showed that the clearance of the IBV from the tracheal mucosa occurred at an early phase of the infection and CTLs at the tracheal mucosa were proposed to be involved in this clearance (19). To date, there is no information available on the tracheal mucosal leukocytes after vaccination with live IBV vaccines. Nevertheless, Okino *et al* (2013) have quantified the relative expression of the CTLs genes in tracheal samples from vaccinated and further challenged birds. The up regulation of these genes, in the tracheal mucosa of the full-dose vaccinated birds, was significantly increased at 24 hours post infection (hpi), demonstrating the development of a CMI memory response (20). However, these researchers did not directly measure the activity of CMI, such as the cytotoxic mechanism of CTLs.

Despite all these reports, the kinetics of, and the relationship between local and systemic HIR and CMI induced by different IBV vaccination regimes, needs to be better understood for protection against emerging IBV strains. Thus, the objective of our study was to measure the local as well as systemic HIR and CMI induced by two different IBV vaccination regimes administered to commercial broiler chicks, and to estimate the protection achieved against a recently isolated virulent Q1 strain.

MATERIALS AND METHODS

Birds

One hundred twenty broiler chicks, aged 1-day-old, were obtained from a commercial hatchery. Birds were allowed *ad libitum* access to feed and drinking water. All procedures were undertaken according to the UK legislation on the use of animals for experiments as

permitted under the project license PPL 40/3723, which was approved by the University of Liverpool ethical review committee.

Challenge virus

The virulent Q1 isolate used in this study was kindly provided by Merial Animal Health. PCR confirmed that the allantoic fluid, from eggs used to propagate the virus, was free of Newcastle disease, avian influenza, infectious bursal disease, infectious laryngotracheitis and avian metapneumoviruses. Q1 IBV was also free of bacterial or fungal contaminants. The virus was titrated in the chicken tracheal organ culture (TOC) as described before and expressed in 50% (median) ciliostatic doses (CD_{50})/ml (21).

Vaccine preparation

As recommended by the manufacturer (Merial Animal Health Limited, UK), the vaccines were prepared, by thoroughly mixing one vial of live IBV H120 (Bioral H 120®) vaccine with 100 ml of sterile water (SW). For combined vaccinations, one vial of each Bioral H 120® and live IBV CR88 (GALLIVAC® IB88) vaccines were mixed together in 100 ml of SW. Immediately after preparation, the vaccines and SW were kept in a cold box (at 0°C). Each chick received a total of 100 µl of the appropriate vaccine ocularly (50 µl) and nasally (50 µl) or SW. To quantify the virus, titration of live IBV vaccine for H120 and CR88 was performed by using 9-11 days of age (doa) specific pathogen free (SPF) embryonated chicken eggs (ECE) inoculated via the allantoic cavity. The ECE were examined for IBV specific lesions (curling and dwarfing) of the embryos up to five days post inoculation. Viral titres were calculated according to Reed *et al.* (22) and expressed as the Egg infective dose (EID_{50}/ml). The titre of the vaccine viruses used was 3.5 $\log_{10}$ EID_{50}/chick and 4.25 $\log_{10}$ EID_{50}/chick for the H120 strain and CR88 strain, respectively.

Experimental design

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One hundred and twenty broiler chicks, aged 1-day-old, were divided into three groups (n=40 chicks/group) (Table 1). Chicks in Group I were inoculated oculonasally with 100 µl of live H120 vaccine alone. In group II, chicks were inoculated oculonasally with 100 µl of both live H120 and CR88 vaccines simultaneously. Chicks in both groups (I and II) were again inoculated with a live CR88 vaccine at 14 doa. Group III received only 100 µl of SW oculonasally and was kept as a control. Samples (5 birds/group) of serum, tears and heparinized blood were collected at 0, 4, 7, 14, 21 and 28 doa before sacrificing the birds. The tears and serum samples were stored at -20ºC, and blood samples were processed immediately for peripheral blood mononuclear lymphocytes isolation. Five chickens from each group per interval were humanely euthanized for the collection of approximately 1 cm of the upper trachea in OCT to be snap-frozen in liquid nitrogen for immunohistochemistry (IHC). The rest of the trachea was used for tracheal washes. At 28 doa, 10 birds from each group were challenged via ocular-nasal route with the Q1 ($10^{4.0}$ CD_{50}/bird) and observed daily for clinical signs. After 5 days post challenge (dpc), all 10 birds from each group were necropsied and tracheal samples were collected; a portion placed in the RNALater® (Qiagen, Crawley, UK) and stored at -70°C until processing for examination of viral RNA load. The remaining portions were examined by histopathology and cilliostasistests. The kidneys from all groups were also taken for histopathology and viral RNA load examination.

Sample collection for antibody detection

The potential of the vaccines to induce antibody production was assessed individually by using samples of sera, tears and tracheal washes. Tears were collected using sodium chloride as described before (23), immediately centrifuged at 3000 x g for 3 min before storing the supernatant at -70°C until used. To collect the tracheal washes, the trachea was clamped with two artery forceps at both the ends, and washed with 1 ml PBS using a syringe

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with 19 gauge needle (24). The collected samples were centrifuged at 3000 x g for 3 min, and the supernatant stored at -70°C until further use.

ELISAs

To detect IBV antibodies, sera samples were tested with a commercial IBV ELISA kit (FlockChek®, IDEXX Laboratories, Inc, Westbrook, ME, USA), and immunoglobulin A (IgA) in tears and tracheal washes was assayed using commercial IgA chicken ELISA kit (Abcam, Cambridge, UK). Both assays were carried out according to the respective manufacturer's instructions.

Haemagglutination inhibition (HI) test

For the HI test, M41 and 793B HA antigens were obtained from GD Animal Health Service (Deventer, Netherlands). The Q1 HA antigen was prepared in our laboratory as described earlier (25). The HI test was conducted according to standard procedures (OIE), using 4 HA units of antigen per well. The HI titres were read as the reciprocal of the highest dilution showing complete inhibition and the HI geometric mean titres were expressed as reciprocal $\log_2$.

Cellular immune responses

Analysis of T lymphocyte subsets (CD4+:CD8+) ratio in peripheral blood

To determine the percentage of T-lymphocyte subpopulations, blood was collected from the cephalic vein in heparin tubes (Sigma Aldrich Co., St. Louis, MO, USA) at final concentrations of 10 USP/ml of blood, and further diluted (1:1) with RPMI 1640 medium (Sigma Aldrich Co., St. Louis, MO, USA). The prepared blood samples (1 ml each) were then over layered onto 0.5 ml of Histopaque –1.077 gradient (Sigma Aldrich Co., St. Louis, MO, USA) and centrifuged in 1.5 ml Eppendorf vial at 8000 x g for 90 sec. After centrifugation, the buffy coat formed of mononuclear cells was gently collected, washed twice with a RPMI 1640 medium and adjusted to 1×10^7 cells/ml. The cells were resuspended

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in 0.5% BSA (Sigma Aldrich Co., St. Louis, MO, USA) in PBS (blocking solution) and incubated at room temperature for 15 min. The sample (100 µl) was incubated with antibodies against surface domains of CD4 (mouse anti-chicken CD4-FITC clone CT-4; 0.5mg/ml; Southern Biotech, Birmingham, AL, USA) and CD8 (mouse anti-chicken CD8a-FITC clone CT-8; 0.5mg/ml; Southern Biotech) receptors of T-lymphocytes (antibody final concentrations as 0.2 µl/100 µl of sample) for 30 min in the dark. The stained cells were detected by flow cytometry (BD Accuri® C6, BD Bioscience San Jose, CA, USA) to count the T lymphocytes. The unstained cell sample was used as a negative control to adjust the threshold.

Immunohistochemical detection of CD4+, CD8+ and IgA-bearing B-cells in tracheal sections

The OCT-embedded tracheal samples were cut into 5 µm sections, fixed in ice-cold acetone for 10 min, air dried at room temperature and stored at -80ºC until staining. Just prior to staining, slides were removed from -80ºC and air dried at room temperature for 10 min. After endogenous peroxidase inhibition using 0.03% hydrogen peroxide in PBS for 20 min, the endogenous biotin or biotin-binding proteins in tissue sections were blocked with blocking serum using VECTASTAIN® Elite ABC kit (Vector Laboratories, Burlingame, USA). Following blocking, tissue sections were stained overnight at 4ºC in the dark to detect CD4+, CD8+ and IgA+ cells by using mouse monoclonal antibodies to chicken CD4 (clone CT-4; 0.5 mg/ml) and CD8a (clone CT-8; 0.5 mg/ml) at 1:1000, and to chicken IgA (clone A-1; 0.5 mg/ml) at 1:2000. All monoclonal antibodies were procured from Southern Biotech, Birmingham, AL, USA. The staining procedure was performed as described earlier (26). For each sample, the average number of positive cells/400× microscopic field was calculated for each cell type (26).

Ciliary protection

At 5 dpc, trachea samples were evaluated according to standard procedure for ciliary movement, and the ciliary protection for each group was calculated (27).

Histopathological evaluation

At 5 dpc, kidneys and tracheas from humanely euthanized birds were collected and fixed in 10% formalin. The tissues were embedded in paraffin wax (50-60ºC) and sections were cut to 7µm thickness. Tissue sections were stained by haematoxylin and eosin (H&E) for microscopic evaluation, the scores attributed according to histopathological severity and determined by recommendations described previously (28, 29).

Real time RT-PCR (RT-qPCR)

Total RNA extractions from the tracheas and kidneys, collected from the challenged birds, were performed immediately using RNeasy® Mini Kit (Qiagen, Crawley, UK) according to the manufacturer's instructions. Quantification of the viral RNA was done by quantitative real-time RT-PCR (RT-qPCR) using IBV 3'untranslated region (UTR) gene-specific primers and probes as described previously (30). The RT-qPCR was performed according to the manufacturer's instructions using the One-Step RT-PCR kit (Qiagen, Crawley, UK) and 40 ng of total RNA per reaction. Amplification plots were recorded and analyzed, the threshold cycle (Ct) determined with Rotor-Gene® Q thermocycler software (Qiagen, Crawley, UK). The Ct values were converted to log relative equivalent units (REU) of viral RNA, done through generation of a standard curve of five 10-fold dilutions of extracted RNA from infective allantoic fluid of a 10^6 EID_{50} dose of M41 as described earlier (31).

Statistical analysis

The comparisons of the means of anti-IBV antibody levels; CD4+:CD8+ ratio in peripheral blood; immunohistochemical detection of CD4+, CD8+ and IgA-bearing B-cells in tracheal sections were performed using one-way analysis of variance (ANOVA), followed by the post-hoc LSD multiple comparison test using GraphPad™ Prism version 6.00 software. Kruskal-Wallis test followed by Dunn's test was used for statistical analysis of the non-parametric RT-qPCR and histopathological evaluation data. Differences were considered significant at $P<0.05$.

RESULTS

Systemic humoral immune response

ELISA

On the day of vaccination, the mean of maternally derived anti-IBV antibody titre was 1750±203. Subsequently, the antibody levels in all three groups declined to below cut-off point (396) by 14 doa. After the booster vaccination with the CR88 at 14 doa, a significant increase in the antibody titres till 28 doa was observed in groups I and II, as shown in Fig. 1. On these time points, the levels of antibodies were not significantly different between the vaccinated groups ($P<0.05$). After 14 doa though, the antibody titres in group III was always less than the cut-off value of 396 in this assay.

HI test

The level of serotype specific antibodies against homologous and heterologous antigens was evaluated by a HI test (Table 2). The HI antibody response against all the antigens used showed no significant difference ($P<0.05$) between the groups from 0 to7 doa. However, a lower antibody response was obtained in all groups when the antigen used in the HI test was heterologous (Q1) to the viruses used in the vaccination. On 14 doa, the mean HI antibody titre to the M41 was significantly higher ($P<0.05$) in group II than group I and III.

Thereafter, at 28 doa, the levels of antibodies to M41 in group I and II were very similar and significantly higher than group III ($P<0.05$). At 21 doa, group II showed significant increase of HI antibody response against CR88, following revaccination with a homologous antigen. A similar increase was observed in group I on the same sample day. Thereafter, at 28 doa, the HI antibody titre to CR88 antigen was overall significantly higher in group II ($\log_2$ 8.2) followed by group I ($\log_2$ 4.4) then group III ($\log_2$ 1.8). At 21 doa, the titres to Q1 in groups I and II were higher compared to group III ($P<0.05$). At 28 doa, the mean HI titre to Q1 was significantly higher ($P<0.05$) in group II than group I with a mean difference of 1.2 $\log_2$.

Mucosal humoral immune responses

In both, groups I and II, the level of IgA in tears increased significantly *($P<0.05$)* compared to control group III from 4 doa, continuing to rise until and initially peaking on 14 doa. In the vaccinated groups, after the second vaccination at day 14, IgA values fell, then increased slightly again through to 28 doa, the day of challenge. The IgA levels in group II were significantly higher *($P<0.05$)* than group I from 14 doa until 28 doa, the end of the observation period (Fig. 2a). The level of IgA in tracheal washes in both vaccinated groups was detected from 4 doa, peaking at 7 doa before declining till 28 doa. No significant *($P<0.05$)* difference in the level of IgA in tracheal washes induced by the two vaccine groups was observed at any doa (Fig. 2b). IgA levels in tears and tracheal washes of both vaccinated groups were significantly higher than the levels from the unvaccinated control group.

Systemic cell-mediated immune response

CD4+:CD8+ ratio in peripheral blood

Flow cytometry results showed that at 7 doa, the CD4+:CD8+ ratios were slightly higher in both vaccinated groups compared to that of the non-vaccinated group, though there was no significant difference ($P<0.05$) between the CD4+:CD8+ ratios of the vaccinated and non-vaccinated groups observed up to 14 doa (Fig. 3). After the booster vaccination with the

CR88 at 14 doa, the ratio of CD4+:CD8+ on 21 doa showed slight increase in both vaccinated groups, being significantly higher ($P<0.05$) in group I compared to that of group II and III. At 28 doa, the ratio was significantly higher ($P<0.05$) in group II compared to groups I and III.

Mucosal cell-mediated immune responses in the trachea

The kinetics of CD4+, CD8+ and IgA-bearing B lymphocytes in the trachea were studied by IHC (Fig. 4). The number of CD4+ lymphocytes in the trachea increased significantly ($P<0.05$) from 4 doa in both vaccinated groups compared to the control (Fig. 5a). The number of CD4+ cells reached its peak at 4 doa in group I and at 7 doa in group II, before gradually decreasing until 14 doa. After the second immunization, CD4+ cells strongly increased in number by 21 doa in comparison to the non-vaccinated controls before declining again. The difference between the vaccinated groups I and II was not statistically significant ($P<0.05$). The CD8+ cells subpopulation in groups I and II started to increase significantly ($P<0.05$) at 4 doa, reaching peak at 7 doa and then declining (Fig. 5b). After revaccination with CR88 at 14 doa, both vaccinated groups showed a strong increase in the number of CD8+ cells. The number of CD8+ cells were significantly higher in group II than group I at 21 and 28 doa ($P<0.05$). Overall, the dynamics of the CD8+ cell subpopulations in both vaccinated groups were more dominant than CD4+ cells. At 7 doa, the IgA-bearing B cells increased in vaccinated groups I and II, peaking at 14 doa and showing significant difference compared with the unvaccinated group ($P<0.05$). The number of IgA-bearing B cells was significantly higher in group II than group I at 21 doa, whereas, no significant ($P<0.05$) difference was observed between both vaccinated groups at 28 doa (Fig. 4c).

Protection

After challenge, no clinical signs were observed in either vaccinated groups. In the unvaccinated group, respiratory signs such as coughing, sneezing, head shaking, tracheal

rales and nasal discharge were observed until 5 dpc. The highest percentage of ciliary protection (97%) was observed in group II, followed by group I (89.75%). The unvaccinated challenged group (group III) showed little protection (12%) compared to the vaccinated challenged groups.

Viral RNA loads, in all tracheal samples collected, were significantly higher (*P<0.05)* (4.416 log REU RNA) in the unvaccinated challenged group (III) compared to the vaccinated groups (I and II)as measured by real time RT-qPCR, at 5 dpc. The vaccinated groups, I and II showed mean log REU of viral RNA of 1.016 and 0.555, respectively, with no significant difference between these groups (Fig. 6). Overall viral RNA in the kidney samples of all the groups were low compared to tracheal samples. The viral RNA load in kidneys in group III was significantly higher (*P<0.05)* than in group II, whereas, group I showed no significant difference (*P<0.05)* in log REU of viral RNA with either of group II and III.

Histopathological lesions in tracheas and kidneys were induced by challenge virus in all the groups at 5 dpc. Marked histopathological changes occurred in group III (non-vaccinated group) with mean scores of 10.2 and showed significant difference with group II (*P<0.05)*, but not with group I (Fig. 7). The mean lesion scores for kidneys in group III was significantly higher (*P<0.05)* than group II, whereas, group I showed no significant difference (*P<0.05)* in mean lesion scores with either group II and III. However, overall mean lesion scores in kidneys were low compared to mean tracheal lesion scores.

DISCUSSION

The vaccination regime, chosen in this study for group I, is based on the research demonstrating that improved protection was seen when two vaccines used were of different serotypes (27, 32). They also emphasized that the vaccination programme used in their experiments may not protect the respiratory tract against challenge with every new IBV

serotype to emerge. It is also evident that despite the use of Mass type vaccine at day 0, followed by 793B type vaccine at 14 doa (same as in group 1 in this study), significant number of new IBVs are still emerging under field conditions e.g. QX, IS/885/00, IS/1494/06 and most recently Q1. Therefore, in order to optimize the use of currently available vaccines, to achieve better immunity and to assess protection against newly emerged Q1 strain, the vaccination regime for group II was also included in this work.

At 1-day-old, chicks had high ELISA anti-IBV antibody titres in all groups, which declined and dropped to below the cut-off point by 14 doa. In the groups that received the vaccine at one day old, these low antibody levels could result from the partial neutralization of the vaccine virus in the target tissues by the maternal antibodies present in the broilers at that age, with a consequently low replication of the vaccine virus and poor stimulation of the humoral response (16, 33, 34). Later, after the second vaccination at 14 doa, an increase in the antibody titres was observed until 28 doa (day of challenge) in groups I and II with no significant difference in antibody levels between these vaccinated groups ($P<0.05$). HI antibody levels declined by 14 doa against the homologous and heterologous virus antigens, showing similar patterns to declining ELISA titres. Interestingly, by 28 doa, there was no significant difference between vaccinated groups I and II in terms of the level of antibodies to M41, whereas, the HI titres to 793B and Q1 were significantly higher in group II than group I ($P<0.05$). The role of antibody in the control of IBV infection remains controversial as workers have shown that circulating antibody titres did not correlate with protection from IBV infection (35-37). However, other studies demonstrated the importance of humoral immunity in disease recovery and virus clearance (38, 39). In our study, as expected, the higher HI titres were obtained using antigen homologous to vaccine strains. However, the chicks also appeared to be protected against heterologous challenge. This could be due to the

presence of local immunity of the upper respiratory tract, induced by vaccination thus reducing the replication of challenge virus.

The role of IgA antibodies is important for mucosal immunity to IBV and its presence in tears following IBV antigen inoculation has been reported earlier (38, 40). In this study, a gradual increase in IgA levels were observed in tears for both vaccinated groups during the first two weeks after vaccination. These results are in agreement with previous research reporting similar kinetics of lachrymal fluid IgA production to H120 vaccination (20, 41). In addition, after the second vaccination, lachrymal IgA levels decreased in both vaccinated groups, though, the levels in group II remained significantly higher *($P<0.05$)* than group I. This observation may indicate a decrease of lachrymal IgA levels after the second vaccination is most likely due to partial neutralization of the anti-IBV IgA. A sharp decrease of IgA-IBV in vaccinated chicks was also observed after challenge (42). In addition, no significant rise in specific lachrymal IgA of vaccinated chickens was detected after subsequent challenge with Ark-IBV isolate, explaining the probable role of neutralizing antibodies in the lachrymal fluid at the time of challenge (43).

IBV-specific IgA can also be found in tracheal washes after an infection with strain of IBV M41 (39, 40). In this study, the pattern of IgA in tracheal washes in both vaccinated groups I and II was closely parallel, reaching peak at 7 doa and thereafter, declining till 28 doa, suggesting a short duration of the local humoral immunity in the trachea. Although there have been conflicting reports on the relative concentrations of IgA in the avian respiratory tract (44-46), our results are consistent with Hawkes *et al* (1983), which showed IgA antibodies in tracheal washes only at day 7 after vaccination (47). Interestingly, in both vaccinated groups, the second vaccination did not cause any rise in tracheal IgA level. Similar findings have also been reported, revealing that the revaccination with homologous

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IBV (M41 or H strains) (45), and secondary M41 IBV exposure (39), did not induce the secondary secretary antibody response in tracheobronchial washings.

Consistent with the notion that CMI is protective against IBV (18, 48), we next sought to study the level of systemic and local cellular immune responses. CD4+ cells may directly produce antiviral cytokines, which increases B cell activity and promotes the proliferation, maturation, and functional activity of CD8+ CTLs, which plays a critical role in controlling IBV infection (49, 50). The ratio of CD4+:CD8+ has been widely shown to be indicative of the general immune system status (51, 52). In this study, the CD4+:CD8+ ratio showed no significant ($P<0.05$) variation among the groups till 14 doa. Nevertheless, the ratio at 28 doa was found significantly higher ($P<0.05$) in group II than in groups I and III indicating that second vaccination at 14 day-old in group II has probably enhanced the cellular immunity by promoting the differentiation and proliferation of CD4+ cells in peripheral blood. There is no specific data regarding the effects of different IBV vaccination on CD4+:CD8+ ratio in peripheral blood so as to compare the present findings, however, Yohannes *et al.* (2012) have reported significantly ($P<0.05$) higher CD4+:CD8+ ratio in IBV infected chicks than in the controls (53). In addition, the high CD4+:CD8+ ratio has also been associated with increased humoral incompetence in chickens, as a low CD4+:CD8+ ratio and a reduced response against sheep red blood cells have been reported earlier (54). In this study, significantly ($P<0.05$) higher HI titres at 28 doa to 793B and Q1 in group II than group I could be attributed to the high CD4+:CD8+ ratio in that group at that time point. However, the significance of this in relation to protection remains to be determined.

The results of IHC in tracheal tissue showed that the number of CD4+ lymphocytes started increasing from 4 doa in both vaccinated groups, as compared to the control. At 28 doa, no significant difference was reported between the vaccinated groups. The CD8+ cells subpopulation in both vaccinated groups started to increase significantly ($P<0.05$) at 4 doa,

reaching peak at 7 doa and then declining in number until 14 doa, suggesting that infiltration and recruitment of these cells occurs in the first two week of initial IBV vaccination. Similar to the findings of the present study, in the trachea, CD8+ cells recruitment in response to infection were at a maximum by 7 days post infection (dpi) and CD4+ cells were not recruited until 5 dpi. This was reported by Dhinakar *et al* (1996) (55). This work also showed an overall higher infiltration of CD8+ cells in numbers compared to CD4+ cells in both vaccinated groups. This observation is consistent with a previous study (55), where CD8+ cells were also found to predominate compared to CD4+ cells in trachea after IBV infection. Moreover, the current study also documents significantly higher number of CD8+ cells in vaccinated chicks of group II compared to group I on 21 and 28 doa, respectively. The IgA-bearing B cells in vaccinated groups reached peak at 14 doa, however, the number of these cells were significantly higher in group II in comparison to group I at 21 and 28 doa ($P<0.05$). This pattern of recruitment of B cells later than either class of T cells is in accordance with earlier studies (56, 57) who contended that local immunity against IBV is mediated mainly by T-cells.

In this study, following the Q1 challenge, ciliary protection was higher in group II, vaccinated with mixed H120 and CR88 vaccines at day-old, than in group I, vaccinated at day old with H120 alone. Furthermore, the results of RT-qPCR showed that the viral RNA load at 5 dpc in the trachea, was higher in group I than group II although the difference was not statistically significant ($P<0.05$). In agreement with this, the scores of histopathology in the trachea showed that the damage caused by the Q1 was higher in group I than II and showed no significant difference in mean lesion scores with either of group II and III. On the basis of these tracheal histopathological assessment, chickens in group II were better protected compared with those in group I and this better protection might be attributed by various factors including those discussed below.

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Although the anti-IBV ELISA antibody titre results indicated that there was no significant difference between the two vaccinated groups at the day of challenge, group II showed higher ciliary protection than group I. This observation is consistent with previous studies which have shown that circulating antibody levels were of minor importance in the protection of the respiratory mucosa against IBV challenge (14, 44).

From our results, it appears that such overall higher protection could be due to significantly higher levels of CD8+ cells in the tracheal tissues in group II than group I at day of challenge. Previous study have shown that CD8+ cells are important contributors to viral clearance in respiratory virus infections, utilizing contact-dependent effector functions, IFN-γ and tumour necrosis factor-α (58). Therefore, we may speculate that the group II's higher CD8+ cell reaction than the group I could have contributed to the faster viral clearance after challenge with Q1, explaining the differences between the vaccinated groups in their tracheal protection. This possible explanation agrees with other studies that emphasized the involvement of local CD8+ cells in the infection of chickens with respiratory pathogens, such as Newcastle disease virus (59) and *Mycoplasma gallisepticum* (60). Additionally, group II's higher levels of IgA in lachrymal fluid, compared to group I, could reduce the tracheal histopathological damage which also corroborates the hypothesis that the traditional role of IgA is to prevent pathogen entry at mucosal surfaces and neutralize virus in infected epithelial cells (61). IBV-specific IgA antibodies in lachrymal fluid were correlated with resistance to IBV reinfection (38, 40, 42). Our results are in agreement with a recent study by Okino *et al* (2013), in which the authors concluded that IBV IgA antibodies in lachrymal secretions and the expression of granzyme-A and CD8 genes in tracheal tissues after H120 vaccination, provides a reliable approach to monitor immune protection status in the trachea, as shown by examination for cilliostasis, histopathology and viral replication (20). For our study, we aimed to stain for a variety of cell-surface markers and thereby identify the T cell

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450 populations infiltrating the trachea. This provides further information about the role of cell-
451 mediated immunity in protection given by different live IBV vaccination regimes against a
452 novel IBV Q1 challenge.

453 The results of RT-qPCR and scores of histopathology in the kidneys showed that the
454 damage caused by the Q1 was higher in group I than II, and showed no significant difference
455 in the mean lesion scores with either of group II and III. Specific cytotoxic T lymphocytes
456 have been shown to be important for the systemic clearance of nephropathogenic IBV and
457 reduction of kidney lesions (15). A plausible explanation is that a higher CD8+ cells response
458 in the tracheal tissues (the portal of entry of challenge virus) in group II compared to group I
459 could have prevented the challenge virus becoming viraemic thus failing to reach the kidneys.
460 This provided an efficient prevention of kidney infection, as measured by viral RNA load and
461 histopathological lesion scores in renal tissue.

462

463 **CONCLUSIONS**

464 Chicks vaccinated with H120 and CR88 at day-old, followed by CR88 at 14 doa, showed
465 significantly higher CD8+ responses in the trachea and higher lachrymal IgA levels compared
466 to those vaccinated with H120 alone. In terms of ciliary protection against Q1, though both
467 vaccinated groups were protected, the combined vaccination of H120 and CR88 of day-old
468 chicks, followed by CR88 at 14 doa, showed higher ciliary protection and less RNA load in
469 trachea and kidneys, wherein histopathological lesions are reduced. This study highlighted
470 the potential modulation of chick immune response with the use of currently available live
471 vaccines so that better protection against variant IBVs can be afforded.

472

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Fig. 1. Anti-infectious bronchitis virus (IBV) antibody titres of the different groups vaccinated with a live H120 alone (group I) or in combination with CR88 (group II) at day-one. Both groups were again vaccinated with CR88 at 14 days of age. One group (group III) was kept as control. Where values were significantly *(P<0.05)* different these are shown with different letters and all other values were not significantly *(P<0.05)* different between the groups on those sampling points.

Fig. 2. Detection of IgA production using ELISA in (a) tears (b) tracheal wash of chickens (n=5 per group) vaccinated with a live H120 alone (group I) or in combination with CR88 (group II) at day-one. Both groups were again vaccinated with CR88 at 14 days of age. One group (group III) was kept as control. The IgA antibody levels in tears and tracheal wash from control chickens (group III) remained below the detectable level. Asterisks indicate values between the two vaccine groups were significantly different *(P<0.05)* on those time point. Error bars indicate standard error of the mean.

Fig. 3. The ratio of $CD4^+$:$CD8^+$analyzed by flow cytometry in peripheral blood of chickens vaccinated with a live H120 alone (group I) or in combination with CR88 (group II) at day-one. Both groups were again vaccinated with CR88 at 14 days of age. One group (group III) was kept as control. Depicted are the mean values (n=5 per group) and one standard error. Where values were significantly *(P<0.05)* different these are shown with different letters and all other values were not significantly *(P<0.05)* different between the groups on those time point.

Fig. 4. Immunohistochemical detection of CD4+ cells in group II at 28 days of age (B), CD8+ cells in group II at 28 days of age (C), IgA-bearing B-cells group II at 28 days of age (D), in tracheas of chickens vaccinated with live H120 alone (group I) or in combination with CR88 (group II) at day-one. Both groups were again vaccinated with CR88 at 14 days of age. One group (group III) was kept as control (A). Magnification (400x). Arrows indicate positive cells.

Fig. 5. Summary of CD4+ cells (a), CD8+ cells (b), and IgA-secreting cells (c) determined by immunohistochemical staining in the trachea of chickens vaccinated with a live H120 alone (group I) or in combination with CR88 (group II) at day-one. Both groups were again vaccinated with CR88 at 14 days of age. One group (group III) was kept as control. Depicted are the mean values (n=5 per group) and one standard error. Where values were significantly *(P<0.05)* different these are shown with different letters and all other values were not significantly *(P<0.05)* different between the groups at those time points.

Fig. 6. Quantification of infectious bronchitis virus (IBV) expressed as log REU of RNA, in trachea and kidney measured by real time RT-PCR after 5 dpc from chickens experimentally challenged at 28 days of age with Q1 strain of IBV (n=10 per group). The chickens were previously vaccinated with a live H120 alone (group I) or in combination with CR88 (group II) at day-one. Both groups were again vaccinated with CR88 at 14 days of age. One group (group III) kept as control received sterile water. Significant differences between the groups were detected by Kruskal-Walis test followed by Dunn's mean test indicated with different letters (*P<0.05).*

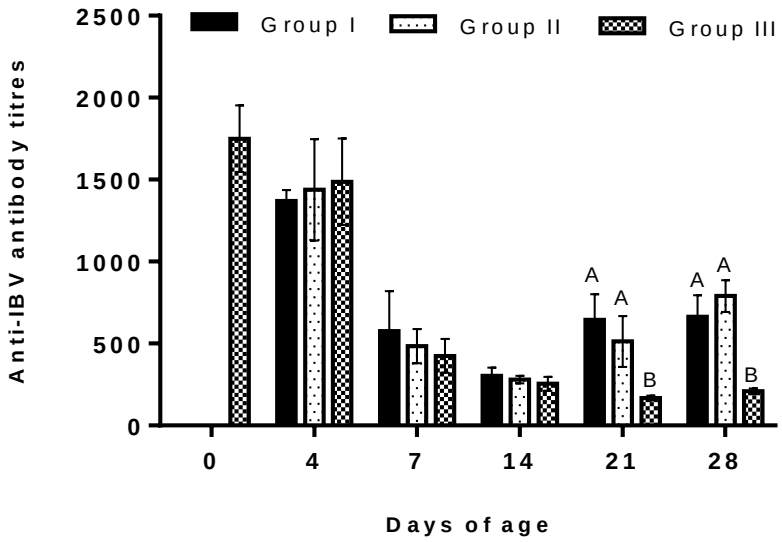
Fig. 7. Means of histopathological scores of lesions in trachea and kidney samples after 5 dpc from chickens experimentally challenged at 28 days of age with Q1 strain of infectious bronchitis virus (n=10 per group). The chickens were previously vaccinated with a live H120 alone (group I) or in combination with CR88 (group II) at day-one. Both groups were again vaccinated with CR88 at 14 days of age. One group (group III) kept as control was inoculated with sterile water. Significant differences between the groups were detected by Kruskal-Wallis test followed by Dunn's mean test indicated with different letters (*P<0.05)*

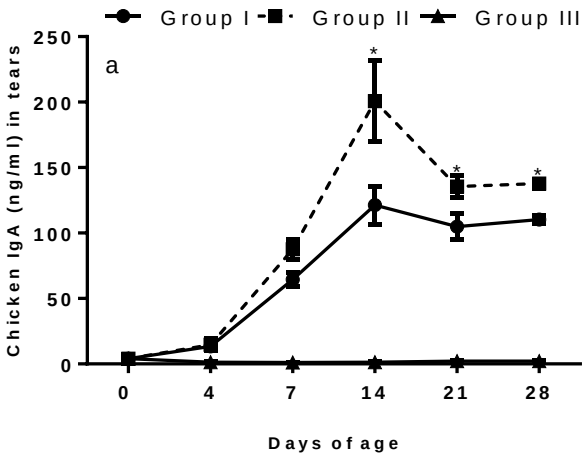
Table 1: Study design showing groups, vaccine and vaccination regimes. At 28 day of age, 10 chicks from each group were challenged with a virulent IBV Q1.

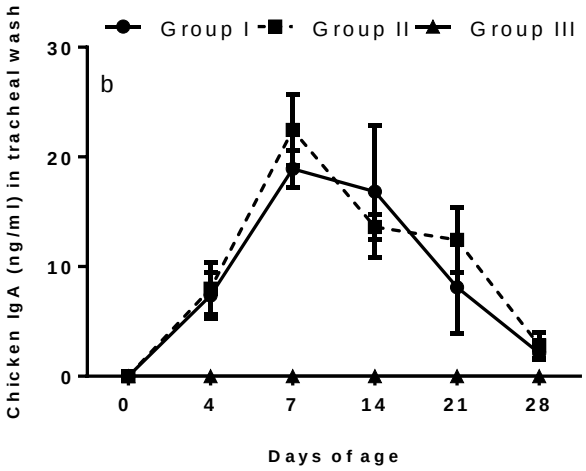
IBV vaccine (dosage/chick in 100 µl)	Group/days of age					
	I		II		III	
	0	14	0	14	0	14
H120 ($3.5 \log_{10} EID_{50}$)	√		√	√		
CR88 ($4.25 \log_{10} EID_{50}$)		√		√		
Sterile water (SW)					√	√

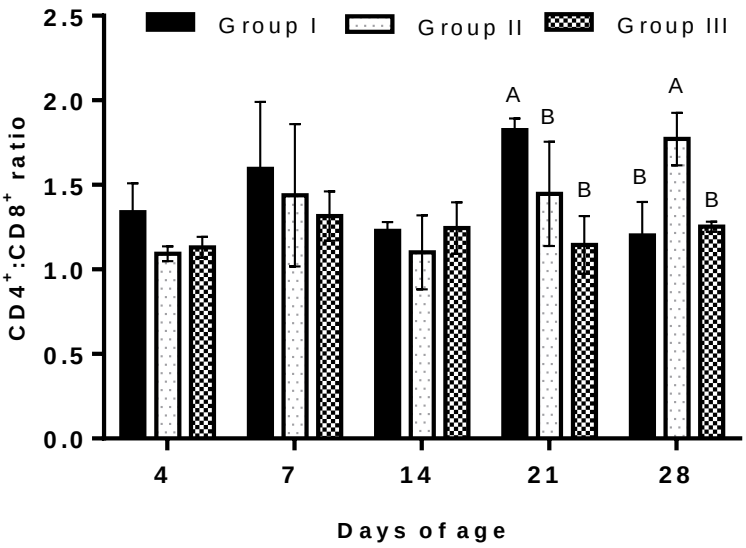
Table 2. Geometric mean anti-IBV HI antibody titre (log_2) in serum of chickens vaccinated with live H120 alone (group I) or in combination with CR88 (group II) at day-one. Both groups were again vaccinated with CR88 at 14 days of age. One group (group III) was kept as control. Significant differences between the groups (n=5 per group) for each homologous as well as heterologous antigen for each interval are represented by different letters ($P<0.05$)

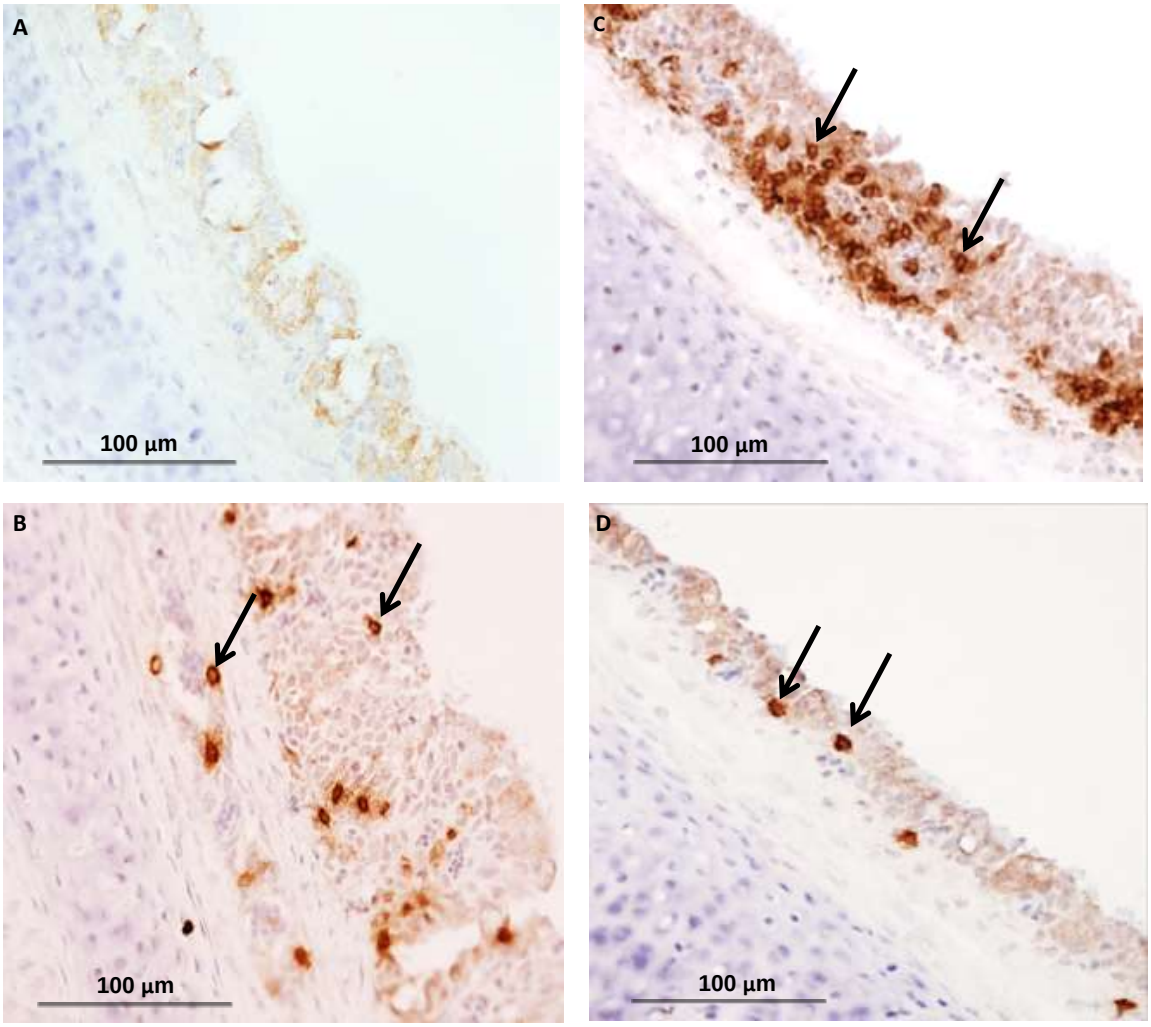
HI antigen	Groups	Days of age					
		0	4	7	14	21	28
M41	Group I	9.2 ± 0.374^{A}	8.2 ± 0.970^{A}	7.4 ± 0.400^{A}	5 ± 0.000^{B}	5 ± 0.548^{A}	4 ± 0.447^{B}
	Group II	9.2 ± 0.374^{A}	8.8 ± 0.374^{A}	7.4 ± 0.400^{A}	6.4 ± 0.510^{A}	5.4 ± 0.400^{A}	4.4 ± 0.400^{A}
	Group III	9.2 ± 0.374^{A}	7.2 ± 0.374^{A}	6.4 ± 0.748^{A}	4.8 ± 0.200^{B}	4 ± 0.632^{A}	2 ± 0.632^{B}
CR88	Group I	8.4 ± 0.400^{A}	7.8 ± 0.374^{A}	6.8 ± 0.490^{A}	5 ± 0.316^{A}	6.8 ± 0.583^{A}	4.4 ± 0.678^{B}
	Group II	8.4 ± 0.400^{A}	8.4 ± 0.245^{A}	6.8 ± 0.374^{A}	5.8 ± 0.200^{A}	7 ± 0.548^{A}	8.2 ± 0.583^{A}
	Group III	8.4 ± 0.400^{A}	7.6 ± 0.400^{A}	7.4 ± 0.245^{A}	4.6 ± 0.600^{A}	3.4 ± 0.245^{B}	1.8 ± 0.490^{C}
Q1	Group I	7 ± 0.316^{A}	3 ± 0.316^{A}	2.4 ± 0.245^{A}	2.2 ± 0.200^{B}	4.4 ± 0.510^{A}	4.4 ± 0.510^{B}
	Group II	7 ± 0.316^{A}	3 ± 0.678^{A}	3.4 ± 0.400^{A}	3.8 ± 0.374^{A}	5 ± 0.548^{A}	5.6 ± 0.245^{A}
	Group III	7 ± 0.316^{A}	3.4 ± 0.316^{A}	2.2 ± 0.200^{A}	2 ± 0.000^{B}	2 ± 0.000^{B}	2 ± 0.000^{C}

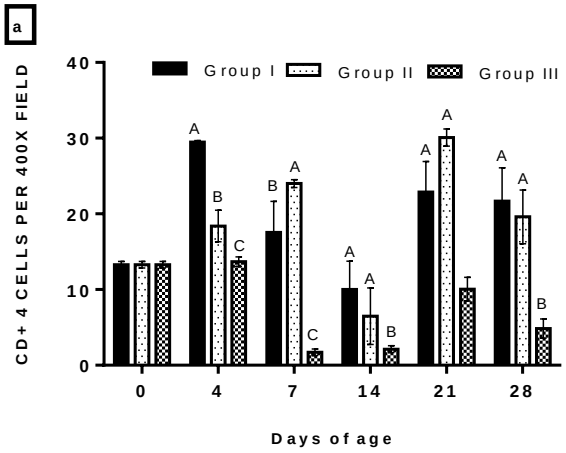


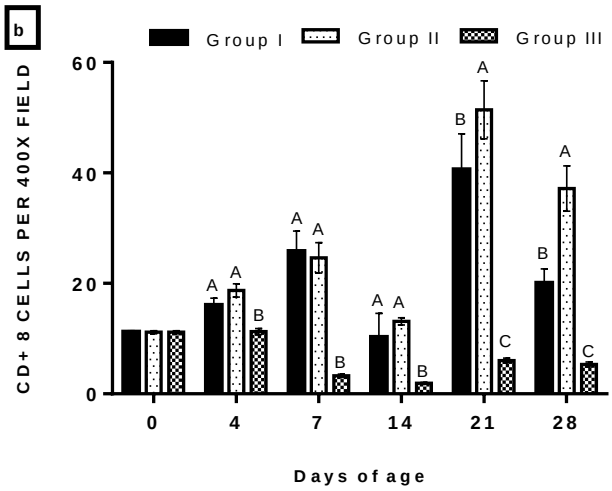


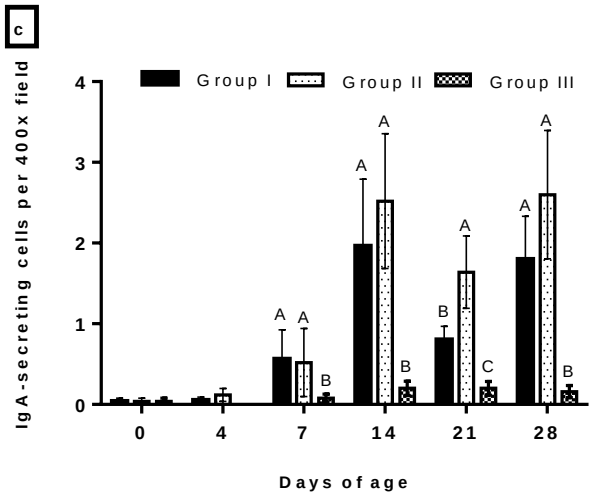












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