

Co-administration of Toll-like Receptor (TLR)-3 Agonist Poly I:C with Different Infectious Bursal Disease (IBD) Vaccines Improves IBD Specific Immune Response in Chicken

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Abstract

Toll-like receptor (TLR) agonists are emerging as promising vaccine adjuvants and immunomodulators in poultry against many diseases. Infectious bursa disease (IBD) still remains as a major threat in poultry industry. Improving the vaccine mediated immune response would help in better protection against IBD virus infection. Adjuvant potential of TLR3 agonist, Poly I:C with different IBD vaccines has been analyzed in chicken in the present study. Intermediate, intermediate plus IBD vaccine, bursaplex vaccine and their respective poly I:C combinations were used for immunization of chicken. IBD specific antibody titre, bursa to body weight ratio, body weight gain and bursal lesion scores were evaluated at weekly interval in different immunization groups. Fold changes in cytokines IL-1 β and IFN- γ mRNA expression levels were also analyzed in different groups. Intermediate IBD plus vaccine induced significantly ($P \leq 0.05$) higher IBD specific antibody response at 35 days of age than other groups with comparatively lower body weight gain and moderate bursal lesion score. Poly I:C co-administration with intermediate IBD vaccine and bursaplex vaccine improved the IBD specific antibody titres, better body weight gain and moderately less bursal lesion score. However, Poly I:C combination with intermediate plus IBD vaccine did not improve the specific immune response. IL-1 β levels were up-regulated in intermediate plus and bursaplex group, whereas IFN- γ mRNA expression levels were upregulated in intermediate IBD with Poly IC group. In conclusion, poly I:C co-administration with intermediate IBD and bursaplex vaccine was beneficial and improved the specific immune response with least immunosuppression and bursal damage.

Introduction

TLR ligands or agonists are considered as promising adjuvant candidates due to their role in self/ non-self differentiation, antigen presenting cell maturation and down-stream cytokine stimulation (Janeway and Medzhitov 2002; Bendelac and Medzhitov 2002). Many TLR agonists including CpG, flagellin, Poly I:C and Pam2CSK4 are currently being tested or employed along with many vaccines to improve immune response (Sayers et al., 2012). The potential of TLR ligands as adjuvants and prophylactic agents has also been established with many poultry vaccines (Bhadouriya et al., 2019).

Infectious bursal disease (IBD) is one of the most important viral disease of poultry caused by IBD virus (IBDV), a non-enveloped ds RNA virus of *Birnaviridae* family (Etteradossi 2020; Muller et al., 1979). IBDV targets the bursa of Fabricius of 3–6 weeks old chicks resulting in immunosuppression and mortality (Hirai et al., 1974). Biosecurity and vaccination are two major effective strategies for IBD control. At present, live attenuated, killed and immune-complex IBD vaccines are available for effective control of IBD (Liew et al., 2016). Live attenuated IBD vaccines are mainly used in broilers at early stages for protection; although killed vaccines are employed in breeders for the induction of maternal antibodies (MDA) and subsequent protection of chicks at early stages. Among the modified live IBV vaccines, intermediate and intermediate plus (hot) vaccines are widely used based on the disease incidences and maternal immunity levels. Although these vaccines are capable of inducing protective IBD specific immunity in host, immunosuppression due to bursal damage by the vaccine viruses is major drawback (Rautenschlein et al. 2007). Alleviating the immunosuppressive effect of IBD vaccines and potentiating their immunostimulation has more implications at field level.

Polinosinic polycytidylic acid (poly I:C), a dsRNA analogue, is a well characterized TLR3 agonists, induce type I IFNs in chickens and act as an antiviral agent (Karpala et al., 2008; Parvizi et al. 2012). Adjuvant effect of poly I:C have been demonstrated with inactivated avian influenza vaccine in chicken and ducks (Liang et al., 2013; Ichinohe et al., 2005; Zhang et al., 2017). Enhanced protection and reduced tumor development were observed in chicken immunized with HVT vaccine for Marek's disease along with poly IC (Parvizi et al., 2012). Earlier we reported the improvement Newcastle disease (ND) specific immune response in chicken upon co-administration of Poly I:C with mesogenic R2B live ND vaccine (Kannaki et al., 2019). However, there is dearth of information on the effect of Poly I:C and its adjuvant potential with IBD vaccines. In the present study, we explored the immunomodulatory potential of poly I:C with different types of IBD vaccines in chicken.

Materials And Methods

Experimental birds

Day old Vanaraja chicks (n = 320) hatched from hatchery of ICAR-Directorate of Poultry Research, Hyderabad were used in the present study. Chicks were housed, fed with standard nutrition and provided *ad libitum* water. All the experiments were carried out following ethical guidelines and approved by Institute animal ethics committee (IAEC/DPR/2017/7). All the chicks received HVT vaccine for Marek's disease at day-old and LaSota vaccine for ND at 5th and 28th day through intranasal route at recommended dose.

IBD vaccines and TLR3 agonist

Commercially available vaccines viz., intermediate, intermediate plus (Venky's India Pvt. Ltd., India) and Bursaplex (Zoetis India Pvt Ltd., India) IBD vaccines were procured and used. TLR3 agonist, Poly I:C (Sigma, MO, USA) dissolved in sterile nuclease free water was used in the study.

Immunization trial

One-day old vanaraja chicks were randomly divided into eight groups of 40 chicks each and immunized as follows. Bursaplex and poly I:C adjuvanted (10 µg/ chick) with Bursaplex vaccine were given by subcutaneous route (s/c) at day old. Other vaccines viz., intermediate, intermediate plus and poly I:C adjuvants of respective vaccines were given on 10th day of age and booster dose on 16th day of age. Intermediate and intermediate plus vaccine were given by oral route either alone or in combination with Poly I:C by intramuscular route (10 µg/ chick). Poly I:C control group received only Poly I:C by intramuscular route. Unimmunized control received only sterile PBS by i/m route (Table 1). All the vaccines were given at recommended dose as per the manufacturer's instruction.

Table 1
Immunization plan in the experimental birds

Groups	Prime ^a	Poly I:C ^b (10µg/ bird)	Booster ^c
I: Int IBD	+	-	+
II: Int IBD plus	+	-	+
III: Bursaplex	+*	-	-
IV: Int IBD + Poly IC	+	+	+
V: Int IBD plus + Poly IC	+	+	+
VI: Bursaplex + Poly IC	+*	+	-
VII: Only Poly IC	+	+	-
VIII: Unimmunized control	-	-	-
+*: Immunization at day-old by i/m route; ^a : prime dose at 10th day of age; ^b : Poly I:C given by i/m route; ^c : booster dose on 16th day of age			

Evaluation of humoral immune response

Blood samples from different groups of immunization trial (n = 6 /each group) were collected at weekly interval, serum separated and stored at -20°C until further analysis. IBD specific serum antibody response from serum samples were analyzed by indirect ELISA (IDEXX laboratories, USA) by following manufacturer's instruction. Briefly, the test was performed on 96-well ELISA plate precoated with IBDV antigen. Diluted (1:500) test sera were dispensed (100 µl/well) in duplicates. Undiluted positive and negative controls (each 100 µl/well) provided along with the kit were also dispensed on the coated wells. After incubating for 30 min at 25°C, the plates were washed with distilled water to remove any unbound material and followed by the addition of 100 µl conjugate. After 30 min at 25°C, the unbound conjugate was washed away and TMB substrate (100 µl) was added. The subsequent color developed was measured by spectrophotometer at 650 nm and corresponding OD values were recorded. The respective antibody titers were calculated as given below:

$$\text{Titer} = \text{Antilog} [1.09 (\log_{10} \text{S/P})] + 3.36$$

Wherein S/P = [Mean OD of test sample-Mean OD of negative control] / [Mean OD of positive control- Mean OD of negative control]

The OD values were converted into titers using their software (xChekPlus, IDEXX). The titer greater than 396 is considered positive.

ND specific antibodies were also analyzed in the serum samples by haemagglutination inhibition (HI) test using 1% chicken RBCs according to OIE protocol. The HI titre was determined as the highest dilution of serum samples that inhibited NDV agglutination of chicken RBCs.

Body weight gain, Bursa bodyweight ratio and bursal lesion scoring

Body weight of three randomly selected birds from each group was recorded at weekly interval (7, 14, 21, 28, 35 & 42 D). The mean difference in body weight of different group birds (n = 3) at 7th day and 42nd day of age was calculated as body weight gain during immunization trial. Three birds from each group were humanely sacrificed at weekly interval; bursa tissue was collected and weighed. Bursa to body weight ratio (B:BW) was calculated as: bursa of Fabricius weight (g) / Body weight (g) x 1000. Bursa tissue from different groups collected on 21st day was subjected to histopathological lesion scoring. The scoring was performed as per Muskett et al. (1979) using following scale: No damage (0); mild necrosis (1); moderate and generalized lymphoid depletion (2); severe lymphoid depletion (3); atrophy of follicles and fibroplasia (4).

RNA extraction and cDNA synthesis

Spleen tissue from different groups of experimental birds were collected aseptically after humane sacrifice at 14th day of age (n = 3 /each group). Total RNA was extracted by using TRIzol (Invitrogen, CA) according to the manufacturer's protocol and treated with DNase I (MBI Fermentas, USA) to remove traces of genomic DNA. Subsequently, 5 ng of purified RNA was reverse transcribed to cDNA using Superscript II first strand cDNA synthesis kit (Invitrogen, CA).

Real time PCR quantification (qRT-PCR) of IL-1 β and IFN- γ cytokine mRNA levels

Cytokine mRNA expression levels of IL-1 β and IFN- γ were analyzed by real -time PCR using Maxima SYBR Green qPCR kit (MBI Fermentas, USA) in diluted cDNA samples using Insta Q96™ real time PCR machine (Himedia, India). The guidelines of MIQE were followed. Primer sequences were given in Table 2. Briefly, each reaction involved a pre-incubation step at 94°C for 30s, followed by 40 cycles of 95°C for 30 s, 55°C for 30 s and extension at 72°C for 30 s. Subsequently melting curve analysis was performed to check the specific product. Each sample was run in triplicate with no template control (NTC). β actin was used as housekeeping gene for normalizing the expression data.

Table 2
Primers used in qRT-PCR

Gene	Forward Primer (5'-3')	Reverse Primer (5'-3')	Amplicon size (bp)	Reference
<i>IL-1β</i>	GGATTCTGAGCACACCACAGT	TCTGGTTGATGTCGAAGATGTC	272	Nang et al. (2011)
<i>IFN-γ</i>	TGAGCCAGATTGTTTCGATG	CTTGCCAGGTCCATGATA	152	Nang et al. (2011)
<i>β-actin</i>	CCGTAAGGACCTGTACGCCAACAC	GCTGATCCACATCTGCTGGAAGG	208	Fan et al. (2013)

Statistical analysis

Humoral immune response data including IBD and ND titers were analyzed by one-way ANOVA with Tukey's post-hoc test to analyze the difference between mean titre between groups at different time intervals. Mean titers were considered significantly different when $P \leq 0.05$. Statistical analysis was performed using SPSS v. 14. Body weight gain, bursa to body weight ratio and bursal lesion scores were also analyzed by one-way ANOVA for any significant differences between groups. Expression levels of IL-1 β and IFN- γ mRNA were calculated after normalizing with housekeeping gene and fold-changes in gene expressions were calculated as $2^{-\Delta\Delta Ct}$ (Livak and Schmittgen 2001). Comparison of fold changes in gene expression between groups were performed using General Linear Model in version 9.2 of SAS software with a P value < 0.05 considered significant.

Results

IBD specific antibody titre

Mean maternal antibody levels of day-old chicks was 3800 ± 356 . In poly I:C and unimmunized control group the maternal antibody levels declined and reached to unprotective (< 396) levels from 21st day of age (Fig. 1). However, the groups which received IBD vaccine either alone or in combination with Poly I:C induced seroconversion and protective antibody titers from 28-35th day (Protective titre cut-off: 396). Among the immunized groups, intermediate IBD plus vaccine induced significantly (Mean titre 3912 ± 1385 ; $P \leq 0.05$) higher levels of IBD specific antibodies on 35th day of age than other vaccine groups; followed by intermediate plus co-administered with poly I:C (1481 ± 914). Intermediate IBD vaccine, Bursaplex vaccine and their combination with poly I:C also induced higher and protective antibody titres on 35th day of age. Poly I:C combination with intermediate and bursaplex vaccine induced better IBD specific antibody titres than the vaccine alone. However, for intermediate plus vaccine, poly I:C combination did not improve the titres.

HI titre

Significant differences ($P \leq 0.05$) in mean HI titre was observed between different groups on 4th and 5th week of age (Fig. 2). Intermediate IBD and intermediate plus vaccines in combination with Poly I:C groups showed significantly lower HI titres on both 4th and 5th week in comparison to other groups. Protective antibody titres were observed in all groups from 5th week onwards. Control group that did not receive any ND vaccine showed waning of ND antibody titer from 2nd to 3rd week and become unprotective for ND from 4th week onwards (data not shown).

Body weight gain, Bursa to Body weight ratio and bursal lesion score

Body weight gain calculated between the period of 7 days to 42 days of age during immunization trial showed significantly lower ($P \leq 0.05$) in intermediate IBD plus and Intermediate IBD plus with Poly I:C group in comparison to other groups and control (Fig. 3).

Bursa to body weight ratio between different groups ranged from 1.95 to 4.96 (Table 3). No significant difference was observed among the different groups on different intervals. Histopathological bursal lesion scoring for different groups on 21st day of age did not show any significant differences among the groups.

However, the lesion score was slightly higher for intermediate plus and intermediate plus with Poly IC vaccine group numerically (Table 4).

Table 3

Bursa to body weight ratio of different immunization groups at weekly interval

Groups	1 st week	2 nd week	3 rd week	4 th week	5 th week
I: Int IBD	1.99	2.36	3.65	3.21	3.00
II: Int IBD plus	2.26	2.54	2.92	3.32	2.36
III: Bursaplex	2.13	2.73	2.45	2.37	1.79
IV: Int IBD + Poly IC	1.99	3.51	2.61	3.97	4.96
V: Int IBD plus +Poly IC	1.95	2.91	2.05	2.81	1.39
VI: Bursaplex + Poly IC	1.72	3.11	2.67	3.13	2.44
VII: Only Poly IC	2.11	2.55	3.71	2.75	3.13
VIII: Unimmunized control	2.17	3.27	4.12	3.46	2.50
BB ratio calculated as (bursa weigh/ body weight) X 1000					

Table 4

Histopathological bursal lesion scoring of different groups
(Mean \pm SEM)

Groups	Histopathological lesion scoring
I: Int IBD	1.5 \pm 0.32
II: Int IBD plus	2 \pm 0
III: Bursaplex	1.5
IV: Int IBD + Poly IC	1.5 \pm 0.75
V: Int IBD plus + Poly IC	2 \pm 0
VI: Bursaplex + Poly IC	1 \pm 0.02
VII: Only Poly IC	0.75 \pm 0.45
VIII: Unimmunized control	0.75 \pm 0.2

Cytokine mRNA expression levels

Fold changes in cytokines IL-1 β and IFN- γ mRNA expression levels in spleen tissue of different groups on 14th day of age are presented in Table 5. IL-1 β levels were up-regulated in intermediate plus and bursaplex group in

comparison to other groups. Whereas IFN- γ mRNA expression levels were upregulated in intermediate IBD with Poly IC group than others.

Table 5
Cytokine IL-1 β and IFN- γ expression levels in spleen by real time PCR

Groups	IL-1 β	IFN- γ
I: Int IBD	2.89	1.21
II: Int IBD plus	4.24	1.80
III: Bursaplex	4.21	3.20
IV: Int IBD + Poly IC	1.02	4.24
V: Int IBD plus + Poly IC	1.21	3.28
VI: Bursaplex + Poly IC	2.87	2.91

*Values are expressed as mean fold changes. Ct values are normalized with β -actin house keeping gene and fold changes are calculated with reference to unimmunized controls. Age: 14th day.

Discussion

IBD is one of the re-emerging threats to poultry industry worldwide. IBD causes severe acute infections in young broilers leading to bursal atrophy, immunosuppression and mortality in the range of 30% and 60% in broilers and layers respectively (Etteradossi 2020). Although vaccines are available to combat the IBD infection, certain issues like maternal antibodies and induction of bursal damage by live vaccines exists. Poly I:C, a TLR3 agonist is a safe and effective adjuvant for improving vaccine induced immune response in birds and animals. Poly I:C binds with TLR3 and activates MyD88 independent pathway through TIR-domain containing adapter-inducing interferon- β (TRIF) and activating the downstream molecules IRF-3 and NF- κ b and results in the production of type I IFN and inflammatory cytokines (Kawai and Akira 2010). Its immunomodulatory potential has been demonstrated with killed avian influenza vaccine, Marek's vaccine and ND vaccine in chicken (Kannaki et al. 2019). Poly I:C alone or in combination with Pam3CSK4, a TLR3 and 2 agonists respectively, alleviated hot IBD vaccine induced immunosuppression in chicken (Bashir et al., 2019).

In the present study, we evaluated the effect of poly I:C upon co-administration with different IBD vaccines in chicken. Humoral antibodies play a major role in the protection of IBDV infection. Positive correlation exists between the IBD antibody titer and the protection (Nakamura et al. 1994). The experimental chicks had intermediate levels of maternal antibodies on day-old. MDAs usually start wane after first week and becomes unprotective from 2–3 weeks of age without any vaccines (Le Gros et al., 2009). We also observed waning of MDAs to unprotective levels around 3rd week in unimmunized control group and Poly I:C group. In the present study, all three vaccines namely intermediate, intermediate plus and bursaplex vaccine alone and in combination with Poly I:C induced seroconversion and protective IBD specific antibody titres at 35 days of age even in the presence of higher levels of maternal antibodies. It is in conformity with earlier studies, wherein the seroconversion was observed around 18 days post-vaccination (Wyeth and Chette 1990; Bose et al., 2003). Among the vaccines studied, intermediate plus vaccine could induce significantly higher antibody

levels than other vaccine groups, nevertheless with moderate bursal lesions and growth suppression indicated by lower body weight gain during the trial period. Body weight gain was significantly lower in intermediate plus vaccine group similar to earlier studies (Ashash et al., 2019).

All these vaccines are shown to induce protective antibody titres in the presence of maternal antibodies (MDA) (Sedeik et al. 2019). Immune-complex vaccine is a mixture of the intermediate plus strain and specific antibodies, that is released after MDA drops off. Moreover, these vaccines offer the practical advantage of hatchery administration as they are given at day-old by parenteral route (Haddad et al., 1997). Further, the recommended single dose avoids handling of birds frequently as in conventional vaccines. We observed poly I:C co-administration improved the IBD specific immune response in intermediate and bursaplex IBD vaccine at 35 day of age, however, it is not the case in intermediate plus vaccine. Replication of vaccine virus in bursa and induction of bursal damages were directly correlated with their induction of humoral immunity (Rautenschlein et al., 2005). However, TLR agonist might reduce the replication of vaccine virus by upregulation of type I IFNs that are antiviral in nature (Cai et al 2012). The effect may be more pronounced with hot vaccine compared to mild to moderate vaccines or immune complex vaccine that are slowly released. In an earlier study also, similar trend was observed and authors speculated the same (Bashir et al. 2019). Intermediate plus and intermediate IBD vaccines induce transient suppression of ND antibody titres (Rautenschlein et al., 2007). Transient suppression in ND titres was observed in the present study too around 35th day of age supporting the earlier notion. *In vitro* stimulation of chicken PBMCs and *in vivo* administration of poly I:C upregulated type I IFN transcripts (Karpala et al., 2008; Parvizi et al., 2012). upregulation of IFN- γ was observed in spleen tissue of poly I:C immunized groups, however, it was not significantly different from IBD vaccine group.

In summary, poly I:C co-administration with different IBD vaccines showed that it improved specific immune response in intermediate IBD vaccine and bursaplex along with comparatively less immunosuppression and bursal damage. Hence, use of poly I: C along with these vaccinations would be beneficial to improve the vaccine response in broilers. Single injection of bursaplex with poly I:C is practically more useful for attaining better vaccine induced immunity.

Declarations

Funding

The research was funded by Indian Council of Agricultural Research and carried out at ICAR- Directorate of Poultry Research, Hyderabad, India.

Compliance with ethical standards

All the experiments involving birds in the current study were carried out following the ethical guidelines and standards given by CPCSEA, India. The experiment was approved by institute animal ethic committee of ICAR- Directorate of Poultry Research (IAEC/DPR/2017/7).

Conflict of interest

The authors declare that they have no conflict of interest.

Data availability

Not applicable

Code availability

Not applicable

Author's contribution

TRK- Conceived the experiment, designed, analyzed the data and prepared manuscript; EP & MA- conducted experiment, analyzed the samples; SH-Critically reviewed the manuscript

Consent to participate

Not applicable

Consent for publication

All authors have read and agreed to the published version of the manuscript.

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Figures

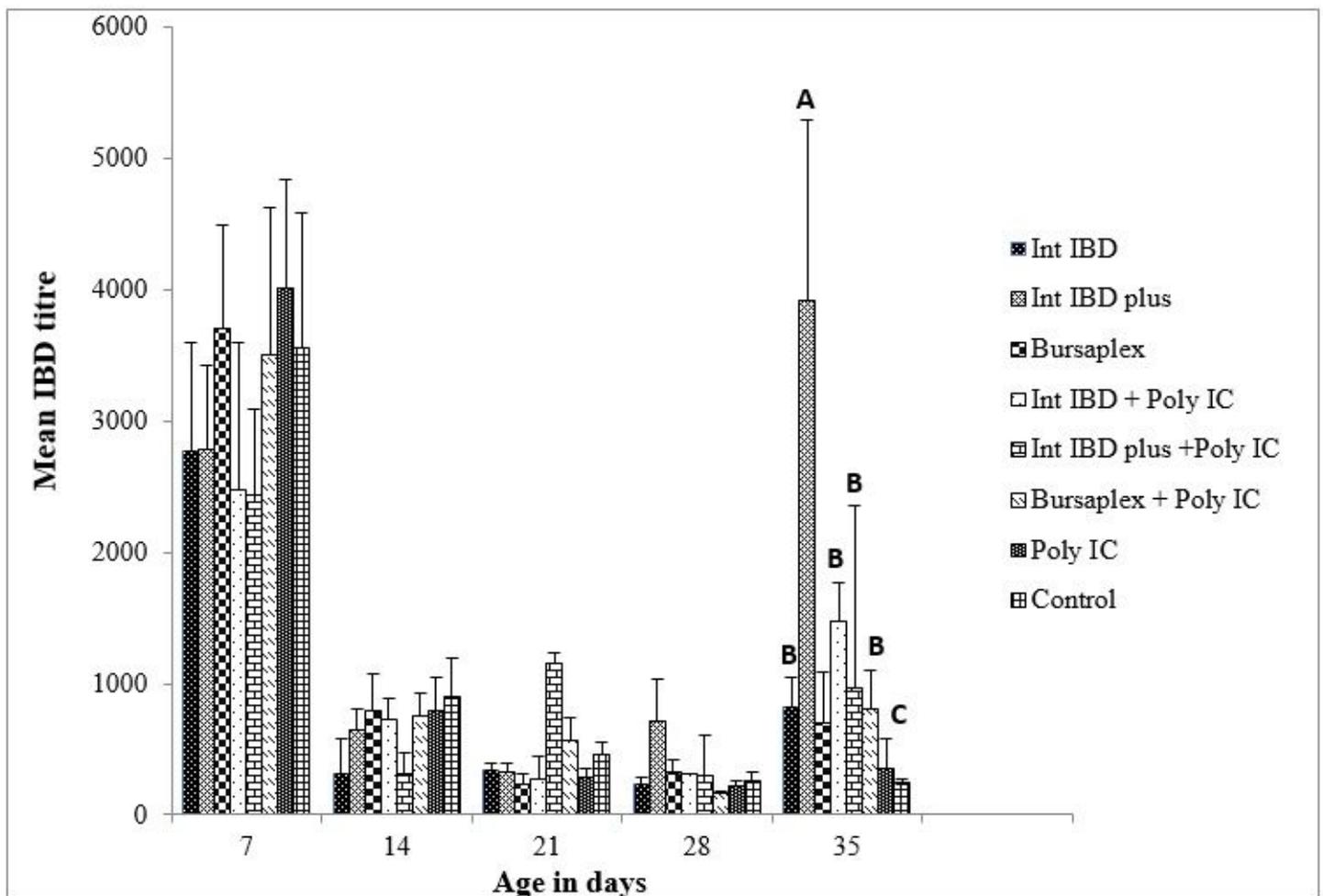


Figure 1

IBD specific antibody titres by iELISA in serum of different groups at weekly interval

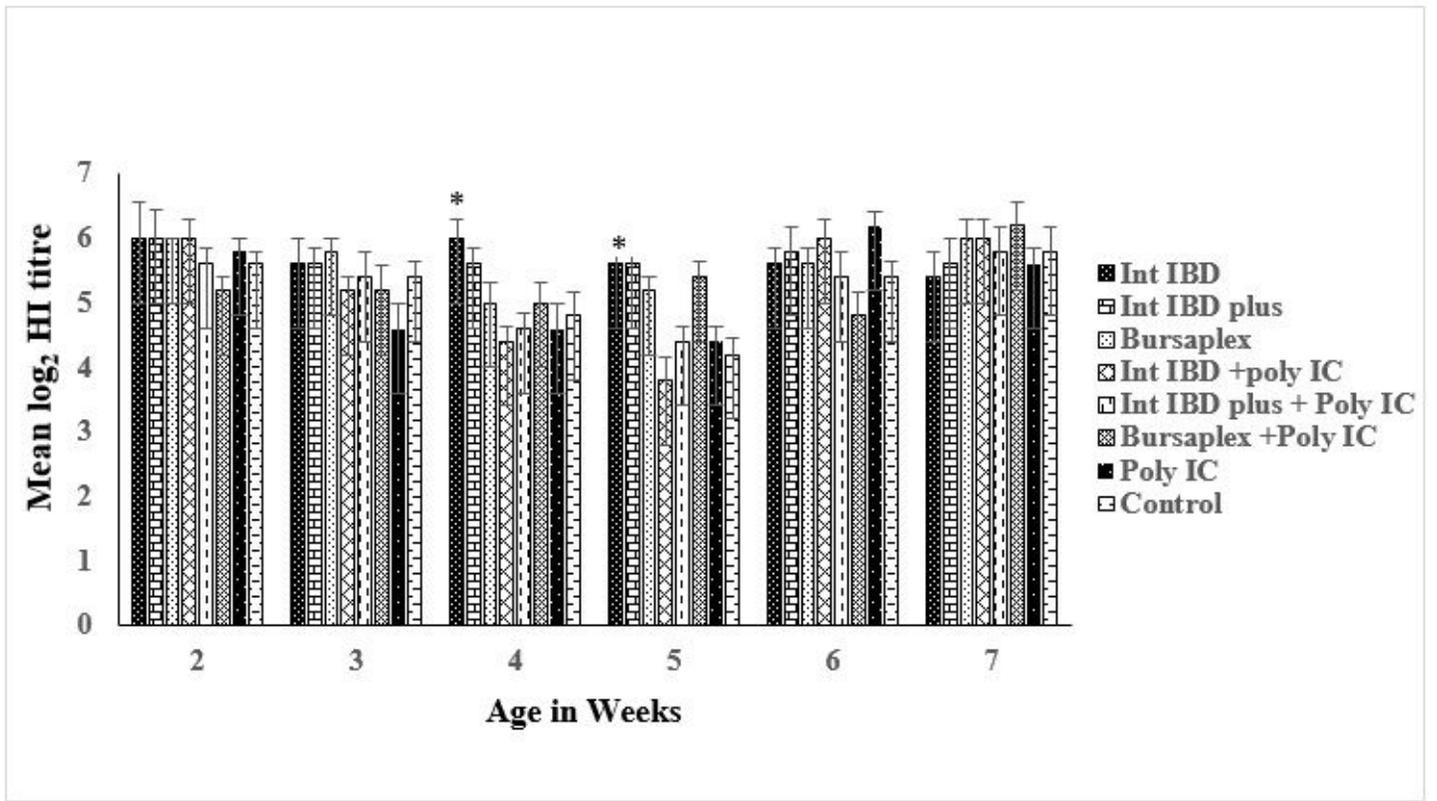


Figure 2

HI (Log2) titres of different immunization groups at weekly interval

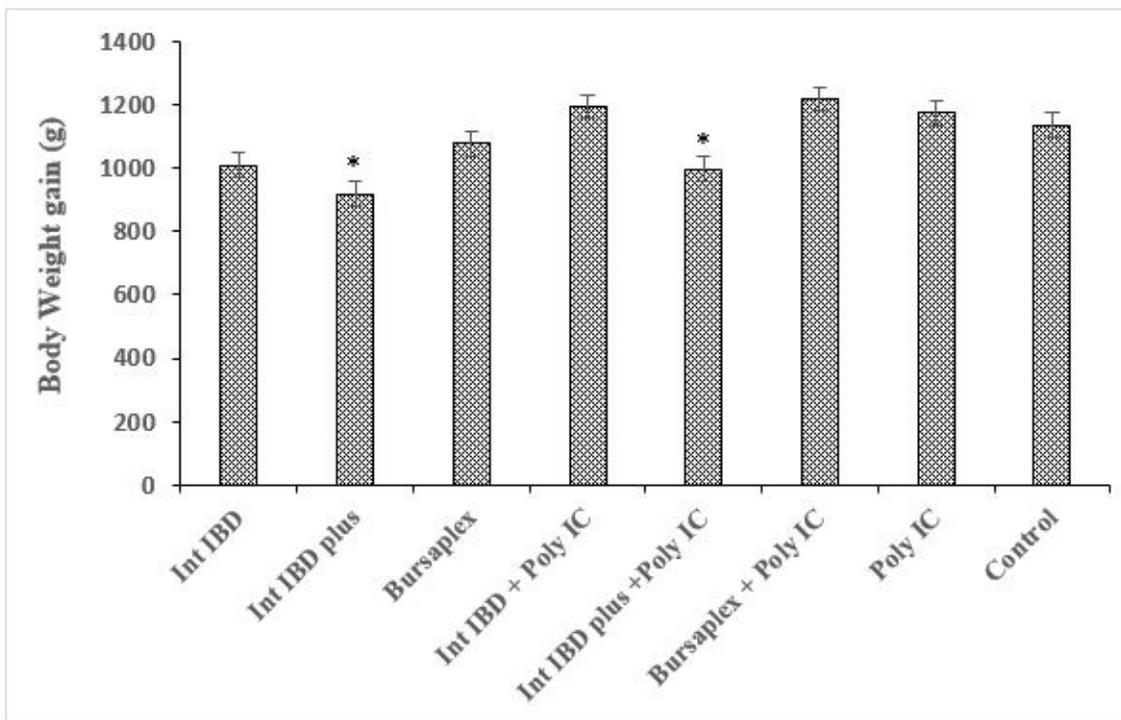


Figure 3

Body weight gain in different immunization groups (Between 7th day and 42nd day)