Pakistan Veterinary Journal

ISSN: 0253-8318 (PRINT), 2074-7764 (ONLINE)
DOI: 10.29261/pakvetj/2020.043

RESEARCH ARTICLE

Evaluation of Immunogenicity and Protective Efficacy of *Eimeria maxima* Immune Mapped Protein 1 with EDA Adjuvant in Chicken

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ARTICLE HISTORY (18-491)

Received: December 15, 2018
Revised: December 12, 2019
Accepted: May 03, 2020
Published online: May 06, 2020

Key words:
Coccidiosis
Immunization
Infection
Molecular adjuvant
Poultry
Subunit vaccines

A B S T R A C T

Immune mapped protein-1 (IMP1) has been shown as a protective protein associated with *Eimeria* (*E.*) *maxima* and cellular fibronectin extra domain A (EDA), the ligand of toll-like receptor 4 (TLR4), has the potential to be used as molecular adjuvant of the vaccine. In the present study, we estimated the protective efficacy of a subunit vaccine comprising of EDA and EmIMP1 against *E. maxima* challenges. For this purpose, the fusion protein EmIMP1-EDA was thoroughly purified by using over-expression of replicated *E. coli* of the protein. Chickens were immunized by the EmIMP1 vaccine having Freund's adjuvant or with the EmIMP1-EDA vaccine without adjuvant. The immunization of chicken by the EmIMP1-EDA vaccine produced stronger IFN-γ secretion as compared to that in other groups ($P<0.05$). The result of the EmIMP1-EDA vaccine was more pronounced on the parameters including weight gain, oocyst shedding, and lesion scores as compared to the EmIMP1 vaccine employing Freund's adjuvant, but there were no statistical differences between the two groups ($P>0.05$). These results showed that EmIMP1-EDA fusion protein could be used as a potent subunit vaccine against *E. maxima* infection.

To Cite This Article: Lin X, Mohsin M, Abbas RZ, Li L, Chen H, Huang C, Li Y, Goraya MU, Huang Z and Yin G, 2020. Evaluation of immunogenicity and protective efficacy of *Eimeria maxima* immune mapped protein 1 with EDA adjuvant in chicken. Pak Vet J, 40(2): 209-213. http://dx.doi.org/10.29261/pakvetj/2020.043

INTRODUCTION

Infectious diseases have been a continuous threat in poultry production systems (Naqvi *et al*., 2017; Rehman *et al*., 2018; Siddique *et al*., 2020; Zhang *et al*., 2020). Among parasitic diseases, coccidiosis is an important disease of poultry having great economic importance (Khater *et al*., 2020). According to an estimate, this disease causes losses of more than three billion dollars every year (Blake and Tomley, 2014). Various factors like the intensity of infection, site of infection, age of the bird, farm management and disease incidence at a particular farm are responsible for its sever mortality and morbidity (Bachaya *et al*., 2015). The usage of anticoccidial drugs in feed or drinking water has been normally practicing against chicken coccidiosis in various parts of the world, however, due to the emergence of drug resistance (Abbas *et al*., 2008, 2011), alternate approaches are being sought for the control of various infectious diseases including coccidiosis (Hussain *et al*., 2017; Abbas *et al*., 2017a, 2017b, 2017c, 2019a, 2019b; Mahmood *et al*., 2018). One of these alternates is the use of essential oils which are helpful in maintaining vital physiological functions (Idris *et al*., 2017; Abbas *et al*., 2018; Khater *et al*., 2018; Ahmad *et al*., 2019; Fayaz *et al*., 2019) but consumption of these oils has been reported for causing cell membrane damage in laboratory animals.

Vaccination against coccidiosis, including adjuvant based vaccine, is an appropriate appealing approach against chicken coccidiosis (Chen *et al*., 2015). Currently, vaccines containing live attenuated (Paracox®, Livacox®) or virulent oocysts (Coccivac®, Immucox®) are available in the market. High cost and of these types of vaccine is a significant reason or their failure in the market. Live oocyst vaccines have some drawbacks including the pathogenicity of virulent oocysts, high cost, low

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production, and virulence reversibility of live attenuated vaccines. So, it is urged to develop new vaccines for use in the market (Chapman *et al.,* 2002).

Immune mapped protein-1 (IMP1) is a well-established immunogenic protein and it confers good immunity against infection with *E. maxima* in chickens (Jenkins *et al*., 2015). Various experiments have shown that cellular fibronectin (FN) extra domain A (EDA) is responsible for activating the TLR4 signalling pathway, and the other portion of FN has no role in this action (Okamura *et al*., 2001). Hence this property of the protein makes it an appropriate candidate as an adjuvant by targeting specified antigen to the dendritic cells (DCs) by TLR4. The interactions of the EDA-TLR4 can activate antigen-presenting cells (APCs), upregulate various co-stimulatory molecules, and also can influence multiple functions mediated by T-cells (Mansilla *et al.,* 2012). This T cell-mediated immune response has shown to be an imperative element of protection against *Eimeria* spp. (Lillehoj *et al.,* 2004).

Based on these reported facts, it is believed that EDA can boost the immunogenic worth of IMP-1 and, hence it can improve the immunity of birds against *Eimeria* spp. Therefore, this research has been planned to examine the adjuvant property of EDA for EmIMP1 target protein and also to analyze the defensive importance of EmIMP1-EDA fusion protein as a vaccine.

MATERIALS AND METHODS

Chickens and Parasites: 150 Day-old chicks were obtained from Fuzhou China. They were reared in clean brooder cages and given feed and water *ad libitum*. To collect oocysts, *E. maxima* oocysts were given to 14-day-old chicks orally (10^4 oocysts per bird). Post infection period of 6 to 9 days, the oocysts were isolated and cleaned from faeces of chickens by saturated NaCl solution flotation method and then sporulated in 2.5% potassium dichromate solution at 27°C in a shaker with a speed of 140 rpm for 48 h (Yin *et al*., 2015).

Cloning of EDA and EmIMP1 gene: Chicken EDA was obtained from Sangon Biotech Company. The EmIMP1 was amplified from the total RNA of *E. maxima* by RT-PCR by utilizing the EmIMP1 specific primers of EmIMP1-f (5'-GAATTCATGGGGGCCGCTTGCGGGA AATC-3') and EmIMP1-r (5'-CTCGAGATCTTGCGAC ACTTTAGTGGG-3'). For sequencing purposes, gene extraction, purification, and cloning were done by pEASY-Blunt Simple Cloning Vector [TransGen Biotech, Beijing, China (Yin *et al*., 2015)].

Expression and purification of recombinant proteins: Chicken EDA fragment was amplified by PCR using the following forward (EDA-f) and reverse primers (EDA-r): 5'-CATATGAATATTGATCGCCCTAAAGGAC-3'and 5'-GAATTCGGTGGACTGGGTTCCAA

TCAGG -3'. The introduction of EmIMP1 constructed the pET-28a-EmIMPI into XhoI and EcoRI digested expression vector pET-28a. Then EDA was introduced into a pET-28a-EmIMPI vector to generate pET-28a-EDA-EmIMPI. The transformed *E. coli (BL21)* were grown in the LB medium (Luria- Bertani) containing tryptone and yeast extract as the main component) overnight to mid-log phase, induced with 1.0 mM of IPTG for 6 h at 37°C. Then, a collection was done by centrifugation and sonication was done on ice for its disruption. For purification of EmIMP1-EDA protein and EmIMPI protein, Hi Trap metal chelating column was used. In the end, protein expression and purification were checked by using SDS-PAGE and western blotting techniques.

Immunization and parasitic infection: Two-week-old male chickens were selected and grouped randomly into 7 groups (n=20). Group 1 and 2 were immunized intramuscularly with 200µg of EmIMP1 protein emulsified in Freund's complete adjuvant (FCA) and 200µg of EmIMP1-EDA protein without adjuvant, respectively (Table 1). Group 3 and 4 were treated with PBS and kept as infected and non-infected control groups. 200µg of EDA vaccinated group 5, while group 6 was treated with 200µl of FCA; both group 5 and 6 were kept as adjuvant control groups. Last group 7 was treated with 200µg of EmIMP1 as a control group. After 14 days the same protocol was repeated. At 14 day, infection of *Eimeria maxima* virulent oocysts was given to all groups except group 4 with a concentration of 1×10^4 oocysts. After this, blood samples were collected and serum was separated and store at -20ºC for further experiment. After 6 to 9 days of infection, body weight and oocyst shedding were determined by the method described by Sun *et al*. (2014) while the intestinal lesion score was checked by the method described by Johnson (1970).

Table 1: Experimental groups of chickens in immunization and challenge experiment

Group	Number of chickens	Immunization dose (µg)	Immunization time (day)	Challenge time (day)
EmIMP1-FCA	20	200	14, 28	42
EmIMP1-EDA	20	200	14, 28	42
PBS-unchallenged	20			
PBS-challenged	20			42
EDA	20	200	14, 28	42
FCA	20	200µl	14, 28	42
EmIMP1	20	200	14, 28	42

Anti-EmIMP1 antibodies and IFN-γ detection: ELISA assay was used to detect the chicken immunoglobulin G (IgG) by the method described by Yin *et al.* (2015). ELISPOT assay was used to check the concentration of IFN-γ in chickens received infection by the method described by Li *et al*. (2015).

Statistical analysis: Statistical analysis was performed by using the ANOVA technique and Tukey's HSD test using a statistical software program [SPSS13.0 Data Editor software, SPSS Inc., Chicago, IL (Yin *et al*., 2015)].

RESULTS

Protein expression: The target 1131 bp cDNA fragment of EmIMP1 was obtained from *Eimeria maxima* total RNA by RT-PCR After sequencing; it was observed that the cDNA sequence shows 100% similarity against EmIMP1 of Weybridge strain of *E. maxima*. For proteomic analysis, SDS-PAGE showed 70kDa and 80kDa protein bands by staining with Coomassie brilliant blue stain (Fig.1A). Mouse anti-His 6 antibody technique can be used for the identification of these proteins. (Fig. 1B).

Table 2: Protective effects of EmIMP1and EDA-EmIMP1 against challenges of *E. maxima* in chickens

Group	Average body weight gain (g)	Relative body weight gain (%)	Oocyst shedding per bird (×10^6)	Oocyst decrease ratio (%)	Lesion score
EmIMP1-FCA	50.82±10.08^a	93.69	4.86±0.40^a	55.41	1.5±0.4^a
EmIMP1-EDA	52.56±10.57^a	96.90	4.29±0.17^a	60.64	1.3±0.2^a
PBS-unchallenged	54.24±7.09^a	100	0	-	0
PBS-challenged	40.77±12.66^b	75.17	10.90±0.23^c	0	3.3±0.2^b
EDA	42.50±10.35^b	78.40	9.80±0.13^c	10.09	3.3±0.2^b
FCA	41.83±9.56^b	77.12	10.50±0.35^c	3.67	3.2±0.4^b
EmIMP1	41.55±11.20^b	76.60	10.20±0.24^c	6.42	3.4±0.3^b

Note: Values are expressed as mean ± SD. Means in the same column with different letters were significantly different between the groups (P<0.05).

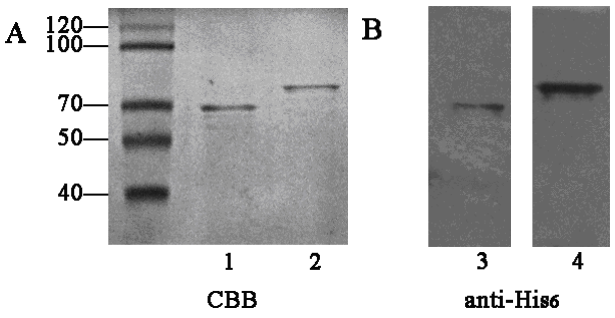

Fig. 1: EmIMP1 and EmIMP1-EDA expression, (A) Use of SDS-PAGE for purification of proteins EmIMP1, EmIMP1-EDA and Coomassie brilliant blue for their mark. (B) EmIMP1, EmIMP1-EDA proteins were established using western blotting method.

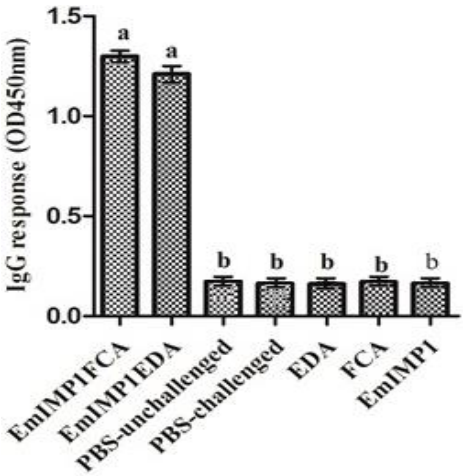

Fig. 2: EmIMP1-EDA encourages effective EmIMP1-specific antibody responses. Anti-IMP1 IgG in the chicken's sera after the second vaccination at 14 days using EmIMP1, EmIMP1-FCA, EmIMP1-EDA, EDA or PBS. Results are expressed as OD450 readings. Bars (mean ± SE, n=6) denoted with different letters are significantly different by the Tukey's HSD test (P<0.05).

Antibodies and cellular immune response against recombinant EmIMP1 and EmIMP1-EDA in chickens: After 2 weeks, EmIMP1-FCA and EmIMP1-EDA vaccinated groups show a higher concentration of IgG specific to EmIMP1 as compared to control groups (P<0.05). However, EmIMP1-FCA vaccinated chicken group shows a higher percentage of IgG as compared to the EmIMP1-EDA vaccinated group with no significant difference was detected. FCA, EDA and EmIMP1 treated groups showed no significant difference than the control groups treated with PBS (Fig. 2). EmIMP1-EDA immunized chickens showed a higher number of IFN-γ-producing cells (70spots/10^{6}cells) that play a role in enhancing the immunity as compared to EmIMP1-FCA group and control groups (12spots/10^6 cells) (P<0.05) (Fig.3).

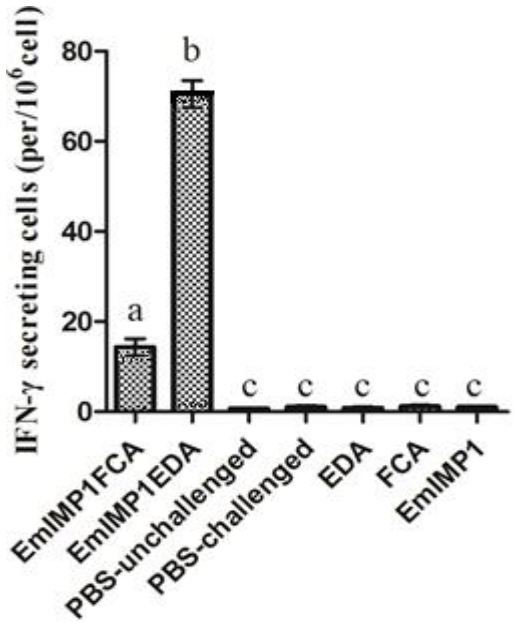

Fig. 3: EmIMP1-EDA protein encourages effective EmIMP1 specific T-cell replies. The Utilization of IFN-γ ELISPOT assay for checking its response. ELISPOT values indicate the quantity of antigen-specific IFN-γ positive spots per 10^6 PBMCs succeeding inspiration through the EmIMP1 protein. Bars (mean ± SE, n=6) denoted with different letters are significantly different by the Tukey's HSD test (P<0.05).

Role of EmIMP1-EDA protein immunization against *E. maxima*: Among chicken groups that were immunized with EmIMP1-FCA or EmIMP1-EDA proteins showed significantly better results for weight gain, decrease oocyst count in faeces and a less intestinal score of lesions as compared to all other groups (P<0.05). However, no significant differences were observed among the EmIMP1-FCA and EmIMP1-EDA groups and the control groups as well (Table 2).

DISCUSSION

For vaccination experiments, FCA is a commonly used adjuvant especially for animals (Grzywa *et al*., 2015) that results in higher immune response in the form of producing higher specific antibodies because of immune stimulant nature (Stills, 2005). Except for these benefits, it is toxic and hard to deal (Fodey *et al*., 2008). However, several studies were conducted to detect non-toxic adjuvant that can be used as a replacer of FCA (Huijbers *et al*., 2012). The current study revealed that EDA showed better results and less toxic effects as compared to FCA. The FCA introduced granulomas and distress in chickens was not detected in the case of EDA adjuvant and EDA showed similar immune-protective results as FCA. Chicken EDA is a simple, low molecular weight protein

having a size of 10 kDa and better combination ability with antigen. Except for these characteristics, the fusion protein can be easily injected, purified and stored. Hence, EDA could be a better alternative to FCA in producing immunity against parasitic antigens.

EDA reported in many studies used as an adjuvant and can increase the immune response against HIV-1 (San Roman *et al*., 2012). EDA can also be used as a specific treatment showing anti-viral, anti-cancer, anti-tumour properties (Lasarte *et al*., 2007) and also against infectious diseases (Arribillaga *et al*., 2013). Furthermore, it can also be used to treat cervical carcinoma by interacting with HPVE7. However, further study is required to check the effect of EDA in chickens by combining with the TLR4 receptor of chicken cells.

The present study concluded that EmIMP1-EDA decreases 60.64% oocyst count showing higher efficacy than EmIMP1 that was emulsified in FCA. Efficacy of the EmIMP1-EDA vaccine is consistent with other studies using recombinant CD40L as a vaccine adjuvant in a chicken vaccine (Yin *et al*., 2015). However, the immunoglobulin level of IgG specific to EmIMP1-EDA was noted the same as in the case of the EmIMP1-FCA treated group. Serum IgG antibodies level indicates the protective immune response against parasite antigen (Guzman *et al*., 2003). Chickens vaccinated by EmIMP1-EDA showed stimulated peculiar cell responses such as more IFN-γ production by PBMCs cells in reaction to EmIMP1 stimulus. In our results, the IgG level was the same in both vaccine candidates when the IFN-γ level was significantly different. The reason is that, as a molecular adjuvant, chicken EDA may be an inducer of innate immune effectors such as cytokines, thereby stimulating the activation of adaptive immune responses, chicken EDA could elicit the IgG response and T-cell response. Meanwhile, FCA mainly enhances antibody IgG responses. Prospective, EDA, fused to EmIMP1 protein, may increase the uptake ability and processing of EmIMP1 by antigen-presenting cells, hence causing stimulation of chicken adaptive immune system responses in affective mode.

Conclusions: In the end, our results demonstrate the possibility of producing two efficacious vaccine candidates against *E. maxima* infection using EmIMP1 with FCA or EDA. This experiment verified by reasonable decline values in the oocysts shedding in chickens infected with *E. maxima*. Hence, the usage of EmIMP1-EDA fusion protein acts as a powerful immunogenic in the improvement of subunit vaccines against the *E. maxima* infection.

Acknowledgments: This research was supported by the National Natural Science Foundation of China (31872466), Discipline Development Grant from College of Animal Sciences FAFU (2018DK004), and Project of Fujian Science and Technology Department (2019N0005).

Authors contribution: GWY and ZJH designed and supervised the research. XLL, LNL, HC, CFH, YKL experimented and analyzed the data. MM wrote this paper, RZA and MUG polished the article.

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