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

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ABSTRACT

Immunomapped 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.

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; Fayaiz 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®6, 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...
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 specific antigen to the dendritic cells (DCs) by TLR4. The interactions of the EDA-TLR4 can activate antigen-presenting cells (APCs), upregulate various costimulatory 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 ACTTTAGGGG-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’-CATATGGAATATGATC GCCCTAAAGGAC-3’ and 5’-GAATTCGTTGACCTGGTTCCAA TCAGG-3’. The introduction of EmIMP1 constructed the pET-28a-EmIMP1 into XhoI and EcoRI digested expression vector pET-28a. Then EDA was introduced into a pET-28a-EmIMP1 vector to generate pET-28a-EDA-EmIMP1. 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 EmIMP1 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 1x10^7 oocysts. After this, blood samples were collected and serum was separated and store at -20°C 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).

**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).
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).

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 that can be used as a replacer of FCA (Huijbers et al., 2020). The current study revealed that EDA 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).

![Image](image.png)

Table 2: Protective effects of EmIMP1 and 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                         | 4.6±0.40^b                       | 55.41                     | 1.3±0.4^a    |
| EmIMP1-EDA     | 52.56±10.57^b                | 96.90                         | 4.29±0.17^c                      | 60.64                     | 1.3±0.2^c    |
| PBS-unchallenged| 54.2±7.09^d                  | 100                           | 0                                | 0                         | 0            |
| PBS-challenged | 40.77±12.66^e                | 75.17                         | 10.90±0.23^i                     | 0                         | 3.3±0.2^e    |
| EDA            | 42.5±10.35^f                 | 78.40                         | 9.80±0.13^g                      | 10.90                     | 3.3±0.2^e    |
| FCA            | 41.83±5.6^b                  | 77.12                         | 10.50±0.35^h                     | 3.67                      | 3.2±0.4^b    |
| EmIMP1         | 41.55±11.20^2                | 76.60                         | 10.20±0.24^i                     | 6.42                      | 3.4±0.3^2    |

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

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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).

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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 HPV7. 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.

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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.

REFERENCES

Abbas RZ, Iqbal Z, Sindhu Z, et al., 2008. Identification of Cross-Resistance and Multiple Resistance in Eimeria tenella Field Isolates to Commonly Used Anticoccidials in Pakistan. J Appl Poult Res 17:361-8.

Abbas RZ, Iqbal Z, Blake D, et al., 2011. Anticoccidial drug resistance in fowl coccidian: the state of play revisited. World Poult Sci J 67:337-50.

Abbas A, Iqbal Z, Abbas RZ, et al., 2017a. In vivo anticoccidial effects of Beta vulgaris (sugar beet) in broiler chickens. Microb Path 111:139-44.

Abbas A, Iqbal Z, Abbas RZ, et al., 2017b. Immunomodulatory activity of Pinus radiata extract against coccidiosis in broiler chicken. Pak Vet J 37:145-9.

Abbas A, Iqbal Z, Abbas RZ, et al., 2017c. Immunomodulatory effects of Comella sinensis against coccidiosis in chickens. J Anim Plant Sci 27:415-21.

Abbas A, Abbas RZ, Khan MK, et al., 2019a. Anticoccidial effects of Trachyspermum ammi (Ajwain) in broiler chickens. Pak Vet J 39:301-4.

Abbas RZ, Abbas A, Raza MA, et al., 2019b. In vitro anticoccidial activity of Trachyspermum ammi (Ajwain) extract on oocysts of Eimeria species of Chicken. Adv Life Sci 7:44-7.

Abbas A, RZ Abbas, S Masood, et al., 2018. Acaricidal and insecticidal effects of essential oils against ectoparasities of veterinary importance. Boletín Latinoamericano Y Del Caribe De Plantas Medicinales Y Aromáticas 17:441-52.

Ahmad S, Ali S, Abbas A, et al., 2019. Effects of dietary supplementation of linseed oil (Omega-3) on quality parameters of Nili Ravi bull spermatozoa. Livestock Sci 224:57-9.

Arribilaga L, Durantez M, Lozano T, et al., 2013. A fusion protein between streptavidin and the endogenous tr4 ligand eda targets biotinylated antigens to dendritic cells and induces t cell responses in vivo. Biomed Res Int 864720.

Bachaya HA, Abbas RZ, Raza MA, et al., 2015. Existence of coccidiosis and associated risk factors in broiler chickens in Southern Punjab, Pakistan. Pak Vet J 35:81-4.

Blake DP, Clark EL, MacDonald SE, et al., 2015. Population, genetic, and antigenic diversity of the apicomplexan Eimeria tenella and their relevance to vaccine development. Proc Natl Acad Sci 112:5343-50.

Blake DP and Tomley FM, 2014. Securing poultry production from the ever-present Eimeria challenge. Trends Parasitol 30:12-9.

Chapman HD, Cherry TE, Danforth HD, et al., 2002. Sustainable coccidiosis control in poultry production: the role of live vaccines. Int J Parasitol 32:617-29.

Chen P, Lv J, Zhang J, et al., 2015. Evaluation of immune protective efficacies of Eimeria tenella Em1c1 polypeptides with different domain recombination displayed on yeast surface. Exp Parasitol 155:1-7.

Fayaz MR, Abbas RZ, Abbas A, et al., 2019. Potential of botanical driven essential oils against Haemochus contortus in small ruminants. Bol Latinoam Caribe Plant Med Aromat 18:533-43.

Fodey TL, Delahaut P, Charlier C, et al., 2008. Comparison of three adjuvants used to produce polyclonal antibodies to veterinary drugs. Vet Immunol Immunop 122:25-34.

Grzywa R, Walczak M, Lupicka-Slowik A, et al., 2015. Adjuvant-dependent immunogenicity of Staphylococcus aureus Efb and Map proteins in chickens. Vet Immunol Immunop 166:50-.

Guzman VB, Silva DAO, Kawazoe U, et al., 2003. A comparison between IgG antibodies against Eimeria acervulina, E. maxima, and E. tenella and their oocyst shedding in broiler breeders vaccinated with live anticoccidial vaccines. Vaccine 21:4225-33.

Huijbers EJM, Femel J, Andersson K, et al., 2012. The non-toxic and biodegradable adjuvant Montanide ISA 720(CpG) can replace Freund's in a cancer vaccine targeting ED-B-a prerequisite for clinical development. Vaccine 30:225-30.

Hussain K, Iqbal Z, Abbas RZ, et al., 2017. Immunomodulatory activity of Glycyrrhiza glabra extract against mixed Eimeria infection in chickens. Int J Agric Biol 19:928-32.

Idris M, Abbas RZ, Masood S, et al., 2017. The potential of antioxidant rich essential oils against avian coccidiosis. World's Poult Sci J 73:89-104.

Jenkins MC, Fetterer R, Miska K, et al., 2015. Characterization of the Eimeria maxima sporozoite surface protein IMPI. Vet Parasitol 211:146-52.
Johnson J and Reid W. 1970. Anticoccidial drugs: lesion scoring techniques in battery and floor-pen experiments with chickens. Exp Parasitol 28:30.

Khater HF, Ali AM, Abouellella GA, et al., 2018. Toxicity and growth inhibition potential of vetiver, cinnamon, and lavender essential oil and their blends against larvae of the sheep blowfly, Lucilia sericata. Int J Dermatol 57:449-57.

Khater HF, Ziam H, Abbas A, et al., 2020. Avian Coccidiosis: Recent Advances in Alternative Control Strategies and Vaccine Development. Agrobiol Rec 1:11-25.

Lasarte JJ, Casares N, Gorraiz M, et al., 2007. The extra domain A from fibronectin targets antigens to TLR4-expressing cells and induces cytotoxic T cell responses in vivo. J Immunol 178:748-56.

Li Z, Tang X, Suo J, et al., 2015. Transgenic Eimeria mitis expressing chicken interleukin 2 stimulated higher cellular immune response in chickens compared with the wild-type parasites. Front Microbiol 6.

Lillehoj HS, Min W and Dalloul RA, 2004. Recent progress on the cytokine regulation of intestinal immune responses to Eimeria. Poult Sci 83:611-23.

Mahmood MS, Amir HW, Abbas RZ, et al., 2018. Evaluation of antiviral activity of Azadirachta indica (Neem) bark extract against Newcastle disease virus. Pak Vet J 38:25-8.

Mansilla C, Berraondo P, Durantez M, et al., 2012. Eradication of large tumors expressing human papillomavirus E7 protein by therapeutic vaccination with E7 fused to the extra domain a from fibronectin. Int J Cancer 131:641-51.

Naqvi MAH, Khan MK, Iqbal Z, et al., 2017. Prevalence and associated risk factors of haemoparasites, and their effects on hematological profile in domesticated chickens in District Layyah, Punjab, Pakistan. Prev Vet Med 143:49-53.

Okamura Y, Watari M, Jerud ES, et al., 2001. The extra domain A of fibronectin activates Toll-like receptor 4. J Biol Chem 276:10229-33.

Rehman SU, Saleemi MK, Khan MZ, et al., 2018. Molecular epidemiology and pathology of chicken infectious anemia in day old broiler chicks in Faisalabad, Pakistan. Int J Agric Biol 20:57-61.

San Roman B, De Andres X, Munoz PM, et al., 2012. The extradomain A of fibronectin (EDA) combined with poly (I: C) enhances the immune response to HIV-1 p24 protein and the protection against recombinant Listeria monocytogenes-Gag infection in the mouse model. Vaccine 30:2564-9.

Stills HF, 2005. Adjuvants and antibody production: dispelling the myths associated with Freund's complete and other adjuvants. Ilar J 46:280-93.

Sun H, Wang L, Wang T, et al., 2014. Display of Eimeria tenella Etmic2 protein on the surface of Saccharomyces cerevisiae as a potential oral vaccine against chicken coccidiosis. Vaccine 32:1869-76.

Yin G, Lin Q, Qiu J, et al., 2015. Immunogenicity and protective efficacy of an Eimeria vaccine candidate based on Eimeria tenella immune mapped protein 1 and chicken CD40 ligand. Vet Parasitol 210:19-24.

Zhang K, Li X, Na C, et al., 2020. Anticoccidial effects of Camellia sinensis (green tea) extract and its effect on Blood and Serum chemistry of broiler chickens. Pak Vet J 40:77-80.