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Effect of a *Mycobacterium* extract as an immunomodulator with necrotic enteritis gel vaccine in chicken

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SUMMARY

This study was carried out to assessment the immune response of broiler chickens orally administered *Mycobacterium phlei* extract as a non-specific immunomodulator prior to vaccination with necrotic enteritis Alhydrogel vaccine. The obtained results cleared that chickens administered *M. phlei* extract and vaccinated with necrotic enteritis vaccine showed an enhancement in their humoral immune response expressed in a higher alpha antitoxin titers (4 IU/ml) by using serum neutralization test , as well as, an enhancement in their cellular immune response at a higher stimulation index of peripheral blood lymphocytes (PBL) by using lymphocytes proliferation test more than those chickens received necrotic enteritis gel vaccine only . In conclusion *M. phei* could be used as immunoenhancer before vaccination in chickens.

INTRODUCTION

Necrotic enteritis (NE) is an economically important enteric disease of chickens caused by *Clostridium perfringens* , alpha toxin of *Closterdium perfringens* types A and C have been associated with NE although most cases appear to be caused by type A mainly (Parish, 1961 ; Hofacre *et al.*, 1986; Prukner and Milakovic 1991 ; Prukner *et al.*, 1995 and Kaldhusdal and Jordan, 2008) .

NE is one of the most common diseases in broiler chickens, young replacement broiler flocks and breeder aged 2-5 weeks, the disease is also regularly found in layers mostly in pullets and young birds that are kept in litter, (Kaldhusdal and Jordan, 2008). It causes high mortality up to 30% in broiler chicken and associated with subclinical chronic intestinal mucosal damage that result in reduced growth and productivity (Bereket *et al.*, 2008.)

A vaccine of NE for chickens may reduce the current need to antimicrobial drugs in order to prevent or treat the disease in broiler chickens (Thompson *et al.*, 2006).

In Egypt , Hussien *et al.*, (2007) , isolated *C. perfringens* type A from the intestine of birds showing symptoms of necrotic enteritis , detected alpha toxin of *C. perfringens* type A in the intestinal filtrate of diseased or dead chickens and prepared a toxoid vaccine adjuvanted with aluminum hydroxide gel from the alpha toxin of the isolated *C. perfringens* type A . Many investigators showed that protection against necrotic enteritis was attained by vaccination with a toxoid vaccine which prepared from an inactivated alpha toxin of *C. perfringens* type A. as reported by EL-Meneisy *et al.*, (2007) and EL- Sehemy *et al.*, (2008).

No specific stimulation of immunological response by biological products has attracted the attention of immunobiologists globally because of its non specificity and wide spectrum nature in respect of biological interception in infections. Mycobacterium antigens are known to produce cell-mediated immune response in mammalian hosts. (Follows *et al.*, 1992). It attack intestinal epithelium and activated gut associated lymphoid tissues, (Aldovini and Young, 1991). The development of a cheaper effective alternatives for immunomodulation is necessary for profitable use in poultry industry, an enzyme treated preparation of *Mycobacterium phlei* which is a fast growing environmental mycobacterium has been used experimentally to potentiate immune response in a variety of animal species and chicken against various bacterial and viral infections in addition to ceceal coccidiosis (Mishra 1970; Jona 1971 and Bera *et al.*, 2010)

Baldridge and Ward, (1997) found that incorporation of *M. phlei* cell wall skeleton (CWS) with oil in water emulsion vaccine containing heat killed *Listeria monocytogenes* enable it to stimulate cell-mediated immune response (CMI) that remain at high level for at least two months . *M. phlei* extract when used as oral immunomodulator in chicken increased non specific effector functions of intestinal epithelial lymphocytes, the major immune cell on gut mucosa, (Sreekumar and Das, 2000). On the other hand, Kamal *et al.*, (2004) reported that *M. phlei* extract may be of potential use in enhancing cell-mediated immunity against infectious coryza vaccine in chickens.

The objective of this study was to evaluate the modulation effect of *M. phlei* extract as a non-specific immunomodulator agent with necrotic enteritis Alhydrogel vaccine in chicken.

MATERIALS AND METHODS

1-Vaccine preparation :-

Concentrated supernatant toxin of a locally toxogenic strain of *C. perfringes* type A (kindly obtained from Anaerobic Vaccine Department, Veterinary Serum and Vaccine Research Institute Abbasia, Egypt), was prepared according to method of Ahmed, (1975) and inactivated by 0.5% formalin. Alhydrogel (Aluminum hydroxide gel (AL $(OH)_3$ - gel) was added in ratio of 20% as an adjuvant (EL-Sehemy *et al.*, 2004). The purity and safety tests of the prepared necrotic enteritis gel vaccine were carried according to British pharmacopoeia, veterinary, 2007

2-Preparation of *Mycobacerium phlei* dead cells :-

A seed culture of standard *M. phlei* strain (kindly obtained from Bacterial Diagnostic Products Research Department , Veterinary Serum and Vaccine Research Institute , Abbasia , Egypt) was grown in Middlebrooke ' s 7H9 medium, the growth was filtered with sterile muslin cloth, the filtered cells washed five times with sterile Phosphate Buffer Saline (PBS) and inactivated by autoclaving, the preparation was tested on nutrient agar and Lowenstein Jensen media for safety and purity from *mycobacteria* and then dried according to method of Sreekumar and Das, (2001).

3-Experimental design :-

Eighty white Leghorn broiler chickens (two weeks old) were used in this study and divided into four groups, (20 chickens / group) then treated as follows:

- **Group 1** : Vaccinated with the prepared necrotic enteritis Alhydrogel vaccine at two weeks old with two doses of 0.5 ml injected subcutaneously (S/c) three weeks apart.
- **Group 2** : Each bird of this group was administered orally (at two weeks old) with 10 mg (dry weigh) of *M. phlei* dead cells in 1 ml PBS in two doses 72 hours apart , after 72 hours of the last dose , the chickens were vaccinated with necrotic enteritis Alhydrogel vaccine as mentioned above (Sreekumar and Das, 2001) .
- **Group 3** : Non-vaccinated control group received the same doses of *M. phlei* only at two weeks old.
- **Group 4** : non-vaccinated and not received *M. phlei* (control negative group).

Twenty blood samples were collected from each group of all four groups before vaccination and after two weeks from the second dose of the vaccine, sera of each 4 chickens of each group were pooled to obtained 5 pooled sera samples for each group which stored at -20 C° until use . Another blood samples were

collected in heparin (Nile Company) and pooled (5 pooled blood samples / group) for measurement of cell-mediated immune response.

4-Serological tests :-

a-serum neutralization test :-

Pooled sera of each group were tested for determination of the alpha antitoxin titers of *C. perfringes* type A. expressed in international unit /ml (IU/ ml) by using the serum neutralization test according to the British Pharmacopoeia , Veterinary , (2007).

b-Lymphoproliferative response :-

MTT colorimetric assay for cellular proliferation was done according to method of Sreekumar and Das, (2001), briefly peripheral blood lymphocytes were isolated from heparinized blood on histopque 1077 (Sigma), after addition of the antigen in appropriate wells in triplicate, the volume in all the wells was made up to 100 ul with RPMI 1640, the plates were incubate at 39 °C in 5% CO_2 in a CO_2 incubator for five days. 20 ul of MTT 3-(4,5-Dimethyle-2-thiazolyl) 2,5 diphenyl-2H-tetrazolium bromide was added to all the wells and incubated further for 4 hr.The OD was taken in an ELISA reader, finally the stimulation index was calculated.

RESULTS AND DISUSSIOIN

Necrotic enteritis is a potentially fatal multifactorial disease of chickens, which under commercial conditions is often associated with increased levels of mortality and reduced bird performance (Crouch et al., 2010). The incidence of *C. perfringens* associated necrotic enteritis in poultry has increased in countries that stopped using growth promoters, (Van Immerseel *et al.*, 2004), Vaccination could be a helpful tool in preventing NE in poultry, and it is a practical preventive measure against *C. perfringens* associated necrotic enteritis in broiler chickens *M.phlei* could induce immunostimulation in different animal species against bacterial and infections (Mishra, 1970).

The results illustrated in Table (1) showed that chicken sera of group (1) vaccinated with necrotic enteritis Alhydrogel vaccine resulted in good immune response represented by 2 IU/ml of alpha antitoxin titers, these findings are in agreement with (Ellis et al., 1991; Heier *et al.*, 2001 and Lovland *et al.*, 2004), who showed that the vaccination of broiler chickens with NE vaccine resulted in strong specific antibodies against alpha toxin of *C. perfringens* type A. Also, these results comes parallel with (EL-Meneisy *et al.*, 2007), who found that chicken vaccinated with NE

Alhydrogel vaccine showed good immune response.

Regarding the results of using *M. phlei* as an oral immunomodulator ,it was clear that, chickens administered orally *M. phlei* and vaccinated with NE vaccine (group 2) yielded higher antitoxin titers (4 IU / ml) as shown in Table (1) as compared to the chicken of group (1) which vaccinated with NE vaccine only. On the other hand, values of antitoxin titers in groups 1 and 2 appeared to be higher than the recommended requirement (0.5 IU/ ml) which gives good protection for chickens against NE disease (Hussien *et al.*, 2007) .

Moreover , The results of cell –mediated immune response as shown in Table (2) supported the previous findings , revealed that *M. phlei* enhanced immune response in chickens of group 2 (vaccinated and fed on *M. phlei*) more than those of group 1 (vaccinated only) , this enhancement was noticed also in group 3 (fed on *M. phlei* only) , these results agree with those found by (Sreekumar and Das, 2000 and 2001) , who reported that *M. phlei* extract modulate specific immune response of chicken against Newcastle disease vaccine in term of heamagglutination-inhibition (HI) antibodies and enhanced CMI due to it activate and increase non specific effector function of intestinal intraepithelial lymphocytes , the major immune cells on gut mucosa . As well as, Lasek (2002) who mention that bacterial immunomodulator that contained killed bacteria or components of bacterial cells will proved to increase the efficacy of immune system response via both specific as well as non-specific effect on cellular and humoral mechanisms.

In conclusion, the results of this study showed that *M. phlei* may be of potential use in enhancing humoral immune response as well as, cell-mediated immunity with chickens necrotic enteritis Alhydrogel gel vaccine and represent a cheaper alternative for non specific immunomodulation.

Table (1): Results of serum neutralization test expressed by (IU/ml) in chickens vaccinated with necrotic enteritis Alhydrogel vaccine with or without *M. phlei* as well as the control group .

Chicken Group No.	No. of chicken	No. of tested pooled s.s.*	Alpha antitoxin titer of C.perfringens type A (IU/ml) of each pooled sera				
			Before vaccination	Two weeks post-vaccination			
1	20	5	0	2	2	2	2
2	20	5	0	4	4	4	4
3	20	5	0	o	o	o	0
4	20	5	0	o	o	o	0

*s.s : sera samples

Group 1:- Vaccinated with the prepared necrotic enteritis Alhydroelgel vaccine.
Group 2:- take *M. phlei* and Vaccinated with necrotic enteritis gel vaccine
Group 3:- Non-vaccinated control group received *M. phlei* only.
Group 4:- Non-vaccinated and not received *M. phlei.* (Control negative group).

Table (2): Results of lymphoproliferation response in chickens vaccinated with necrotic enteritis gel vaccine with or without *M. phlei* as well as the control group using the colorimetric assay.

Chicken Group No.	No. of chicken	No. of tested pooled blood samples	Mean Stimulation Index (SI) of each pooled sera				
			Before vaccination	Two weeks post-vaccination			
1	20	5	0.035	0.468	0.392	0.471	0.384	0.411
2	20	5	0.036	1.013	0.911	0.855	0.907	0.974
3	20	5	0.036	0.559	0.511	0.438	0.531	0.581
4	20	5	0.035	0.035	0.036	0.035	0.036	0.035

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تاثير مستخلص من الميكوبكتيريم كمحفز مناعي مع لقاح الالتهاب المعوي التنكرزى في الدجاج

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أجريت هذه الدراسة لتقييم الاستجابة المناعية لبدارى التسمين للقاح الالتهاب المعوي التنكرزى مع استخدام مستخلص الميكوبكتيريم فيللى كمحفز مناعي غير نوعى عن طريق الفم قبل التحصين فى المجموعات المختلفة . أوضحت النتائج ان مجموعة الدجاج التي أخذت مستخلص الميكوبكتيريم فيللى مع اللقاح أظهرت زيادة في الأجسام المناعية وذلك باستخدام اختبار المصل التعادلى بالاضافة الى زيادة معنوية فى تحفيز المناعة الخلوية معبرا عنها بمعامل الخلايا البلعميه الطرفيه وذلك بالمقارنة بمجموعة الدجاج التي أخذت اللقاح فقط .

من هذه النتائج يتضح أنه من الممكن استخدام مستخلص الميكوبكتيريم فيللى كمحفز مناعي غير نوعى قبل التحصين لزيادة الكفاءة المناعية للدجاج.