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Using of Phospholipase D of Corynebacterium Pseudotuberculosis Ovis as Immunostimulant with Some Poultry Vaccines

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ABSTRACT

The immunopotentiating effect of Phospholipase D (PLD) of Mycobacteria pseudotuberculosis with the Newcastle disease (ND) and Mycoplasma gallisepticum (MG) inactivated oil adjuvant vaccines was investigated by measuring the antibody titers following immunization comparing the inactivated vaccines alone as control.Two groups of chickens were inoculated with PLD in the 1st group at two weeks before vaccination with ND and MG inactivated oil adjuvant vaccines , while in the 2nd group chickens were simultaneously inoculated with PLD at the same time with vaccination against ND and MG. The ND- HI titer was higher in the 1st group (2^9) than in the 2nd group ($2^{8.3}$) 4-7 weeks postvaccination .In case of MG vaccine primed by PLD 2 weeks before vaccination induced higher and earlier antibodies from the 1st week postvaccination when examined by ELISA test , while inoculation with both MG vaccine and PLD at the same time induced lower titers from the 2nd week postvaccination .The levels of antibody titers in the vaccinated birds and protection level in the challenged birds were enhanced in the presence of PLD inoculated 2 weeks before vaccination. In conclusion PLD was found to be safe and efficacious immune stimulator suitable for use with inactivated vaccines to produce early ,better and prolonged immune response.

INTRODUCTION

Immunopotentiators used to enhance the immune response of killed vaccines are of continous increasing interest. Corynebacterium Pseudotuberculosis has clearly showed its capacity as a non-specific immune stimulant capable of raising the resistance against infection with potential pathogens, in manner comparable with that produced by BCG *(1)*. The mechanisms by which microbial products act as adjuvants to improve the host antibody response were discovered by *(2)*. The Phospholipase D (PLD) is the most important determinant identified in Corynebacterium Pseudotuberculosis that considered immunogenic soluble protein antigen because it has a molecular weight 31.4 KDa adequate to stimulate both humeral and cell mediated immune response without conjugation to a carrier protein *(2)*. There are many common and important respiratory diseases that can affect the respiratory system of poultry. Newcastle disease is one of the highly contagious and lethal disease affect all birds of all ages and so causes great economic losses *(3)*. It causes high mortality,high morbidity, decreased egg production and hatchability *(4)*. At the same time, Mycoplasmosis is one of the most important respiratory diseases and causes significant economic losses in chickens either directly or indirectly. It is caused by Mycoplasma gallisepticum (MG) with or without complicating factors *(5)*.

The objective of this study aims to investigate the effect of PLD of C. Pseudotuberculosis to improve the humeral immune response of chickens postvaccination with Mycoplasma gallisepticum and Newcastle disease virus inactivated vaccines.

MATERIAL AND METHODS

Chicks

Two hundred and forty chickens of 21 days old were used in safety test and potency test. Random blood samples were taken and sera were proved to be free from antibodies against NDV and MG.

Microbial Strains

Corynebacterium Pseudotuberculosis Ovis: An identified local strain was supplied by Department of Bacterial Diagnostic Products, Veterinary Serum and Vaccine Research Institute (VSVRI), Abbasia, Cairo, Egypt. It was used for preparation of the PLD.

Mycoplasma gallisepticum strain: M. gallisepticum R. strain was obtained from the Central Lab. for Evaluation of Vet. Biologics, Abbasia, Cairo. It was used in titer of (3.8×10^6 cfu/ml) for challenge test in chickens vaccinated with mycoplasma vaccine.

Newcastle disease virus (NDV) strain Velogenic viscerotropic NDV strain (VVNDV) was obtained from Newcastle Department, VSVRI, Abbassia, Cairo. It was used in titer of (10^6 EID_{50})for challenge test in chicks vaccinated with ND vaccine.

Vaccines

Inactivated Newcastle disease virus oil adjuvant vaccine (Merial)®.

Inactivated M. gallisepticum oil adjuvant vaccine (Nobilis, MSD)®.

Preparation of phospholipase D toxoid from C. pseudotuberculosis

The phospholipase D was prepared and purified according to *(6)*. The inactivation of phospholipase D was done according to *(7)*. It was inoculated subcutaneously (S/C) in dose of 100 ug per bird according to *(8)*.

Safety test for PLD

According to *(8)* fifteen chickens were inoculated each with double dose of the prepared PLD (200 ug/ chick). The chicks were kept under observation for two weeks for any local or general reactions post inoculation.

Experimental design

Seven groups of chickens were used, and treated as illustrated in the following :

Groups of chickens	No. of chickens	Inoculation	Dose/bird, Route	*Challenge
1	25	Inoculated with inactivated oil adjuvant ND vaccine alone	0.3 ml, I.M.	+ve with NDV
2	40	Inoculated with inactivated oil adjuvant MG vaccine alone	0.5 ml, I.M.	+ve with MG
3	25	Inoculated with inactivated oil adjuvant ND vaccine and PLD (100ug/bird, S/C) at the same time	0.3 ml, I.M.	+ve with NDV
4	40	Inoculated with inactivated oil adjuvant MG vaccine and PLD (100ug/bird, S/C) at the same time	0.5 ml, I.M.	+ve with MG
5	25	Inoculated with PLD (100ug/bird, S/C) 2 weeks before vaccination with inactivated ND vaccine	0.3 ml, I.M.	+ve with NDV
6	40	Inoculated with LPD (100ug/bird, S/C) 2 weeks before vaccination with inactivated MG vaccine	0.5 ml, I.M.	+ve with MG
	45	Left as unvaccinated control group then subdivided at the time of challenge:		
7	30	** 30 chickens were inoculated with virulent R-strain of MG	0.1 ml, I.M.	+ve with MG
	15	* 15 chickens were inoculated with VVNDV strain of ND	0.5 ml, I.M.	+ve with NDV

** Challenge with virulent R-strain of MG for groups 2,4,6 and 30 bird from group7.

* Challenge with virulent NDV for groups 1,3,5 and 15 bird from group 7.

Challenge test

Challenge against ND: Ten birds from each group No. 1, 3, 5 and 7 were challenged four weeks post vaccination (PV) with 0.5 ml (IM) of the VVNDV (106 EID50). Chickens were kept under observation for 15 days post challenge and dead birds were subjected to postmortem examination as described by ***(10)***.

Challenge against MG: Thirty birds from group No. 2, 4, 6 and 7 were challenged four WPV with 0.1 ml of virulent R-strain of M. gallisepticum containing (3.8 x106 CFU/ ml), chickens were kept under observation for 3 weeks post challenge and examined for air sac lesions according to ***(11, 12)***.

Serological tests

Blood samples were collected from all chicken groups weekly for two months then every two weeks up to 4 months post vaccination. Serum samples were harvested and tested individually to each vaccine and then average titre for each group was estimated.

For monitoring the humeral immune response against ND vaccination the heamagglutination inhibition (HI) test was used according to ***(13)***.

For monitoring the humeral immune response against Mycoplasma vaccination the enzyme linked immunosorbant assay (ELISA) kits (BioS., Inc., USA) was used according to manufacturer's recommendations.

RESULTS

Table 1. Results of safety of PLD in chickens

Number of chickens	Dose of PLD/chick	Route of inoculation	Response of birds to PLD 2 week postinoculation
15	200µg	S/C	It was found that PLD safe for chickens when injected in double field dose , no abnormalities or disease symptoms postinoculation, no lesion at seat of inoculation

Table 2. Results of ND HI- titers postvaccination with inactivated ND vaccine expressed as mean log_2 HI titer

Chicken Groups	Weeks post vaccination											
	1	2	3	4	5	6	7	8	10	12	14	16
1	2.0	3.0	4.0	6.0	7.0	7.3	7.0	6.3	6.3	6.0	5.0	5.0
3	3.0	4.0	5.3	7.0	8.3	8.3	8.3	7.7	7.7	7.0	7.0	6.3
5	3.7	5.0	6.7	9.0	9.0	9.0	9.0	8.3	8.3	8.3	8.0	7.0
7	0	0	0	0	0	0	0	0	0	0	0	0

Table 3. Results of challenge with VVNDV in vaccinated and non-vaccinated chickens

Chicken Groups	Number of challenged birds	Number of survival birds	Protection %
1	10	9	90%
3	10	10	100%
5	10	10	100%
7	10	0	0%

Table 4. Results of ELISA test postvaccination with inactivated Mycoplasma gallisepticum vaccine expressed as ELISA mean titer

Chicken Groups	Weeks post vaccination											
	1	2	3	4	5	6	7	8	10	12	14	16
2	130	202	212	262	345	371	353	313	304	321	284	222
4	178	231	293	370	391	395	374	359	333	351	323	289
6	202	293	371	433	428	435	429	411	416	395	382	333
*7	0.39	0.41	0.39	0.42	0.33	0.35	0.39	0.31	0.33	0.37	0.41	0.34

Positive titer > 185 as the cut-off value of the tested sampels.
*Control group.

Table 5. Air sac lesion scores in chickens vaccinated with inactivated MG vaccine and challenged with virulent MG- R strain.

Chicken groups	No. of birds	Air sac lesions (Degrees)					Mean score lesion
		0	1	2	3	4	
2	30	12	8	5	3	2	1.20
4	30	12	11	6	1	0	0.83
6	30	14	10	4	2	0	0.77
*7	30	0	2	2	10	16	3.30

*Control group.
According to (17):
0 Normal air sacs, sparkling clear and thin.
1 Only cloudiness or gray areas with slight thickening or flecks of yellowish exudates, involving very limited areas of one or two air sacs.
2 Readily visible grayish to yellow exudates, sometimes foaming with thickening other air sac involving one or portions of two air sacs.
3 Some what more severe exudative, thickened air sacculitis, but mainly more extensive, involving essentially three air sac regions.
4 Severe air sacculitis with consiberable exudates and thickening of all air sac regions.

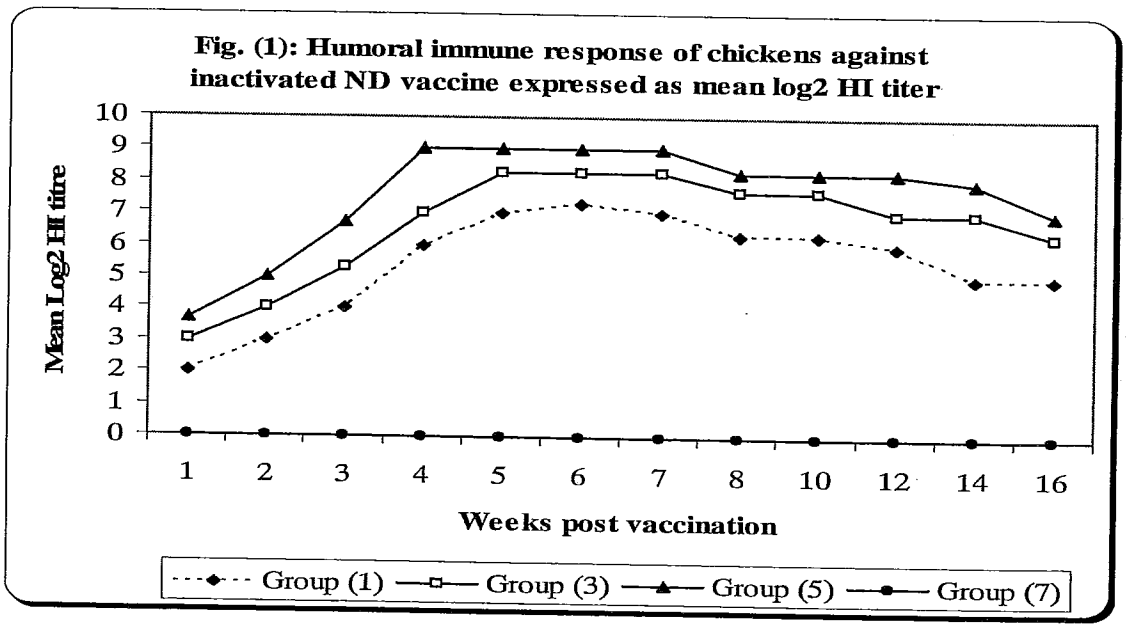
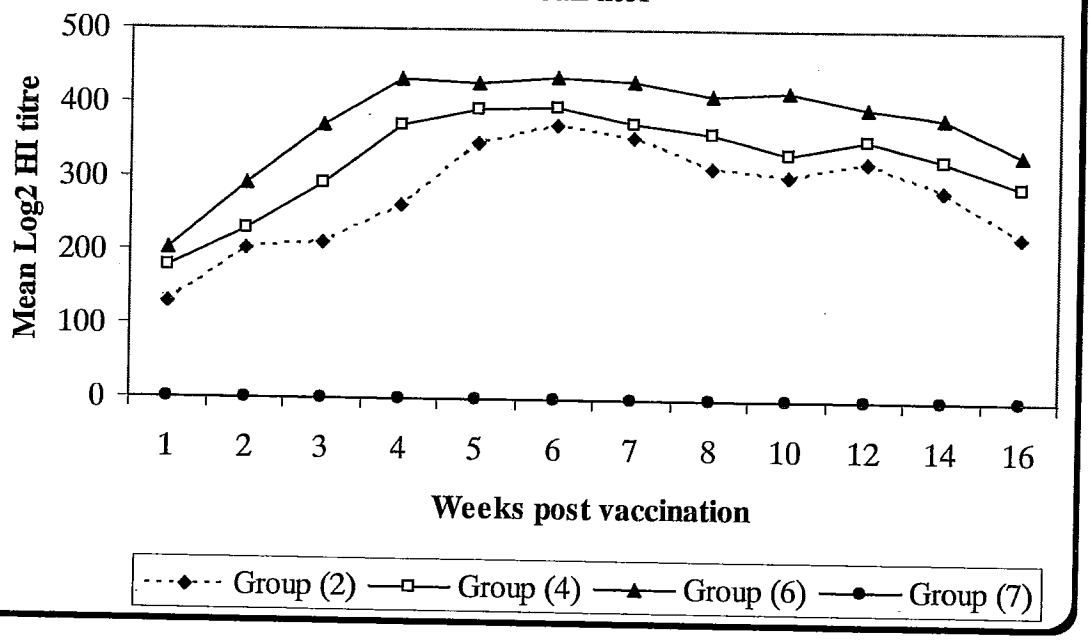


Fig. (2): Humoral immune response of chickens against inactivated Mycoplasma gallisepticum vaccine expressed as ELISA mean titer



DISCUSSION

Immunopotentiation is an increase in the intensity of the immune response, prolongation of its duration or even the development of a response to non-immunogenic substance .There is a continuous search for such immunopotentiators to enhance the immune response of inactivated vaccines in the medical field. Corynebacterium ovis properties as a non-specific immune stimulant has been studied *(1)*. Mycoplasma gallisepticum and Newcastle disease infections are among the most important respiratory diseases affecting poultry causing great economic losses *(14)*.In this study the use PLD of C. ovis as an immune potentiator in association with inactivated Mycoplasma gallisepticum and inactivated Newcastle virus vaccines was studied.

Safety test for PLD in chickens confirmed that PLD is safe for chickens when inoculated in double field dose ,its injection did not cause any disease symptoms, abnormal reaction nor lesion at seat of inoculation table (1).

The HI - ND titer postvaccination with inactivated ND vaccine alone (group 1) while in (group 3) taking PLD with ND vaccine and in (group 5) taking PLD 2weeks before ND vaccine , the ND antibodies titer was (27.3 ,28.3 and 29) respectively, table (2) and fig. (1). The highest antibody titer post vaccination was detected in group (5) from the 1st week post vaccination (WPV), it reached the maximum titer *(29)* and maintain the higher level on the 4th - 7th WPV, followed by the group (3) which showed maximum titer (28.3) on the 5th - 7th WPV that was in agreement with *(13)* who stated that the protective HI antibody titres reached 7.65 + 1.182 and this titre could protect chickens against ND infection under farm conditions. The ND -HI titer in group (1) was lower than in group (3) and group (5) (27.3) on the 6th WPV. These results indicated that inoculation of PLD 2 weeks before vaccination induces higher and earlier antibody response against NDV inactivated vaccine than the inoculation at the same time or vaccine alone.

The challenge test with virulent NDV for vaccinated and non-vaccinated groups , results in 100% protection in groups (3 and 5) which received PLD with ND inactivated vaccine , while 90% protection was post ND inactivated vaccine alone, group (1) table (3) , these results indicated that PLD injection increase the protection of ND inactivated vaccine. These results agree with *(13, 15)* who recorded that the high level of antibody titer has always been associated with better protection against ND.

The results of ELISA test for MG titer of chickens vaccinated with inactivated MG vaccine alone (group 2) ,(group 4) taking PLD with MG vaccine and (group 6) taking PLD 2weeks before MG vaccine the titer was (371, 395, 435) respectively , table(4) and fig.(2) . The results showed that the positive titer appeared on the 2nd week PV in groups (2 and 4), while it was earlier on (group 6) from the 1st WPV, also it could be noticed that the protection curve by ELISA maintained the highest level in (group 6) till the end of the experiment. These results agree with *(16)* who reported that the ELISA mean protective titer in birds vaccinated with MG vaccine appeared as early as 1st WPV and reaching its maximum level at the 4th week PV and continued to 6th WPV.

The challenge by MG virulent R-strain was evaluated by air sacculitis scores which was compared in the vaccinated chicken groups in comparison with control unvaccinated group after challenge *(17)* the scores in (group 6) showed lowest grade of air sacculitis (0.77) followed by (group 4) (0.83) then (group 2) (1.20)when compared with control unvaccinated group which recorded highest and several grades of airsacculitis (3.30) table (5). These results agree with *(18, 19)* who illustrated that inactivated immunostimulating complex vaccine of M. gallisepticum induced protective immunity and significantly reduced air sacs lesions after challenge with virulent M. gallisepticum strain and the air sacculitis depends on the immune status of the birds. These results agree with *(16, 20)* who reported that the different grades of air saculitis obtained after MG challenge depending upon the immune status of the birds and correlated with the antibody levels.

In conclusion it is clear that inoculation of chickens with PLD two weeks before vaccination with inactivated ND and MG vaccines gave better immune response and higher protection percent than those inoculated with PLD and the vaccines at the same time than using the vaccine alone. Also PLD improves the vaccine effectiveness and promotes an earlier peak of antibodies that increases rapidly and lasts for a long time.

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الملخص العربى

استخدام الفوسفوليبيز دى المستخلص من بكتيريا السل الكاذب للأغنام كمحفز مناعى مع بعض لقاحات الدواجن

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فى هذه الدراسة تم تقييم تأثير مادة الفوسفوليبازدى (PLD) لميكروب السل الكاذب كمحفز مناعي على رد الفعل المناعى فى الطيور عند استخدام لقاحي مرض النيوكاسل والميكوبلازما جاليسبتكم المثبط الزيتى. تم اختبار مستوى الاجسام المناعية الناتجة بعد التحصين عند حقن (PLD) مع اللقاح المختبر في نفس الوقت او قبل التحصين باسبوعين بالمقارنة بمستوى الاجسام المناعية الناتجة بعد التحصين عند استخدام اللقاح المثبط وحده.

تم قياس المناعة الدموية بإختبار منع التلازن الدموي (HI) لفيروس النيوكاسل وجد ان مستوى الاجسام المناعية في مجموعة الطيور المحصنة فقط ،والمحقونة بمادة PLD مع اللقاح والمحقونة PLD قبل اللقاح باسبوعين كان $2^{7.3}$ ، $2^{8.3}$ ، 2^{9} على التوالي كما تم استخدام اختبار الإليزا لتقييم مستوى الاجسام المناعية في مجموعات الطيور المحصنة بلقاح الميكوبلازما جاليسبتكم المثبط ،وجد أن مستوى الاجسام المناعية في مجموعة الطيور المحصنة فقط ، والمحقونة بمادة PLDمع اللقاح والمحقونة PLDقبل اللقاح باسبوعين كان ٣٧١، ٣٩٥، ٤٣٥على التوالي . بالنسبة لاختبار التحدي بعد اربع أسابيع من التحصين دلت نتيجة إختبار التحدي على زيادة نسبة الحماية فى الطيور المحصنة مع مادة الفوسفوليبازدى عن تلك المحصنة باللقاح وحده. فى مجموعات الطيور المحقونة بمادة PLDسواء بالتزامن مع حقن لقاح النيوكاسل المثبط أو قبل حقن اللقاح باسبوعين كانت نسبة الصد ١٠٠% .وكذلك فى مجموعات الطيور المحقونة بلقاح الميكوبلازما المثبط بالتزامن مع حقن مادة PLDأو بعد حقن ال PLDباسبوعين كان متوسط الحماية من الاصابة بالتهاب الحويصلات الهوائية أعلى من نسبة الحماية فى حالة استخدام اللقاح وحده.

نستنتج من هذة النتائج ان مادة الفوسفوليبازدى يمكن ان تستخدم كمحفز مناعى مناسب مع اللقاحات المثبطة لتعطى وقاية مبكرة قوية و لفترة أطول.