PREPARATION AND EVALUATION OF ADJUVANTED LIVE VACCINE AGAINST INFECTIOUS BRONCHITIS

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Abstract

Live vaccines are widely used in poultry industry. Such vaccines can be either injected or delivered onto the bird mucosa and most probably not adjuvanted. The herein study was devoted to focus on the potential role of gel adjuvant when added to infectious bronchitis live attenuated vaccine (IB). Montanide gel 01 is a polymer adjuvant that was recommended to be added to live vaccines improving their immunogenicity and efficacy. It was noted that augmentation of IB live vaccine with Montanide gel 01 resulted in superior quality vaccine when utilized as spray as well as the intranasal methods. Both humoral and cellular immunity were higher upon using the IB- Montanide gel 01 more than the conventional non- adjuvanted IB vaccines. Moreover, the gel 01 IB vaccine showed higher protection than the ordinary vaccines when challenged with the virulent IB strains. It was also demonstrated that gel IB vaccine could be successfully used as spray and intranasal delivered vaccine especially in mass vaccination in huge poultry industry. The results also proved that the use of Montanide gel 01 vaccine increased the safety margin of the IB vaccine and enhanced the IB vaccine efficacy.

Key words: Live Vaccine, Infectious Bronchitis, Adjuvant, Mucosal Delivery, Montanide

INTRODUCTION

Avian Infectious Bronchitis (IB) is an acute highly contagious viral infection of chickens. The young chickens are manifested clinically by severe respiratory signs and marked drop in egg production with poor egg quality in laying hens (Cavanagh and Gelb, 2008). It is also associated with nephritis in affected chicken (Abdel-Moneium *et al*., 2006). Since the first description of infectious bronchitis (IB) in the late 1930s, its first recognition in Egypt, it was associated with various respiratory, renal and egg production problems (Ahmed, 1954). IBV is a virus member of genus Coronavirus, family Coronaviridae and order Nidovirales (Cavanagh and Naqi, 2003). The virus possesses a positive stranded RNA genome that encodes phosphorylated nucleocapsid protein (N), membrane glycoprotein (M), spike glycoprotein (S) and small membrane protein (E). Diversity in S1 probably results from mutation and recombination (Song *et al*. 1998). The disease causes major economic losses not only because of poor performance or decreased egg production and quality, but also because of secondary

infections (Cavanagh and Naqi, 2003). The only practical means of controlling IB is vaccination, which is routinely used throughout the intensive poultry industry.

Both live and inactivated virus vaccines are used for IB immunization. Live vaccines are widely employed in veterinary practice and generally used to vaccinate young animals. The live pathogens delivered by vaccine inoculums are thought to initiate both humoral and cellular protective response after one delivery. Under experimental conditions, 100% efficacy has been demonstrated for live vaccine application. However, in field practice up to 10% of vaccinated animals present a lack of protection after live vaccine delivery (Cavanagh, 2003).

Several improvements are expected from the addition of adjuvants in live vaccine formulations. The most important improvement would be a reduction of the antigenic dose delivered, which would lead to safety improvements, cost limitations and a better risk control linked to the vaccination procedure. Adjuvant benefits in relation to this type of vaccine technology have already been demonstrated with model adjuvant molecules (Rauw *et al.*, 2010). However, laboratory studies on adjuvants are hardly compatible with industrial-scale production. On the contrary, the Montanide™ range of adjuvants is a well established brand of vaccine adjuvants (Aucouturier *et al.*, 2000) which is already used in all farm animal models at industrial scale in combination with diverse types of antigens.

Live vaccines against IB are widely used in chicken breeding lypholyized with stabilizer as milk and not adjuvanted and chose this model to study the effect of adjuvant on the improvement of an IB commercial live vaccine in chicken delivered by individual intranasal or collective spray methods. Montanide™ Gel 01 ST (Gel 01) is a polymer based adjuvant. The adjuvant was used as extemporaneous diluents for the lyophilized IB antigen. Sebastien *et al*. (2012) showed that gel 01 adjuvanted formulation improved the antibody titers and protection provided by intranasal delivery of live IB vaccine, whereas only the polymer adjuvanted vaccines showed better efficacy compared to prepared live IB vaccine in the spray assay.

This work aims to study the efficacy and safety of Montanide™ adjuvanted mucosal live infectious bronchitis (IB) vaccines in poultry were assessed.

MATERIALS AND METHODS

1. Chicken embryos and chicks:

Specific pathogen free (SPF) chicks and embryonated chicken eggs (ECEs) were purchased from Ministry of Agriculture, Specific Pathogen Free Farm, Koum Osheim, Fayoum. Embryonated chicken eggs 9-11 days old were used for propagation

and titration of live IBV strains and vaccines. One day old SPF chicks were housed in positive pressure stainless steel isolation cabinets, and used for vaccines evaluation.

2. Viruses:

Two strains of avian infectious bronchitis virus (IBV); IBV M41 and IBV H120 strains with initial virus titer of $10^{7.5}$ and 10^{8} EID_{50}/ml respectively were kindly provided from Newcastle Disease Department, Veterinary Serum and Vaccine Research Institute (VSVRI), Abbasia, Cairo, Egypt. The titers of H120 and M41 IBV were estimated according to OIE (2008)

3. Preparation of IBV live vaccine:

It was carried out according to OIE (2008).

a. IB virus propagation: Preparation of the live liquid vaccine of strain H120 (Cunningham, 1973).

b. Titration of IBV strains: The titre of the virus used was 8 $\log_{10}$ EID_{50}/ml for the H120 strain

c. Mixing the harvested allantoic fluid 40 hours post ECE inoculation of IBV strains H120 and add 20% skimmed milk as stabilizer to the mixture.

d. Lyophilization of H120 strain live vaccine.

4. Preparation of adjuvanted IB live vaccine:

Montanide gel 01 adjuvant was formulated extemporary to vaccination as 10% ratio by manual shaking according to the manufacturer directions.

5. Quality control: Sterility and safety tests were applied on the prepared vaccines according to OIE (2008).

5.1. Sterility test:

The prepared vaccines were tested according to United State Code of Federal Regulations (2008) to be proved free from bacterial and viral contaminants.

5.2. Safety test:

One day old chickens were inoculated with ten of the recommended dose of prepared vaccine in groups each one consisted of 5 chicks and 5 chicks were left as blank control. The chicks were observed for 2 weeks for any signs of local reactions.

5.3. Potency and efficacy test: (OIE, 2008):

Fifty one day old, SPF chicks were vaccinated intranasal with one field dose of the prepared live IB vaccine and prepared adjuvanted IB live vaccine. The blood serum samples were collected weekly for 3 weeks post vaccination. The vaccinated and non-vaccinated control groups were challenged at 3rd week post vaccination via intranasal route with IBV M41 (10^{3} EID_{50}/bird). All the birds were observed daily for 10 days post challenge (PC). Morbidity and mortality rates were recorded for

vaccinated and non-vaccinated groups till the end of the observation period, to measure the protection %.

6. Experimental Design:

In this study, two hundred and fifty, one day old SPF chicks were used to evaluate the efficacy of prepared live and adjuvanted IB live vaccines. The birds were divided into 5 experimental groups (50 birds/each) and all groups were vaccinated with the prepared vaccines as shown in Table (1).

Table 1. Groups vaccinated with prepared vaccine

Groups	Number of birds	Type of used vaccine
1	50	Vaccinated with prepared live vaccine by spray route.
2	50	Vaccinated with prepared live vaccine by intranasal route
3	50	Vaccinated with adjuvant live vaccine by Montanide gel 01 by spray route.
4	50	Vaccinated with adjuvant live vaccine by Montanide gel 01 by intranasal route.
5	50	Non-vaccinated control group

7. Enzyme linked immunosorbant assay (direct ELISA):

It was carried out for estimation of antibodies against IBV vaccines according to kit of manufacturer (Affinitech, LTD, Suit 2, Bentonville, Catalog No. IBV-0500 and Serial No. 5548). IBV -Ab ELISA kit was used for in vitro diagnostic detection of serum antibody to IBV in chicks.

8. Cellular immunity assay:

Whole blood sample collected at 3rd, 5th, 7th, 14th and 21st for evaluation of the cell mediated immune response to the prepared vaccines in vivo was carried out using lymphocyte transformation assay (MTT kit) in chicks according to kit of manufacturer .(Beijing Mesochem Technology Co. , Ltd-China).

9. Virus reisolation from tracheal swabs and kidneys:

The test was carried out at the 5th day post challenge according to Gelb and Jackwood (1998).

RESULTS AND DISCUSSION

New tools are increasingly needed to manage the biosafety of avian production regarding viral and bacterial diseases. Indeed, viral diseases can cause zoonotic risks and bacterial diseases may become difficult to be controlled due to the recent regulations and the stronger restrictions on antibiotic utilization.

Vaccination is one of the most powerful tools to save the farming efficiency and improve the protection of herds (Glisson and Kleven, 1993).

Adjuvants also can prolong the immune response and stimulate specific components of the immune response either humoral or cellular mediated immunity (SEPPIC,2002).

Chun *et al*. (2011) concluded that an effective vaccine needs not only good antigens but also preferable adjuvant to enhance the immunogenicity of antigen. The adjuvant was used to enhance humoral and cellular immune responses.

However, it is not possible to add multiple injection vaccination steps in avian farming procedures due to the cost and time consumed by such steps in farms containing several thousands of birds. Mucosal spray vaccination could then be a very useful tool for time and cost efficient. Humoral response to IB vaccines were estimated by ELISA at zero day prevaccination and at 8 time points 1st, 2nd, 3rd, 4th, 5th, 6th, 7th and 8th weeks post vaccination as shown in table (2).

Table 2. The antibody titer response to different IB vaccines using ELISA

Weeks Post Vaccination (WPV)	Prepared live IB vaccine		adjuvant live vaccine by montanide™ gel 01		Non-vaccinated control group (group 5)
	Spray route (group 1)	Intranasal route (group 2)	Spray route (group 3)	Intranasal route (group 4)	
Prevaccination (0 day)	33	34	24	33	33
1	44	84	400	976	20
2	353	513	796	1364	21
3	575	585	1100	1994	18
4	676	860	1200	2285	16
5	864	1640	1302	2300	14
6	949	1645	2305	2303	14
7	1200	1854	2305	2313	84
8	1405	1854	2400	2505	91

The minimum positive level serum is $\geq$ 400 in ELISA antibody titre for IB living vaccine according to Kit of manufacturer.

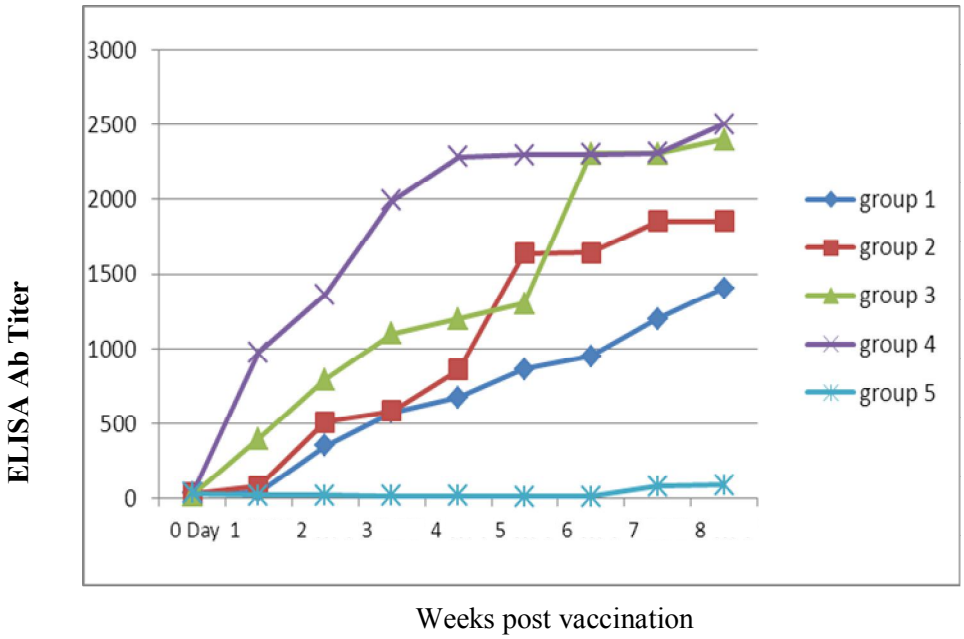

Fig. 1. The antibody titer response to different IB vaccines using ELISA test

Table (2) revealed that groups (3 and 4) that received adjuvant live vaccine by montanide gel 01 vaccine (spray route and intranasal route) induced antibody value alternates between 400 and increased gradually till 8 weeks reach 2400 in group (3) and from 976 till 2505 in group (4). Regarding to group that received prepared live IB vaccine (spray route (group 1)) and intranasal route (group 2), the ELISA antibody titers increased gradually from 1st week (44) and reached 1405 in group (1) and from 84 to 1854 in group (2) at 8th week.

Table (2) revealed that groups received adjuvant IB vaccine by intranasal route gave higher antibody response than groups which received the vaccine by spray route, and faster onset of response of adjuvant IB vaccine than the prepared IB live vaccine at first week (400, 769) and (44, 84) in groups vaccinated with adjuvanted and prepared vaccines either by spray or intranasal route respectively. These results were in agreement with Sebastien *et al*. (2012) who said that using intranasal delivery Gel 01 adjuvanted vaccines were able to trigger a significantly stronger humoral immune response than the non-adjuvanted live vaccine, gel 01 adjuvanted vaccines conferred an improved protection to the vaccinated animals compared to the commercial reference.

Spray delivery was not as efficient as IN delivery. Antibody titers obtained after spray vaccination were lower than the IN induced titers. A faster onset of the response could still be observed (as soon as day 14 for adjuvanted vaccines, at day 21 for the prepared live vaccine).

All vaccinated groups showed lymphocyte proliferation rates higher than control groups which can be explained by the fact that spraying and intranasal routes affect directly on the Harderian and paranasal glands that evolve a better immune responses **(**Okwor *et al.,* 2013)

Also, the effect on cell mediated response of T-cell expressed as Delta optical density (OD) was recorded for all groups . Lymphocyte proliferation rates were estimated by lymphocyte blastogenesis assay at 5 time points 3rd, 5th, 7th, 14th and 21st days post vaccination as shown in Table (3). The mean lymphocyte blastogenesis (proliferation) increased from the 3rd day and still high till the 21st day in group received Montanide gel 01 adjuvant vaccine either by spray route or by intranasal route while group which received prepared live IB vaccine increased more than control group but less than group which received Montanide gel 01 adjuvanted vaccine.

Table 3. Results of lymphocyte blastogenesis expressed as optical density assay

Days Post Vaccination	Prepared IB vaccine		Montanide gel 01 adjuvant vaccine		Non vaccinated group (group 5)
	Spray route (group 1)	Intranasal route (group 2)	Spray route (group 3)	Intranasal route (group 4)	
3rd	0.140	0.75	1.051	1.301	0.48
5th	0.531	0.847	1.014	1.283	0.43
7th	0.687	0.873	1.22	1.418	0.50
14th	0.791	0.83	1.243	1.667	0.31
21st	0.681	0.61	1.106	1.179	0.28

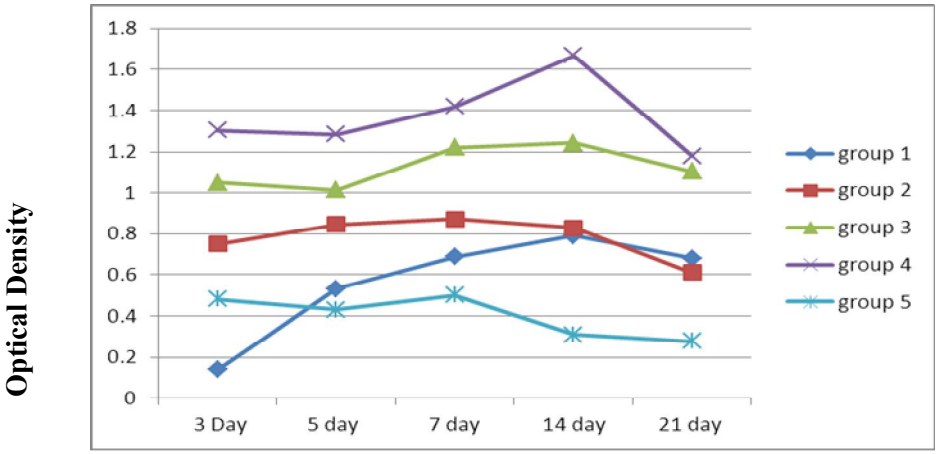

Fig. 2. Lymphocyte blastogenesis response expressed as optical density to different IB vaccines

Challenge test revealed that the clinical signs started to appear at 3rd day post challenge in the form of mild to moderate rales in non-vaccinated challenged group revealed protection 0%. It was concluded that the protection % is considered valid if the challenge virus is isolated from not less than 80% of non-vaccinated control group and less than 20% of vaccinated birds according to European Pharmacopeia Reference Standards (Council of Europe, 2007).

The protection % of chicks after the challenge test by observing the clinical signs on chicks were illustrated in table (4) which implies that prepared live vaccine by spray route protected the chicks with 60% but 80% for intranasal route, while in adjuvant live vaccine by montanide gel 01 either by spray route or by intranasal route, the protection rates were 100%. The non-vaccinated control group the protection rate was 0% in both kidney and trachea.

Table (4) showed the results of protection % in chicks after proceeding the challenge test using viral reisolation from both kidney and trachea. The table implies that prepared live vaccine by spray route protected the kidney with 80% but it protected trachea with 90%. In case of prepared live vaccine by intranasal route, the protection was 90% for the kidney and 100% for trachea, while in adjuvant live vaccine by montanide gel 01 either by spray route or by intranasal route, the

protection rates were 100% for both kidney and trachea. While the non-vaccinated control group it was 0% in both kidney and trachea.

The above data indicates that adjuvant vaccine is highly effective and could protect the chicks from being infected upon the exposure to different strains of IBV. These results agree with Sebastien *et al*. (2012) who reported that all the unvaccinated animals presented clinical signs at a high score. The commercial vaccine formulations reduce the scoring but 89% of the chickens were still positive for clinical signs at day 10 post challenge. The scoring per group was strongly reduced for adjuvant vaccine groups either by spray or intranasal route. Illness duration was also reduced to less than one day using Montanide™ adjuvants while unvaccinated controls were sick for almost a week on average and chickens receiving the commercial formulation for 2-3 days after challenge. The Montanide™ Gel 01 based formula was the only formula able to induce a high rate of protection when used in spray (88% of chickens without symptoms at day 10 post challenge, and to have a significantly higher efficacy than the commercial formulation.

Table 4. Re-isolation results at five days post challenge with IBV M41

Groups	Type of used vaccine and delivery route	No. of diseased birds / Total	Protection %	Kidney		Trachea	
				Virus isolation		Virus isolation	
				No. of +ve samples*	Protection %**	No. of +ve samples	Protection %
G1	Vaccinated with prepared live vaccine by spray route.	4/10	60%	2/10	80%	1/10	90%
G2	Vaccinated with prepared live vaccine by intranasal route.	2/10	80%	1/10	90%	0/10	100%
G3	Vaccinated with adjuvant live vaccine by montanide gel 01 by spray route	0/10	100%	0/10	100%	0/10	100%
G4	Vaccinated with adjuvant live vaccine by montanide gel 01 by intranasal routes	0/10	100%	0/10	100%	0/10	100%
G5	Non-vaccinated control group	10/10	0%	10/10	0 %	10/10	0%

In this study it was concluded that efficacy of adjuvant formulations containing live viral vaccines polymer based adjuvant technologies were able to improve the immune response and protection against IB challenge. Montanide™ Gel 01 ST adjuvant conferred enhanced protection to challenge compared to the non-adjuvant commercial vaccine in SPF chicks (Jang *et al*., 2010).

In this study, chicks behavior including feeding and movement would be observed after vaccination in normal agree with Sebastien *et al*. (2012) who reported that no modification of chicken behavior (social, movements, feeding) could be observed after vaccine delivery. Furthermore, no IB specific clinical signs could be observed after vaccine delivery. At last, no local reactions on the mucosa (eye, nostril, mouth) to vaccine delivery could be observed after IN vaccination.

Further studies should also assess if the protective stimulation using spray vaccination that was observed using polymer adjuvanted vaccine was restricted to the

nasal mucosa or was also linked to the oral and on eye surfaces which would lead to different immune system / antigen contact (Purswell, 2010).

Other criteria still remain to be studied, such as the ability to manage the antigen load or to combine several antigens in the same spray vaccine. Benefits anticipated from the use of adjuvants in live vaccines concern both safety and efficacy improvements. The use of adjuvants in live vaccine could improve the efficacy and lead to a better management of the antigen load per vaccine dose. Such efficacy improvement could also improve the safety of the vaccine as the possible adverse reactions observed after delivery of live infectious vaccines could be lowered. Moreover, the use of adjuvants should reduce the number of low or not responding animals and therefore reduce the possible reservoir for the disease (Cavanagh, 2003)

Finally, it would be interesting to study whether adjuvants such as polymer formulae can also improve the survival and transmission of live vaccines.

This work underlines the ability to use polymer adjuvants in mass vaccination for avian species, opening doors to improvements some of live avian vaccines safety and efficacy.

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تحضير وتقييم لقاح زيتي حي ضد مرض الالتهاب الشعبي المعدي

سماح السيد علي أبودلال

معهد بحوث الامصال و اللقاحات البيطرية – العباسية – القاهرة

تستخدم اللقاحات الحية بصورة كبيرة فى صناعة الدواجن . تُعطى هذه اللقاحات الحية للطيور اما عن طريق الحقن أو عن طريق استثارة الغشاء المخاطى وهى عادة ما تكون بدون استخدام مادة حاملة. تمت هذه الدراسة لإستبيان الدور المتوقع لمادة جل المونتانيد المتبلمر 01 الحاملة عند إضافتها للقاح فيروس الإلتهاب الشعبى المعدى الحي المستضعف وموصي بإضافتها للقاحات الحية لتحسين مناعتها وكفاءتها. لوحظ أنه بإضافة مادة المونتانيد جل 01 للقاح الإلتهاب الشعبي الحي أدي إلي إرتفاع جودة اللقاح عند إعطاءه عن طريق الرش وكذلك طريقة الغشاء المخاطي. كلا من المناعة الخلطية والخلوية كانت أعلي عند إستخدام المونتانبد جل 01 مع اللقاح منها عند إستخدام اللقاح التقليدي الغير محمل. بالإضافة الى ذلك وجد أن لقاح الإلتهاب الشعبي المحمل بالجل 01 أعطي حماية أعلي من اللقاح التقليدى عند التحدي بفيروس الالتهاب الشعبي المعدي الضاري. كان من الملاحظ أيضا أن لقاح فيروس الألتهاب الشعبي المعدي المحمل بالجل يمكن استخدامه بنجاح عن طريق الرش و استثارة الغشاء المخاطى و خاصة في حالة تحصين القطعان الكبيرة من الدواجن. برهنت هذه النتائج على زيادة حد الأمان للقاح فيروس الألتهاب الشعبي المعدي المحمل بالجل مونتانيد 01 وحسن الكفاءة.