Immunostimulant Activity of Levamisole to Polyvalent FMD Vaccine in Buffaloes.

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Abstract
In Egypt, many challenges facing eradication strategy of FMD concerning the short term immunity induced by inactivated polyvalent FMD vaccine. The aim of the study was directed to ascertain whether post vaccination antibodies against FMDV in buffaloes could be enhanced by administration with levamisole. To achieve the goal, 24 buffaloes were divided into 4 groups, G1 is non vaccinated non adjuvanted group, G2 is vaccine control group, G3 is simultaneously FMD vaccinated and levamisole stimulated group and G4 is FMD vaccinated 7 days after levamisole stimulation. Blood samples were examined for 16 succesive weeks post vaccination for FMD antibodies to serotypes O, A and SAT-2 by ELISA test. The results revealed that animalssimultinously vaccinated and treated with levamisole was higher than the group adjuvinated with levamisole 7 days before FMD vaccination. Levamisole has stimulant effect on the vaccinalimmunity of buffaloes to FMD serotypes O, A and SAT2. Antibody titers of FMD in animal groups were parallel in each group with a high titer in serotype A than O and SAT2. Further investigations are needed to explore the effect of levamisole on cell mediated immune response and challenge test in buffaloes.

Introduction:

Foot and mouth disease (FMD) is a highly contagious and important disease affecting a lot of domestic and wild animals particularly cloven hoofed animals. The disease has a major economic impact concerning the loss of production and constrains of international trade ***(Ko et al., 2009).*** FMD caused by +ve sense, ssRNA, small sized, non enveloped virus belongs to genus Aphtho viruses, family Picornaviridae***(Knowles and Samuel, 2003).***

FMDV occurs as seven serologically distinct serotypes (A, O, C, SAT-1, SAT-2, SAT-3 and Asia-1). No cross antigenic relationship between them***(Radostits et al., 2010).*** In Egypt, FMD is enzootic disease and many outbreaks had occurred since 1950 and onwards. FMDV serotype O was the most prevalent until serotype A appeared in 2006, then

in 2012 serotype SAT-2 was emerged and distributed in different governorates of Egypt ***(Farag et al., 2005).***

Control strategy of FMD in Egypt depends on compulsory and regular vaccination of susceptible animals with polyvalent inactivated vaccine. Emergency vaccination may be employed in the event of an exotic FMDV serotype ***(Chinsangram et al., 1998).***Many challenges facing eradication strategy of FMD such as highly infectious nature and antigenic diversity of the virus, multiple susceptible animal species and short term immunity induced by inactivated vaccine ***(Paton et al., 2005).***To overcome the disadvantages of FMD vaccine used for vaccination of animals in Egypt, adjuvant is very important issues taken in considerations for production of high and long term FMD immune response ***(Dalsgarrd et al., 1990).***

Levamisole is antithelmentic agent that used regularly in human and animals ***(Renoux, 1980).***It can be used as immunostimulant***(Stellata et al., 2004).*** The immuno-stimulatory effect of levamisole was first reported by ***Renoux and Renoux, 1971*** and was found to enhance the protection of a Brucella vaccine in mice. Levamisolehas a potent immune stimulants in modulation of leukocyte cytotoxic activity ***(Cuesta et al., 2002),*** phagocytosis ***(Findlay and Munday, 2000),*** and macrophage activation***(Mulero et al., 1998).***

Levamisole is extensively studied as immunostimulant in human ***(Johnkoski et al., 1996)***, in chicken ***(Habibi et al., 2012)***, in fish ***(Sajid et al., 2011),***In FMD vaccinated cattle ***(Jin et al., 2004)*** andin FMD vaccinated sheep ***(Shawky et al., 2014).*** In buffaloes, the effect of levamisole on the immune response to FMD vaccine is poorly understood, hence, the aim of this study was directed to highlights on the effect of levamisole as immunostimulant in vaccinated buffaloes with polyvalent inactivated FMD vaccine and to evaluate the humeral immune response in levamisole treated and non treated animals by ELISA test.

Material and methods:

1-Animals:

Twenty four apparently healthy native breed buffaloes aged 3 -5 years and weight 300-400 kg were used in this study. The animals were proved to be free from FMDV type "A, O and SAT-2" antibodies by using ELISA.

2- **vaccine**:

The animals were vaccinated with polyvalent inactivated FMD vaccine. The vaccine contain six FMD strains (O manisa, O 3039, A Iran 05, A Saudi 95, Asia-1 and SAT- 2) and was purchased from Meryal company, United Kingdom. The vaccine was injected subcutaneously in neck region of

buffaloes and was boosteredof dose of 2ml/dose/animal after 14 days.

3-Levamisole:

Levamisole hydrochloride 10% (Avisole) is brood spectrum antiparasitic agents regularly used in buffalo farms. It contains levamisole hydrochloride 10% (100mg/ml). The drug was purchased from Arab Vet. Company (Avico), Jordan. Levamisoleis used as immunostimulator with FMD vaccine to potentiate the immune response of buffaloes to FMDV. The immune stimulant dose used in buffaloes is 2 ml /50 kg body weight injected subcutaneously in the neck region.

4-Serum samples:

A total of 384 blood samples were collected in sterile tubes without anticoagulant from buffaloes under experiment at different intervals after vaccination and/or levamisole stimulation. Serum samples were prepared and collected after one week post vaccination then weakly for 16 successive weeks. Clear non-haemolysedsera were collected in sterile tubes for evaluation of FMD humeral immune response in vaccinated and/or stimulated animals by using ELISA technique.

5-Experimental design of vaccination and immune stimulation :

Twenty four sero-negative buffaloes were randomly divided into four groups six animals / each (G 1-4), G1 was non vaccinated control group (-ve control). G2 was vaccinated without levamisole(+ve control). G3 wassimultaneously vaccinated and levamisole stimulated group and was subdivided into 2 subgroup, subgroup B3 contain 3 animals were injected with sensitizing dose of FMD vaccine and levamisole dose at the same time (0 day) and subgroup A3 was contained 3 animals were simultaneously injected with FMD vaccine and levamisole at 0 day and booster dose of vaccine at 15 days later. G4 is vaccinated animals 7 days after levamisole injection and was subdivided into 2 subgroups B4 and A4. The schedule of group 4 is the same as group 3 except that group receive levamisole 7 days before vaccination.

6- **ELISA kits for detection of FMD antibodies to structural and non structural proteins:**

Two ELISA kits were used in this study. The PrioCHECK FMD NS (non structural ELISA kits) was purchased from Prionics AG company, Switzerland and kindly supplied by FMD department, Animal Health Research Institute, Dokki, Cairo.Solid phase competitive ELISA (structural protein ELISA kits) were used for detection of serotype specific FMDV antibodies. The kits was purchased from Izsler Biotechnology company, Italy and kindly supplied by FMD department, Animal Health Research Institute, Dokki, Cairo.

Results and discussion:

I-Evaluation of mean antibody titer (PI) to FMD non structural protein by ELISA

As shown in table (1), ELISA test for examination of FMD non structural protein proved that all experimental buffaloes are negative.

II-Evaluation of antibody titers to FMD structural protein by ELISA

1-Evaluation of antibody titers in FMD vaccinated buffaloes (G 2):

As shown in Table (2) and Figure (1) ELISA titer for FMD serotype A began higher in the first week post vaccination and remained relatively higher till the eighth week than serotype O and SAT-2.The mean antibody titer of A and SAT-2 vaccine components are often considerably higher than the mean antibody titer of O component as examined by ELISA test. The same results obtained by ***Black et al. 1984 and Doel and Pullen, 1990 who***reported that A 24 Cruzeiro antigen was thirty-fold more immunogenic than O*1* Campos, howeverFMD antigen integrity and immunogenicity are crucially dependent on the type of adjuvant used and type of strain of virus incorporated in vaccine preparation.

2-Immunostimulant effect of Levamisole simultaneously injected with FMD vaccine in buffaloes (G3):

From the results illustrated in Table 3 and Figure (2), it is observed that levamisole simultaneously injected with FMD vaccine can enhance the FMD antibody response either when the animals administered only one vaccine dose (G3B) or administered vaccine dose and boostered with another dose 15 days later (G3A). Animal group was vaccinated with FMD sensitizing dose and boostered shown higher increase in ELISA titer than group administered only FMD sensitizing dose when compared to either +ve control (vaccinated group without immunostimulant) and –ve control (non vaccinated).Levamisole has stimulant effect on the vaccinal immunity of FMD serotype O, A and SAT-2.

Data shown in Table 3 and Figure (2,3) revealed that antibody response of FMD serotype O, A and SAT-2 have the same increasing pattern during the period of experiment when FMD vaccine injected in animals with or without booster dose of FMD vaccine in the same time with levamisole. Duration of antibody titers after the second revaccinations showed a high levels of ELISA titer and usually recorded from 21 to 28 days after vaccination and/or levamisolestimultion (G3A and C4A).The same results obtained by ***Doel and Chong, 1982*** who mentioned that levamisole can stimulate the immune response of FMD serotypes A, O and SAT2 .when injected with FMD vaccine.

When levamisole injected in buffaloes 7 days before FMD vaccine (Table 4) can also enhance the antibody response but with a little limits than simultaneous group

(G3) (table 3,figure 2,3) either when the animals administered only one sensitizing vaccine dose (G4B) or administered sensitizing dose and boostered with another dose 15 days later (G4A). The results proved by ***Qurashi et al. 2000.***Who mentioned that the immunostimulating activity oflevamisole resulted in higher serum antibody titres and long-lasting immunity.

3- Immunostimulant effect of Levamisole injected 7 days before FMD vaccine in buffaloes (G4):

From the results illustrated in **(Table4, Figures 4,5)**, it was cleared that when levamisole injected 7 days before FMD vaccination can enhance the antibody response either when the animals administered only one vaccine dose (G4B) or administered sensitizing dose and boostered with another dose 15 days later(G4A). Animal group vaccinated with FMD vaccine dose and boosteredwith another dose showed higher increase in ELISA titer than group administered only FMD sensitizing dose **(Table 4)**. In G4B FMD type "O" ELISA titer was began high (73.08) in the first week post treatment compared to –ve control (17.68), or vaccinated group (43.87) then progressively increased in the next 2 weeks reached up 87.61 then decreased in the next 14 weeks**(Table 4)**. A significant rise in ELISA titer were shown when serum examined for serotype A and SAT-2 throughout the experimental period. Levamisole-treated animals showed a progressive rise in antibody titre until week 3, reaching a peak value of 96.24 (serotype A) and 92.65 (serotype SAT-2).

In G4A FMD type O, ELISA titer was begin high (70.68) in the first week post treatment compared to –ve control (17.68), then progressivly increased in the next 3 weeks reached up 74.44 then decreased in the next 13 weeks. A significant rise in ELISA titer were shown when serum examined for serotype A and SAT-2 throughout the experimental period**(Table 4)**. Levamisole-treated animals showed a progressive rise in antibody titre throughout the experimental period, reaching a peak value of 95.373 (serotype A) and 88.65 (serotype SAT-2).

From the results illustrated in Tables 3 and 4, it was cleared that levamisole simultaneously injected with FMD vaccine and boosterd with another dose of FMD vaccine can enhance the FMD antibody response with a progressive increase in ELISA titer for serotype O, A and SAT-2 throughout the experimental period. Levamisole treated animals showed a progressive rise in antibody titre until the 4^{th}week, reaching a peak value of 96.53 (serotype A) and 95.61 (serotype SAT-2). These data coincided with ***(Qureshi et al., 2000)*** who mentioned that levamisole-treated animals showed a progressive rise in FMD antibody titre until the 6^{th}weekin serum of pregnant buffaloes, reaching a peak

value of 70.0 ± 4.3 during that same week..

Table 3 and Figure 2,3 revealed that, ELISA antibody titer of the three FMD serotypes in animal groups simultaneously vaccinated and treated with levamisole were higher than group stimulated with levamisole 7 days before vaccination **(Table 4)**either the animals vaccinated with one dose or double dose. These data confirmed before for BVD, PI-3 and IBR vaccine by ***(Ahmed et al. 2015)***who mentioned that serum neutralizing antibody titer to pneum-3 vaccine in calves were increased at 28 days post initial vaccination and reach the highest level by the day 60 post initial vaccination.

Levamisole enhances macrophage and T-lymphocyte function and reduces suppressor T-cell function. Antibody formation to most infectious agents is T-lymphocyte-dependent, so the augmentation of the helper functions of these cells could enhance antibody production ***(Babiuk and Misra, 1981).***Levamisole hydrochloride has effect on humoral and cell-mediated immune response in several diseases have been examined ***(Confer et al., 1985).***Levamisoleis considered asantiproliferative and affects both adhesion and MHC class I molecule expression ***(Kimball and Fisher, 1996).***Levamisole (LMS) is proved to have immune stimulant properties ***(Cuesta et al., 2002).*** It stimulates T cell activation and increases the production of antibody when co-adminstered with DNA vaccine or inactive vaccine ***(Jin et al., 2004 and Kang et al., 2005).***

Levamisole showed to be more advantageous than other conventional adjuvants for many reasons; i) it can used not only as immunostimulant but also to eradicate the parasitic infestations in livestock animals, ii) it is non specificimmunostimulant not only used to enhance the vaccinal immunity to FMDV but also for other viral and bacterial vaccines, iii) it administered oral mixed with food in chicken and fish farm or by injection, iv) it is injected alone not incorporated with vaccine preparation that might affect the vaccine antigenicity, v) it is safe agent have no skin reaction or granuloma formation when given subcutaneously.

Levamisole have shown to increase humeral antibody response of chickens to Lasota vaccine ***(Kulkarni et al., 1973)***andcan promote recovery from immune suppression states ***(Sakai, 1999)*** and also can enhance both the innate and specific humeral and cellular immune responses ***(Li et al., 2006).***It was proved thatLevamisole could increase serum lysozyme activity, serum antibody titers after immunization, the number of leucocytes, phagocyte activities, the expression of cytokines by macrophages, lymphocyte proliferation and anti-

tumor responses ***(Tempero et al., 1995; Holcombe et al., 2001).***

In summary, the results presented in our study demonstrated that levamisole as an immunostimulant can activate immune system of buffaloes to FMD vaccine. Levamisole have a stimulant effect on the vaccinal immunity to FMD serotype O, A and SAT-2.Simultaneous administration of levamisole with FMD vaccine or administered 7 days before vaccination can enhance the antibody response of buffaloes to FMD vaccine either vaccinated with one dose or with double dose. Increasing pattern of antibody titer through experimental time in the three FMD serotypes were parallel in each buffalo groups with a high titer in serotype A than O and SAT-2.

Finally, the study recommend that, the regular use of levamisole simultaneously with each FMD vaccine dose is very important to enhance the immune system of buffaloes to different FMD virus serotypes in addition to its usage as a conventional antiparasitic agents.

Table (1): *ELISA check test for evaluation of non structural proteins for FMDV in buffaloes*

groups / Mean of ELISA titer %	(G1)N=6 non adjuven ated nono vaccinated	(G2)N=6 Vaccinate d only	(G3A)N=3 Simultinously adjuvenated with booster vaccine dose	(G3B)N=3 Simultinously adjuvenated with single dose	(G4A)N=3 Vaccinated after 7d of levamisole and boostered	(G4B)N=3 Vaccinated after 7d of levamisole No booster
PI %	49	34	36	45	37	27
Result	-ve	-ve	-ve	-ve	-ve	-ve

PI: Percent of inhibition PI *less than 50% is negative****PI*** *more than 50% is positive*

Table (2): *ELISA meanantibody titer of FMD serotype O, A and SAT-2 in vaccinated buffaloes (G2):*

Time / week	Neg. Control	Mean serotype O titer(PI)	Mean serotype A titer(PI)	Mean serotype SAT2 titer(PI)
1	17.684	43.876^{NS}	70.089^{*}	62.422^{*}
2	48.463	48.370^{NS}	75.752^{*}	72.792^{*}
3	34.563	60.731^{*}	80.261^{*}	70.742^{*}
4	44.952	81.451^{*}	70.728^{*}	63.059^{*}
8	15.511	69.257^{*}	76.879^{*}	39.435^{NS}
12	45.362	52.478^{*}	61.429^{*}	42.772^{NS}
16	39.457	43.272^{NS}	56.670^{*}	44.168^{NS}

PI= Percent of Inhibition protective titer more than 50%

**=significant NS=non significant*

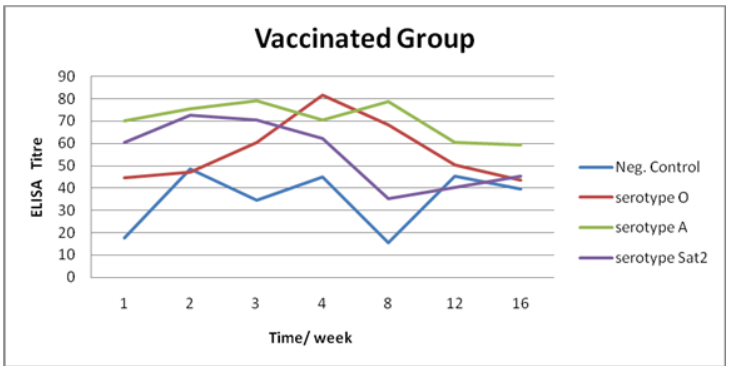

Figure (1): Progression of ELIZA titer (PI) in buffaloes during the period of experiment (16 weeks).

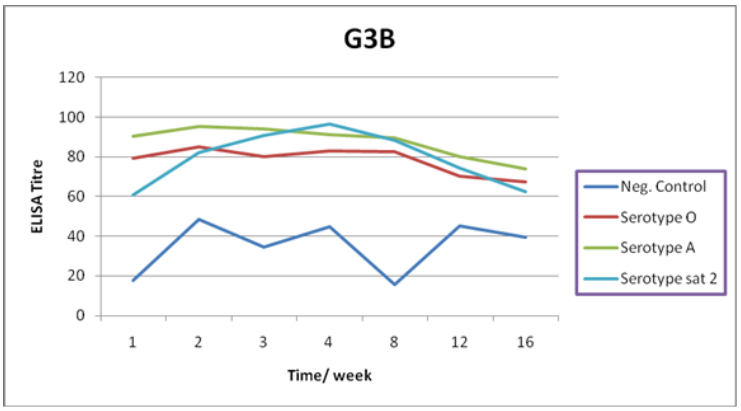

Figure (2): ELISA titer of FMD serotypes in buffaloes vaccinated with one dose and treated simultaneously with levamisole (G3B):

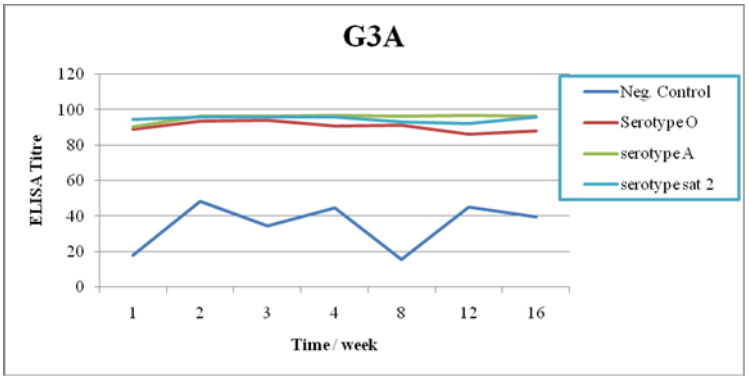

Fig. (3): ELISA titer of FMD serotypes in buffaloes vaccinated with booster dose and treated simultaneously with levamisole (G3A):

Table. (3): *ELISA mean antibody titers(PI) of FMD serotype O, A and SAT-2 in vaccinated buffaloes and simultaneously stimulated with levamisole (G 3):*

Time/ week	Neg. Control	Mean serotype O titer (PI)		Mean serotype A titer (PI)		Mean serotype SAT 2 titer (PI)	
		G3A	G3B	G3A	G3B	G3A	G3B
1	17.684	89.167*	79.244*	90.445*	90.495*	94.570*	72.613*
2	48.463	93.854*	85.574*	96.542*	95.557*	95.677*	82.580*
3	34.563	94.167*	80.529*	96.565*	94.523*	95.516*	90.597*
4	44.952	90.568*	82.964*	96.501*	91.358*	95.616*	96.417*
8	15.511	91.622*	82.578*	96.531*	89.555*	93.286*	85.380*
12	45.362	86.443*	70.359*	96.501*	80.273*	92.577*	74.067*
16	39.457	88.301*	67.632*	96.534*	74.577*	95.455*	62.653*

Table. (4): *ELISA antibody titer of FMD serotype O, A and SAT-2 in vaccinated buffaloes and stimulated with levmisole 7 days before vaccination (G4):*

Time/week	Neg. Control	Mean serotype O titer		Meanserotype A titer		Mean serotype SAT 2 titer	
		G4A	G4B	G4A	G4B	G4A	G4B
1	17.684	70.689*	73.087*	85.257*	89.601*	60.490*	63.717*
2	48.463	72.440*	87.611*	94.353*	96.247*	65.607*	90.547*
3	34.563	74.449*	80.669*	87.737*	95.820*	77.597*	92.650*
4	44.952	68.632*	72.515*	93.670*	85.233*	88.653*	81.901*
8	15.511	51.032*	59.516*	95.373*	90.570*	86.693*	74.613*
12	45.362	42.924NS	53.163*	92.160*	77.420*	78.170*	53.957*
16	39.457	44.916NS	50.623*	92.290*	83.407*	53.167*	59.487*

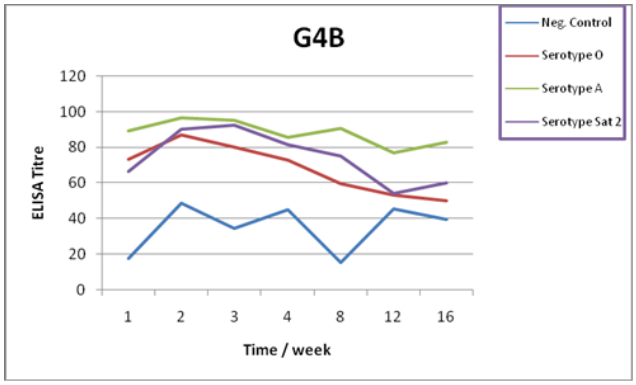

Fig.(4): *ELISA titer of FMD serotypes in buffaloes vaccinated with one dose and treated with levamisole 7 days pre vaccination*

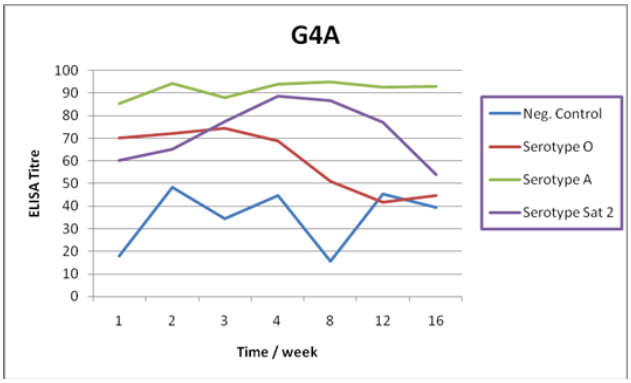

Fig.(5): *ELISA titer of FMD serotypes in buffaloes vaccinated with booster dose and treated with levamisole 15 days pre vaccination*

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تاثير الليفاميزول كمحسن مناعي علي لقاح الحمي القلاعية المتعدد في الجاموس

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الملخص العربي: في مصر نواجهة الكثير من التحديات للقضاء علي مرض الحمي القلاعية بسبب المناعة قصيرة المدي للقاح الحمي القلاعية المتعدد الغير نشط . ولذلك هدفنا من هذة الدراسة هو التاكد من ان الاجسام المضادة لفيروس مرض الحمي القلاعية في الجاموس تزداد مع حقن الليفاميزول. ولتحقيق الهدف ٢٤ جاموس وتم تقسيمة الي ٤ مجموعات . المجموعة الاولي غير محصنة وغير محفزة مناعيا ، المجموعة الثانية هية المجموعة الضابطة، المجموعة الثالثة تم حقنها بالليفاميزول في نفس يوم حقنها باللقاح ، المجموعة الرابعة تم حقنها بالليفاميزول قبل تحصينها باللقاح ب ٧ ايام . تم فحص عينات الدم لمدة ١٦ اسبوع متتاليا بعد التحصين علي الاجسام المضادة الثلاثة للامصال بواسطة اختبار الاليزا . وقد اوضحت النتائج ان الاستجابة المناعية للحيوانات اللتي حقنت باللقاح مع الليفاميزول اعلي من الحيوانات التي تم حقنها بالليفاميزول قبل اللقاح ب ٧ ايام . وقد وجد ان الليفاميزول يحفز الاستجابة المناعية للقاح الحمي القلاعية للامصال الثلاثة . ونحتاج المزيد من الدراسة لمعرفة تاثير الليفاميزول علي الخلايا المناعية واختبار التحدي في الجاموس .