Trial For Preparation And Evaluation Of Inactivated Multicomponent Vaccine Containing BVD, IBR, PI-3 Viruses, *Salmonella typhimurium* And *Salmonella Dublin* For Calves

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ABSTRACT

An inactivaated multicomponent vaccine adjuvanted with aluminum hydroxide gel against Bovine Viral Diarrhea (BVD), Infectious Bovine Rhinotracheits (IPR), and Para influenza-3 (PI-3) viruses with *S. typhimurium* and *S. dublin* bacteria was prepared. The vaccine was pure and safe. Potency evaluation was applied on 12 adult apparently healthy calves divided into 4 groups 3 animals for each. The immune responses were monitored using serum neutralization, agglutination and mouse protection tests. The results revealed a lack of interaction and antagonistic effect among the component of the prepared vaccine. The humeral immune responses gave antibody titers over the permissible limit of protective level and persist for 24 weeks post vaccination (period of study).

INTRODUCTION

Pneumo-enteritis diseases complex syndrome are of paramount economic important because of high loses and deaths rate, so respiratory and enteric disorders are often cited as the significant cause of economic losses probably more costly than all other combined diseases ***(1)***.

Pneumo-enteritis diseases complex syndrome is considered as multifactorial diseases syndrome caused by a number of infectious agents either viral and/or bacterial. Bovine viral diarrhea (BVD), Infectious bovine rhinotracheits (IBR) and parainflunza-3 (PI-3) viruses have been all incriminated in the etiology of bovine viral pnumo-enteritis complex syndrome among cattle and buffalo causing severe economic losses ***(2-4)***.

Bovine salmonellosis is of worldwide public health concern and is an economically important disease. Although cattle of all ages can be infected with salmonella, serious infections and deaths are most often seen in calves up to 10 weeks of age ***(5,6)***. The most common serotypes involved are *salmonella typhimurium* and *salmonella dublin* ***(7-10)***. Numerous attempts have been made to protect calves by immunization ***(11-13)***.

Concurrent infection with salmonella and BVDV would be expected to cause more serious disease than infection with either agent alone. Therefore the two agents should be considered during disease outbreak ***(14-16)***.

Vaccination has proved to be by far, the most effective and cost effective method for controlling of infectious diseases. Vaccination is the undersigned, support immunization as the safest, most effective way to control infectious diseases. The two or more separate immunogens in a single product and administrated through the same way, have many potential advantages in the form of decreased of injecting risks, decreased of clinical visits and easier introduction of vaccine. So to facilitate the vaccination program, the multicomponent vaccines are being economic by reducing costs of storage transport and administration ***(17)***.

This investigation was designed to prepare and asses the new locally inactivated multicomponent vaccine named (Pneumo-Sal) containing pneumo-enteric viral and bacterial

etiological agents (BVD, IBR, PI-3, *S. typhimurium* and *S. dublin)* for using in calves against respiratory and enteric diseases.

MATERIAL AND METHODS

1. Laboratory animals

1.1. Mice

A total of 100 Swiss albino mice (18-20 grams weight) were used in this work. Eighty mice were used for protection test in case of inactivated combined bacterial vaccine while twenty mice were used for safety test in locally prepared multicoponent (Pneumo-Sal) vaccine.

1.2. Guinea pigs

Ten adult male guinea pigs (300-400 grams weight) were classified into 2 groups (5 for each) and used in the safety test of the locally prepared multicoponent (Pneumo-Sal) vaccine.

1.3. Calves

A total of 18 cross breed apparently healthy male calves (6-9 months' old ages) were used for this study. Calves were housed in an isolation facility at Veterinary Serum and Vaccine Research Institute. All calves were tested to confirm that they are free from antibodies against BVD, IBR, PI-3, *S. typhimurium* and *S. dublin* prior to vaccination. Six calves were used in safety test while the other 12 were used for potency evaluation of the prepared vaccine.

All experimental animals used in this work were obtained from Veterinary Serum and Vaccine Research Institute (VSVRI) Abbassia, Cairo.

2.Vaccines

2.1.Preparation of inactivated combined viral vaccine (Pnemue-3)

The vaccine was prepared from 3 reference Egyptian viral strains. BVD (Iman strain) with a titer of 7 log 10 $TCID_{50}$ / ml. which was isolated and identified *(18)*. IBR (Abo-Hammad strain) with a titer of 8 log 10 $TCID_{50}$ / ml. which was isolated and identified *(19)*. While PI-3 with a titer of 8 log 10 $TCID_{50}$ /ml (Strain 45) which was isolated and identified *(20)*. The 3 viruses (BVD, IBR and PI-3) were initially adapted and propagated in Madin Darby Bovine Kidney (MDBK) cell culture. One ml of each virus was inoculated in roller bottles containing healthy and confluent sheet of MBDK cell culture using MEM as maintenance media. All roller bottles inoculated virus were kept in an incubator at 37 ^{0}C and examined daily for follow up the specific CPE related to each virus. After complete CPE showing 70 % of whole sheet of cell culture the harvesting was done by rapid freezing and thawing. The process of inactivation was made by adding 0.01 M of Binary Ethylene amine and mixed together by stirring at 37 ^{0}C for optimum times according to the described techniques *(21-23).* The stoppage action of BEI was performed by adding of Sodium thiosulphate 20 % with the final concentration of 2 %.

2.2.Preparation of inactivated combined bacterial vaccine

The vaccine was prepared from 2 local bacterial field isolates (S. *typhimurium* and *S. Dublin*). The bacterial isolates were serologically identified by somatic and flagellar standard antisera *(24)*. Both bacterial strains (S. *typhimurium* and *S. dublin)* were grown in nutrient broth enriched with casein and yeast extract then incubated at 37 ^{0}C. The cultures were shaken at 120 CPM and harvested during the logarithmic growth phase. Washed saline growth suspension was standardized optically to contain 2 mg dry weight of cells / ml *(25)*. Cold ethanol was added *(26)*. Equal volume of both inactivated culture suspensions were mixed well.

2.3.Preparation of inactivated multicomponent vaccine (Pneumo-Sal)

The inactivated viral and bacterial suspensions in equal volume were mixed together. Then Aluminum hydroxide gel 30 % (Al-hydrogel solution) was added as an adjuvant and mixed well by using of magnetic stirrer for obtaining of homogenized solution. Thiomersal was added as a vaccine preservative (0.01%) and PH was adjusted to 7.8. The final vaccine product was distributed in sterile glass bottles of 100 ml capacity capsulated and labeled.

3.Evaluation of the locally prepared inactivated multicomponent vaccine (Pneumo-Sal)

3.1. Sterility test

It was performed according to American protocol *(27)*. It was done to be proved that the vaccine is free from bacteria, mycoplasma, and fungi.

3.2.Safety test

3.2.1. Safety test in mice

A group of 20 Swiss albino mice were classified into 2 groups (10 for each). The first group were inoculated I/P with a dose of 0.2 ml of the prepared multicombonent vaccine (Pnumo-Sal.). The second group was used as control and inoculated I/P with 0.2 ml of physiological saline. Mice were kept under observation for 2 weeks to detect undesired post vaccinal side effects.

3.2.2. Safety test in guinea pigs

Ten adult male guinea pigs were used in this experiment allocated into 2 groups. The first group consists of 5 guinea pigs that inoculated I/P with 0.5 ml of the prepared vaccine (Pneumo-Sal) while the second group was left as control and inoculated with 0.5 ml of physiological saline. All animals were kept under observation for 2 weeks post inoculation.

3.2.3. Safety test in calves

It was achieved using six adult male calves. Three calves of them were inoculated I/M with ten times of the vaccinal dose (Pnumo-Sal.) 75 ml of the prepared vaccine were distributed to be inoculated in different places of animal body. The sofety test was applied *(27)*. The other 3 calves were inoculated with the same dose (75 ml) at the same time with physiological saline solution and kept as a non-vaccinated control. All animals were kept under observation for 2 weeks post inoculation for detection of any abnormalities, also daily rectal temperature has been recorded.

3.3. Potency test

Potency evaluation of the prepared multicomponent inactivated vaccine (Pnumo-Sal) was carried out *(27)*. Twelve adult male calves were used in this study and divided into four groups, 3 animals for each (Table 1).

4.Blood sampling

Blood samples were collected from each calve of all groups at different intervals. Before vaccination then 2 weeks post 1st vaccination and every 2 weeks after booster vaccination till 24 weeks later.

5.Serological investigations

5.1.Serum neutralization test (SNT)

SNT was applied in microtiter plate for detection of viral antibody titers following the procedures described *(28,29)*. SNT antibody titers were expressed as log 10 $TCID_{50}$ of the reciprocal serum dilutions following the calculation formula *(30)* for viral antibody evaluation.

5.2. Serum agglutination test (SAT)

The test was used for detection of antibody titers against S. *typhimurium* and *S. dublin (31)*.

5.3.Mouse protection test

The test was used for evaluation of both combined bacterial and multicomponent (Pnumo-Sal.) vaccines through challenge test. Eighty mice were used for this experiment and divided into 8 groups; ten mice were used for each group. All groups exposed to challenge with both virulent S. *typhimurium* (2656 V) and *S. Dublin* (2560 V) 2 weeks post vaccination following the reported technique *(25)*. Dead and survived mice in every group were registered post challenge over a period of 14 days. The deaths of challenged mice were used as criteria for lack of immunity and the percentage of protection was calculated according to the formula:

$$= \frac{\text{Survivors in vaccinated group} - \text{Survivors in the control group}}{10} \times 100$$

Table 1. Experimental Design for potency evaluation of pnumo – sal inactivated vaccine

Calves group	No. of calves	Vaccination protocol				
		Type	First dose	second dose	Route	Interval period
Group 1	3	Inactivated combined viral vaccine (Pneumo-3)	5 ml	5 ml	I/M	2 weeks
Group 2	3	Inactivated combined bacterial vaccine	2.5 ml	2.5 ml		
Group 3	3	Inactivated multicomponent vaccine (Pneumo-Sal.)	7.5ml	7.5ml		
Group 4	3	Control non-vaccinated calves				

- Inactivated combined viral vaccine (Pneumo-3) = Containing BVD, IPR and PI-3 viruses
- Inactivated combined bacterial vaccine = Containing S. *Typhymurium & S. dublin.*
- Inactivated multicomponent vaccine (Pneumo-Sal.) = Containing both viral and bacterial components.

RESULTS AND DISCUSSION

Salmonella infections in cattle are of world wide public health concern, therefore numerous attempts had been made to protect cattle *(5,32)*. Perhaps the most frightening aspect of salmonellosis is the rapid strain resistance to drugs and despite the application of vaccine and availability of therapeutic agents; bovine salmonellosis remained a serious and wide spread problem *(33)*. *S. typhimurium* and *S. dublin* appears to be the commonest serovars isolated from cattle, hence the great demand for preparation of vaccine which may have a greatest part to play in the control of disease outbreaks *(34)*.

Acute BVDV infection of cattle was closely associated with temporary suppression of the immune function which predispose to endogenous bacteraemia *(35)*. BVD infection can aggravate and possibly predispose to salmonella infection, this situation could assume greater significance in calves stressed by colostrum deprivation and also stressed by movement through markets, and hence the etiological interaction of the two should be considered during outbreaks of salmonella in cattle. Also the super infection or combined infection of salmonella and BVDV in calves is more severe than salmonella infection alone *(14)*. In addition the severe disease was observed in pregnant dairy heifers that had natural BVDV and *S. typhimurium* DT 104 infection *(15)*.

So the use of vaccine will continue to be a basic tool used to reduce the risk of infection of viral disease alone (BVD, IBR, PI-3 viruses) or accompanied with bacterial infection (*S. typhimurium* or *S. dublin)*. It will be necessary to produce of a broad spectrum of protection against a range of viral and bacterial agent *(36)* and high level of specific antibodies prior to infection may reduce the risk of disease *(37)*.

The evaluation of the locally prepared inactivated multicomponent vaccine (Pnumo-Sal) for purity, safety and potency showed that the vaccine was free from any aerobic and anaerobic bacteria as well as mycoplasma and fungal contamination through inoculation on the specific media for 15 days. Belonging to the safety test of vaccine in mice, guinea pigs and calves, it was recorded to be safe as there were no post vaccinal reaction, no abnormalities or deaths during the period of observation (14 days). Also the vaccine was completely safe for calves as there were no local or systemic reaction has been observed during the period of the experiment *(38)*.

Regarding to the potency evaluation of the prepared vaccine (Pneumo-Sal) through

immunization of adult calves with 2 immunizing doses at 2 weeks interval,: the immunogenicity of the inactivated vaccine was rapidly diminished when used a single dose, while 2 doses virtually eliminate this phenomena *(39)*. The fact that the vaccine in an adjuvant was capable of inducing an immune response that revealed the active infection *(26)*, as aluminium hydroxide gel exerts its effect by protracted release from the site of injection *(40)*

The results obtained in Tables 2, 3 indicated that each component of vaccinal strains either viral (BVD, IBR and PI-3 viruses) or bacterial (*S. typhimurium* and *S. dublin*) used in the preparation of inactivated multicomponent vaccine (Pnumo-Sal) proved that there was no antagonistic effect between each component of the prepared vaccine. Such data was similar to that obtained in several studies *(41,42)*.

The results of serum neutralization antibody titers illustrated in Table 2 and figures 1A, B, and C proved that calves vaccinated with Pnumo-Sal (group 3) have antibody titers nearly similar to that titers observed in calves vaccinated with Pnumo-3 (group 1). The level of the recorded antibody titers were effectively sufficient to combat such active infection which remained within minimum acceptable titer of protective level till 6 months post vaccination. BVD antibody level of 1: 8 dilutions (Log 0.9) was protective *(43)*. Also it has been recorded that the minimum acceptable titers was 1: 4 dilutions or 0.6 Log was protective against PI-3 and IBR viruses *(44,45)*.

Table 2. Mean of serum neutralizing antibody titers in calves vaccinated with inactivated multicomponent (Pneumo-Sal) and inactivated combined (Pneumo-3) vaccines.

Type 0f Vaccine	Viral antigens	Mean serum neutralizing antibody titers expressed in log 10											
			weeks post-vaccination										
		* 0 day	** 2	4	6	8	10	12	14	16	18	20	22	24
Pneumo-Sal Vaccine	BVD	0.20	0.30	1.20	1.30	1.80	1.85	1.87	1.77	1.60	1.60	1.55	1.35	1.20
	IBR	0.25	0.40	1.40	1.45	1.90	2.00	2.00	1.95	1.75	1.65	1.55	1.30	1.25
	PI-3	0.26	0.30	1.20	1.35	1.86	1.90	1.93	1.90	1.70	1.57	1.50	1.35	1.20
Pneumo-3 Vaccine	BVD	0.25	0.30	1.45	1.55	1.85	1.95	1.87	1.75	1.70	1.70	1.60	1.40	1.30
	IBR	0.30	0.40	1.45	1.50	1.95	2.1	1.95	1.95	1.80	1.66	1.55	1.35	1.30
	PI-3	0.25	0.40	1.35	1.35	1.80	1.95	1.90	1.90	1.75	1.65	1.55	1.40	1.25
Control Non vaccinated	BVD	0.20	0.20	0.20	0.30	0.20	0.20	0.20	0.25	0.20	0.20	0.25	0.20	0.20
	IBR	0.20	0.20	0.25	0.20	0.30	0.20	0.26	0.30	0.30	0.30	0.20	0.30	0.20
	PI-3	0.50	0.30	0.30	0.30	0.20	0.20	0.20	0.20	0.20	0.20	0.30	0.20	0.20

- Protective serum neutralizing (SN) antibody for BVD is 0.90 *(43)*.
- Protective serum neutralizing (SN) antibody for IBR is 0.60 *(45)*.
- Protective serum neutralizing (SN) antibody for PI-3 is 0.60 *(44)*.

* First vaccination

** Second vaccination

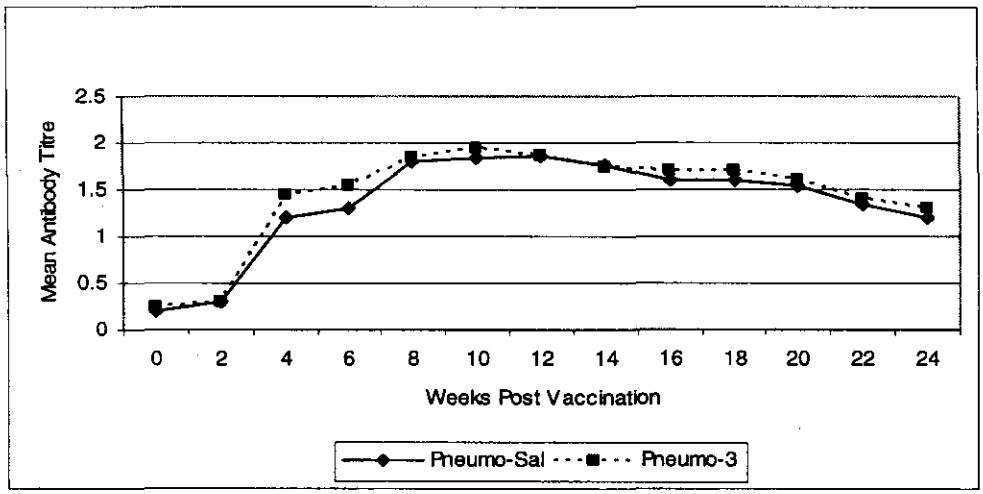

Fig. 1A. Mean serum neutralizing antibody titers against BVD in both pneumo-sal and pneumo-3 vacinated calves

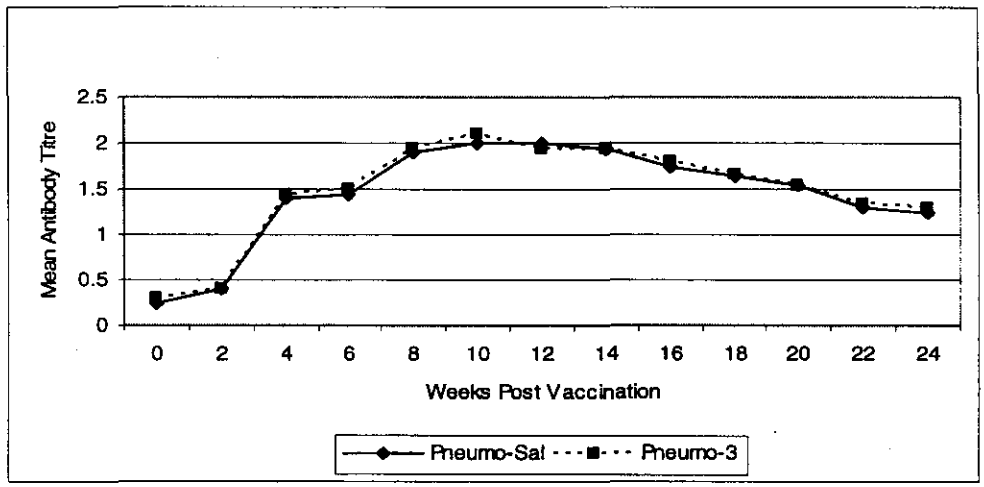

Fig. 1B. Mean serum neutralizing antibody titers against IBR in both pneumo-sal and pneumo-3 vacinated calves

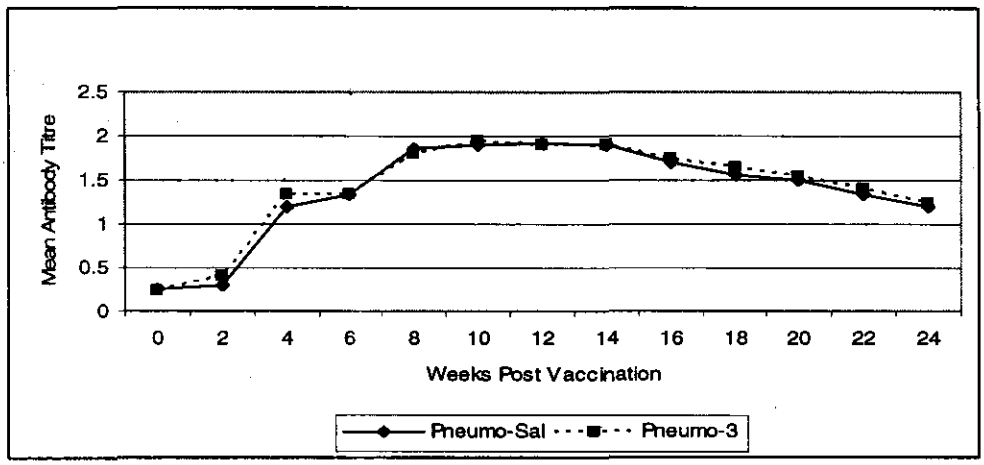

Fig. 1C. Mean serum neutralizing antibody titers against PI-3 in both pneumo-sal and pneumo-3 vacinated calves

The results of serum agglutination test presented in Table 3 and figures 2A and B revealed that there were higher agglutinating antibodies titers in calves vaccinated with Pneumo-Sal or inactivated combined bacterial vaccine in comparison to the control non vaccinated group *(39)*. Moreover the antibody titers were slightly similar in both Pneumo-Sal and combined bacterial vaccinated groups *(25, 40)*.

Table 3. Mean of serum agglutinating antibody titers against *S. typhimurum* and *S. dublin* in calves vaccinated with inactivated multicomponent (Pneumo-Sal) and inactivated combined bacterial vaccines.

Type 0f Vaccine	Bacterial antigens	Mean serum agglutinating antibody titers (geometric mean)												
		* 0 day	weeks post-vaccination											
			** 2	4	6	8	10	12	14	16	18	20	22	24
Inactivated multicomponent (Pneumo-Sal) vaccine	*S. Typhimurium*	4.6	160	320	640	1015	1015	1280	1280	1015	640	160	80	80
	S. Dublin	4.6	201	640	1015	1280	1280	1015	1015	640	640	320	160	160
Inactivated Combined bacterial vaccine	*S. Typhimurium*	4.6	320	640	1015	1280	1280	1280	1280	1015	640	320	160	80
	S. Dublin	4.6	320	1015	1280	1280	1280	1280	1015	1015	640	320	160	160
Control non vaccinated	*S. Typhimurium*	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0
	S. Dublin	4.6	4.6	4.6	4.6	4.6	4.6	4.6	4.6	4.6	4.6	4.6	4.6	4.6

* First vaccination

** Second vaccination

➢ 80 considered positive titer for salmonella *(13)*.

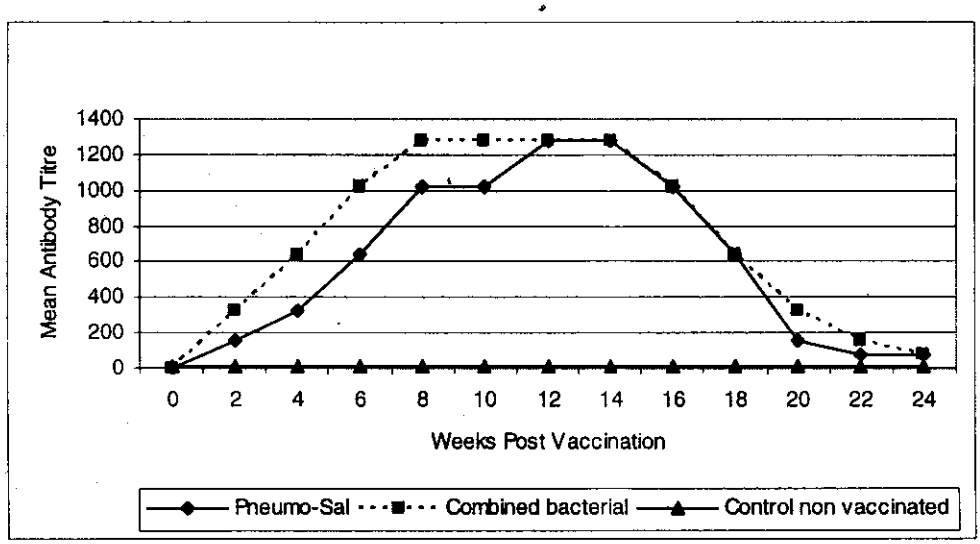

Fig. 2A. Mean serum agglutinanting antibody titers against *S. typhimurium* in both pneumo-sal and pneumo-3 vacinated calves

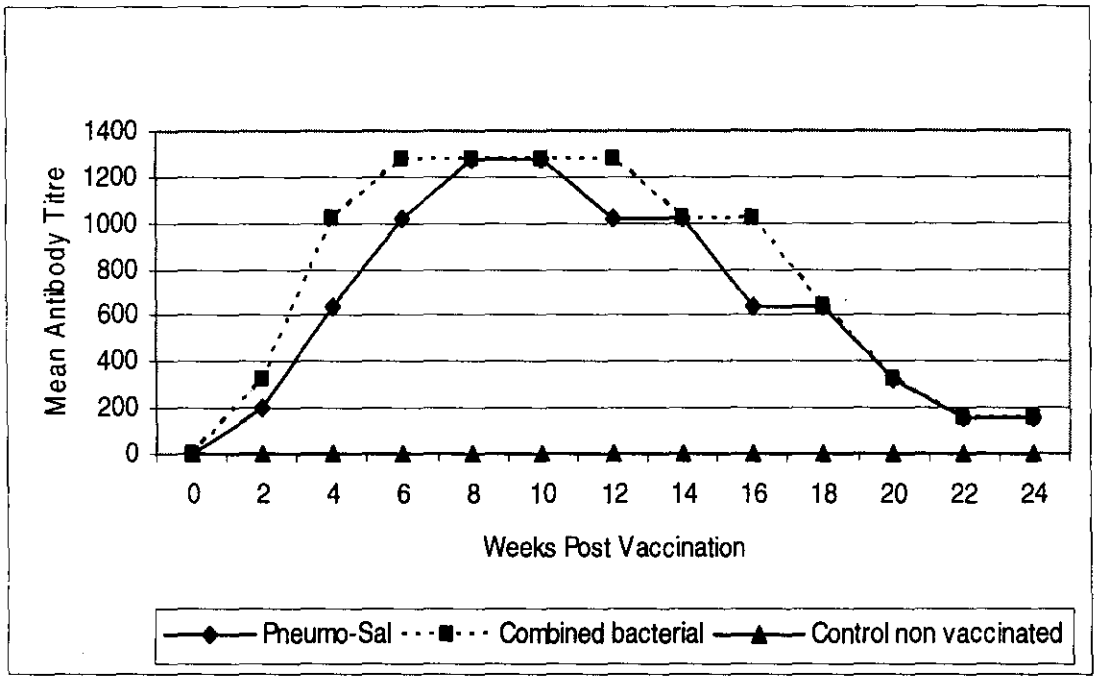

Fig. 2B. Mean serum agglutinanting antibody titers against *S. dublin* in both pneumo-sal and pneumo-3 vacinated calves

The results depicted in Table 4 showed that the protection percentage in mice vaccinated with inactivated combined bacterial vaccine and challenged with *S. typhimurum and S. Dublin* were 80 % and 70 % while it was 70 % in case of mice vaccinated with Pnumo-Sal vaccine respectively.

The humeral antibody response induced by Pnumo-3 (group 1), combined salmonella (group 2) and Pnumo-Sal (group 3) vaccines, revealed that there is no interference or competition observed between each fraction consisting the prepared vaccine. The combined vaccine of BVD, *S. typhimurium* and *S. dublin* gives adequate protection against such component and recommended for field use ***(25)***.

From the previous results it could be concluded that, the locally prepared inactivated multicomponent (Pnumo-Sal) vaccine containing BVD, IBR, PI-3, *S. typhimurium* and *S. dublin* has been proved to be pure, safe and potent in calves against respiratory and enteric illness inducing immune response lasting for 24 weeks against such viral or bacterial causative agents and it is recommended practically to be used effectively in the field application.

Table 4. Results of challenge test for *S. typhimurum* and *S. dublin* in mice vaccinated either with inactivated combined bacterial or multicomponent (Pneumo-Sal) vaccines

Days post Challenge	Inactivated combined bacterial vaccine				Inactivated multicomponent (Pneumo-Sal) vaccine			
	Mice challenged with *S. typhimurum*		MiceChallenged with *S. dublin*		Mice challenged with *S. typhimurum*		Mice challenged with *S. dublin*	
	No. of survived mice		No. of survived mice		No. of survived mice		No. of survived mice	
	Vaccinated group	Control group	Vaccinated group	Control group	Vaccinated group	Control group	vaccinated group	Control group
1	10/10	10/10	10/10	9/10	10/10	10/10	10/10	9/10
2	10/10	9/10	10/10	9/10	10/10	9/10	10/10	9/10
3	10/10	9/10	9/10	8/10	10/10	9/10	10/10	8/10
4	9/10	7/10	9/10	8/10	10/10	7/10	10/10	8/10
5	9/10	7/10	9/10	8/10	10/10	7/10	9/10	8/10
6	9/10	7/10	8/10	7/10	10/10	7/10	9/10	7/10
7	9/10	5/10	8/10	7/10	10/10	5/10	9/10	7/10
8	9/10	5/10	8/10	5/10	9/10	5/10	9/10	5/10
9	9/10	5/10	8/10	2/10	9/10	5/10	9/10	2/10
10	9/10	2/10	7/10	2/10	9/10	2/10	8/10	2/10
11	9/10	2/10	7/10	2/10	9/10	2/10	8/10	2/10
12	8/10	1/10	7/10	1/10	9/10	1/10	8/10	1/10
13	8/10	0/10	7/10	0/10	8/10	0/10	8/10	0/10
14	8/10	0/10	7/10	0/10	7/10	0/10	7/10	0/10
Protection %	80 %	0 %	70 %	0 %	70 %	0 %	70 %	0 %

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الملخص العربى

محاولة تحضير و معايرة لقاح جامع مثبط يحتوى على فيروسات مرض الأسهال البقرى الفيروسى ومرض ألتهاب القصبة الهوائية الرغامى المعدى و فيروس البارا أنفلونزا-٣ و بكتيريا السالمونيلا تيفيميوريوم و السالمونيلا دبلن فى العجول

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المعمل المركزى للرقابة على المستحضرات الحيوية البيطرية

تهدف هذة الدراسة إلى تحضير و معايرة لقاح جامع مثبط بالبينارى يحتوى على فيروسات مرض الأسهال البقرى الفيروسى (بى فى دى)

و فيروس مرض إلتهاب القصبة الهوائية الرغامى المعدى (أى بى أر) و فيروس البارا إنفلونزا-٣ (بى أى-٣) و بكتيريا السالمونيلا تيفيميوريوم و السالمونيلا دبلن و محمل بهيدروكسيد الألمونيوم الجيل كعامل مساعد لتفعيل اللقاح.

تمت معايرة اللقاح الجامع المخمد المحضر عن طريق أختبارات النقاوة و الأمان و الكفاءة المناعية . أثبتت الأختبارات أن اللقاح المحضر محليا أنه نقى و أمن تماما للأستحدام فى العجول بدون ظهور أى أعراض أو رد فعل غير مستحب سواء عضوى أو موضعى فى مكان الحقن.ولتقييم كفاءة اللقاح مناعيا تم إستخدام عدد ١٢ عجل بقرى بالغ سليم ظاهريا تم تقسيمهم إلى ٤ مجموعات تحتوى كل مجموعة على ٣ عجول. أستخدمت فى تقييم الكفاءة المناعية للعجول (أختبارات المصل التعادلى- التلازن الدقيق و أختبار الحماية للفئران). أتضح من النتائج أنه لا يوجد أى تفاعل أو تضاد بين أى من مكونات اللقاح المحضر. كذلك أستمرت الأستجابة المناعية للعجول بمعدل حماية مرتفع حتى نهاية التجربة (الأسبوع ٢٤ بعد التحصين).

أستنتجت من النتائج السابقة أن اللقاح المحضر محليا هو لقاح نقى و أمن و ذو كفاءة مناعية عالية لذلك ينصح بأستخدامة فى الحقل البيطرى لتحصين العجول لحمايتها من الأسهال و الأمراض التنفسية.