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PRODUCTION AND EVALUATION OF A MULTICOMPONENT INACTIVATED RESPIRATORY VIRAL VACCINE (BVD, IBR, PI-3 AND BOVINE ADENO VIRUS TYPE 3 (PNEUMO-4) IN CATTLE

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Abstract

An inactivated virus vaccine (MDBK-dextran) containing strains of bovine viral diarrhoea (BVD), infectious bovine rhinotracheitis (IBR), parainfluenza type 3 (PI-3) and bovine adeno virus type-3 was conveniently produced by propagation on MDBK-dextran-roller system, then, the viruses were inactivated with binary ethyleneimine (BEI), lower concentration of inactivants which killed the viruses in the shortest period of time was selected for preparing the corresponding vaccine. Alhydrogel was added in a final concentration of 30% to the vaccine as adjuvant. Effectiveness of each fraction of the prepared vaccine was studied in challenged immunity studies. Cattle vaccinated with any fraction of vaccine followed by challenge with a disease causing strain of that fraction, resisted infection well by both forms; qualitative (clinical aspect) and quantitative points of view (virus isolation and temperature). All antigens stimulated high antibody titres detected by ELISA immuno assay (EIA) and serum neutralization test (SNT) against all component of the vaccine and haemagglutination inhibition test (HIT) aganist PI-3. Immunity obtained was long-lasting and even persisted up to 6 months post-vaccination.

INTRODUCTION

Respiratory virus affections as bovine viral diarrhoea (BVD), infectious bovine rhinotracheitis (IBR), parainfluenza-3 (PI-3) and bovine adeno virus serotype 3 represent the most important and drastic upset among fattening calves, and are widely spread throughout the world. These affections constitute major causes of morbidity and mortality in feedlot cattle despite the high prevalence and economic importance of respiratory virus infection. The incidence of pneumoenteritis appears to increase in recent years. The development of intensive beef production may have largely contributed to the increase in the size of the problem (El-Sebaie *et al.*, 1986).

Environmental stressors including hunger, thirst, extreme heat cold climatic

temperature fear and anxiety during transport, weaning, dehorning, dipping, castration, highly parasitized animals, deficiencies of vitamins such as vitamin A, and vaccination play a role in predisposing of outbreaks of respiratory and alimentary disease, and the climatic stress may determine or influence the occurrence, frequency of occurrence or the severity of various diseases (Schwabe *et al.*, 1977).

The clinical patterns within a herd depend on interaction of several factors, including strains of the virus, age and immune status of the animals resulting in increasing of susceptibility to disease (Radostits, 1994). The immunosuppressive effect of acute BVD infections compromises the immune system and can enhance the clinical disease of other pathogens and precipitate illness by opportunistic infections that are of an importance in calf respiratory disease complex. However, many primary postnatal infections, whether subclinical or not, render the animals transiently immunosuppressed (Reggiardo, 1979).

In Egypt, El-Sabbagh *et al.* (1995) succeeded in preparing a safe and potent combined inactivated respiratory virus vaccine against BVD, IBR and PI-3, and Wahid (1998) prepared inactivated bovine adeno virus type 3 vaccine.

The present study was performed to prepare and evaluate the efficacy of the ɔ-cal combined inactivated respiratory virus vaccine containing BVD, IBR, PI-3 and BAV-3 vaccine from viruses propagated in MDBK cell roller system in the presence of DEAE dextran used to increase the yield of viruses (infective power).

MATERIALS AND METHODS

1. Animals

a. Laboratory animals : Four healthy adult guinea pigs and thirty adult mice were used for safety test of the vaccine.

b. Calves : Twenty susceptible male cross breed calves aged 8 months old were used to study the safety, potency and duration of immunity tested to be free from antibodies against IBR, BVD, PI-3 and adeno virus before vaccination with the prepared vaccine.

2. Cell culture

Madin Darby Bovine Kidney (MDBK) cell line culture which was tested to be free from the non cytopathic (NCP) BVD-MD virus by fluorescent test was used in this study.

3. Inoculum

a. IBR virus, Abou Hammad strain ($10^{-7.5}$ $TCID_{50}$/ml).
b. PI-3 virus : strain 45 (10^{-8} $TCID_{50}$/ml).
c. BVD virus : Iman strain ($10^{-6.5}$ $TCID_{50}$/ml).
d. Adeno-virus (type 3) : 10^{-6} $TCID_{50}$/ml.

These local virus strains were used in the preparation of combined inactivated respiratory virus vaccine. Virulent viruses were used in challenge exposure test in calves.

4. Inactivants

Two bromoethyleneimine hydrobromide (BEI) Aldrish Chemical Company, Melwaukeewis, USA and used for preparation of BEI.

5. Sodium thiosulphate

It was obtained from Difco Laboratories and it was prepared as 20% solution. It was used to stop the action of binary ethyleneimine inactivant.

6. Alhydrogel solution

It was used as stabilizer and adjuvant on the vaccine preparation. It was sterilized by autoclaving for 20 minutes at 140°C and the pH was adjusted to 6.6 then it was bottled and stored at 4°C.

7. Preparation of multicomponent inactivated vaccine comprising BVD, IBR, PI-3 and BAV-3 viruses (Pneumo-4)

i. Four blind passages of MDBK cell culture to measure viable virus and were observed for any CPE that may appear, then, the cells were superinfected with live cytopathic strain of BVD virus three or four days after inoculation in an effort to determine the presence of any non-cytopathic virus (NCP) (homologous interference).

ii. Four roller bottle of MDBK cells each was inoculated with 2ml of 1.5 ml of each virus + 0.5ml of DEAE dextran (100ug/ml) and were mixed together to get a final concentration of 25ug/ml of DEAE dextran. After the CPE was advanced, the virus was inactivated by 0.01 M of binary ethyleneimine at the optimum times after studying the inactivation rate (Table 1).

iii. 10% of sodium thiosulphate were added to stop the action of BEI and,then, centrifuged at 4°C for 10 minutes to remove BEI and sodium thiosulphate. Equal amounts of each inactivated virus suspension were mixed together and stirred in a magnetic stirrer to obtain a homogenized solution. The virus suspension was adjusted to 6.0 pH just before the addition of adjuvant. Alhydrogel was added as adjuvant in 30%, and stirred for 24 hours at 4°C, then, pH was adjusted to 7.5 (thiomersal was added as a vaccine preservative at final concentration of 0.01%) and distributed in sterile bottle 100ml capacity then capsulated and labelled well.

8. Experimental evaluation of the vaccine

a. Purity in accordance with the US Code of Federal Regulations (1987) testing CFR 113-26, 113.27, 113,27, 113.30 and 113.25.

b. Safety According to 9 CFR (1987) testing 113.41 in calves and 9 CFR 113.38 in Guinea pigs were used in this study.

c. Potency evaluation and challenge exposure test

Eighteen cross breed apparently healthy male calves (Fresian and local) about 6-9 months, of an average body weight 150 kilograms were used in this study. Ca..es were housed individually in an isolation facility on arrival at the Veterinary Serum and Vaccines Research Institute (VSVRI), Abbasia, Cairo, Egypt.

Before experimental studies, Serum samples were collected for titration of antibody against BVD, PI-3, IBR and adeno-3 (BAV-3) viruses, nasal swabs and buffy coats were obtained for viral isolation. Eighteen calves were randomly assigned to 2 groups :

I. Group A : This group consisted of 8 animals, each calf was immunized with intramuscular, 5ml of the locally produced inactivated pneumo -4 vaccine (BVD, PI-3, IBR, and BAV-3) then received a booster dose of vaccine 4 days apart according to Urban *et al.* (1995). This group was divided into two subgroups, 4 calves each, as follows:

Subgroup 1: This group was kept for collection of blood, serum samples, for studying the duration of immunity. Samples were collected at 0, 7, 10, 14, 21, 28, 45, 60 days and, then, monthly up to 6 months post-vaccination.

Subgroup 2: The other 4 calves of this group were challenged at 28 days post-injection of the second dose of vaccination with each pathogenic virulent strain of BVD, IBR and PI-3. The $TCID_{50}$ of challenge viruses was 10^6/0.2 ml, and calves were in-

fected with 5 ml intravenously and 5ml instilled intranasally according to Ballasch (1993), and the effectiveness of the vaccine was evaluated on the basis of clinical observation and virus isolation for 3 weeks post challenge.

II. Group B: This group also consisted of 8 animals and divided into 4 subgroups, 2 animals in each group, inoculated with 10ml of each virulent virus according to Ballasch (1993) and Urban *et al.* (1995).

III. Group C: This group consisted of 2 calves which were left as non-infected and non-vaccinated control group.

Body temperature was recorded daily for two weeks post infection. Monitoring of specific antibody titre was followed up for vaccinated groups of calves for 6 months post-vaccination (PV).

Samples

a. Nasal, conjunctival and rectal swabs for trials of virus isolation : Swabs were collected from different experimental groups daily through the first 3 weeks post-vaccination (PV) and post challenge (PCh).

b. Blood samples : Non clotted blood samples for virus reisolation from buffy coat and clotted blood samples for preparation of serum for studying the immune response post-vaccination.

Methods of examination

A. Virological studies

1. Trials of virus reisolation was conducted on nasal, conjunctival and rectal swabs, buffy coat. The used method was described by Ballasch (1993).
2. Identification of isolated agents; serum neutralization test was utilized as described by Ballasch (1993) for the identification of the reisolated viral agents.

B. Serological studies

1. Serum neutralization test : The test was performed for the measurement

of specific BVD, IBR, PI-3 and BAV-3 serum neutralizing antibodies in vaccinated calves the method adapted by Fulton *et al.* (1995).

2. The haemagglutination inhibition test : was conducted in microtitre plates against eight haemagglutination units of PI-3 virus (strain 45) which was described by Cho *et al.* (1985).

3. Enzyme linked immuno-sorbent assay (ELISA) : for antibodies to IBR, BVD, PI-3 and BAV-3 viruses according to Voller *et al.* (1976).

4. Immunofluorescent technique : This test was processed according to Fernelius and Lambert (1969).

RESULTS

1. Purity or sterility tests revealed that the local vaccine was free from bacteria and fungi and mycoplasma contamination.

2. Safety tests revealed that no clinical abnormalities were observed throughout 10 days of observation among mice and Guinea pigs.

3. Post-vaccination and challenge exposure test Neither elevation of body temperature nor development of clinical signs of illness were recorded in all calves during 21 days of observation. Vaccinated calves with pneumo-4 (subgroup 1) did not show any clinical abnormalities. The vaccinated challenged calves (subgroup 2) showed a slight increase in body temperature on the 2nd, 3rd and 4th day post-challenge. The body temperature returned to its normal values on the fifth day post-challenge (P.Ch.). All calves of this group did not show any clinical abnormalities post-challenge.

The calves of the group (B) (positive infected control calves) showed elevation of body temperature for eight days post-infection. The observed clinical signs of this group were pneumo-enteric in nature, firstly, were in the form of dullness, off-food, then, serous nasal discharge began to appear within two days, and faeces became soft and of the fifth day. It was watery. On the 8th day post-infection (PI), the animals showed hurried respiration excessive salivation and congestion and erosions on the buccal mucosa. The condition relieved after few days. Two weeks later, the calf became anoretic and shed watery diarrhoea. Pneumoenteritis respiratory syndrome of

cattle varied from an inapparent disease to a rapidly developing fatal pneumonia. Signs of the disease might appear within 2 to 21 days. Control calves in group (C) did not show any clinical abnormalities post challenge.

4. Detection of viruses post vaccination and challenge exposure test

Cytopathogenicity of reisolated viruses on MDBK cell culture revealed that :

A. BVD virus causing elongation of the cells granular with intracytoplasmic vaculoses, then, vacuoles coalesce together giving large vacuoles.

B. IBR virus caused rounding, shrinkage of cells and increase of granularity and progressed to form a bunch of grapes, and then, cell destruction and detachment of cell sheet from the glass surface.

C. PI-3 virus caused rounding of cells with progressive syncytial formation. The infected cultures caused haemadsorption to Guinea erythrocytes.

D. BAV serotype 3 causing cytopathic changes characterized by rounding of the individual cells and separation from each other. Shrinkage of the cell wall with an increase in the granularity of the cytoplasm, then, progress rapidly and complete destruction of the cells. The detachment and sloughing of the cells from the wall of the flasks were recorded.

As shown in Table 2, there was no reisolation of BVD, IBR, PI-3 and Adeno virus type-3 viruses from the vaccinated calves throughout the experiment. In the vaccinated challenged groups the viruses were recovered only on the first eight days post-challenge. In the non-vaccinated infected calves, the viruses were recovered throughout 21 days post-infection (DPI).

5. Immune response against vaccine

a. The mean serum neutralizing antibody titres expressed as $\log_{10}$ of vaccinated animal in Table 3 indicated that the prepared local vaccine induced protective titres against BVDV, IBR, PI-3 and BAV-3 neutralizing antibodies; starting with a mean $\log_{10}$ values of (1.10, 1.4, 1.5 and 1.2, respectively) at the 3rd WPV reaching their maximum values of (1.7, 1.75, 1.85 and 1.75, respectively) between the 6th and 12th WPV and declined to their minimal accepted titres of (0.9. 1.00, 1.2 and 0.90) at the 24th WPV, respectively in sera of calves vaccinated with the prepared vaccine.

b. Also, PI-3 Table 4. haemagglutinating antibodies of acceptable titres were recorded at mean log_{10} values of (1.35 and 1.85) at the 2nd WPV, 3rd WPV and reaching their maximum values of (2.40 log_{10} $TCID_{50}$) at 6th WPV and declined to their minimal accepted titres of (1.30) at the 28th WPV.

c. The average ELISA antibody titres against each component of the vaccine in sera of vaccinated calves are shown in Table 5.

DISCUSSION

Prevention of respiratory affections to eliminate viruses associated reproduction losses and the birth of persistently infected calves is essential for control of disease attributable to respiratory viruses.

Therefore, the present study was planned for preparation and evaluation of (MDBK-dextran roller) combined inactivated respiratory virus vaccine (Pneumo-4). The vaccine proved to be safe when tested in adult mice, guinea pigs and calves.

This paper described the clinical observation and the detection of the vaccinal viruses in body secretion and excretions of experimental animals for 21 days post-vaccination and post-challenge. It also described the immune response towards each constituent of the vaccine during the following 6 months after vaccination.

Clinical observation indicated that calves of vaccinated groups remained in a good condition for 21 days post-vaccination. The virus was not recovered from these animals throughout the experiment. This agreed with Granston and Welter (1968) Fulton *et al.* (1995) and Urban *et al.* (1995). No clinical abnormalities throughout the challenged period were observed. These findings go along with those of Stalloness *et al.* (1967).

Regarding the period of immunity conducted by the prepared inactivated vaccine, the vaccinated calves developed adequate level of neutralizing antibody titres against 4 constituents of the vaccine at 4 weeks post-vaccination higher than the minimum accepted titre and was sufficient to protect susceptible animals from infection till 6 months post-vaccination (Table 3). This agreed with Bittle (1968) who reported that BVD antibody level of 1:8 dilution (log 0.9) was protective. Mihajlovic *et al.* (1979) and Zuffa and Feketeova (1980) reported that the minimum accepted neutralizing titres

was 1:4 or 0.6 $\log_{10}$ against PI-3 and IBR viruses. Mattson *et al.* (1987) reported that 1:4 is the immune accepted SN titre for adeno virus type 3.

Mean haemagglutinating antibodies of acceptable titres were recorded post-vaccination (Table 4) against PI-3 virus as recorded by Mohanty and Lilly (1964).

The results of ELISA in Table 5 showed that the average of ELISA titres of vaccinated calves against 4 antigens (BVD, virus, PI-3 and BAV-3) was protective at 4 weeks post-vaccination according to Suribabui (1984) and sufficient to protect susceptible animals from infection with the virulent viruses.

Determination of the immune response to BVD, virus, PI-3 and BAV-3 viruses by using serum neutralization, ELISA and haemagglutination inhibition tests is considered to be valid. These findings were as expectation from tables (3, 4 and 5). This results agreed with those obtained by Valla (1985), Fulton *et al.* (1995) and Urban *et al.* (1995).

No viral isolation could be detected from vaccinated and contact control calves during 21 days post-vaccination. Absence of specific antibodies in sera of control non infected calves ascertained to the safety of the vaccine, and indicated no shedding virus. These results are going in harmony with those obtained by Granston and Welter (1965).

In vaccinated challenged calves, viruses were recovered from 1-8 days post challenge and from 1-21 days from infected calves. These results explained the higher immunogenicity against each fraction of the vaccine (Ballasch, 1993).

In conclusion, the main line in control of respiratory affection should include, firstly, isolation and prevenion of movement of animals affected and adaptation of symptomatic treatments. Secondly, vaccination of clinically normal calves and prevention of any contact between diseased and healthy animals, must be adopted.

Table 1. Determination of the optimum time needed for BEI to inactivate BVD, IBR, PI-3 and BAV-3 viruses (inactivation rate).

Original titre expressed in log_{10} $TCID_{50}$	BEI conc. 0.1 M in 0.15 N NaOH	Temperature of inactivation process	Titre after exposure expressed in log_{10} $TCID_{50}$ 1ml / hour								
			0	1	2	3	4	5	6	7	8
BVD $10^{6.5}$	0.01	37°C	6.5	5.0	4.5	3.5	2.5	1.0	-	-	-
IBR $10^{7.5}$	0.01	37°C	7.6	5.5	4.5	2.5	1.0	-	-	-	-
PI-3 10^{8}	0.01	37°C	8.5	6.0	3.0	1.5	-	-	-	-	-
BAV-3 $10^{6.0}$	0.01	37°C	6.5	5.0	4.5	2.5	1.75	1.0	-	-	-

- No cytopathic effect (CPE).

Table 2. Results of virus recovery by the cytopathogenicity in MDBK, FA technique and serum neutralizing of antibody titres of calves sera following challenge with virulent viruses (BVD, IBR, PI-3 and BAV-3) in vaccinated calves and non vaccinated control infected calves.

		Virus recovery in MDBK cells by cytopathogenic on post-infection days (PID)				Virus detection by FA on post-infection days (PID) and post-challenge days								Neutralizing antibody titres on post-challenge days (PChD) and post-infection days (PID)		
		B.C.	N.S.	C.S.	R.S.	CD	2	6	8	10	12	14	21	0 D.	30 D.	60 D.
BVD	Challenged calves	2-6	2-5	-	2-6	-	+	+	-	-	-	-	-	32	64	128
	Infected calves	3-10	1-14	4-8	3-18	-	+	+	+	+	+	+	-	0	128	256
IBR	Challenged calves	3-6	1-8	3-6	3-8	-	+	+	+	-	-	-	-	44	128	128
	Infected calves	2-11	1-12	3-9	4-10	-	+	+	+	+	+	+	-	0	256	512
PI-3	Challenged calves	2-6	2-7	3-8	3-6	-	+	+	+	-	-	-	-	64	128	256
	Infected calves	2-10	1-13	2-9	4-9	-	+	+	+	+	+	+	-	0	128	512
BAV-3	Challenged calves	3-8	2-8	1-7	4-7	-	+	+	+	-	-	-	-	64	64	128
	Infected calves	2-9	1-21	3-9	4-11	-	+	+	+	+	+	+	+	< 4	256	256
Control non-infected		0/2	0/2	0/2	0/2	-	-	-	-	-	-	-	-	-	-	-

No virus isolation could be detected from vaccinated groups and control non infected groups post infection by the virulent viruses.
BC : Buffy Coat. C.S. : Conjunctival Swabs. N.S. : Nasal Sawbs. R.S. : Rectal Swabs.
+ : Positive for the presence of viruses using FA. - : No virus present using FA.

Table 3. The immune response of calves vaccinated with combined inactivated respiratory virus vaccine containing BVD, IBR, PI-3 and BAV-3 (pneumo-4) using serum neutralization test.

Weeks post-vaccination	Log_{10} serum neutralizing antibody titres on post-vaccination				
		BVD	IBR	PI-3	BAV-3
1	First vaccination initial dose	0	0	0	0
2		0.55	0.6	0.7	0.8
3	Second vaccination Booster dose	1.1	1.4	1.5	1.2
4		1.5	1.65	1.7	1.7
6		1.7	1.75	1.82	1.75
8		1.7	1.75	1.8	1.65
12		1.6	1.75	1.65	1.5
16		1.4	1.5	165	1.2
20		1.1	1.3	1.5	1.15
24		0.9	1	1.2	0.9
28		0.8	0.9	0.9	0.6

Table 4. Results of haemagglutination test in vaccinated calves with (pneumo-4) against PI-3.

Weeks post-vaccination	Log_{10} haemagglutination inhibiting titres / post-vaccination
Zero day	0.25
1	0.65
2	1.36
3	1.85
4	2.25
6	2.40
8	2.20
12	2.10
16	2.0
20	1.80
24	1.60
28	1.30

Table 5. Average ELISA titre of calves post vaccination by local combined inactivated respiratory virus vaccine (pneumo-4).

Type of virus	Titre range	Weeks Post-Vaccination									
		0	2	4	6	8	12	16	20	24	28
BVD	2000-5000	< 1000	1826	2697	3665	4326	5652	5286	4236	3021	2024
IBR	3000-6000	< 1000	1495	2256	2056	4322	7125	7586	5286	4560	2834
PI-3	3500-7000	< 1000	1945	2858	3206	4804	6964	5670	4960	3954	2924
BAV-3	3000-5000	< 1000	1858	2062	3400	4018	5520	5074	3184	3180	2620
Control non-infected	< 1000	< 1000	< 1000	< 1000	< 1000	< 1000	< 1000	< 1000	< 1000	< 1000	< 1000

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تم دراسة إمكانية تحضير وتقييم اللقاح التنفسي الجموعي الرباعي الميت الذي يحتوي على عترات الإسهال الفيروسي في الأبقار والتهاب القصبة الهوائية المعدي والبارا إنفلونزا-٣ وكذلك فيروس الادينو-٣. تم تحضير اللقاح بتمرير الفيروسات في خلايا الزرع النسيجي الدوار (MDBK) مع بعض إضافة مادة الديادكستران وذلك لزيادة القوة العيارية للفيروس المنتج ودراسة معدل التثبيط بمادة البينري إيثيلين امين وإضافة الهيدروجل بنسبة ٣٠٪ كمادة مدمصة لتكثيف الأجسام المضادة وزيادة الاستجابة المناعية. وبعد ذلك تم تقييم اللقاح بدراسة درجة النقاوة والسلامة في الفئران وخنازير غينيا والعجول البقري، وقد اختبرت القوة المناعية للقاح المنتج عن طريق تحصين العجول بجرعة مزدوجة من اللقاح.

وأوضحت الاختبارات المختلفة عن وجود تباين في القوى التكوينية للأجسام المناعية المعادلة المضادة باستخدام اختباري السيرم المصلي المتعادل والاليزا وكذلك أجسام التلازن الدموي. وعند إجراء اختبار التحدي للقاح بحقن تلك الحيوانات المحصنة بالعترات الضاربة بعد شهر من الجرعة الثانية لم تظهر أيه اعراض مرضية بينما ظهرت اعراض مرضية على الحيوانات الأخرى التي لم تحصن. كذلك تم عزل الفيروسات والتأكد من وجودها باستخدام اختبار الفلورسين المشع.