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Research Article

Immunomodulating Effect of Zylexis on the Immune Response of Cattle Vaccinated with (Pneumo-4) Vaccine

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ABSTRACT

Bovine respiratory diseases (BRD) are a viral diseases of cattle, It causes a risk to the cattle industry in Egypt especially BVD as It causes diarrhea, reproductive disorders, immunosuppression and mortalities. So the present study zylexis was used as immunomodulator to enhance the immune response of calves to the inactivated Pneumo-4 virus vaccine. Twenty calves were allotted into four groups (five animals /group) where the first group was inoculated with Zylexis (2ml/calve) two days before vaccination with the field dose (5ml/ I/M) of inactivated pneumo-4 vaccine; the second group was inoculated with Zylexis simultaneously with the vaccine; the third calves 'group was inoculated with the vaccine only while the fourth group was kept as non-vaccinated and non-inoculated control. Booster dose of the vaccine was inoculated 15 days later. Blood samples were collected at heparin as anticoagulant at 0.3rd, 7th, 10th, 14th, 21th, 28th for lymphocyte proliferation and Nitric Oxide assay, while serum samples had been collected weekly then monthly till the end of the experiment period for monitoring of the induced antibodies using SNT and ELISA. The obtained results of serological tests demonstrated that calves inoculated with zylexis with the vaccine exhibited higher humoral and cellular immunity than those inoculated with zylexis two days before vaccination and other inoculated with the vaccine only. These findings suggested that zylexis has an immune stimulating action in calves vaccinated with the inactivated pneumo-4 vaccine.

Key words: BVD, IBR, BRSV, PI-3, SNT, Zylexis, ELISA and BRD

INTRODUCTION

Bovine respiratory diseases (BRD) are Bovine viral diarrhea virus- 1 (BVDV-1), Bovine herpes virus 1 (BHV-1), Bovine respiratory syncytial virus (BRSV) and Bovine parainfluenza virus 3 (BPIV-3) and combinations of these viruses have a great economic impact on beef and dairy cattle industry (Hay *et al*., 2016) as they affect calves of 2-5 months of age (Radostitis *et al*., 2000). BRD causes acute febrile respiratory diseases, decreased milk production, weight loss and abortion (Chi *et al*., 2016) but BVDV is one of the most economically significant diseases in the bovine industry in Egypt, it causes losses due to diarrhea, reproductive disorders, immuno-suppression and mortalities (Soltan *et al*., 2015). Prevention and control of BRD is much more successful and economically feasible than treatment (Samira *et al*., 2001).BVDV was recorded at farms in Ismailia and Kafr El- Sheikh while IBR was reported at Behera governorate, PIV-3 at Kafer El Sheikh, Alexanderia, El Behaira, Port Said, Damiatta, El-Qalubia and Giza governorate while BRSV was recorded at Assiut Governorate, Egypt ((Jehan *et al*., 2009, Elshemey and Hassan 2010, Elsayed *et al*., 2014 and Soltan *et al*., 2015).

It was noticed that calves' vaccination by inactivated Pneumo-4 vaccine induces short duration of immunity (4-6 months) So the use of immune-modulators (immune stimulants) is of beneficial value to improve animal immune response (El-Sabbagh *et al*., 2001 and Palomares *et al*., 2016). Zylexis is inactivated Para pox virus that acts as immune-modulators agent stimulates cell mediated immunity.

The present study aims to enhance the humoral and cellular immune response for calves which vaccinated with inactivated Pneumo-4 vaccine.

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MATERIALS AND METHODS

Viruses

Local strains of Bovine viral diarrhea virus (BVDV) (Iman strain) with a titer of 10^6 $TCID_{50}$/ml (Thanaa, 1975); Infectious Bovine Rhinotracheitis Virus (IBRV) Bovine Herps "Abou Hammad strain" with a titer of 10^8TCID_{50}/ml (Hafez *et al*., 1976); Parainfluenza-3 virus (PI-3V (Strain 45) with a titer of 10^8 $TCID_{50}$/ml (Singh and Baz, 1966) and Bovine respiratory syncytial virus (BRSV) (Reference strain "375L") of a titer 10^6TCID_{50}/ml were used for vaccine preparation and serological assays. These viruses were supplied by the Department of Rinder pest like Diseases Research (DRLDR), Veterinary Serum and Vaccine Research Institute (VSVRI).

Tissue culture

Madin Darby Bovine Kidney (MDBK) cell line was used for virus propagation, titration; vaccine preparation and serum neutralization test, it was obtained from Strain bank at CLEVB.

Na heparin

It was used as anticoagulant in sterile plastic centrifuge tube (200 IU/ml) used for collection of blood for lymphocytic proliferation assay, it was obtained from Central Laboratory for Evaluation of Veterinary Biologics, Abbassia, Cairo.

Zylexis

Inactivated Parapox Ovis virus strain D107 (commercially known as zylexis - Zoetis, Animal Health) was used to enhance the immune response of calves to the inactivated Pneumo-4 vaccine using a dose of 2ml/ animals according to Ulgen *et al*. (2014).

Inactivated Oil adjuvant gel pneumo-4 vaccine: The vaccine is commercially produced and supplied by Rinderpest like Diseases Dept, Veterinary Serum and Vaccine Research Institute (VSVRI). according to Bartling *et al*. (1990).

Virus titration

Titration of IBR, BVD, PI3 and RSV was carried out using infectivity method according to Mohanty and Lillie (1965) and the virus titer was expressed as $TCID_{50}$/ml according to Reed and Muench (1938).

Experimental design

Twenty native breed calves, had been proven to be free from BVDV, IBRV, PI3 and BRSV antibodies as screened by Serum Neutralization Test (SNT). These calves were allotted in to four groups (Five animals/ group).

Group 1: injected I/M with Zylexis **(**2ml/dose/calve) two days before vaccination with pneumo-4 (5ml/I/M)

Group 2: inoculated with Zylexis simultaneously with Pneumo-4vaccine.

Group 3: inoculated with vaccine (5ml / I/M) only.

Group 4: was kept as control negative.

The blood samples were collected at weekly intervals for first month then monthly till the experiment period post vaccination for serological tests (SNT, ELISA) and at 0, 3, 7, 10, 14, 21 and 28 days post vaccination for evaluation of cellular immunity.

Evaluation of the humoral immune response

Serum Neutralization Test (SNT): Serum samples were tested for BVD, IBR, PI3, RSV antibodies using SNT and the antibody titer was expressed as neutralizing index according to Fulton *et al*. (1995).

Enzyme Linked Immunosorbent Assay (ELISA): The test was performed by MONO SCREEN Ab ELISA kit for sero diagnosis of bovine IBR, BVD, PI3, RSV supplied by Bio-X Diagnostics (Ref no. BIO K 061\2, LOT no. BRS 17G06).The results were expressed as antibody titers.

Evaluation of the cellular immune response

Lymphocyte cell proliferation assay (MTT): The peripheral mononuclear cells (PMNCs) separation was done from heparinized whole blood samples according to Macpherson and Stocker (1962) and Mayer *et al*. (1974) using cell proliferation kit with Tetrazolium salts (MTT) reagent (AppliChem GmbH cat # A8088 Germany). The optic density (O.D) of the developed colons was then measured by ELISA reader at 450◦ A with reference of 630◦ A and this method according to Lucy (1974).

Determination of Nitric Oxide (NO) levels: It was carried out according to the assay described by Rajaraman *et al*., (1998) using ELISA reader at 570nm

RESULTS

Propagation of BVD; BRS; IBR and PI-3 in MDBK cell culture revealed that these viruses had the titers of 6.0; 6.0; 8.0 and 8.0 log_{10}TCID50/1ml respectively for several successive passages in MDBK cell culture as shown in Table 1.

Table 1: Virus propagation on MDBK cell culture

Propagated viruses	Virus titer ($log_{10}TCID_{50}$/1ml)
BVD and BRS virus	6.0
IBR and PI-3 virus	8.0

The observed CPE was characterized by granulation of the cytoplasm, vaculation and cell elongation (72hr) for BVDV; cell rounding, shrinkage of the cell wall and granulation of the cytoplasm (36hr) for IBRV and cell rounding, giant cell formation and syncytial information (72hr) for both of PI-3V and BRSV as demonstrated in Figure 1.

Evaluation of cellular and humeral immunity

Evaluation of cellular immune response by lymphocyte cell proliferation assay and Nitric Oxide and humoral immunity by SNT and ELISA revealed that calves inoculated with Zylexis at the same time of vaccination with gel adjuvant Pneumo-4 vaccine (group-2) showed higher cellular immunity than those inoculated with zylexis two days before vaccination (group-1) and those inoculated with the vaccine only (group-3) as shown at Tables 2, 3, 4 & 5 and Fig. 2 & 3.

Table 2: Lymphocyte cell proliferation assay **(MTT)** for calves inoculated by Pneumo-4 inactivated vaccine and Zylexis

Time of sampling	Lymphocyte proliferation assay measured by MTT			
	Group 1 (Mean)	Group 2 (Mean)	Group 3 (Mean)	Group 4 (control) (Mean)
Zero day	0.076	0.073	0.079	0.077
3rd DPV*	0.456	0.684	0.406	0.068
7th DPV	0.589	0.870	0.519	0.063
10th DPV	0.674	1.03	0.654	0.060
14th DPV	0.660	0.982	0.623	0.059
21th DPV	0.457	0.865	0.489	0.054
28th DPV	0.343	0.631	0.329	0.049

*DPV: Days post vaccination. Group 1: inoculated with Zylexis 2 days before vaccination (Pneumo-4). Group 2: inoculated simultaneously with Zylexis and Pneumo-4 vaccine. Group 3: inoculated with Pneumo-4 vaccine only. Group 4: control negative.

Table 3: Nitric Oxide records (µm/ml) for calves inoculated by Pneumo-4 inactivated vaccine and Zylexis

Time of sampling	Nitric Oxide reading (µm/ml)			
	Group 1 (Mean)	Group 2 (Mean)	Group 3 (Mean)	Group 4 (control) (Mean)
Zero day	2.25	2.40	2.10	0.077
3rd DPV*	4.23	5.67	3.77	0.068
7th DPV	5.28	6.32	4.48	0.063
10th DPV	5.78	6.82	3.09	0.060
14th DPV	4.02	5.25	2.65	0.059
21th DPV	2.27	3.79	1.26	0.054
28th DPV	1.18	2.34	0.98	0.049

*DPV: Days post vaccination. Group 1: inoculated with Zylexis 2 days before vaccination (Pneumo-4). Group 2: inoculated simultaneously with Zylexis and Pneumo-4 vaccine. Group 3: inoculated with Pneumo-4 vaccine only. Group 4: control negative.

Table 4: Serum neutralization indexes for calves inoculated by pneumo-4 inactivated vaccine and Zylexis

Time of Sampling	Serum neutralization index															
	Group 1				Group 2				Group 3				Group 4 control			
	BVD	IBR	RSV	PI3	BVD	IBR	RSV	PI3	BVD	IBR	RSV	PI3	IBR	BVD	RSV	PI3
Pre	0.3	0.3	0.15	0.21	0.3	0.23	0.15	0.3	0.3	0.3	0.22	0.3	0	0	0	0
Vaccination (Zero day)																
2 WPV*	0.35	0.55	0.37	0.45	0.65	0.8	0.65	0.75	0.35	0.5	0.35	0.45	0	0	0	0
Booster dose of vaccine																
04 WPV	1.7	1.80	1.72	1.95	1.95	2.05	1.92	2.1	1.65	1.75	1.62	1.8	0	0	0	0
08 WPV	1.65	1.75	1.69	1.82	1.9	2.0	1.91	2.08	1.6	1.7	1.61	1.78	0	0	0	0
12 WPV	1.5	1.60	1.54	1.73	1.8	1.95	1.83	2	1.5	1.65	1.53	1.7	0	0	0	0
16 WPV	1.3	1.45	1.42	1.6	1.55	1.6	1.56	1.7	1.25	1.3	1.26	1.4	0	0	0	0
20 WPV	1.1	1.3	1.21	1.4	1.3	1.4	1.4	1.6	1.0	1.1	1.1	1.3	0	0	0	0
24 WPV	0.9	1.1	1.0	1.23	1.2	1.25	1.23	1.4	0.9	0.95	0.93	1.1	0	0	0	0

*WPV: Weeks post vaccination. Group 1: inoculated with Zylexis 2 days before vaccination (Pneumo-4). Group 2: inoculated simultaneously with Zylexis and Pneumo-4 vaccine. Group 3: inoculated with Pneumo-4 vaccine only. Group 4: control negative. **N.B.:** Protective level of BVD 0.9 $\log_{10}$ - IBR, PI3 and BRSV=0.6 $\log_{10}$.

Table 5: ELISA optical density for calves inoculated by Pneumo-4 inactivated vaccine and Zylexis

Time of Sampling	ELISA Optical Density (OD)															
	Group 1				Group 2				Group 3				Group 4 control			
	BVD	IBR	RSV	PI3	BVD	IBR	RSV	PI3	BVD	IBR	RSV	PI3	BVD	IBR	RSV	PI3
Pre	0.3	0.3	0.22	0.15	0.3	0.23	0.15	0.3	0.3	0.3	0.22	0.3	0	0	0	0
Vaccination (Zero day)																
2 WPV*	0.45	0.5	0.7	0.55	0.65	0.7	0.9	0.75	0.35	0.4	0.6	0.45	0	0	0	0
Booster dose of vaccine																
04 WPV	1.75	0.7	0.92	1.9	1.95	0.9	1.12	2.1	1.65	0.6	0.82	1.8	0	0	0	0
08 WPV	1.7	1.59	1.63	1.88	1.9	1.79	1.83	2.08	1.6	1.49	1.53	1.78	0	0	0	0
12 WPV	1.6	1.53	1.52	1.8	1.8	1.73	1.72	2.0	1.5	1.43	1.42	1.7	0	0	0	0
16 WPV	1.35	1.38	1.42	1.5	1.55	1.58	1.62	1.7	1.25	1.28	1.32	1.4	0	0	0	0
20 WPV	1.1	1.2	1.3	1.4	1.3	1.4	1.5	1.6	1.22	1.15	1.2	1.3	0	0	0	0
24 WPV	0.9	1.03	1.1	1.2	1.2	1.23	1.3	1.4	0.86	0.93	1.03	1.15	0	0	0	0

*WPV: Weeks post vaccination. Group 1: inoculated with Zylexis 2 days before vaccination (Pneumo-4). Group 2: inoculated simultaneously with Zylexis and Pneumo-4 vaccine. Group 3: inoculated with Pneumo-4 vaccine only. Group 4: control negative.

DISCUSSION

Bovine respiratory diseases (BVDV, IBRV, BRSV and PI-3) are the most important causes of clinical diseases and deaths in feedlot cattle. BRD is multi-factorial, where a variety of physical and physiological stressors combining to predispose cattle to pneumonia. Affected calves are at highest risk shortly after transportation. Bad environment, accumulation of ammonia and excessively high humidity in closed areas, which lower the resistance of animal, which enhanced the multiplication of microorganisms (Taylor *et al.*, 2010).

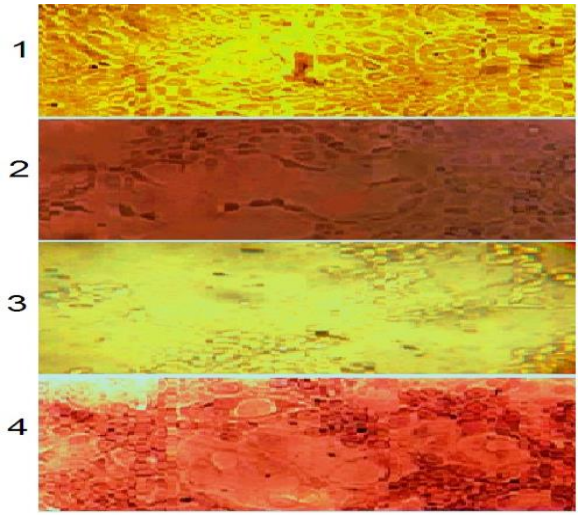

Fig. 1: Characteristic Cytopathic effect (CPE) of BVD, IBR, PI-3 and BRS Viruses on MDBK cell line. 1: Normal MDBK cell line. 2: CPE of BVDV showing granulation of the cytoplasm, vaculation and cell elongation (72hr). 3: CPE of IBRV showing cell rounding, shrinkage of the cell wall and granulation of the cytoplasm (36hr). 4: CPE of PI-3 and BRSV showing cell rounding, giant cell formation and syncytial information (72hr).

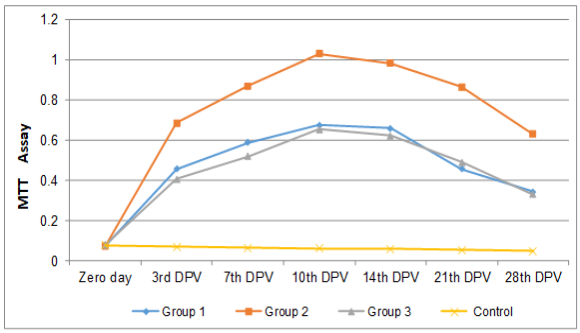

Fig. 2: Lymphocyte cell proliferation assay (MTT) of calves inoculated by Pneumo-4 inactivated vaccine and Zylexis.

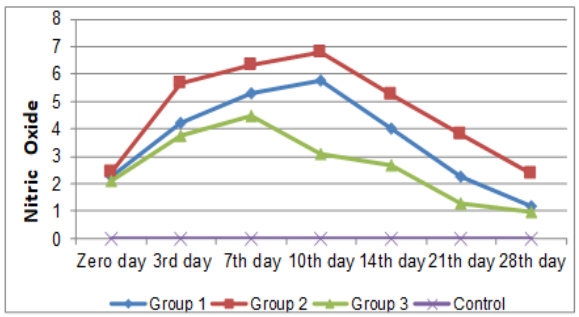

Fig. 3: Nitric Oxide records (µm / ml) of calves inoculated by Pneumo-4 inactivated vaccine and Zylexis.

Good vaccination program is considered very important way to control the disease. This study highlights the beneficial use of an immunomodulator (Zylexis) to evade immunity to produce a variety of immunomodulatory proteins that support active immune response (Paillot, 2013). Zylexis is currently used in equine medicine where it has a supportive effect on their cellular immunity and an immunomodulatory effect against equine viral infections (Ulgen *et al.*, 2014). Such activity is based on the activation of innate cells and consequent cytokine production (Anzilero *et al.*, 2014).

Table 1 showed the result of propagation of BVD, IBR, PI-3and BRS viruses on MDBK cell lines. The virus titers were expressed as the $\log_{10}TCID_{50}$/ml as described by Reed and Munech (1938). Photo (1) demonstrated the cytopathic effect which was characterized by granulation of the cytoplasm, vaculation and cell elongation (72hr) for BVDV; cell rounding, shrinkage of the cell wall and granulation of the cytoplasm (36hr) for IBRV and cell rounding, giant cell formation and syncytial information (72hr) for PI-3 and BRSV. These results agree with those of Marcus and Moll (1968) and Samira *et al.* (2001) who reported that MDBK cell lines were used successfully for a daptation, propagation and titration of these viruses.

Concerning the cellular assay, Tables (2 & 3) and Fig. (2 & 3) clarified that the use of Zylexis at the same time of vaccination with inactivated gel adjuvant Pneumo-4 vaccine evoked higher cellular immune responses in inoculated calves than in those inoculated with Zylexis two days before vaccination. These responses elevated by lymphocyte proliferation assay and Nitoric Oxide reading recorded peak values at 10 DPV (1.03) for group-2 and (0.674) for group-1 and persisted to 21 DPV (0.865) in group-2 and (0.457) in group-1 as confirming the immunomodulator effect of zylexis. The results were agreeable with those of Ulgen *et al.* (2014) and Fachinger *et al.*, (2000) who reported that the immunomodulator activate innate immune cells and support animals having suboptimal immune function and response or stressed (suppressed).

Table (4) showed an increasing of the neutralizing index (NI) started from two weeks post vaccination (BVDV=0.35, IBRV=0.5, BRSV=0.35 and PI-3=0.45) to reach its peak at the 4th week post vaccination ((BVDV=1.65 , IBRV=1.75, BRSV=1.62 and PI-3=1.8) with great elevation in groups-2 and 1(receiving zylexia) than non immunomodulated (group-3) coming in agreement with Bittle (1968) who reported that BVD virus antibody level 0.9 $\log_{10}$ was protective. Mihajlovic *et al.*, (1979) and Zuffa and Feketeova (1980) reported that the minimum accepted neutralizing titers was 1:4 ($\log_{10}$ 0.6) against PI-3 and IBR viruses. Fulton *et al.*, (1995) mentioned that the minimum accepted neutralizing titer for BRSV virus was ($\log_{10}$ 0.6).

Table (5) illustrated that ELISA antibody titer of immunomodulated calves in group (1&2) are much higher than those of group(3) in the same manner of that obtained by SNT as the results started to increase from two weeks post vaccination to reach the peak at 4th week post vaccination (BVDV=1.95,IBRV=0.9, BRSV=1.12 and PI-3=2.1). These results are in agreement with OIE (2015) which approved that the vaccine is considered protective where there is a significant increase of neutralizing antibody titer of vaccinated calves in parallel to the ELISA antibody titer (IgG). The cellular and humoral investigations showed an elevation in nearly parallel manner for groups 2and 1 immunomodulated before and at the time of vaccination comparing with group 3 that vaccinated with inactivated gel adjuvant Pneumo-4 vaccine alone.

Conclusions

It is obvious that administrations of Zylexis as immune-modulator agent potentiate the cellular and humoral immune response and enhance the duration of immunity induced by the inactivated gel adjuvant Pneumo-4 vaccine.

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